Herpes simplex virus type 1 variants arising after selection with an antiviral carrageeenan: lack of correlation between drug-susceptibility and syn phenotype

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Running title: Carrageenan susceptibility of HSV-1 syn variants

Summary. Natural carrageenans of diverse structural types isolated from the red seaweed *Gigartina skottsbergii* have been recently identified as potent and selective inhibitors of herpes simplex virus (HSV) types 1 and 2. The μ/ν carrageenan 1C3 was tested in vitro for its ability to select resistant variants. After serial passages of HSV-1 strain F in Vero cells in the presence of increasing concentrations of 1C3, there emerged viruses which were approximately 2-10 fold more resistant to 1C3 inhibition than parental virus and formed large plaques with an altered syncytial phenotype (1C3-syn). Plaque-purified syncytial variants isolated from passages 13 and 14 have shown variable levels of resistance to 1C3 as well as to the other antiviral carrageenans isolated from *G. skottsbergii*, and to other sulfated polysaccharides with known antiviral activity, such as heparin and dextran sulfate 8000, but all the clones were susceptible to acyclovir. The syn phenotype was not related to polysaccharide-resistance. All the 1C3-syn variants formed large syncytia in Vero and CV-1 cells, but did not induce fusion in other cell types. The growth efficiency in Vero cells as well as the virulence for mice by intracerebral or intraperitoneal inoculation of 1C3-syn variants showed no significative alterations in comparison to the parental virus. The syncytial properties were not affected by cyclosporin A or melittin, suggesting that an alteration on glycoprotein gB could be responsible of the syn phenotype induced by 1C3.

Introduction

Herpes simplex virus type 1 (HSV-1) is associated with primary and recurrent mucocutaneous infections, mainly localized to the oropharynx, which are usually self-limiting in the immunocompetent host. However, in the immunocompromised patient recurrence of HSV-1 may cause persistent and severe lesions that are refractory to repeated antiviral chemotherapy [7, 9, 16]. Although the life cycle of HSV-1 offers many opportunities for the development of antiviral strategies, at present the only target of successful antiherpetic therapy is DNA replication, through the use of nucleoside analogues such as acyclovir (ACV), valacyclovir, penciclovir and famciclovir [8]. ACV-resistant HSV isolates are recovered frequently from immunocompromised subjects receiving this drug for a prolonged period of time [6, 20, 25]. Thus, the increasing problem of resistance in transplant recipients or patients with AIDS has prompted the development of new antiherpetic drugs.

Sulfated polysaccharides may represent a promising class of future therapeutic agents against HSV infections, either alone or in combination with drugs in clinical use [27]. Recently, we have reported that diverse structural types of natural carrageenans, isolated from the red seaweed *Gigartina skottsbergii*, proved to be potent and selective inhibitors of HSV-1 and HSV-2 in Vero cells and also in cells of neural origin such as murine astrocytes [5]. Among them, the μ/ν carrageenan 1C3 showed the best relationship between antiviral efficacy and lack of cytotoxicity, with a selectivity index greater than 1000, and without anticoagulant action [3]. Mechanistic studies performed with this compound have shown that the main target of the antiviral action of 1C3 was the initial binding of HSV to the host cell [4], a process mediated by interaction of the virion glycoproteins gC and/or gB with heparan sulfate residues on cell surface proteoglycans [12, 22].

The aim of the present study was to test the ability of 1C3 to generate resistance by serial passages of HSV-1 strain F in Vero cells in the presence of the compound and to characterize the variants arising during the selection process.

Materials and Methods

Compounds

The λ-carrageenan 1T1, the κ/ι-carrageenan 1C1 and the μ/ν-carrageenan 1C3 were extracted and purified from cystocarpic and tetrasporic stages of *Gigartina skottsbergii*, a red seaweed collected in Camarones Bay, Chubut, Argentina, as previously described [3]. Heparin, dextran sulfate with average molecular weight of 8000 (DS8000), cyclosporin A and melittin were purchased from Sigma-Aldrich (USA).

Cells and viruses

Vero cells were grown as monolayers in Eagle`s minimum essential medium (MEM) (GIBCO, USA) supplemented with 5 % inactivated calf serum and 50 μg/ml gentamycin. For maintenance medium (MM) the serum concentration was reduced to 1.5 %. Cultures of murine astrocytes were obtained from 2-3 day-old OF1 (Iffa Credo, Lyon, France) mice as previosuly described [5] and maintained in MEM supplemented with 10% fetal calf serum. Hep-2 cells, CV-1 cells and human foreskin fibroblasts (PH) were grown and maintained as Vero cells. HSV-1 strain F was propagated and assayed by plaque formation in Vero cells.

Viral passages for 1C3 selection and plaque purification

To select virus variants in the presence of 1C3, Vero cells were infected with HSV-1 at a moi of 0.1 in the presence of a starting concentration of 1C3 of 2 μg/ml (twice the IC_{50}). The carrageenan was present at virus adsorption and thereafter during all the period of incubation. When 50-70 % cytopathic effect was observed the cell culture was frozen and thawed twice, centrifuged and the supernatant was used to infect a fresh monolayer in the presence of 1C3. The concentration of 1C3 was raised two-fold when massive cytopathic effect was observed and virus appeared to be growing to high titers. Twenty serial pasagges were continued in this manner and the titer and IC_{50} of each viral passage against 1C3 was determined by plaque reduction assay. Simultaneously, control serial passages of HSV-1 in Vero cells without 1C3 were also performed.

Virus corresponding to passages 13 and 14 in the presence of 1C3 was plaque-purified in 6-well microplates. Confluent Vero cells were infected with limiting dilutions of the virus stocks and covered with MM containing 0.6 % agarose. After 3 days of incubation,

the cell cultures were stained with neutral red and the clones were picked up. The plaque-purified viruses were amplified in Vero cells. The clone stocks were then titrated and subsequently tested for their in vitro susceptibilities to antiviral compounds.

Antiviral assay

Antiviral activity was evaluated by a plaque reduction assay. Vero cell monolayers grown in 24-well plates were infected with about 80 PFU of virus/well in the absence or presence of various concentrations of the compounds. After 1 h adsorption, residual inoculum was replaced by MM containing 0.7 % methylcellulose and the corresponding dose of compound. Plaques were counted after 2 days of incubation and the inhibitory concentration 50% (IC_{50}) was calculated as the concentration required to reduce virus plaques by 50 %.

Virulence assays

Six-week old OF1 mice were inoculated by intraperitoneal route with 100 µl of ten-fold serial dilutions of each virus in MM. Suckling 2 day old OF1 mice were used for intracerebral inoculation with 20 µl of virus serial dilutions. Six to eight animals were used for each dilution. Titers of inocula were verified by simultaneous plaque assays. Morbidity and mortality were recorded daily for 2 weeks. The dose of each virus that resulted in 50 % lethality (lethal dose 50 %, LD_{50}) was calculated by the method of Reed and Muench and the PFU/LD_{50} ratios were obtained.

Results

To evaluate the ability of the natural carrageenan 1C3 to generate resistant variants of HSV-1, serial passages of the F strain in Vero cells in the presence of increasing concentrations of 1C3 were performed as described in Materials and Methods. Twenty serial passages were performed and each passage was titrated by plaque formation and for antiviral susceptibility against 1C3. The IC_{50} values of the passages were variable, but after 11 passages in the presence of 1C3 the IC_{50}s were 3-7 fold higher than those for the original F strain or this strain passaged similarly in parallel in the absence of the compound. Thus, only a partial 1C3 resistance could be generated by the carrageenan. In contrast, parallel passages of HSV-1 strain F in Vero cells in the presence of ACV

induced the appearance of resistant strains, with IC_{50} values 60-fold higher than the wt virus, only after 6 virus transfers (data not shown).

Noticeably, viruses grown in the presence of 1C3 showed an alteration in their cytopathogenic properties in Vero cells, developing a syncytial (syn) phenotype and plaques with larger size than wt virus. After passage 16, 100 % of plaques were large and syncytial, whereas there was no enrichment in syncytial variants after sequential passages of HSV-1 in the absence of drug.

To further characterize the mutants selected with 1C3 and assess the homogeneity of the viral population, virus samples corresponding to passages 13 and 14 (named 1C3-syn 13 and 1C3-syn 14, respectively) were biologically cloned and several plaque-purified clones were isolated and then propagated in Vero cells. All the tested clones maintained their stable syncytial phenotype and large plaque size after cloning and amplification in Vero cells. These clones induced a variable but always extensive cell-cell fusion producing giant polykaryocytes with an average of 500-700 nuclei/syncytium (Fig. 1), In contrast, the parental HSV-1 F strain induced the typical quick cell rounding and detachment (Fig. 1). The syn phenotype was dependent on the cell type: the variants formed syncytia with the same efficiency in Vero as well as in the CV-1 monkey kidney cell line, but cell rounding was observed in HEp-2 cells, human foreskin fibroblasts and murine astrocytes.

The patterns of susceptibility of five clones isolated from 1C3-syn 13 and 1C3-syn 14 to 1C3 as well as to the other two antiviral carrageenans isolated from *G. skottsbergii*, 1T1 and 1C1 [4], and to other sulfated polysaccharides with known antiviral activity, such as heparin and dextran sulfate 8000, are presented in Table 1. The level of virus susceptibility was expressed as the ratio of IC_{50} of each virus clone to that of HSV-1 strain F taken as reference. Interestingly, the degree of resistance to polysaccharide inhibition did not correlate in a simple way with the syn phenotype: although all the variants were consistent in their ability to induce large syncytia in Vero cells, they showed variable levels of resistance to 1C3. Variant 1C3-syn 13-8 was as sensitive to 1C3 as the parental virus, the variant 1C3-syn 14-1 showed the highest level of resistance (IC_{50} 10-fold higher than the parental virus) and variants 1C3-syn 13-3, 1C3-syn 13-9 and 1C3-syn 14-3 exhibited an intermediate behavior with a 3-4-fold relative resistance to inhibition by 1C3. These results allowed to clearly conclude that the syn phenotype was not entirely related to drug resistance. Furthermore, 1C3-syn variants

resistant to 1C3 showed cross resistance to the other types of carrageenans and sulfated polysaccharides. Finally, because some HSV-1 syn mutants are thymidine kinase deficient [21], we also tested the ACV susceptibility of the 1C3-syn variants. Both the wt strain and the either polysaccharide-sensitive or resistant 1C3-syn variants were similarly sensitive to ACV (Table 1).

To determine whether the altered cytopathogenicity and large plaque size of syn variants was due to an enhanced viral replication, one-step growth curves of variants 1C3-syn 13-8 and 1C3-syn 14-1 and parental virus in Vero cells were studied. As seen in Fig. 2, variants grow as efficiently as the wt virus in Vero cells, with slight differences during the earliest times, but similar final yields at 15 h post-infection. Similarly, the kinetics of the early stages of virus replication, such as adsorption and internalization, did not show any alteration for the variants in comparison to the parental F strain (data not shown).

To obtain a preliminary mapping of the mutations responsible for the syn phenotype of 1C3-syn variants, two inhibitors of HSV-induced syncytium formation were used. Cyclosporin A inhibits fusion caused by syncytial virus mutants if the responsible mutation is not in the cytoplasmic tail of gB [26], whereas melittin, a bee venom that blocks the Na+/K+ ATPase, inhibits syncytium formation when the responsible mutation maps to the glycoprotein gK [2]. When titered in the presence of melittin or cyclosporin A the syncytial plaque morphology of 1C3-syn variants was not affected, suggesting that an alteration on gB could be responsible for the syn phenotype induced by 1C3.

As it has been described that an alteration in the carboxy terminus of gB of a syn HSV-1 strain selected with heparin results in both its syncytial phenotype and an altered pathogenesis for mice [11], we tested the inoculation of mice with our variants by two routes. Following intracerebral or intraperitoneal inoculation, the 1C3-syn variants were as pathogenic as the parental virus, showing no significant differences neither in the values of LD_{50} to PFU ratio nor in the survival time after inoculation (Table 2).

Discussion

The results presented here further support the known properties of sulfated polysaccharides as slow and poor inducers of virus-drug resistance [27]. Several passages in the presence of concentrations of 1C3 highly increasing the IC_{50} were required to obtain HSV-1 variants with a reduced susceptibility to this carrageenan and to other polysaccharides.

The cytopathic effects induced by HSV are manifested by either rounding or fusion of cells. Infection of cells with most of the fresh isolates of HSV leads to cell rounding, but mutations in at least six syn loci in viral genome, syn 1 to 6, induce a syncytial plaque phenotype. Only three of the genes involved have been identified: the syn 1 mutation alters the gene encoding gK [14]; the syn 3 mutation affects the carboxy-terminus of gB [10], whereas syn 5 mutation seems to affect the UL24 gene [23]. Little is known about the genes or encoded proteins altered by the other syn mutations. Although the in vivo emergence of syncytial variants is rather exceptional, they may be selected in vitro by some selective forces [18]. The presence of the carrageenan 1C3 has shown to be a positive pressure for the appearance of syn variants. The acquisition of a strong fusogenic ability responsible to produce large polykaryocytes in Vero and CV-1 cells was a stable character of HSV-1 variants, even after several subsequent passages in cell culture in the absence of the carrageenan. No alterations in other biological properties were here detected in the HSV-1 variants selected with 1C3. In addition, the behavior of the clones 1C3 syn 14-1 and 1C3 syn 13-8 (Table 1) clearly indicated that the selected syn phenotype was independent of the drug susceptibility.

Previous studies have reported the selection of HSV-1 syn mutants by serial passage in the presence of heparin, a glycosaminoglycan which potently inhibits the infectivity of HSV and other enveloped viruses [1, 28]. Goodman and Engel [11] described a syncytial mutant named 17 hep syn, sixfold more heparin resistant than its parent, and the syn phenotype of this virus was mapped to the carboxy terminus of gB. By contrast, the syncytial phenotype of HSV-1 mutants 2- to 20- fold more resistant to heparin inhibition than original virus isolated by Pertel and Spear [19] mapped to a mutation in gK, a glycoprotein which does not reach the plasma membrane [15]. The mutation responsible for heparin resistance was not mapped, although the presence of heparin favored the selection of gC-negative mutants resistant to heparin inhibition. In our case, the failure of cyclosporin A and melittin to affect the syncytial properties of 1C3-syn

variants seems to be indicative of a probable alteration on gB as responsible of the syn phenotype induced by 1C3. Strains with a mutation in the syn 3 locus affecting gB induce fusion in the presence of cyclosporin A at a concentration up to 150 µM, whereas all other syncytial strains induce cell rounding after infection of cells in the presence of cyclosporin A [17, 26]. Consequently, gB would be a main target for the antiviral activity of 1C3. Due to the lack of correlation between carrageenan susceptibility and syn phenotype in the variants selected with 1C3, the 1C3-syn variants partially resistant to 1C3 may also have another alteration responsible of the resistance. At least two proteins of HSV-1 envelope, gC and gB, were demonstrated to be able to bind heparin in vitro and to interact with heparan sulfate in the initial stage of the virus cycle [12, 13, 22, 24]. It is conceivable that 1C3, which prevents virus adsorption, may interact with gC and/or gB. Thus, the pressure exerted by 1C3 can select by a mutation in gB, responsible of syn character, as indicated by melittin and cyclosporin A results, and also by other mutation in gC or gB leading to drug resistance. To elucidate the exact target site (s) of 1C3, further analyses are in progress.

Our previous studies ascertained that 1C3 is able to inhibit the replication of tk^- HSV-1 strains resistant to ACV [3]. Concomitantly, here it is shown that the 1C3-syn variants resistant to 1C3 are fully sensitive to ACV (Table 1). Therefore, 1C3 may be a good candidate drug in patients who require alternative therapy due to the emergence of ACV resistant variants. The efficacy of carrageenans in the therapy of viral infections remains to be demonstrated both in animal models and humans, but due to their mode of antiherpetic action as inhibitors of the initial binding of virions to the host cell, it is important to consider these compounds as promising candidates not only for treatment of patients who are already infected with HSV, but also for prophylaxis and prevention from infection.

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Table I. Drug-susceptibility of HSV-1 syn variants arising after 1C3 selection in vitro

HSV-1 clone	IC_{50}[a] (RR[b])					
	1C3	1T1	1C1	Heparin	DS8000	Acyclovir
F	1.1	0.4	3.2	1.3	1.8	0.013
1C3-syn 13-3	3.9 (3.5)	0.8 (2.0)	8.4 (2.6)	6.8 (5.2)	5.2 (2.9)	0.010 (0.8)
1C3-syn 13-8	1.2 (1.1)	0.6 (1.5)	2.6 (0.8)	6.3 (4.8)	4.4 (2.4)	0.018 (1.4)
1C3-syn 13-9	4.5 (4.1)	3.9 (9.7)	9.7 (3.0)	10.0 (7.7)	3.4 (1.9)	0.006 (0.5)
1C3-syn 14-1	11.6 (10.5)	2.9 (7.2)	40.0 (12.5)	13.2 (10.1)	10.0 (5.5)	0.026 (2.1)
1C3-syn 14-3	3.9 (3.5)	1.2 (3.0)	6.8 (2.1)	3.1 (2.4)	1.0 (0.6)	ND[c]

[a]IC_{50} (Inhibitory concentration 50%): concentration in µg/ml required to reduce plaque number by 50%. Data are the mean value of two experiments.

[b]RR (Relative resistance): ratio between IC_{50} for each syn variant and the IC_{50} for F strain.

[c]ND: not done.

Table II. Virulence for mice of 1C3-syn variants

Virus	Intraperitoneal inoculation[a]		Intracerebral inoculation[b]	
	PFU/LD_{50}[c]	Survival time[d]	PFU/LD_{50}	Survival time
F wt	$3.3x10^5$	13.5 ± 0.7	0.54	4.3 ± 1.0
1C3-syn 13-3	$1.7x10^5$	10.3 ± 3.4	0.21	4.6 ± 0.5
1C3-syn 13-8	$1.1x10^5$	12.3 ± 5.7	0.20	3.8 ± 0.9

[a]4-6 week old OF1 mice were intraperitoneally inoculated with serial dilutions of each virus. [b]1-3 day old OF1 newborn mice were intracerebrally inoculated with serial dilutions of each virus. [c]LD_{50} values were determined by the method of Reed and Muench, and the PFU/LD_{50} ratios were then calculated. [d]Average survival time (days) ± standard deviation of dead mice inoculated with ? PFU

Fig. 1. Morphological aspect of syncytia in Vero (left) and CV-1 (right) cell cultures: control cells (upper), cells infected with HSV-1 F (middle) or 1C3-syn 13-8 (bottom).

Fig. 2. Vero cells were infected with syn variants and F strain (moi = 0.1). At different times after infection, one culture from each series was frozen and thawed, and virus yields were determined