Short Communication

Intraspecific bovine herpesvirus 1 recombinants carrying glycoprotein E deletion as a vaccine marker are virulent in cattle

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Received 22 February 2006
Accepted 20 March 2006

Vaccines used in control programmes of *Bovine herpesvirus 1* (BoHV-1) utilize highly attenuated BoHV-1 strains marked by a deletion of the glycoprotein E (gE) gene. Since BoHV-1 recombinants are obtained at high frequency in experimentally coinfected cattle, the consequences of recombination on the virulence of gE-negative BoHV-1 were investigated. Thus, gE-negative BoHV-1 recombinants were generated *in vitro* from several virulent BoHV-1 and one mutant BoHV-1 deleted in the gC and gE genes. Four gE-negative recombinants were tested in the natural host. All the recombinants were more virulent than the gE-negative BoHV-1 vaccine and the gC- and gE-negative parental BoHV-1. The gE-negative recombinant isolated from a BoHV-1 field strain induced the highest severe clinical score. Latency and reactivation studies showed that three of the recombinants were reexcreted. Recombination can therefore restore virulence of gE-negative BoHV-1 by introducing the gE deletion into a different virulence background.

The virulence of any given viral strain is the result of the effects of the constellation of genes carried by that strain. How these genes and their products interact with each other and interact with host proteins determine the outcome of the disease. Glycoprotein E (gE) is considered as a virulence factor of all studied members of the subfamily *Alphaherpesvirinae*. The gE-minus mutants generated hitherto from herpes simplex virus (HSV), pseudorabies virus, equine herpesvirus 1 and *Bovine herpesvirus 1* (BoHV-1) were shown to be attenuated in mice, swine, foals and calves, respectively (Jacobs *et al.*, 1993; Dingwell *et al.*, 1994; van Engelenburg *et al.*, 1994; Matsumura *et al.*, 1998). BoHV-1 gE and gI form a non-covalent-linked heterodimer in infected cells and in the virion envelope (Whitbeck *et al.*, 1996; Yoshitake *et al.*, 1997). *In vitro*, the analysis of mutant BoHV-1 viruses lacking gE, gI or both has shown that the complex is involved in cell-to-cell spread (Baranowski *et al.*, 1996; Rebordosa *et al.*, 1996; Tyborowska *et al.*, 2000; Mahony *et al.*, 2002; Trapp *et al.*, 2003).

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Detection of latent BoHV-1 DNA in trigeminal ganglion is available as supplementary material and figures in JGV Online.

BoHV-1, classified as an alphaherpesvirus, is a major pathogen of cattle. Primary infection is accompanied by various clinical manifestations such as infectious bovine rhinotracheitis, infectious pustular vulvovaginitis, abortion and systemic infection (Tikoo *et al.*, 1995; Kaashoek *et al.*, 1996b). When an animal survives a latent infection is established in the sensory ganglia. Reactivation from latency can occur after natural stimulus exposure or corticosteroid treatment culminating in recurrent virus transmission to uninfected animals. In regards to the significant losses incurred by disease and trading restrictions, several European countries have initiated BoHV-1 control programmes based on the use of marker vaccines deleted in the gE gene. These marker vaccines, either inactivated or live attenuated, used together with a serological detection of gE-specific antibody (Ab), allow differentiation between infected and vaccinated animals (van Oirschot *et al.*, 1997; Lehmann *et al.*, 2002). However, all the gE-negative BoHV-1 tested hitherto have been generated from weakly virulent BoHV-1 strains (van Engelenburg *et al.*, 1994; Chowdhury *et al.*, 1999). Therefore, there is a concern about the virulence of 'vaccine-like' gE-negative BoHV-1 issued from recombination with virulent BoHV-1 strains.

Intramolecular recombination is a mechanism of genetic material exchange closely related to the alphaherpesvirus

replication cycle (Thiry *et al.*, 2005). Previous data supported the frequent rise of recombinants in cattle after concomitant nasal infections with two BoHV-1 mutants (Schynts *et al.*, 2003). The present study aimed at characterizing the virulence of gE-negative BoHV-1 recombinants generated *in vitro* from several strain backgrounds. Coinfection experiments have involved a weakly virulent BoHV-1 strain deleted in the gC- and gE-encoding genes and several wild-type BoHV-1 strains. A biological characterization led to the scoring of the BoHV-1 recombinants possessing the vaccine gE-negative phenotype (Muylkens *et al.*, 2006). Based on this *in vitro* screening, four gE-negative BoHV-1 recombinants were selected for testing their virulence in the natural host.

An experiment was designed to assess the virulence of these gE-negative BoHV-1 recombinants by inoculating blindly seven BoHV-1 to seven groups of four calves (Table 1). Two mock-infected calves were used as control. Four gE-negative BoHV-1 recombinants, namely rIowaΔgE, rED1ΔgE, rCineyΔgE and rCooperΔgE, were tested. The virulence of their respective wild-type strains had been assessed *in vivo* (Table 1). The wild-type BoHV-1 strain Iowa was used as a highly virulent comparison strain; a BoHV-1ΔgE vaccine and BoHV-1ΔgCΔgE, the parental strain of all the recombinants, were inoculated as weakly virulent comparison strains (Table 1). The description of the BoHV-1 viruses used in this study is available as Supplementary Fig. S1 in JGV Online. The differences in the clinical, virological and serological data were tested in the form of mixed models for repeated measurements by SAS procedure (procedure MIXED) (Littell *et al.*, 1998). The animal study was accredited by the local ethics committee (folder 115).

The serological status of calves provided prerequisite evidence that the observed clinical signs were induced by the inoculated viruses. Indeed, all the calves infected by gE-negative BoHV-1 recombinants remained seronegative to gE (data not shown). Seroconversion to gB was recorded in all the BoHV-1-infected calves. No seroconversion was observed in the mock-infected group. Calves were clinically examined and rectal temperatures were measured daily for 17 days post-infection (p.i.). All the calves inoculated with the gE-negative BoHV-1 recombinants showed clinical signs typical for BoHV-1 infection (Fig. 1a). A global score was obtained by scoring the following clinical signs: apathy, anorexia, quality and quantity of nasal discharge, cough, dyspnoea, rhinitis, lesions of nasal mucosa, ocular discharge and conjunctivitis (Gogev *et al.*, 2004). The gE-negative BoHV-1 recombinants induced higher scores than the BoHV-1ΔgE vaccine ($P<0{\cdot}05$) (Fig. 1a), but lower scores than the wild-type BoHV-1 (WT; $P<0{\cdot}001$). The recombinant issued from BoHV-1 strain Ciney showed a higher virulence than the other recombinants ($P<0{\cdot}001$) (Fig. 1a). This recombinant also induced hyperthermia above 39·5 °C, apathy and anorexia for 3 consecutive days in the four inoculated calves (Fig. 1b). At days 2 and 3, the mean temperatures of the calves infected with the CineyΔgE recombinant were higher than the mean temperatures of the calves infected with either the BoHV-1ΔgE vaccine or the other recombinants ($P<0{\cdot}01$). A video-endoscope examination performed at day 3 p.i. on two calves in each group allowed investigating the lesions induced by the different viruses in the upper respiratory tract. Data are available as Supplementary Fig. S2 in JGV Online. Six of eight calves inoculated by BoHV-1 recombinants showed more severe lesions than the calves inoculated either by the parental BoHV-1ΔgCΔgE or BoHV-1ΔgE vaccine. One calf infected by rCineyΔgE and one infected by rED1ΔgE exhibited lesions comparable to the lesions induced by wild-type BoHV-1.

The results presented above indicated that the gE-negative recombinants issued from virulent BoHV-1 retained the ability to induce the disease caused by BoHV-1. To determine the level of virus excretion of gE-negative BoHV-1 recombinants, nasal swabs were taken daily from each animal for 17 days p.i. The presence of BoHV-1 was detected and titrated by plaque assay on Madin-Darby bovine kidney cells (ATCC CCL-22) as described previously (Lemaire *et al.*, 1999). BoHV-1 was isolated from the nasal swabs of all infected calves, whereas no virus was isolated from the mock-infected calves (Fig. 1c). The recombinants were excreted as the same amount as the wild-type BoHV-1 from days 1 to 5. The calves infected with the BoHV-1ΔgE vaccine excreted less than calves infected with wild-type or recombinant BoHV-1 ($P<0{\cdot}001$). The parental strain BoHV-1ΔgCΔgE was excreted for the shortest period

Table 1. BoHV-1 viruses inoculated in this study

Inoculated BoHV-1	Virus characteristic		Virulence of the wild-type strain
rCineyΔgE	–	−Ciney	High (Lemaire *et al.*, 1999)
rED1ΔgE	Recombinant between BoHV-1ΔgCΔgE and	−ED1	High (Edwards *et al.*, 1990)
rIowaΔgE	–	−Iowa	Very high (Miller & van der Maaten, 1984)
rCooperΔgE	–	−Cooper	Weak (Chowdhury *et al.*, 1999)
BoHV-1 vaccine	–	Vaccine ΔgE/US9	Weak (Rijsewijk *et al.*, 1999)
BoHV-1ΔgCΔgE	–	Parental ΔgCΔgE	Weak (Schynts *et al.*, 2003)
Iowa	–	Parental wild type	Very high (Miller & van der Maaten, 1984)

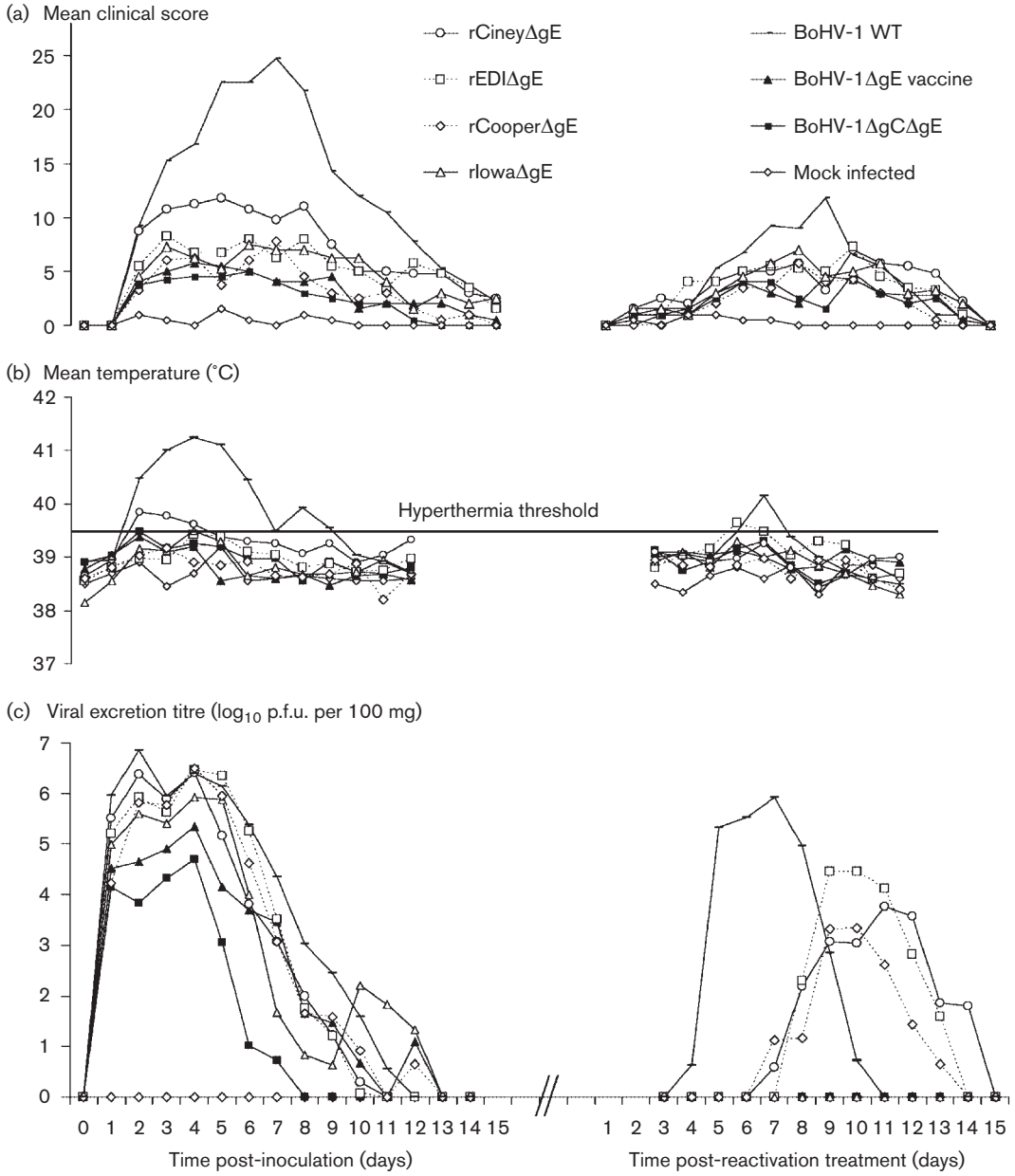

Fig. 1. Mean clinical scores (a) mean rectal temperatures (b) and mean BoHV-1 excretion titres (c) recorded during primary infection and reactivation of seven groups of calves infected by the four gE-negative recombinants, the highly virulent BoHV-1 Iowa, the BoHV-1ΔgE vaccine and the BoHV-1ΔgCΔgE. (a) To obtain a global score, the evaluated clinical signs included apathy, anorexia, quality and quantity of nasal discharge, cough, dyspnoea, rhinitis, lesions of nasal mucosa, ocular discharge and conjunctivitis. (c) BoHV-1 excretion titres were expressed as $\log_{10}$ p.f.u. per 100 mg nasal secretions.

(8 days). (Fig. 1c). Shedding of the gE-negative BoHV-1 recombinants was not reduced at the peak of excretion. This massive virus excretion contrasts with virus shedding recorded with gE-negative BoHV-1 mutants analysed hitherto, LamΔgE, and CooperΔgE were excreted in 100-fold smaller amounts (Kaashoek *et al.*, 1996a, 1998; Chowdhury *et al.*, 1999). With regards to the CooperΔgE mutant, the presence of the *β*-gal gene under the control of a strong promoter from human cytomegalovirus (HCMV) could affect viral cycle regulation and therefore progeny virus production. In our study, the gE-negative recombinant Cooper, which was devoid of the *β*-gal gene and the HCMV promoter, was excreted similarly as wild-type BoHV-1. The phenotype of the gE-negative recombinants was confirmed positive for gC expression and negative for gE expression by double immunofluorescence staining (Schynts *et al.*, 2001) (data not shown). The virus identity was further confirmed by *Hin*dIII restriction analysis using the disappearance of

the K fragment as evidence of the gE deletion (data provided as Supplementary Fig. S3 available in JGV Online).

In order to investigate the latency-reactivation properties of gE-negative BoHV-1 recombinants, calves were injected intravenously with sodium orthophosphate of dexamethasone (Rapidexon) at 0·1 mg kg^{-1} body weight from days 90 to 95 p.i. A slight increase in the rectal temperature was recorded in all the inoculated calves post-reactivation treatment (PRT) (Fig. 1b). Serous and/or mucous nasal discharges and lesions in the nasal mucosa were observed in all inoculated calves within days 2–13 PRT (Fig. 1a). All the calves inoculated with the wild-type BoHV-1 reexcreted at days 5–8 PRT, and some calves of the groups infected with rCineyΔgE, rED1ΔgE and rCooperΔgE reexcreted from days 7 to 14 PRT (Fig. 1c). There was a marked temporal delay between the reexcretion periods of the recombinants and the wild-type BoHV-1 (Fig. 1c). No virus was isolated from the mock-infected calves, nor from any calf infected with rIowaΔgE, BoHV-1ΔgCΔgE or BoHV-1ΔgE vaccine (Fig. 1c). The reexcretion of some of the gE-negative recombinants indicates that these recombinants are able to establish latency and could disseminate the infection under reactivation conditions. Calves were euthanized at days 112, 113 and 114. Trigeminal ganglia were dissected within 2 h after death and immediately stored at −80 °C. Latent BoHV-1 DNA was detected and characterized by PCR as described in Supplementary material available in JGV Online. In the presence of trigeminal ganglion extracts, no cell culture presented cytopathic effect. This prerequisite allowed exclusion of any positive result through PCR due to infectious replication competent BoHV-1. The electrophoretic pattern of PCR products is available as Supplementary Fig. S4 in JGV Online. The calves infected by BoHV-1ΔgCΔgE were positive for gD amplicon but negative for gC and gE amplicons, showing the establishment of latency of this strain, even if no virus was recovered PRT. All calves infected by recombinants gave positive results in gC and gD amplification and no signal in gE amplification. This demonstrated that all the gE-negative BoHV-1 recombinants established latency. Finally, all the calves infected by the wild-type BoHV-1 were positive in the three BoHV-1-specific PCR assays.

Serological monitoring was performed on blood samples collected weekly from each animal. BoHV-1-neutralizing antibodies (NAb) titres were measured in one large-scale assay where the BoHV-1 strain Iowa has been used (Lemaire *et al.*, 2000). No effect of the viral strain on the NAb titre was evidenced by performing cross neutralization assay on two sera per group with four BoHV-1 strains: Iowa wt, Lam wt, LamΔgE and LamΔgC. The titres of BoHV-1 NAb (Fig. 2) showed that all the BoHV-1-inoculated groups reached a plateau at day 21 p.i. except for the BoHV-1ΔgE vaccine (plateau at day 28 p.i.). The mean Ab titre of wild-type was higher than titres obtained in the groups infected with the different gE-negative BoHV-1 recombinants ($P<0·001$). The Ab titres remained at slightly decreased levels until reactivation. After reactivation stimulus, the Ab titres fell before rising again from days 98 to 112 p.i. (Fig. 2). The strongest stimulation of the humoral immune response was observed in the four reexcreting groups of calves, likely due to this reexcretion.

This study reports the first description of a virulent gE-negative alphaherpesvirus generated by recombination. This study shows that attenuation with a single attenuating marker deletion may not be sufficient. The virulence of gE-negative BoHV-1 recombinants demonstrated in this study is clearly in opposition with the pronounced attenuation observed after the inoculation of calves with genetically modified gE-negative BoHV-1 (van Engelenburg *et al.*, 1994; Kaashoek *et al.*, 1996a, 1998; Chowdhury *et al.*, 1999). In a view of straightforward approach to design new generation vaccines, these gE-negative BoHV-1 mutants were constructed from weakly virulent BoHV-1 strains, namely Lam (van Engelenburg *et al.*, 1994) and Cooper (Chowdhury

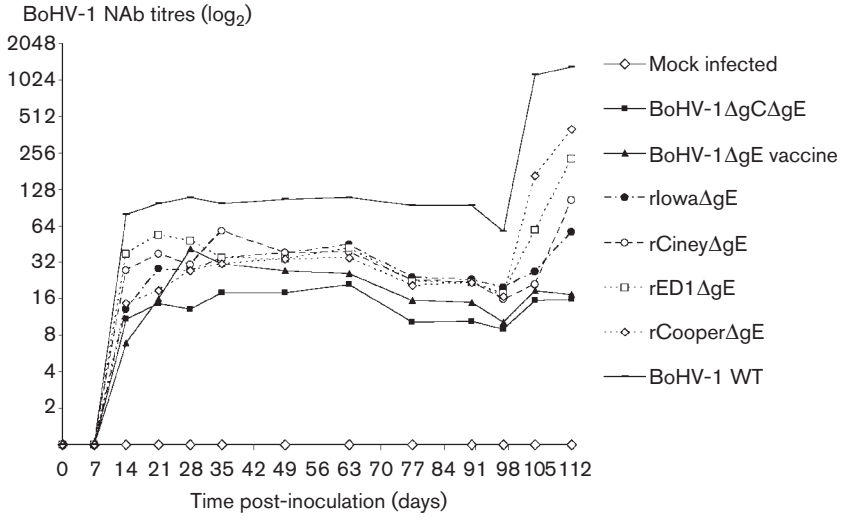

Fig. 2. Evolution of the BoHV-1 NAb titres in seven groups of calves infected at day 0 by the four gE-negative recombinants, the highly virulent wild-type BoHV-1 Iowa, the vaccine BoHV-1ΔgE and the BoHV-1ΔgCΔgE. Calves were reactivated at day 90. NAb titres are expressed as the initial dilution of serum that inhibits viral cytopathic effect in 50 % of wells. Filled symbols label the viruses that remained negative in cell culture upon reactivation; open symbols the viruses that produced infectious viruses following the reactivation treatment.

et al., 1999). However, in our study, three gE-negative recombinants originated from two virulent wild-type BoHV-1, namely ED-1 and Ciney, and one from a highly virulent BoHV-1, namely Iowa (Kaashoek *et al.*, 1996b; Lemaire *et al.*, 1999). Because virulence determinants are highly interdependent, recombination is susceptible to restore the loss of virulence due to the absence of one virulence factor (gE) by implementing this deletion character in another strain's background.

The generated recombinants tested here were characterized on short genome stretches. Immunofluorescence staining and restriction endonuclease analysis showed that the gC ORF was acquired from the wild-type BoHV-1 used in each coinfection situation, while the gE ORF deletion was inherited from the parental gC–gE-negative BoHV-1. It cannot be ruled out that some recombinants arose from multiple crossing over events, as described previously in both HSV-1 and HSV-2 (Brown *et al.*, 1992). Because the distance between the two deletion markers used in this study does not allow us to explore the recombination along the entire genome, some recombinants could not have been detected. Nevertheless, our data support the hypothesis that a single gE deletion is not sufficient to provide an avirulent phenotype.

In conclusion, we have demonstrated the virulence of BoHV-1 recombinants having acquired the gE deletion vaccine marker. Latency and reactivation monitoring suggests that these recombinants are susceptible to perpetuation in the cattle population but further experiments are needed to study their dissemination after reactivation. The present study contributes to the assessment of the consequences of recombination between vaccine and field strains of BoHV-1. The next step will be the investigation of the rise and spread of virulent gE-negative BoHV-1 recombinants in natural populations.

Acknowledgements

The authors are thankful to Christelle Ghyse and Laetitia Wiggers for their technical assistance, to Tess Laidlaw and Katalin de Fays for careful reading, to S. Edward and L. A. Babiuk for BoHV-1 strains. This study was supported by the Service Public Fédéral, Santé Publique, Administration Recherche et Développement and by the Fonds National Belge de la Recherche Scientifique (FNRS) (FRFC 2.4508.02 and grant 1.5.105.03). Benoît Muylkens is Research Fellow of the FNRS.

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