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Antiviral activity of an extract from leaves of the tropical plant *Acanthospermum hispidum*

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Abstract

Incubation of the alphaherpesviruses pseudorabiesvirus (PRV) and bovine herpesvirus 1 during infection of cell cultures with an extract prepared from the leaves of *Acanthospermum hispidum* impaired productive replication of these viruses in a concentration-dependent manner whereas propagation of classical swine fever virus, foot-and-mouth disease virus and vaccinia virus was not affected. The 50% inhibitory concentration for cell growth (IC_{50}) was 107 ± 5 μl/ml, and the concentration reducing PRV yield by 1 $\log_{10}$ (90% effective concentration, EC_{90}) was 8 ± 3 μl/ml. The selectivity index calculated as the IC_{50}/EC_{90} ration was 13 ± 4. Delineation of the mechanism of the antiviral activity demonstrated inhibition of alphaherpesvirus attachment to and, to a lesser extent, penetration into the cells. In contrast, viral gene expression was not inhibited by the extract when added after entry of virions into the target cells. Reduced antiviral activity of A.h. against PRV deletion mutants lacking glycoprotein C (gC) or glycoproteins gC, gE, gG and gI altogether indicated that gC alone and/or viral attachment complexes of which gC is a component constitute the target structures for *A. hispidum*. © 1997 Elsevier Science B.V.

Keywords: *Acanthospermum hispidum*; Alphaherpesviruses; Inhibition of adsorption

1. Introduction

Acanthospermum hispidum is a tropical plant whose leaves are used in traditional medicine in Benin, Africa, for therapy of infectious diseases. The biological and molecular basis of this activity

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are still unknown, and it was therefore the objective of the present study to investigate and characterize a possible antiviral activity, specially against herpesviruses.

HSV-1 and HSV-2 are members of the alphaherpesvirus subfamily of the Herpesviridae. This subfamily of viruses comprises a number of important human and animal pathogens including varicella-zoster virus (VZV), bovine herpesvirus 1 (BHV-1) and pseudorabiesvirus (PRV) (Roizman et al., 1992). Herpes virions consist of nucleoprotein core containing the linear double-stranded DNA genome associated with proteins, an icosahedral capsid shell, an amorphous proteinaceous tegument structure, and a lipid envelope (Ward and Roizman, 1994). Within the envelope, virus-encoded glycoproteins are inserted. These viral glycoproteins have been shown to be instrumental in mediating crucial steps in the initiation of herpesvirus infection which is a rather complicated process involving several glycoproteins. Virus attachment is mediated by glycoprotein C (gC) which interacts with cell surface proteoglycans carrying heparan sulfate carbohydrate chains. Initial binding ensues into a stable attachment, a process which is dependent on the presence of gD. Membrane fusion between virion envelope and plasma membrane of the target cell requires glycoproteins D, B, H and L, which probably act in combination. After fusion, the naked nucleocapsid is released into the cytoplasm and is subsequently translocated to nuclear pores, and genomic DNA is released into the nucleus. Interestingly, gC has been demonstrated to be dispensable for viral replication, whereas gB, gD, gH, and gL are essential for virus infectivity. In total, herpes virions contain at least 10 envelope glycoproteins including gE and gI for which no function in the initial steps of infection could be assigned (Mettenleiter, 1994; Spear, 1993).

In this report we demonstrate that water-soluble material extracted from leaves of *A. hispidum* exhibited antiviral activity by interaction with alphaherpesvirus particles resulting in inhibition of attachment to and penetration into target cells.

2. Materials and methods

2.1. Extract from A. hispidum

The extract from *A. hispidum* was kindly provided by Dr M. Wolf (Merck, Darmstadt Germany). Homogenized leaves were extracted with distilled water, and lyophilized. Prior to use, aliquots were reconstituted with distilled water, centrifuged at $100\,000 \times g$, and the supernatant was passed through 0.2-μm filters after dialysis against phosphate-buffered saline. The extract used in this report originated from one purification batch.

2.2. Cell proliferation assay

Subconfluent MDBK cells were incubated with the *A. hispidum* extract at different concentrations for 24 h, then [^{3}H]thymidine (5 μCi/ml final concentration) was added and 8 h later radioactivity incorporated into newly synthesized DNA was determined after harvesting on glass fibre filters by scintillation counting.

2.3. Viruses and cells

PRV and BHV-1 strains were grown on MDBK cells. Classical swine fever virus (CSFV) strain Alfort, kindly provided by H.-J. Thiel (Institut für Virologie, Justus-Liebig-Universität Giessen, Germany), was propagated on PK15 cells. Foot-and-mouth-disease virus (FMDV) strain O1 Lausanne, kindly provided by R. Ahl (Federal Research Centre for Virus Diseases of Animals, Tübingen, Germany), was propagated on BHK-21 cells and β-galactosidase (β-gal) expressing vaccinia virus mutant VaclacZ (G.M. Keil, unpublished) was grown on CV-1 cells. All cells were cultivated in DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 0.1 mg/ml streptomycin in a humidified, 5% CO_2 atmosphere at 37°C. The PRV strains used were Phylaxia (wild-type), glycoprotein deletion mutants for gC (Mettenleiter et al., 1988), gE (Mettenleiter et al., 1987), gG (Mettenleiter et al., 1990), gI (Zuckermann et al., 1988) and a mutant deficient for all four men-

tioned glycoproteins (Mettenleiter and Rauh, 1990).

For determination of virus titers, the respective cells in 6- or 24-well tissue culture dishes were incubated with serially diluted infected cell culture supernatants for 1 h at 37°C and, after removal of the inoculum, were overlaid with culture medium containing 1.6% methyl cellulose or 0.6% agarose containing BluoGal (Fehler et al., 1992) for better visualization of VaclacZ-induced plaques. Plaques were counted 2–3 days later and titers calculated as plaque forming units (PFU) per ml.

2.4. Determination of β-gal activity

Quantitative determination of β-gal activity induced by β-gal expressing BHV-1 mutants (BHV-1res) and gG-negative PRV mutants (Mettenleiter et al., 1990) was performed as described previously (Fehler et al., 1992).

2.5. Adsorption assay

Radiolabelled PRV virions were kindly provided by A. Karger (Institute of Molecular and Cellular Virology, D-17498 Insel Riems, Germany), and BHV-1 was radiolabelled as described in a previous report for PRV (Karger and Mettenleiter, 1993). Briefly, MDBK cells preincubated with 20 μg/ml 5-fluorouracil and 5 μg/ml thymidine for 16 h were then infected with BHV-1. After 1 h adsorption the inoculum was removed and replaced by medium containing 50 μCi/ml [3H]thymidine. The cell lysate harvested after 48 h was loaded on a discontinous sucrose gradient for virus purification as described (Karger and Mettenleiter, 1993). For the adsorption assay confluent MDBK cells were washed with PBS supplemented with 1% FCS and incubated in the same buffer for 30 min at 4°C. [^{3}H]Thymidine-labeled PRV or BHV-1 virions were added for 2 h at 4°C to allow adsorption. Unbound virus was then removed by washing with ice-cold PBS. Cells were lysed and cell-associated radioactivity was determined as described (Karger and Mettenleiter, 1993).

3. Results

3.1. Effect of A. hispidum on the proliferation of MDBK cells

To assay for possible toxic effects of *A. hispidum*, MDBK cells were incubated with increasing amounts of the extract and [^{3}H]thymidine was added 24 h later for 8 h. Cells were harvested by trypsinization and radioactivity incorporated into newly synthesized DNA was measured by liquid scintillation counting. The results from a representative experiment shown in Fig. 1 indicate that *A. hispidum* concentrations up to 25 μl/ml did not impair the proliferation of MDBK cells in contrast to higher concentrations. The 50% growth inhibitory concentration (IC_{50}) for *A. hispidum* calculated from several experiments was 107 ± 5 μl/ml cell culture medium. Toxic effects

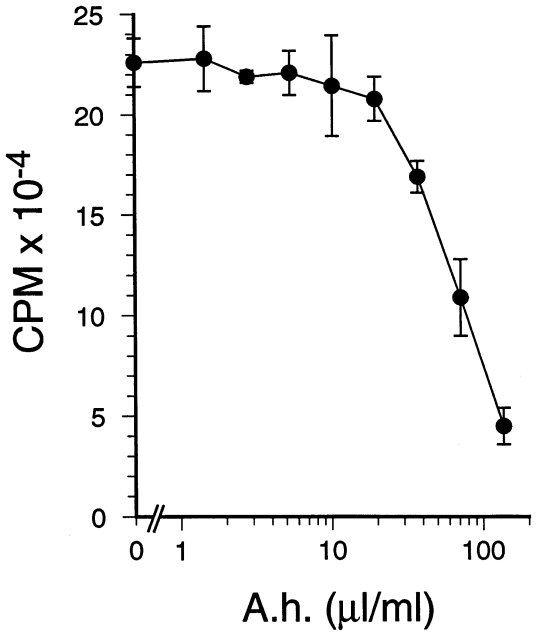

Fig. 1. Influence of the *A. hispidum* extract on the proliferation of MDBK cells. Subconfluent MDBK cells were incubated without or with 1, 2, 6, 12, 25, 50, 100, and 200 μl extract per ml culture medium for 24 h. Newly synthesized DNA was labeled for 8 h with 5 μCi [^{3}H]thymidine per ml. Cells were harvested by trypsinization and incorporated radioactivity was determined by scintillation counting. Data are the means of triplicates ± S.D.

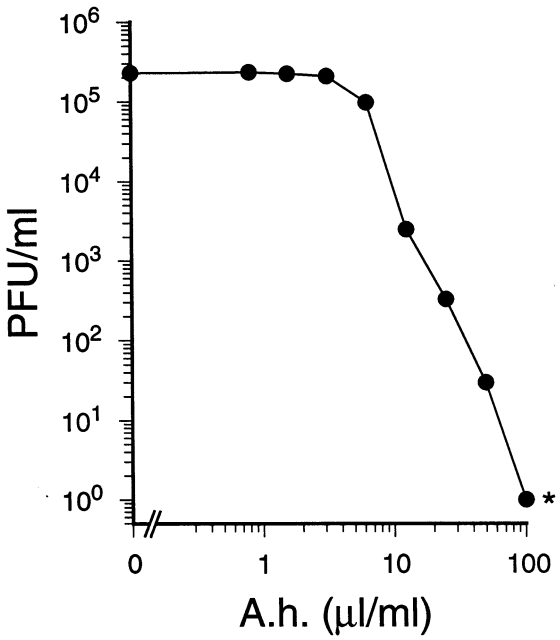

Fig. 2. The *A. hispidum* extract inhibits PRV replication. MDBK cells were infected with PRV at an M.O.I. of 2 PFU/cell without or in presence of 0.8, 1.5, 3, 6, 12.5, 25, 50, and 100 μl extract per ml culture medium. Infectivity released into the supernatants at 24 h p.i. was determined by titration. The asterisk indicates infectivity below the detection limit of 1 PFU per ml. A representative experiment out of three is shown.

of *A. hispidum* were also detected by microscopic examination of cultures in which rounding and detachment of cells was observed 48 h after addition of 100 and 200 μl extract per ml, respectively (not shown).

3.2. Antiviral activity of A. hispidum

To test for the effect of *A.hispidum* on alphaherpesvirus replication, MDBK cells were infected with PRV at a multiplicity of infection (M.O.I.) of 2 PFU/cell in the presence of different concentrations of extract. Infectious virus released 24 h post infection (p.i.) into the culture supernatants was determined by titration. A representative experiment shown in Fig. 2, demonstrates that the presence of the *A. hispidum* extract at concentrations of 12.5 μl/ml and higher interfered with the production of infectious virus in a concentration-dependent manner and resulted in an approximately 10^4-fold titer reduction at 50 μl/ml. No infectivity was detected in the media from cultures treated with 100 μl extract/ml. The average concentration inhibiting virus yield by 1 $\log_{10}$ (90% effective concentration, EC_{90}), calculated from 3 experiments was 8 ± 3 μl/ml. The average selectivity index (IC_{50}/EC_{90}) of the extract in these experiments was 13 ± 4. Essentially, the same results were obtained with BHV-1 infected MDBK cells (data not shown).

In contrast, no effect of *A. hispidum* was detected using the same protocol for BHK-21 cells infected with FMDV, PK15 cells infected with CSFV, and CV-1 cells infected with vaccinia virus (not shown). These results demonstrate that *A. hispidum* inhibits replication of alphaherpesviruses and indicate a specific mode of action since productive infection of cells by the picorna-, pesti- and poxviruses tested was not affected.

3.3. A. hispidum inhibits adsorption and penetration of alphaherpesviruses

Alphaherpesvirus replication is characterized by a sequence of coordinately regulated events. To identify the step at which alphaherpesvirus replication is inhibited, MDBK cells were infected with PRV at an M.O.I. of 2 PFU/cell after pre-incubation of the cells or the virus with *A. hispidum*. Virus titers in the culture supernatants were determined 24 h later. Pretreatment of the cells followed by removal of the extract had no effect on the production of infectious virus (Fig. 3, ●) whereas pre-incubation of the inoculum resulted in a concentration-dependent reduction of progeny virus yield (Fig. 3, ▲), suggesting that *A. hispidum* binds to virion envelope structures, thereby inhibiting virus entry, and not cell surface molecules which is in accordance with the failure of the *A. hispidum* extract to interfere with CSFV, FMDV or vaccinia virus replication.

To confirm these conclusions, to examine possible effects of *A. hispidum* on intracellular gene expression, and to further delineate the step in virus entry that is blocked, MDBK cells were infected with BHV-1res either without extract, or with extract added during adsorption only, or

during penetration, or after penetration, or after pre-incubation of the inoculum with *A. hispidum*. BHV-1 res is a β-galactosidase (β-gal) expressing BHV-1 mutant (Fehler et al., 1992) for which it has been shown that virus-induced intracellular β-gal activity correlates with the amount of penetrated virions. The results obtained are shown in Fig. 4 and demonstrate that the *A. hispidum* extract did not affect intracellular β-gal expression when added after penetration of virions into the cells. In contrast, pre-incubation of virus suspension with *A. hispidum* extract reduced β-gal expression to the level found in non-infected cells. The extract was removed by ultracentrifugation before infection of the cells without losing the antiviral activity, suggesting a binding to the virus

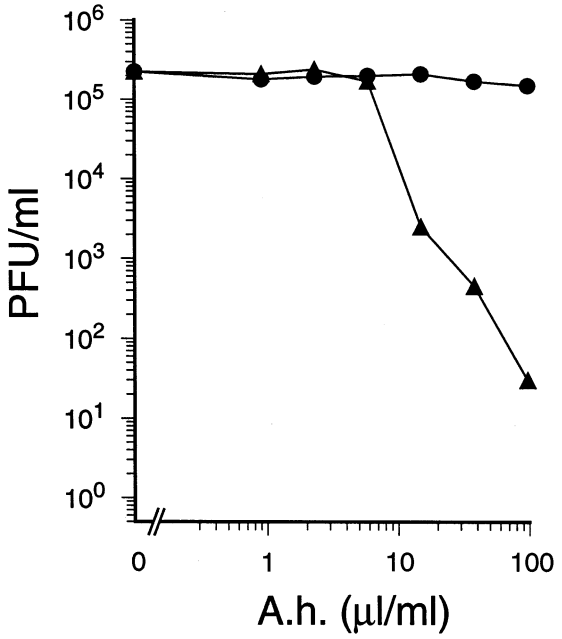

Fig. 3. The *A. hispidum* extract is effective on virions. MDBK cells (circles) or PRV virus stock (triangles) were incubated without or with 3, 6, 12.5, 25, 50, and 100 μl extract per ml for 1 h at 37°C. Cells were washed with PBS and infected with untreated PRV at an M.O.I. of 2 PFU/cell. The extract was removed from the virus by ultracentrifugation. Virus pellets were resuspended in the original volume with PBS, before addition to untreated MDBK cells. Infectivity released into the supernatants at 24 h p.i. was determined by titration. A representative experiment out of three is shown.

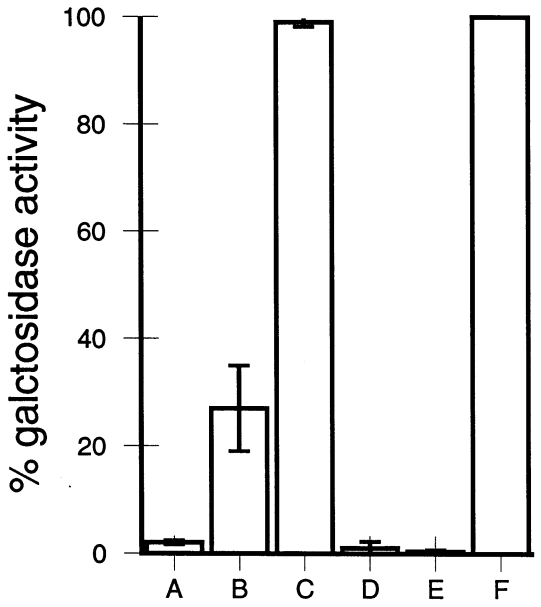

Fig. 4. Attachment of virions is inhibited by the *A. hispidum* extract. MDBK cell cultures were incubated at 4°C for 30 min and infected with BHV-1res at an M.O.I. of 2 PFU/cell. After 2 h at 4°C to enable adsorption of virions to the cells, cultures were transferred to 37°C for 1 h to allow penetration. Then, non-penetrated virions were inactivated by pH 4 treatment with citrate buffer for 1 min and after 16 h of culture intracellular β-gal activity was determined. The *A. hispidum* extract (25 μl per ml culture medium) was present only during the attachment phase (column A); the penetration phase (column B); or added after the penetration phase (column C). β-gal activity shown in column D was induced by BHV-1res incubated with 25 μl of the extract per ml virus stock followed by ultracentrifugation to remove the extract as described in the legend to Fig. 2. Columns E and F show β-gal activities in non-infected MDBK cells and MDBK cells infected with BHV-1 res without the extract, respectively. Induced β-gal activities are indicated in % of the activity induced by BHV-1res without addition of extract. The results shown are the mean of three different experiments $\pm$ S.D.

and/or an irreversible alteration of the virions structure responsible for the antiviral activity. Furthermore, presence of extract during the attachment phase resulted in a similar strong reduction in β-gal activity which was more pronounced than that seen when *A. hispidum* extract was added after attachment but before penetration occurred. The same kind of experiments were repeated with β-gal expressing gX-negative PRV mutant and similar results were obtained.

These data indicate that *A. hispidum* exerts its antiviral activity by inhibiting early steps in initiation of herpesvirus infection, presumably during attachment of free virions to target cells. This assumption was supported by direct analysis of binding of radiolabelled PRV and BHV-1 virions to target cells. To this end, MDBK cells were incubated with purified [3H]thymidine-labeled virions for 2 h in the presence or absence of *A. hispidum* extract. Thereafter, unbound virions were removed by stringent washes with PBS, and bound virus was measured by liquid scintillation counting of cell-associated radioactivity. Results showed that attachment of PRV was reduced by 90% in the presence of 10 μl of *A. hispidum* extract per ml, and by 98% in the presence of 100 ml *A. hispidum* extract per ml of virus suspension. When the same experiments were performed using BHV-1 instead of PRV, the reduction in virus adsorption found was 91% in the presence of 10 μl/ml and 99% in the presence of 100 μl/ml *A.h.*

3.4. Evidence that gC might be a target for the A. hispidum extract

Adsorption of alphaherpesvirus particles to heparan sulfate carrying proteoglycans is the initial step of infection. This process is mediated by gC, a nonessential component of the virion envelope (Mettenleiter, 1994; Spear, 1993). To test the role of gC in the antiviral activity of *A. hispidum*, wild-type PRV, as well as PRV mutants deficient in expression of glycoproteins gC, gE, gG, and gI either singly or altogether (mutant 2915) were titrated on MDBK cells in the presence of 25 μl of extract per ml of inoculum during the attachment process. As control, cells were infected in the absence of *A. hispidum* extract. As shown in Fig. 5, the effect of the extract on a gC-deficient mutant was decreased compared to wild-type PRV, and mutant 2915 exhibited an even more pronounced resistance against the antiviral activity of *A. hispidum*. In contrast, mutants deficient in gE, gG or gI were similar or more sensitive than wild-type PRV towards inhibition by *A. hispidum*.

4. Discussion

Aqueous extracts prepared from leaves of *A. hispidum* exhibit antiviral activity against herpesviruses (this report) as well as retroviruses, e.g. Maedi-Visna virus and caprine arthritis-encephalitis virus (data not shown). Interestingly, this activity was resistant against digestion with trypsin, chymotrypsin, pronase and aminoglycosidase, but proved to be sensitive towards oxidation by periodate (M. Wolf, personal communication). Chemical analysis of the extract was performed which demonstrated presence of fucose, rhamnose, ribose, arabinose, xylose, mannose, glucose, and galactose with only little (0.2%) amino acids. Taken together, this suggests that the antiviral molecule(s) should be carbohydrate(s). To date, the exact nature of the biologically active molecule(s) is still unknown (M. Wolf, personal communication).

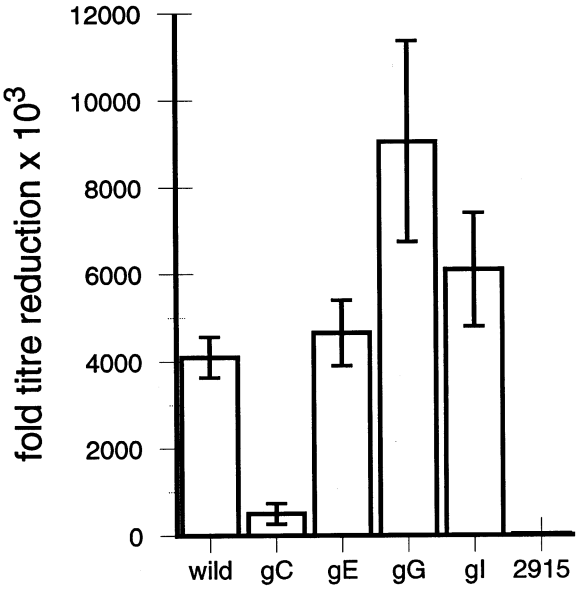

Fig. 5. Evidence that gC might be involved in the *A. hispidum*-mediated antiviral activity. MDBK cells were infected with wild-type PRV (wt) or PRV deletion mutants lacking glycoproteins gC (gC-), gE (gE-), gG (gG-), gI (gI-) or mutant 2915 with deletions of gC, gE, gG and gI in absence or presence of 25 μl extract per ml. Infectivity released into the supernatants at 24 h p.i. was determined by titration. Fold reduction was calculated from the respective titers obtained without and in presence of the extract. Data represent the mean of three different experiments $\pm$ standard deviation.

Reports on the antiviral activity of the *A. hispidum* extract against the alphaherpesviruses HSV-1 and HSV-2 (University of Benin, M. Wolf, personal communication) prompted our studies to pinpoint the step during viral replication at which *A. hispidum* extract exerts its inhibitory influence. We used the well-studied animal alphaherpesviruses PRV and BHV-1 as targets, as well as the pestivirus CSFV, the picornavirus FMDV and the poxvirus vaccinia virus to assay for specificity of the observed effect. In contrast to CSFV, FMDV, and vaccinia virus, which proved to be insensitive towards the *A. hispidum* extract at the concentrations used, both alphaherpesviruses were inhibited by *A. hispidum* in a dose-dependent manner. By dissecting the replicative cycle, especially by directly assaying initiation events such as attachment and penetration, we show here that *A. hispidum* acts by binding to viral particles and, thereby, inhibits attachment of virions to target cells. Pre-incubation of cells with *A. hispidum* did not reveal any inhibition, whereas pretreatment of virions showed a dramatic reduction of infectivity. Electron microscopic evaluation of virion particles treated with *A. hispidum* extract did not reveal any overt alterations in virion morphology or virion envelope ultrastructure (data not shown). Therefore, *A. hispidum* extract does not simply destroy or damage the virion envelope. In addition, immuno-electron microscopic analysis revealed that pretreatment of PRV virions with *A. hispidum* did not abolish subsequent binding of monoclonal antibodies against gB, gC, or gE, indicating that the antigenic structure of these glycoproteins was not seriously altered (data not shown).

Attachment studies using radiolabelled virions pretreated with A. hispidum extract showed that it was able to abolish adsorption of virions to target cells. Addition of extract after the attachment phase performed at 4°C, but prior to the penetration phase initiated by a temperature-shift to 37°C, indicated also an effect on the membrane fusion process. Thus, both processes are sensitive towards the *A. hispidum* extract-mediated inhibition, but the very first step in virus infection, i.e. initial contact between virion and target cell, was shown to be more sensitive to the inhibitory activity of the extract. It is unclear at present, whether the effect of the extract on attachment and penetration is due to different mechanisms, or whether the same target structure may be involved in both processes.

Adsorption of alphaherpesviruses is primarily mediated by gC which binds to heparan sulfate moieties present on proteoglycans on the surface of target cells (Mettenleiter, 1994; Spear, 1993). To test whether gC may act as a target molecule for the extract, sensitivity of PRV mutants unable to express gC, gE, gG, gI, either singly or in combination, was analyzed. The results showed that of the single glycoprotein mutants, only the gC mutant exhibited a decreased sensitivity towards inhibition by the *A. hispidum* extract. The absence of gE, gG, and gI, in addition to the absence of gC increased resistance against inactivation to an even higher degree. Since gG is not a component of the PRV virion but constitutes a glycoprotein which is released from infected cells into the surrounding medium in large amounts, the synergistic effect may be attributed to the absence of gE and gI. This is particularly striking, since gE and gI have been shown to form a non-covalently linked complex in PRV virions. It is, however, puzzling that neither the gE nor the gI mutant exhibited an increased resistance against the extract. In addition, neither of the two glycoproteins has so far been shown to play a role in entry of virions, but rather mediate direct spread from primary infected to adjacent non-infected cells (Mettenleiter, 1994). The gC dependence of the anti-herpesvirus activity is in contrast to the fact that also a anti-retro virus activity has been observed. This may be attributed to the fact that the *A. hispidum* extract is composed of many different molecules, and that the anti-herpes virus molecule differs from the anti-retro virus molecules. Fractionation and purification of the extract will have to be performed to answer this question.

Given the overall similarities in initiation of infection by different alphaherpesviruses, such as, e.g. HSV-1, HSV-2, PRV, BHV-1 or equine herpesvirus 1 (Mettenleiter, 1994; Spear, 1993), it appears reasonable to deduce from the findings with PRV and BHV-1 a mode of action of *A. hispidum* extract common to alphaherpesviruses. Thus, our results could be indicative of an even

broader antiviral spectrum of *A. hispidum* extract. Further analysis of this activity requires identification of the inhibitory molecule(s). The use of the A.h. extract as an antiviral drug has to take toxic components of the plant into consideration (Ali and Adam, 1978; Lemonica and Alvarenga, 1994). These could be responsible for the low selectivity index found in the present analysis. Whether the activity of *A. hispidum* extract may open new ways for therapy of alphaherpesvirus infections in animals and humans depends on the demonstration of its efficacy and safety in the relevant target organism, be it animal or human.

Acknowledgements

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