Capture of Influenza Viruses and Prevention of Their Infection by Coral Mineral Powder (Sango Mineral Powder)

KIYOSHI TACHIKI, MIKA KURAMOTO, MISUZU KANEKO, MAYUKO NAWA, YUSUKE NIWA, AND MASAE ITOH

Department of Microbiology, Faculty of Bioscience, Nagahama Institute of Bio-Science and Technology
1266 Tamura-cho, Nagahama, Shiga 526-0829, Japan

Received 7 June, 2011/Accepted 29 September, 2011

The anti-influenza virus activity of fossilized marine coral powder (sango mineral powder, SMP) was studied. SMP is composed in terms of mass of around 25% of calcium and 10% of magnesium, respectively, principally as dolomite (CaMg(CO₃)₂) but not as calcium oxide (CaO) or magnesium oxide (MgO). By mixing the influenza virus with SMP, the infectivity of the virus substantially decreased and there was more than a 10⁴ reduction on the 3rd d of infection. The antiviral effect was observed against all the type A and B strains of the influenza virus examined including the H1N1 2009 pandemic and H5N1 avian viruses. The surface structure of SMP was highly porous and the anti-influenza activity was explained by the adsorption of the viral particles onto its surface. The binding of viruses to SMP was strong and stable in the physiological condition, and the attached viruses detached only in the presence of a high concentration of phosphate. This was similar to the binding of protein to hydroxyapatite, suggesting an ionic interaction between SMP and the viral proteins. SMP maintained its activity to capture influenza viruses even after being immobilized on a non-woven textile. SMP would be useful as a practical anti-influenza tool especially in preparation for the next pandemic virus.

Key words: Coral sands powder / Antiviral activity / Influenza virus.

INTRODUCTION

An outbreak of influenza-like respiratory illness started in Mexico in mid-February of 2009 and spread rapidly to other countries and regions around the world by the end of April through human-to-human transmission, resulting in the first influenza pandemic in the 21st century (Dawood et al., 2009; Smith et al., 2009; Zimmer and Burke, 2009). The causative virus was determined as H1N1 influenza virus containing a unique combination of gene segments originating from the North American avian virus, human H3N2 virus, classical swine virus and Eurasian avian-like swine virus (Neumann et al., 2009; Horimoto et al., 2010). Although the new H1N1 virus (H1N1pdm) was not a new subtype of type A influenza virus in human beings, a large proportion of the human population was susceptible to infection and the 2008-2009 seasonal vaccine could not provide protection against it (Hancock et al., 2009).

The present medicinal methods for the prevention and treatment of influenza include vaccination and anti-viral medication; neither, however, is entirely satisfactory. The current vaccines do not provide a complete solution because the ability of a vaccine based on non-live components is essentially limited in preventing infection (Ihara, 2010), and the yearly occurring genetic variations further lower such a vaccine’s efficacy (Boni, 2008; Hay et al., 2001). Production of a vaccine takes several months during which a virus, in the case of a newly emerging strain, could spread around the world.

*Corresponding author. Tel: +81-749-64-8168, Fax: +81-749-64-8140, E-mail: m_itoh(a)nagahama-i-bio.ac.jp
One of the most promising alternatives is the use of antiviral drugs (Hayden, 2009; Jackson et al., 2011). The recently developed specific inhibitors for the influenza virus neuraminidase (NA) such as zanamivir and oseltamivir are now widely utilized, but there are certain possibilities that resistant viruses will be generated (Collins et al., 2008; Reece, 2007; Thorlund et al., 2011). In fact, most of the human H1N1 viruses that had been prevailing up to 2009 before the H1N1pdm virus appeared were reported to have already acquired resistance against oseltamivir (Ujike et al., 2010). Considering the situation of the pandemic in 2009, there is a great concern that there might be an insufficient supply of antiviral drugs in case of the next pandemic. Should a pandemic of a new influenza virus with high pathogenicity and new antigenicity occur, it would be extremely important to take a high level of precautionary measures.

In recent years, attention has been paid to the use of inorganic antimicrobial agents for the control of microbes because of their remarkable advantages in safety and stability as compared with organic antimicrobial agents. Among them, metallic oxides such as calcium oxide (CaO), magnesium oxide (MgO) and zinc oxide (ZnO) have exhibited strong antibacterial or antifungal activities (Koper et al., 2002; Sawai, 2003; Sawai and Yoshikawa, 2004; Sawai et al., 2001, 2007). A natural mineral dolomite composed of calcium and magnesium carbonate (CaMg(CO₃)₂) was reported to display antiviral as well as antibacterial activities when heated at high temperatures (Okouchi et al., 1998; Yamana et al., 2007). The strong activity was related to the CaO and MgO produced by the thermal decomposition of CaMg(CO₃)₂ (Motoike et al., 2008), though native dolomite before heating exhibited no such activities.

Natural fossilized coral grains (coral sands) are composed primarily of the skeletons and shells of reef-building coral or the foraminifera. Since these organisms ingest minerals from the ocean to form their skeletons and shells, coral sands are constituted mainly of calcium (Ca) and magnesium (Mg). Sango mineral powder (SMP) is prepared from fossilized marine coral grains collected from ocean floor around subtropical Okinawa, Japan. Although the Ca and Mg of SMP exist principally as dolomite (CaMg(CO₃)₂) or carbonate (CaCO₃ or MgCO₃) (Table 1), we found that native SMP efficiently prevented the infection of various subtypes of type A and type B influenza viruses without heat decomposition to CaO or MgO. In this study, we investigated the mechanism of the strong antiviral activity of natural SMP. The report also discusses the ability of SMP immobilized on non-woven fabric to interfere with influenza virus infection, taking aim at the application of SMP in the practical use. This study can help us to evaluate the usefulness of SMP as a compound that prevents influenza virus infection.

**MATERIALS AND METHODS**

**Sango mineral powder (SMP)**

SMP was prepared from fossilized marine coral grains collected from the ocean floor in certain locations in Okinawa, Japan. The chemical composition of SMP is shown in Table 1. SMP contains by mass around 25% of Ca and 10% of Mg principally as dolomite (CaMg(CO₃)₂) or carbonate (CaCO₃ or MgCO₃). If there is no mention about the particle size hereafter, SMP of 1.5 μm diameter was used.

**Cells and viruses**

Madin-Darby canine kidney (MDCK) cells were grown in Dulbecco’s modified Eagle medium (DMEM, Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) supplemented with 5% fetal bovine serum.

Strains of influenza virus used in this study were as follows: A/New Caledonia/20/99 (H1N1), A/Hyogo/73/02 (H1N1), A/WSN/33 (H1N1), A/Osaka/159/09 (H1N1pdm), A/Panama/2007/99 (H3N2), A/R/Hokkaido/1/04 (H5N1) and B/Shanghai/361/2002. A/Osaka/159/09 (H1N1pdm) was one of the new emerging viruses isolated during the pandemic in 2009 (kindly provided by Dr. Kase, Osaka Prefectural Institute of Public Health) and A/R/Hokkaido/1/04 (H5N1) was an avirulent reassortant of the avian influenza virus that originated from duck viruses (kindly provided by Dr. Kida, Hokkaido University). All the viruses but A/R/Hokkaido/1/04 (H5N1) were inoculated into MDCK cells and incubated in DMEM supplemented with 2.5 μg/ml purified trypsin (Sigma-Aldrich Co., St. Louis,

<table>
<thead>
<tr>
<th>TABLE 1. Chemical composition of SMP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>(A)</strong></td>
</tr>
<tr>
<td>Analyte</td>
</tr>
<tr>
<td>Calcium (Ca)</td>
</tr>
<tr>
<td>Carbon (C)</td>
</tr>
<tr>
<td>Magnesium (Mg)</td>
</tr>
<tr>
<td>Sillicon (Si)</td>
</tr>
<tr>
<td>Sulfur (S)</td>
</tr>
<tr>
<td>Vanadium (V)</td>
</tr>
<tr>
<td>Sodium (Na)</td>
</tr>
<tr>
<td>Chloride (Cl)</td>
</tr>
<tr>
<td><strong>(B)</strong></td>
</tr>
<tr>
<td>Component</td>
</tr>
<tr>
<td>CaMg(CO₃)₂</td>
</tr>
<tr>
<td>(Ca, Mg)CO₃, or CaCO₃</td>
</tr>
</tbody>
</table>
MO) at 34 °C for 3 d. Culture medium was harvested and stored at -80 °C after centrifugation at 1500 g for 10 min. A/V/Hokkaido/1/04 (H5N1) was grown in the allantoic cavity of 10-d-old embryonated hen's eggs. Allantoic fluid was harvested, centrifuged at 1500 g for 10 min and stored at -80 °C. Titers of the virus stocks were estimated as described before by the indirect immunofluorescent method using MDCK cells and expressed as cell-infesting units (CIU)/ml (Kashiwazaki et al., 1965). Antibodies used were the mouse monoclonal antibody against influenza A virus nucleoprotein (Serotec, Oxford, UK) followed by FITC-conjugated anti-mouse immunoglobulin G (IgG) goat serum (Medical & Biological Laboratories Co. Ltd., MBL, Nagoya, Japan) for H1N1 and H5N1 viruses, anti-influenza A virus goat polyclonal antibody (Millipore Co., Billerica, MA) followed by FITC-conjugated anti-goat IgG rabbit serum (MBL) for H3N2 virus, and anti-influenza B virus goat polyclonal antibody (Millipore) followed by rhodamine-conjugated anti-goat IgG rabbit serum (MBL) for B/ Shanghai.

Assay of influenza virus infection inhibition

SMP was suspended in phosphate-buffered saline (PBS) at a concentration of 10 mg/ml and serially diluted by 1:2, 1:4, 1:8, and 1:16. A volume of 40 μl of each diluted SMP suspension was mixed with 360 μl of influenza virus (1.0 × 10⁶ CIU/ml), incubated at 25 °C for 30 min with occasional mixing, and then SMP was removed by centrifugation at 1500 g for 5 min. Fifty μl of each supernatant was then inoculated in duplicate onto a monolayer of MDCK cells in a 96-well plate. When the virus infectivity in the SMP precipitate was titrated, 300 μl of DMEM was added to the precipitate and 50 μl of the suspension containing SMP was laid over the MDCK cells. After 1 h of adsorption at 37 °C in 5% CO₂, 150 μl of DMEM supplemented with 5 μg/ml of soybean trypsin inhibitor (Sigma-Aldrich) was applied as a layer. The infected cells were incubated at 37 °C in 5% CO₂ for 14 h and were then fixed by 1% paraformaldehyde in PBS for 1 h, followed by treatment with 1% Triton X-100 in PBS for 15 min. After being washed three times with PBS, cells were stained by the indirect immunofluorescent method. The antibodies used for each virus were the same as that used in the virus titration. Positive cells were counted using a fluorescent microscope (Carl Zeiss, Inc., Thornwood, NY) (N = 4, two fields per each of two wells) and the infectivity was expressed as CIU/ml.

Detection of the genome of the influenza virus

Virus genome RNA was extracted using NucleoSpin™ RNA II (Macherey-Nagel GmbH & Co. KG, Düren, Germany) following the manufacturer’s instructions and then cDNA was prepared by reverse transcription with oligonucleotides (agcagaagcgg) complementary to the consensus sequence of 3'-terminus of each influenza genome segment as the primer. A set of primers (caccacacgtcttacgaacaga and ttaagctgctctgacagtct) was then used to amplify the part of N gene of influenza virus by PCR reaction.

Anti-influenza virus effect of SMP immobilized on non-woven fabric

The surface of non-woven fabric samples was coated with SMP of 1.5 μm particle size. We used two types of fabric made of different materials. One was hydrophobic polypropylene (cloth A) and the other was hydrophilic polyethylene (cloth B). The SMP fixed on the fabric would not detach through ordinary physical challenges such as being touched by fingers, being shaken in some aqueous solution or being subjected to centrifugation at a low speed. The amount of SMP applied on the non-woven fabric varied from 1.5 to 7.0 g/m².

SMP-coated non-woven fabric was cut into pieces of 2 × 2 cm in size and DMEM containing 3.0 × 10⁶ CIU of influenza virus was applied on it. After incubation at 25 °C for 30 min, the SMP-coated non-woven fabric samples were centrifuged at 80 g for 1 min. Infectivity of the virus remaining in the oozing solution was titrated as the virus that was not adsorbed to the SMP immobilized on the base fabric.

RESULTS

Effect of SMP on the growth of influenza virus

First, we confirmed the inhibitory effect of SMP on influenza virus infection. Influenza virus strain A/WSN/33 (H1N1) treated with SMP was inoculated into MDCK cells and its growth was monitored every day. As shown in Fig. 1, the infectious virus was not detected in the culture medium of MDCK cells inoculated with the SMP-treated virus before 4 d after inoculation, while the control virus not treated with SMP grew rapidly, reaching as high as 10⁶ CIU/ml 3 d after infection. The delay of the virus growth suggested that the infectious virus levels in the inoculum had decreased after the treatment with SMP.

We next examined the viral infectivity directly in the supernatant after SMP treatment. Fig. 2(A)
demonstrates that the titer of the infectious virus in the supernatant decreased as the amount of SMP mixed with virus increased, and most of the infectivity disappeared when more than 5 mg/ml SMP was used. Contrarily, viruses associated with SMP in the precipitated fraction increased in inverse proportion to those remaining in the supernatant. The results indicated that influenza virus was not inactivated by SMP but was cleared out from the supernatant through adsorption to SMP. Fig. 2(B) shows the effect of SMP against other influenza virus strains. SMP effectively cleared out type A influenza strains including the 2009 pandemic virus (H1N1pdm) and type B virus with a little less efficiency from viral solutions.

**Dependence of particle size**

Motoike et al. (2008) reported that the antiviral activity of heated dolomite strongly depended on the crystal sizes of CaO and MgO, and then the particle size. Though the mechanism of anti-influenza effect of SMP seemed to be different from that of heated dolomite, if SMP strongly adsorbed the influenza virus and interfered with its infection in the host cells, the particle size of the SMP should affect the activity, since the size of the surface area might be concerned. Therefore, we then examined the effect of the particle size of SMP on the anti-influenza activity. As demonstrated in Fig. 3, the smaller the particle size of SMP, the higher its ability was to capture influenza viruses, and SMP of 1.5 μm diameter cleared away more than 90% of the infectious viruses.

**FIG. 1.** Suppressive activity of SMP on the growth of influenza virus. Influenza virus strain A/WSN/33 (H1N1) was mixed with 5 mg/ml SMP and the suspension was kept at 25 °C for 30 min. The virus in the supernatant after precipitation of SMP was inoculated into MDCK cells in a 6-well plate. As a control, the same virus without SMP treatment was similarly inoculated, and the infected cells were incubated at 37 °C. Viral growth was monitored every day by titrating the infectious virus in the culture medium. ●: SMP treated virus, △: virus without SMP treatment. The dashed line indicates the limit of detection.

**FIG. 2.** Capture of the infectious influenza virus by SMP. (A) Effect of the concentration of SMP on the clearance of the infectious virus through adsorption. After serially diluted SMP was mixed with influenza virus strain A/Hyogo/73/02 (H1N1), the SMP precipitate was separated from the supernatant fraction as described in MATERIALS AND METHODS and the infectious viral titer in each fraction was assayed. The viral titer in each fraction was demonstrated as the ratio (%) to that of the initial virus mixed with SMP. ●: virus not adsorbed by SMP and remaining in the supernatant, ○: SMP-adsorbed virus. (B) Decrease in the infectivity of various strains of the influenza virus after SMP treatment. Infectious virus not adsorbed by SMP and remaining in the supernatant fraction was titrated after treatment with SMP (2.5 or 10 mg/ml) as in (A). The remaining viral titer was demonstrated as the ratio (%) to that of the initial virus mixed with SMP. A/H1N1pdm; A/Okao/159/09 (H1N1pdm), A/H3N2; A/Panama/2007/99 (H3N2), A/H5N1; A/R/Hokkaido/1/04 (H5N1), B; B/Shanghai/361/2002.
**FIG. 3.** Effect of the particle size of SMP on its virus-capturing activity. Suspensions of 5 mg/ml SMP possessing various particle sizes, 1.5, 6.0 or 10.0 μm in diameter, were prepared and examined for their ability to remove the infectious influenza virus as described in FIG. 2 (B). ● A/New Caledonia/20/99 (H1N1), ◆; A/R/Hokkaido/1/04 (H5N1).

**FIG. 4.** Time course of the inactivation of the influenza virus captured by SMP. Influenza virus strain A/Osaka/159/09 (H1N1pdm) was mixed with 5 mg/ml SMP, and the SMP fraction carrying the virus was prepared by centrifugation as described in MATERIALS AND METHODS. Then the SMP fraction was incubated at 4 or 20 °C for 10 d and the residual viral infectivity was assayed. As a control, a solution of free virus not adsorbed by SMP was subjected to the assay equally. Residual viral infectivity was demonstrated as the ratio (%) in each fraction after setting the titers on the starting day (d 0) as 100 %. ●; SMP-adsorbed virus incubated at 4 °C, Δ; free virus incubated at 4 °C as a control, ◆; SMP-adsorbed virus incubated at 20 °C, □; free virus incubated at 20 °C as a control.

**Infectivity of the SMP-adsorbed virus**

To study whether the influenza virus captured by SMP would be inactivated more rapidly or not, the influenza virus was kept at 4 °C or 20 °C as we monitored the SMP-adsorbed or the not adsorbed free virus for the time course of the inactivation. At 20 °C, the influenza virus lost more than 90 % of its infectivity by d 3 regardless of whether it had been adsorbed or not by SMP (Fig. 4). At 4 °C, though inactivation occurred more gradually than at 20 °C, again the SMP-adsorbed virus lost its infectivity as slowly as did the free virus, with about 70 % of the initial infectivity remaining 3 d after the incubation started. The results indicated that SMP possessed strong activity to capture various types of the influenza virus, but no ability to facilitate the inactivation of the attached viruses.

**Mechanism of the adsorption of the influenza virus by SMP and conditions required for the release of the virus from SMP**

We then tried to investigate the mechanism of how SMP adsorbed the influenza virus by seeking the conditions under which the attached virus would detach from SMP. When SMP carrying the influenza virus was suspended in PBS, no released virus was detected in the supernatant after occasional shaking (data not shown). However, as the concentration of phosphate increased, infectious virus came to be observed in the supernatant depending on the concentration (Fig. 5). About 9 % of attached virus was recovered by a phosphate buffer of 1 M. On the other hand, as shown in Fig. 5, no virus infectivity was observed after the incubation with 3 M solutions of MgCl₂ or CaCl₂. Since the infectivity assay can count only living viruses, there was a possibility that the released virus had been inactivated by the hypertonic effect in 3 M solution and could not be proved. Therefore, to confirm the absence of viral particles released from SMP in the salt solution of a high concentration, we performed RT-PCR to detect the viral genome. Viral genome RNA was observed in the supernatant after the treatment with higher concentrations than 0.25 M of phosphate. Contrarily, the presence of the genome of the influenza virus was not confirmed at all when SMP carrying influenza virus was treated with PBS, 3M MgCl₂ or 3M CaCl₂, denying the existence of inactivated virus particles. No other salt solutions including NaCl and KCl of high concentrations would cause the attached virus to detach (data not shown). These data indicated that attachment of the influenza virus to SMP was probably due to the ionic interaction between the two, which would be interrupted only in the presence of a high...
FIG. 5. Detachment of the captured influenza virus from SMP in the presence of a high concentration of phosphate. The SMP fraction capturing the influenza virus strain A/Hyogo/73/02 (H1N1) was prepared according to the method in Fig. 4, and then was suspended in 100 μl of 3 M MgCl₂, 3 M CaCl₂, and 1, 0.5, 0.25 or 0.125 M phosphate buffer (pH 7.0) followed by incubation at 25 °C for 30 min with occasional mixing. After centrifugation at 8,000 g for 5 min at 4 °C, the supernatant was divided into two fractions, one of which was inoculated into MDCK cells for the infectivity assay. The other was subjected to RT-PCR as described in MATERIALS AND METHODS for detection of the influenza virus genome. Primer sets used in this study amplified the DNA fragment (194 bp) derived from the influenza virus NP gene. The detached virus was demonstrated as the ratio (%) of the infectivity to that of the initial virus mixed with SMP.

Concentration of phosphate.

Adsorption of the influenza virus by immobilized SMP

In the expected practical use of SMP, for example, the application of SMP to masks or filters of air-conditioners, SMP should be immobilized on some base material without losing the capacity to capture viruses after fixation. Then, we examined various textiles as a base onto which SMP could be fixed and found that the activity of SMP to capture the influenza virus depended on the character of textile as well as that of the adhesives mixed with SMP for fixation. Fig. 6 shows one of the results. Under the selected conditions, SMP-coated cloth A made of hydrophobic polypropylene demonstrated efficient ability to adsorb the influenza virus. The effect of SMP depended on the amount fixed on the fabric, giving nearly complete clearance of the influenza virus at more than 3.5 g/m². On the other hand, when another non-woven cloth B made of hydrophilic polyethylene was tested, SMP failed to exhibit its full activity to capture the influenza virus, and around half of the virus escaped at the concentration of 3.5 g/m².

DISCUSSION

As shown in Table 1, SMP is principally composed of CaMg(CO₃)₂ containing 25% of Ca and 10% of Mg, which is quite similar to the chemical composition of a natural mineral dolomite, CaMg(CO₃)₂, with 30% of Ca and 15% of Mg (Motoike et al., 2008). Dolomite exhibited its anti-influenza virus activity only after being heated at above 800 °C and Motoike et al. (2008) concluded that CaO and MgO created by thermal decomposition were responsible. An alkaline effect caused by the hydration of CaO and MgO is considered to be the primary mechanism of the antimicrobial activity and an oxygen radical is also suggested to be involved (Sawai, 2003). In contrast, native SMP demonstrated similar antiviral effects without any heating (Fig. 1). Therefore, the mechanism of the preventive effect of SMP should be different from that of heated dolomite.

In this paper, we demonstrated that SMP strongly captured the influenza virus and that the adsorption
of the virus by SMP was disturbed only by the presence of a high concentration of phosphate. The phenomenon was quite similar to protein-binding to and elution from hydroxyapatite, \( \text{Ca}_{10} (\text{PO}_4)_6 (\text{OH})_2 \), the mechanism of which has been already established (Gorbunoff, 1984a, 1984b; Gorbunoff and Timasheff, 1984). In case of acidic proteins like influenza virus hemagglutinin (HA) or NA proteins (Bosch, 1985), the binding of protein to hydroxyapatite occurs by the specific complexing of the carbonyl groups of protein with the Ca loci on hydroxyapatite, and elution can take place as the result of the specific displacement of phosphate ions for carbonyl groups of bound protein. Adsorption of viral particles by SMP probably could be explained by the interaction between the negative charge of the carbonyl groups on the HA or NA proteins protruding from the viral envelope and the positive charge of Ca on the surface of SMP. Once captured, influenza viruses detached only in the presence of a high concentration of phosphate above 0.25 M, indicating that they would not be released in the physiological conditions where the concentration of phosphate is around 1 mM (Lewis III, J.L., 2009).

SMP powder from fossilized coral retains its porous quality, carrying a greater surface area because it is full of microscopic holes when compared with the mineral dolomite (\( \text{CaMg}(\text{CO}_3)\cdot6\)) or calcium carbonate (\( \text{CaCO}_3 \)) as can be seen in Fig. 7. This means that greater number of calcium atoms is exposed to the surface of SMP, allowing the efficient interaction with proteins on viral particles. It might be possible that the porous structure partially explains the anti-influenza effect of native SMP, whereas unheated dolomite lacks the activity. Supposing that the SMP particle is a sphere, the SMP of 1.5 \( \mu \)m diameter theoretically would possess nearly 7 times a larger surface area than SMP of 10 \( \mu \)m diameter per weight. In accordance with this, SMP of 1.5 \( \mu \)m diameter demonstrated an activity that was 7 or more times stronger in capturing the influenza virus as shown in Fig. 3, suggesting the importance of the size of the surface area.

Capturing viral particles is an excellent strategy for prevention of viral infection. Taking advantage of the binding affinity of the HA protein of influenza virus to the specific sialic acids-containing receptors, we recently developed a sialyglycopolymer and proved the efficiency of the compound to interfere with the infection by arresting the viral particles before they could bind to the cellular receptors (Umemura et al., 2008, 2010). SMP could possibly be a useful tool to prevent influenza viral infection. For practical use, however, SMP needs to be immobilized on some supporting base since it is a fine powder with a particle diameter of \( \mu \)m in size. There may be two requirements: firstly, since adsorption of viral particles is due to the interaction between the cationic Ca of SMP and the anionic carboxyl moiety of the viral proteins, the ionic interaction should not be disturbed by the fixation procedure, and secondly, SMP should be completely immobilized on the base material and not be released because native SMP does not facilitate inactivation of the influenza virus. After examining materials for use as the supporting base and adhesive materials to mix with SMP, we succeeded in immobilizing SMP on a non-woven fabric. The efficiency of immobilized SMP to capture the influenza virus depended on the character of the base and we obtained good results with hydrophobic polypropylene (cloth A). Whereas 40 \( \mu \)l

![Fig. 7. Porous structure of SMP. Scanning electron microscope observation of the surface of SMP (A), dolomite (B) and calcium carbonate, \( \text{CaCO}_3 \) (C). Bars in the photographs indicate 1 \( \mu \)m.](image-url)
of 5 mg/ml (total 0.2 mg) SMP powder, for example, cleared away almost all of the influenza virus of 3.6 x 10^5 CFU (Fig. 2), a 2 x 2 cm of polypropylene cloth coated with 3.5 g/m² of SMP carrying 1.4 mg of SMP captured the influenza virus of 3.0 x 10^4 CFU (Fig. 6). Fixed SMP exhibited a sufficient effect of capturing the same influenza virus (A/H1N1pdm), though the efficiency somewhat decreased compared with that of SMP powder.

Here we demonstrated the possibility of using SMP as an anti-influenza virus material by capturing the viral particles and interfering with infection by them. The influenza virus is adsorbed very tightly due to ionic interaction, and viral particles once attached to SMP are not released under normal physiological conditions. SMP is utilized as a food additive to fortify nutrition and to improve the quality and taste of processed foods. Therefore, it is quite a safe and harmless powder without side effects to our health in its ordinary use. When SMP was laid over the cells in this study, cytotoxic effects such as rounding or detachment of the cells were not observed. The anti-influenza virus effect of SMP is not limited to a certain virus strain whereas that of vaccines depends strictly on the strain. Moreover, SMP does not give rise to antigenic mutations or resistant mutants against influenza virus inhibitors. SMP possesses the potential to be utilized against a broad range of influenza viruses and especially is expected to give us a benefit in case the next pandemic occurs, since it cannot be predicted which virus strain will be involved and when it will emerge.

ACKNOWLEDGMENTS

We would like to thank Dr. Kida, Graduate School of Veterinary Medicine, Hokkaido University, Hokkaido Japan, and Dr. Kase, Osaka Prefectural Institute of Public Health, Osaka Japan, for kindly providing the influenza viruses A/R/Hokkaido/1/04 (H5N1) and A/Osaka/159/09 (H1N1pdm), respectively. We also wish to acknowledge the electron microscope photographs kindly provided by the Marine Bio Co., Ltd., Tokyo Japan.

REFERENCES


