

Short communication

Protective effect of a natural carrageenan on genital herpes simplex virus infection in mice

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Abstract

In the present study, the protective effect of 1T1, a λ -carrageenan extracted from the red seaweed *Gigartina skottsbergii* was evaluated in a murine model of herpes simplex virus type 2 (HSV-2) genital infection. Six to eight-week-old female BALB/c mice were intravaginally inoculated with a lethal dose of HSV-2 (MS strain) and pre- or post-infection treated with different doses of a 10 mg/ml solution of 1T1. A single topical administration of 1T1 shortly before infection of BALB/c mice with HSV-2 protected 9 out of 10 mice from HSV-2-induced lesions and mortality, compared with only 10% survival in control mice. In addition, 1T1 produced a total blockade in virus shedding in the vaginal secretions. When 1T1 pre-treatment was reinforced with a second dose 2 h after infection, total protection was observed even when the prophylactic administration had taken place at 60 min before infection. The irreversible virucidal action of 1T1 against herpes virus seems to be responsible of its protective effect against virus replication and mortality following vaginal HSV-2 infection.

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In spite of the challenge represented by the emergence of the human immunodeficiency virus (HIV) and the concomitant epidemic of the acquired immune deficiency syndrome (AIDS), there has only been limited success in controlling the spread of sexually transmitted diseases (STD). In fact, the prevalence of STD pathogens has increased in recent years. This is the case for herpes simplex virus type 2 (HSV-2), a virus causing recurrent genital lesions and also responsible for increasing the risk and severity of HIV infection (Arvin and Prober, 1997; Cohen, 1998; Corey and Handsfield, 2000). Consequently, the development of topical microbicides for preventive use against a wide spectrum of STD is considered a high priority for public and private organizations (Spieler Trager, 2003; Keller et al., 2003).

A topical microbicide active against viruses should prevent virus transmission either by inactivation of virions or by

blocking virus entry into the host cell. In addition, it must be safe and well tolerated even after frequent applications. Much work has been focussed on detergents such as nonoxynol-9 (N-9), a surfactant spermicide, which inactivates STD pathogens, including HSV-2 and HIV, by disruption of lipid viral envelopes (Jennings and Clegg, 1993). However, as this compound has the same effect on the cell membrane, the repeated use of N-9 resulted in vaginal irritation, which could increase the risk of HIV infection (Stafford et al., 1998). In search of less toxic microbicides, polysulfates have become attractive candidates. In vitro studies have shown that diverse classes of polysulfates are selective inhibitors of HSV, HIV and other enveloped viruses (Witvrouw et al., 1994) and, particularly, their antiherpetic in vivo effectiveness has been evaluated (Neyts and De Clercq, 1995; Zacharopoulos and Phillips, 1997; Zeitlin et al., 1997; Bourne et al., 1999b; Piret et al., 2000; Herold et al., 2000; Maguire et al., 2001).

In previous studies, we have identified carrageenans of different structural types, isolated from the red seaweed *Gi-*

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gartina skottsbergii, as selective inhibitors of herpes viruses, in Vero cells as well as in human and neural cells (Carlucci et al., 1997, 1999b). Among these compounds, the λ -carrageenan 1T1 showed the highest selectivity index and a dual mode of action, leading to virus inactivation and blockade of virus adsorption (Carlucci et al., 1999a). In the present study, the protective efficacy of 1T1 against HSV-2 mouse genital infection was evaluated.

The T crude extract of carrageenans was obtained from the tetrasporic stage of the red seaweed *G. skottsbergii*, collected in Bahia Camarones (Provincia de Chubut, Argentina), and the λ -carrageenan 1T1 was purified from T as previously described (Carlucci et al., 1997). An aqueous solution of 1T1 (10 mg/ml) was prepared in PBS and sterilized by autoclaving. A 1T1 gel formulation was prepared by mixing equal volumes of 1T1 solution and 1% Carbopol 974P NF (B.F. Goodrich, Cleveland, OH).

Vero cells were grown as monolayers in Eagle's minimum essential medium (MEM) (GIBCO, Carlsbad, CA) supplemented with 5% inactivated calf serum and gentamycin. The MS strain of HSV-2, provided by Dr. F. Benencia (Immunochemistry Laboratory, UBA, Argentina) was propagated and titrated by plaque formation on Vero cells. The plaque reduction assay to determine the antiviral activity of 1T1 against MS and the virus inactivation assay to assess its virucidal activity were conducted as previously described (Carlucci et al., 1997, 1999a).

Six to eight-week-old female BALB/c mice were used for vaginal inoculation with HSV-2. Prior to virus inoculation, animals were injected subcutaneously with 20 μ l of medroxyprogesterone acetate (Medrosterona; Gador, Argentina), prepared as 25 mg/ml solution in PBS, a treatment known to increase the susceptibility of mice to HSV-2 infection (Parr et al., 1994). Five days later, 100 μ l of 1T1 (10 mg/ml in PBS) was instilled into the vagina. One minute after receiving the carrageenan, mice were inoculated intravaginally with 1×10^5 PFU of HSV-2 in 20 μ l. Control animals received PBS. Animals were examined for morbidity and mortality during 20 days. In other set of experiments, variations in the time of administration of the compound were assayed.

To study virus shedding, samples of vaginal secretions from all animals in each group were collected at 3 days post-infection. Briefly, 10 μ l of PBS containing 5% calf serum was pipetted in and out of the vagina five times, then the lavages of each group of animals were pooled, and frozen at -70°C until titration of HSV-2 infectivity by plaque assay. Data were analysed by using the chi-square method. A *P*-value of 0.05 or less was considered significant.

The model of herpes vaginal infection used to assay the in vivo microbicidal potential of 1T1 consisted of the inoculation of BALB/c mice with the MS strain of HSV-2. As the antiviral activity of 1T1 against this strain has not been previously assayed, we first evaluated the in vitro inhibitory effect of 1T1 against MS strain. In a virus plaque reduction assay, 1T1 showed a strong dose-dependent inhibitory action, and

the antiviral effective concentration 50% (EC_{50}) was calculated to be 0.5 $\mu\text{g/ml}$, comparable to previous values of EC_{50} obtained for this carrageenan against the G strain and clinical isolates of HSV-2 (Carlucci et al., 1997).

In a previous report of our laboratory about the virucidal properties of natural carrageenans, the λ -carrageenan 1T1 showed a differential behavior in comparison with μ/ν - or κ/ι -carrageenans, exhibiting a direct inactivating effect against HSV-1, F strain (Carlucci et al., 1999a). The results of a virucidal test performed by incubation of HSV-2 (MS strain) with variable concentrations of 1T1 confirmed the ability of this compound to destroy virion infectivity, and the virucidal concentration 50% (VC_{50}) was 1.2 $\mu\text{g/ml}$, comparable to the antiviral EC_{50} .

In preliminary experiments, BALB/c mice were inoculated with variable doses of HSV-2 to determine the adequate virus dose to be used in protection studies. In contrast to other murine models of herpes vaginal infection, the system BALB/c-MS was characterized by a total correlation between morbidity and mortality at all virus doses assayed, in the range 10^2 – 10^5 PFU/mouse. In all cases, mice developing disease signs at 7–9 days after infection finally died, but the mean day of death varied among 8 and 16 days post-infection, inversely with the amount of virus inoculated. Disease signs evolved from inflammation and redness, to hair loss, ulcerous lesions in the genital region, and finally flaccid paralysis. A dose of 10^5 PFU/mouse was chosen for protection studies because this dose produced a high rate of morbidity/mortality (consistently around 90% in repeated experiments), with a short time to death (<9 days) and a considerable amount of virus shedding in vaginal secretions (approximately 10^4 PFU/ml at 3 days post-infection). This dose would be rigorous enough to assess the putative protective effect of 1T1.

To study the effectiveness of 1T1 on this vaginal infection, in a first experiment mice were treated with 1T1 shortly, 1 min, before infection with 1×10^5 PFU of HSV-2. As shown in Table 1, under these treatment conditions a highly significant level of protection was observed in comparison with control infected animals similarly treated with PBS (10% mortality in group 2, as compared with 90% in group 1, $P < 0.005$). Only one animal developed typical vaginal lesions and died, but the day of death was delayed from 9.3 in the control group to 16.0 in the 1T1-treated mouse. Furthermore, no infectious HSV-2 was detected at 3 days post-infection in the vaginal washings indicating that the compound afforded effective protection against HSV-2 replication in the genital tract.

To increase the effectiveness of 1T1 against HSV-2 genital infection, the pre-treatment was reinforced with post-infection administration of the compound. Three schedules of post-treatment were assayed, consisting in one dose at 2 h post-infection (group 3), two doses at 2 and 6 h post-infection (group 4), and three doses at 2, 6, and 24 h after HSV-2 inoculation (group 5). When the 1T1 pre-treatment at 1 min before infection was combined with just only one second dose administered 2 h later, the genital infection with HSV-2

Table 1
Effect of 1T1 treatment on vaginal mouse infection with HSV-2

Group	Treatment ^a	Mortality no. dead mice/total (%)	Mean day of death \pm S.D.	Virus shedding ^b (PFU/ml)
1	PBS –1 min	9/10 (90)	9.3 \pm 0.7	1.1 \times 10 ⁴
2	1T1 –1 min	1/10 (10) ^c	16.0	<10
3	1T1 –1 min, +2 h	0/10 (0) ^c	S ^d	<10
4	1T1 –1 min, +2 h, +6 h	0/10 (0) ^c	S	<10
5	1T1 –1 min, +2 h, +6 h, +24 h	0/10 (0) ^c	S	<10
6	1T1 + 974P NF –1 min	0/10 (0) ^c	S	<10
7	974P NF –1 min	8/10 (80)	8.6 \pm 1.2	6.1 \times 10 ³
8	1T –1 min, +2 h	0/10 (0) ^c	S	<10

^a BALB/c mice were intravaginally inoculated with HSV-2, at time 0. Doses of 1T1 or 1T (group 8) (100 μ l of a 10 mg/ml solution) were instilled into the vagina at the indicated times pre- (–) or post-infection (+). For group 6, 1T1 was previously formulated with 974P NF and then inoculated before infection. Animals were monitored daily for morbidity and mortality.

^b Vaginal washings were collected at day 3 and titrated by PFU on Vero cells.

^c $P < 0.005$, each treated group compared with the corresponding control group.

^d S: survival.

was completely prevented (Table 1). None of the mice inoculated developed any sign of vaginitis, no infectious virus was detected in vaginal lavages and all animals survived until the end of the experiment. Same results were obtained when two or three 1T1 doses of post-treatment were assayed (Table 1). No toxicity was detected in the 1T1-treated animals by macroscopic observation: there was no induction of redness, irritation, inflammation, or ulceration to the genital mucosa, and the amount of vaginal mucus produced was similar to untreated mice.

Although the aqueous solution of 1T1 exhibited an apparent viscosity adequate to remain active in the vagina until virus inoculation, a gel formulation of 1T1 in Carbopol 974P NF was also tested. A slight improvement in 1T1 protective activity was observed with respect to aqueous solution, since the administration of one dose of the formulation before virus inoculation protected all animals (Table 1, group 6).

Interestingly, a more crude preparation of the seaweed carrageenans named 1T (Carlucci et al., 1997) was as effective as the homogeneous 1T1 against the HSV-2 vaginal infection (Table 1, group 8).

An effective ideal microbicide will need not only to protect immediately after application, but also to retain activity

in the genital tract for a more prolonged period of time. Thus, the effect of the time of administration of 1T1 on the efficacy against vaginal infection was examined. The different pre-treatment times were combined with a post-treatment dose at 2 h post-infection. Results obtained showed that the administration of 1T1 from 15 to 60 min before virus challenge afforded the same level of protection as 1T1 treatment immediately before HSV-2 inoculation (Table 2, $P < 0.005$). The effect of post-infection treatment alone was also examined. As shown in Table 2, when 1T1 was given 15 min after infection, protection was reduced to non-significant levels, and only a delay in the mean day of death was registered. Finally, the administration of 1T1 2 h after infection failed to provide any protection against HSV-2 vaginal inoculation, although again a prolongation in survival time was observed.

These studies demonstrated the high microbicidal efficacy of the seaweed λ -carrageenan 1T1 against murine genital HSV-2 infection, as evaluated by the protection afforded against virus-induced lesions and mortality and the blockade of virus shedding in the vaginal secretions. The prophylactic administration of 1T1 shortly before infection was required to achieve nearly 100% protection, and when 1T1

Table 2
Effect of the time of treatment with 1T1 on vaginal mouse HSV-2 infection

Treatment ^a	Mortality no. dead mice/total (%)	Mean day of death \pm S.D.	Virus shedding ^b (PFU/ml)
PBS –1 min	9/10 (90)	10.5 \pm 1.0	2.6 \times 10 ³
1T1 –1 min, +2 h	0/10 (0) ^c	S ^d	<10
1T1 –15 min, +2 h	0/10 (0) ^c	S	<10
1T1 –30 min, +2 h	1/10 (10) ^c	23.0	<10
1T1 –45 min, +2 h	1/10 (10) ^c	10.0	<10
1T1 –60 min, +2 h	0/10 (0) ^c	S	<10
1T1 +15 min, +2 h, +4 h	8/10 (80)	14.7 \pm 4.8	N.D. ^e
1T1 +2 h, +4 h	10/10 (100)	14.7 \pm 5.6	N.D.

^a BALB/c mice were intravaginally inoculated with HSV-2, MS strain, at time 0. Doses of 1T1 (100 μ l of a 10 mg/ml solution) were instilled into the vagina at the indicated times pre- (–) or post-infection (+). Animals were monitored daily for mortality.

^b Vaginal washings were collected at day 3 and titrated by PFU on Vero cells.

^c $P < 0.005$, each treated group compared with the control group.

^d S: survival.

^e N.D.: not done.

pre-treatment was reinforced with a second dose of 1T1 after infection, total protection was observed even when the prophylactic inoculation was extended to 60 min before infection (Tables 1 and 2).

The in vitro interaction between 1T1 and herpes virus has been shown to be virucidal and irreversible, leading to virion inactivation after pre-incubation of 1T1 with virions (Carlucci et al., 1999a). The irreversibility of 1T1 treatment was also evidenced by a plaque reduction assay under different conditions: removal of the compound immediately after virus adsorption by repeated washings did not affect the inhibitory effect on plaque formation in Vero cells (Carlucci et al., 1999a). This irreversible virucidal mode of action of 1T1 against HSV may be responsible of its in vivo efficacy here demonstrated, allowing to maintain a very stable virus–compound complex at the vagina, without release of the compound from the viral envelope glycoprotein. In fact, when we assayed in the BALB/c-MS model the μ/ν carrageenan 1C3, a polysaccharide isolated from the seaweed *G. skottsbergii* with in vitro potent antiviral activity against HSV-1 and HSV-2 but lacking virucidal properties (Carlucci et al., 1999a), it was unable to confer complete protection against mouse vaginal infection (data not shown). Similar negative results were reported for dextran sulfate in mice intravaginally inoculated with HSV-2 (Neyts and De Clercq, 1995). As occurs with 1C3, dextran sulfate has a non-virucidal interaction with herpesviruses and HIV (Baba et al., 1988; Neyts et al., 1992), pointing to the importance of the inactivating properties of polysulfates such as 1T1 in perspective of their eventual in vivo administration.

Our results are in agreement with other studies about the efficacy and preliminary safety of commercial λ -carrageenans (Zeitlin et al., 1997; Zacharopoulos and Phillips, 1997; Bourne et al., 1999a; Coggins et al., 2000; Maguire et al., 2001). Some remarkable features of the protective effect here described for a natural carrageenan deserve consideration: (a) 100% protection against HSV-2 mortality and replication was achieved in a very strict model of murine infection at a high dose of virus; (b) it is unlikely that protected surviving animals remained latently infected because neither virus nor neutralizing antibodies against HSV-2 were detected in serum until 3 weeks after infection (data not shown); (c) multiple doses (at least four) of compound during a period of 24 h did not induce any sign of vaginal irritation in the animals; (d) the protective ability of the crude extract of λ -carrageenans obtained from *G. skottsbergii* was as effective as the homogeneous carrageenan 1T1. The small animal model presented in this study warrants the availability of 1T1 to protect the whole infectable surface of the mouse vagina. The assay of this carrageenan in a larger animal, such as guinea pig, will be the next step to confirm its effectiveness for topical applications. Taking into account the real need of a microbicidal preparation obtainable at low cost in large quantities to be used in Third World countries to prevent STD, further clinical evaluations of 1T and 1T1 seem warranted.

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References

- Arvin, A.M., Prober, C.G., 1997. Herpes simplex virus type 2 – a persistent problem. *N. Engl. J. Med.* 337, 1158–1159.
- Baba, M., Pauwels, R., Balzarini, J., Arnout, J., Desmyter, J., De Clercq, E., 1988. Mechanism of inhibitory effect of dextran sulfate and heparin on replication of human immunodeficiency virus in vitro. *Proc. Natl. Acad. Sci. U.S.A.* 85, 6132–6136.
- Bourne, K.Z., Bourne, N., Reising, S.F., Stanberry, L.R., 1999a. Plant products as topical microbicide candidates: assessment of in vitro and in vivo activity against herpes simplex virus type 2. *Antiviral Res.* 42, 219–226.
- Bourne, N., Bernstein, D.I., Ireland, J., Sonderfan, A.J., Profy, A.T., Stanberry, L.R., 1999b. The topical microbicide PRO 2000 protects against genital herpes infection in a mouse model. *J. Infect. Dis.* 180, 203–205.
- Carlucci, M.J., Pujol, C.A., Ciancia, M., Nosedá, M.D., Matulewicz, M.C., Damonte, E.B., Cerezo, A.S., 1997. Antiherpetic and anticoagulant properties of carrageenans from the red seaweed *Gigartina skottsbergii* and their cyclized derivatives: correlation between structure and biological activity. *Int. J. Biol. Macromol.* 20, 97–105.
- Carlucci, M.J., Ciancia, M., Matulewicz, M.C., Cerezo, A.S., Damonte, E.B., 1999a. Antiherpetic activity and mode of action of natural carrageenans of diverse structural types. *Antiviral Res.* 43, 93–102.
- Carlucci, M.J., Scolari, L.A., Damonte, E.B., 1999b. Inhibitory action of natural carrageenans on herpes simplex virus infection of mouse astrocytes. *Chemotherapy* 45, 429–436.
- Coggins, C., Blanchard, K., Alvarez, B., Brache, V., Weisberg, E., Kilmarx, P.H., Lacarra, M., Massai, R., Mishell Jr., D., Salvatierra, A., Witwatwongwana, P., Elias, C., Ellertson, C., 2000. Preliminary safety and acceptability of a carrageenan gel for possible use as a vaginal microbicide. *Sex. Transm. Infect.* 76, 480–483.
- Cohen, M.S., 1998. Sexually transmitted diseases enhance HIV transmission: no longer a hypothesis. *Lancet* 351, 5–7.
- Corey, L., Handsfield, H.H., 2000. Genital herpes and public health: addressing a global problem. *JAMA* 283, 791–794.
- Herold, B.C., Bourne, N., Marcellino, D., Kirkpatrick, R., Strauss, D.M., Zaneveld, L.J.D., Waller, D.P., Anderson, R.A., Chany, C.J., Barham, B.J., Stanberry, L.R., Cooper, M.D., 2000. Poly(sodium 4-styrene sulfonate): an effective candidate topical antimicrobial for the prevention of sexually transmitted diseases. *J. Infect. Dis.* 181, 770–773.
- Jennings, R., Clegg, A., 1993. The inhibitory effect of spermicidal agents on replication of HSV-2 and HIV-1 in vitro. *J. Antimicrob. Chemother.* 32, 71–82.
- Keller, M.J., Klotman, M.E., Herold, B.C., 2003. Development of topical microbicides for prevention of human immunodeficiency virus and herpes simplex virus. *Am. J. Reprod. Immunol.* 49, 279–284.
- Maguire, R.A., Bergman, N., Phillips, D.M., 2001. Comparison of microbicides for efficacy in protecting mice against vaginal challenge with herpes simplex virus type 2, cytotoxicity, antibacterial properties, and sperm immobilization. *Sex. Transm. Dis.* 28, 259–265.
- Neyts, J., De Clercq, E., 1995. Effect of polyanionic compounds on intracutaneous and intravaginal herpesvirus infection in mice: impact on the search for vaginal microbicides with anti-HIV activity. *J. Acquir. Immune Defic. Syndr. Hum. Retrovirol.* 10, 8–12.

- Neyts, J., Snoeck, R., Schols, D., Balzarini, J., Esko, J.D., Van Schepdael, A., De Clercq, E., 1992. Sulfated polymers inhibit the interaction of human cytomegalovirus with cell surface heparan sulfate. *Virology* 189, 48–58.
- Parr, M.B., Kepple, L., McDermott, M.R., Drew, M.D., Bozzola, J.J., Parr, E.L., 1994. A mouse model for studies of mucosal immunity to vaginal infection by herpes simplex virus type 2. *Lab. Invest.* 70, 369–380.
- Piret, J., Lamontagne, J., Bestman-Smith, J., Roy, S., Gourde, P., Désormeaux, A., Omar, R.F., Juhasz, J., Bergeron, M.G., 2000. In vitro and in vivo evaluations of sodium lauryl sulfate and dextran sulfate as microbicides against herpes simplex and human immunodeficiency viruses. *J. Clin. Microbiol.* 38, 110–119.
- Spieler Trager, R., 2003. Raising new barriers against HIV infection. *Science* 299, 39.
- Stafford, M.K., Ward, H., Flanagan, A., Rosenstein, I.J., Taylor-Robinson, D., Smith, J.R., Weber, J., Kitchen, V.S., 1998. Safety study of nonoxynol-9 as a vaginal microbicide: evidence of adverse effects. *J. Acquir. Immune Defic. Syndr. Hum. Retrovirol.* 17, 327–331.
- Witvrouw, M., Desmyter, J., De Clercq, E., 1994. Antiviral portrait series: 4. Polysulfates as inhibitors of HIV and other enveloped viruses. *Antiviral Chem. Chemother.* 5, 345–359.
- Zacharopoulos, V.R., Phillips, D.M., 1997. Vaginal formulations of carageenan protect mice from herpes simplex virus infection. *Clin. Diagn. Lab. Immunol.* 4, 465–468.
- Zeitlin, L., Whaley, K.J., Hegarty, T.A., Moench, T.R., Cone, R.A., 1997. Tests of vaginal microbicides in the mouse genital herpes model. *Contraception* 56, 329–335.