A Brazilian glycoprotein E-negative bovine herpesvirus type 1.2a (BHV-1.2a) mutant is attenuated for cattle and induces protection against wild-type virus challenge

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ABSTRACT - Franco A.C., Spilki F.R., Esteves P.A., Lima M., Weiblen R., Flores E.F., Rijsewijk F.A.M. & Roehe P.M. 2002. A Brazilian glycoprotein E-negative bovine herpesvirus type 1.2a (BHV-1.2a) mutant is attenuated for cattle and induces protection against wild-type virus challenge. Pesquisa Veterinária Brasileira 22(4):135-140. Centro de Pesquisas Veterinárias Desidério Finamor, Fepagro-Saúde Animal, Cx. Postal 2076, Porto Alegre, RS 90001-970, Brazil. E-mail: proehe@ufrgs.br

The authors previously reported the construction of a glycoprotein E-deleted (gE-) mutant of bovine herpesvirus type 1.2a (BHV-1.2a). This mutant, 265gE-, was designed as a vaccinal strain for differential vaccines, allowing the distinction between vaccinated and naturally infected cattle. In order to determine the safety and efficacy of this candidate vaccine virus, a group of calves was inoculated with 265gE-. The virus was detected in secretions of inoculated calves to lower titres and for a shorter period than the parental virus inoculated in control calves. Twenty one days after inoculation, the calves were challenged with the wild type parental virus. Only mild signs of infection were detected on vaccinated calves, whereas non-vaccinated controls displayed intense rhinotracheitis and shed virus for longer and to higher titres than vaccinated calves. Six months after vaccination, both vaccinated and control groups were subjected to reactivation of potentially latent virus. The mutant 265gE- could not be reactivated from vaccinated calves. The clinical signs observed, following the reactivation of the parental virus, were again much milder on vaccinated than on non-vaccinated calves. Moreover, parental virus shedding was considerably reduced on vaccinated calves at reactivation. In view of its attenuation, immunogenicity and protective effect upon challenge and reactivation with a virulent BHV-1, the mutant 265gE- was shown to be suitable for use as a BHV-1 differential vaccine virus.

INDEX TERMS: BHV-1, differential vaccine, gE deletion, IBR.

1Aceito para publicação em 7 de agosto de 2002.
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RESUMO.- [Um mutante gE-negativo de herpesvírus bovino tipo 1.2a é atenuado para bovinos e induz proteção frente ao desafio com vírus de campo.] Em estudo prévio os autores reportaram a construção de um mutante do Vírus da Rinotraqueíte Infecciosa Bovina (IBR) ou Herpesvírus Bovino tipo 1.2a (BHV-1.2a), do qual foi deletado o gene que codifica a glicoproteína E. Esse mutante (265gE-) foi construído a partir de uma amostra autóctone do vírus, tendo como objetivo seu uso como amostra vacinal em vacinas diferenciais, capazes de permitir a diferenciação entre animais vacinados e infectados com vírus de campo. Para determinar a atenuação e eficácia do 265gE-
INTRODUCTION

Bovine herpesvirus type 1 (BHV-1), a major pathogen of cattle, is the agent of infectious bovine rhinotracheitis/infectious pustular vulvovaginitis (IBR/IPV). The virus is associated with a number of other clinical syndromes, including pustular balanopostitis, conjunctivitis, infertility and abortion (Gibbs & Rweyemamu 1977). BHV-1 strains have been subdivided into three distinct genotypes, BHV-1.1, BHV-1.2a and BHV-1.2b (Metzler et al. 1985). BHV-1.1 and 1.2a seem to be the most pathogenic, being associated with respiratory disease and abortion, while BHV-1.2b usually displays moderate to low pathogenicity and has not so far been associated to abortion (Metzler et al. 1985).

The BHV-1 genome encodes several glycoproteins (gps) that are expressed on the viral envelope and membranes of infected cells. While some of these are essential for viral replication and mediate different biological functions (Rebordosa et al. 1996), other gps are not essential for virus multiplication, thus representing potential targets for deletions aiming the development of differential vaccines. Glycoprotein E (gE), is one of such non-essential gps (Mettenleiter et al. 1987, Balan et al. 1994, Dingwell et al. 1994, Dingwell et al. 1995, Olson & Groose 1997, Dingwell & Jonhson 1998, Maresova et al. 2001). The gE gene is located in the unique (U) region along the BHV-1 genome, consisting of a 1800 base pair (bp) fragment which codes for a polypeptide of 575 amino acids (Balan et al. 1994). BHV-1.1 gE mutants reported so far displayed reduced pathogenicity in calves and were excreted for shorter periods than wild type virus (Van Engelenburg et al. 1994, Chowdhury et al. 1999). In view of those findings, such mutants have been chosen for the development of differential BHV-1 vaccines (Van Engelenburg et al. 1994, Chowdhury et al. 1999). As the mutant viruses do not express gE, vaccinated animals do not develop antibodies to this protein, thus, making it possible to differentiate the immune responses of naturally infected from vaccinated animals with serological tests based on the detection of gE antibodies.

The differential BHV-1 vaccines available to date have been prepared with BHV-1.1 strains, the most common subtype associated with IBR and abortions in Europe and North America (Metzler et al. 1985). In Brazil, such vaccines have not become available yet. In addition, the differential vaccine viruses prepared in the northern hemisfere (Kaashoek et al. 1994, Chowdhury et al. 1999) have not been tested to determine their protective potential against viruses circulating within Brazil, where most BHV-1 isolates examined so far are of the 1.2a genotype (Souza et al. 2002).

Focusing on the development of a differential vaccine virus to be used within the country, a gE BHV-1.2a mutant virus was constructed from an autochtonous virus (Franco et al. 2001). In the present study, the in vivo behaviour of such mutant was examined in experimentally inoculated calves, in order to determine its attenuation and its potential use as a differential BHV-1 vaccine.

MATERIAL AND METHODS

Experimental design

A group of four calves was inoculated with the mutant virus 265gE-. These will be referred to as "vaccinated" calves. A group of three calves was kept as non-vaccinated controls. Twenty one days after vaccination, vaccinated and control calves were challenged with the parental (wild type) virus by intranasal inoculation and monitored for fourteen days. Six months later, all calves under study were subjected to dexamethasone administration in attempting to reactivate latent viruses.

Cells and viruses

The wildtype BHV-1 strain SV265 (SV265 wt) was isolated from a heifer with signs of respiratory infection in São Borja county, Rio Grande do Sul, Brazil. The virus was used for the construction of the mutant virus (265gE-) as described previously (Franco et al. 2001). Madin Darby bovine kidney (MDBK) cells were maintained in Eagle’s minimal essential medium (EMEM) supplemented with 5 % to 10 % fetal bovine serum (FBS, Nutricell), 2 mM glutamine and antibiotics (100 IU/ml penicillin and 100 mg/ml streptomycin).

Animal inoculation

Four calves of mixed European breeds, three to four months old, seronegative to BHV-1 and BHV-5, were inoculated (vaccinated) with 10^{6.9} fifty percent tissue culture infective doses (TCID_{50}) of the 265gE- mutant, in 2 mL of virus suspension, administered intranasally into the right nostril. Three calves were kept as non-vaccinated controls for subsequent challenge. Three additional calves were kept as non-vaccinated, non-challenged controls throughout. Clinical signs and virus specimens were collected as described below. Twenty one days later, vaccinated and non-vaccinated calves were challenged with 10^{9} TCID_{50} of SV265wt, in 5 mL of suspension administered intranasally, 2.5 mL into each nostril. Six months after challenge, all calves were subjected to corticosteroid administration to reactivate the latent infection. Dexamethasone (0.1 mg per kg of bodyweight) was administrated by intranasal route twice a day for five days. The previous inoculated calves were challenged 21 days after first challenge. The animals were monitored for 21 days after challenge.
body weight) was administered intravenously for 5 consecutive days. Calves were kept under observation and samples collected as below.

**Clinical examination**

Clinical examinations were performed daily from day 12 prior to vaccination up to day 14-post challenge (pc). The same examination protocol was followed after dexamethasone administration. Signs recorded were rectal temperature, respiratory rate, cough, congestion of the nasal mucosa, conjunctivitis, ocular and nasal discharges, lesions on the nasal and oral mucosa and changes in behaviour and appetite. The clinical scoring method was adapted from Collie (1992), with modifications based on our previous experience with BHV-1 experimental inoculations. Thus, scores were determined by attributing different weights to different signs of disease. Apathy, anorexia, dyspnoea and alterations at auscultation were scored on an arbitrary scale up to 100. Nasal and ocular secretions, conjunctivitis, erosions of the nasal mucosa, enlargement of lymph nodes and cough were scaled up to 10. Body temperature (TR) was included in individual scores using the following formula:

\[
\text{Scored temperature rate (TR)} = 100 \times (\text{body temperature} - \text{mean of calf's body temperature on the 12 days before inoculation})
\]

Mean clinical scores were calculated daily for each group.

Six months later, during reactivation attempts, clinical and virological examinations were recorded as described above. In view of the aging of calves, a new record for standard body temperatures was obtained by measuring temperatures for 12 days before reactivation.

**Virological examination**

Samples for virological examination were collected on day 12 prior to inoculation, day 0 (date of inoculation) and daily from days 1 to 14 post vaccination (pv), as well as on days 1 to 14 pc and post reactivation (pr). Nasal and ocular swabs were eluted in 2 mL of sample medium (EMEM supplemented with 10 times the usual concentration of antibiotics and 2 % FBS) for one hour at room temperature. The samples were vigorously shaken, the swabs removed, drained, the medium clarified by low speed centrifugation and stored at -70 °C. Virus titrations were performed on microtitre plates. Infectious titre were calculated and expressed as \( \log_{10} \text{TCID}_{50} \) per 50 mL of nasal or ocular fluid. Serum samples were collected by jugular venipuncture on days 0, 7, 14 pv, pc and pr. Sera were tested in serial twofold dilutions in a standard BHV-1 neutralizing antibody test against SV265wt (House & Baker 1971).

**Identification of the recovered virus**

To confirm the identity of the viruses recovered from inoculated calves, an immunoperoxidase monolayer assay (IPMA) was used, with either a monoclonal antibody (Mab) directed to gE as primary antibody, or an anti-BHV-1 Mab that recognizes both SV265wt and 265gE (Mab 2G5). The IPMA followed essentially the method described previously (Souza et al. 2002), using initially the anti-gE Mab (Kaashoek et al. 1995) followed by an anti-mouse peroxidase/ IgG conjugate. In case uncoloured viral plaques were observed, a second reaction was performed with the Mab 2G5 (Rohe et al. 1997), following the same protocol.

**Statistical analysis**

Statistical analysis was performed using the Student t-test or the analysis of variance (ANOVA) where the least significance difference for \( p \leq 0.05 \) was determined. Comparisons were made daily within the groups. Statistical analysis was performed with Minitab® for Windows Release 11.1 (Minitab Inc., State College, PA, USA). The term “significant” (statistically significant) in the text means \( p \leq 0.05 \).

**RESULTS**

**Clinical signs and virus shedding after vaccination**

Clinical signs after inoculation of the vaccine virus were very mild. Serous nasal discharges were detected from day 2 to 5 pv, especially on the right nostril, where the virus had been inoculated. Rectal temperatures of vaccinated calves did not exceed 39.5°C, except for one calf, which presented pyrexia (39.8°C) on day 6 pv. The vaccinated calves shed virus for up to 8 days in their nasal secretions. The highest virus titre was recorded in one calf on day 2 pv (10^5 TCID_{50}/50 µL). In ocular secretions, the 265gE mutant was only isolated from one calf on day 4 pv.

**Clinical signs after challenge**

After challenge, all non-vaccinated calves developed severe clinical signs, which included apathy, anorexia, rhinitis, serous to mucopurulent nasal discharges and serous to...
mucous ocular discharges, starting from day 3 pc (Fig. 1). In
the nostrils, formation of vesicles which tended to coalesce
and eventually gave rise to erosions of the mucosae were
detected. Conjunctivitis, nasal stridor, sneezing and sponta-
naneous coughing were also recorded. Enlargement of the
retropharyngeal, submandibular and cervical lymphnodes
were observed from days 7 to 12 pc. Dyspnoea and tracheal
stridor were detected in all non-vaccinated calves. Pyrexia
(>39.5°C) was also a consistent finding (Fig. 2).

In the vaccinated group, all calves had some serous nasal
discharge and a few small erosions on the nasal mucosae.
Clinical scores on the vaccinated group were very low,
in comparison to the non-vaccinated group. Pyrexia was not
observed on calves from the vaccinated group.

Both vaccinated and control calves shed virus in nasal
secretions. However, the amount of virus shed by the group
of vaccinated calves was reduced to a maximum of 10²
TCID₅₀, whereas on the non-vaccinated group virus was
excreted for longer and to higher titres (up to 10⁹ TCID₅₀; Fig. 3).
Non-vaccinated calves shed virus in ocular secretions
for 12 days, with peaks of infectious titres at day 4 post
challenge (10¹⁵ TCID₅₀/50 µl; Fig. 4), whereas only two of the
vaccinated calves shed SV265wt virus in their ocular
secretions, albeit intermittently and to low titres on days 1,
3, 6 and 8 pc.

Clinical signs after reactivation

Vaccinated calves showed only very mild clinical signs
at reactivation, whereas clearly noticeable respiratory signs
of disease were observed in non-vaccinated calves (Fig. 1b).
These signs were less intense than those observed during
acute infection following challenge. However, one of the non-
vaccinated calves died on day 11 after reactivation. This
calf had low titres of virus in the nasal horns, trachea,
lung, mediastinic and periportal lymphnodes, liver and spleen
(data not shown). Post mortem examination of this calf
revealed mild pneumonia and hepatic focal necrosis. In such
lesions, BHV-1 antigen was detected by immunohistoche-
mistry (not shown). Nasal virus shedding in the group
of non-vaccinated calves was detected from day 2 to 9 after
reactivation, and to higher titres than on vaccinated
calves (Fig. 3).
Glycoprotein E-negative bovine herpesvirus type 1.2a mutant attenuated for cattle induces protection against wild-type virus

In the group of vaccinated calves, reactivation was followed by a shorter period of virus shedding, which lasted three days (from day 4 to 6 pr). Ocular shedding was detected from day 5 to 7 pr. All virus samples recovered from secretions obtained from vaccinated calves were confirmed as SV265wt, as evidenced by IPMA with the anti-gE monoclonal antibody (Souza et al. 2002). The mutant 265gE- was not recovered from any of the calves after dexamethasone administration.

The neutralizing antibody profile of calves was similar in both vaccinated and non-vaccinated calves (Fig. 5). Differences in antibody titres were never greater than fourfold between vaccinated and non-vaccinated animals, either after challenge or after reactivation.

**Analysis of virus shed during the experiments**

After vaccination, only gE-negative plaques were detected by IPMA. After challenge, no gE-negative viral plaques were detected. Following dexamethasone induced reactivation, only gE-positive viral plaques were recovered from nasal and ocular samples.

**DISCUSSION**

The 265gE- vaccine virus candidate evaluated in the present study was shown to be attenuated when inoculated intranasally into three to four months old calves. After inoculation, the calves showed only very mild clinical signs, as evidenced by the low clinical scores recorded. The virus was excreted in lower titres and for a significantly shorter period of time than the SV265wt virus. Other studies have also shown reduction in virulence and viral excretion with BHV-1 gE mutants (Kaashoek et al. 1994, Van Engelenburg et al. 1994, Kaashoek et al. 1998, Chowdhury et al. 1999). Likewise, in the present study, the deletion of gE from SV265wt appeared to be responsible for the reduced pathogenicity of the mutant when inoculated into seronegative calves, since calves at the same age inoculated...
with SV265wt were severely ill. We have not been able to detect any other significant changes along the genome of 265gE, except for the absence of the gE gene (Franco et al. 2001). Thus, it is very likely that the attenuated phenotype was a consequence of the gE deletion.

Challenge of vaccinated calves with wild type virus gave rise to very mild clinical signs of infection, whereas unvaccinated calves developed severe rhinitritis. Although nasal and ocular virus shedding post challenge could not be prevented by the vaccination, infectious titres and the duration of virus shedding were significantly reduced on vaccinated calves. Both aspects are significant for pathogen eradication and disease control programs (Hage et al. 1994), as pointed out by others with similar immunogens (Kaashoek et al. 1994, Chowdhury et al. 1999).

Clinical signs and virus shedding were also greatly reduced on the group of vaccinated calves after reactivation. Besides, only the wild type virus was recovered after reactivation. Although a 265gE latent infection may have been present, BHV-1 gE virus have been reported not to reactivate readily (Van Engelenburg et al. 1995). This has been regarded as a contributing factor to the safety of gE vaccines. Moreover, the reduction of wild type virus excretion in vaccinated animals is also of interest, and probably reflects a reduced colonization of the trigeminal ganglia by wild type virus, as pointed out by Galeota et al. (1997).

In the present study, it was demonstrated that the 265gE mutant virus is attenuated for calves, induces protection upon challenge and reactivation with a large infectious dose of the parental wild type BHV-1.2a. Therefore, 265gE behaved as a suitable candidate for a vaccine virus. Additional experiments are in progress to further examine its immunogenic potential in cattle and to evaluate its protective effect upon challenge, not only against BHV-1.2a, but also against other herpesviruses of cattle.

Acknowledgements - Research carried out by A.C.F. for the partial fulfillment of the requirements for the PhD degree at the Programa de Pós-graduação em Ciências Veterinárias (PPG-CIVET), Universidade Federal do Rio Grande do Sul (UFRGS), RS. E.F.F., W.R. and P.M.R. are CNPq research fellows. This work was supported by PRONEX, CNPq, FAPERGS and IRFA Biotecnologia Industrial Ltda.

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