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Hydrogels containing monocaprin have potent microbicidal activities against sexually transmitted viruses and bacteria in vitro

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Objective: To investigate the in vitro microbicidal and cytocidal potency of monocaprin dissolved in pharmaceutical hydrogel formulations and to evaluate their potential use as vaginal microbicides against sexually transmitted pathogens such as herpes simplex virus type 2 (HSV-2), human immunodeficiency virus type 1 (HIV-1), *Chlamydia trachomatis*, and *Neisseria gonorrhoeae*.

Methods: Gel formulations were mixed with equal volumes of virus/bacteria suspensions in culture medium and incubated for 1 and 5 minutes. The reduction in virus/bacteria titre was used as a measure of microbicidal activity. Similarly, gels were mixed with human semen to study their effect on leucocytes. The toxicity of the gels was tested in rabbits by the standard vaginal irritation test.

Results: Gels containing 20 mM of monocaprin caused a greater than 100 000-fold inactivation of HSV-2 and *Neisseria* in 1 minute and of *Chlamydia* in 5 minutes. Similarly, the gels caused a greater than 10 000-fold inactivation of HIV-1 in semen in 1 minute. They caused more than a 10 000-fold reduction in the number of viable leucocytes in semen in 1 minute. No toxic effect on the vaginal mucosa of rabbits was observed after daily exposure for 10 days.

Conclusions: Hydrogels containing monocaprin are potent inactivators of sexually transmitted viruses and bacteria in vitro. This simple lipid seems to be a feasible choice as a mucosal microbicide for prevention of sexually transmitted infections. It is a natural compound found in certain foodstuffs such as milk and is therefore unlikely to cause harmful side effects in the concentrations used.

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Introduction

The microbicidal effect of lipids has been extensively studied in recent years. Several free fatty acids and 1-monoglycerides have been found to kill enveloped viruses and a variety of both Gram negative and Gram positive bacteria.[1–5] A recent study has shown that *Chlamydia trachomatis* is rapidly inactivated in vitro by certain medium chain fatty acids and 1-monoglycerides.[6] These studies suggest that lipids can be used as intravaginal microbicides for protection against sexually transmitted diseases (STD). In order to prevent infection by a sexually transmitted pathogen it is important that the microbicide is fast acting and kills the infectious agent before it has time to infect cells of the genital mucosa. It is also important that the microbicide can be solubilised in an acceptable pharmaceutical formulation which can be easily and effectively applied to the genital tract of women and to other mucosas or skin areas which can serve as entry sites for infectious agents. Novel hydrogel formulations have recently been designed which contain 1-monoglyceride of capric acid as the active ingredient and which cause a greater than 100 000-fold reduction in the infectivity titre of herpes simplex virus type 1 (HSV-1) in vitro in 1 minute or less (unpublished data). The formulations have now been tested in vitro against four sexually transmissible agents—namely, human immunodeficiency virus type 1 (HIV-1), herpes simplex virus type 2 (HSV-2), *Chlamydia trachomatis*, and *Neisseria gonorrhoeae*. They were found to kill these agents rapidly and at high titres.

Materials and methods

HYDROGEL FORMULATIONS

Three active hydrogel formulations were used. Formulation 1A (pH 7) is based on a solution of sodium carboxymethylcellulose (NaCMC) and polyvinylpyrrolidone (povidone K30) in water to which is added 1-monoglyceride of capric acid (monocaprin) dissolved in glycofurol 75. Formulation 1B has the same composition as 1A except that the pH is adjusted to 5.0 by addition of lactic acid. Formulation 2A is based on a solution of hydroxypropylmethylcellulose (HPMC) and a carbomer (Carbopol 934) in water to which a solution of monocaprin is added, as in formulations 1A and 1B. The pH is adjusted to 5.0 by addition of sodium hydroxide. 1-Monoglyceride of capric acid, NaCMC, glycofurol 75, and Povidone K30 were purchased from Sigma Chemical Co, St Louis, MO, USA; Carbopol 934 from Nomeco, Copenhagen, Denmark; and HPMC from Aldrich Chemical Company Inc, Milwaukee, WI, USA. All the ingredients were reagent grade.

VIRUSES AND BACTERIA

A strain of HSV-2 was kindly provided by the Department of Medical Virology, National University Hospital, Reykjavik, Iceland. The virus was isolated from a genital herpes lesion and identified as HSV-2 by staining with a type specific fluorescent antibody. It was passed five times in Vero cells before use in experiments. HIV-1 strain III$_B$ was obtained from persistently infected H9 cells and was provided by R C Gallo, when at NCI, Bethesda, MD, USA.[7] *C trachomatis* serotype K, originally isolated from human cervix, was obtained from the American Type Culture Collection (ATCC VR 887). A penicillin resistant strain of *Neisseria gonorrhoeae* was used. It was isolated from the urethra of a male patient with gonorrhoea and identified by the oxidase test and Gram staining, confirmed by standard sugar fermentation[8] and staining with fluorescent monoclonal antibodies (Difco Laboratories, Detroit, MI, USA).

CELL CULTURES AND MEDIA

Monolayers of Vero cells (African green monkey kidney cell line, Flow Laboratories, Inc) were used in experiments with HSV-2. They were grown in Dulbecco's modified Eagle medium (D-MEM) with 10% heat inactivated fetal bovine serum (FBS) and maintained in D-MEM with 2% FBS. MT-4 cells,[9] a T4+ lymphocyte cell line, were used in experiments with HIV-1. They were grown and maintained in RPMI 1640 medium with 2 mM L-glutamine, 50 µg per ml of gentamicin, and 10% FBS. McCoy cells, a heteroploid mouse fibroblast cell line, were used in the experiments with *C trachomatis*. They were grown in RPMI 1640 medium with 5% FBS.[6] All media were purchased from Gibco, Paisley, Scotland. *N gonorrhoeae* were grown on chocolate agar medium and passed daily.

ASSAY OF VIRUCIDAL ACTIVITY OF HYDROGELS AGAINST HSV-2

The gels were thoroughly mixed with equal volumes (100 µl) of HSV-2 suspensions in 12×75 mm polystyrene round bottomed tubes (Falcon) and incubated for 1 minute at room temperature. Virus mixed with maintenance medium served as control. The virus titres of the mixtures were determined by the serial dilution end point method in Vero cell monolayers in 96 well microtitre tissue culture plates (Nunc, Roskilde, Denmark).[4] The titre ($\log_{10}$) of a gel-virus mixture was subtracted from the titre ($\log_{10}$) of the control mixture and the difference—that is, the reduction in viral infectivity, was used as a measure of the virucidal activity of the gel.

ASSAY OF VIRUCIDAL ACTIVITY OF HYDROGELS AGAINST HIV-1

A volume of 100 µl of virus was mixed with 100 µl of gel in polystyrene tubes for 1 minute at room temperature. Virus mixed with culture medium served as control. The mixtures were diluted in culture medium and titrated in fivefold dilutions in 96 well microtitre plates to which a suspension of 3×10^4 MT-4 cells per well was added. The cytopathogenic effect was examined microscopically after incubation at 37°C for 5 days and the virus titres were calculated by the method of Reed and Muench.[10] The reduction of virus titre in a gel-virus mixture was calculated in the same way as for HSV-2 and used as a measure of the virucidal activity of the gel. Virucidal activity of gels against HIV-1 in semen was tested in the same way except that the virus was diluted 1:10 in fresh (< 2 hours) liquefied human semen before it was mixed with the gels. Virus spiked semen mixed with culture medium was used as control.

ASSAY OF MICROBICIDAL ACTIVITY OF HYDROGELS AGAINST *C TRACHOMATIS*

Equal volumes (100 µl) of gels and bacteria suspensions were mixed in polystyrene tubes at 37°C for a given time. The mixtures were then diluted in culture medium and viable bacteria titrated in monolayers of McCoy cells as described previously.[6] Bacteria mixed with culture medium served as control. The titre, expressed in $\log_{10}$ inclusion forming units (IFU), of gel-bacteria mixtures was subtracted from the titre of the control mixture and the difference used as a measure of antichlamydial activity of the gel.

ASSAY OF MICROBICIDAL ACTIVITY OF HYDROGELS AGAINST *N GONORRHOEAE*

A bacterial suspension in trypticase soy broth was prepared from a 24 hour old culture at a density of about 10^8 colony forming units (CFU) per ml. The suspension was mixed with an equal volume (100 µl) of gel formulation and incubated at room temperature for 1 minute. Samples were then diluted in 10-fold dilutions in sterile physiological saline and the number of surviving bacteria determined on chocolate agar plates by the time exposure method described by Singh and Cutler.[11] Control samples without gel formulations were tested in the same way. The titres ($\log_{10}$ CFU) of gel-bacteria mixtures were subtracted from the titre of the control mixture and the difference used as a measure of the microbicidal activities of the gels.

ASSAY OF CYTOCIDAL ACTIVITY OF GEL FORMULAS IN HUMAN SEMEN

Human white blood cells (WBC) were separated from heparinised blood by sedimentation on Histopaque-1077 (Sigma Chemical Co, St Louis, MO, USA). The washed pellet was suspended in liquefied human semen (< 2 hours) at a density of 4×10^7 WBC per ml. The semen with added WBC was mixed with an equal volume (200 µl) of gel formulation in a polystyrene tube at room temperature and thoroughly mixed with the gel for 1 minute. A 100 µl sample was then immediately diluted 1:10 in maintenance medium, stained with an equal volume of 0.5% trypan blue and viable cells counted. Semen mixed in the same way with medium instead of gel served as control.

VAGINAL IRRITATION TEST IN RABBITS

The test was performed as recommended by the International Working Group on Vaginal Microbicides.[12] Briefly, 4–5 month old white rabbits, weighing 3–3.5 kg, were used. About 0.5 ml of gel formulation was injected into the vagina with a tuberculin syringe daily for 10 days. Control rabbits were either injected with a sterile physiological saline solution or not treated. Two rabbits were used for each sample. After treatment, the rabbits were killed, the genital tract removed by dissection, and the mucosa examined for redness or other abnormalities. The vaginas were then fixed in 4% formaldehyde in phosphate buffered saline and embedded in paraffin. Sections were cut at three different levels and stained with haematoxylin and eosin.

Results

ANTI-HSV-2 ACTIVITY OF GEL FORMULATIONS

Gel formulations 1A, 1B, and 2A were mixed with HSV-2 in maintenance medium at a titre of $10^{7.5}$ TCID$_{50}$ (50% tissue culture infective dose) per ml and incubated at room temperature. Formulations 1B and 2A containing 20 mM of monocaprin (10 mM in the gel-virus mixture) caused a 700 000-fold or greater ($\geqslant 5.5$ log$_{10}$) reduction in virus titre in 1 minute. Gels containing 10 mM monocaprin reduced the virus titre by 100 000-fold in 1 minute. Formulation 1A was somewhat less active than 1B and 2A at monocaprin concentrations of 20 mM and 10 mM. Gels with 5 mM monocaprin caused little inactivation—that is, from 100-fold in gel 1A to 2000-fold in gel 2A. Formulations without monocaprin had a minor effect except for gel 2A which caused about 30-fold reduction in virus titre.

ACTIVITY OF GEL FORMULATIONS AGAINST HIV-1 IN HUMAN SEMEN

HIV-1 was diluted 1:10 in human semen to a final titre of about 10^7 TCID$_{50}$ per ml. It was then mixed with gels 1A and 2A containing no monocaprin or 20 mM monocaprin, incubated at room temperature, and titrated as described. The control gels caused a less than 10-fold inactivation in 1 minute. Gels with 20 mM of monocaprin (10 mM in the gel-virus mixture), on the other hand, caused a greater than 10 000-fold reduction in virus titre in 1 minute. The active gels were toxic to MT-4 cells at a 20-fold dilution and slightly toxic at a 100-fold dilution. The semen was toxic to MT-4 cells at a 100-fold dilution. Virus diluted in medium rather than in semen was inactivated by greater than 60 000-fold ($\geqslant 10^{4.8}$ log$_{10}$ reduction in titre) in 1 minute by gels containing 20 mM monocaprin. Gel 1B was tested in one experiment which showed that it inactivated HIV-1 in semen by more than 8000-fold in 1 minute.

ANTI-CHLAMYDIAL ACTIVITY OF GEL FORMULATIONS

Gel formulations 1A, 1B, and 2A containing a series of monocaprin concentrations and the corresponding control gels without monocaprin were tested against $10^{7.5}$ to 10^8 IFU per ml of *C trachomatis*. Exposure for 5 minutes to gel formulations 1B and 2A containing monocaprin at a concentration of 10 mM (5 mM in the mixture) led to a greater than 600 000-fold inactivation of the bacteria. Formulation 1A was slightly less active. Gels with a monocaprin concentration of 5 mM were less active, particularly gel formulation 2A. Treatment for 2.5 minutes with formulations containing 20 mM of monocaprin also effectively inactivated the bacteria. Control formulations without monocaprin had only a minor effect.

KILLING OF *N GONORRHOEAE*

Gel formulations 1A, 1B, and 2A containing 10 mM of monocaprin (5 mM in the mixture) caused a 250 000-fold or greater reduction in the number of viable bacteria in 1 minute at room temperature. Formulations without monocaprin had only a minor effect.

KILLING OF WBC IN HUMAN SEMEN

Considerable evidence suggests that sexual transmission of viruses such as HIV can be mediated by mononuclear cells.[13] Gel formulations which are virucidal against HIV-1 in semen were therefore tested for cytocidal activity. The WBC counts in most normal ejaculates are below 10^6 cells per ml.[14] Since this number is too low to demonstrate a significant cytocidal effect of the gels, WBC from human blood were added to the semen at a concentration of 4×10^7 WBC per ml as described in Materials and methods. After incubation with gel formulations 1A (10 mM) and 2A (20 mM) for 1 minute no viable WBC could be detected under the microscope, while dead cells were observed in large numbers. From the number of microscopic fields examined it was estimated that the WBC counts in semen samples treated with the gels were less than 4000 cells per ml, with a reduction in viable WBC count greater than 10 000-fold. Gels without monocaprin had no effect on the cell count.

EFFECT OF GEL FORMULATIONS ON THE VAGINAL MUCOSA OF RABBITS

A preliminary experiment in the vaginal mucosa of mice showed no adverse effect of gel formulation 1A containing 10 mM or 20 mM of monocaprin after daily application for 10 days. Gel formulations 1A and 2A with 20 mM of monocaprin or without the active compound were therefore tested in rabbits by the standard vaginal irritation test. No abnormalities were detectable in the mucosa either by macroscopic examination of fresh tissue or by microscopic examination of stained sections from various areas of the vagina.

Discussion

A number of different microbicides have been shown to kill sexually transmissible bacteria and viruses including HIV-1, HSV, *C trachomatis*, and *N gonorrhoeae*.[15–21] The microbicidal activity of the non-ionic surfactant nonoxynol-9 has been studied most extensively, both in an animal model and in vivo.[22 23] The results of clinical trials have been controversial[24 25] and when used frequently or

at a high dose nonoxynol-9 may cause vaginal and cervical lesions.[26–28] There is therefore a need for new microbicidal products which are less toxic for mucosal membranes and could be used to prevent or counteract sexual transmission of bacteria and viruses, including HIV.

In this report we describe the microbicidal activities of novel pharmaceutical formulations, in the form of gels of various compositions which contain a simple lipid, monocaprin, as the active ingredient. Monocaprin was selected after a comparison of the microbicidal activities of several fatty acids and monoglycerides against HSV-1 and *C trachomatis*,[6] which were used as the test virus/bacterium. In addition to monocaprin, other lipids such as lauric acid, palmitoleic acid, and monolaurin suspended in maintenance medium caused a rapid inactivation of HSV-1, and lauric acid and capric acid were active against *C trachomatis*. However, the activities of these lipids in gel formulations have not been studied.

Since semen is thought to be an important vehicle for sexual transmission of HIV-1, gel formulations containing 20 mM monocaprin were tested against HIV-1 suspended in human semen and found to cause a 10 000 fold or greater reduction of virus titre in 1 minute. No virus activity was detectable in the lowest dilution (10^{-3}) which was not toxic to the MT-4 cells. Since a recent clinical isolate was not available for this study, the laboratory strain HIV-1 III$_B$ was used. Whether or not different strains of HIV-1 show differences in their sensitivity against monocaprin or other virucidal lipids is unknown. The gels showed a remarkably high activity against *C trachomatis* after incubation for 5 minutes and somewhat less after 2.5 minutes. A comparison of monocaprin with nonoxynol-9 indicated that the 50% effective concentration against *C trachomatis* was at least 20-fold lower for monocaprin under similar conditions.[6] A standard strain of C *trachomatis* was used in these experiments. However, monocaprin was found to be equally active against two strains of *C trachomatis* newly isolated from patients.[6] Various fatty acids and monoglycerides had similar activity profiles against two different isolates of *N gonorrhoeae*, with monocaprin being the most active (unpublished data). Similar to HSV, monocaprin kills *N gonorrhoeae* at very high titres in only 1 minute, whereas a longer time is required to kill the elementary bodies of *Chlamydia*.

Cell associated HIV-1 has been found more frequently than cell free HIV-1 in semen from HIV infected individuals,[29] and there is experimental evidence suggesting that sexual transmission of HIV-1 can occur via cell to cell contact between infected mononuclear cells and CD4 negative epithelial cells.[13] The efficacy of virucidal gel formulations in killing WBC in semen was therefore tested and it was found to be very high, with more than a 10 000-fold reduction in viable WBC count within 1 minute. This may be very important, since cell to cell transmission of HIV appears to be very rapid, taking place in a few minutes.[13]

Because of the high cytotoxicity of the gel formulations in semen, it is necesssary to evaluate their toxicity in the vaginal mucosa of experimental animals. They should preferably be less damaging to mucosal membranes than the spermicidal products currently in use. In an in vitro experiment they were found to be about 10-fold less cytotoxic in Vero cell monolayers than the commercial spermicidal product, Gynol-plus, containing 2% nonoxynol-9 (unpublished data). A preliminary experiment in the vaginal mucosa of mice showed no adverse effect of gel formulation 1A after daily application for 10 days. This was confirmed by testing the gel formulations 1A and 2A in the standard rabbit vaginal irritation test, either in New Zealand White rabbits (gel 2A) or in rabbits of mixed breed (gel 1A). The results indicate that the gel formulations containing 20 mM monocaprin are not toxic to the vaginal mucosa despite the fact that they are highly cytocidal in human semen and inactivate sexually transmitted viruses and bacteria rapidly and in large numbers in vitro. Preliminary experiments have shown that gel formulations containing 20 mM monocaprin are highly spermicidal. Thus, the motility of spermatozoa decreased by 100% after incubating the gels with semen in a 1:3 ratio for 10 minutes at room temperature, and the viability was reduced by 96% to 100%. This suggests that the formulations could be useful as contraceptives in addition to being active against sexually transmitted pathogens.

Monocaprin is a natural compound present in certain foodstuffs such as milk. In contrast with non-ionic and cationic detergents, which have been proposed as vaginal microbicides, it is therefore not likely to cause harmful side effects in the concentration used.

Tables giving the results of the experiments can be seen on the journal's website (www.sextransinf.com).

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