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The influence of oseltamivir carboxylate and oseltamivir on hemagglutinin inhibition and microneutralization test

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ABSTRACT

The administration of oseltamivir in humans is suggested to affect the results of hemagglutinin–inhibition test. To investigate this phenomenon, the concentrations of oseltamivir and oseltamivir carboxylate (OC) in sera obtained from oseltamivir-administered individuals were quantified by the liquid chromatography–tandem mass spectrometry (LC–MS/MS) system. The analysis revealed that the concentrations of OC in sera obtained at 4 and 7 h after administration were greater than those at 24 h after administration. Flow cytometry analyses revealed that OC or oseltamivir added in the sera affects the expression level of sialic acid α2,3-Gal linkages on horse erythrocytes; however, no effect was observed on the expression level of these linkages on chicken erythrocytes. Moreover, the addition of oseltamivir or OC may yield pseudopositive results in hemagglutinin–inhibition assays. These results suggest that the pseudopositive results obtained in hemagglutinin–inhibition assays occurred by the presence of OC, and that it is very important to take care of the patients in the prescription of oseltamivir when anti-influenza investigations are performed.

1. Introduction

Influenza viruses belong to the family Orthomyxoviridae and are classified as influenza A, B, and C viruses (Lamb and Krug, 2001). Influenza A viruses are harbored by a wide range of host species, including mammalian and avian species (Webster et al., 1992). The origin of new pandemic viruses that infect humans is expected to be derived from avian species, suggesting that interspecies transmission of avian influenza viruses into humans will occur in the near future (Horimoto and Kawaoka, 2005). In recent times, the most notable event of influenza virus infection in humans is the infection of H5N1 viruses that occurred in 1997 in Hong Kong (Subbarao et al., 1998); furthermore, the number of influenza patients is increasing every year (World Health Organization, 2008). It is said that influenza viruses of other subtypes have also been shown to infect humans, which makes it important to focus attention on the emergence of influenza viruses from species other than humans. In 2005 in Japan, a large outbreak of H5N2 avian influenza virus occurred in commercial chickens (Okamatsu et al., 2007a,b), which resulted in great economic losses and public health concern in the population engaged in epidemic control (Kyodo News Agency, 2006). The prototype strain A/Chicken/Ibaraki/1/2005 (Okamatsu et al., 2007a) was isolated and designated as a "low pathogenic" strain on the basis of its virological and genetic properties (Okamatsu et al., 2007a,b). In this outbreak, serological tests were conducted to determine whether the employees had been infected with H5N2 viruses. These tests revealed that some of the samples (20.5%) were seropositive for H5N2 influenza viruses; this was the first evidence suggesting the infection of H5N2 avian influenza viruses in human population (Kyodo News Agency, 2006).

In this outbreak, many individuals who tested positive in microneutralization test (MNT) were prescribed the anti-influenza drug oseltamivir (OP; commercial name: Tamiflu®) to prevent the infection of influenza viruses. OP is an orally active prodrug of oseltamivir carboxylate (OC), which is particularly effective against influenza virus neuraminidase (Ward et al., 2005); it is designed to target conserved residues at the active site of neuraminidase enzyme of influenza A and B viruses (Von Itzstein et al., 1993). To determine whether the prescription of Tamiflu® influences the result of serological tests, healthy human volunteers were administered Tamiflu®, and blood samples were collected to perform serological tests (Yamazaki et al., 2008). These tests revealed that hemagglutinin inhibition (HI) was observed when mammalian erythrocytes (horse, guinea pig, and human O type) were used and

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suggested that oseltamivir administration might affect the result of HI test. However, in this previous study, it was not clearly shown whether oseltamivir did influence the result of serological investigations. In the present study, we addressed the question of whether oseltamivir administration influences the result of serological investigations.

2. Materials and methods

2.1. Reagents and standards

OC and OP were kindly provided by F. Hoffmann, La Roche Ltd. (Basel, Switzerland). Both were dissolved in distilled water at a final concentration of 1000 μg/ml. Methanol, formic acid, and ammonium acetate were obtained from Kanto Chemicals (Tokyo, Japan). Animal erythrocytes (horse, guinea pig, chicken, goose, and turkey) were purchased from Nippon Bio-Test Laboratories Inc. (Tokyo, Japan), and human erythrocytes were contributed by our laboratory staff. Mammalian erythrocytes were prepared at a final concentration of 0.75% in phosphate buffered saline {PBS (−)} supplemented with 0.01% gelatin (horse) or without gelatin (guinea pig and human). Avian erythrocytes were prepared at a final concentration of 0.5% in PBS (−).

2.2. Sera samples

The human volunteers (10) included 6 males and 4 females (Yamazaki et al., 2008). They received one capsule of Tamiflu®, and their blood samples were collected at 0 h (prior to administration of the drug) and 4, 7, and 24 h after administration. Before serological testing, the sera samples were treated with a receptor-destroying enzyme (RDE II; Denka Seiken, Tokyo, Japan).

2.3. Cells and viruses

Madin-Darby canine kidney (MDCK) cells (Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan) were used for MN assay. The cells were grown in Eagle's minimum essential medium (E-MEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Invitrogen), 2 mM of L-glutamine (Invitrogen), 100 U/ml of penicillin–streptomycin (Invitrogen), and 0.25 μg/ml of Fungizone (Invitrogen). For MNT, viruses were grown in E-MEM supplemented with 2 mM of L-glutamine, 10 mM of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; Invitrogen), 1% bovine serum albumin (Sigma, St. Louis, MO, USA), 100 U/ml of penicillin–streptomycin, and 10 μg/ml of acetylated trypsin (Sigma). The influenza virus strain A/chicken/Ibaraki/1/2005 (H5N2) (Okamatsu et al., 2007a,b) was kindly provided by the Division of Virology, National Institute of Animal Health (Ibaraki, Japan). The virus was propagated in 10-day-old hen eggs.

2.4. Extraction of OC and OP from sera

Extraction of OC and OP from sera samples was performed according to the methods described previously (Wiltshire et al., 2000) with slight modifications. Briefly, 100 μl aliquots of sera sample were mixed with 900 μl of 5 mM ammonium acetate buffer (pH 3.5, Kanto Chemicals) in a glass tube (Iwaki, Tokyo, Japan). Solid phase extraction disc cartridges (Oasis MCX cartridge, 1 cc, 30 mg; Waters Corporation, Milford, MA, USA) were conditioned with 1 ml of methanol (Kanto Chemicals), followed by 3× 1 ml of 9:1 methanol–50 mM ammonium acetate buffer (pH 3.5) and 1 ml of 5 mM ammonium acetate buffer (pH 3.5). Diluted sera samples were drawn through cartridges, which were washed with 1 ml of methanol and 1 ml of 9:1 methanol–water. The trapped materials were then eluted with 1 ml of 9:1 methanol–50 mM aqueous ammonium acetate buffer (pH 3.5) and were evaporated with nitrogen gas at 50 °C. The resultant extracts were resuspended in 100 μl of distilled water, and 1 μl of this suspension was injected into the liquid chromatography–tandem mass spectrometry (LC–MS/MS) system.

2.5. Separation and detection of OC and OP

The LC–MS/MS system consisted of TSQ Quantum Ultra quadrupole mass spectrometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA) coupled to a Nanospace SI-2 HPLC system (Shiseido, Tokyo, Japan). OC and OP were separated using a Hypersil GOLD CN column (2.1 mm × 150 mm, particle size 3 μm, Thermo Fisher Scientific) protected by a Hypersil GOLD CN drop-in guard cartridge (2.1 mm × 10 mm, particle size 3 μm, Thermo Fisher Scientific) at 40 °C. The mobile phase used was 50% methanol–50% 80 mM aqueous formic acid (pH 3) (Wiltshire et al., 2000) at a flow rate of 0.2 ml/min. The retention time of the two compounds was approximately 3.50 min (OC) and 3.70 min (OP). After chromatographic separation, the analytes were introduced into the mass spectrometer, and quantification of OC and OP was performed with selected reaction monitoring (SRM) for transition of m/z 285.3–179.8 for OC and m/z 313.1–208.0 for OP. Data were analyzed by Xcalibur software (Thermo Fisher Scientific). The LC–MS/MS system was maintained at the following parameters: spray voltage, 1000 V; vaporizer temperature, 350 °C; sheath gas pressure, 50 psi; capillary temperature, 350 °C; collision gas pressure, 1.5 mTorr; collision energy, 14 V (OC, m/z 179.8) and 12 V (OP, m/z 208.0).

2.6. Flow cytometry analysis

Flow cytometry analyses were performed using the digoxigenin (DIG) glycan differentiation kit (Roche) according to the methods described previously (Govorkova et al., 1999; Ito et al., 1997; Medeiros et al., 2001) with slight modifications. Briefly, 100 μl of RDE-treated sera samples were mixed with an equal volume of virus antigens adjusted to 4 hemagglutinin (HA) units and incubated at 37 °C for 60 min in a 1.5-ml microtube (Treff Lab, Degersheim, Switzerland). Subsequently, 200 μl of horse (0.75%) or chicken (0.5%) erythrocytes was added to the mixture and incubated at room temperature for further 60 min. After incubation, the tubes were centrifuged at 4 °C at 640 × g for 10 min, and pellets were resuspended in 1 ml of PBS (−). Next, the solutions were incubated at 4 °C for 60 min with 0.1 μg/ml of DIG-labeled *Sambucus nigra* agglutinin (SNA), which possesses specific binding affinity to the Neu5Ac/Neu5Gc-SAα2,6-Gal/GalNAc residues (Shibuya et al., 1987), or 0.5 μg/ml of DIG-labeled *Maackia amurensis* agglutinin (MAA), which binds with a high affinity to the Neu5Ac/Neu5Gc-SAα2,3-Gal residues (Wang and Cummings, 1988). As a control, virus–cell mixture was incubated with PBS (−). The cells were washed twice with cold PBS (−) and then incubated with fluorescein isothiocyanate (FITC)-conjugated anti-DIG antibodies {diluted with PBS (−) at 1:200, Roche} for 30 min at 4 °C. After washing with cold PBS (−) for two times, the fluorescence intensity of the cells was analyzed on the FACScalibur fluorospectrometer (Becton–Dickinson). As a positive control, a hyperimmunized anti-A/duck/Hong Kong/342/78 strain (H5N2 subtype) goat serum was used.

2.7. Influence of OC and OP on MN assay results

OC (100 μg/ml) or OP (1000 μg/ml) was twofold diluted with 1× E-MEM. The resultant solution was mixed with an equal volume of

Table 1
Quantification of OC and OP in human sera samples

	Hours after administration/concentrations of compounds (ng/100 μl)			
	0	4	7	24
OC[a]	ND[b]	37.97 ± 14.47[c]	30.94 ± 16.64[c]	4.48 ± 3.06[c,d]
OP[e]	ND	2.20 ± 2.05[c]	–[f]	–

[a] Oseltamivir carboxylate.
[b] Not determined.
[c] Values are averages of 10 samples.
[d] Detection limit is 10 ng/ml (1 ng/100 μl); this value is reference value.
[e] Oseltamivir.
[f] Less than 1 ng/ml (0.1 ng/100 μl).

1.0×10^2 50% tissue culture infection dose ($TCID_{50}$) of virus antigens, transferred onto MDCK cells grown in a 96-well flat bottom plate (Greiner GmbH, Frickenhausen, Germany) and incubated at 37 °C in 5% CO_2 for 96 h. After incubation, infected cells were fixed with 10% formalin/PBS (−), and the remaining cells were stained with naphthol black solution (containing 0.1% of naphthol black (Sigma) and 9% of acetic acid). The stained cells were decolored with 50 μl of 0.1N sodium hydroxide, and optical density was determined by measuring absorbance at 630 nm by using a microplate reader (Model 680; Bio-Rad Laboratories, Hercules, CA, USA). The value was expressed as the highest dilution of OC or OP that inhibited $TCID_{50}$ of virus.

2.8. Influence of OC and OP on HI assay results

OC and OP (1000 μg/ml) were twofold diluted with PBS (−) at a final volume of 25 μl. Next, 4 HA unit of virus antigen/25 μl was added and incubated at 37 °C for 30 min. After incubation, an equal volume of erythrocytes was added and incubated further for 30 min (chicken, goose, and turkey erythrocytes) or 60 min (horse, guinea pig, and human O type erythrocytes). HI titer was expressed as the highest dilution of serum that inhibited hemagglutination.

3. Results

3.1. Quantification of OC and OP in sera samples

OC and OP concentrations in the sera samples obtained from oseltamivir-administered individuals were measured by the LC–MS/MS system. As shown in Table 1, the average concentration of OC in the samples obtained at 4, 7, and 24 h after administration was 37.97, 30.94, and 4.48 ng/100 μl, respectively. The average concentration of OP in the samples obtained at 4 h was 2.20 ng/100 μl.

3.2. Influence of OC and OP on MN and HI assay results

To assess the effect of OC and OP on the result of serological investigations, OC and OP were used to perform HI and MN assays (Table 2). In the MN assay, the OC solution inhibited virus infection at a concentration of 6,250 ng/ml, while the OP solution inhibited virus infection at a concentration of 500,000 ng/ml. In the HI assay, no HI was observed with OC and OP solutions for avian cells; however, HI was observed for mammalian cells. The minimum concentrations of OC were <1 ng/ml, and those of OP were ranging from 488 to 1950 ng/ml, respectively.

3.3. Flow cytometry analysis of SAα2,3-Gal and SAα2,6-Gal linkage on the cell surface

It has been reported that the amount or distribution patterns of SAα2,3-Gal and SAα2,6-Gal linkages on erythrocytes are different in each animal species (Ito et al., 1997). To investigate the distribution pattern of these moieties on horse and chicken erythrocytes, relative amounts of SAα2,3-Gal and SAα2,6-Gal were measured with the glycan differentiation kit. As shown in Fig. 1, the expression level of SAα2,3-Gal on erythrocytes in the virus-horse RBC mixture was significantly greater than that in the control (virus alone) when 500 ng of OC and OP were added to the former. Furthermore, the expression level of SAα2,3-Gal on erythrocytes was higher than that in the negative control when positive control serum was reacted with the virus antigen (Fig. 1). On the other hand, the distribution pattern of SAα2,3-Gal and SAα2,6-Gal on chicken erythrocytes did not show significant difference in all combinations.

Next, the distribution patterns of these sialyloligosaccarides in the sera samples from oseltamivir-administered individuals were determined. As shown in Fig. 2, the expression level of SAα2,3-Gal linkages on horse erythrocytes with the sera from an oseltamivir-administered individual (volunteer number 7; Yamazaki et al., 2008) obtained at 4, 7, and 24 h was significantly higher than that at 0 h. However, the distribution patterns of these linkages on chicken erythrocytes showed no significant change in all samples.

4. Discussion

OC and OP were detected in the sera samples of oseltamivir-administered individuals by the LC–MS/MS system (Table 1). In a previous study, the concentrations of OP and OC in the sera were reported to be 6.73 and 380 ng/ml, respectively (Lindegardh et al.,

Table 2
Effects of OC and OP in serological assays

	Number of wells that showed hemagglutination inhibition with different erythrocytes (concentrations of compounds: ng/ml)						Concentrations of compounds that inhibited $TCID_{50}$ virus infection (ng/ml)
	Horse	Guinea pig	Human (O)	Chicken	Goose	Turkey	MDCK cell
OC[a]	20 (<1[c])	22 (<1[b])	22 (<1[b])	<1[c] (>1,000,000)	<1[c] (>1,000,000)	<1[c] (>1,000,000)	6,250
OP[d]	9 (1950)	10 (975)	11 (488)	<1[c] (>1,000,000)	<1[c] (>1,000,000)	<1[c] (>1,000,000)	500,000

[a] Oseltamivir carboxylate.
[b] Less than 1 ng/ml.
[c] Less than well number 1.
[d] Oseltamivir.

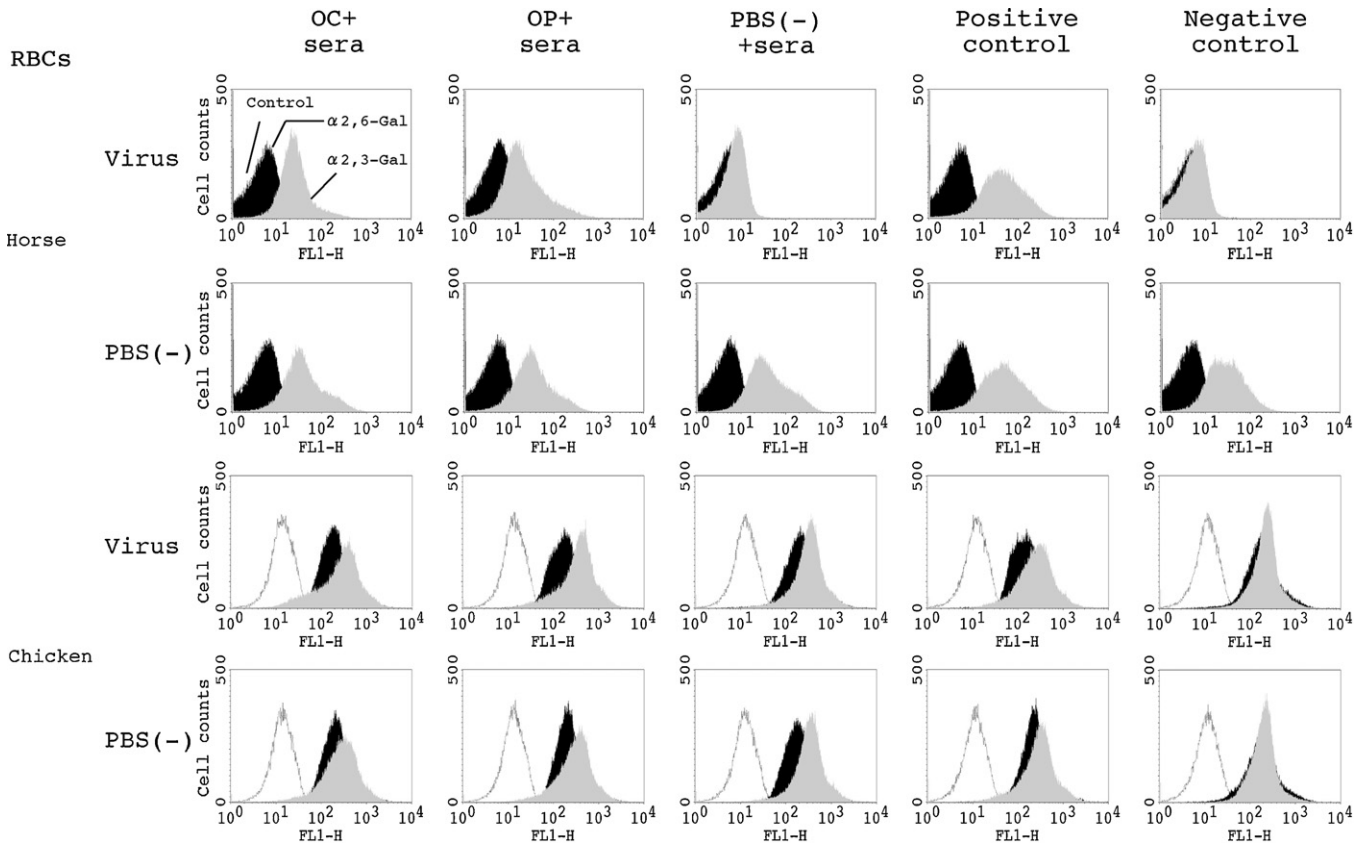

Fig. 1. Comparison of SAα2,3-Gal and SAα2,6-Gal linkages expressed on the surface of horse and chicken erythrocytes that were reacted with RDE-treated serum. Cells were incubated with 4 HA unit of virus suspensions and the following: 500 ng of OC and pooled normal human sera (consisting of five normal human sera samples); 500 ng of OP and sera; PBS (−) and sera; positive control serum; and PBS (−) (negative control). The mixtures were treated as described in Section 2. Histograms are shown as gray (SAα2,3-Gal), black (SAα2,6-Gal), and colorless (control).

2007), which is consistent with our results. The concentration of OC was relatively high in the samples obtained at 4 and 7 h after oseltamivir administration and decreased at 24 h after administration (Table 1). Yamazaki et al. (2008) showed that HI titers at 4 and 7 h after administration were higher than those at 0 and 24 h in all volunteers. These results suggest that the presence of OC or OP, mainly OC, is related to the attainment of pseudopositive results in HI test.

In the present study, it was also observed that the relative amounts of SAα2,3-Gal in horse erythrocytes were retained in the serum samples added with OC or OP as compared to normal distribution pattern (Fig. 1). These results suggest that the presence of oseltamivir may influence the expression of SAα2,3-Gal receptor on horse erythrocytes and thereby prevent the absorption of virus particles onto the cell surface.

And this study clearly showed that the expression level of SAα2,3-Gal linkages on horse erythrocytes did not decrease in the samples obtained from oseltamivir-administered individuals after 4, 7, and 24 h of administration (Fig. 2). This result suggests that the presence of OC or OP in sera may influence the distribution pattern of SAα2,3-Gal linkages on horse erythrocytes. The influenza virus strain A/chicken/Ibaraki/1/2005 (Okamatsu et al., 2007a) used in this study is an H5N2 subtype derived from avian species; this is in accordance with the fact that avian-derived influenza viruses preferentially recognize SAα2,3-Gal linkages rather than SAα2,6-Gal linkages (Matrosovich et al., 2000; Rogers and Paulson, 1983). The above-mentioned results suggest that the binding of viral HA and sialic acid receptors located on the cell surface is influenced by the presence of OC or OP in the reaction mixtures.

Recently, Ohuchi et al. showed that the number of virus particles located on guinea pig erythrocytes was not significantly decreased by the presence of neuraminidase inhibitors (zanamivir); additionally, by performing an electron microscopy investigation, they showed that the virus particles were sunk into the cell membrane (Ohuchi et al., 2006). Taken together, their data and our new data suggest the binding conditions between sialic acid receptors and viral HA, which are illustrated in Fig. 3. In the absence of oseltamivir, virus particles attach onto the sialic acid receptor and may interrupt the binding of DIG-labeled lectins exposed on the surface of erythrocytes (Fig. 3b), and viral HA may form a bridge with other erythrocytes. However, in the presence of oseltamivir, virus particles are sunk into the cell membrane; as a result, the receptors on the cell surface are accessible, and DIG-labeled lectins may be able to attach the cellular receptors (Fig. 3a), which may result no or little bridge formation.

In Table 2, it is shown that the presence of OC or OP causes the pseudopositive results in MNT. However, their minimum inhibitory concentrations are higher than the values when an adult takes 75 mg of oseltamivir (380 ng/ml in OC and 6.73 ng/ml in OP; Lindegardh et al., 2007). These data and our results suggest that a greater amount of drug is required to cause the pseudopositive results in MNT with human sera samples in comparison to the usual values. Therefore, it is suggested that these events may not be observed in MNT with OP-administered human samples. However, these results also suggest that further study will be needed to understand these events further.

The results shown in Table 2 suggest that the presence of OC and OP yields pseudopositive results in HI assay with mammalian

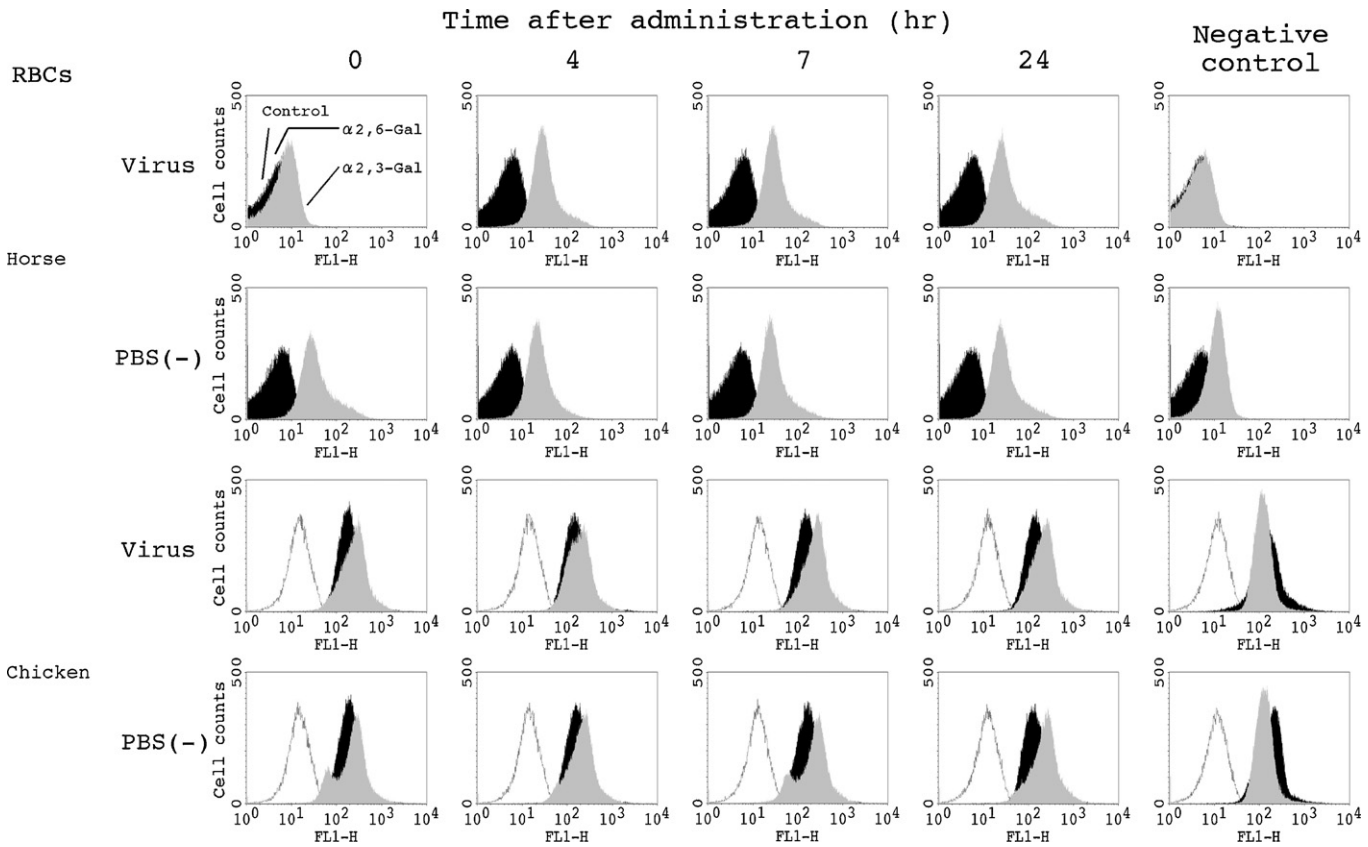

Fig. 2. Comparison of SAα2,3-Gal and SAα2,6-Gal on the surface of horse and chicken erythrocytes that were reacted with human sera after administration of oseltamivir. Sera samples were obtained before administration (0 h) and 4, 7, and 24 h after administration. RDE-treated samples were mixed with an equal volume of 4 HA unit of virus suspensions and then reacted with erythrocytes. The mixtures were then treated as described in Section 2. Histograms are shown as gray (SAα2,3-Gal), black (SAα2,6-Gal), and colorless (control).

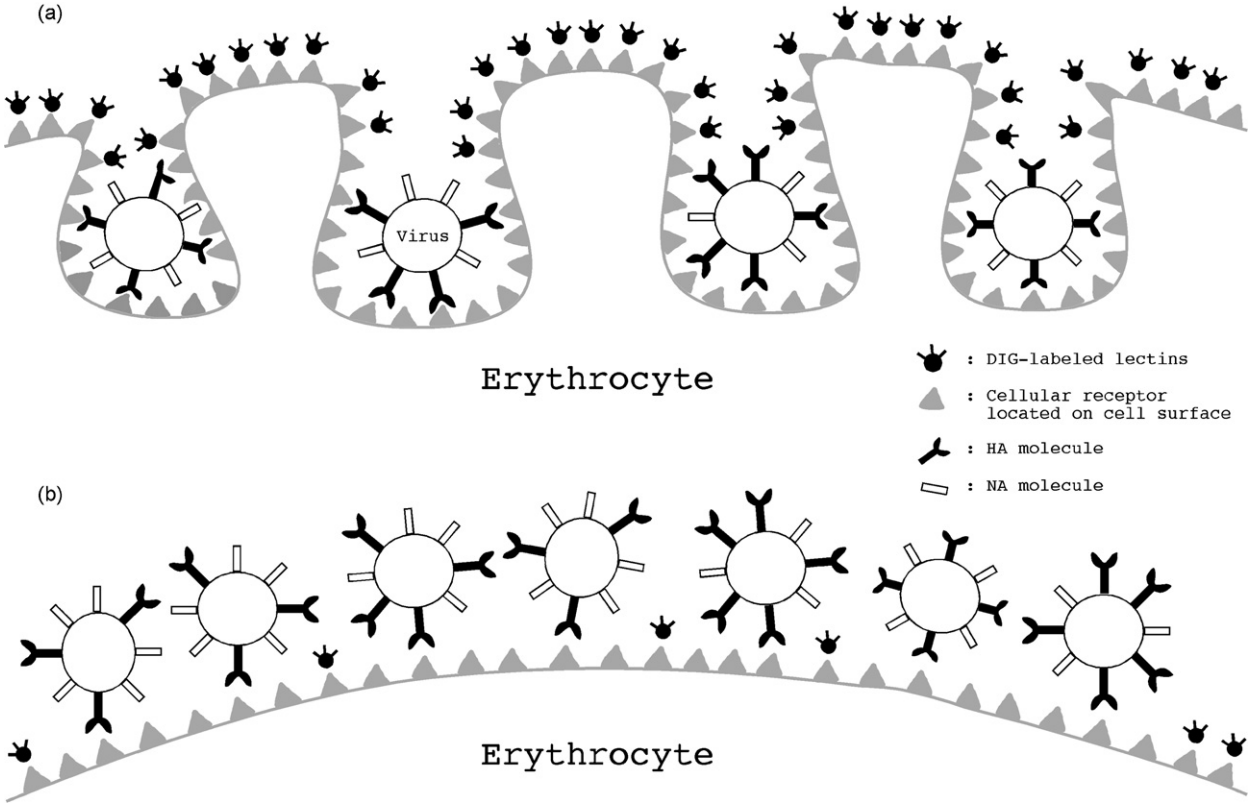

Fig. 3. Schematic illustration of the binding condition between virus particle, DIG-labeled lectins, and cellular receptors. (a) In the presence of neuraminidase (NA) inhibitor and (b) in the absence of NA inhibitor.

erythrocytes; it should also be noted that the minimum effective dose of OP is 1000-fold greater than that of OC. This suggests that the pseudopositive result is due to the presence of OC, which is supported by the fact that the amount of OC in serum is greater than that of OP (Table 1). However, this study does not clearly address the question of why pseudopositive results were observed in HI test with mammalian cells but not with avian cells. A possible explanation for this issue is that different concentrations of virus suspensions were used for erythrocytes of each species. Since 4 HA units of virus suspensions were prepared, the concentrations of virus suspensions used for erythrocytes of mammalian species were higher than those used for avian species, i.e., 64 or 128 times of dilutions are required to prepare a 4 HA unit of virus antigens with the A/chicken/Ibaraki/1/2005 strain and avian erythrocytes, but only two times of dilution is required with the same virus strain and horse erythrocytes. This implies that the concentration of virus antigens used for the test varied greatly between the erythrocytes of these species. Furthermore, avian erythrocytes are larger in diameter than mammalian erythrocytes are and usually possess a nucleus; this suggests that the speed of sedimentation of avian erythrocytes in the solution is faster than that of mammalian erythrocytes. It is unclear that whether these differences are related to the expression of this phenomenon, and further studies are required to clarify this issue.

In the near future, a new pandemic influenza virus is expected to emerge in human population. This will lead to increased administration of antiviral drugs, including oseltamivir, to prevent influenza infection in humans. In this scenario, it is important to raise the issue of obtaining misleading results in serological investigations. Furthermore, this issue will occur with other subtypes of influenza virus possibly due to the pharmacological properties of oseltamivir (Von Itzstein et al., 1993). Hence, it would be essential to take care of the condition of patients when investigations are performed, for example, maintaining the record of medications and checking the present status of administration; this information can enable to obtain accurate results for the evaluation of this phenomenon.

In summary, this study shows that oseltamivir administration increases titers in HI test. The causative agent of this increase is OC, which is supported by the results that OC is retained in the sera obtained from oseltamivir-administered individuals and that the distribution pattern of SAα2,3-Gal linkages on erythrocytes was changed by the addition of OC.

Furthermore, our results also suggest that it is very important to take care of the prescription of oseltamivir in patients when anti-influenza investigations are performed, which leads to obtain exact inspection results in the clinical scene.

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