In Vitro Inactivation of Chlamydia trachomatis by Fatty Acids and Monoglycerides

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Chlamydia trachomatis is the most common sexually transmitted bacterial pathogen. Annually, there are an estimated 50 million new cases of C. trachomatis infection worldwide (4), with more than 4 million occurring in the United States and 3 million occurring in Europe (2). Although they are treatable with antibiotics, many Chlamydia infections go undetected, particularly in women, and can cause severe permanent damage to the female genital tract, which may lead to infertility (18). A vaccine against C. trachomatis has not been developed, and other means of prevention except for the use of condoms are not available. In recent years there has been considerable interest in the use of microbicidal compounds for the prevention of sexually transmitted diseases (STDs) (3). Vaginal spermicides, which have been used as contraceptives for a number of years, have been shown to kill sexually transmissible bacteria and viruses in vitro and in vivo (13). The microbicidal activity of the nonionic surfactant nonoxynol-9 has been studied most extensively. Two studies found that nonoxynol-9 inactivates C. trachomatis in vitro (1, 9), whereas one study found that it did not (8). There is evidence from clinical trials that it may have some effect against chlamydial infections in vivo (12). However, because of the toxicity of nonoxynol-9 to mucosal membranes, particularly when it is applied frequently, there is a need for other less toxic microbicidal compounds which could be used to provide protection against STDs (3).

The microbicidal effects of a variety of lipids have been extensively studied in recent years. A number of free fatty acids and their 1-monoglycerides have been found to inactivate enveloped viruses and various bacteria, both gram-negative and gram-positive bacteria (5, 7, 14, 15, 17). These lipids are commonly found in natural products, for example, in milk, and can therefore be assumed to be nontoxic to mucosas, at least at low concentrations. It has therefore been suggested that they might be useful as intravaginal microbicides for protection against STDs (6). In order to prevent infections caused by sexually transmitted viruses, it is important that the microbicidal lipid is fast acting and kills the virus before it has time to infect cells of the genital mucosa. The same is true for bacteria such as Chlamydia, which, like viruses, replicate intracellularly. In the present study several fatty acids and their 1-monoglycerides which have previously been found to inactivate enveloped viruses (15) were tested for their microbicidal activities against C. trachomatis (15). A short inactivation time of 10 min or less was selected as a criterion for a fast and effective killing of the bacteria.

MATERIALS AND METHODS

Cell culture. Monolayer cultures of McCoy cells, a heteroploid mouse fibroblast cell line, were used for cultivation of C. trachomatis and in antichlamydial assays. They were grown in RPMI 1640 medium (GIBCO) containing 5% (vol/vol) heat-inactivated fetal calf serum, 45 mM sodium bicarbonate, 2 mM L-glutamine, and 0.05 mg of gentamicin per ml. This was called the base medium (BM).

The cell cultures were maintained by weekly trypsinization and passage in 260-ml tissue culture flasks (Nuncolon). The cultures were passaged in the following manner. The medium was removed and the monolayer was rinsed twice with 10 ml of Hank's balanced salt solution (GIBCO). The monolayer was covered with 1 ml of trypsin-EDTA solution (GIBCO) and was carefully shaken until the cells came into suspension. The trypsin activity was then stopped by adding 10 ml of BM, and the cells were evenly suspended by pipetting. They were counted, and 105 cells in 20 ml of BM were seeded into each flask. Cell cultures were kept at 37°C in a humidified incubator with 5% CO2 in air.

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**RESULTS**

**Activities of fatty acids.** A comparison of the antichlamydial activities of six fatty acids is presented in Fig. 1. The open bars represent the reduction in infectivity titers \( \log_{10} \) IFU of *C. trachomatis* after incubation with an equal volume of 20 mM fatty acid for 10 min at 37°C. Caprylic acid (8:0), myristic acid (14:0), and the unsaturated fatty acids palmitoleic (16:1) and oleic acid (18:1) did not cause a significant inactivation of the bacteria, with the reduction in titer varying from zero to 0.5 \( \log_{10} \). In contrast, capric acid (10:0) and lauric acid (12:0) reduced the titer by greater than 10,000-fold (≥4 \( \log_{10} \)).

To compare the activities of capric and lauric acids, they were tested at lower concentrations and with shorter incubation times. The data in Table 1 indicate that capric acid lost most of its activity when it was diluted to a concentration of 10 mM, whereas lauric acid was still very active at that concentration, causing a 500,000-fold reduction in titer (5.7 \( \log_{10} \)). Lauric acid was therefore more active than capric acid against *C. trachomatis*. This was confirmed by testing lauric acid at 5 mM, which caused a 2,000-fold (3.3 \( \log_{10} \)) reduction in infectivity titer. Both acids were active at 20 mM for 5 min, but incubation for 1 min had no effect.

**Activities of monoglycerides.** The antichlamydial activities of five 1-monoglycerides were tested, with monomyristin (14:0) being omitted. The results are presented in Fig. 1. Monocaprylin (14:0), monopalmitylamine (16:1), and monosonoien18:1) had no significant effect, and monolinolein (12:0) caused only a three-fold reduction in titer (0.5 \( \log_{10} \)). Monocaprin, on the other hand, caused 40,000-fold or greater reductions in the infectivity titer of *C. trachomatis* (≤4.6 \( \log_{10} \)). The high level of antichlamydial activity of monocaprin was confirmed by low-
Dilution tested because of toxicity to cell cultures.

To 1:1 dilution with the bacteria.

seen that most of the bacteria were recovered after washing, not washed with those in samples that were washed, it can be caprin was further confirmed by staining with fluorescein-la-

$\text{active at 5 mM with an incubation time of 5 min, causing a time to 5 min.}$

As indicated in Table 2, monocaprin was still ering the concentration to 10 and 5 mM and the incubation time to 5 min. As indicated in Table 2, monocaprin was still active at 5 mM with an incubation time of 5 min, causing a greater than 100,000-fold reduction in the infectivity titer ($\approx 5.1 \log_{10}$). The high level of antichlamydial activity of monocaprin was further confirmed by staining with fluorescein-la-

beled monoclonal antibodies specific for Chlamydia. Monocaprin-treated Chlamydia samples showed no inclusions at the lowest dilution tested ($10^{-1}$), whereas control samples without monocaprin showed fluorescent Chlamydia inclusions at a dilution of $10^{-6}$ and had a final infectivity titer of $10^{-3}$ IFU per ml. The reduction in titer was therefore 400,000-fold or greater ($\approx 5.6 \log_{10}$). In order to show that the antichlamydial activity of monocaprin was not limited to the one bacterial strain obtained from the American Type Culture Collection, two Chlamydia strains recently isolated from patients were also tested. Both strains were inactivated by monocaprin at a concentration of 10 mM and incubation for 5 min, with the reductions in titer being $\approx 3.4$ and $\approx 3.7 \log_{10}$, respectively.

In another experiment the lipid was removed by centrifugation and the bacteria were washed in culture medium before inoculation of 10-fold dilutions onto cell monolayers. The results are presented in Table 3. By comparing the titers ($\log_{10}$ numbers of IFU) of the untreated control samples that were not washed with those in samples that were washed, it can be seen that most of the bacteria were recovered after washing.

<table>
<thead>
<tr>
<th>Monoglycerides</th>
<th>Conc (mM)$^a$</th>
<th>Reduction of IFU ($\log_{10}$) at the following times (min):</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Monocaprin (10:0)$^b$</td>
<td>20</td>
<td>$\approx 5.9$</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>ND$^d$</td>
</tr>
<tr>
<td>Lauric acid (12:0)</td>
<td>20</td>
<td>$\approx 4.2$</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>5</td>
</tr>
</tbody>
</table>

$^a$ Concentration of fatty acids mixed with Chlamydia and incubated at 37°C for 1, 5, and 10 min. The concentration in the mixtures was reduced by half.  
$^b$ No inclusions were detected in the $10^{-2}$ dilution, which was the lowest dilution tested.  
$^c$ No inclusions were detected in the $10^{-1}$ dilution.  
$^d$ ND, not done.

that is, recoveries of 100 and 50% in the samples obtained at 5 and 10 min, respectively. As expected from previous experiments (Table 2), no inclusion-forming bacteria were detected in the 1:10 dilution of unwashed samples after treatment with monocaprin for 5 and 10 min. Thus, the loss of infectivity was about 100,000-fold or greater, i.e., $\approx 4.9 \log_{10}$ IFU after 5 min and $\approx 5.0 \log_{10}$ IFU after 10 min. The same result was obtained after washing of the bacteria treated with monocaprin for 10 min. In this sample no inclusion-forming bacteria were observed in the 1:10 dilution. The titer was therefore $\leq 1.7 \log_{10}$ IFU per ml, whereas the titer in the washed untreated control was 6.4 $\log_{10}$ IFU per ml. On the other hand, in the sample treated with monocaprin for 5 min, inclusion-forming bacteria became detectable in the 1:10 dilution after washing. The sample incubated for 5 min still showed an 8,000-fold (3.9 $\log_{10}$) reduction in infectivity titer compared to that for the untreated control.

Electron microscopy of monocaprin-treated Chlamydia. Figure 2 shows electron micrographs of elementary bodies (EBs) of Chlamydia treated with 10 mM monocaprin for 1, 5, and 10 min. Untreated bacteria are shown for comparison (Fig. 2A). Bacteria treated for 1 and 5 min (Fig. 2B and C, respectively) are not visibly different from the untreated bacteria. On the other hand, after treatment for 10 min (Fig. 2D) the EBs appear deformed and shrunken, and there is some indication that some of them are disintegrating (Fig. 2D, inset). At a lower magnification it could be seen that in both treated and untreated samples the bacteria are well dispersed on the grid and there is no aggregation of EBs (data not shown).

MICS. The effects of a series of monocaprin concentrations on Chlamydia upon contact for 2 h at 37°C are presented in Table 4. A concentration of 50 µg per ml (0.2 mM) caused a 96% inactivation of the bacteria, whereas after treatment with 25 µg per ml, 38% of the bacteria were unable to form inclusions. There was less inactivation at lower concentrations. The 50% effective concentration was about 30 µg per ml. Monocaprin at a concentration of 100 µg per ml caused complete lysis of the cell layers in 2 h, whereas at 50 µg per ml and lower concentrations, no lysis was observed by the trypan blue exclusion test. The bacteria were therefore about 2.5 times more sensitive to the lipid than the host cells. It should be noted that mixtures of Chlamydia and monocaprin were diluted 100-fold before inoculation onto McCoy cells. In the tests, the cell monolayers were therefore exposed to monocaprin at concentrations of 25 µg per ml or less.

**DISCUSSION**

Previous studies have shown that medium-chain saturated fatty acids and long-chain unsaturated fatty acids and their 1-monoglycerides are potent inhibitors of enveloped viruses and
bacteria, both gram-negative and gram-positive bacteria (5, 14). In this study we have shown that *C. trachomatis*, a sexually transmitted, gram-negative bacterium, is effectively inactivated by exposure for 10 min to 10 mM (final concentration) lauric acid, a 12-carbon saturated fatty acid (12:0), and to capric acid (10:0) and its 1-monoglyceride. The monoglyceride of lauric acid had much less of an effect, and a number of other fatty acids and their monoglycerides, i.e., caprylic acid (8:0), myristic acid (14:0), and the unsaturated fatty acids palmitoleic acid (16:1) and oleic acid (18:1), had no effect or only a negligible effect.

The narrow range of activities of the fatty acids and monoglycerides against *Chlamydia* is notable and suggests that these lipids have specific antichlamydial effects. A somewhat wider range and higher levels of activity of fatty acids and monoglycerides have been found against herpes simplex virus type 1 (HSV-1), against which, in addition to monocaprin and lauric acid, palmitoleic acid and, to some extent, oleic acid cause a rapid inactivation of the virus (10). Capric acid, on the other hand, had no activity against HSV-1 under the same conditions.

The question of how monocaprin inactivates the infectivity of *Chlamydia* was addressed by studying whether or not removal of the lipid before inoculation into cell cultures restored the infectivity. The results (Table 3) indicate that the loss of infectivity was not caused by an effect of the lipid on host cells and that the viability of the bacteria was irreversibly lost by treatment with monocaprin. After treatment for 5 min a small viable fraction became detectable by washing, showing that the bacteria were not fully inactivated at this time. This is in agreement with the electron microscopy study (Fig. 2), which showed no visible changes in the EBs after treatment with 10 mM monocaprin for 5 min, whereas after 10 min the EBs appeared deformed and partly disintegrated. We therefore hypothesize that the lipid kills the bacteria by affecting the outer membrane, leading to disruption of the membrane(s) in 5 to 10 min. This is supported by a previous electron microscopy study of the effect of linoleic acid on vesicular stomatitis virus and on Vero cells, in which the viral envelope and the cellular membrane were disrupted by the fatty acid (15).

In a recent study (11) ethers of 6- and 8-carbon fatty acids were tested against *C. trachomatis*. 2-O-Octylglycerol, which was the most active of the four ethers tested, caused a complete inactivation of the bacteria in 2 h at a concentration of 7.5 mM but was apparently not fully active at lower concentrations or with shorter exposure times. In our study we chose to use short exposure times of 10, 5, or 1 min in order to detect the rapid inactivation of *Chlamydia*. Monocaprin at a final concentration of 2.5 mM reduced the infectivity titer by \( >5 \log_{10} \) IFU in 5 min at 37°C and was the most active of all the lipids tested in this study (Table 2). An exposure time of 1 min had only a

### Table 4. Viability of *Chlamydia* after treatment with monocaprin at concentrations of 2.5 to 2,500 µg/ml for 2 h

<table>
<thead>
<tr>
<th>Monocaprin concn (µg/ml [mM])</th>
<th>No. of inclusions</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5 (0.01)</td>
<td>151</td>
<td>4</td>
</tr>
<tr>
<td>5 (0.02)</td>
<td>134</td>
<td>15</td>
</tr>
<tr>
<td>10 (0.04)</td>
<td>123</td>
<td>22</td>
</tr>
<tr>
<td>25 (0.1)</td>
<td>98</td>
<td>38</td>
</tr>
<tr>
<td>50 (0.2)</td>
<td>6</td>
<td>96</td>
</tr>
<tr>
<td>100 (0.4)</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>200 (0.8)</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>500 (2)</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>1,000 (4)</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>2,500 (10)</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

*Mean number of inclusions in six wells.*

*Compared with the level of inhibition of the untreated control preparation, in which the mean inclusion count in four wells was 157.*
minor effect. This is in contrast to the case for HSV-1, which is inactivated 100,000-fold or more (≥5 log_{10}) by exposure to monocaprin for 1 min (10). The rapid in vitro killing of large numbers of sexually transmitted bacteria and viruses by microbicides is an essential prerequisite for their possible use in the prevention of STDs. Even if the number of infectious bacteria or viruses transmitted to genital mucous is several orders of magnitude lower than the number used in vitro tests, the conditions for the killing of the microbe in vivo are likely to be less favorable due to less effective mixing with the microbicide and an uneven distribution of the microbe on the mucosa. A large margin of microbicidal activity is therefore necessary. Due to the high in vitro efficacy of monocaprin against C. trachomatis and sexually transmitted enveloped viruses, this lipid may be useful as a microbicidal agent for the prevention of the transmission of STDs. Pharmaceutical formulations which contain monocaprin as the active ingredient have been developed and are potent inactivators of C. trachomatis, HSV-2, and human immunodeficiency virus in vitro (16). In vivo testing of these formulations is needed to establish whether or not they may be used for the prevention of STDs.

Several studies have shown that spermicides such as nonoxynol-9 inactivate C. trachomatis and other sexually transmitted bacteria and viruses (13), and it has been suggested that they may be used for protection against STDs. Benes and McCormack (1) studied the effects of various concentrations of nonoxynol-9 on large numbers of C. trachomatis upon contact for 120 min and found a 50% inhibition of inclusion formation at a concentration of between 500 and 1,000 μg per ml. In a comparable study of the activity of monocaprin against Chlamydia, a greater inhibitory effect was found (Table 4), with a 50% effective concentration of about 30 μg per ml. Although toxic in cell cultures, monocaprin at a concentration of 5 mg per ml (20 mM) has been shown not to cause irritation of the vaginal mucosa of mice and rabbits (16). The low level of toxicity in vivo and the high level of antichlamydial activity in vitro suggest that monocaprin may be more useful than nonoxynol-9 as protection against chlamydial infections.

ACKNOWLEDGMENT

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REFERENCES