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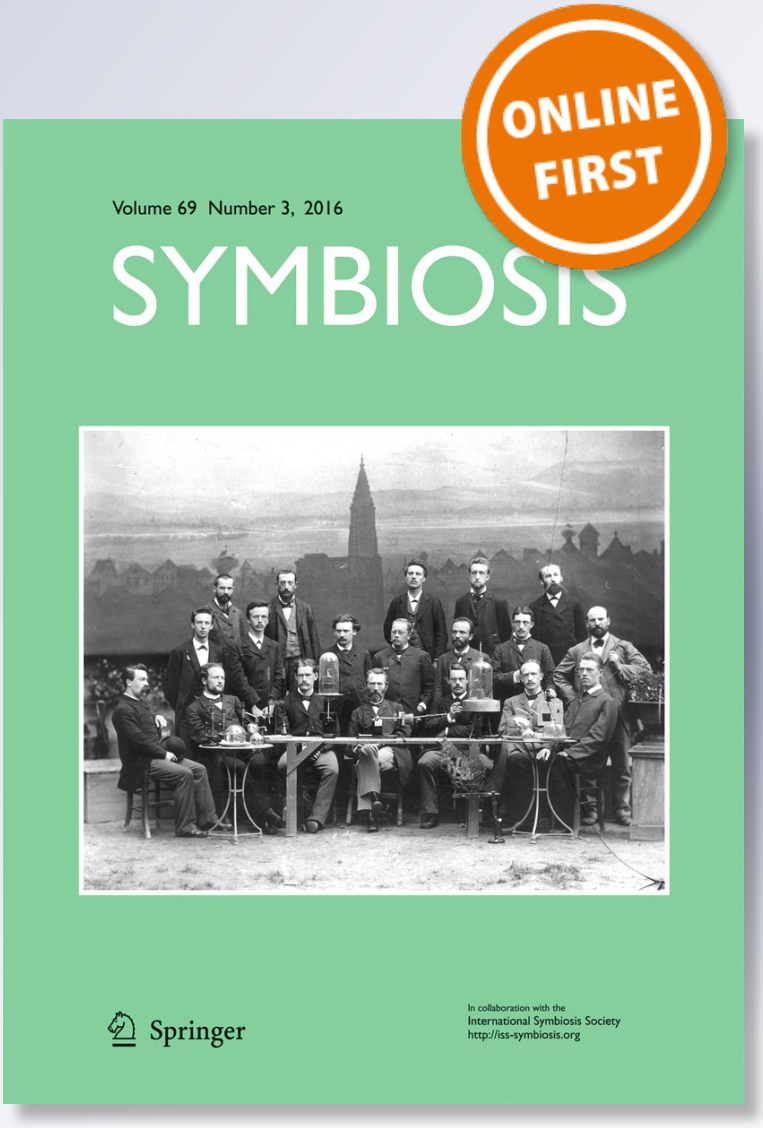
Symbiosis

ISSN 0334-5114

Symbiosis
DOI 10.1007/s13199-016-0437-4



Springer

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In vitro isolation of variants of herpes simplex virus attenuated with altered thymidine kinase and DNA polymerase genes using carrageenans as selection agents

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Received: 27 April 2016 / Accepted: 23 June 2016

Abstract Natural polysaccharides known as carrageenans are potent and selective inhibitors of herpes simplex virus through blocking the interaction of the virus with the cellular receptor. Passaging the virus in vitro in the presence of carrageenans types k and ι enabled us to generate variants of herpes simplex type 2. At passage 22, four clones were selected for further characterization: ι22-9, ι22-12, k22-12 and k22-13. Variants showed a syncytial phenotype, grew at similar titers when compared to parental virus and exhibited moderate resistance to carrageenans. These were found to have a mutation in the thymidine kinase gene in the case of k22-13 (aa 149 Val to Ala) and in the DNA *pol* gene in the case of ι22-12 (aa 789 Met to Thr). In variant k22-12, three substitutions in the DNA *pol* gene were identified. Variants were less virulent than parental strain when tested intravaginally or intranasally in mice. Attenuation correlated with higher levels of IL-6 and TNF-α in animals inoculated with the variants. Selective pressure on the external glycoproteins of the virus may generate viruses with alterations in genes unrelated to the target of action of the selection agent. Study of this type of variation might help us to understand the basis of persistent viral strategies, which in turn may play a role in the development of virus-host symbioses.

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Keywords Herpesvirus · Pathogenicity · Syncytial variant · Carrageenan · Virulence · Mutants

1 Introduction

In 1878 Anton de Bary defined symbiosis as "the living together of differently-named organisms" (Sapp 1994). Since only one of the partners needs to benefit, symbiosis includes parasitism and commensalism as well as mutualism. Indeed mutualism, in which two or more partners benefit, very often evolves from parasitism, with many observed examples of symbiosis lying somewhere in between the extremes of parasitism and mutualism. Virus-host symbioses are common in nature and play an important and underestimated role in evolution (Ryan 2007). In such virus-host symbioses this change, from outright parasitism to the more evolutionarily creative mutualism, is more likely to develop where the viral symbionts adopt "persistent" strategies in their interactions with the host (Villarreal 2007). Complementary to our understanding of the evolutionary dynamics of Darwinian natural selection and neo-Darwinism are other forces leading to hereditary changes, such as symbiogenesis, hybridogenesis, and horizontal gene transfer in prokaryotes and epigenetics, which collectively expand our horizons to a diversity of possibilities (Villarreal and Ryan 2011).

Sulfated polysaccharides may also have played a special role in contributing to the mechanisms of generating hereditary change. These molecules are highly abundant and accessible in nature, notably from sources such as marine algae. They exhibit a wide range of physico-chemical properties that have found useful applications in food science, and the medical and pharmacological fields. The cell walls of marine algae are rich in sulfated polysaccharides, and, in particular, those with chemical structures similar to the glycosaminoglycans

Published online: 02 July 2016

(GAGs) have exhibited significant antiviral activity (Damonte et al. 2004; Pujol et al. 2007; Shao et al. 2015). Polysaccharide moieties of proteoglycans constitute complex molecules that may be capable of storing functional information for the cell. A number of investigations have shown that carbohydrate chains of proteoglycans are chemically heterogeneous and structurally irregular, and that the informational content of proteoglycans provides a chemical basis for their intricate and highly specific functions in cellular physiology (Gagneux and Varki 1999; Lord and Whitelock 2013; Paulson 1989). Other studies have shown that these same compounds can play important roles in the interactions of microbes and important human diseases (Tiwari et al. 2012). For example, sulfated polysaccharides can block the infection of herpes simplex virus (HSV) by chemically imitating heparan sulphate, the cellular receptor for this virus, thus impairing the attachment of the virus to the cellular surface (Herold et al. 1994; Liu and Thorp 2002; Tal-Singer et al. 1995).

Red algae contain large quantities of polysaccharides in their cellular walls, most of which are sulfated galactans. Among these galactans, the carrageenans (CGNs) are one of the most active antivirals against HSV (Carlucci et al. 1999a, b). Given that the frequent use of antivirals, such as acyclovir, against HSV may generate resistance, we initiated a series of studies that proposed to examine the behavior of the viral population when subjected to selective pressure using increasing concentrations of natural bioactive compounds during in vitro replication. In earlier studies we found that such selective pressure, employing the 1C3 CGN (precursor of ι and k CGNs), generated variants of HSV-1 with low resistance to CGNs and altered virulence when tested in mice by different routes (Artuso et al. 2014; Carlucci et al. 2002; Mateu et al. 2011). Variants of HSV-2 were also obtained under similar selective pressure using nonsulfated compounds. These HCV-2 variants showed resistance to the drug used for selection, as well as to heparin and to the $1C_3$ CGN in the order of 2.6 to 6.7 times with respect to the control virus (Mateu et al. 2011). However, in these studies, we found that, in vivo, the same HSV-2 variants showed no difference in virulence when compared to the parental strain.

In the present study we set out to obtain and characterize HSV-2 variants by passaging parental virus in the presence of increasing concentrations of CGNs. This enabled us to calibrate the rate of in vitro replication and the relative resistance of the virus to CGNs and other anti HSV compounds. The virulence of the isolated variants was also tested in vivo in BALB/c and C57BL/6 mouse models. Our first aim was a practical one: to assess if sulfated polysaccharides from marine algae could be used as a driving force for the emergence of attenuated viruses, thus enabling a practical approach for preventive therapies for herpes simplex virus infection. Our second aim was more theoretical: we looked to the possibility that such a study might provide useful information on the transition of viral behavior in relation to its host from a transient to a more persistent strategy, which would have implications for our understanding of viral symbiosis.

2 Materials and methods

Cells and viruses Vero cells were grown as monolayers in Eagle's minimum essential medium (EMEM; Gibco, Gaithersburg, MD) supplemented with 5 % inactivated calf serum and 50 μg/ml gentamicin. For maintenance medium, the serum concentration was reduced to 1.5 %.

HSV-2 strain MS was obtained from the American Type Culture Collection and was propagated and assayed by plaque formation in Vero cells.

Compounds For this work we used the k and ι CGNs whose structures are present in the 1C3 CGN, a 'hybrid' k/ι- and partially cyclized μ/ν CGN. k and ι CGNs, heparin, acyclovir (ACV) and foscarnet (PFA) were purchased from Sigma-Aldrich, Co (St Louis, Mo, USA).

Selection and characterization of viral variants Viral passages were performed as previously described (Carlucci et al. 2002). Briefly 22 passages of HSV-2 (MS) were performed in Vero cells with increasing concentrations of k or ι CGNs. The concentration of CGNs in the first passage should be less than the IC_{50} of said amenable virus when it is in wild type state. Virus corresponding to passages 22 in the presence of k or ι CGNs were plaque-purified in 6-wells microtiter plates. k22-12, k22-13, ι22-9 and ι22-12 are the variants used throughout this paper. In addition 6 passages of HSV-2 (MS) were performed with ACV to obtain the non-syncytial variant ACV-p6MS to employ as a control. Also, 1C3 14–1 and 1C3 17–2 are viral variants used as controls and derived from HSV-1 strain F. Amplification of clones was performed in Vero cells.

Syncytium staining assay Vero cells grown on coverslips were infected with HSV-2 (MS strain) or variants with 100 PFU. At 24 h p.i., the medium was collected for viral quantification and cultures were washed with phosphate-buffered saline (PBS), fixed with ice cold methanol, stained with Giemsa (0.4 %), and examined microscopically.

One step growth curve Fitness of variants in vitro was inferred through a one step growth curve. For the one step growth curve, Vero cells as previously described (Carlucci et al. 1999a) grown in 24-well microtiter plates were infected with HSV-2 (MS strain) and variants with 100 PFU per well. At different times post-infection (6, 10, 18 and 24 h) the monolayers were frozen and thawed, and virus yield was determined by a plaque assay.

Drug susceptibility Drug susceptibility of variants was evaluated by a plaque reduction assay. Vero cells grown in 24-well microtiter plates were infected with 80 PFU/well in the absence or presence of a range of concentrations for each compound. Plaques were counted after 2 days of incubation at 37 °C. The inhibitory concentration 50 % (IC50) was calculated as the concentration of the drug able to reduce the number of plaques by 50 %. All determinations were performed twice and each in duplicate.

Thymidine kinase (TK) and DNA *pol* genes sequence In order to analyze the TK and DNA *pol* gene sequences, Vero cells were infected with HSV-2 (MS strain) and HSV-2 variants at an MOI of 1 PFU/cell. Infected cells were harvested when a marked CPE was observed. DNA was then extracted with QIAampDNA Mini Kit (QIAGEN) according to the manufacturer's instructions and PCR amplified. The primers used for sequencing of TK were according Andrei et al. (Andrei et al. 2005). All primers for DNA *pol* amplification and sequencing were according to Chibo et al. (Chibo et al. 2004). The results were analyzed with DNABaser 3.5.4.2 programs and DNAMAN 4.15.

Mice infection For the intravaginal infection, female BALB/c and C57BL/6 mice (18–21 g weight, 8–10 week-old) were previously injected subcutaneously with 0.05 ml (25 mg/ml) of medroxyprogesterone acetate (Medrosterona, Gador, Argentina). Five days later, the animals were inoculated intravaginally with 20 μl (10^6 PFU) of HSV-2 (MS strain) or the variants or PBS (mock infected control) (10 mice per group). Vaginal lavages were obtained on days 1, 2 and 5 post-infection using 100 μl of sterile saline solution, pooled (from 5 animals), and stored at −80 °C for cytokine quantification by ELISA (BD optEIA, Biosciences, USA). Vaginal lavages from day 2 were also used for virus titer determination by plaque assay in Vero cell monolayers. For the intranasal infection, groups of 10 C57BL/6 mice were anesthetized by intraperitoneal injection of 2 mg ketamine hydrochloride (Ketalar, Parke Davis, Argentina) and 0.04 mg xylazine (Rompun, Mosby, Argentina) in 0.1 ml of PBS. The animals were infected by placing 20 μl (10^6 PFU) of viral variants suspensions onto the tip of both nasal nares (10 μl in each nostril) with a micropipette. In both cases, groups of ten animals were used for each virus. Morbidity and mortality were recorded daily for 2 weeks. Experimental animals were maintained and handled in accordance with national and international laws and policies (Regulation for the care and the use of laboratory animals and Health Department and Human Service, Public Health Service, NIH, USA, and regulations for care and use of test animals from Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, [CD 140/00]).

3 Results

3.1 Selection of HSV-2 variants with carrageenans

After 22 passages of HSV-2 in the presence of carrageenans, four viral clones were isolated as variants and these were further characterized by their ability to grow in vitro in Vero cells. Daily inspection of the infected cultures to detect CPE revealed that all the variants showed a syncytial (syn) phenotype in contrast with parental virus, which showed a CPE characterized by cell rounding and detachment (data not shown). Syn phenotype was a stable trait that persisted even after 8 to 10 serial passages of the variants in Vero cells in the absence of the drug. To rule out a deficient replication of the variants in these cells, one-step growth curves were performed. For this purpose, released infectivity in supernatants of infected cultures was quantified by plaque assay. Variants replicated equally well and similarly to the parental strain, reaching titers that ranged 10^8 to 10^9 PFU/ml at 24 h p.i. (data not shown). Next, we determined if resistance to the compound was developed during selection, assaying replication of variants in the presence of CGNs. The susceptibility of each variant to the compound was expressed as relative resistance (RR) (Table 1). Variants were also characterized according to the pattern of susceptibility to ACV and PFA, compounds that interfere with the replication of viral DNA. Controls included the parental MS strain, the ACV-p6MS variant and the MS strain after serial propagation for 22 passages in Vero cells in the absence of CGNs (the latter designated as MS-control passage 22, or MS-cp 22). As can be seen in Table 1, variants showed different levels of relative resistance (RR) to the corresponding CGN ranging from 1.5 to 10.4, as previously observed for HSV-1 variants (Carlucci et al. 2002). Surprisingly, HSV-2 variants showed resistance to ACV and PFA. ι22-12 showed the highest level of resistance to ACV and PFA with RR values between 8.8 and 14.7 respectively, meanwhile k22-13 variants only showed resistance to ACV (RR: 8.6). Low resistance to PFA and ACV was observed for k22-12 (RR 1.8 and 2.2, respectively) and ι22-9 (RR 3.4 and 1.7 respectively). The virus that had been passsaged 6 times in the presence of ACV (ACV-p6MSL) showed a high resistance to this drug and a moderate resistance to PFA, with RR values of 52 and 12 respectively, meanwhile showing no resistance to CGNs. For MS-cp 22, the RR values for all the assayed compounds indicated that serial passaging of the virus in Vero cells in the absence of ι or k CGNs did not affect its susceptibility, whether to the polysaccharides or to the DNA replication inhibitors. These results demonstrate that HSV-2 variants were able to grow in the presence of ACV and PFA at

Table 1 Drug susceptibility of HSV-2 variants

Inhibitory concentration 50 % (IC_{50}) [a] (Relative resistance) (RR)[b]					
Virus	Kappa	Iota	Heparin	ACV	PFA
HSV-2 (MS) parental strain	0.19 ± 0.08	0.19 ± 0.03	0.81 ± 0.04	0.78 ± 0.02	1.02 ± 0.10
K22-12	1.20 ± 0.09	0.30 ± 0.05	0.83 ± 0.05	1.42 ± 0.03	1.83 ± 0.02
	(6.3)	(1.5)	(1.0)	(1.8)	(1.8)
K22-13	1.23 ± 0.05	0.30 ± 0.02	0.60 ± 0.03	6.77 ± 0.09	3.62 ± 0.16
	(6.4)	(1.5)	(0.7)	(8.6)	(3.5)
ι22-9	0.60 ± 0.07	1.98 ± 0.03	1.59 ± 0.02	1.39 ± 0.05	3.46 ± 0.11
	(3.2)	(10.4)	(1.9)	(1.7)	(3.4)
ι22-12	0.60 ± 0.07	0.60 ± 0.04	0.80 ± 0.09	6.89 ± 0.09	15.0 ± 0.10
	(3.2)	(3.2)	(0.9)	(8.8)	(14.7)
ACV-p6MS	0.17 ± 0.10	0.15 ± 0.30	0.60 ± 0.10	40.8 ± 0.10	12.2 ± 0.10
	(1.0)	(1.0)	(1.0)	(52.3)	(12.0)
MS-cp 22	0.22	0.23	0.90	0.80	1.3
	(1.15)	(1.21)	(1.11)	(1.02)	(1.27)

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

[b] RR (relative resistance) is the ratio between IC_{50} for each variant and the IC_{50} for the MS parental strain

MS-cp 22 is the parental strain with 22 pasages on Vero cells without compound

ACV-p6MS is the parental strain with 6 successive passages on Vero cells with increasing concentrations of ACV

doses that were two to eight fold higher than those necessary to inhibit virus yield by 50 %.

3.2 Analysis of the TK and DNA *pol* genes in HSV-2 variants

Acknowledging the results obtained with the variants and ACV and the fact that more than 95 % of HSV variants that showed resistance to ACV in previous accounts have been associated with mutations occurring in the TK gene (Frobert et al. 2005; Piret and Boivin 2011), we analyzed the complete sequence of the gene encoding for TK in our variants. The only change detected in clones k22-12 and ι22-9 that were susceptible to ACV was an Asp to Gly substitution in amino acid 6, suggesting that this change would not be relevant for the development of resistance. This would also apply to the k22-13 variants that proved to be resistant to ACV (RR 8.6), where a Val to Ala substitution in the amino acid 149 was the only change detected in the non-conserved region of the TK gene. In variants of ι22-12 that proved resistant to ACV (RR 8.8), no changes at all were found in the TK gene sequence. Resistance to ACV linked to mutations that occur in the DNA *pol* gene has been less commonly described (Frobert et al. 2005). In this study we identified amino acid substitutions in both conserved and non-conserved regions of the DNA *pol* gene. The only change detected for variant ι22-12 in comparison with the control strain was a Met to Thr substitution in amino acid 789. This mutation is located in the conserved region VI. In addition, for variant k22-12 (ACV-sensitive), three substitutions in highly conserved sequence motifs were detected: two in region IV (Exo II) from DNA pol, and one in a non-conserved gene region (Cassai et al. 1975). These changes corresponded to a Leu to Thr in amino acid 439, a Phe to Leu in amino acid 462 and an Ala to Glu in 691 amino acid position. Despite these mutations, this variant continued to be susceptible to ACV, suggesting that these changes are unlikely to be involved in the resistance. Also, in ACV-p6MS (RR 52.0) a Val to Ile in amino acid 806 was detected.

3.3 Virulence of HSV-2 variants for mice

HSV-2 variants were also characterized in accordance to their virulence for mice. To emulate the natural routes of infection for humans, virulence of variants was evaluated in two strains of mice experimentally infected either via intravaginal or intranasal. Intravaginally inoculated mice were examined daily for signs of disease and the resultant morbidity and mortality was recorded. As can be seen in Table 2, the virulence of the variants differed from the parental strain when mortality and survival time were analyzed. The MS strain proved to be highly virulent for BALB/c mice inoculated intravaginally, with 100 % mortality and a survival time of 8 days pi. In mice inoculated with the clonal variants, the mortality was reduced to 80 % for k22-12, 40 % for k22-13 and ι22-9 and 20 % for ι22-12. ACV-p6MS, showed a similar behavior to k22-12 with 40 % mortality rate. For C57BL/6 mice inoculated

Table 2 Virulence of HSV-2 syn variants for BALB/c and C57BL/6 mice inoculated intravaginally

Virus	BALB/c		C57BL/6	
	Mortality (%)	Survival Time (day)	Mortality (%)	Survival Time (day)
MS	100	8 ± 1	80	13 ± 1
K22-12	80	13 ± 1	40	13 ± 1
K22-13	40	9 ± 1	0	Survival
ι22-9	40	11 ± 1	20	13 ± 1
ι22-12	20	13 ± 1	0	Survival
ACV-p6MS	40	9 ± 1	20	14 ± 1

BALB/c or C57BL/6 female mice of 6 to 8 weeks old were inoculated intravaginally with the different viral variants (10^6 PFU). Mortality was recorded daily for 2 weeks

intravaginally with the parental strain, 80 % mortality was recorded. No mortality was observed after inoculation of k22-13 and ι22-12. The variants k22-12 and ι22-9 showed a moderate mortality of 40 and 20 %, respectively. Despite these differences in virulence, infectious virus was detected in vaginal lavages in both strains of animals infected either with the variants or the control virus (Fig. 1).

When the intranasal route of inoculation was employed in C57BL/6 mice, the mortality was reduced from 80 % for the parental strain to 20 % for k22-13 and ι22-12, meanwhile k22-12 proved non virulent by this route, with 100 % survival of inoculated mice. The strain ι22-9, that produced 20 % mortality by intravaginal route, showed little difference in mortality in comparison with the control virus when introduced by intranasal inoculation, with an overall mortality of 80 %. These results suggest that although the route of inoculation and the genetic background of mice are important determinants for HSV-2 virulence in this host, all variants showed attenuation of virulence when compared to parental virus.

When cytokines, IL-6 and TNF-α, were analyzed in vaginal lavages of BALB/c mice at day 1 and 5 p.i., it was observed that mice inoculated with the variants produced higher levels of both pro-inflammatory cytokines than those inoculated with the parental virus. In the case of IL-6, assayed at day 1 p.i., lavages from animals inoculated with MS strain showed a concentration of 10 pg/ml, whereas those infected with the variants presented values more than 30-fold higher, with the exception of variant-k22-12-inoculated mice, the latter responding in a similar way to those inoculated with the MS strain (Fig. 2a). At day 5 p.i., only MS strain and variant ι22-9 produced lower values of the cytokines (36.81 and 10 pg/ml, respectively) when compared with those obtained for the other variants (>300 pg/ml). Mice inoculated with ACV-p6MS showed a moderate level of IL-6 production with values of 42.17 and 303 pg/ml at days 1 and 5 p.i, respectively. Animals inoculated with the MS strain showed low values of TNF-α (Fig. 2b) at both 1 and 5 days p.i., showing 10 pg/ml. A similar response for TNF-α was detected in those inoculated with k22-12 and ι22-9 at days 1 and 5, whereas mice inoculated with ACV-p6MS showed the highest figures for production of this cytokine, with values of 1033 and 2220 pg/ml at days 1 and 5 p.i.. In general, we found that the HSV-2 variants strongly stimulated the production of TNF-α, either at day1 and/or day 5, with values ranging from 100 to 1000 pg/ml. These results suggest that pro-inflammatory cytokines, like IL-6 and TNF-α, might play a protective role in mice inoculated intravaginally with the variants. This is supported by the findings of infective virus in vaginal lavages of mice inoculated intravaginally with the variants and control virus at day 2 p.i., suggesting that differences in virulence were not directly associated with the quantity of virus produced at the inoculation site (Fig. 2).

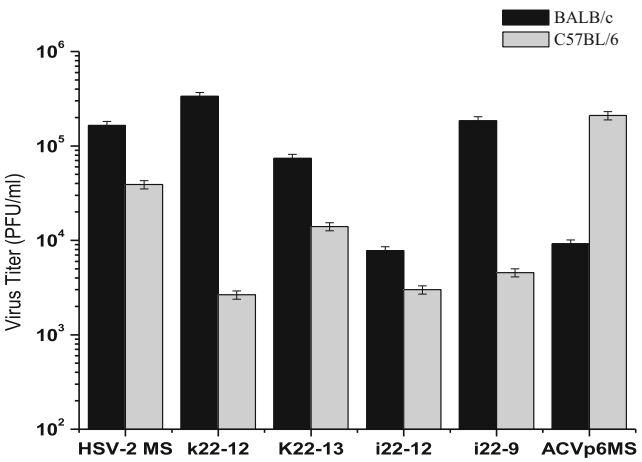

Fig. 1 Virus yield in vaginal lavages of BALB/c mice (day 2 p.i). Vaginal lavages were obtained on day 2 post-infection using 100 μl of sterile saline solution, pooled (5 mice per group), and stored at −80 °C. Virus titers (PFU/ml), were determined by plaque assay in Vero cell monolayers

4 Discussion

A common characteristic of HSV-2 variants isolated through this approach was the fact that they exhibited a stable syn phenotype (Artuso et al. 2014; Carlucci et al. 2002) in contrast to the cytolitic effect induced by wild type HSV. Although syn

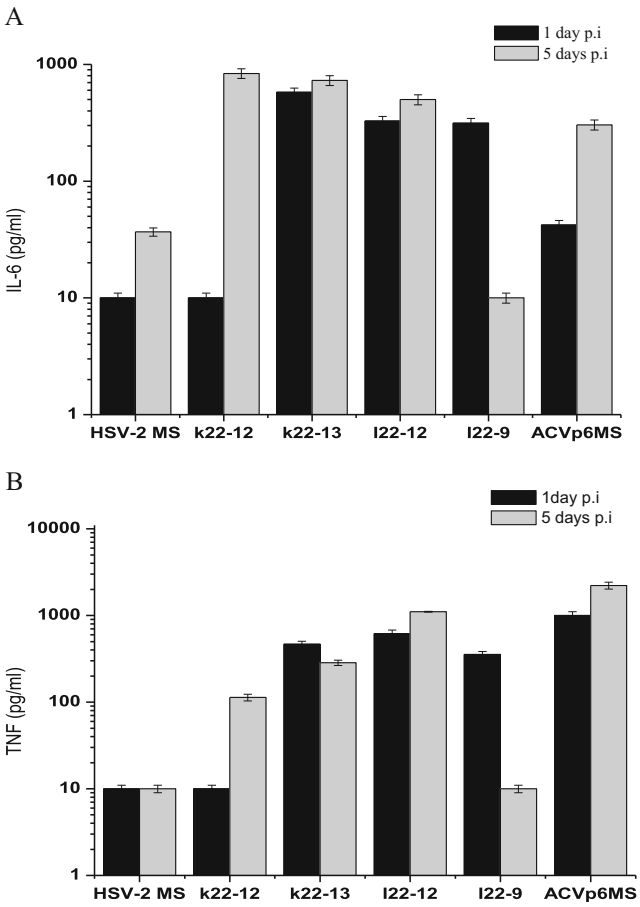

Fig. 2 Cytokine determination in vaginal lavages of BALB/c mice (days 1 and 5 p.i). Vaginal lavages were obtained on days 1 and 5 p.i., using 100 μl of sterile saline solution, pooled (5 mice per group), and stored at −80 °C. The cytokines were determinated by ELISA, (A) IL-6 quantification (pg/ml), (B) TNF-α quantification (pg/ml)

variants can be readily isolated from laboratory HSV strains (Carlucci et al. 2002; Cassai et al. 1975; Muggeridge et al. 2004), they are rarely found in clinical isolates, suggesting that the syn phenotype may represent a deleterious trait for in vivo HSV infections (Muggeridge et al. 2004). This speculation is given some support by our results which demonstrate that, although they replicate as readily as parental virus, HSV-2 variants proved to be attenuated in terms of virulence (Artuso et al. 2014; Bestman-Smith et al. 2001; Schneweis et al. 1984; Tognon et al. 1991; Wheeler 1964). Our results would appear to tally with the findings of seroepidemiological studies of HSV in humans that reported more than 80 % of patients with genital herpes as either unrecognized as such even though they had symptomatic genital herpes or as asymptomatic disease (Marchant and Roe 1997). Thus while unapparent persistent infections are a common manifestation, there is little current information on which to propose the cooperative interactions that might constitute virus-host symbiosis (Villarreal 1999). When we evaluated the resistance of the viral strains to CGNs and other antiviral compounds we found that the four variants showed a moderate resistance to CGNs. We were also surprised to discover that two of the variants were also resistant to ACV. This finding led us to investigate the possibility of a genetic explanation. When we did so this confirmed changes in the sequences of TK and DNA *pol* genes of both variants. In the case of the TK gene we found a change at position 6 (Asp →Gly) in variants that were ACV-sensitive, in addition to a change in position 149 (Val →Ala) in the k22-13 variant that was ACV-resistant (RR 8.6). It is noteworthy that changes in position 6 have been previously been reported for viruses isolated from clinical cases of HSV, but these have not been associated with ACV resistant strains (Bestman-Smith et al. 2001; Morfin and Thouvenot 2003). In the case of the DNA *pol* gene, a change at position 789 (Met →Thr) was detected for the ι22-12 variant that was ACV resistant. This mutation is located in the conserved region VI. It is interesting that, in clinical isolates, mutations conferring resistance to ACV through single amino acid substitutions located in regions VI have been also reported (Schmit and Boivin 1999). For the ACV-sensitive k22-12 variant, three changes were detected: at positions 439 (Leu →Thr), 462 (Phe →Leu) and 691 (Ala →Glu).

It was both surprising and unexpected that, in the case of BALB/c infections, cytokines appeared to play a protective role, with high levels of pro-inflammatory cytokines correlating with the improved survival of the mice inoculated with the variants. This contrasts with the opposite effect reported for HSV-1 syn variants under selective pressure with CGNs (Artuso et al. 2014). In this study HSV-1 variants failed to induce strong cytokine responses, suggesting that differences in the innate immune response might be a key factor in the observed responses associated with different strains of HSV. On the other hand, it is known that the wide range of symptoms, tropism and severity of herpetic disease is closely related to the complexity and size of the herpes virus genome. When one considers that this virus has co-evolved with a broad variety of hosts over a long period of time (Carlucci et al. 2011, 2012; McGeoch et al. 2006), it seems likely that virulence does not correlate with the expression of a single gene but with a complex combination of many genes. Our results also favor the hypothesis that this slow process might be accelerated by an experimental approach of the sort as we have described here and in previous reports (Artuso et al. 2014; Carlucci et al. 2011).

One key feature to consider during in vivo HSV evolution is the selective pressure exerted by the immune system that prompted HSV to develop multiple immune evasion strategies. This is supported by extensive literature reporting changes in gB, gD and gC (Novak and Peng 2005). It is also possible that alterations in the viral glycoproteins might, through their interaction with polysaccharides, contribute to the changes in virulence. Thus we suggest that evolution may be determined not only by interactions at the level of proteins and

nucleic acids, but also involve polysaccharides (Zimnitskii 2012), and that analysis of biological systems focused on all three types of molecules might help to clarify key aspects of cell biology.

In conclusion we suggest that CGNs may be useful tools in obtaining attenuated HSV-2 variants with prospective prophylactic or therapeutic applications. We also demonstrate, for the first time, that a selective pressure directed to the external glycoproteins of the HSV with CGNs may give rise to viruses with alterations in genes not related to the target of action of the selection agent. We furthermore suggest that such genetic evolutionary change, which appears to be related to change in the immune responsiveness of the host, may provide an original example of the key transition from acute to persistent viral strategies that are thought to be important for the development of mutualistic viral symbiosis.

Acknowledgments This work was supported by grants from the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT) BID-PICT 719 and Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICET) PIP 11420080100529.

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