Antiviral activity of hypothiocyanite produced by lactoperoxidase against influenza A and B viruses and mode of its antiviral action

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Summary. – Hypothiocyanite (OSCN-) is a natural component of human saliva and is produced by the lactoperoxidase (LPO)/thiocyanate/hydrogen peroxide (H2O2) system. OSCN- has been previously shown to exhibit antiviral activity against influenza viruses (IFV) A/H1N1/2009 and A/H1N2/2009 in vitro as well as antimicrobial and antifungal activities. We elucidated the antiviral activity of OSCN- against both IFV types A and B and the mode of its antiviral action. OSCN- was produced constantly at 900 ± 200 μmol/l in Na3PO4 buffer solution containing NaSCN and LPO in the presence of H2O2 as an original OSCN- solution. In a plaque reduction assay, IFV A/PR/8/34 (H1N1), A/Fukushima/13/43 (H3N2), B/Singapore/222/97, and B/Fukushima/15/93 were exposed to various concentrations of OSCN- for 0 to 30 min before adsorption to MDCK cells, and plaque formation was examined. OSCN- exhibited significant similar antiviral activities against all four viruses without cytotoxicity, and the EC50 values for them were from 57 ± 16 to 148 ± 27 μmol/l regardless of the exposure times. The exposure of MDCK cells to OSCN- before viral adsorption did not affect its anti-IFV activity (EC50: more than 450 μmol/l), but the exposure after viral adsorption affected it moderately (EC50: 380 ± 40 μmol/l). Moreover, the exposure of virus particles to OSCN- at 450 μmol/l did not affect the hemagglutinin activity of IFV in hemagglutination inhibition assay. These results suggest that the attachment of OSCN- to the viral envelope critically contributes to the mode of antiviral action of OSCN- without interfering with viral adsorption.

Keywords: hypothiocyanite; influenza virus type A; influenza virus type B; lactoperoxidase; antiviral activity

Introduction

Hypothiocyanite (OSCN-) is a natural component of human saliva and a major oxidation product of the lactoperoxidase (LPO)/thiocyanate (SCN-)/hydrogen peroxide (H2O2) reaction (Aune and Thomas, 1977; Wijkstrom-Frei et al., 2003). This reaction is referred to as the LPO system. OSCN- produced by the LPO system inhibits bacterial metabolism by the reaction with bacterial sulphydryls (Thomas and Aune, 1978; Thomas et al., 1983). Shin et al. (2011) showed that ingestion of a tablet containing LPO reduces the volume of the oral bacteria that cause oral malodor and periodontal disease. Moreover, several studies have reported that OSCN- possesses inhibitory properties against fungi such as Candida albicans and viruses such as influenza virus (IFV), herpes simplex virus type 1, respiratory syncytial virus, echovirus 11, and human immunodeficiency virus (Pourtois et al., 1990; Lenander-Lumikari, 1992; Mikola et al., 1995; Cegolon et al., 2014; Gingerich et al., 2016). Therefore, OSCN- produced by the LPO system surely contributes to host defense against infections.
IFV is a respiratory RNA virus enclosed by a lipid envelope that embeds two glycoproteins, hemagglutinin and neuraminidase. These glycoproteins assist virus adsorption to cells and virus budding during infection. IFV types A (H1N1, H1N2, and H3N2 subtypes) and B commonly infect humans and cause seasonal epidemics of disease (Steel and Lowen, 2014). Especially IFV type A causes severe pandemics involving an unpredictable morbidity and mortality in humans (Simonsen, 1999; Thompson et al., 2004). Therefore, prophylaxis of IFV infection is important (Aiello et al., 2010; World Health Organization, 2017). In a recent study, Gingerich et al. (2016) indicated that OSCN formation by the LPO system inactivates the H1N2 subtype of IFV type A. Moreover, OSCN has a virucidal effect against pandemic IFV (A/H1N1/2009) in vitro (Cegolon et al., 2014). However, the antiviral effects of OSCN on the H3N2 subtype of IFV type A and type B still remain unclear.

In the present study, we compared the antiviral activities of OSCN against IFV types A and B and elucidated the mode of its antiviral action. We confirmed that OSCN exhibits similar antiviral activity against both IFV types A and B, and that the attachment of OSCN to the viral envelope without interfering with viral adsorption to host cells critically contributes to the mode of antiviral action of OSCN.

Materials and Methods

Viruses and cells. IFV A/PR/8/34 (H1N1), A/Fukushima/13/43 (H3N2), B/Singapore/222/97, and B/Fukushima/15/93 were propagated in Madin-Darby canine kidney (MDCK) cells as described previously (Sawamura et al., 2010). The infected cultures were frozen and thawed three times and then centrifuged at 880 × g for 15 min. The supernatants were stored at -80°C as virus stock solution until use. MDCK cells were grown and maintained in Eagle’s minimum essential medium (EMEM; Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 8% and 2%, respectively, heat-inactivated fetal bovine serum (FBS; Hyclone Laboratories, Inc., South Logan, Utah, USA) (Kurokawa et al., 1990).

OSCN preparation by LPO system. OSCN was enzymatically generated by the LPO system (Lloyd et al., 2008). In the system, 50 mmol/l NaPO₄ buffer solution (pH 6.6, Nacalai Tesque, Inc., Kyoto, Japan) containing 7.5 mmol/l NaSCN (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 1.5 mmol/l LPO (Morinaga Milk Industry Co., Ltd., Tokyo, Japan) was used as a reaction solution that is negative control without H₂O₂. OSCN was generated in the reaction solution by the addition of H₂O₂ (Wako Pure Chemical Industries, Ltd.) to make a final concentration of 3.75 mmol/l in a volume of 40 μl for 15 min at room temperature. Then, catalase (1.0 ml, 140 U/ml, Wako Pure Chemical Industries) was added to remove unreacted H₂O₂, and the enzyme reaction mixture was centrifuged in filter tubes (Amicon Ultra Centrifugal Filters Ultracel-10K, Merck Millipore Ltd., County Cork, Ireland) at 2,000 x g for 5 min at 4°C. The filtered solution was used as an original OSCN solution.

Measurement of OSCN concentration. The OSCN concentration produced in the reaction solution was measured in the presence of 5,5’-dithiobis-2-nitrobenzoic acid (DTNA, Nacalai Tesque) as reported by Tenovuo et al. (1985). The original prepared OSCN solution or the reaction solution of the LPO system (as a reference) (25 μl) was mixed with 1.975 ml of 49 mmol/l Tris buffer solution (pH 8.0, Nacalai Tesque) containing 32 μmol/l DTNA, 30 μmol/l 2-mercaptopethanol (2-ME, Wako Pure Chemical Industries), and distilled water. The absorbance of 5-thio-2-nitrobenzoic acid (TNA) was measured at 412 nm within 30 min after the mixing to determine the concentration of OSCN, because TNA in the mixture is produced from DTNA by 2-ME, and 2 moles of TNA react with 1 mole of OSCN to produce DTNA again. The OSCN concentration was calculated from the measured absorbance using a molar absorbance coefficient of 14,150 (μmol/l)⁻¹·cm⁻¹ of TNA according to the following formula (Tenovuo et al., 1985):

\[
\text{OSCN (μmol/l)} = \frac{A_{412} \times 1/14,150 \times 1/2 \times 2,000/25 \text{ mol/l} \times 10^6}{A_{412}}
\]

where \(A_{412}\) is the reference absorbance – (original OSCN solution absorbance); 14,150 = the molar absorbance coefficient of TNA; 2 = correction factor (2 moles TNA requires 1 mole OSCN to produce 1 mole DTNA); 2,000 = total volume (μl); 25 = sample volume (μl).

Stability of OSCN. To examine the stability of OSCN produced by addition of H₂O₂ in original OSCN solution, we assessed the change of OSCN concentrations in the mixture of equal parts of the original OSCN solution and phosphate buffered saline (PBS) containing 1% bovine serum albumin (BSA; Sigma-Aldrich Corp., St. Louis, MO, USA), which was used to dilute the original virus solution as described below. The OSCN concentrations in the mixture were measured in the presence of DTNA at 0, 15, 30, 45, 60, 75 and 90 min after the mixing at room temperature as described above. As a reference, we used the mixture of equal parts of the reaction solution without H₂O₂ and PBS containing 1% BSA. The OSCN concentrations were calculated as described above.

Plaque reduction assay. We examined the antiviral activity of OSCN against IFV A/PR/8/34, A/Fukushima/13/43, B/Singapore/222/97, and B/Fukushima/15/93 by a plaque reduction assay using MDCK cells. The OSCN concentrations produced were constantly 900 ± 200 μmol/l in the original OSCN solution. The original prepared OSCN solution was serially diluted 2-fold with the reaction solution of the LPO system, and each dilution was mixed with an equal volume of virus solution at 200 plaque-forming units (PFU)/0.2 ml prepared from virus stock solution by the addition of PBS containing 1% BSA. The mixtures were incubated for 0, 15, and 30 min at room temperature. Then, 0.2 ml of each mixture (100 PFU) was added to confluent MDCK cells grown in 60 mm plastic dishes, and virus was adsorbed to MDCK cells at room temperature for 1 h. The inoculum was removed, and the cells were overlaid with 5 ml of a nutrient agarose (0.8 %) medium for a plaque assay and maintained in a humidified atmosphere containing 5% CO₂ for 3 to 4 days as described previously (Sawamura et al.,...
The infected cells were fixed with 5% formalin solution and stained with 0.03% methylene blue. The number of plaques was counted, and the effective concentrations for 50% plaque reduction (EC_{50}) were determined from a curve relating the plaque number to the concentrations of OSCN’ solutions (Shimizu et al., 2008).

**MDCK cells exposure to OSCN before and after virus adsorption.** We examined the effects of exposure of MDCK cells to OSCN’ before and after the adsorption of IFV A/PR/8/34 to MDCK cells. The original prepared OSCN’ solution was serially diluted 2-fold, and each dilution was mixed with an equal volume of PBS containing 1% BSA. The highest concentration of OSCN’ in the mixture was 450 μmol/l (as a half concentration of the original OSCN’ solution). To examine the effect of exposure of MDCK cells to OSCN’ before viral adsorption, 0.2 ml aliquots of OSCN’ dilutions were added to confluent MDCK cells in 60 mm plastic dishes and incubated for 1 h at room temperature. The cells were rinsed with PBS and inoculated with 100 PFU/0.2 ml of IFV A/PR/8/34 diluted from the virus stock solution by PBS containing 1% BSA. After adsorption at room temperature for 1 h, a plaque assay was performed as described before. On the other hand, to examine the effect of OSCN’ exposure after viral adsorption, confluent MDCK cells in 60 mm plastic dishes were incubated with 100 PFU/0.2 ml of IFV A/PR/8/34 for 1 h at 4°C to avoid virus entry into the cells. The cells were rinsed with cold PBS, and 0.2 ml of the mixtures of diluted OSCN’ solutions or the reaction solution of the LPO system (as a control) was added to the infected cells. After incubation of 1 h at room temperature, a plaque assay was performed as described before. After fixing and staining the infected cells, the number of plaques was counted, and EC_{50} values were determined as described before.

**Cytotoxicity assay.** The cytotoxicity of OSCN’ toward IFV-infected MDCK cells was evaluated by a trypan blue exclusion test as described previously (Tsurita et al., 2001). Briefly, MDCK cells were seeded at 3.5 × 10^5 cells in a 60 mm dish and grown with 8% FBS-EMEM at 37°C for 3 days. The OSCN’ concentration in the original OSCN’ solution was 900 ± 200 μmol/l, which was the highest concentration that could be produced in our LPO system. The original OSCN’ solution or the reaction solution of the LPO system as a control was mixed with an equal volume of virus solution (IFV A/PR/8/34 at 200 PFU/0.2 ml) as described before. The concentration of OSCN’ in the mixture prepared was 450 μmol/l. Immediately after mixing, 0.2 ml of the mixture (100 PFU/0.2 ml) was added to MDCK cells grown in 60 mm plastic dishes and virus was adsorbed to MDCK cells at room temperature for 1 h as described before. The cells were rinsed twice with PBS and treated with trypsin, and then the number of viable cells was examined by the trypan blue exclusion test. The cytotoxicity of OSCN’ was determined from the ratio of the number of viable cells to total cells.

**Hemagglutination assay and hemagglutination inhibition assay (HAI).** The effect of OSCN’ on virus-induced hemagglutinin activity was evaluated. Viral stock solutions of IFV A/PR/8/34, A/Fukushima/13/43, B/Singapore/222/97, and B/Fukushima/15/93 were serially diluted 2-fold with saline in 96-well V-bottom plates. Each diluted viral solution was mixed with an equal volume of a 1% suspension of guinea pig red blood cells (RBC) and incubated at 4°C. After 60 min, hemagglutination titers were determined. The hemagglutination titer of the original IFV stock solution of A/PR/8/34 was 256 and those of A/Fukushima/13/43, B/Singapore/222/97, and B/Fukushima/15/93 were 512. For the HAI, four hemagglutinin viral solutions of the four viral strains were prepared. The HAI was performed in 96-well V-bottom plates as described previously (Reading et al., 2008; Chan et al., 2011). Briefly, serial 2-fold dilutions of the original OSCN’ solution or the reaction solution of the LPO system (as a control) was mixed with each of four hemagglutinin viral solutions and incubated at room temperature for 30 min. Then, a 1% guinea pig RBC suspension was added to the mixtures and incubated at 4°C for 60 min. Finally, hemagglutination inhibition titers in the presence or absence of OSCN’ were compared.

**Statistical analysis.** All data are expressed as means ± standard deviation (SD). Differences between or among the individual groups were statistically evaluated by the Mann-Whitney test or ANOVA with the Kruskal-Wallis test, respectively, using GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA, USA). A P-value below 0.05 was considered to indicate statistical significance (n indicates the number of samples).

**Results**

**Antiviral activity of OSCN’ against IFV type A and B**

The antiviral activity of OSCN’ against IFV A/PR/8/34, A/Fukushima/13/43, B/Singapore/222/97, and B/Fukushima/15/93 was examined by a plaque reduction assay using MDCK cells. The OSCN’ produced by H_2O_2 was stable in the mixture of original OSCN’ solution and PBS containing 1% BSA used as the dilution buffer of virus stock solution at least up to 90 min after their mixing as shown in Fig. 1. IFV was exposed to various concentrations (less than 450 μmol/l) of OSCN’ prepared from the original OSCN’ solution (900 ± 200 μmol/l) at room temperature for 0, 15, and 30 min and then adsorbed to MDCK cells in the presence of OSCN’ for 1 h. Fig. 2 shows a representative test of plaque formation of IFV A/Fukushima/13/43 exposed to OSCN’ for 30 min. OSCN’ dose-dependently decreased the number of plaques. At concentrations of OSCN’ higher than 196 μmol/l, the plaque formation was inhibited by more than 98%. Table 1 shows the plaque formation of IFV types A and B strains exposed to OSCN’ before and during viral adsorption. The EC_{50} values of OSCN’ for A/PR/8/34, B/Singapore/222/97, and B/Fukushima/15/93 were not significantly affected by the exposure times of 0, 15, and 30 min before viral adsorption. The EC_{50} values at 0 and 30 min of the four IFV strains were similar, and the differences were not significant. At 15 min, there were no significant differences in the EC_{50} values for A/PR/8/34, B/Singapore/222/97, and B/Fuku-
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Shima/15/93, although the EC_{50} value (148 ± 27 μmol/l) for A/Fukushima/13/43 was significantly higher than that for B/Fukushima/15/93 but not those for A/PR/8/34 and B/Singapore/222/97. Antiviral activities of OSCN against IFV types A and B were not markedly different. For A/Fukushima/13/43, the EC_{50} value (73 ± 1 μmol/l) of OSCN at 30 min was significantly lower than that at 15 min but not at 0 min. For all four IFV examined, the antiviral activity of OSCN was likely to be independent of its exposure time to IFV before viral adsorption. Thus, OSCN exhibited similar antiviral activity against IFV types A and B, and the effect of OSCN on viral particles was suggested to occur shortly before viral adsorption.

Effect of OSCN treatment of MDCK cells before and after viral adsorption

Because the antiviral activities of OSCN against IFV types A and B were similar, we used the A/PR/8/34 strain as a representative of IFV types A and B to evaluate the effects of OSCN exposure on MDCK cells before and after viral adsorption. In both cases, MDCK cells were exposed to OSCN for 1 h. As shown in Fig. 3, the exposure to OSCN at 450 μmol/l before viral adsorption did not affect the plaque-forming ability of the A/PR/8/34 strain, but the exposure to OSCN at 450 μmol/l after viral adsorption significantly reduced the plaque-forming ability. The EC_{50} values of OSCN for MDCK exposure before and after viral adsorption were more than 450 μmol/l and 380 ± 40 μmol/l, respectively, and significantly higher than EC_{50} values in the case of exposure to OSCN before and during viral adsorption, as shown in Table 1 (0.078 ± 0.014, 0.083 ± 0.001, and 97 ± 13 μmol/l). Exposure of MDCK cells to OSCN before viral adsorption did not affect the antiviral activity of OSCN but did moderately after viral adsorption.

Cytotoxicity of OSCN to MDCK cells

The cytotoxicity of OSCN was examined to characterize its potential antiviral activity, as shown in Table 1. MDCK cells were exposed to OSCN for 1 h, corresponding to the time of viral adsorption in the plaque reduction assay,
and the ratios of viable cells in the presence and absence of OSCN were compared. The OSCN concentration of 450 μmol/l was prepared from the highest concentration (900 ± 200 μmol/l) that we could produce in the LPO system and was much higher than the EC₅₀ values shown in Table 1. As shown in Fig. 4, there was no significant difference in the ratios of viable cells in the presence and absence of OSCN at 450 μmol/l. Therefore, OSCN is potentially not cytotoxic in vitro.

Effect of OSCN on hemagglutinin activity

The effect of OSCN on hemagglutinin activity of IFV was examined to characterize the inhibitory effect of OSCN on the adsorption of IFV to MDCK cells. As shown in Fig. 5b, hemagglutinin activities of IFV [A/PR/8/34 (H1N1), A/Fukushima/13/43 (H3N2), B/Singapore/222/97, and B/Fukushima/15/93] at four hemagglutinin units were not inhibited in the reaction solution used as a control. As shown in Fig. 5a, hemagglutinin activities of the four IFVs were not inhibited in the presence of OSCN either, even at the highest

<table>
<thead>
<tr>
<th>Exposure time (min)</th>
<th>EC₅₀ (µmol/l) A/PR/8/34</th>
<th>EC₅₀ (µmol/l) A/Fukushima/13/43</th>
<th>EC₅₀ (µmol/l) B/Singapore/222/97</th>
<th>EC₅₀ (µmol/l) B/Fukushima/15/93</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>97 ± 13</td>
<td>111 ± 37</td>
<td>133 ± 25</td>
<td>88 ± 5</td>
</tr>
<tr>
<td>15</td>
<td>78 ± 14</td>
<td>148 ± 27</td>
<td>84 ± 12</td>
<td>57 ± 16*</td>
</tr>
<tr>
<td>30</td>
<td>83 ± 1</td>
<td>73 ± 1*</td>
<td>70 ± 15</td>
<td>76 ± 19</td>
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EC₅₀, effective concentrations for 50% plaque reduction. *P <0.05 vs A/Fukushima/13/43 at 15 min among the groups after 15 min exposure of IFV to OSCN or among three different times of OSCN exposure of A/Fukushima/13/43 by the Kruskal-Wallis test (3 independent experiments). Data are shown as means ± SD.

Effect of OSCN on hemagglutinin activity

The effect of OSCN on hemagglutinin activity of IFV was evaluated by HAI. (a) OSCN (900 μmol/l) or (b) reaction solution (as a control) was serially diluted 2-fold and added to 96-well plates. Four hemagglutinin units of each IFV strain [A/PR/8/34 (H1N1), A/Fukushima/13/43 (H3N2), B/Singapore/222/97, and B/Fukushima/15/93] were added to the 96-well plates. Then, 1% guinea pig RBC suspension was added to the mixtures as described in the Material and Methods.
concentration (450 μmol/l) of OSCN which exhibited anti-
IFV activity. OSCN concentrations up to 450 μmol/l had no
effect on hemagglutinin activity of the four IFVs examined.

**Discussion**

In the present study, we showed that OSCN exhibits anti-
viral activity against not only IFV type A but also type B. The
exposure of MDCK cells to OSCN before IFV adsorption
had no effect on the viral growth, and the exposure to virus
particles did not affect hemagglutinin activity of IFV either.
However, the exposure of cells following virus adsorption
to OSCN moderately affected the viral growth, and the ex-
posure of viral particles to OSCN before viral adsorption to
host cells was significantly effective in inhibiting viral growth.
Thus, the attachment of OSCN to the components of the
viral envelope without interfering with viral adsorption to
host cells was suggested to contribute critically to the mode
of antiviral action of OSCN.

The LPO system converts SCN to OSCN in the pres-
ence of LPO and H$_2$O$_2$, and OSCN acts as the host defense
against infection. Shin et al. (2008) reported that the forma-
tion of OSCN in saliva shows bactericidal activity against
oral bacteria and contributes to a reduction of breath odor.
OSCN inhibits a wide variety of viruses as well as bacteria
(Pourtois et al., 1991; Mikola et al., 1995; Shin et al., 2002).
In addition, recent studies suggested that OSCN inactivates
pandemic IFV (A/H1N1/2009) and IFV A/H1N2/2009 in
vitro (Cegolon et al., 2014; Gingerich et al., 2016). Our results
confirmed the inhibitory effect for IFV type A. However, the
effect of OSCN against type B IFV has been unknown.
The present study found that OSCN exhibited antiviral activity
against both types A and B IFV in vitro. This is the first study
demonstrating the antiviral activity of OSCN against both
IFV types A (H3N2 subtype) and B. In this study, the EC$_{50}$
values of OSCN for four IFV strains were determined by
plaque reduction assay and 57 to 148 μmol/l. These EC$_{50}$ val-
ues were higher than the value (2 μmol/l) for A/H1N1 2009
pandemic IFV determined by a specific reverse transcriptase
real-time-PCR, which was reported by Cegolon et al. (2014).
The plaque reduction assay is quantitatively much better way
than real-time-PCR to assess the number of infective viral
particles. Thus, our results would be useful and valuable to
evaluate anti-influenza virus activity in vivo, especially in
saliva containing OSCN. Antiviral activity of OSCN against
IFV was independent of the exposure time of IFV to OSCN in
the plaque reduction assay. It is probable that OSCN reacted
quickly with some component of the influenza viral envelope.

OSCN is the final product of the LPO system and is
normally present in the human body with SCN (Pruitt
et al., 1982). OSCN is efficiently generated from SCN by
peroxidase in the presence of H$_2$O$_2$ in vivo because H$_2$O$_2$ is
contained in and/or generated from various kinds of foods
and additives (Tenovuo et al., 1985; Akagawa et al., 2003).
Shin et al. (2005) have shown that oral administration of
LPO attenuated pneumonia in a murine IFV infection model.
Thus, OSCN produced by LPO in vivo might contribute to
the alleviation of pneumonia. Previous reports showed that
SCN and OSCN were detected in whole saliva at concentra-
tions of 0.5–4 mmol/l and 10–212 μmol/l, respectively
(Tenovuo and Makinen, 1976; Tenovuo et al., 1981; Thomas
et al., 1980; Pruitt et al., 1983). The EC$_{50}$ values of OSCN for
the IFV types A and B examined in this study were from 57
to 148 μmol/l. Actually, our results are within the range of
OSCN in saliva supplemented with H$_2$O$_2$ and SCN (Ten-
ovuo et al., 1981). No cytotoxic effect was observed at con-
centrations higher than the EC$_{50}$ values. Thus, OSCN may be
an anti-IFV component in vivo, and the sufficient generation
of OSCN in vivo probably contributes to the prophylactic
management of IFV infection.

When MDCK cells were exposed to OSCN before IFV
A/PR/8/34 adsorption, the plaque-forming ability of IFV
was not affected. This indicates that exposure of MDCK
cells to OSCN did not inhibit the viral attachment to the
cells and/or the growth in the cells. On the other hand, the
exposure of virus-adsorbed MDCK cells to OSCN reduced
the plaque-forming ability of IFV A/PR/8/34 by more than
50%, when the highest concentration (450 μmol/l) of
OSCN that could be produced by the LPO system was
used. It is possible that the exposure to OSCN interfered
with viral entry into MDCK cells after the attachment and/
or the stages of viral replication after the entry. However,
the EC$_{50}$ value (380 ± 40 μmol/l) for OSCN exposure after
viral attachment to MDCK cells was much higher than the
value (78–97 μmol/l) for A/PR/8/34 in the plaque reduction
assay. In the plaque reduction assay, IFV particles were first
adsorbed to MDCK cells in the presence of OSCN. Thus, the
exposure of viral particles to OSCN was a critical step in
the plaque formation, and some component of the viral envelope
associated with OSCN might largely have contributed to the
reduction of plaque formation.

Virus adsorption to cells is an initial step in IFV infection
and is performed by the binding between hemagglutinin of
IFV and sialic acid on the cell surface (Suzuki et al., 1986;
Skehel and Wiley, 2000). Exposure to OSCN did not affect
the hemagglutinin activity of IFV. OSCN was indicated not
to interfere with the binding of hemagglutinin molecules
and sialic acid. Because the exposure of MDCK cells to
OSCN showed no effect on plaque formation, OSCN did
not interfere with the binding of hemagglutinin molecules
and sialic acid to the surface of host cells. Therefore, it was
suggested that OSCN associated with some component on
the viral envelope without interfering with viral adsorption
to host cells and reduced the plaque formation of IFV.
In conclusion, our results showed that OSCN produced by an LPO system possesses antiviral activity against both IFV types A and B. The mode of the antiviral action of OSCN was suggested to be based on the association of OSCN with some component on the viral envelope without interfering with viral adsorption to host cells.

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