Killing of *Chlamydia trachomatis* by Novel Antimicrobial Lipids Adapted from Compounds in Human Breast Milk

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The development of new methods for prevention of sexually transmitted *Chlamydia trachomatis* infection is a top public health priority. Topical self-administered vaginal microbicides represent one such approach in which the organism is eradicated at the time of initial exposure. To this end, we examined the activity of five synthetic lipids adapted from naturally occurring compounds found in human breast milk. *C. trachomatis* serovar D or F elementary bodies were added to serial dilutions of the lipids and incubated for various times. Aliquots were then cultured in monolayers of McCoy cells, and inclusions were counted. A 7.5 mM concentration of 2-0-octyl-sn-glycerol completely prevented growth of *C. trachomatis* after 120 min of contact with the organism. The remaining lipids, 1-O-octyl-, 1-O-heptyl-, 2-O-hexyl-, and 1-O-hexyl-sn-glycerol, showed less activity. On electron microscopic examination, the lipids were shown to have disrupted the chlamydial inner membrane, allowing leakage of the cytoplasmic contents from the cell. Lipid activity was unaffected by the presence of 10% human blood or alterations in pH from 4.0 to 8.0, conditions reflecting those sometimes found in the vagina. Our results suggest that these lipids, especially 2-0-octyl-sn-glycerol, may be effective as topical microbicides in preventing the transmission of *C. trachomatis*. Further efficacy and toxicity studies with these lipids and assessment of their activity against other sexually transmitted disease pathogens are in progress.

As many as 12 million individuals in the United States are infected with sexually transmitted diseases (STDs) each year, and many of these infections result in serious reproductive sequelae (3). Since current prevention strategies have been only partially successful, the use of topical microbicides offers a new approach to the prevention of STDs that is particularly attractive in that they are broad spectrum in their activity and can be self administered by women before sexual contact. An ideal microbicidal would prevent STDs caused by bacterial pathogens such as *Chlamydia trachomatis* and *Neisseria gonorrhoeae*, as well as those caused by human immunodeficiency virus, human papillomavirus, herpes simplex viruses, and the parasitic *Trichomonas vaginalis*. In addition, such preparations should be spermicidal but should not disrupt the normal flora or be toxic to the vaginal epithelium. Since *C. trachomatis* is the most common bacterial cause of STDs in the United States (1), accounting for at least one-third of all these infections, we have focused our efforts on this pathogen.

Human breast milk contains numerous antimicrobial compounds (5), some of which are lipid based. We have modified some of these antimicrobial lipids to increase their stability and aqueous solubility while maintaining their antimicrobial activity and then synthesized these compounds for use in antimicrobial assays. These novel lipids have been shown to have inhibitory activity against *Escherichia coli*, *Salmonella enteritidis*, and *Staphylococcus epidermidis* (4), but their activity against *Chlamydia* is unknown. *C. trachomatis* is transmitted from an infected to an uninfected individual in genital secretions via sexual contact. Even though *C. trachomatis* is an obligate intracellular bacterium, the elementary body (EB) or infectious form of the organism is found extracellularly in secretions. EBs are adapted for survival in cell-free conditions but are susceptible in vitro to various antimicrobials such as deterrents (7), peptides (11), whole human milk, and fractions of it (2). We undertook these studies to examine whether genital strains of *C. trachomatis* were killed by these novel antimicrobial lipids adapted from human breast milk. Five lipids, including 1- and 2-0-hexyl-sn-glycerol, 1-O-heptyl-sn-glycerol, and 1- and 2-0-octyl-sn-glycerol, were examined in an in vitro *C. trachomatis* viability assay and assessed for their morphologic effects upon *C. trachomatis* EBs by electron microscopy.

![Chemical structures of antimicrobial lipids](http://aac.asm.org/)

**FIG. 1.** Structures of 1-O-hexyl-, 2-O-hexyl-, 1-O-heptyl-, 1-O-octyl-, and 2-O-octyl-sn-glycerol.
controls, respectively, at an initial concentration of 2 mg/ml in the preinoculation
15 to 30 s to ensure their even distribution.
(10.2 mg/ml) in SPG or EMEM. After each dilution, the lipids were vortexed for
routinely once per month for mycoplasma contamination.
medium with 10% fetal calf serum (EMEM). The McCoy cells were checked
(157C CRL 1696) were maintained in antibiotic-free Eagle’s minimal essential
stored at 270°C in SPG (219 mM sucrose, 3.8 mM KH2PO4, 8.6 mM Na2HPO4,
4.9 mM glutamic acid [pH 7.0]). Immediately prior to use, the purified organisms
were thawed and diluted in SPG.
Novel lipids. Five lipids, including 1- and 2-O-hexyl-sn-glycerol, 1-O-heptyl-
sn-glycerol, and 1- and 2-O-octyl-sn-glycerol, were tested. The structures of these
lipids are shown in Fig. 1. The lipids were designed by C. E. Isaacs and synthe-
sized by Deva Biotech (Hatboro, Pa.). Each lipid was received as a 100
solution in 100% ethyl alcohol (EtOH). The 1- and 2-O-hexyl-sn-glycerol ethers
were diluted to an initial concentration of 15 mM (2.64 mg/ml), and the 1-O-heptyl-
sn-glycerol ether and 1- and 2-O-octyl-sn-glycerol ethers were diluted to 50 mM
(10.2 mg/ml) in SPG or EMEM. After each dilution, the lipids were vortexed for
15 to 30 s to ensure their even distribution.
Controls. Polymyxin B and penicillin G were used as positive and negative
controls, respectively, at an initial concentration of 2 mg/ml in the preinoculation
assays. EMEM and EtOH were used as controls in the alamarBlue cell toxicity
assay. All controls were prepared fresh on the day of the assay.
Preinoculation assay. C. trachomatis serovars D or F (106 inclusion-forming
units [IFU]) in SPG was added to dilutions of each lipid or positive or negative
counterpart organisms (polymyxin B and penicillin G) and incubated for 0, 30, 60, 90,
or 120 min. After each time period, a 5-μl aliquot of the organism-drug mixture
was diluted 1:40 in 195 μl of SPG. One hundred microliters of this dilution was
then added to a McCoy cell monolayer in a 96-well microtiter plate and centri-
fuged for 1 h to inoculate the tissue culture cells. The organism-drug mixture
was removed and replaced with EMEM containing 1 μg of cycloheximide per
ml. The cultures were incubated for 48 h and stained with the Chlamydia genus-
specific fluorescein isothiocyanate-labeled monoclonal antibody CF2, and the
chlamydial inclusions in three fields were counted. All assays were performed in
triplicate, the inclusion counts were averaged, and the polymyxin B positive
counterpart and the penicillin G negative control were run in parallel. Additional
wells were inoculated either with the C. trachomatis organisms only, which served
as an organism control, or with SPG only, to monitor McCoy cell morphology. Percent killing in assays with lipid, the polymyxin B positive control, and the
penicillin G negative control were calculated by the following formula: [([mean
IFU of organism control — mean IFU of test]) — (mean IFU of organism control)]
× 100. The lowest concentration of lipid which showed 100% killing was defined
as the minimal cidal concentration (MCC). One hundred percent killing repre-
sents a decrease of at least 104 organisms, from the original 106 IFU in the
inoculum to 100 IFU, the minimum number of IFU which can be counted in our
assay.
alamarBlue cytotoxicity assay. The preinoculation assay was duplicated except
that no Chlamydia organisms were added. Lipid dilutions were further diluted
1:40 in EMEM as in the preinoculation assay and then added to 24-h monolayers
of McCoy cells in 96-well microtiter plates and incubated for 1 h. Lipid dilutions
were aspirated from the wells and replaced with EMEM containing 1 μg of
cycloheximide per ml, and cultures were incubated for 4 h. alamarBlue (Alamar
Biosciences, Inc., Sacramento, Calif.) was then added to each well, and the cells
were incubated for 4 h. alamarBlue is chemically reduced by the metabolic
activity of growing cells, which causes the fluorometric-colorimetric REDOX
indicator to change from an oxidized, nonfluorescent blue form to a reduced,
fluorescent red form. The optical densities of the wells (OD570 and OD600) were
read, and percent inhibition of McCoy cells was calculated in comparison to that
of EMEM negative controls by the following formula: percent inhibition
= [(mean OD570 — mean OD570 of negative cell control) — (mean OD570 — mean
OD570 of test)]/[(mean OD570 — mean OD570 of negative cell control)] × 100 (9).

Preinoculation assay in the presence of 10% human blood. The preinoculation
assay described above was used with the addition of 10% whole human blood.
Blood was collected from one of the authors who was not receiving antibiotics
and who has no anticlamlmlid antibodies, as measured by microimmunofluo-
rescence (10). The pH of the lipid dilutions was adjusted to pH 7.0. Assays were
performed only at 0- and 120-min time points. Ten percent human blood was also
added to the organism controls.

Preinoculation assay with pH alterations. The preinoculation assay described
above was followed except that lipid dilutions were adjusted to pH 4, 5, 6, 7, or
8 with 1 M Na2HPO4 or 1 M KH2PO4 before C. trachomatis IFU were added.
Again, assays were performed only at 0- and 120-min time points and all controls
were tested at each different pH value.
Electron microscopic examination of *C. trachomatis* exposed to lipids. The lowest concentration of each of the lipids showing 100% killing in the preinoculation assay was incubated with *C. trachomatis* EBs for 90 min. The treated organisms were pelleted, fixed with 2% glutaraldehyde, postfixed in 1% osmium tetroxide in deionized water, dehydrated in a series of alcohols, embedded in EMBed 812 (Electron Microscope Supplies, Chestnut Hill, Mass.), thin sectioned, stained with uranyl acetate and lead citrate, and examined in a Philips (Eindhoven, The Netherlands) model CM-10 transmission electron microscope. Organisms exposed to SPG were treated as described above and examined for typical morphology.

RESULTS

Comparison of the antichlamydial activities of the five lipids. The antichlamydial activities of all five lipids against *C. trachomatis* serovars D and F are shown in Fig. 2. It is important to note that the 2- and 1-octyl-sx-glycerol derivatives showed 100% killing at lower concentrations (7.5 and 15 mM respectively) whereas the 1-heptyl-sx-glycerol and 1- and 2-hexyl-sx-glycerol derivatives showed 100% killing only at higher concentrations (25 to 50 mM). Table 1 summarizes the MCCs of each lipid against *C. trachomatis* serovars D and F.

**TABLE 1. MCCs for all four lipids against *C. trachomatis* serovars D and F**

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Time (min)</th>
<th>MCC(^a)</th>
<th>Time (min)</th>
<th>MCC(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-O-octyl-sx-glycerol</td>
<td>90</td>
<td>7.5</td>
<td>120</td>
<td>7.5</td>
</tr>
<tr>
<td>1-O-octyl-sx-glycerol</td>
<td>30</td>
<td>15</td>
<td>120</td>
<td>7.5</td>
</tr>
<tr>
<td>1-O-heptyl-sx-glycerol</td>
<td>90</td>
<td>25</td>
<td>ND(^c)</td>
<td>ND(^c)</td>
</tr>
<tr>
<td>2-O-hexyl-sx-glycerol</td>
<td>90</td>
<td>50</td>
<td>120</td>
<td>12.5</td>
</tr>
<tr>
<td>1-O-hexyl-sx-glycerol</td>
<td>120</td>
<td>&gt;50</td>
<td>120</td>
<td>50</td>
</tr>
</tbody>
</table>

\(^a\) Time that organisms were exposed to lipid prior to inoculation.  
\(^b\) Concentration of lipid which caused 100% killing of *C. trachomatis* at indicated time point.  
\(^c\) ND, not done.

Determination of lipid cytotoxicity under preinoculation MCC assay conditions. The preinoculation assay described above includes a 1:40 dilution of the lipid-organism mixture prior to centrifugation of the inoculum. To ensure that the diluted lipid contained no residual toxicity for the McCoy cells used to culture *Chlamydia*, all dilutions of the lipids were tested in the alamarBlue cytotoxicity assay. Values were calculated in comparison to that of the organism-only control as described in Materials and Methods. Results for the polymyxin B positive and penicillin G negative controls are also shown.

**TABLE 2. 2-O-Octyl-sx-glycerol cytotoxicity under preinoculation MCC assay conditions**

<table>
<thead>
<tr>
<th>Concentration of lipid (mM)(^a)</th>
<th>Mean OD(<em>{570}) (\pm) mean OD(</em>{600}) (SD)</th>
<th>% Inhibition of McCoy cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3750</td>
<td>0.405 (0.064)</td>
<td>2.0</td>
</tr>
<tr>
<td>0.1875</td>
<td>0.366 (0.045)</td>
<td>11.5</td>
</tr>
<tr>
<td>0.0938</td>
<td>0.410 (0.052)</td>
<td>1.0</td>
</tr>
<tr>
<td>0.0469</td>
<td>0.406 (0.039)</td>
<td>1.9</td>
</tr>
<tr>
<td>0.0234</td>
<td>0.414 (0.041)</td>
<td>0.0</td>
</tr>
<tr>
<td>0.0118</td>
<td>0.414 (0.007)</td>
<td>0.0</td>
</tr>
<tr>
<td>0.0058</td>
<td>0.414 (0.0045)</td>
<td>0.0</td>
</tr>
<tr>
<td>0.0029</td>
<td>0.414 (0.038)</td>
<td>0.0</td>
</tr>
<tr>
<td>0.0014</td>
<td>0.406 (0.058)</td>
<td>1.9</td>
</tr>
<tr>
<td>0.0008</td>
<td>0.405 (0.032)</td>
<td>2.2</td>
</tr>
<tr>
<td>0 (no lipid)</td>
<td>0.414 (0.052)</td>
<td>0.0</td>
</tr>
</tbody>
</table>

\(^a\) Values determined by alamarBlue cytotoxicity assay.  
\(^b\) These values represent the millimolar concentrations of the lipid, diluted 1:40, that were plated directly onto McCoy cell monolayers.

FIG. 3. Comparison of 2-O-octyl-sx-glycerol activity against *C. trachomatis* serovars D and F. The percent killing of each serovar after exposure to 2-O-octyl-sx-glycerol for 0 and 120 min in the preinoculation assay is plotted, and standard deviations from triplicate tests are indicated by error bars. Values were calculated in comparison to that of the organism-only control as described in Materials and Methods. Results for the polymyxin B positive and penicillin G negative controls are also shown.
The development of a topical microbicide that is safe, inexpensive, easy to use, and effective against the most common STD pathogens could be of great importance in reducing the transmission of STDs. The lipids we have examined in these studies may contribute toward achieving this goal. The 2-O-octyl-sn-glycerol lipid was the most active of the five closely related lipids tested.

**DISCUSSION**

The fluid environment of the female genital tract, particularly the vagina, poses a unique challenge for the design and development of prophylactic agents to prevent sexually transmitted infections. The vaginal pH can vary widely, from acidic in the follicular and luteal phases of the menstrual cycle to alkaline during the menstrual period. The lipids we have examined in these studies may contribute toward achieving this goal. The 2-O-octyl-sn-glycerol lipid was the most active of the five closely related lipids tested.

**Lipid activity at pH 4, 5, 6, 7, and 8.** Because the pH can vary widely in the human vagina, we tested the activity of the lipids at pH 4, 5, 6, 7, and 8 against *C. trachomatis* serovar D for 0 and 120 min. As shown in Fig. 5, 2-O-octyl-sn-glycerol at a few pH-concentration combinations showed increased (pH 8, 3.75 mM) or decreased (pH 6, 7.5 mM) killing of *C. trachomatis* serovar D when the organism was exposed to the lipid for 120 min. In general, however, differences in pH did not have a major impact on 2-O-octyl-sn-glycerol activity (Table 3).

**Examination by electron microscopy of *C. trachomatis* exposed to lipids.** *Chlamydia* organisms exposed to the lowest concentration of each of the lipids that resulted in 100% killing in the preinoculation assay were examined by transmission electron microscopy. After incubation with the most active 2- and 1-O-octyl-sn-glycerol derivatives, only *C. trachomatis* ghosts containing outer membrane shells and no cytoplasmic contents remained (similar to the organism visible on the left in Fig. 6B). After incubation with the less active lipids, 2- and 1-O-hexyl-sn-glycerol derivatives, not only hollow ghosts but also organisms in the process of leaking their cytoplasmic contents could be seen (organism visible on the right in Fig. 6B). In both cases, it appeared that the inner membrane of *Chlamydia* was disrupted while most of the outer membrane remained intact. We did not examine the 1-O-heptyl-sn-glycerol activity by electron microscopy.
related lipids tested. A 7.5 mM concentration of this lipid killed *C. trachomatis* after 90 min of exposure to the organism. A 15 mM concentration of 1-O-octyl-sn-glycerol also killed *C. trachomatis* after 30 min of contact. The 1-O-heptyl-sn-glycerol and 1- and 2-O-hexyl-sn-glycerol derivatives were less active, either requiring higher concentrations or longer exposure times to kill *C. trachomatis* or showing incomplete killing at the concentrations and times examined. A 7.5 mM concentration of 2-O-octyl-sn-glycerol can be easily synthesized and incorporated into a vehicle suitable for self administration and is currently being formulated. Preliminary studies have shown no vaginal irritation in the rabbit model by lipid concentrations as high as 120 mM (5a). Since this concentration is well above the minimum necessary to kill *C. trachomatis* and can readily be synthesized, millimolar lipid concentrations are physiologically relevant for a topical microbicide. A further advantage of such a lipid preparation is its markedly lower cost in comparison to that of other antimicrobials such as peptides.

Lipids, which disrupt the lipid bilayers of pathogens, might also disrupt cell membranes they come into contact with, including the epithelial cells lining the human vagina. Toxicity of this type caused by lipids applied to mucosal surfaces would likely be at least partly prevented by the mucous layer. Under the conditions of our assay utilizing 1 h of contact of a 1:40 dilution of the lipid concentrations tested, we found that the lipids were not toxic to McCoy cells as determined by the alamarBlue test. However, toxicity of these lipids is an important issue which needs to be examined in future studies of humans and in animal models.

Since there are numerous different *C. trachomatis* serovars which can cause urogenital disease, we wanted to determine whether these antimicrobial lipids were active against different serovars. Of the lipids tested, none showed significant variability in activity when two different serovars, D and F, were tested. For example, the same concentration of 2-O-octyl-sn-glycerol necessary to kill serovar D (7.5 mM) was also completely active against serovar F. While additional strains and serovars need to be tested, these results suggest that a topical 2-O-octyl-sn-glycerol preparation would likely be broadly active against many or most of the *C. trachomatis* serovars associated with STDs.

In addition to lipids, there are other classes of antimicrobials such as naturally occurring antimicrobial peptides that could be used as topical microbicides. One potential problem with defensins and other antimicrobial peptides is their reduced activity in the presence of serum (8). During the menstrual period, topically applied defensins would come in contact with serum proteins contained in menstrual blood, thus diminishing their activity. The short-chain monoglycerides examined in these studies could bind to proteins such as albumin or to other proteins with fatty acid binding sites that are found in blood (4). However, the activity of the lipids examined in these studies was not decreased by the presence of 10% whole human blood. These results indicate that a topical lipid preparation would likely remain active even during the menstrual period, when other antimicrobial compounds such as defensins might have reduced activity.

The pH of the human vagina varies greatly, from pH 4 in healthy women to pH 5 to 6 in women with bacterial vaginosis, pH 7 in women who are postmenopausal, and pH 8 after

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>pHs</th>
<th>MCC (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (&lt;74% inhibition)</td>
<td>4, 5, 7</td>
<td>15</td>
</tr>
<tr>
<td>0</td>
<td>6, 8</td>
<td>15</td>
</tr>
<tr>
<td>120</td>
<td>4, 7, 8</td>
<td>7.5</td>
</tr>
<tr>
<td>120</td>
<td>5, 6</td>
<td>15</td>
</tr>
</tbody>
</table>
It is important to note that the inner membrane has lost its structural integrity.

-O-sn-glycerol for 90 min appear as hollow ghost-like structures. (A) C. trachomatis serovar D EBs exposed to SPG only and processed for microscopy. It is important to note the intact outer membrane structures and electron-dense cytoplasmic mass. (B) EBs exposed to 50 mM 1-O-hexyl-sn-glycerol at pH 7.1. It is important to note that the inner membrane has lost its structural integrity.

FIG. 6. Transmission electron micrographs of C. trachomatis serovar D treated with 1-O-hexyl-sn-glycerol. (A) C. trachomatis serovar D EBs exposed to SPG only and processed for microscopy. It is important to note the intact outer membrane structures and electron-dense cytoplasmic mass. (B) EBs exposed to 50 mM 1-O-hexyl-sn-glycerol for 90 min appear as hollow ghost-like structures. It is important to note that the inner membrane has lost its structural integrity.

semen is deposited. Since the ideal topical antimicrobial should be effective at all of these different pH values, we examined the activity of the lipids at pH 4, 5, 6, 7, and 8. There are some changes in the activity of 2-O-octyl-sn-glycerol at pH values other than 7, but these changes are likely not biologically significant. Our results indicate that a topical lipid preparation would be active under a wide variety of pH conditions in the female genital tract.

Antimicrobial lipids are thought to be active because they disrupt lipid bilayers. The electron micrographic examination of Chlamydia treated with these lipids confirms the results in the preinoculation MCC assays. The lipids were observed to disrupt the chlamydial inner membrane, allowing the cytoplasmic contents to leak out of the cell and confirming that the lipids are directly active against the organism. The absence of cellular toxicity observed in the alamarBlue test is also consistent with the conclusion that the antichlamydial activity of these lipids results directly from disruption of the chlamydial membrane and not from toxicity to the McCoy cells.

In conclusion, these lipids, which are related to the antimicrobial monoglyceride esters found in human breast milk (6), are able to kill C. trachomatis directly; 2-O-octyl-sn-glycerol is the most active of those tested. Further tests to determine whether they are active against other chlamydial strains and other STD pathogens are under way. Given further testing of toxicity and efficacy in humans, it is possible that these lipids would make an effective topical microbicide to kill sexually transmitted Chlamydia on contact at the time of sexual transmission, before infection occurs. These naturally occurring compounds, found in humans at mucosal membranes, are likely to be relatively nontoxic and may be well suited for this proposed use.

ACKNOWLEDGMENTS

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REFERENCES