A glycoprotein E deletion mutant of bovine herpesvirus 1 infects the same limited number of tissues in calves as wild-type virus, but for a shorter period

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To gain insight into the role of glycoprotein E of bovine herpesvirus 1 (BHV-1), we compared the distribution of wild-type (wt) BHV-1 with that of a gE deletion mutant (gE-) in calves after intranasal inoculation. The wt-infected calves had severe clinical signs, but the gE--infected calves were virtually free of clinical signs. At 3, 4, 7, 8, 44, 45, 50 and 51 days post-infection (p.i.), one calf from each group was killed and tissues were collected for virus isolation and PCR analysis. At 3, 4, 7 and 8 days p.i., infectious virus could be isolated only from the nasopharyngeal mucosa, parotid gland and nearby lymphoid tissues for both the wt- and gE-infected calves. At 3 and 4 days p.i., virus titres in these tissues were comparable in both the wt- and gE-infected calves. However, the virus titres were significantly reduced at 7 and 8 days p.i. in the gE--infected calves, but not in the wt-infected calves. Semi-quantitative PCR analysis revealed that for the entire infection period (3 to 51 days p.i.) significantly more BHV-1 DNA was detected in the trigeminal ganglia (TG) of the wt-infected calves than in those of the gE--infected calves. We conclude that the gE--mutant infects the same limited number of tissues as wt BHV-1, but for a shorter period.

Bovine herpesvirus 1 (BHV-1) causes infectious bovine rhinotracheitis, infectious pustular vulvovaginitis and infectious pustular balanoposthitis in cattle (Gibbs & Rweyemamu, 1977). After acute infection, BHV-1 establishes a latent infection, which can be reactivated. The genome of BHV-1 consists of a linear double-stranded DNA molecule of about 140 kb which codes for approximately 75 proteins including several glycoproteins. Until now seven glycoprotein genes of BHV-1 have been identified: glycoproteins gB, gD, and most probably gH are essential, whereas gC, gE, gG and gI are not essential for in vitro growth (Spear, 1993; F. A. M. Rijsewijk and others, unpublished data).

The in vitro properties of gE deletion mutants of the related alphaherpesviruses herpes simplex virus 1 (HSV-1) and suid herpesvirus 1 (SHV-1) indicate that gE is involved in cell-to-cell spread of the virus (Dingwell et al., 1994; Zsak et al., 1992). In vivo, gE deletion mutants of HSV-1 and SHV-1 are reduced in virulence (Küdelová et al., 1991; Kimman et al., 1992a) and show impaired neuroinvasiveness (Racjáni et al., 1990a, b; Kimman et al., 1992b; Card et al., 1991). Previously, we showed that deleting the gE gene from BHV-1 strongly reduces its virulence in calves (Van Engelenburg et al., 1994). To gain further insight into the role of gE of BHV-1, we compared the distribution of a gE deletion mutant (gE-) and the parental wt strain in calves.

The gE--mutant was produced by deleting the complete gE open reading frame from the wt BHV-1 isolate Lam (Van Engelenburg et al., 1994). This gE--mutant has the same in vivo phenotype as a gE--frameshift mutant (M. J. Kaashoek and others, unpublished results), demonstrating that the phenotype of the gE--mutant was due to inactivation of the gE gene and was not affected by altering the expression of the gE flanking genes. A revertant virus from the gE--mutant has the same in vitro phenotype as wt BHV-1 (F. A. M. Rijsewijk and others, unpublished results). The inocula were produced from virus infected Madin–Darby bovine kidney cells and for virus isolations and virus titrations embryonic bovine trachea (EBTr) cells were used as described previously (Van Engelenburg et al., 1994).

Sixteen calves were delivered by caesarean section, all within 1 week, and were randomly allotted to two

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isolation stables at our institute and was fed and treated similarly. The calves were colostrum deprived and had no BHV-1 antibodies at the start of the experiment. At the age of 7 weeks, the calves were inoculated with the gE− mutant (group 1) or the parental strain Lam (group 2) by spraying 1 ml of virus suspension (10^8 TCID_{50}/ml) into each nostril. At 47 days post-infection (p.i.) the calves received 0.1 mg dexamethasone/kg body weight (Dexaderon, Intervet) intramuscularly for 4 consecutive days to reactivate putatively latent virus.

For each calf, rectal temperatures were measured and nasal swabs were taken daily during the whole experiment and ocular swabs were taken daily until 15 days p.i. The nasal cavities were inspected daily until 15 days p.i. for the presence of lesions as described by Van Engelenburg et al. (1994). Sera and ACD (acid-citrate–dextrose, solution B; Becton Dickinson) blood were collected for a BHV-1 neutralization test and BHV-1 PCR analysis, respectively, several times throughout the whole experiment. Before storage at −70 °C the ACD blood samples were diluted with 1 vol. of Glycigel (Kaye et al., 1991). Virus isolation and titration from the nasal and ocular swabs and the BHV-1 neutralization test were done as described by Kaashoek et al. (1994).

To determine the distribution of wt BHV-1 and the gE− mutant, one calf from each group was killed at 3, 4, 7, 8, 44, 45, 50 and 51 days p.i. by administering intravenously an overdose of barbiturate (Dolethal, Vétouquimol). From each calf we collected tissue samples from the upper respiratory tract (nasal mucosa, olfactory mucosa and oropharyngeal mucosa); lower respiratory tract (trachea and lung); upper digestive tract (parotid gland and oesophagus); lower digestive tract (ileum, jejunum and rectum); lymphoid tissues (tonsil, thymus, spleen, mandibular lymph node, retropharyngeal lymph node and parotid lymph node); peripheral nervous system (trigeminal ganglion, a pool of two cervical, two thoracic and two lumbar ganglia, and a pool of four sacral ganglia); central nervous system (pons cerebri, medulla oblongata, spinal cord, olfactory bulb, cerebrum and cerebellum); endocrine organs (adrenal gland); urinary tract (kidney); and genital tract (penile or vaginal mucosa, and testis or ovary). The collected tissues were stored separately at −70 °C for virus isolation and PCR analysis.

To detect infectious BHV-1, 0.4 to 4 g of a tissue sample was tested by virus isolation. After adding sterile sea-sand (Merck), the sample was homogenized in a mortar and was suspended in 5 ml of tissue culture medium. A 200 µl volume was tested by virus isolation (Kaashoek et al., 1994). The limit of sensitivity of the virus isolation was 0.8 TCID_{50}/ml. Virus titres were measured in samples found positive by virus isolation and were expressed as TCID_{50}/g tissue. To detect BHV-1 DNA, total DNA was isolated from 50 to 150 mg of a tissue sample. After adding sterile sea-sand, the tissue sample was homogenized in a micro-centrifuge tube. The piston of a 1 ml syringe was used as a disposable micropipette. Next, 1-2 ml of lysis buffer [0.15 M-NaCl, 20 mM-Tris- HCl (pH 7.5), 10 mM-EDTA (pH 8.0), 0.5% sodium N-lauroylsarcosine, 1 mg/ml proteinase K (Boehringer) and 40 mM-dithiothreitol] was added and the sample was incubated at 50 °C for 16 h. After centrifugation of the lysate at 12000 g for 30 s, 300 µl of the supernatant was added to a micro-centrifuge tube containing 300 µl 6 M-NaI. After mixing, the sample was extracted twice with 1 vol. chloroform–isooamyl alcohol (24:1) and the DNA was precipitated by adding 1 vol. isopropanol and centrifuging at 12000 g for 30 min. The DNA pellet was washed with 70% ethanol and resuspended in 100 µl of TE (10 mM-Tris–HCl, pH 7.5, 1 mM-EDTA) buffer. One µg of the isolated DNA was tested by PCR. To detect BHV-1 DNA in whole blood, we isolated total DNA from 600 µl of a whole blood sample as described above and tested 0.5 µg of the isolated DNA by PCR. The isolated DNA was amplified by PCR as described by Van Engelenburg et al. (1993).

The amount of BHV-1 DNA present in a tissue sample was determined by competitive PCR analysis (Van Engelenburg et al., 1993). One µg of isolated DNA from a tissue sample was spiked with either 15, 150 or 1500 molecules of control template and was amplified. The amount of viral DNA present in a tissue sample was estimated by comparing the amount of the control PCR product with that of the viral PCR product. The results of the semi-quantitative PCR analysis were analysed statistically by the Mann–Whitney–Wilcoxon test (P < 0.05).

All wt-infected calves had fever (Fig. 1a) and developed severe and widespread necrotic lesions in the nasal mucosa. None of the gE−-infected calves had fever but some of them had mild and restricted lesions in the nasal mucosa. During the acute infection high virus titres were detected in nasal fluid from both the wt- and gE−-infected calves (Fig. 1b). In the wt-infected calves significantly (P < 0.05) higher virus titres were detected in nasal fluid than in the gE−-infected calves at 6 to 8 days p.i., but not on the other days. Virus titres up to 4 TCID_{50}/ml were measured in the ocular fluid of wt- and gE−-infected calves (data not shown).

After the acute infection, none of the wt- and gE−-infected calves had fever and none of them excreted BHV-1 in nasal fluid on any day. Also, after the dexamethasone treatment that was started at 47 days p.i. no recurrent BHV-1 infection could be detected in these calves. Usually, infectious virus can be detected in nasal fluid of BHV-1-infected calves 4 to 5 days after the start.
Fig. 1. Temperature, nasal virus excretion and BHV-1 neutralizing antibody response of the wt BHV-1- (■) and gE--mutant- (○) infected calves. (a) Mean daily rectal temperature; (b) mean virus titres expressed as log_{10} of the TCIDs_{50}/ml nasal fluid; (c) mean BHV-1 neutralizing antibody titres expressed as 10^{10} of the reciprocal of the highest serum dilution that inhibited the BHV-1 cytopathic effect. Dex, start of dexamethasone treatment. The data shown are, for 0-42 days p.i., from the four calves of each group that were not killed during the course of acute infection, whereas for 47-50 days p.i. the data are from two calves of each group, and for 51 days p.i. from one calf of each group. The results were statistically evaluated by analysis of variance for the difference between wt BHV-1 and the gE--mutant determining the least significant difference for daily pairwise comparisons.

Table 1. Virus isolation from tissues of wt- and gE--infected calves at 3, 4, 7 and 8 days p.i.

<table>
<thead>
<tr>
<th>Tissue*</th>
<th>wt BHV-1</th>
<th>gE-- mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nasal mucosa</td>
<td>2.3-4.9</td>
<td>5.8-6.0</td>
</tr>
<tr>
<td>Olfactory mucosa</td>
<td>1.4-3.4</td>
<td>2.7-3.6</td>
</tr>
<tr>
<td>Oropharyngeal mucosa</td>
<td>4.8-5.0</td>
<td>4.3-2.0</td>
</tr>
<tr>
<td>Parotid gland</td>
<td>1.6-1.9</td>
<td>1.6-3.0</td>
</tr>
<tr>
<td>Tonsil</td>
<td>5.3-5.5</td>
<td>5.5-2.5</td>
</tr>
<tr>
<td>Mandibular lymph node</td>
<td>0.9-0.9</td>
<td>-</td>
</tr>
<tr>
<td>Retropharyngeal lymph node</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Trigeminal ganglion</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pons cerebri</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* In total 29 different tissues from each calf were examined by virus isolation. The results for nine tissues are presented in this Table. The mean mass (±SD) of a tissue sample was 1.7 (±0.98) g and the mean limit of sensitivity was 1.3 TCIDs_{50}/g. Samples found negative by virus isolation are indicated by ‘-’. The results for the nasal, olfactory and oropharyngeal mucosae, parotid gland and tonsil were statistically analysed by Friedman’s method for randomized blocks and by Monte Carlo simulations for other experimental designs. We introduced a statistic ‘time’, which as the value ‘early’ (3 and 4 days p.i.) and ‘late’ (7 and 8 days p.i.). Using Monte Carlo simulations, we did a two-way analysis of variance (virus, time) with repeated measures (tissues). All other tissues were negative for virus isolation for both the wt- and gE--infected calves at 3, 4, 7 and 8 days p.i.

† Each time point represents a different calf.

of treatment (Kaashock et al., 1994). Why we could not detect reactivation after dexamethasone treatment in this study is not clear.

BHV-1 neutralizing antibodies were first detected in sera of both the wt- and gE--infected calves at 10 days p.i. (Fig. 1c). From 10 to 20 days p.i., the titres of BHV-1 neutralizing antibodies of the wt-infected calves increased more rapidly than those of the gE--infected calves. The titres of the wt-infected calves were significantly (P < 0.05) higher than those of the gE--infected calves.

At 3, 4, 7 and 8 days p.i., infectious virus was detected one or more times in the nasopharyngeal mucosa, parotid gland and nearby lymphoid tissues in the wt-infected calves (Table 1). High titres (>10^5 TCIDs_{50}/g tissue) were found in nasopharyngeal mucosa and tonsils. In the gE--infected calves infectious virus was detected in the same tissues as in the wt-infected calves, but not in the retropharyngeal lymph node. Also, in the gE--infected calves, high titres were found in the nasal mucosa at 3 and 4 days p.i. and in the tonsils at 4 days p.i. However, most of the tissues found positive at 3 or 4 days p.i. were negative by virus isolation at 7 or 8 days p.i. At 44, 45, 50 and 51 days p.i., no infectious virus was detected in any tissue of both the wt- and gE--infected calves.

Because each time point was represented by only a single calf, the virus titres of the tissues are influenced by animal to animal variation. Statistical analysis that takes this variation in consideration showed that in gE--infected calves the mean of the virus titres of the tissues at 7 and 8 days p.i. was significantly (P < 0.05) lower than at 3 and 4 days p.i. In the wt-infected calves, the mean virus titres of the tissues at 7 and 8 days p.i. were not significantly (P < 0.05) different from those at 3 and...
Table 2. PCR analysis of tissues of wt- and gE- infected calves*

<table>
<thead>
<tr>
<th>Tissue</th>
<th>wt BHV-1 (Days p.i.)</th>
<th>gE- mutant (Days p.i.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nasal mucosa</td>
<td>++ + + + + - + + +</td>
<td>+ + + + + + - + +</td>
</tr>
<tr>
<td>Olfactory mucosa</td>
<td>++ + + + - + + + +</td>
<td>++ + + - + + + +</td>
</tr>
<tr>
<td>Oropharyngeal mucosa</td>
<td>+ + + + - + + + +</td>
<td>+ + + - + + + +</td>
</tr>
<tr>
<td>Parotid gland</td>
<td>- - + - - + + -</td>
<td>- - + - - + + -</td>
</tr>
<tr>
<td>Tonsil</td>
<td>+ + + + - + + + +</td>
<td>+ + + - + + + +</td>
</tr>
<tr>
<td>Mandibular lymph node</td>
<td>+ + - + + + + +</td>
<td>+ + + - + + + +</td>
</tr>
<tr>
<td>Retropharyngeal lymph node</td>
<td>- - - - + + + +</td>
<td>- - - - + + + +</td>
</tr>
<tr>
<td>Trigeminal ganglion</td>
<td>+ + + + + + + +</td>
<td>+ + + + + + + +</td>
</tr>
<tr>
<td>Pons cerebri</td>
<td>- - - - - - - -</td>
<td>- - - - - - - -</td>
</tr>
</tbody>
</table>

* A sample found positive by PCR analysis is indicated with a ‘+’, a sample found negative with a ‘-’. BHV-1 DNA could not be detected in any of the samples of olfactory bulb and medulla oblongata.
† From 47 to 51 days p.i. the calves were treated with dexamethasone.
‡ One of the four negative controls that were amplified along with the samples of tonsil from the wt- and gE- infected calves killed at 3, 4, 7 and 8 days p.i. was positive. Contamination due to carryover of viral templates during the processing of the samples can therefore not be excluded.

4 days p.i. We therefore conclude that the gE- mutant infects the same tissues as wt BHV-1, but for a shorter period.

The analysis of the tissues by virus isolation indicated that wt BHV-1 spread by blood, we tested by PCR analysis several whole blood samples that were collected from two wt-infected calves at 0, 2, 4, 6, 8, 10, 12, 14 and 46 days p.i. All these samples tested negative (data not shown), indicating that wt BHV-1 did not spread by blood or only at a level below the detection limit of the PCR analysis. This limit was approximately three BHV-1 DNA molecules/10^8 cells (data not shown). Spread of BHV-1 by blood can occasionally be observed (Brenner et al., 1989; M. J. Kaashoek and others, unpublished data) and is probably dependent on the strain and dose of the virus, and on the age and condition of the calf.

To further analyse the distribution of wt BHV-1 and the gE- mutant, we performed PCR analysis of tissue samples. Because the results of the virus isolation on tissues and the PCR analysis of whole blood samples indicated that the wt BHV-1 infection remained local, we limited the PCR analysis to 11 tissues from the head and neck region including several nervous tissues (Table 2). At 3, 4, 7 and 8 days p.i., in the wt-infected calves, BHV-1 DNA was detected in all tissues that were also found positive by virus isolation, except the retropharyngeal lymph node. Moreover, BHV-1 DNA was detected in the trigeminal ganglia (TG). At 44, 45, 50 and 51 days p.i., BHV-1 DNA was detected in all tissues that were also found positive by virus isolation, except the retropharyngeal lymph node. Moreover, BHV-1 DNA was detected in the trigeminal ganglia (TG). At 44, 45, 50 and 51 days p.i., BHV-1 DNA was detected in the same tissues as in the wt-infected calves, but not in the parotid gland. At 44, 45, 50 and 51 days p.i., BHV-1 DNA was detected in the same tissues as in the wt-infected calves, but not in the tonsils and pons cerebri. The PCR analysis showed that the distribution of BHV-1 DNA was similar in the wt- and gE- infected calves.

The presence of high numbers of BHV-1 DNA molecules, and the simultaneous absence of infectious virus in TG during the acute infection, suggest that BHV-1 does not cause a productive infection in the TG. In contrast, in animals infected with the related alphaherpesviruses BHV-5, HSV-1 and SHV-1, high titres of infectious virus are measured in TG during the acute infection (Bagust & Clark, 1972; Roizman & Sears, 1987; Gustafson, 1986).

We also estimated the BHV-1 DNA content of the other tissues of the wt- and gE- infected calves in which
BHV-1 DNA was detected at 44, 45, 50 or 51 days p.i. Using semi-quantitative PCR analyses, we found that in all these tissues only small amounts of BHV-1 DNA (3 to 30 molecules of BHV-1 DNA/10^6 cells) were present (data not shown). The detection of viral DNA at extraneuronal sites long after primary infection has not been reported for BHV-1 before.

This study showed that a BHV-1 gE^- mutant infects the same limited number of tissues as wt BHV-1, but for a shorter period.

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**References**


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