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Research in Veterinary Science 81 (2006) 327–334
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BHV-1 vaccine induces cross-protection against BHV-5 disease in cattle

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Accepted 26 January 2006

Abstract

Protection against BHV-5 disease induced by inactivated BHV-1 or BHV-5 based vaccines was analysed. Two groups of calves were subcutaneously immunized with an inactivated BHV-1 or BHV-5 based vaccine. A third group was not vaccinated and used as control. In the post-vaccination period, we studied the humoral and cellular immune response resulting similar to both groups. The efficacy of the vaccines was tested after intranasal challenge of the calves with a virulent Argentinean BHV-5 isolate (A-663). All control animals developed neurological signs associated with BHV-5 infection and high levels of virus shedding. Calves immunized with the BHV-1 and BHV-5 inactivated vaccines were protected against BHV-5 disease. Our study provides evidence that strongly support the existence of cross-protection between BHV-1 and BHV-5 in calves. Even though this has already been suggested by previous works, this is the first time an exhaustive study of the immune response is performed and typical clinical BHV-5 meningoencephalitis signs are reproduced in an experimental BHV-5 challenge trial.

Keywords: Bovine herpesvirus 1 and 5; Immune response; Experimental infection; Cross-protection

1. Introduction

Bovine herpesvirus types 1 (BHV-1) and 5 (BHV-5) are two closely related α-herpesviruses with a recognized narrow host range, rapid growth in cell culture, ability to lyse infected cells and to establish latency. BHV-1 is distributed worldwide and it is the causative agent of Infectious Bovine Rhinotracheitis (IBR) in cattle. It is responsible for considerable economic losses due to decreased milk production, weight loss and abortion. The wide spectrum of clinical symptoms includes rhinotracheitis, infectious pustular vulvovaginitis, balanopostitis, conjunctivitis, abortion, enteritis, and encephalitis (Wyler et al., 1989). Bovine herpesvirus 5 (BHV-5) is the etiological agent of fatal meningoencephalitis in cattle. Outbreaks of this disease have been reported in Australia (Gardiner and Nairn, 1964; Johnston et al., 1962), Hungary (Bartha et al., 1969), Canada (Gough and James, 1975), United States (Barenfus et al., 1963; Eugster et al., 1974; Reed et al., 1973), Italy (Moretti et al., 1964), Brazil (Riet-Correa et al., 1989; Salvador et al., 1998) and Argentina (Carrillo et al., 1983). This virus affects calves from birth to approximately 18 months of age and can reach 100% of mortality in young cattle (Schudel et al., 1986). The prevalence of BHV-5 is unknown because outbreaks are sporadic and routine serologic tests do not discriminate between antibodies against BHV-1 or BHV-5. Cross-protection by naturally occurring or vaccination-induced BHV-1 antibodies has been suggested as a possible explanation for the rare occurrence of BHV-5-associated disease in BHV-1 endemic areas (d'Offay et al., 1995).

Several studies have demonstrated a certain degree of molecular and antigenic polymorphism differences between BHV-1 and BHV-5 by restriction endonuclease mapping (Brake and Studert, 1985; Engels et al., 1986), PCR fol-

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0034-5288/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.rvsc.2006.01.004

lowed by restriction analysis (Ros and Belák, 1999), immunoassays using monoclonal antibodies (MAbs) and protein foot printing techniques (Collins et al., 1993; Friedli and Metzler, 1987; Metzler et al., 1986). These differences could explain the differential tropism between both viruses. On the other hand, BHV-1 and BHV-5 show a sequence nucleotide identity of 85% approximately (Engels et al., 1986). The analysis of genes and encoded proteins of BHV-5 glycoprotein C (gC) (Chowdhury, 1995), gD (Abdelmagid et al., 1995), gG (Engelhardt and Günther, 1996), gH (Meyer and Thiry, 1999), gE and Us9 (Chowdhury et al., 2000, 2002) demonstrate a high degree of homology. The close genetic and antigenic relationship between BHV-1 and BHV-5 presents implications for diagnosis as well as BHV-1 vaccination and eradication programs.

Protection against BHV-5 encephalitis induced by BHV-1 vaccines might be evaluated in vaccination and challenge trials. Although some authors have already performed this kind of experiments (Bratanich et al., 1991; Cascio et al., 1999) they did not succeed in reproducing clinical encephalitis in BHV-5 infected animals which were used as control. Those circumstances do not allow an appropriate interpretation of those results and emphasize the difficulty of BHV-5 trials under experimental conditions. In addition, information concerning the immune response induced by BHV-5 inactivated vaccines is insufficient.

Here we present a complete and comparative study of the humoral immune response that was developed by inactivated BHV-5 and BHV-1 vaccines and protection against the disease with the Argentinean A-663 BHV-5 strain. Besides, this is the first time that neurological symptoms associated with BHV-5 infection are reproduced in an experimental cross-protection trial. This study provides relevant information concerning cross-protection between BHV-1 and BHV-5.

2. Materials and methods

2.1. Virus and cells

BHV-1 Los Angeles (LA) strain and BHV-5 663 isolate (Carrillo et al., 1983; Metzler et al., 1986) were propagated in Madin Darby bovine kidney (MDBK) cells grown in Eagle Minimal Medium (MEM), supplemented with 2% fetal bovine serum (FBS) (Romera et al., 2000). We selected two virus suspensions (BHV-1 and 5) with the same virus titre (10^7 tissue-culture infective dose per ml ($TCID_{50}$/ml)) for vaccine formulations. BHV-5 used for the challenge procedure was prepared at a concentration of 10^7 $TICD_{50}$/ml titre.

2.2. Vaccine formulations

BHV-1 or BHV-5 preparations were inactivated with 1 mM binary bromoethylenimine for 25 h at 37 °C as described by Romera et al. (2000). The virus inactivation was tested according to the procedure previously described by Smitsaart et al. (1989). One volume of inactivated virus suspension was mixed with one volume of mineral oil adjuvant (formulated with Arlacel C, Markol 52 and Tween 80) resulting in a water-in-mineral oil formulation.

2.3. Experimental design

Seventeen 4–6 month-old Holstein calves serologically negative to BHV-1 and BHV-5, which were determined by virus neutralisation and ELISA, were used in the experiment. They were separated into three groups at random, two groups consisting of 5 animals and the other of 7 mock-vaccinated calves, which was the control group. The animals were subcutaneously immunized with 3 ml of inactivated vaccines at days 0 and 21 and maintained in an experimental field facility with controlled feeding and sanitary conditions. Animal care was in accordance with institutional guidelines.

At day 71, all animals were challenged in type II bio security boxes. Blood samples and nasal swabs were collected at different post-vaccination and post-challenge intervals. The humoral immune response was determined at 0, 7, 21, 28, and 71 days post-vaccination (dpv) and the limphoproliferation test (LPT) was performed at 0, 7, 28 and 71 dpv. Samples from day 14 pv were spoilt and therefore could not be tested.

2.4. Virus neutralisation (VN) test: cross-neutralisation

We performed the protocol described previously (Romera et al., 2000). A cross-neutralisation test was performed to evaluate sera from both groups of vaccinated animals at different dpv. The method was used to test sera from the BHV-1 vaccine group against BHV-5 virus (A-663) and vice versa (cross-neutralisation).

2.5. Enzyme-linked immunosorbent assay (ELISA)

The cut-off values of the ELISAs describe below were determined using the method based on the frequency distributions of test results from uninfected and infected reference animals (OIE, 2004). Briefly, using an hyperimmune serum or nasal secretion we performed the assays with all the infected and uninfected samples in one dilution. Providing the hyperimmune serum a 100% value, we normalized the absorbance (A) in relation to the former. The frequency distribution plots obtained allowed us to establish the cut-off value for serum and nasal secretion isotype ELISA. The plot for the total antibodies ELISA had been already done in our laboratory.

2.5.1. Indirect ELISA for determining serum total antibodies

Total antibodies in serum were determined using an indirect ELISA test described before (Romera et al., 2000). The titre was expressed as $\log_{10}$ of the reciprocal of the highest serum dilution showing an adjusted A_{405}

value (A_{405} BHV + MDBK − A_{405} MDBK) greater than 40% of the adjusted A_{405} of the positive control.

2.5.2. Indirect ELISA test to detect anti BHV isotype specific antibodies

Immunoglobulin G_1(IgG_1) and IgG_2 isotypes were detected using an indirect ELISA (Romera et al., 2000). Titres were expressed as $\log_{10}$ of the reciprocal value of the highest serum dilution showing an A_{490} higher than 35% (IgG_1) or 15% (IgG_2) of the A_{490} of the positive control.

2.5.3. Mucosal immunity: detection of IgG_1/IgA antibodies in nasal swabs

The mucosal immune response was determined using a specific ELISA for the detection of IgG_1 and IgA. Briefly, nasal swabs were collected and frozen at −70 °C. The antigen was prepared as described before (Romera et al., 2000) and polystyrene microtitre plates (Immulon IB, Dynatech Laboratories) were incubated with BHV-1 (positive antigen) or FBT (negative antigen) in alternate wells. Samples were added in 4-fold dilutions (starting with dilution 1:8) and negative and positive nasal swabs were included in the test as controls. After incubation and washing the monoclonal antibody against IgG_1 (kindly provided by Dr. S. Srikumaran, University of Nebraska, USA) or a polyclonal antibody against IgA (Bethyl Lab. Inc.) was added. IgG_1 plates were then incubated with peroxidase labelled anti-mouse IgG (KPL). The titre was calculated as the $\log_{10}$ of the reciprocal of the highest sample dilution showing an adjusted A_{490} value greater than 10% or 30% of the adjusted A_{490} of the positive control for IgG_1 and IgA, respectively.

2.6. BHV specific lymphocyte proliferation

The lymphoproliferation test was performed as describe previously (Romera et al., 2000) but incubating lymphocytes with BHV-1 or BHV-5 for BHV-1 or BHV-5 vaccinated animals, respectively. Each test was performed in triplicate. Results were obtained as mean counts per minute (CPM) and expressed as stimulation index (SI = mean CPM of culture with antigen/mean CPM of MDBK controls). We considered as positive samples those obtaining a SI > 3.

2.7. Challenge procedure

All animals in each vaccinated group and seven mock-vaccinated calves were challenged with 10^7 $TCID_{50}$ BHV-5 A-663 isolate. The inoculum (1.5 ml into each nostril of individual animal) was administered by inhalation, using an ultrasonic nebulizer (ELECTROLAB AP-300).

Prior to challenge, the calves were examined clinically and the rectal temperature was recorded. Blood samples and nasal swabs were collected to establish basal values. Nasal swabs were obtained by inserting tampons into the ventral meatus of the nasal passage and they were dipped immediately in 5 ml MEM containing 5000 IU penicillin/ml, 2500 μg streptomycin/ml and 10 μg amphotericin B/ml (Romera et al., 2000). Samples were collected at 0, 2, 3, 5, 7, 9, 11, 13, 15, 17, 20 and 22 days post-challenge (dpc).

2.8. Virus isolation

Nasal swabs were inoculated immediately after collection onto MDBK cell monolayers: 0.1 ml of the nasal fluids were inoculated onto 96 well microtitre plates and 10-fold dilutions were tested in four wells. Monolayers were inspected two days later for the appearance of cytopathic effects (CPE) and virus titres were calculated by the Reed and Muench method (Reed and Muench, 1938).

2.9. Clinical examination

After challenge, a veterinarian who was not aware of the individual animals treatment, examined calves clinically for 22 consecutive days. The parameters evaluated included anorexia, body temperature, rhinitis, vulvovaginitis and neurological symptoms such as depression, circling, tremors, bruxism and incoordination (Carrillo et al., 1983).

2.10. Statistical analysis

A comparison among the vaccine profiles through the experimental period was performed using the analysis of variance (ANOVA) for repeated measures with the Greenhouse and Geisser correction of significance levels (fixed at 1%). The post-ANOVA comparisons were performed using Bonferroni test with the same level of significance. All the statistical calculations were performed using SAS program (released 6.04), following G.M.L. procedure (SAS User's guide, 1988).

In the proliferative test, the LPT positive animals proportion was compared using the Fisher's exact test (with a significance level of 5%).

3. Results

3.1. Humoral immune response in serum

Antibodies (Abs) against BHV-1 or BHV-5 were measured by ELISA test (Fig. 1). Both inactivated vaccines induced similar profiles of total IgG in the period studied (there were not significant differences). The maximal response was reached seven days after revaccination (28 dpv), presenting levels around 4 ($\log_{10}$). Specific antibody titres consisted of 3 $\log_{10}$ at the moment of challenge (71 dpv). As expected, a typical secondary response was observed with an antibody titre increase after challenge. Mock-vaccinated animals did not develop any antibody titres in the post-vaccination period and seroconverted 14 days after challenge.

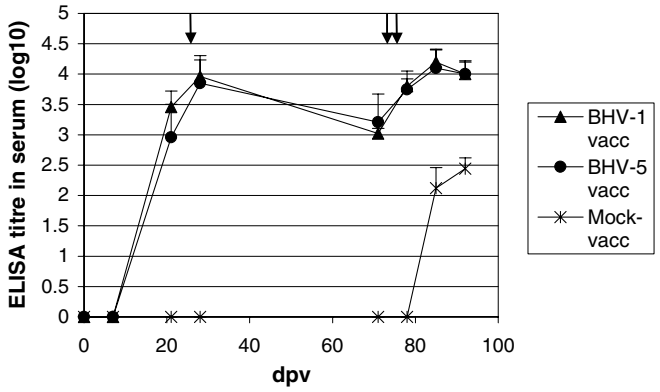

Fig. 1. Antibody responses induced by vaccination and challenge. Total IgG antibodies in serum from BHV-1-vaccinated ($n = 5$), BHV-5-vaccinated ($n = 5$) and mock-vaccinated ($n = 7$) bovines were measured by ELISA. Results correspond to mean titres within each experimental group ± SD. The same groups were used throughout the study. One arrow indicates revaccination day and two arrows challenge day.

3.2. Systemic IgG isotype response in vaccinated animals

Fig. 2 shows IgG_1 and IgG_2 profiles from both vaccinated groups. After the vaccination booster, BHV-1 and BHV-5 vaccinated animals showed the greater titres in both isotypes. There were no significant differences in both isotypes levels between the two immunized groups in the post-vaccination and post-challenge period.

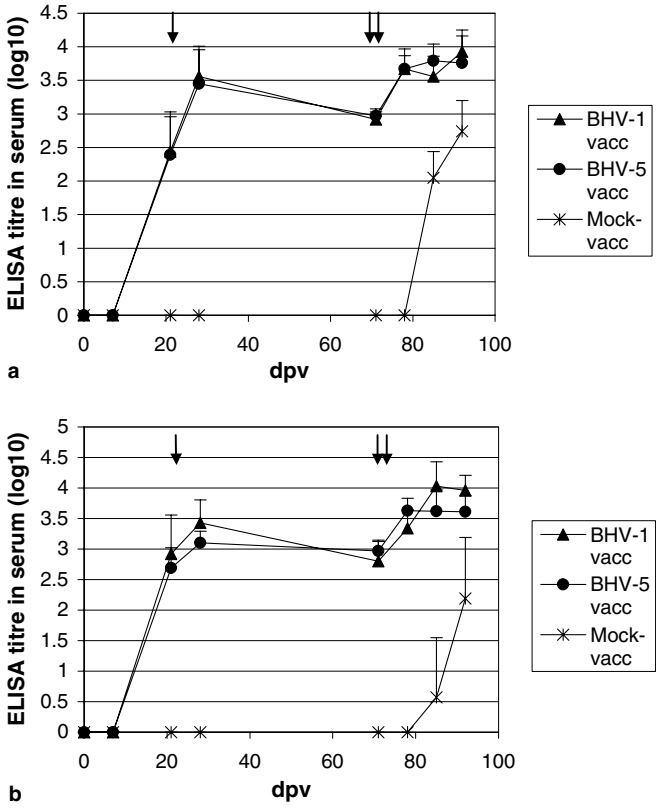

Fig. 2. IgG_1 (a) and IgG_2 (b) antibody levels in serum from vaccinated animals at different days post-vaccination and post-challenge. Results correspond to mean titres within each experimental group ± SD. One arrow indicates revaccination day and two arrows challenge day.

3.3. Humoral immune response in nasal mucosa

Animals immunized with the inactivated BHV-1 or BHV-5 based vaccines developed the same IgG_1 profiles in nasal mucosa samples (Fig. 3a). IgG_1 Abs were detected in only one animal at 21 dpv while all individuals showed this isotype at 28 dpv with a 2 (log_{10}) average value. We found this particular isotype in vaccinated animals until the day of challenge while control animals did not develop any antibody response. IgG_1 antibody levels were significantly different ($p < 0.01$) between both vaccinated groups only at the moment of challenge.

We did not detect IgA Abs in nasal swabs in none of the vaccinated groups in the post-vaccination period. However, we found this isotype at 7 and 14 dpc in BHV-5 and BHV-1 vaccinated animals, respectively (Fig. 3b).

Mock-vaccinated animals developed only IgG_1 from day 14 pc.

3.4. Neutralizing antibody responses in vaccinated animals: cross-neutralisation assay

Neutralizing Abs profiles in serum were similar in both vaccinated groups (Fig. 4). Abs peaked at 28 dpv with titres

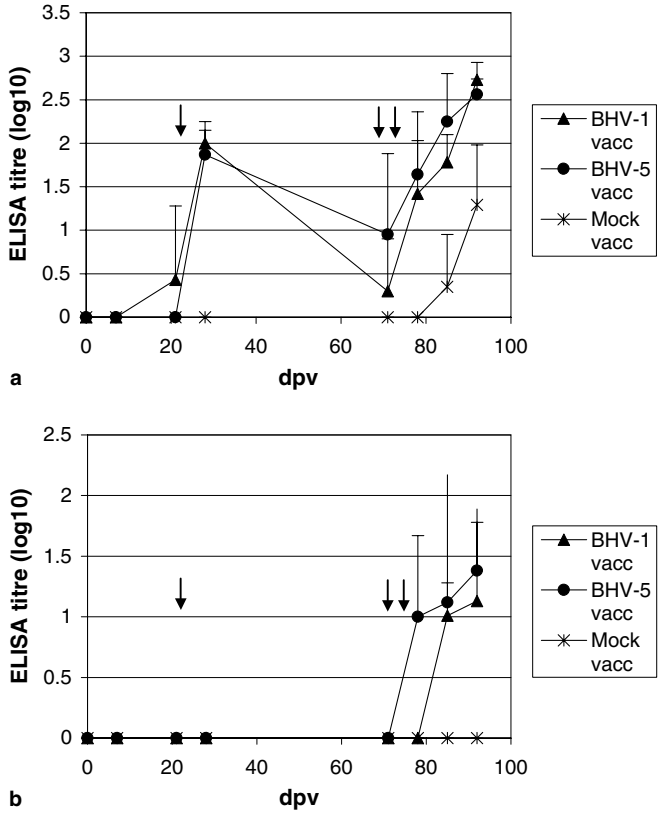

Fig. 3. IgG_1 (a) and IgA (b) antibody levels (means ± SD) in nasal swabs of BHV-1-, BHV-5- or mock-vaccinated animals at different days post-vaccination and post-challenge. Results correspond to mean titres within each experimental group ± SD. One arrow indicates revaccination day and two arrows challenge day.

of 1.7 (log10) and these levels were maintained until the moment of challenge.

Cross-neutralisation assays were performed. We did not detect significant differences in serum titres when the samples were evaluated with the homologous or heterologous virus. There is only a significant difference at day 21pv between the group of BHV-5 vaccine neutralized with BHV-1 and the one vaccinated with BHV-1 and neutralized with BHV-1.

3.5. Cellular immune response

Only one animal from each vaccinated group developed a positive lymphoproliferation response (SI > 3) in the post-vaccination period (Table 1). Although the BHV-1 vaccine group showed a positive response at 7 dpv and the BHV-5 at 28 dpv, there were not significant differences in all the period observed due to the small number of positive animals.

3.6. Virus shedding after BHV-5 challenge

All control animals (mock-vaccinated) showed high levels of viral excretion confirming the virulence of the virus used. The virus shedding period was between 4 and 12 days long and the higher levels were reached at 9 dpc with an average value of $10^{4.23}$TCID$_{50}$%/ml (Fig. 5).

Only one animal immunized with the inactivated BHV-1 vaccine presented virus shedding at 5 and 13 dpc with titres of 2.3 and 2, respectively. Within the group vaccinated with inactivated BHV-5, 3 animals presented virus shedding. Two of them excreted virus for only one day and the other one showed intermittent viral excretion (levels of $10^{2.5}$ TCID$_{50}$%/ml at days 7 and 13 pc).

As shown in Fig. 5, the virus shedding kinetics was significantly different ($p < 0.01$) between vaccinated and control animals. However, both vaccinated groups did not differ in those parameters.

3.7. Clinical signs

After challenge with the BHV-5 A-663 strain, all animals (vaccinated or unvaccinated) presented seromucous and, in some cases, mucopurulent rhinitis between 7 and 20 dpc. Conjunctivitis was detected in animals belonging to all groups. However respiratory signs detected between vaccinated animals were less severe than those observed in control animals and were present for a shorter period.

Neurological signs were observed in all unvaccinated animals. Symptoms were first detected at day 11 pc with 2 animals moving their head (Nos. 133 and 188). Six calves (Nos. 88, 133, 162, 176, 187 and 188) showed bruxism from day 13 pc until day 20/22 pc. Four of these animals, Nos. 161, 162, 187 and 188, developed muscle trembling at 20 dpc and animal No. 161 also presented anorexia at day 22 pc. In bovines Nos. 88 and 187 pressing of the head against objects was observed at day 17 pc and in No. 133 at day 20 pc. The most severe neurological signs were observed at day 20 pc.

No severe symptoms were observed in both groups of vaccinated animals. Only 3 out of 9 vaccinated calves presented slight neurological signs (moderate depression and bruxism). One animal immunized with the BHV-1 vaccine showed depression at day 11pc and bruxism for 5 consecutively days. Two animals of the BHV-5 vaccine group developed bruxism for 2/7 days. Compared with control animals, both vaccinated groups showed less severe neurological signs.

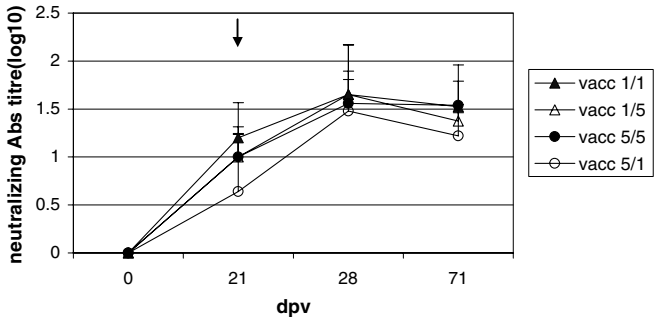

Fig. 4. Neutralizing antibody titres (means ± SD) at different days post-vaccination. BHV-1 and BHV-5 vaccine sera were evaluated with either BHV-1 and BHV-5 virus. Results correspond to mean titres within each experimental group ± SD. The arrow indicates revaccination day.

Table 1
Lymphoproliferation response of vaccinated animals at different days post-vaccination

dpv	BHV-1 vacc	BHV-5 vacc
0	0/5	0/5
7	1/5	0/5
21	1/5	1/5
71	1/5	1/5

Positive animals number (SI > 3) out of the total in each group is indicated.

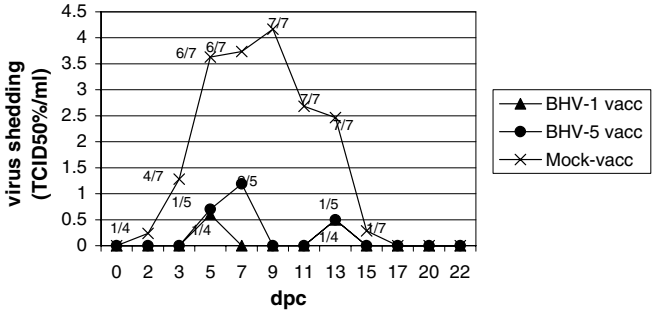

Fig. 5. Mean nasal virus excretion of both vaccinated and control groups after challenge with BHV-5 663 isolate. Titres are expressed as log TCID$_{50}$%/ml of nasal secretions. Shedding animals number out of the total in each group is indicated.

4. Discussion

BHV-1 and BHV-5 are two closely related α-herpesvirus that share similarities at the antigenic and molecular level. A possible cross-immunity between these two viruses with implications in infectivity and latency of BHV-5 has been suggested. It has been reported that BHV-5 can establish latency, i.e., reactivate and spread sub-clinically in calves vaccinated with a live attenuated BHV-1 vaccine (Cascio et al., 1999). Similarly, calostrum-fed newborn calves can become latently infected with BHV-5 without developing neurological signs (Belknap et al., 1994; Perez et al., 2002). In this context, we decided to study the humoral and cellular immune responses induced by inactivated vaccines (formulated with BHV-1 or BHV-5) and the protection they elicit against BHV-5 infection.

Serum antibodies against BHV-1 are considered to be more important in secondary virus exposures, as they appear late after infection, probably preventing reinfection and aiding in the clearance of virus through various mechanisms (Babiuk et al., 1996). Humoral immune response could participate in the protection against BHV-5 challenge. The humoral response induced by BHV-1 and BHV-5 inactivated vaccines in this study showed parallel profiles of total antibodies, IgG_1/IgG_2 isotypes and neutralizing antibodies. This is the first time that serum isotypes were studied in BHV-5 vaccinated animals giving additional information regarding BHV-5 immune response. In contrast to our results, a previous study performed with inactivated vaccines showed that total antibodies induced by a BHV-5 vaccine appeared after receiving a booster and 30 days later than with the BHV-1 vaccine (Bratanich et al., 1991). Although they detected neutralizing Abs 15 days after revaccination, that study showed that seroneutralising response was similar for both vaccines. Experimental design and/or vaccine formulation could be responsible for these differences.

It is assumed that IgA in nasal mucosa is effective in conferring protection against respiratory virus infections (Haan et al., 2001; Israel et al., 1992; Nedrud et al., 1987; Renegar and Small, 1991a,b) and therefore, prevents its spread to susceptible hosts. IgA induction largely depends on the immunogen, the immunization route and adjuvant (Gao et al., 1995). Although there is no available information about nasal antibodies in BHV-5 vaccinated or infected animals, several studies reported IgA antibodies in nasal mucosa after immunization of calves with different BHV-1 vaccines (Gao et al., 1994, 1995; Israel et al., 1992; Zhu and Letchworth, 1996). We did not detect IgA in nasal swabs in none of the vaccinated groups during the post-vaccination period. These results are consistent with those reported by Mars et al. (2000) where all vaccinated animals, which were inoculated intramuscularly with a marker BHV-1, vaccine did not develop IgA antibodies in nasal mucosa supporting the fact that parenteral immunization does not induce mucosal immunity generally. After BHV-5 challenge, we observed a strong response in vaccinated animals indicating the existence of mucosal memory. However, we did not detect IgA in the control group during the studied period. Even though Madic et al. (1995a) does find this isotype after an experimental infection, this might be due to sensibility differences between our detection systems. On the other side, we found that both formulations induced a high IgG_1 post-vaccination response. IgG_1 Abs could be selectively transported from serum to nasal mucosa (Madic et al., 1995a,b) and this isotype might probably contribute to the mechanism which is involved in protection during secondary virus exposures (Madic et al., 1995a,b; Haan et al., 2001). We observed a rapid and high secondary response that could help to reduce virus shedding by neutralizing virus particles. BHV-5 infected animals showed IgG_1 Abs from day 14pi consistently with observations with BHV-1 infected animals (Madic et al., 1995a).

A cellular immune response is thought to be critical for protection against BHV-1 infections (Rouse and Babiuk, 1974; Gerber et al., 1978; Romera et al., 2000). Since only one animal per group was positive to LPT we consider that further experiments will be necessary to test the importance of cellular immune response against BHV-5 infections. We consider that the results presented here constitute the first attempt to examine this specific aspect of the immune response.

BHV-5 infection leads to meningoencephalitis in young cattle with different degrees of severity and it is commonly observed in populations with a high percentage of BHV-1 seropositive animals. Meyer et al. (2001) infected 3-month-old calves with N-569 BHV-5 strain and animals were euthanized due to severe neurological symptoms induced by the virus. Ashbaugh et al. (1997), Cascio et al. (1999) and Vogel et al. (2003) performed experimental infections in 4–6 month old calves with BHV-5 field isolates and they observed that animals did not develop neurological symptoms associated with virus infection. However, histopathological examination of the brain revealed evidence of encephalitis. Ashbaugh suggested that different BHV-5 isolates are neuroinvasive but not always neurovirulent developing different phenotypes in calves depending on viral factors, age and immunological status of the animals (Ashbaugh et al., 1997). The development of an experimental challenge with a BHV-5 strain that reproduces neurological signs of infection is extremely important to test cross-protection with BHV-1. This is the first time that control animals infected with the A-663 strain developed BHV-5 neurological disease in this kind of cross-protection experiments.

All unvaccinated control calves showed viral excretion confirming the viability of the virus used. The period of excretion and titres detected in nasal swabs were comparable to other reports of experimental infections with BHV-5 (Belknap et al., 1994; Cascio et al., 1999; Meyer et al., 2001; Perez et al., 2002). Our study confirmed the fact, previously described by others (Ashbaugh et al., 1997; Belknap et al., 1994), that different strains of BHV-5 replicate in the respi-

ratory tract at rates 10–100-fold lower than BHV-1. In agreement with several reports on BHV-5 neurological disease (Belknap et al., 1994; Meyer et al., 2001; Perez et al., 2002), the present study showed that control calves had ocular and seromucous nasal discharge prior to neurological signs. The first signs of neurological disease were observed 11–13 days after infection and all the animals were recovered by day 24 pc. These results are similar to other assays that reported meningoencephalitis in young cattle at 10–12 days after virus inoculation (Bagust and Clark, 1972; d'Offay et al., 1993; Flores et al., 1998). All together, these results confirm the neuropathogenicity of the BHV-5 A-663 strain.

In BHV challenge experiments with calves, protection is commonly evaluated in terms of virus shedding levels, excretion periods after infection with the virulent strain and severity of clinical disease compared with unvaccinated animals. Since only one BHV-1 vaccinated animal developed slight neurological symptoms and showed intermittent virus excretion, it seems that vaccination with BHV-1 induces protection against virus shedding and neurological signs associated with BHV-5 infections.

Cross-protection could explain the rare occurrence of BHV-5 outbreaks especially in regions where infections with BHV-1 and BHV-5 coexist and cattle are vaccinated against BHV-1. Several in vitro experiments showed that BHV-1 and BHV-5 share common epitopes (D'Arce et al., 2002; Metzler et al., 1986; Suarez Heinlein et al., 1993) that might explain these observations. Nevertheless, it is important to identify viral factors implicated in BHV-5 induced neuropathogenesis since there is no complete in vivo protection between these viruses. In this context, Chowdhury et al. (2000, 2002) had identified a number of glycoproteins implicated in the differential tropism of BHV-1 and BHV-5 in a rabbit model. Besides, a glycine-rich gE-specific epitope was suggested to be required for expression of the full virulence potential of BHV-5 (Al-Muharraq et al., 2004). Further experiments are necessary to investigate the causes of the differential pathogenesis of BHV-5 and BHV-1 in the natural host.

To sum up, our results suggest that the immune mechanisms involved in cattle protection against BHV-1 infection could also be implicated in BHV-5 protection. Additionally, we performed an experimental BHV-5 trial that induced evident disease, fact that allowed us to conclude that cattle immunized with inactivated vaccines based on BHV-1 or BHV-5 were protected against virus replication and neurological disease associated with BHV-5 infection.

Acknowledgements

The authors are grateful to Marta Radolci for technical assistance, Laura Marangunich for her help in the statistical analysis, Francisco Cristo and Ricardo Arias for their dedication and care of the calves used in the experiments and Anselmo Odeón and Ana Bratanich for the careful reading of the manuscript and helpful comments. This work was sponsored by Grant BID No. 802 OC/AR PMT-PIT No. 53 from SECYT-CONICET Argentina.

References

Abdelmagid, O.Y., Minocha, H.C., Collins, J.K., Chowdhury, S.I., 1995. Fine mapping of bovine herpesvirus type 1 (BHV-1) glycoprotein D (gD) neutralizing epitopes by type-specific monoclonal antibodies and sequence comparison with BHV-5 gD. Virology 206, 242–253.

Al-Muharraq, A., Zhou, Y., Chowdhury, S.I., 2004. A glycine-rich bovine herpesvirus 5 (BHV-5) gE-specific epitope within the ectodomain is important for BHV-5 neurovirulence. J. Virol. 78 (9), 4806–4816.

Ashbaugh, S.E., Thompson, K.E., Belknap, E.B., Schultheiss, P.C., Chowdhury, S., Collins, J.K., 1997. Specific detection of shedding and latency of bovine herpesvirus 1 and 5 using a nested polymerase chain reaction. J. Vet. Diagn. Invest. 9, 387–394.

Babiuk, L.A., van Drunen Little-van den Hurk, S., Tikoo, S.K., 1996. Immunology of bovine herpesvirus 1 infection. Vet. Microbiol. 53, 31–42.

Bagust, T.J., Clark, L., 1972. Pathogenesis of meningo-encephalitis produced in calves by infectious bovine rhinotracheitis herpesvirus. J. Comp. Pathol. 82, 375–383.

Barenfus, M., Delli Quadri, C.A., McIntyre, R.W., Schoeder, R.J., 1963. Isolation of infectious bovine rhinotraqueitis virus from calves with meningoencephalitis. J. Am. Vet. Med. Assoc. 143, 725–728.

Bartha, A., Hajdu, G., Aldasy, P., Paczolay, G., 1969. Occurrence of encephalomyelitis caused by infectious bovine rinotracheitis virus in calves in Hungary. Acta Vet. Acad. Scient. Hung 19, 145–151.

Belknap, E.B., Collins, J.K., Ayers, V.K., Schulteiss, P.C., 1994. Experimental infection of newborn calves with neurovirulent bovine herpesvirus type 1.3. Vet. Pathol. 31, 358–365.

Brake, F., Studert, M.J., 1985. Molecular epidemiology and pathogenesis of ruminant herpesviruses including bovine, buffalo and caprine herpesvirus 1 and bovine encephalitis herpesvirus. Aust. Vet. J. 62, 331–334.

Bratanich, A.C., Sardi, S.I., Smitsaart, E.N., Schudel, A.A., 1991. Comparative studies of BHV-1 variants by in vivo and in vitro tests. J. Vet. Med. B 38, 41–48.

Carrillo, B.J., Ambrogí, A., Schudel, A.A., Vazquez, M., Dahme, E., Pospischil, A., 1983. Meningoencefalitis caused by IBR virus in calves in Argentina. J. Vet. Med. B 30, 327–332.

Cascio, K.E., Belknap, E.B., Schultheiss, P.C., Ames, A.D., Collins, J.K., 1999. Encephalitis induced by bovine herpesvirus 5 and protection by prior vaccination or infection with bovine herpesvirus 1. J. Vet. Diagn. Invest. 11, 134–139.

Chowdhury, S.I., 1995. Molecular basis of antigenic variation between the glycoprotein C of respiratory bovine herpesvirus type 1 (BHV-1) and neurovirulent BHV-5. Virology 213, 558–568.

Chowdhury, S.I., Lee, B.J., Weiss, M.L., 2000. Bovine herpesvirus type 5 glycoprotein E is important for neuroinvasiveness and neurovirulence in the olfactory pathway of the rabbit. J. Virol., 2094–2106.

Chowdhury, S.I., Onderci, M., Bhattacharjee, P.S., Al-Mubarak, A., Weiss, M.L., Zhou, Y., 2002. Bovine herpesvirus type 5 (BHV-5) Us9 is essential for BHV-5 neuropathogenesis. J. Virol., 3839–3851.

Collins, J.K., Ayers, V.K., Whetstone, C.A., van Drunen Little-van Der Hurk, S., 1993. Antigenic differences between the major glycoproteins of bovine herpesvirus type 1.1 and bovine encephalitis herpesvirus type 1.3. J. Gen. Virol. 74, 1509–1517.

d'Offay, J.M., Mock, R.E., Robert, W., Fulton, D.V.M., 1993. Isolation and characterization of encephalitic bovine herpesvirus type 1 isolates from cattle in North America. Am. J. Vet. Res. 54 (4), 534–539.

d'Offay, J.M., Ely, R.W., Baldwin, C.A., Whitenack, D.L., Stair, E.L., Collins, J.K., 1995. Diagnosis of encephalitic bovine herpesvirus type 5 (BHV-5) infection in cattle: virus isolation and immunohistochemical detection of antigen in formalin-fixed bovine brain tissues. J. Vet. Diagn. Invest. 7, 247–251.

D'Arce, R.C.F., Almeida, R.S., Silva, T.C., Franco, A.C., Spilki, F., Roehe, P.M., 2002. Restriction endonuclease and monoclonal antibody analysis of Brazilian isolates of bovine herpesvirus types 1 and 5. Vet. Microbiol. 88, 315–324.

Engelhardt, T., Günther, M.K., 1996. Identification and characterization of the bovine herpesvirus type 5 Us4 gene and gene products. Virology 225, 126–135.

Engels, M.C., Giuliani, C., Wild, P., Beck, P., Loepf, T.M., Wyler, R., 1986. The genome of bovine herpesvirus 1 (BHV-1) strains exhibiting a neuropathogenic potential compared to known BHV-1 strains by restriction site mapping and cross-hybridization. Virus Res. 6, 57–73.

Eugster, A.K., Angulo, A.A., Jones, L.P., 1974. Herpesvirus encephalitis in range calves. Proc. Annu. Meet. Am. Assoc. Vet. Lab. Diagn 17, 267–281.

Flores E, Silva A, Weinblen R. 1998. Neuropatogenicidade do herpesvirus bovino tipo 5 (HVB-5) (avalicao de ovinos e cohelos como modelos experimentais). In: Proceedings of the Simposio Internacional sobre Herpesvirus bovino (tipo 1 e 5) e virus da Diarréia Viral Bovina (BVDV), Santa María, Brasil, pp. 127–137.

Friedli, K., Metzler, A.E., 1987. Reactivity of monoclonal antibodies to proteins of a neurotropic bovine herpesvirus type 1 (BHV-1) strain and to proteins of representative BHV-1 strains. Arch. Virol. 94, 109–122.

Gao, Y., Leary, T.P., Eskra, L., Splitter, G.A., 1994. Truncated bovine herpersvirus-1 glycoprotein I (gpI) initiates a protective local immune response in its natural host. Vaccine 12 (2), 145–152.

Gao, Y., Daley, M.J., Splitter, G.A., 1995. BHV-1 glycoprotein 1 and recombinant interleukin 1β efficiently elicit mucosal IgA response. Vaccine 13 (9), 871–877.

Gardiner, M.R., Nairn, M.E., 1964. Viral meningoencephalitis in calves in Western Australia. Aust. Vet. J. 40, 225–228.

Gerber, J.D., Marron, A.E., Kucera, C.J., 1978. Local and systemic cellular and antibody immune responses of cattle to infectious bovine rhinotracheitis virus vaccines administered intranasally or intramuscularly. Am. J. Vet. Res. 39 (5), 753–760.

Gough, A., James, D., 1975. Isolation of IBR virus from a heifer with meningoencefalitis. Can. Vet. J. 16, 313–314.

Haan, L., Verweij, W.R., Holtrop, M., Brands, R., van Scharrenburg, G.J., Palache, A.M., et al., 2001. Nasal or intramuscular immunization of mice with influenza subunit antigen and the B subunit of *Escherichia coli* heat-labile toxin induces IgA- or IgG-mediated protective mucosal immunity. Vaccine 19 (20–22), 2098–2907.

Israel, B.A., Herber, R., Gao, Y., Letchworth, G., 1992. Induction of a mucosal barrier to bovine herpesvirus-1 replication in cattle. Virology 188, 256–264.

Johnston, L.A.Y., Simmons, G.C., McGavin, M.D., 1962. A viral meningoencephalitis in calves. Aust. Vet. J. 38, 207–215.

Madic, J., Magdalena, J., Quak, J., van-Oirschot, J.T., 1995a. Isotype-specific antibody response in sera and mucosal secretions of calves experimentally infected with bovine herpesvirus 1. Vet. Immunol. Immunopathol. 46 (3–4), 267–283.

Madic, J., Magdalena, J., Quak, J., van-Oirschot, J.T., 1995b. Isotype-specific antibody responses to bovine herpesvirus 1 in sera and mucosal secretions of calves after experimental reinfection and after reactivation. Vet. Immunol. Immunopathol. 47 (1–2), 81–82.

Mars, M.H., de Jong, M.C.M., van Oirschot, J.T., 2000. A gE-negative bovine herpesvirus 1 vaccine strain is not re-excreted nor transmitted in an experimental cattle population after corticosteroid treatments. Vaccine 1 (18), 1975–1981.

Metzler, A.E., Schudel, A.A., Engels, M., 1986. Bovine herpesvirus type 1: molecular and antigenic characteristics of variant viruses isolated from calves with neurological disease. Arch. Virol. 87, 205–217.

Meyer, G., Thiry, E., 1999. Identification and characterization of bovine herpesvirus type 5 glycoprotein H gene and gene products. J. Gen. Virol. 80 (11), 2849–2859.

Meyer, G., Lemaire, M., Ros, C., Belak, K., Gabriel, A., Cassart, D., et al., 2001. Comparative pathogenesis of acute and latent infections of calves with bovine herpesvirus types. Arch. Virol. 146 (4), 633–652.

Moretti, B., Orfei, Z., Mondino, G., Persechino, A., 1964. Infectious bovine rinotracheitis, clinical observations and isolation of virus. Vet. Ital. 15, 676.

Nedrud, J.G., Liang, X., Hague, N., Lamm, M.E., 1987. Combined oral/nasal immunization protects mice from Sendai virus infection. J. Immunol. 139, 3484–3492.

OIE. 2004. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals. Part I. On-line edition, Section I.1, Chapter I.1.3, Stage 3, Item 2.

Perez, S.E., Bretschneider, G., Leunda, M.R., Osorio, F.A., Flores, E.F., Odeón, A.C., 2002. Primary infection, latency, and reactivation of bovine herpesvirus Type 5 in the bovine nervous system. Vet. Pathol. 39, 437–444.

Reed, C.J., Muench, H.A., 1938. A simple method of estimating fifty per cent end points. Am. J. Hyg. 27, 493.

Reed, D.E., Bicknell, E.J., Bury, R.J., 1973. Systemic form of infectious bovine rhinotracheitis in young calves. J. Am. Vet. Med. Ass. 163, 753–755.

Renegar, K.B., Small Jr., P.A., 1991a. Immunoglobulin A mediation of murine nasal anti-influenza virus immunity. J. Immunol. 65, 2146–2148.

Renegar, K.B., Small Jr., P.A., 1991b. Passive transfer of local immunity to influenza virus infection by IgA antibody. J. Immunol. 146, 1972–1978.

Riet-Correa, F., Vidor, T., Schild, A.L., Mendez, M.C., 1989. Meningoencefalite e necrose do córtex cerebral em bovinos causadas por herpes virus bovino-1. Pesq. Bras. 9, 13–16.

Romera, S.A., Hilgers, L.A., Puntel, M., Zamorano, P.Z., et al., 2000. Adjuvant effects of sulfolipo-cyclodextrin in a squalane-in-water and water-in-mineral oil emulsions for BHV-1 vaccines in cattle. Vaccine 19, 132–141.

Ros, C., Belák, S., 1999. Studies if genetic relationships between bovine, caprine, corvine and rangiferine alphaherpesviruses and improved molecular methods for virus detection and identification. J. Clin. Microbiol. 37 (3), 1247–1253.

Rouse, B.T., Babiuk, L.A., 1974. Host defense mechanism against infectious bovine rhinotracheitis virus: in vitro stimulation of sensitized lymphocytes by virus antigen. Infect. Immunity 10, 681–687.

Salvador, S.C., Lemos, R.A.A., Riet-Correa, F., Roehe, P.M., Osorio, A.L.A.R., 1998. Meningoencefalite em bovinos causadapor herpesvirus bovino 5 no Mato Groso do Sul e Sao Paulo. Pesq. Vet. Bras. 18, 76–83.

STAT User's guide 1988. SAS Institute Inc., SAS/STAT User's guide, Release 6.04. Cary, NC 27512, USA: SAS Institute.

Schudel, A.A., Carrillo, B.J., Wyler, R., Metzler, A.E., 1986. Infections of claves with antigenic variants of bovine herpesvirus type 1 (BHV-1) and neurological disease. J. Vet. Med. B. 33, 303–310.

Smitsaart, E.N., Bratanich, A.C., Ruiz, M., Sardi, M., Blanco Viera, F.J., León, E., González, O., Pugliese, M., Schudel, A.A., 1989. Comportamiento inmunogénico de vacunas inactivadas polivalentes para Herpesvirus Bovino 1. Rev. Med. Vet. (Bs As) 68 (3), 148–154.

Suarez Heinlein, A., Metzler, A.E., Weiblen, R., Berrios, P., Schudel, A.A., Rodriguez, M., 1993. Molecular characterization of South American bovine herpesvirus isolates with monoclonal antibodies and SDS–PAGE. J. Vet. Med. B 40, 125–130.

Vogel, F.S., Caron, L., Flores, E.F., Weinblen, R., Winkelmann, E.R., Mayer, S.V., et al., 2003. Distribution of bovine herpesvirus type 5 DNA in the central nervous systems of latently, experimentally infected calves. J. Clin. Microbiol. 41 (10), 4512–4520.

Wyler, R., Engels, M., Schwyzer, M., 1989. Infectious bovine rhinotracheitis/vulvovaginitis BHV-1. In: Wyttman, G., Becker, I. (Eds.), Herpesvirus Diseases of Cattle, Horses and Pigs. Developments in Veterinary Virology. Kluwer Academic Publishers, Boston, pp. 1–72.

Zhu, X., Letchworth, G.J., 1996. Mucosal and systemic immunity to bovine herpesvirus-1 glycoprotein D confer resistance to viral replication and latency in cattle. Vaccine 14 (1), 61–69.