The Inhibitory Effect of Disodium Cromoglycate on the Growth of Chlamydia pneumoniae in Vitro

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Chlamydia pneumoniae is associated with asthma and several other respiratory illnesses. Disodium cromoglycate (DSCG) is known to inhibit both immediate and late asthmatic responses. In this study, the inhibitory effect of DSCG on the growth of C. pneumoniae was examined by minimum inhibitory concentration (MIC) and pre-inoculation minimal cidal concentration (MCC) assays using HL cells and C. pneumoniae AR-39. DSCG below the clinically relevant concentration inhibited the growth of C. pneumoniae in a dose-dependent manner in both the MCC and MIC assays. The inhibitory effect was also time-dependent in the MCC assay at 20 mg/ml of DSCG. These results warrant further clinical study on the connection between C. pneumoniae infections and use of DSCG.

Key words Chlamydia pneumoniae; disodium cromoglycate; asthma; minimum inhibitory concentration; pre-inoculation minimal cidal concentration

Chlamydia pneumoniae (C. pneumoniae) is a pathogen associated with respiratory illness such as pneumonia,1,2 bronchitis,3 asthma,4–6 chronic obstructive pulmonary disease1,2,4,5 and pharyngitis.1,2 Antimicrobials such as tetracyclines, macrolides and fluoroquinolones are effective against C. pneumoniae infections.1,2,4,5 However, C. pneumoniae is a ubiquitous pathogen and eradication of this organism is difficult.5 There is no established strategy for the treatment of chronic infections with C. pneumoniae. Disodium cromoglycate (DSCG) inhibits the release of chemical mediators and has been used for the prevention of allergic diseases such as asthma.6,7 The aim of this study was to clarify whether DSCG inhibits the growth of C. pneumoniae in vitro.

MATERIALS AND METHODS

C. pneumoniae AR-39, and the human line (HL) cells were used in this study. The chlamydia strain was propagated in HL cells, purified on a renografin density gradient and stored at –80 °C in sucrose phosphate glutamate solution (SPG) until use. DSCG was kindly provided by Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan). Minimum inhibitory concentration (MIC) and pre-inoculation minimal cidal concentration (MCC) assays were used to determine the susceptibility of C. pneumoniae to DSCG.

One milliliter of culture medium (containing 2×10⁵ HL cells) was dispensed into each well of a plastic 24-well culture plate and incubated in 5% CO₂ at 37 °C for 24 h to form a confluent monolayer. Then, 1×10⁴ inclusion-forming units (IFU) of C. pneumoniae were inoculated onto a monolayer of HL cells. After centrifugation onto the cells at 1500 rpm for 60 min, the inoculum was removed and 1 ml of the culture medium (Eagle’s Minimum Essential Medium [MEM] plus 10% fetal calf serum and 0.6 µg/ml of cycloheximide) containing serially diluted DSCG was added. Cells were incubated in 5% CO₂ at 35 °C for 72 h. Cultures were then fixed with methanol and stained with the Chlamydia genus-specific FITC-conjugated monoclonal antibody (Denka Seiken Co., Ltd., Tokyo, Japan). Inclusions were counted by fluorescence microscopy and the condition of the cells was noted. The MIC was defined as the lowest concentration at which no inclusions were found.

We also tested whether DSCG had any effect on HL cells before infection with C. pneumoniae. A 1 ml aliquot of DSCG (20 mg/ml) was added to HL cells and incubated at 35 °C for 60 min. After removing DSCG, the HL cells were then infected with C. pneumoniae as described earlier.

The pre-inoculation MCC assay was performed as described previously.8 Briefly, the 1.0×10⁴ IFU of C. pneumoniae strain was incubated with serial dilutions of DSCG at 35 °C for 30, 60, 120, and 180 min. Controls were incubated with SPG buffer containing no DSCG. The pre-treated mixture was inoculated and centrifuged onto the HL cells as described for the MIC method. After the inoculum was removed, culture medium without DSCG was added and incubated for 72 h. The pre-inoculation MCC was defined as the lowest concentration of DSCG at which no inclusions were found. At least three wells per dilution were tested and each experiment was repeated at least three times.

The toxic effect of DSCG on HL cells was evaluated by a CK01 cell counting kit (Dojindo Laboratory Co., Ltd., Kumamoto, Japan), incorporating a colorimetric assay for cell proliferation and viability. Serial dilutions of DSCG were dispensed onto microtiter plates containing HL cells and incubated at 37 °C for 72 h. After removal of the DSCG solution, cell activity was determined by monitoring the optical density. The culture medium without DSCG was dispensed into a microtiter plate and used as a control. The condition of the monolayer cells was also assessed by microscopy (×400 magnification).

RESULTS

The results of MIC and MCC assays are shown in Table 1. The number of C. pneumoniae inclusions in the MIC assay

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at of DSCG. Although no decrease in cell activity was observed Fig. 1. Cell activity decreased with increasing concentrations (data not shown).

20 mg/ml of DSCG for 60 min prior to infection with significantly when HL cells were pre-incubated with 20 mg/

20 mg/ml of DSCG. After pre-incubation for 30 min with

in the MCC assay decreased with increasing concentrations of DSCG. The inhibitory effect was also time-dependent at concentration of 20 mg/ml DSCG in the MCC method, the inhibitory effect was both dose- and time-dependent. The precise mechanism of the inhibitory effect of DSCG on C. pneumoniae requires further investigation.

In conclusion, DSCG at concentrations below the clinically relevant range inhibited the growth of C. pneumoniae dose-dependent manner. The inhibitory effect was also time-dependent at concentration of 20 mg/ml DSCG in the MCC assay. These results warrant further clinical study on C. pneumoniae infections and use of DSCG.

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Table 1. Inhibitory Effect of Disodium Cromoglycate (DSCG) on
Chlamydia pneumoniae, AR-39

Inclusion count (% of control)\textsuperscript{a} after exposure to the indicated concentrations of DSCG (mg/ml)

<table>
<thead>
<tr>
<th>Concentration (mg/ml)</th>
<th>0.5%</th>
<th>1%</th>
<th>2%</th>
<th>5%</th>
<th>10%</th>
<th>20%</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCC (30 min)</td>
<td>74 (±12)</td>
<td>58 (±11)</td>
<td>46 (±12)</td>
<td>27 (±5)</td>
<td>20 (±11)</td>
<td></td>
</tr>
<tr>
<td>MCC (60 min)</td>
<td>105 (±4)</td>
<td>70 (±12)</td>
<td>50 (±6)</td>
<td>21 (±9)</td>
<td>6 (±6)</td>
<td></td>
</tr>
<tr>
<td>MCC (90 min)</td>
<td>89 (±8)</td>
<td>63 (±1)</td>
<td>52 (±5)</td>
<td>24 (±16)</td>
<td>1 (±1)</td>
<td></td>
</tr>
<tr>
<td>MCC (120 min)</td>
<td>109 (±8)</td>
<td>75 (±10)</td>
<td>44 (±6)</td>
<td>7 (±5)</td>
<td>1 (±1)</td>
<td></td>
</tr>
<tr>
<td>MIC</td>
<td>95 (±7)</td>
<td>110 (±4)</td>
<td>97 (±7)</td>
<td>80 (±7)</td>
<td>31 (±10)</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} Inclusion count (% of control) = inclusion forming unit (IFU) in test sample / IFU in control x 100%. MIC, minimum inhibitory concentration. MCC, pre-inoculation minimal cidal concentration. Values are the mean (± S.D.) of three experiments. At least three wells per dilution were tested for one experiment.

Fig. 1. Cytotoxic Effect of Disodium Cromoglycate (DSCG) on HL Cells Using the Cell Counting Kit

- Cell activity decreased with increasing concentrations of DSCG. Values are mean ± S.D. of 4 wells of a microtiter plate.

decreased with increasing concentrations of DSCG, although C. pneumoniae was not completely inhibited at concentrations less than 20 mg/ml. Similarly, the number of inclusions in the MCC assay decreased with increasing concentrations of DSCG. The inhibitory effect was also time-dependent at 20 mg/ml of DSCG. After pre-incubation for 30 min with 20 mg/ml of DSCG, the number of inclusions was 20% that of the control. The number of inclusions did not decrease significantly when HL cells were pre-incubated with 20 mg/ml of DSCG for 60 min prior to infection with C. pneumoniae (data not shown).

The cytotoxic effects of DSCG on HL cells are shown in Fig. 1. Cell activity decreased with increasing concentrations of DSCG. Although no decrease in cell activity was observed at <2.5 mg/ml of DSCG, activity decreased at concentrations >10 mg/ml. However, no morphological changes were apparent in the HL cells treated with <20 mg/ml DSCG.

DISCUSSION

Our results demonstrate that DSCG inhibits the growth of C. pneumoniae at clinically relevant concentrations. It is possible that the clinical effects of DSCG for asthma are due to the inhibition of C. pneumoniae infection as well as prevention of release of chemical mediators. Our data also shows that 20% of inclusions remained after 30 min pre-incubation with a clinically relevant concentration of DSCG (20 mg/ml). These data indicate that repeated topical application of DSCG would be beneficial.

Åberg et al.\textsuperscript{9} treated adult patients with upper respiratory tract infections (URTIs) using DSCG administered both by inhalation and nasal spray for 7 d. The symptoms such as nasal running, sore throat and cough, resolved faster in patients treated with DSCG than with placebo. C. pneumoniae is a pathogen that causes pharyngitis,\textsuperscript{1} which is often observed in the early stages of pneumonia and bronchitis.\textsuperscript{1} Therefore treatment with DSCG could be beneficial for URTIs with C. pneumoniae.

Recently the inhibitory effect of DSCG on influenza virus has been reported. Hidari et al.\textsuperscript{10} showed that DSCG treatment of cells concurrent or after viral absorption resulted in significant reduction of influenza viral infection. They also showed that prior treatment of cells with DSCG did not inhibit infection, suggesting DSCG might inhibit viral neuraminidase and fusion activities. Our data indicates that the inhibitory effect of DSCG for C. pneumoniae occurs predominantly outside, rather than inside, the HL cells. Although no morphological changes were apparent in the HL cells, decreased cell activity suggested the decrease of inclusions in the MIC method could be caused by DSCG acting on the HL cells. At 20 mg/ml of DSCG in the MCC method, the inhibitory effect was both dose- and time-dependent. The precise mechanism of the inhibitory effect of DSCG on C. pneumoniae requires further investigation.

In conclusion, DSCG at concentrations below the clinically relevant range inhibited the growth of C. pneumoniae dose-dependent manner. The inhibitory effect was also time-dependent at concentration of 20 mg/ml DSCG in the MCC assay. These results warrant further clinical study on C. pneumoniae infections and use of DSCG.

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