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A throat lozenge containing amyl meta cresol and dichlorobenzyl alcohol has a direct virucidal effect on respiratory syncytial virus, influenza A and SARS-CoV

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A potent virucidal mixture containing amyl metacresol and dichlorobenzyl alcohol at low pH inactivated enveloped respiratory viruses influenza A, respiratory synctial virus (RSV) and severe acute respiratory syndrome coronavirus (SARS-CoV) but not viruses with icosahedral symmetry, such as adenoviruses or rhinoviruses. A titre of approximately 3.5 $\log_{10}$ $TCID_{50}$ was reduced to below the level of detection within two minutes. Electron microscopy of purified influenza A virus showed extensive clumping and morphological changes in spike configuration after contact with the virucidal mixture, but no overt destruction of the viral membrane. We conclude that, formulated as a lozenge, the mixture could have significant effects in reducing the infectivity of certain infectious viruses in the throat and presumably in cough droplets, thus reducing, theoretically, opportunities for person-to-person transmission.

Keywords: throat lozenge, respiratory syncytial virus, influenza A, SARS-CoV, rhinovirus

Introduction

Respiratory viruses such as influenza A, rhinoviruses and respiratory syncytial virus are transmitted from person to person predominantly by coughing virus-contaminated saliva and throat droplets (reviewed by Tyrrell, 1965; Collier & Oxford, 2002). Moreover, such droplets can settle on surfaces, cups and doorknobs and thereby contaminate and infect another person (Gwaltney & Hendley, 1982). Most scientific attention has been directed towards synthetic inhibitors which block the replication of viruses intracellularly and two such classes of drug(s) have been discovered with activity against influenza A, the M2 blockers and the neuraminidase inhibitors (Oxford & Galbraith, 1987; Von Itzstein *et al.,* 1993; Kim, *et al.,* 1997; Gubareva *et al.,* 2000).

At present, severe acute respiratory syndrome coronavirus (SARS-CoV)-infected patients are treated only by supportive nursing, as the efficacy of ribavirin and corticosteroids in SARS-CoV infection are unproven. Most recently, Haagmans *et al.* (2004) have described the effectiveness of pegylated interferon alpha on SARS-CoV in a monkey model but such studies have not been initiated in humans. However, other molecules may destroy virus extracellularly or attach to viruses and prevent subsequent adsorption. Mild detergents can disrupt lipid-enveloped viruses whereas certain externally positioned virus spikes and proteins are susceptible to configuration changes at low pH. Additionally a range of more traditional antimicrobial compounds may bind to viruses and cause inactivation.

Here we describe the inactivation *in vitro* of three respiratory viruses by a lozenge formulation of amyl meta cresol and dichlorobenzyl alcohol at low pH, dissolved in artificial saliva. Phenols and cresols have long been recognized as antimicrobials and have membrane-active properties, whilst alcohols also exhibit broad-spectrum antimicrobial activity (McDonnell & Russell, 1999). A range of these molecules relieve the symptoms of sore throats (Pitts & Vincent, 1988). However there have been relatively few virucidal investigations using specific human viruses, with the exception of microbicides against HIV (reviewed by Keller *et al*, 2003). A number of sore throat lozenges, including Strepsils®, contain agents that are known to have an antibacterial effect. However, given that sore throat is a symptom of an upper respiratory tract infection and is known to be mostly associated with viral infection, it was of interest to determine whether such products exert any antiviral effect.

Materials and methods

Viruses

Three enveloped viruses [respiratory syncytial virus (RSV) and influenza A viruses, A/Taiwan/78 (H1N1) &

A/Beijing/ 353/89 (H3N2)] and two non-enveloped respiratory viruses (adenovirus type subgroup C and rhinovirus type 2) were propagated using standard techniques with Madin Darby canine kidney (MDCK), human HELA, or green monkey cells BSC-1. (Al-Jabri *et al.*, 1996). Cells were propagated in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum and 2 mM L-glutamine in 50 ml Nunc tissue culture flasks. Cells were plated into 96-well tissue culture plates and incubated at 37°C. SARS-CoV virus was cultivated in Vero cells E6 (Peiris *et al.*, 2003a). Virus was kindly provided by A Osterhaus, University of Rotterdam, the Netherlands. Virus endpoints were calculated from cytopathogenic changes recorded visually in duplicate cultures after incubating virus-infected cells for 5–7 days.

Virucidal test and titration for residual virus infectivity

One lozenge (2,4-dichloro benzyl alcohol 1.2 mg and amyl meta cresol 0.6 mg incorporating tartaric acid) Strepsils® was dissolved in 5 ml of artificial saliva (0.1% $NaHCO_3$, 0.1% gastric mucin pH 6.0–6.5) or in DMEM.

Each virus at a known infectious titre (approximately $10^{5.2}$ $TCID_{50/ml}$ for influenza virus, $10^{5.2}$ $TCID_{50/ml}$ for RSV, 10^9 $TCID_{50/ml}$ for adenovirus 10^5 $TCID_{50/ml}$ for rhinovirus and $10^{4.5}$ $TCID_{50/ml}$ for SARS-CoV) was diluted 1/10 in the artificial saliva (or in medium) and incubated for 2–16 min at 37°C. Any residual virus infectivity was detected by quantitative titration in the respective cell line. A positive control in the SARS-CoV experiment was 20% ethanol and 1% tween 20 in phosphate buffered saline and in the influenza experiments 0.1% triton X-100. All experiments were performed in duplicate with equivalent results.

No cytotoxicity of the lozenge solution was observed during the titrations for residual virus infectivity which, if detected, could have altered the interpretation of residual virus infectivity.

Electron microscopy

One lozenge was dissolved in 5.0 ml of artificial saliva, while artificial saliva alone was the control. The virucidal mixture or saliva control was placed in Eppindorf tubes, and purified influenza A/USSR/90/77 (H1N1); A/Taiwan/77 (H1N1) and B/Beijing/184/93 viruses (10 mg/ml viral protein) were added and incubated as above for 1–20 minutes.

Subsequently, 100 µl of 3% glutaraldehyde/0.1M cacodylate buffer was added to each tube to stop the interaction. In another experiment, the virus was exposed to either virucidal mixture or saliva in the same way without adding glutaraldehyde. After exposure, EM grids were prepared by placing 8 µl virus suspension on a 2×2 mm agarose square, following which the grid was placed down over the suspension and allowed to remain until the fluid was absorbed through the agarose. The grid was then washed in distilled water, negatively stained with 2% sodium phosphotungstate at pH 6.0, and allowed to dry. The grids were examined in a Phillips EM301 transmission electron microscope and micrographs were taken at either 9.1 k or 45 k magnification.

Effect of the virucidical lozenge mixture on the infectivity of respiratory viruses

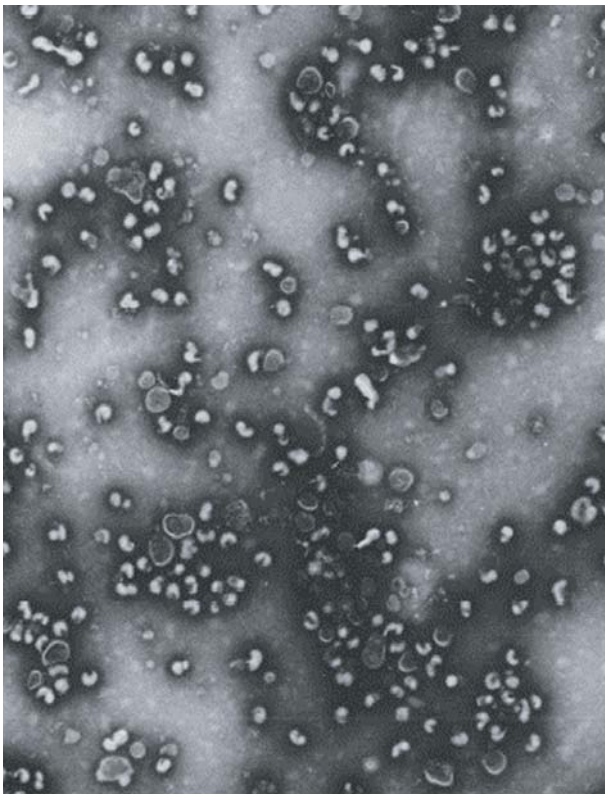
Incubation of influenza A virus with either the virucidal mixture or a non-ionic detergent (0.1% triton ×–100) as a control for 2 min or longer at 37°C reduced the virus infectivity to undetectable levels. Thus, the infectivity titre of the influenza virus A/Taiwan/78 (H1N1) incubated with the artificial saliva approximated to $10^{5.2}$ $TCID_{50/ml}$. After incubation with the lozenges, this reduced to less than 1.2 $\log_{10}$ $TCID_{50/ml}$ (Table 1). Similarly, after 2 min incubation at 37°C with the virucidal mixture a reduction of infectivity was detected for RSV of ≥4.0 $\log_{10}$ $TCID_{50/ml}$. Greater than 3.5 log $TCID_{50}$ per ml inactivation of SARS-CoV virus was also detected (Table 1). Virucidal activity commenced after 2 min incubation when 1.0 $\log_{10}$ $TCID_{50}$/µl of SARS-CoV was destroyed. No additional virucidal effects were noted after the initial 2 min inactivation. The positive control mixture of 20% alcohol and 1% tween also inactivated SARS-CoV virus after 2 min at 37°C (≥2.5 $\log_{20}$ $TCID_{50}$).

Table 1. Inactivation of infectivity of influenza A/Taiwan/77 (H1N1), RSV and SARS-CoV viruses after contact with the virucidal lozenge mixture

Virus	Infectious titre $\log_{10}$ $TCID_{50}$ ml before incubation*	Infectious titre $\log_{10}$ $TCID_{50}$ ml after incubation*	Log_{10} $TCID_{50}$ ml reduction in infectious titre
Influenza, A/Taiwan	5.2	≤1.2	≥4.0
RSV	5.2	≤1.2	≥4.0
SARS-CoV	4.5	≤1.0	≥3.5

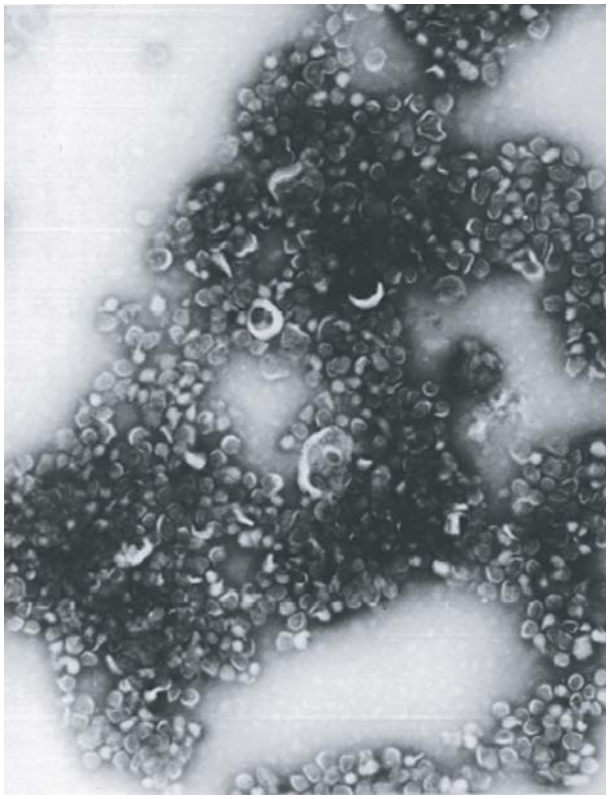
*Incubation was for 2 min at 37°C.

Figure 1. Control virions incubated for 4 min in artificial saliva buffer solution



Virions are evenly distributed. Magnification 9k approximately.

Figure 2. Virions incubated for 4 min with the virucidal lozenge mixture



Note clumped virions. Magnification 9k approximately.

No virucidal effects were detected after varying times of incubation (up to 16 min) at 37°C with the lozenge solution and adenovirus or rhinovirus.

Electron microscopy of influenza A viruses and the virucidal lozenge mixture

The dissolved lozenge virucide caused very significant clumping and aggregation of the influenza virus (Figures 2 and 3) compared to the evenly distributed control virions in the artificial saliva buffer solution (Figure 1). The size of the clumps varied greatly from small clumps of a few tens of influenza particles to very large masses that were too electron-dense to estimate their size. There were also some particles that were not clumped. The clumping was consistent over the time range of the experiment from 2–16 min incubation at 37°C. Essentially identical data were noted with A/USSR/90/77, A/Taiwan/77 and B/Beijing/184/93 viruses.

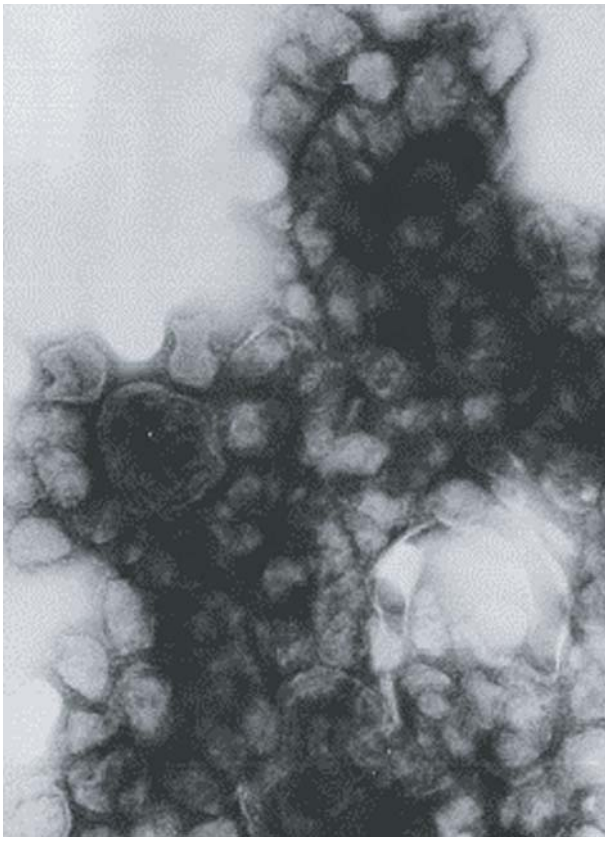
Another morphological effect was distortion of the morphology of the virus particles. After incubation with the lozenge for 1 min or longer at 37°C the virus spikes were indistinct and distorted (Figure 3). The outline of the particles, usually round or fusiform (Figure 4), became angular perhaps as the result of distortion during clumping.

The negative stain penetrated a low and equivalent proportion of virions at each of the times of incubation with the virucide or saliva control (1–16 min at 37°C), indicating no significant destructive effect on the viral lipid membrane by the virucide. Thus, quantitation of stain penetrated viruses from electron micrographs showed 78/287 penetrated virions in Strepsils®-treated virus compared to 56/259 stain penetrated virions in the control experiments.

Discussion

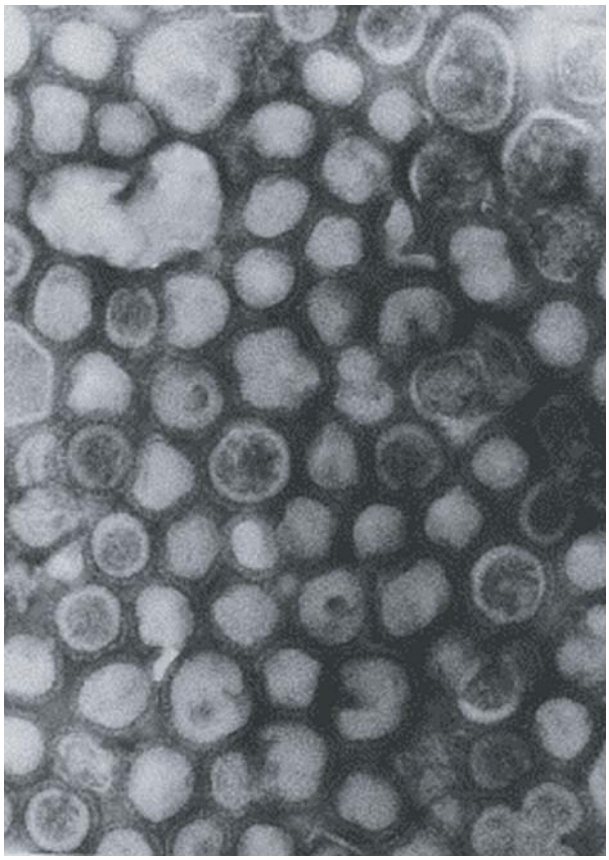
The study has established a virucidal destructive effect of a dissolved throat lozenge upon respiratory viruses of three distinct families, namely influenza, RSV and SARS-CoV. The virus destructive effect is rapid, occurring within 2 min of contact of the virus with the mixture at 37°C. Molecules which interact to solubilize the lipid bilayer of a virus such as cationic or non-ionic detergents would also be expected to destroy infectivity (Oxford *et al.*, 1971, 1994). However, the latter molecules would be expected to cause a complete

Figure 3. Clumped influenza A/Taiwan/77 (H1N1) virions and also virions showing distortion of shape after incubation for 4 min with the virucidal lozenge mixture



Magnification 45k approximately.

Figure 4. Control virions incubated for 4 min with artificial saliva buffer solution



Virions show clear and typical morphology with a fringe of HA and NA spikes. Magnification 45k approximately.

breakdown of the structure of a lipid virus such as influenza. Complete destruction of influenza virions was not visualized by electron microscopy in the present study, although we cannot exclude destruction of a minority of the virions which may not have been quantified. In contrast electron microscopy established that immediate clumping of virions occurred in the presence of the lozenge solution. In addition, some distortion of virion morphology was detected and there was some penetration of viral membrane by the negative stain.

Theoretically, there are several morphological explanations for the reduction in virus infectivity after incubation with the lozenge. Viral membrane damage by itself is not considered to be the main mode of action because there was only a low proportion of strain-penetrated virions (M Addawe, unpublished data) after incubation with the lozenge or control saliva for varying periods from 1–16 min at 37°C. Mild denaturation of the external protein spikes by χ metacresol and dichlorobenzyl alcohol may have led to clumping in the low pH conditions and may be a significant factor. The lozenge solution is at a low pH induced by tartaric acid and this could affect the structure of the influenza haemagglutinin, causing rearrangement of the protein's tertiary structure. This could, in turn, affect the infectivity of the virus and also result in virus clumping. Additionally, we cannot exclude osmotic pressure changes or stickiness induced by the sugar of the lozenge. Further studies are required to analyse the contribution of the individual constituents of the lozenge and, in particular, the effects of low pH and whether tartaric acid is an essential constituent, and also to investigate any effects of the constituents on viral replication *per se*.

The absence of virion-destructive effects on the non-enveloped viruses (adenoviruses and rhinoviruses) would also suggest some selective effect of the lozenge mixture on viral lipid membranes or viral protein–lipid interaction. The non-enveloped viruses are, in general, more heat and reagent stable (reviewed by Collier & Oxford, 2002).

Of particular interest would be deductions about the clinical relevance of the *in vitro* studies. It has been

commonly observed that individuals infected with influenza virus excrete 10^3 or 10^4 influenza virions per ml of throat washing (reviewed by Stuart-Harris *et al*., Schild & Oxford, 1985). It could be deduced that contact of the lozenge with these quantities of virus in the throat could cause rapid virus inactivation. The practical and clinical effect would be a potential reduction of spread of the virus to a contact. In addition, it is possible that reduction of virus infectivity titre *per se* in the throat could lead to a reduction in spread of the virus to the lower respiratory tract in the patient. Obviously, the lozenge would need to be administered over a period of 2 days. These clinical and virological parameters could be studied in a small pilot clinical trial with volunteer patients who have sore throats caused by influenza virus. Recently quarantine experiments with influenza A and B viruses have been recommenced in the UK [Fries *et al*., (2003) An activated trivalent influenza vaccine for intranasal administration, is protective in human challenge with A/Panama/2007/99 (H3N2) virus. *5th International Conference on the Options for the Control of Influenza.* October 2004, Bankoku Shinryokan, Okinawa, Japan].

SARS virus RNA has been detected in saliva and sputum in high concentrations (Drosten *et al.,* 2003, reviewed by Oxford *et al.,* 2003). This finding is unique for a respiratory virus but the cell type's precise localization for virus replication has yet to be established. However, inactivation of SARS virus in sputum by a lozenge may theoretically be expected to reduce any transmission by this route.

The recent outbreaks of emerging enveloped viruses such as SARS-CoV (Peiris *et al.,* 2003b; Lee *et al.*, 2003) and chicken influenza A H5 and H7 (Bridges *et al.*, 2002; Von Kolfschooten, 2003; Hien *et al.*, 2004) viruses has highlighted how quickly new pandemics may arise. In the case of SARS-CoV, which has a low reproductive factor whereby an infected person normally infects only two or three contacts, increased hygiene and cleanliness resulted in an abrupt termination of the outbreak. However, should either virus of the Myxoviridae or Coronaviridae re-emerge, a complete armentarium of antiviral drugs (reviewed by Oxford *et al.*, 2002), vaccines and virucides, either for surface disinfection or as throat lozenges, may have important public health applications.

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