Disodium cromoglycate (DSCG) is one of the safest drugs for the prevention of bronchial asthma and allergic rhinitis attacks. The effect of DSCG on acute upper respiratory tract viral infection is still controversial. Here we investigated DSCG inhibition of influenza virus infection in vitro and in vivo. In vivo effects of DSCG on viral infection were assessed using a murine model of respiratory tract infection. Intranasal administration of DSCG protected mice from death induced by infection with influenza virus A/PR/8/34. We analyzed DSCG anti-viral effects in vitro by either (i) treating cells prior to viral adsorption, (ii) treating cells concurrently with viral adsorption, or (iii) treating cells after viral adsorption. DSCG treatment of cells during or after, but not before, viral adsorption significantly inhibited influenza viral infection, indicating DSCG acts on events late in viral infection. DSCG exerts anti-influenza effect both in vitro and in vivo at the doses compatible with treatment for asthma. DSCG marginally inhibited influenza viral neuraminidase and membrane fusion functions, suggesting that DSCG inhibition of viral neuraminidase and fusion activities may partially mediate this anti-influenza effect. Our results indicate that treatment of patients including children with DSCG may take advantages for prevention from influenza virus infection.

**Key words** sodium cromoglycate; influenza virus infection; anti-influenza drug

Disodium cromoglycate (DSCG) is a safe and widely used drug for the prevention of bronchial asthma and allergic rhinitis attacks.1—3) DSCG inhibits the release of chemical mediators, including histamine and tumor necrosis factor, from mast cells.4) While DSCG is known to induce intracellular signaling events associated with physiological and pathological processes, the molecular mechanisms of DSCG-induced signaling have remained unclear.5,6) Recent evidence, however, also demonstrates that DSCG has an antiviral effect on upper respiratory tract infection. In in vivo studies, DSCG significantly reduced expression of ICAM-1, a receptor for rhinoviruses.7,8) This drug also inhibits the cytopathic effects induced by certain viruses in vitro.9) The mechanisms of DSCG viral inhibition have not been elucidated. Inhalation or intranasal administration of DSCG was effective for the treatment of acute upper respiratory tract infection in children.10) Additional studies, however, have suggested that DSCG is not effective against viral infection either in vitro or in vivo.11) A recent randomized trial examining the effect of DSCG on acute upper respiratory tract viral infection showed that intranasal administration of DSCG was not effective against viral infection.12) Thus, the effect of DSCG on acute upper respiratory tract viral infections remains controversial.

The chemical structure of DSCG is similar to flavonoids and related derivatives of cromolyn compounds (Fig. 1). Several flavonoids demonstrate anti-influenza effects and inhibition of influenza virus neuraminidas.13) In this study, we investigated the effects of DSCG on viral infection, particularly on human influenza virus infection in vivo. We determined that DSCG exerts anti-influenza virus effects both in vitro and in vivo. These findings indicate that DSCG may be an effective agent for prevention of human including children from influenza virus infection.

**MATERIALS AND METHODS**

**Reagents** Disodium cromoglycate (DSCG) (Intal; disodium 5,5’-(2-hydroxytrimethenedioxy)bis 4-oxo-4H-1-benzoypyan-2-carboxylate) was obtained from Fujisawa Pharmaceutical Co., Ltd. Zanamivir (Relenza; 5-acetylamino-2,6-anhydro-4-guanidino-3,4,5-trideoxy-D-glycerol-D-galactonon-2-enic acid) was synthesized by Fujisawa Pharmaceutical Co., Ltd. Ribavirin (Virazid; 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamid) was supplied by Viratek (Costa Mesa, CA, U.S.A.). All chemicals were of the highest purity available.

**Animals** BALB/c mice were purchased from Charles River Japan, Inc.

**Viruses** The human influenza virus strains used in this study, A/PR/8/34 (H1N1), A/Memphis/1/71 (H3N2), and B/Lee/40, were propagated and purified as described previously.14,15)

**Influenza Virus Infection in Mice** Mouse-adapted in---
fluenza A/PR/8/34 virus was used in this study to evaluate drug anti-viral effects. Briefly, female BALB/c mice (four weeks old, 15—17 g) were anesthetized with halothane and inoculated intranasally with 20 μl of viral suspension (2.0×10^5 PFU/mouse). Four hours after infection, mice were again anesthetized, then intranasally administered 20 μl of a test compound dissolved in H2O. Mice were then treated twice daily for days 1—4. Control mice were treated intranasally with 20 μl vehicle (H2O) alone. Mice were observed for 14 d after infection. Anti-influenza agent efficacy was assessed by lethality at the 50% effective dose (ED50) of virus, calculated by the survival rate at 9 d after infection. Protection against weight loss during the experimental period was also assessed.

**Cell Culture and Viral Inoculation** Madin–Darby canine kidney (MDCK) cells were seeded at 4×10^5 cells/well in 6-well plates and cultured in MEM supplemented with 10% fetal bovine serum at 37 °C. After three washes in serum-free MEM, cells were inoculated with the virus at 80—100 plaque-forming units (pfu). After an additional incubation for 1 h at 34.5 °C, we assayed antiviral activity. Treatment with inhibitors was evaluated using three different protocols (Fig. 2).

**Plaque Assay** The assay measuring human influenza virus infection to MDCK cells was performed as previously described.10 Viral infection was quantitated by counting the plaques formed on the infected cells. Briefly, after viral inoculation, cells were washed twice in serum-free MEM. The cells were then overlaid with serum-free MEM containing 0.8% agarose, 8.3 mg/ml bovine serum albumin, and 10 μg/ml trypsin. Cells were cultured further for 48 h at 34.5 °C in the presence or absence of test agents. The cells were then fixed for 1 h with ethanol/acetic acid (5 : 1, by vol.). Fixed cells were stained with 0.5% amide black 10B solution. Each experiment was performed in triplicate of wells. Significant analysis of means of plaque numbers between control and treatment was performed by t-test.

**Neuraminidase Assay** A fluorometric assay was used to determine influenza virus neuraminidase activity.17 Viral suspensions (each 28 of HA units) were preincubated for 30 min at 4 °C with inhibitory agents diluted at the indicated concentrations. The enzymatic reaction was initiated by addition of the substrate, 2′-((4-methylumbelliferyl)-α-D-N-acetylneuraminic acid. Following incubation of reaction mixtures for 30 min at 37 °C, the reaction was terminated by addition of 500 mM carbonate buffer (pH 10.7). The fluorescence resulting from substrate cleavage by the viral neuraminidase was measured at 355 nm (excitation) and 460 nm (emission). Agent concentrations causing 50% inhibition (IC50) were calculated by plotting the percent inhibition of neuraminidase activity against the agent concentration.

**Hemagglutination Inhibition (HAI) Assay** Hemagglutination inhibition (HAI) assay was carried out as described previously.18 Briefly, virus suspension (22 of HA units) was incubated for 1 h at 4 °C with agents serially diluted two-fold with 0.01% gelatin-containing phosphate-buffered saline in 96-well microtiter plates. After 0.5% (v/v) of guinea pig erythrocytes had been added to each well, the plates were kept for 1 h at 4 °C. The maximum dilution of the agents showing complete inhibition of hemagglutination was defined as the titer of hemagglutinin inhibition.

**Hemolysis Inhibition Assay** Hemolysis inhibition assay was carried out as described previously.18—20 Briefly, influenza viruses (28 HAU) were preincubated for 1 h at 4 °C with DSCG in 50 μl of saline. The mixtures were then reacted for 30 min at 4 °C with 0.5 ml of saline containing 2.5% guinea pig erythrocytes. Erythrocytes were sedimented and resuspended in 0.5 ml of 20 mM acetate-buffered saline at the optimum pH for hemolysis mediated by each virus. The hemolysis was carried out for 30 min at 37 °C and terminated by addition of an equal volume of phosphate-buffered saline. The mixtures were centrifuged, and the concentrations of hemoglobin in the supernatants were determined by measuring the absorbance at 492 nm.

**RESULTS**

We examined the protective effect of DSCG in a murine model of respiratory tract viral infection with influenza A/PR/8/34 virus. Zanamivir and Rivabirin were used as control reagents with a known activity against influenza infection. These compounds are active intratracheally in animal models following intranasal administration, the same route of administration used clinically in DSCG treatment of asthma. The anti-influenza agent Zanamivir, administered at 10 mg/kg twice a day, reduced mortality even after delay of

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**Fig. 2. Diagrams of Infection Experiments in Vitro in This Study**

For treatment of cells before viral adsorption, cells were cultured for 20 to 24 h at 37 °C in the presence or absence of inhibitor. For treatment of cells during viral adsorption, viral solutions were first preincubated for 30 min at room temperature with the indicated inhibitor concentrations. Cells were then inoculated with the virus-inhibitor mixtures. For treatment of cells after viral adsorption, cells were first inoculated with the virus, then treated with or without the indicated concentrations of inhibitor.
treatment to 4 h postinfection (Fig. 3). The anti-respiratory syncytial virus agent Rivabirin, administered at a dose of 32 mg/kg, also showed activity in this model prolonging the survival of infected mice. DSCG, a potent drug used to prevent bronchial asthma attacks, also provided protection against lethal infection with influenza A/PR/8/34 (Fig. 3). The $ED_{50}$ of DSCG at nine days after infection was 15.5 mg/kg. In mice treated with DSCG at a dose of 32 mg/kg, we also observed an improvement in the weight gain of mice surviving to day 9 post-infection. Thus, DSCG showed a significant protective effect in this murine model of influenza virus infection. The protective effect of this drug is comparable to that of Ribavirin and 14-times less potent than that of Zanamivir (Table 1). Drug-related toxicity was not detected in any of the groups examined.

To determine the inhibitory effects of DSCG on influenza virus infection of cells, we treated MDCK cells with DSCG at different points in viral infection (Fig. 2). An anti-viral effect following the pretreatment of cells with drug would demonstrate that DSCG has direct action on the host cells (before viral adsorption), possibly through signaling events induced by DSCG producing a heightened cellular resistance to infection. Pretreatment of cells with DSCG for 20 h (before viral adsorption), however, did not inhibit the infection of A/PR/8/34 (Fig. 4). The result suggests that putative signals evoked by DSCG treatment do not mediate the anti-influenza effect. Inhibition upon concurrent viral inoculation and administration of test agents would determine that DSCG acts directly on cells in the early stages of viral infection, perhaps affecting adsorption, endocytosis, or membrane fusion (during viral adsorption). The agent demonstrated only marginal protection from infection with the virus tested in a dose-dependent manner. DSCG at a concentration of 10 mM suppressed viral infection by 25% of the levels seen in untreated cells (Fig. 4B). The size of plaques under this condition was not affected by the treatment with DSCG (Fig. 4A). Virally-infected cells were also cultured in medium con-

![Fig. 3. Protection of Mice by Intranasal Administration of DSCG from Death Induced by the Infection of Influenza A/PR/8/34](image)

Comparative studies were tested using anti-viral compounds. Open circle, 32 mg/kg DSCG; open square, 10 mg/kg Zanamivir; open triangle, 32 mg/kg Ribavirin; closed circle, vehicle ($n=8$).

![Fig. 4. DSCG Inhibits Plaque Formation by the Infection of Influenza A/PR/8/34](image)

Plaque assays were performed as in Materials and Methods. For treatment of cells before viral adsorption, the cells were cultured for 20 to 24 h at 37°C in the presence or absence of the inhibitors. Following extensive washing, the cells were inoculated with the virus for 1 h. For treatment of cells during viral adsorption, viral solutions were preincubated for 30 min at room temperature with the indicated concentrations of inhibitors. The cells were then inoculated with the preincubated virus-inhibitor mixture for 1 h. Following washing, cells were then treated with or without the indicated concentrations of the inhibitors. Panel A (left) shows plaques on plates treated with or without DSCG. The figure shows one representative result of two independent experiments. Before, application of DSCG before viral adsorption; During, application of DSCG during viral adsorption; After, application of DSCG after viral adsorption. Graph B (right), summary of the number of plaques on plates treated with DSCG at the indicated concentrations. The graph shows one representative result of two independent experiments. Values are indicated as means of relative ratios of viral infection in treated to untreated cells. Bars indicate standard deviations. Closed circle, DSCG treatment of cells before viral adsorption; closed square, DSCG treatment of cells during viral adsorption; closed triangle, DSCG treatment of cells after viral adsorption. Significant differences from viral infection without agents are indicated by * $p<0.001$; ** $p<0.01$.

### Table 1. Intranasal Administration of DSCG Protects Mice against a Lethal Challenge with Mouse-Adapted Influenza Virus A/PR/8/34 (H1N1)

<table>
<thead>
<tr>
<th>Tested compound $a$</th>
<th>$ED_{50}$ (mg/kg) $b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSCG</td>
<td>15.5</td>
</tr>
<tr>
<td>Zanamivir</td>
<td>1.16</td>
</tr>
<tr>
<td>Ribavirin</td>
<td>16.2</td>
</tr>
</tbody>
</table>

$ a$ All tested compounds were dissolved in sterilized water and were administrated intranasally with 20 μl at 4 h after infection and then twice daily on days 1—4 after infection.  
$ b$ $ED_{50}$ values were calculated at day 9 post-infection.
The chemical structure of DSCG is analogous to that of flavonoid (Fig. 1), suggesting that this agent may function in a similar manner to inhibit influenza infection. To clarify the mechanisms by which DSCG inhibits viral infection, we investigated its effects on viral neuraminidase activity. DSCG exhibited slight inhibitory activity against the neuraminidase activity of both influenza A and B viruses, A/PR/8/34 (H1N1), A/Memphis/1/71 (H3N2) and B/Lee/40 at fifty percent inhibitory concentrations of DSCG, 3.5, 5.0 and 5.6 mM, respectively. Zanamivir, as a positive control, exhibited strong inhibition of viral neuraminidases as described previously [21]. Fifty percent inhibitory concentrations of zanamivir were 2.5, 10 and 170 nM against A/PR/8/34 (H1N1), A/Memphis/1/71 (H3N2) and B/Lee/40, respectively.

Next, we examined the effect of DSCG on virus-mediated hemagglutination. Erythrocyte hemagglutination is mediated by the interaction of viral hemagglutinin with sialic acid-containing glycoconjugates on the surfaces of erythrocytes. Following the incubation of erythrocytes with influenza virus pretreated with 10 mM DSCG, no inhibition of virus-mediated hemagglutination was observed (Fig. 5). Fetuin-treated viruses, however, demonstrated a significant reduction in erythrocyte hemagglutination. This result strongly suggests that DSCG does not interfere with the attachment of virions to host cell surfaces.

We also determined the inhibitory effect of DSCG on virus-mediated membrane fusion by hemolysis inhibition. Viral hemagglutinin (HA) is proteolytically cleaved into HA1 and HA2 subunits; under acidic conditions, the cleaved HA2 subunit mediates membrane fusion between host cells and viruses. To determine if DSCG affects membrane fusion mediated by viral HA, we investigated the inhibitory effect of DSCG on erythrocyte lysis (hemolysis) induced by viruses. First, the optimum pH for hemolysis induced by each virus was determined. A/PR/8/34 (H1N1), A/Memphis/1/71 (H3N2), and B/Lee/40 strains exhibit maximal hemolytic activity at pH 5.2, 5.0, and 5.4, respectively. At 10 mM, DSCG inhibited influenza-mediated hemolysis at most by 20% of control levels. The inhibition of hemolysis was weak, correlating well with the inhibition of viral infection seen when administered concurrently, indicating that the major anti-viral activity of DSCG occurs at a later stage in infection.

DISCUSSION

Previously, studies have shown that viral inoculation in the presence of DSCG or administration of DSCG within 24 h of viral infection effectively inhibits viral infection in vitro and in vivo. In this study, we have tried to determine the mechanism of action governing the inhibitory effects of DSCG on influenza virus infection in vitro and in vivo. DSCG, when administered after, but not before, influenza infection significantly reduced viral infectivity of MDCK cells. Also, in vivo experiments using a murine model of respiratory tract infection demonstrated that serial administration of DSCG to mice infected with influenza virus protected animals from viral-induced death. In previous studies demonstrated that DSCG had no effect on upper respiratory tract infection, including influenza, the cells were pretreated with DSCG followed by viral inoculation in the absence of
DSCG.\textsuperscript{11} In our experiments, pretreatment of cells with DSCG had little effect on the influenza infectivity of MDCK cells. These findings likely exclude the possibility that intracellular signaling events evoked by DSCG affects the infectivity of influenza viruses \textit{in vitro} and \textit{in vivo}. We instead examined DSCG inhibition of influenza virus interactions with host cell surface, such as budding. The function of influenza viral neuraminidases is indispensable for budding of progeny viruses from host cells.\textsuperscript{21} Several inhibitors of neuraminidases have been developed and are currently used clinically.\textsuperscript{21—23} We tested the ability of DSCG to inhibit the neuraminidase activities of influenza viruses. Although DSCG inhibited influenza viral neuraminidase, its activity against viral neuraminidases was much lower than the known neuraminidase inhibitor, zanamivir and seemed not to be enough to exert anti-influenza activity \textit{in vivo} (Table 3). Thus, the inhibition of neuraminidases by DSCG may partially contribute to the inhibition of viral infection. There may exist other unknown actions of the agent related with late events of influenza viral infection. To exclude the possibility that DSCG affects the adsorption of influenza viruses to host cell surfaces, we examined hemagglutination induced by influenza viruses. DSCG had no inhibitory effect on hemagglutination, indicating that DSCG does not interfere with the interaction of influenza viruses and host cell surface receptors. Hemagglutinin, a viral glycoprotein, mediates membrane fusion and facilitates the interactions between viruses and host cells. We also examined the effect of DSCG on membrane fusion, an essential event for influenza virus infection.\textsuperscript{30} As the agent marginally inhibited fusion activity, the inhibitory activity of DSCG was in good agreement with data obtained from the inhibition of MDCK cell infections. Thus, DSCG may exhibit a combination of effects, including an inhibition of viral neuraminidase activities and inhibition of membrane fusion, partly contributing to a significant anti-influenza effect \textit{in vivo}. In a recent study refuting that DSCG is clinically useful for the treatment of patients with acute viral upper respiratory tract infections, DSCG was intranasally administered to patients suspected to be infected with viruses for longer than 24 h.\textsuperscript{12} Two possibilities might explain these discrepancies. First, acute upper respiratory tract infections in that study may have included multiple viruses, not only influenza, but also other infectious diseases such as parainfluenza viruses and adenoviruses. DSCG may have an effect on influenza, but not on other viral infections. Also, the anti-viral effect of DSCG may act within the first 24 h of influenza-viral infection, perhaps a crucial exposure period missed in this study. As recent advances in diagnostic methods have enabled doctors to distinguish infection with influenza viruses from infections with other viruses, further clinical trials evaluating the effect of DSCG on influenza will be required. Up to date, neuraminidase inhibitors have successfully used for treatment of influenza patients. Although the effective doses of DSCG on anti-influenza activity \textit{in vivo} and \textit{in vitro} clarified in this study have been apparently higher than those of neuraminidase inhibitors, they are still very comparable for clinical administration to asthma and allergic rhinitis attacks. As DSCG has been clinically used as one of the safest drugs for administration to human including children for a long time, the anti-influenza virus effect elucidated in this study will enable physicians to take into consideration to apply the agent for prevention of human from influenza.

In conclusion, DSCG exerts an anti-influenza virus effect both \textit{in vitro} and \textit{in vivo}, exhibiting a significant anti-viral effect in an \textit{in vivo} animal model of influenza infection. These observations strongly encourage the potential use of DSCG as an anti-influenza virus chemotherapeutic agent.

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