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Vaginal Formulations of Carrageenan Protect Mice from Herpes Simplex Virus Infection

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The observations from the present study indicate that vaginal formulations of the sulfated polysaccharide carrageenan are highly effective in protecting mice from herpes simplex virus type 2 (HSV-2) infection. Test formulations were placed in the vaginas of progestin-treated mice prior to inoculation with HSV-2. Infection was determined by the presence of inflammation in the genital region and death. At a dose of virus that infected half of the control animals, 1% solutions of either lambda, kappa, or iota carrageenan prevented infection of almost all of the animals. Concentrations as low as 0.05% protected a large majority of the mice. At a dose of virus that infected all of the control mice, 1% solutions of carrageenans protected 85% of the inoculated mice. Other sulfated polysaccharides were less effective or showed no efficacy in preventing HSV-2 infection. These findings suggest that a vaginal formulation of carrageenan may be effective in blocking sexual transmission of HSV-2 in women.

Despite the efforts of numerous workers, there is no vaccine for any sexually transmitted pathogen except hepatitis B virus. Thus, prevention strategies are limited to the use of behavior modification and condoms. Results of recent in vitro and animal studies suggest that it may be feasible to develop a topical formulation that would prevent infection by various sexually transmitted pathogens (11, 12, 16).

The majority of genital herpes simplex virus (HSV) infections are caused by HSV type 2 (HSV-2). This virus infects many millions of people, causing persistent and recurrent painful genital lesions. Although humans are the natural host for HSV-2, infection can be experimentally induced in many species including mice, which can be infected through vaginal inoculation (10, 19). Infection of mice by this route results in lethal neurological disease which is preceded by easily recognizable symptoms. The initial symptom is usually inflammation in the genital area.

It has previously been shown that certain sulfated polysaccharides can block infection of cultured cells by HSV and other enveloped viruses (2, 6, 9). In the present study we have explored the possibility that sulfated polysaccharides can block HSV-2 infection in mice. Our results suggest that certain of these compounds are very effective. We speculate that these compounds in a topical formulation may prove useful at preventing sexual transmission of HSV-2 and possibly other enveloped viruses, including human immunodeficiency virus (HIV).

MATERIALS AND METHODS

Mice. Six- to 8-week-old female BALB/c mice were maintained on a 12-h light cycle at The Rockefeller University laboratory animal care facility. Animals were injected subcutaneously, 5 days prior to inoculation, with 0.1 ml of medroxyprogesterone acetate (Depo-Provera; Upjohn, Kalamazoo, Mich.) at 25 mg/ml in phosphate-buffered saline (PBS). Treatment with medroxyprogesterone acetate has been shown to increase the susceptibility of mice to HSV-2 infection (10, 19).

HSV-2. HSV-2 strain G obtained from the American Type Culture Collection (ATCC; Rockville, Md.) was propagated in Vero cells (ATCC) as described by McDermott et al. (7). Virus titer was assayed by plaque formation on Vero cells as described by Rawls et al. (13). Virus stock was aliquoted into 0.8-ml Eppendorf tubes, and the tubes were stored at −70°C. The same stock virus was used for all experiments.

Compounds. Sulfated polysaccharides and nonoxynol-9 (N-9), purchased from Sigma (St. Louis, Mo.), were diluted in PBS (Gibco BRL). The sulfated polysaccharides used were 8,000-molecular-weight (MW) dextran sulfate (D-4911), 500,000-MW dextran sulfate (D-6001), heparin (H-3393), fucoidan (F-5631), iota carrageenan (C-4014), lambda carrageenan (C-3889), kappa carrageenan (C-1263), and chondroitin sulfate A (C-8529).

Infection. Mice were inoculated 5 days after progestin treatment. The mice were not anesthetized because BALB/c mice are not difficult to handle. Twenty microliters of the test compound was carefully instilled into the vagina by using a P20 Pipetman (Rainin, Woburn, Mass.). For 1% formulations of iota carrageenan and kappa carrageenan, which are somewhat viscous, the end of the pipet tip was cut off to a diameter of approximately 1 mm for vaginal delivery. Five minutes after receiving the formulation, the mice were inoculated intravaginally with 10^3 PFU of HSV-2 in a volume of 10 μl. Six to 7 days after inoculation, the mice were scored as infected or uninfected, depending on the presence or absence of inflammation in the genital region. The mice were sacrificed after being examined.

Assay for HSV-2 in vaginal secretions. Two groups of 10 mice each were used for studying vaginal shedding and latency: 10 PBS-treated control mice inoculated with 10^4 PFU of HSV-2 and 10 mice treated with 0.1% lambda carrageenan prior to HSV-2 inoculation. Samples of vaginal secretions were collected by swabbing the vagina as described by McDermott et al. (7). HSV-2 was detected by a plaque assay (13).

Assay for latency. Four dorsal root ganglia were removed from each mouse as described by Waltz et al. (18). Explanted ganglia were rinsed in medium and were cultured on Vero cell monolayers in 12-well plates for 2 weeks. HSV-2 was detected by a plaque assay (13).

RESULTS

Determining infectious dose. In preliminary experiments, we followed the course of infection of animals that displayed vaginal inflammation at 6 days postinfection and those who did not. In all cases mice with vaginal inflammation developed more severe symptoms, including hair loss and paralysis. We also caged inoculated and uninoculated animals together to determine whether HSV-2 was transmitted among cage mates. Vaginal inflammation was never observed in uninoculated animals.

A dose-range study was performed to determine a dose of virus for blocking studies (Table 1). We chose a dose of 10^3 PFU, because this dose resulted in infection of about half the animals. We reasoned that an infection rate of 50% would allow us to detect both inhibition and enhancement.

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TABLE 1. Number of mice that were infected by various doses of HSV-2

PFU/mouse	No. of infected mice/total no.
7×10^4	10/10
1×10^4	9/10
1×10^3	5/10
1×10^2	1/10
0	0/10

Blocking studies. We have carried out separate experiments with different vaginal formulations. In each case 20 animals were used per treatment group. Because there could be unknown variables, we used a control group of 20 animals treated with PBS in each of the nine experiments. The results for the controls were very consistent; the number of animals infected was about 50% in all nine experiments. We have combined these data in Table 2.

N-9. N-9 is the active ingredient in all vaginal spermicide products in the United States. A 4% N-9 product has been reported to block HSV-2 infection in mice (19). Since N-9 had been shown to protect mice from infection, we used this surfactant as a positive control in our blocking experiments. We observed that 0.5% N-9 slightly inhibited infection, 1% N-9 prevented infection in more than half the animals, and 2% N-9 completely blocked infection (Table 2).

Carrageenan. We have previously found that a number of polysaccharides are effective in blocking cell-mediated infection of cervix-derived epithelial cells by HIV (12, 16, 17). The most effective of these was dextran sulfate. Heparin, fucoidan, and carrageenan were also very effective. We studied the effect of 0.5 and 1% aqueous solutions of these compounds on HSV-2 infection.

Carrageenans were by far the most effective in blocking HSV-2 infection. Of the first 120 animals that we tested with iota, lambda, or kappa carrageenan, two mice became infected, whereas the infection rate of control mice was about 50% (Table 2).

We next carried out a study with decreasing doses of iota and lambda carrageenans. Iota carrageenan prevented infection in all of 60 animals at concentrations of 1, 0.5, and 0.25% and was highly effective at a concentration as low as 0.05% (Table 3). Results of the dosage study with lambda carrageenan were very similar (Table 4).

TABLE 2. Number and percentage of mice that became infected when the test formulation was placed in the vagina prior to inoculation with HSV-2

Treatment	No. of infected mice/total no. (%)
PBS	44/100 (44)
0.5% N-9	7/20 (35)
1% N-9	5/40 (12.5)
2% N-9	0/20 (0)
1% chondroitin sulfate A	9/20 (45)
1% dextran sulfate (high MW)	9/20 (45)
0.5% dextran sulfate (high MW)	8/20 (40)
1% dextran sulfate (low MW)	4/20 (20)
0.5% fucoidan	5/20 (25)
1% heparin	7/20 (35)
0.5% heparin	3/20 (15)
1% kappa carrageenan	1/40 (2.5)
1% iota carrageenan	1/40 (2.5)
1% lambda carrageenan	0/40 (0)

TABLE 3. Number and percentage of mice that became infected after different doses of iota carrageenan were placed in the vagina prior to inoculation with HSV-2

Treatment	No. of infected mice/total no. (%)
PBS	9/20 (45)
Iota carrageenan	
1%	0/20 (0)
0.5%	0/20 (0)
0.25%	0/20 (0)
0.1%	2/20 (10)
0.05%	2/20 (10)

To determine if carrageenan would protect animals from high doses of HSV-2, we increased the dose of HSV-2 to 10^4 PFU, a 10-fold higher dose. Animals treated with different doses of lambda and iota carrageenans were compared to PBS-treated control animals. In this experiment we did not sacrifice the animals after the first week but kept them and examined each surviving mouse once a week on days 14, 21, 35, and 42 postinoculation. All of the control mice died. However, both 1% lambda carrageenan and 1% iota carrageenan protected 85% of the animals. Doses of 0.1% also afforded some protection (Table 5).

Of the total of 140 animals used in this experiment, infection was detected in 74 animals on day 7. Eleven more infected mice were detected on day 14, and 4 more infected mice were detected on day 21. All animals judged to be infected died. No further infected animals were detected on day 35 or 42 (Table 5).

Dextran sulfate. Dextran sulfate is the most commonly used sulfated polysaccharide in in vitro blocking studies of enveloped viruses and is usually the most efficacious of the sulfated polysaccharides (12, 17). Several groups are interested in using this compound as a vaginal "microbicide" for preventing HIV infection. A phase I safety trial has been carried out on a low-dose formulation of this sulfated polysaccharide (14). Although we found no inhibition of high-MW dextran sulfate on HSV-2 infection, low-MW dextran sulfate prevented infection in half of the animals (Table 2). We therefore carried out a dose study to determine if higher doses of low-MW dextran sulfate would block infection. In this experiment there was no significant effect at doses of 0.5 and 1%. However, doses of 2.5 and 5% protected the majority of the animals (Table 6).

Other sulfated polysaccharides. The sulfated polysaccharides heparin and fucoidin are efficacious in blocking infection,

TABLE 4. Number and percentage of mice that became infected after different doses of lambda carrageenan were placed in the vagina prior to inoculation with HSV-2

Treatment	No. of infected mice/total no. (%)
PBS	9/20 (45)
Lambda carrageenan	
1%	0/20 (0)
0.5%	0/20 (0)
0.25%	2/20 (10)
0.1%	2/20 (10)

TABLE 5. Number and percentage of mice that became infected after PBS or different doses of lambda or iota carrageenan were placed in the vagina prior to inoculation with HSV-2[a]

Treatment	No. of infected mice/total no. (%)				
	Day 7 p.i.	Day 14 p.i.	Day 21 p.i.	Day 35 p.i.	Day 42 p.i.
PBS	17/20 (85)	20/20 (100)	NC	NC	NC
Lambda carrageenan					
1%	2/20 (10)	3/20 (15)	NC	NC	NC
0.1%	9/20 (45)	NC	11/20 (55)	NC	NC
0.01%	14/20 (70)	17/20 (85)	18/20 (90)	NC	NC
Iota carrageenan					
1%	3/20 (15)	NC	NC	NC	NC
0.1%	13/20 (65)	16/20 (80)	NC	NC	NC
0.01%	16/20 (80)	17/20 (85)	18/20 (90)	NC	NC

[a] In this experiment we increased the dose of HSV-2 to 10^4/PFU, a dose which we had previously determined kills 90 to 100% of the mice. Animals were examined on days 7, 14, 21, 35, and 42 to determine if more infected animals would be detected after the first week. On day 7 we determined that 74 of the 140 animals in the experiment were infected. Eleven more infected animals were detected on day 14, and 4 more infected animals were detected on day 21. All 51 carrageenan-treated animals that did not show signs of infection on day 21 showed no signs of infection on days 35 or 42. NC, no change in the number of animals infected from the previous week; p.i., postinoculation.

but they are not as effective as carrageenan. Chondroitin sulfate did not protect animals from infection (Table 2).

HSV-2 in vaginal vault and ganglia. Although HSV-2 infection in mice is generally fatal, it has been shown that HSV-2 can spread from the vagina and cervix to lumbosacral ganglia and establish a latent infection (5, 15, 18). To determine if the vaginas of inoculated mice contained infectious virus or if dorsal root ganglia were latently infected, we infected control (PBS) and 0.1% lambda carrageenan-protected animals with a high dose (10^4 PFU) of HSV-2.

To detect virus in the vagina, swabs were cultured on days 2, 5, 7, 9, 14, and 28 postinoculation. All cultures of Vero cells which were inoculated with vaginal contents from 10 control animals were positive on days 2 and 5. No further swabs were taken because 9 of the 10 control animals were dead on day 7 and the 10th animal was very sick. Of the carrageenan-treated animals 7 of 10 were positive on day 2; 6 of 10 were positive on day 5. On day 7 postinfection two of the animals with positive assay results had died and one of the remaining animals tested positive. This animal was dead on day 9. Cultures of swabs from the six remaining animals were negative on days 9, 14, and 28.

As a positive control, explants of dorsal root ganglia from 10 PBS-treated animals were cultured 7 days after inoculation. All cultures showed viral plaques. Explanted ganglia from six protected animals were cultured 28 days following inoculation. All of these cultures were negative for viral plaques.

DISCUSSION

Sulfated polysaccharides have been shown to block infection of cultured cells by enveloped viruses. In this report we present the first evidence that a sulfated polysaccharide is highly effective in inhibiting HSV-2 infection in an animal model. We have shown that 0.25 to 1% solutions of the sulfated polysaccharide carrageenan are nearly 100% efficacious in preventing infection in mice at a dose of virus that infects half of the control mice. Furthermore, the three types of carrageenan, kappa, iota, and lambda, all appear to have similar abilities to block infection. When used at concentrations as low as 0.05%, carrageenan still protects the large majority of animals that were inoculated with HSV-2. At doses of virus that infect all of the control animals, 1% lambda carrageenan or 1% kappa carrageenan still protect 85% of the animals.

It should be noted that in the experiments with low doses of virus we detected infection only on day 7. When we examined animals on later days in a subsequent experiment we observed that infection was detected on day 14 or 21 in about 20% of the animals that had not shown symptoms on day 7. Thus, the percentage of animals infected in the low-dose experiments is likely to be somewhat lower than would be the case if we had followed the animals further.

HSV-2 typically causes latent infections in humans, although in very rare cases HSV can infect the central nervous system (3). In mice the same virus typically kills the host, although it can cause latent infections in ganglia (5, 15, 18). It is unlikely that the carrageenan-protected animals were latently infected because virus was not detected in cultures of ganglia or in vaginal smears. In addition, in the experiment in which we followed animals for 42 days, all of the 51 mice that did not display signs of infection on day 21 postinfection still had no sign of infection on day 42.

The carrageenans are large galactose-linked polysaccharides that form a natural structural component of red seaweed. They are particularly attractive candidates for a vaginal formulation to prevent HSV-2 infection because they are considered GRAS (generally regarded as safe) by the U.S. Food and Drug Administration. In fact, they are used as thickeners in soups and ice cream at a concentration of about 1%, the same concentration used in these experiments. These compounds are very stable, not substrates for bacteria, not absorbed, and inexpensive and are used as inert ingredients in pharmaceutical products. The observations that carrageenans block HIV infection of cervix-derived epithelial cells (12) suggests that they may also find utility in protecting women from infection by HIV.

We compared the efficacy of sulfated polysaccharides to that of N-9. Carrageenan at 0.5 to 1% is considerably more effective than N-9 in blocking infection. N-9 has been shown to be highly effective in vitro in inactivating enveloped viruses including HSV (1), HIV (4), and feline leukemia virus (8). In comparison, carrageenan is far less cytotoxic than N-9. In vitro, N-9 is toxic to lymphoma cells at a concentration similar to the effective dose (11), whereas carrageenan is effective at a dose more than 1,000 times lower than the cytotoxic dose (12). We have recently completed a phase I safety trial of a 2% formulation of carrageenan. We observed no damage to the vaginal or cervical epithelia by colposcopic examination (unpublished data).

Although the results presented here are encouraging, we

TABLE 6. Number and percentage of mice that became infected after different doses of dextran sulfate (MW, 8,000) were placed in the vagina prior to inoculation with HSV-2

Treatment	No. of infected mice/total no. (%)
PBS	13/20 (65)
Dextran sulfate	
5%	5/20 (25)
2.5%	3/20 (15)
1%	10/20 (50)
0.5%	9/20 (45)

caution that it is premature to conclude that carrageenan will block infection of HSV-2 in women. In addition, the evidence presented here and in our in vitro studies with HIV is not sufficient to conclude that carrageenan will block HIV infection in women. Because human trials of the efficacy of carrageenan for the prevention of infection in humans would be demanding and expensive, we believe that the prudent approach in developing a vaginal formulation for the prevention of sexually transmitted diseases is to develop more animal models for the testing of carrageenan as well as other candidate compounds. We are pursuing this line of work in our laboratory.

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