Intranasal delivery of formulations containing virucidal lipids for treatment of respiratory syncytial virus (RSV) infection in rats

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A pharmaceutical formulation was designed which reduces the viral load of RSV in the nasal mucosa of rats after application immediately before inoculation and then daily for 4 days. The formulation did not cause irritation in the mucosal membranes or any other ill effects in intranasally treated rats. The formulation is composed of lauric acid and monocaprin as virucidal lipids, propylene glycol as a solvent, a polyisorbate as a surfactant and Carbopol 974P, which has bioadhesive properties causing the formulation to adhere to mucosal membranes. Formulations without lipids were also found to exhibit antiviral activity in the nasal mucosa although apparently less than the formulation with lipids which caused a significant reduction in the viral load compared with controls receiving salting (P < 0.01). The formulation may have potential as a nasal spray to suppress or ameliorate respiratory infections by RSV and other enveloped viruses.

Keywords: Respiratory syncytial virus — Nasal mucosa — Respiratory infections — Antimicrobial — Virucidal lipids — Lauric acid — Monocaprin — Pharmaceutical formulations — Nasal spray.

Respiratory syncytial virus (RSV) is a common cause of serious lower respiratory tract infection in infants and elderly people. There is no effective treatment for RSV infection. The drugs presently used against RSV and other viruses causing respiratory infections are only recommended for treatment of high-risk children or severely ill infants due to efficacy and safety concerns [1, 2]. Therefore, new prophylactic or therapeutic compounds for general use against respiratory viruses would be desirable.

Lipids, particularly fatty acids and monoglycerides, are known to have potent antimicrobial activity which have been thoroughly studied in vitro [3]. Thus, a recent study of the virucidal activities of fatty acids against RSV showed that the 12-carbon saturated (C12:0) lauric acid in a concentration of 10 mM inactivated the virus below a detectable level in 1 min. Similarly, 10 mM monocaprin, the 1-monoglyceride of capric acid (C10:0), inactivated RSV in 1 min and was the most active monoglyceride tested [4, 5]. Because of the high antiviral activity of lauric acid and monocaprin in vitro and their relatively low toxicity on mucous membranes [6, 7] it was considered of interest to study whether or not a formulation containing these lipids would reduce the titer of RSV on the nasal mucosa of rats experimentally infected with RSV. The formulation chosen for the nasal delivery of the lipids was a solution that contained a mucoadhesive polymer in order to increase the residence time of the lipid solution on the mucous membrane.

I. MATERIALS AND METHODS

1. Materials

Lauric acid (preamble grade) was purchased from Sigma Chemical Co., St. Louis, MO, United States. Monocaprin was obtained from Danisco/A/S, Copenhagen, Denmark as emulsifier TS-PH003 glycerol monooctenate (pharmaceutical grade). Propylene glycol was obtained from NMD, Norway. Carbopol 974P was purchased from BF Goodrich Europe, United Kingdom, and Tween 20 (polyoxyethylene sorbitan monolaurate) and Tween 40 (polyoxyethylene sorbitan monopalmitate) were obtained from ICN Biomedicals Inc. (Aurora, OH, United States) and Merck.

2. Nasal formulations

The nasal formulations used in the rat experiments contained 10 mM lauric acid and 10 mM monocaprin as virucidal ingredients, propylene glycol as a solvent, Tween 20 or 40 as a solubilizing agent and Carbopol 974P, which has bioadhesive properties causing it to adhere to mucosal membranes. The pH was adjusted to 7.0 by dropwise addition of 1 M NaOH.

3. Virus strains, cell culture and media

RSV strain A2 was obtained from Imperial College of Science, Technology and Medicine at St Mary’s Hospital, London, United Kingdom. The virus was grown in MA-104 cells (African green monkey kidney cell line). The cell culture medium was RPMI-1640 (Gibco) with 2 mM L-glutamine, 0.05 mg/mL gentamicin, 0.375 % (w/v) sodium bicarbonate and 10 % heat-inactivated fetal bovine serum (FBS). The maintenance medium (MM) contained 2 % FBS and was used in cell cultures for growth and titration of virus.

4. Testing of virucidal activity

Samples of nasal formulations were thoroughly mixed for 1 min with an equal volume (100 µL) of virus in polystyrene tubes (Falcon). The virucidal action was then stopped by dilution of the mixture in MM and the virus titer determined by inoculation of 10-fold dilutions in MM onto monolayers of MA-104 cells in 96-well microtiter tissue culture plates (Nunc, Roskilde, Denmark). One hundred microlitres of each dilution were inoculated into quadruplicate wells. The plates were incubated at 37 °C in a humidified incubator with 5 % CO2 in air and examined for cytotoxic effect (CPE) for 10 days. Virus titers (log10 50 % cell culture infective dose (CCID50/100 µL) were calculated by the method of Reed and Muench [8]. The virucidal activity of nasal formulations was measured by the reduction in virus titer compared to a virus control in MM.

5. Rats

Female Sprague Dawley (SD) rats (Taconic, Denmark, bred at the Institute for Experimental Pathology at Keldur, University of Iceland)
were used in the experiments at the age of 12-20 weeks. The animals were housed in the veterinary care facility of the Institute at Keldar and all experiments were approved by the Icelandic Food and Veterinary Authority (MAST).

6. Treatment of rats before and after inoculation with RSV

Rats were randomized in groups of \( n = 2-4 \) in separate cages and lightly anesthetized by subcutaneous injection with 400 \( \mu \)L of hypnorm (Vetapharm Ltd., Leeds, United Kingdom), diluted 10 fold in water. They were inoculated intranasally with 50 \( \mu \)L of RSV A2 in each nostril by a pipette tip. The titer of the virus was approximately 5.5 log10 CCID50/100 \( \mu \)L. The virus inoculation was performed 3-4 min after intranasal injection of 50 \( \mu \)L of nasal formulation with or without lipids, using a pipette tip. The rats were weighed daily and treated intranasally with nasal formulations for 4 days. They were euthanized on day 5 by injection with pentobarbital. Following euthanasia, the nasal mucosa was immediately removed and homogenized in Downs glass homogenizers in 1 mL of MM. Alternatively, the mucosae were stored in 200 \( \mu \)L of Depo-water (Merck) under liquid nitrogen for later processing. The mucosal homogenates were placed in Eppendorf tubes and spun for 4 min at 10,000 rpm at 4 °C. Samples of the supernatant were titrated by inoculation of 10-fold dilutions in MM onto MA-104 cells as described previously. Alternatively, supernatants were assayed by plaque assay.

7. Plaque assay of viable RSV from rat nasal mucosa

Ten-fold dilutions of supernatants from homogenized mucosal tissue were inoculated into 12-well microtiter tissue culture plates (Nunc) with monolayers of MA-104 cells. Four hundred microlitres of each sample dilution were inoculated into duplicate wells and incubated at 37 °C in an atmosphere of 5% CO2. After 2 h, the sample dilutions were removed with a pipette, each well washed with 0.5 mL of MM and covered with an overlay of 1% CMIC (sodium carboxymethyl cellulase, Gibco) in MEM-HEPS with 2% FBS. After 5 days of incubation at 37 °C, 1.5 mL of 1% formaldehyde (Merck) and 20% ethanol (Merck) in PBS were added to the overlay and the cultures fixed for 1 h at room temperature. The overlay was then removed and 0.5 mL of a 0.04% solution of crystal violet (Merck) in water was added to the wells for staining. After incubation for 10 min at room temperature, the plates were washed three times with PBS and the plaques counted under a light microscope.

8. Statistical analysis

Differences in viral titers among samples were analyzed by ANOVA. Values of \( P < 0.05 \) were considered statistically significant.

II. RESULTS AND DISCUSSION

The composition and in vitro activities of two lipid-containing formulations, one with 0.8% Tween 40 (A) and the other with 1% Tween 20 (B), are shown in Table I. Both were active in killing RSV, causing a reduction in virus titer of 4 log10 (A) and 4.5 log10 or greater (B) upon contact for 1 min, compared with the MM control. Formulations without lipid caused a less than 1 log10 reduction in virus titer. Testing of the formulation ingredients in vitro against the virus showed no virucidal effect of Carbopel 974P, PG or Tween in the concentrations used (results not shown).

Table II summarizes the results of two experiments carried out in rats using formulations A and B. In both experiments, the virus in infected mucosa was quantified by endpoint titration on MA cells. There was an apparent but not significant difference in RSV titers in nasal mucosa from rats treated with lipid-containing formulations compared with rats receiving formulations without lipids. Infection with virus and treatment with nasal formulations did not affect the weight of the rats (data not shown) and macroscopic examination of

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**Table I** - Composition of formulations A and B used in rat experiments (Table II). All contained 0.1% Carbopol 974P, 5% propylene glycol and were adjusted to pH 7. Titors (log10 CCID50/100 \( \mu \)L) from 1 min in vitro testing of the formulations against RSV A2 are shown.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Lauric acid</th>
<th>Monocaprin</th>
<th>Tw 40 (%)</th>
<th>Tw 20 (%)</th>
<th>RSV A2 titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>10 mM</td>
<td>10 mM</td>
<td>0.8</td>
<td>0</td>
<td>2.0</td>
</tr>
<tr>
<td>B</td>
<td>10 mM</td>
<td>10 mM</td>
<td>0.8</td>
<td>1.0</td>
<td>5.3</td>
</tr>
<tr>
<td>B</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>≤1.52</td>
</tr>
<tr>
<td>RSV control</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>6.0</td>
</tr>
</tbody>
</table>

*Formulations A and B used in rat experiments (Table II). *ar|t indicates that no virus was detected in 100 \( \mu \)L of the 10° dilution which was the lowest dilution tested due to toxic effects on MA-104 cells. Virus control was incubated with MM instead of formulation for 1 min.

**Table II** - Treatment of rats with formulations A and B (Table I). Mean virus titers ± SD are shown as log10 CCID50/100 \( \mu \)L of extract from nasal mucosa.

<table>
<thead>
<tr>
<th>Rat experiment, ( n = 2 )</th>
<th>With lipids</th>
<th>Without lipids</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSV titer</td>
<td>0.85 ± 0.21</td>
<td>1.50 ± 0.28</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Rat experiment B, ( n = 3 )</th>
<th>With lipids</th>
<th>Without lipids</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSV titer</td>
<td>1.60 ± 0.61</td>
<td>3.23 ± 0.38</td>
</tr>
</tbody>
</table>

The number of rats in each group. *A significant difference in the virus titers between groups treated with formulations with or without lipids (ANOVA).

In these experiments, viable virus from harvested nasal mucosa was measured by CCID50 titration, effected by the inoculation of 10-fold dilutions of mucosal extracts onto MA-104 cells and evaluation of the CPE. The MA-104 cell culture plates were examined daily for 10 days before the final virus titers were calculated. In both studies, CPE appeared earlier and was more widespread in the cell monolayers inoculated with dilutions of extracts from rats treated with formulations without lipids. Since endpoint titration used in these experiments is based on the presence or absence of CPE, it did not accurately quantify the viral load in the extracts. This raised the question of measuring the viral load by a more accurate assay. Therefore, a rat experiment was carried out in which virus was quantified by plaque assay. In this experiment, two groups of four rats each were used. One group was treated with formulation B containing lipids. The second group received 0.9% saline injected into the nostrils and served as a control. This experiment was carried out in the same way as the previous experiment (Table II), except that the extracts from nasal mucosa were tested in plaque assay. The results are shown in Table III. The group treated with the nasal formulation showed a significant reduction in viral plaque forming units compared with the saline group (P < 0.01). Therefore, treatment with a formulation containing lauric acid and monocaprin

**Table III** - Treatment of rats with virucidal formulation B. The control group received 0.9% saline. Virus titers (means for 4 rats ± SD) are shown as plaque forming units (pfu)/100 \( \mu \)L of extract from nasal mucosa.

<table>
<thead>
<tr>
<th>Rat experiment, ( n = 4 )</th>
<th>Formulation B</th>
<th>Control (0.9% saline)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSV pfu</td>
<td>1.82 ± 0.50</td>
<td>3.32 ± 0.13</td>
</tr>
</tbody>
</table>

The number of rats in each group. *A significant reduction in the virus titer of the group treated with formulation B compared to the control group (P < 0.01) ANOVA.
as virucidal ingredients, propylene glycol as a solvent, Tween 20 as a solubilizing agent and Carbopol 974P as a bioadhesive significantly reduced the viral load in the nasal mucosa of rats intranasally infected with RSV. The nasal mucosa of rats treated with the virucidal formulation showed no signs of abnormalities in contrast to the nasal mucosa of rats receiving saline which showed redness and a slight swelling, indicating active infection. Thus, the formulation containing lauric acid and monacaprin was well tolerated by the animals and did not cause weight loss or other physical abnormalities.

Several studies have addressed the question of whether or not lipids, particularly free fatty acids, may serve in the natural defense of the skin and mucosal membranes against pathogens. These studies have recently been reviewed [9, 10]. Antimicrobial lipids have been found in bronchoalveolar lavage of many animals, including humans, in lung surfactants and in breast milk. Investigations suggest that they may be active in extracellular clearance of bacteria in the lungs, and free fatty acids have been found in rat bronchoalveolar lavage in sufficient amounts to show antibacterial activities in vitro [11, 12]. The idea of applying free fatty acids, or other microbial lipids which inactivate RSV and parainfluenza virus, to respiratory mucosa may therefore not seem farfetched since this might enhance a natural defense by lipids already present in the mucosa.

The nasal mucosa is known to be an important site of RSV entry into the body [13], the infection spreading from there through the pharyngeal entrance to the lower respiratory tract. A reduction in the RSV load in the nasal mucosa can therefore be assumed to decrease the possibility of viral spread to the lungs. The present experiments show that intranasal administration of a formulation containing virucidal lipids significantly (P > 0.01) lowers the RSV load in the nasal mucosa of experimentally infected rats compared with saline, particularly if given as a prophylactic measure. Such treatment may therefore reduce the likelihood of the virus entering the lungs and resulting in serious infection. Formulations without virucidal lipids, or their separate ingredients, did not inactivate RSV in vitro or had only a minor effect, whereas lipid formulations were highly virucidal (Table I). In contrast, there was no significant difference in the viral titers of inoculated nasal mucosa treated with either formulation, although those treated with formulations without lipids apparently contained less virus after treatment for four days (Table I). Possibly, the non-lipid ingredients inhibit viral adsorption or replication in the infected mucosa and thus enhance the antiviral activity of the formulation. Since lipids are active against other respiratory viruses, such as parainfluenza and influenza viruses [4, 5], a topical treatment with formulations containing virucidal lipids may have a prophylactic effect against these viruses as well. More studies need to be conducted to further explore the use of formulations with virucidal lipids in topical treatments against respiratory infections in experimental animals or in humans.

REFERENCES


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