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ORIGINAL ARTICLE

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Inactivation of influenza A virus by gentian violet (GV) and GV-dyed cotton cloth, and bactericidal activities of these agents

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Abstract Recently we have heard warnings of an outbreak of a highly pathogenic avian influenza virus (H5N1). Although, to prevent such infections we must prepare antiviral drugs and type-specific vaccines against influenza, we need various simple and effective protection methods, such as the use of face masks for public health. Also, in any consideration of bacterial infections, methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococci* (VRE), and multidrug-resistant *Pseudomonas aeruginosa* (MDRP) also pose serious concerns which must be addressed. I examined the antiviral activity of gentian violet (GV) and GV-dyed cloth against the influenza A (H1N1) virus. Time-kill studies were carried out, and the virus titer was determined based on the 50% tissue culture infective dose ($TCID_{50}$). The minimum inhibitory concentrations (MICs) of GV against bacteria were also determined, and the killing activities of the GV-dyed cloth were judged from viable cell counts. GV immediately killed the influenza A virus and this was confirmed by electron microscopy. Moreover, cloth dyed with a combination of GV and copper showed not only excellent antiviral activity but also prominent bactericidal activities.

Key words Influenza virus · MRSA · VRE · MDRP · Gentian violet

Introduction

An influenza pandemic is an epidemic of an infectious disease with serious worldwide consequences. The 1918 influenza pandemic was severe and killed over 30 million people. During 2004, a highly pathogenic avian influenza A virus (H5N1) appeared, causing poultry disease in a large part of Asia.[1–3] This avian H5N1 strain has already infected at least 89 persons and killed 52 who had close contact with birds.[2,4,5] It was naturally thought that this avian virus would not infect humans, because humans have receptors specific for human viruses, not for avian viruses. However, recent studies have shown that H5N1 seems to have aquired the ability to infect mammals such as pigs and cats.[6,7] Although none of the H5N1 strains have shown evidence of having aquired genetic changes with an efficient ability to transmit from human to human, it may be possible for a reassortment between an avian and a human influenza virus to occur in time.[4] If H5N1 evolves to become easily transmittable among humans, we would likely face an even more severe pandemic than that of 1918. Influenza is an infective respiratory disease, so some of the infection control efforts should be directed at shutting off the airborne route or at eliminating the source of infection with antiseptics.

Regarding bacterial infections, methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococci* (VRE), and multidrug-resistant *Pseudomonas aeruginosa* (MDRP) are known causes of hospital infections in patients with risk factors. Recently, we have had several disturbing reports that MRSA infections have been found in patients without risk factors in the community.[8–10] Now, it seems that these nosocomial infections are going to spread gradually into the community at large, as community-associated infections. In order to prevent such troubling developments, we need cost-effective methods to eliminate the source of infection with antiseptics, in addition to precision in the selection of antimicrobial agents for the treatment of these infections.

Materials and methods

Gentian violet (GV) and dyeing of cotton cloth

Gentian violet (hexamethyl-pararosaniline chloride; GV) was purchased from Sigma-Aldrich (Tokyo, Japan). GV and $CuCl_2$ stock solutions were prepared at a concentration

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of 4 mg/ml (0.4%) in distilled water. This triphenylmethane dye is widely used as a stain, and as a dye for silk, cotton, and leather (GV color index [CI], 42555). Therefore, simple common procedures were used for dyeing the cloth. Briefly, washed 20-mm-square pieces of cotton cloth were boiled in 3% tannic acid for 10 min, cooled, and then dyed in 0.005–0.001% GV heated to 100°C. After vigorous washing with soap and hot water, they were color-fixed in 0.01% $CuCl_2$ (or other metallic mordants).

Bacterial susceptibility tests and bactericidal activity tests

MRSA (strain ATCC29213), VRE (*Enterococcus faecium* strain ATCC51559), MDPR (a clinical isolate of Fukuoka University Hospital strain FP1), and strain ATCC27853 as a standard strain of *P. aeruginosa* were used. The minimum inhibitory concentrations MICs of GV and metals were determined by the agar dilution of brain heart infusion (BHI) agar, according to the Clinical and Laboratory Standards Institute (CLSI) method, and expressed as percentages (%) instead of micrograms per milliliter. Bactericidal activities of the dyed cloth against MRSA, VRE, and MDRP were determined on cotton cloths treated with tannic acid only, $CuCl_2$ only, GV alone, and a combination of GV and $CuCl_2$. Samples of 50 µl, of about 10^6 colony forming units (cfu)/ml, of MRSA, VRE, or MDRP in BHI broth were dropped on triplicate sample cloths, and they were kept in enough moisture to exclude drying effects at 37°C. After 18 h of incubation, a piece of cloth was put into 5 ml of BHI broth in a 50-ml tube and shaken vigorously with a vortex mixer to release bacteria. Bacterial suspensions were diluted with BHI broth and plated on BHI agar to determine cfu/ml.

Virus and cells

Influenza A / WSN / 33 (H1N1) virus was kindly provided by Dr. N. Hamada (Department of Virology, School of Medicine, Kurume University, Kurume, Japan). Mardine-Darby canine kidney (MDCK) cells (ATCC CCL-34) were purchased from Dainippon Pharmaceutical (Osaka, Japan). Cells were maintained in Dulbecco's Modified Eagle minimum essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS).

Preparation of virus stocks and virus titration

Confluent monolayers of MDCK cells in one hundred 60-mm tissue culture plates were infected with 1.0 hemagglutinin units (HA) of influenza virus. After 4 days, when cytopathic effects (CPE) were prominent, the cells, together with culture media, were harvested and frozen at –80°C. After two cycles of freezing and thawing, the cells were centrifuged for 15 min at 3000 *g*. The supernatants were frozen as virus crude stock for another infection and further purification. Standard procedures of HA testing, using a 96-well U-plate, were carried out at 4°C.

The virus titer was determined based on the 50% tissue culture infective dose ($TCID_{50}$) with MDCK cells in 96-well flat culture plates, with a tenfold serial dilution of virus stock or experimental samples. The $TCID_{50}$ was calculated from the results of the CPE in six wells at each dilution, using the methods of Reed and Muench.[11] The virus stock showed 512 HA, corresponding to $10^{7.50}$ $TCID_{50}$/ml.

Antiviral activity of GV and $CuCl_2$ and the killing activity of GV-dyed cloth against influenza virus

An amount of 500 µl of 64 HA H1N1, which corresponded to $10^{6.80}$ $TCID_{50}$/ml, was added to 500-µl alignets of serial dilutions of GV or $CuCl_2$ with Dulbecco's phosphate-buffered saline (DPBS) in 5-ml Assist-Sarstedt tubes (Assist Trading Co., Ltd., Tokyo, Japan). Samples were left at room temperature for 1 h and virus titers were measured as described above.

Time-kill assays were carried out in 5-ml tubes containing 500 µl of 64 HA of H1N1 and an equal volume of GV, GV, and $CuCl_2$, $CuCl_2$ alone, and povidone-iodine (PVP-I) as a positive control. Samples were removed at 30, 60, 180, and 360 min for quantitative culture to determine the presence of viable virus.

Time-killing studies of the GV-dyed cloths were carried out on four kinds of cotton cloths. Samples of 50 µl of 64 HA H1N1 in DMEM were dropped onto the sample cloths and they were kept in enough moisture at room temperature. A piece of cloth was put into 5 ml of DMEM in a 50-ml tube at 60 and 180 min and shaken vigorously to release virus particles. Then virus suspensions were diluted and plated onto MDCK cells to determine the $TCID_{50}$.

Virus purification and electron microscopy

MDCK-grown influenza A (H1N1) stock was purified according to the method described by Laver.[12] Briefly, 40 ml of virus stock was centrifuged for 10 min at 6000 *g* and the supernatant was adsorbed with 2 ml of packed chicken red blood cells at 4°C. The cells were washed twice in ice-cooled DPBS, using low-speed centrifugation; then adsorbed virus particles were eluted at 37°C into 10 ml of DPBS. The elutate was clarified by centrifugation for 10 min at 6000 *g* and then the virus was deposited for 60 min at 30000 *g*. The pellet was resuspended in 1 ml of DPBS. A small amount of purified and concentrated H1N1 was mixed with an equal volume of 0.0125% GV, 0.0063% GV containing 0.0031% $CuCl_2$, or 0.0125% $CuCl_2$. After 10 min at room temperature, specimens were dropped onto a copper grid covered with Formvar (polyvinyl formal), washed, and then negatively stained with 1% phosphotungstic acid (PTA). Final photographs were taken using a Hitachi H-7000 electron microscope (Hitachi, Ltd., Tokyo, Japan) operating at 100 Kv.

Table 1. MICs of CuCl2, GV, and MG against MRSA, VRE, and MDRP (%)

	$CuCl_2$	GV		MG	
		Alone	+$CuCl_2$[a]	Alone	+$CuCl_2$[a]
MRSA	0.1	<0.00005	<0.00005	<0.00005	<0.00005
VRE	0.2	<0.00005	<0.00005	<0.00005	<0.00005
MDRP (FP1)	0.2	0.1	0.0125	>0.1	0.0063
Pseudomonas aeruginosa	0.1	0.05	0.0125	0.1	0.0125

MRSA, ATCC29213
VRE, *E. faecium* ATCC51559
MDRP (FP1), clinical isolate from Fukuoka University Hospital
P. aeruginosa, ATCC27853
[a] Cu^{2+} was added at a 1:10 ratio to GV or MG

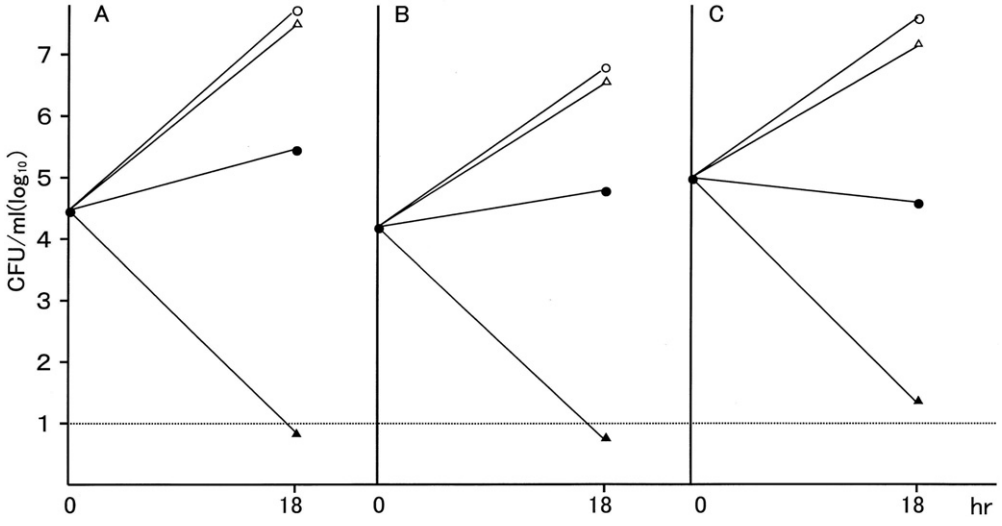

Fig. 1A–C. Bactericidal activities of dyed cloths. **A** Methicillin-resistant *Staphylococcus aureus* (MRSA); **B** varcomycin-resistant enterococci (VRE); **C** multidrug-resistant *Pseudomonas aeruginosa* (MDRP). Viable cell counts were determined as cfu/ml on brain heart infusion (BHI) agar plates. Expermental samples were as follows. Tannic acid treatment only (*open circles*), $CuCl_2$ (*open triangles*), gentian violet GV; (*closed circles*), GV and $CuCl_2$ (*closed triangles*). The results represent the means for three different pieces of cloth in each of the cases. The detection limit for viable cell counts was 10^1 CFU/ml. The maximum SD of the data points shown was less than $\log 10^{0.20}$

Results

GV has been used as a bactericidal and mycostatic agent[13] since before the discovery of antibiotics, and it is also used as a colorant for the Gram staining of bacteria in our microbiological field. First, I determined the MICs of GV against MRSA, VRE, and MDRP. It is well known that GV has excellent bactericidal activity against Gram-positive bacteria but not against Gram-negative ones. Therefore, I searched for chemicals which could enhance the killing activity of GV against Gram-negative bacteria. Metals such as Cu^{2+}, Zn^{2+}, and Ag^{2+} have weak bactericidal activity alone, but when GV and $CuCl_2$ were combined at a ratio of 10:1, the killing effect of GV on MDRP showed results eight times better than that of GV alone against MDRP (Table 1). Of course, no inhibitory effects were seen against Gram-positive bacteria with the combination of GV and Cu^{2+}. A closely related triphenylmethane dye, malachite green (MG) and Cu^{2+} showed augmentation of antimicrobial activity similar to that seen with GV (Table 1). Therefore, next, I examined whether GV-dyed cloth had bactericidal activities against MRSA, VRE and MDRP.

Samples of 50 µl of about 6×10^6 cfu/ml of MRSA, VRE, or MDRP in BHI broth we dropped on sample cloths. After 18 h of incubation, approximately 3×10^4 cfu/ml of MRSA and MDRP multiplied to over 10^7 cfu/ml, and VRE multiplied to 4×10^6 cfu/ml in the control cloth and the cloth treated with $CuCl_2$ alone, whereas these three bacterial strains were killed and viable cells dropped below 20 cfu/ml in cloth dyed with a combination of GV and $CuCl_2$ (Fig. 1). In cloth dyed using GV alone, these three strains did not multiply, but about 10^4 cfu/ml viable cells were still able to survive (Fig. 1). It was obvious that a combination of GV and $CuCl_2$ augmented the bactericidal activity of GV in dyed cloth.

As Gawande[14] has emphasized, washing the hands is a most effective method for halting hospital epidemics of various infections. However, it is difficult to get clinicians and other medical staff to wash their hands frequently. Another way to eliminate hospital infections is to reduce the number of drug-resistant bacteria in the hospital surroundings. Given this background, we may anticipate good results from the use of GV-dyed cloths which are effective against MRSA, VRE, and MDRP for dressing materials, gowns, sanitation items, mats in the wards, etc.

When tissue culture cells were exposed to a low concentration of GV (0.01% or less), cells were easily killed by destruction of the cell membrane. Consequently, I examined GV's antiviral activity against the influenza virus (the envelope of which originates mainly from the host cell membrane) and its application in dyeing cloths in order to prevent the spread of influenza.

Initially, GV concentrations between 0.25% and 0.0004% were tested for antiviral activity. When about $10^{6.80}TCID_{50}$ influenza A / WSN / 33 (H1N1) virus was exposed to 0.0063% GV for 1 h, the remaining viable virus was reduced to below $10^{3}TCID_{50}$. In the field of antimicrobial chemotherapy, the minimum bactericidal concentration (MBC) or minimum lethal concentration (MLC) are terms often used for evaluating antimicrobial agents. Accordingly, in this study, I designated as the MLC the minimum concentration which reduced the original viral infectivity to less than 10^{-4}. The MLCs of GV and $CuCl_2$ were 0.0063% and 0.025%, respectively.

To determine the kinetics of inactivation of viral infectivity, mixtures of H1N1 with GV, GV and $CuCl_2$, $CuCl_2$ alone, I_2, and DPBS were incubated at room temperature and assayed for residual infectivity at various time intervals. In untreated suspensions of DPBS, viable virus remained for 6 h within $1\log_{10}$ below the viable counts observed immediately after the experiment (Fig. 2), whereas GV and GV with $CuCl_2$ produced excellent antiviral activity against the H1N1 influenza virus. When $10^{6.80}TCID_{50}$ virus was exposed to 0.0063% GV, the residual viable count decreased to below $3\log_{10}$ within 30 min and below $5\log_{10}$ at 60 min (Fig. 2). This indicates that the interaction of GV with the influenza virus is very rapid and GV completely destroys the infectivity of the influenza virus within 60 min.

This finding was confirmed by electron microscopy. Purified influenza A virus particles showed two major glycoprotein projections on the surface: hemagglutinin and neuraminidase (Fig. 3A). When virus particles were treated with 0.0063% GV for 10 min, phosphotungstic acid (PTA) penetrated into them and it was obvious that the surface envelopes were destroyed (Fig. 3B). Because the envelopes were broken, inner nucleocapsids were released, and this was the reason why infectivity was lost. Under these conditions, the suspension became slightly viscous, and virus particles easily made aggregations. At this stage, some traces of surface projections were observed (Fig. 3B, arrows). After 60 min, the number of virus-like particles observed in Fig. 3B decreased and small pieces of membrane-like structures and their aggregates were prominent. GV and $CuCl_2$ in combination, at a ratio of 2:1 (0.0031% GV with 0.0016% $CuCl_2$), had antiviral activity similar to that of 0.0063% GV alone and more effective than that of 0.0031% GV or 0.0063% $CuCl_2$ (Fig. 2). The 0.0016% $CuCl_2$ was a 16-times lower concentration of $CuCl_2$ than the $CuCl_2$ MLC (0.025%), so it was clear that the combination of GV and $CuCl_2$ had a synergistic antiviral effect. Thus, GV, or a combination of GV and $CuCl_2$, was shown to be an excellent disinfectant against the influenza A virus, and these findings could shed further light on applications for prophylaxis against influenza.

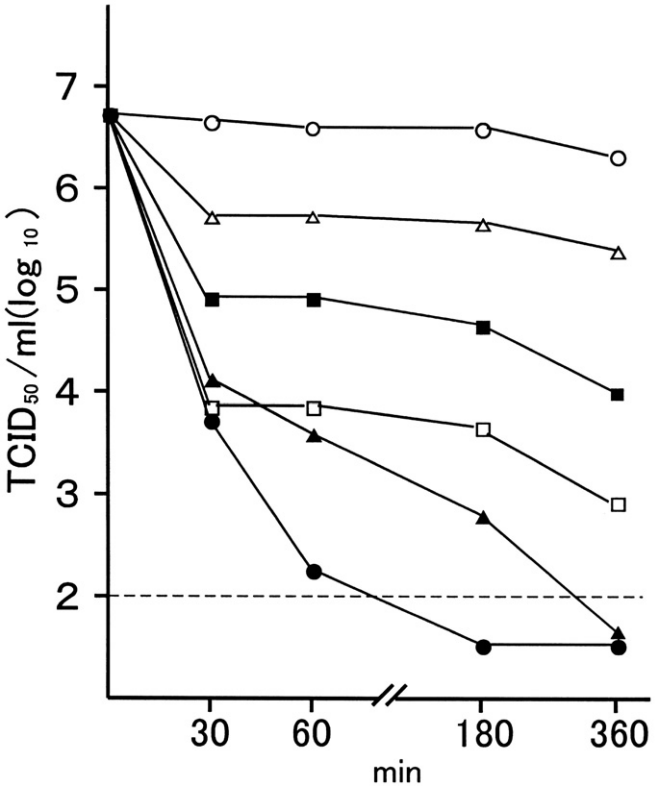

Fig. 2. Time-Kill curves. Changes in viable H1N1 exposed to GV, GV, with $CuCl_2$, $CuCl_2$, and povidone-iodine (PVP-I). Infective virus was titrated by using Mardine-Darby carine kidney (MDCK) cells, and the 50% tissue culture infective dose (*$TCID_{50}$*) was determined. Dulbecco's phosphate-buffered (DPBS); control; (*open circles*), 0.0063% $CuCl_2$ (*open triangles*), 0.0031% GV (*closed squares*), 0.5% PVP-I (*open squares*), 0.0031% GV and 0.0016% $CuCl_2$ (*closed triangles*), 0.0063% GV (*closed circles*). The results were means of three independent experiments. The detection limit of infectivity was $10^{2}TCID_{50}$/ml. The maximum SD of the data points shown was less than $\log 10^{0.20}$

Time-killing studies for these dyed cloths were carried out with H1N1. Triplicate samples were diluted and virus viability was plotted for each experiment (Fig. 4). Cloths dyed with a combination of GV and $CuCl_2$ led to a remarkable decrease of viable virus within 60 min. Residual infectivity fell below $3\log_{10}$ in 60 min and below the detection limit in 180 min (Fig. 4). The killing ability of the cloth dyed by GV alone was greater than that of the cloth dyed with tannic acid only (control cloth) or that dyed with $CuCl_2$ alone, but was less than that of the cloth dyed with a combination of GV and $CuCl_2$ (Fig. 4). In the dyed cloths, a

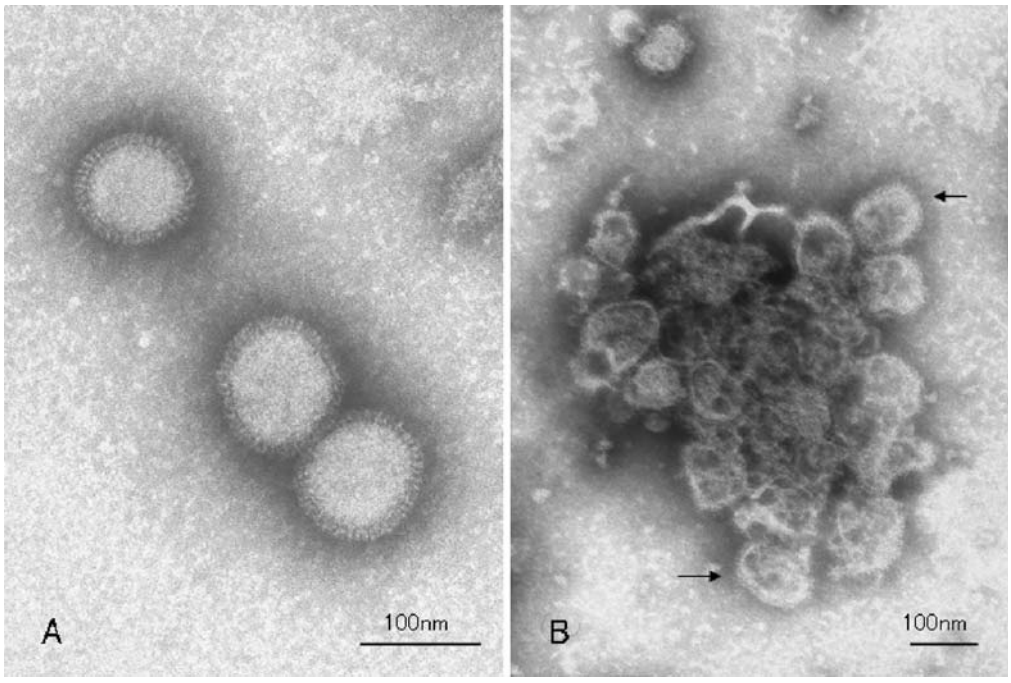

Fig. 3. **A** Electron micrograph of purified intact influenza virus (H1N1) embedded in phosphotungstic acid (PTA). The particles show well-defined surface projections. **B** Aggregated broken virus particles 10 min after treatment with 0.0063% GV. Some traces of surface projections are still observed (*arrows*). With a combination of 0.0031% GV and 0.0016% $CuCl_2$, similar broken virus particles were seen. **A** ×160 000; **B** ×80 000

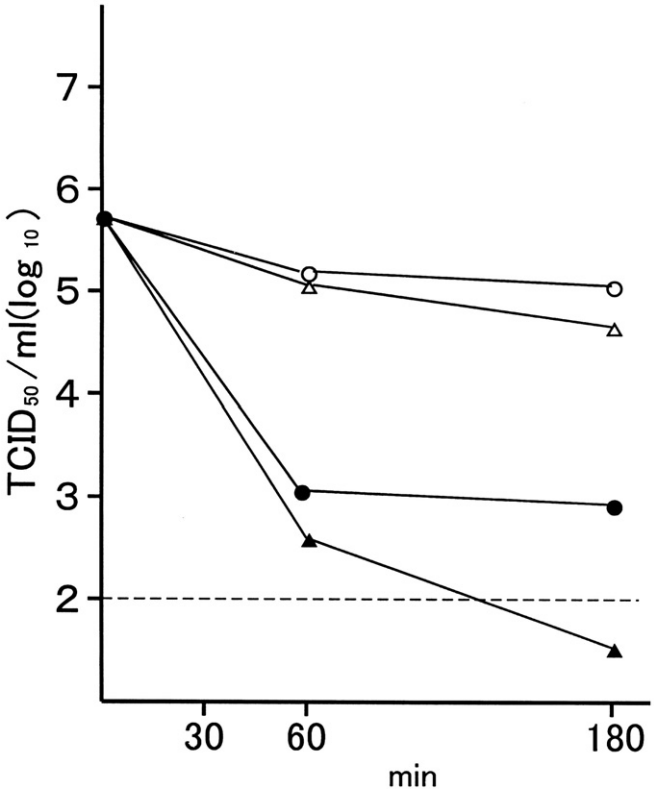

Fig. 4. Kinetics of inactivation of H1N1 by dyed cloths. Infective titers were determined as described for Fig. 2. Tannic acid treatment only (*open circles*), $CuCl_2$ (*open triangles*), GV (*closed circles*), GV and $CuCl_2$ (*closed triangles*). The results represent the means for three different pieces of cloth in each of the cases. The maximum SD of the data points shown was less than $\log 10^{0.20}$

combination of GV and $CuCl_2$ seemed to work synergistically against H1N1, and $CuCl_2$ was necessary as a mordant. As for mordants, zinc and silver had similar synergistic effects on the antiviral activity of GV against H1N1 (data not shown).

These results document a major breakthrough, because this is the first time that anyone has demonstrated the antiviral activity of GV and GV-dyed cloth against influenza virus.

Discussion

It has been suggested that the 1918 pandemic influenza virus originated from an avian virus adapted to human transmission.[15] Early in July of 2005, it was reported that thousands of migratory birds had been infected and died carrying a new H5N1 variant.[16,17] This is a very dangerous signal that H5N1 is evolving and being scattered widely, and the emergence of a worldwide pandemic strain poses a great risk. If this dangerous avian influenza virus should morph into a pandemic strain among humans, how would we face such a pandemic? As antibodies against envelope structures such as hemagglutinin and neuraminidase are essential to protect from infection, vaccination would be an ideal way to reduce the pandemic.[18–21] However, a pandemic virus will be different from the strains that have previously been prevalent, and so current vaccines will be useless. Moreover, influenza viruses are easily able to mix genomes with other strains (reassortment), so this poses a significant risk for the manufacturers of developing and stocking large volumes of such vaccines, which may subsequently prove unreliable against pandemic strains.

A second strategy to prevent pandemics is the use of

antiviral drugs.[18,22] Tamiflu (Oseltamivir; Roche, Basel, Switzerland) and related compounds, called neuraminidase inhibitors, reduce the release of virus particles from infected cells. It has been reported that drug-resistant strains have already appeared, and the proportion of resistant endemic strains has reached 18% in Japan;[23] such resistance has also happened for a pandemic strain.[24] In any case, both the development and stockpiling of vaccines or drugs entail political and financial difficulties.

When severe acute respiratory syndrome (SARS) was in the news in 2003, various face masks, particularly N95 masks, were in urgent demand. As the N95 masks physically filter viruses out by fitting tightly over the mouth and nose, users have to breathe hard and so are unable to wear the masks for extended periods. Therefore, the N95 mask is not practical except in situations such as when healthcare workers or patients' family members are in close contact with patients in an emergency room or a ward. Based on experience with the SARS corona virus, Seto et al.[25] pointed out that cheaper polypropylene surgical masks were quite effective to prevent transmission among healthcare workers, and the masks were crucial for protection from droplet transmission. Kariwa et al.[26] studied the efficacy of several povidone-iodine (PVP-I) products for inactivating the SARS corona virus.[26]

Here, I have compared the antiviral activity of GV with 0.5% PVP-I diluted from Isodine (Meiji Seika, Tokyo, Japan), which contained 10% PVP-I, corresponding to 1% effective I_2. As shown in Fig. 2, 0.0063% GV had a much greater killing effect than 0.5% PVP-I on the H1N1 virus. Certainly, PVP-I is a good disinfectant and it has been used for disinfection against various bacteria and viruses, but it seems to be difficult to dye cloth using I_2 products. On the other hand, using GV, we can dye cloth and retain the capacity to kill influenza viruses (Fig. 4). When a reassortment has occurred, the unexpected variant influenza virus aquires new antigenicity, which voids the effectiveness of vaccines now in use. Even if this happens, however, GV may destroy not only the envelopes of H1N1 but also those of H5N1 and those of new variant strains. This is because the envelopes of influenza viruses, like cellular membranes which are easily damaged by GV, are essential and are made up of universal membrane components, i.e., lipid bilayers and proteins. Very recently, we have been given a warning of the increasing threat of an avian influenza A virus (H5N1) pandemic, issued by the writing Committee of the WHO.[27] Moreover, in articles in *Science*[28] and *Nature*[29], it has been reported that the virulent 1918 influenza virus has been resurrected, and this constructed virus could be useful for preventing another pandemic. If the dyed cloth described here is made use of in cheaper masks, which easily become wet with exhalation and saliva, they might be able to capture respiration droplets. Consequently, I believe that a next influenza pandemic could be reduced if such GV masks (called Flu Mask) were used not only by health professionals but also by the general public worldwide. Moreover, GV seems to attack the envelope of influenza viruses (Fig. 3B), so GV and GV-dyed cloth might be effective in preventing infections with other respiratory enveloped-viruses, such as the SARS corona virus.

In conclusion, GV-dyed cloths can be widely applied for use in sanitation supplies, medical equipment, and other materials, constituting a low-cost method for preventing not only bacterial but also viral infections.

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