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Serological responses in sheep injected with plasmids encoding bovine herpesvirus 1 (BHV-1) gD glycoprotein

Resposta sorológica de ovinos inoculados com plasmídeos codificando a glicoproteína D do herpesvírus bovino 1 (BHV-1)

A.L. Cândido[I]; M. Resende[II,*]; L.R.G. Bessa[I]; R.C. Leite[III]

[I]Pós-graduando em Microbiologia – ICB da UFMG
[II]Departamento de Microbiologia do Instituto de Ciências Biológicas Universidade Federal de Minas Gerais Caixa Postal 485 30161-970 – Belo Horizonte, MG
[III]Escola de Veterinária da UFMG

ABSTRACT

A genetic vaccine consisting of the bovine herpesvirus-1.2a (BHV-1.2a) glycoprotein D (gD) gene under the control of the cytomegalovirus immediate-early promoter/enhancer was generated and administered to sheep intramuscularly in the neck. All animals developed serum antibodies which recognized the homologous antigen (BHV-1.2a strain BH-83) and also exhibited cross-reactivity against the heterologous antigen (BHV-5 strain EVI-190). Three intramuscularly injections were given but serological responses were not improved after the second inoculation. Specific antibodies were detected against BHV-1.2a until at least 12 months after the first inoculation. However, the capacity to induce antibodies against BHV-5 was lower and of shorter duration than to BHV-1.2a.

Keywords: bovine herpesvirus-1.2a, glycoprotein D gene, DNA vaccine, ELISA

RESUMO

Uma vacina geneticamente preparada com o gene da glicoproteína D do herpesvirus bovino-1.2a (BHV-1.2a) controlado com o promotor/potenciador imediatamente precoce de citomegalovírus foi administrada no pescoço de ovinos. Todos os animais produziram anticorpos séricos que reconheceram, em ELISA, o antígeno homólogo (BHV-1.2a, amostra BH-83) e também mostraram reatividade cruzada contra um antígeno heterólogo (BHV-5 amostra EVI-190). Foram aplicadas três inoculações, mas não houve melhora de resposta com a terceira inoculação. Resposta específica contra BHV-1.2a foi detectada pelo menos até 12 meses após a primeira

inoculação, entretanto, a resposta sorológica contra o BHV-5 foi de menor intensidade e duração do que aquela contra o BHV-1.2a.

Palavras-chave: herpesvírus bovino-1.2a, gene da glicoproteína D, vacina de DNA, ELISA

INTRODUCTION

Bovine herpesvirus 1 (BHV-1) is an important pathogen of cattle and signs and clinical manifestation are dependent on virus strain-specific virulence. To reduce economical losses, vaccination with attenuated live or inactivated vaccines is widely performed. However, live attenuated vaccines may cause immunesuppressions or abortions if not attenuated sufficiently. Killed vaccines are often unable to generate protective levels of immunity for reasons of antigen load or loss of important epitopes during inactivation (Gibbs, Rweyemamu, 1977; Monaco, 1992; den Hurk et al., 1993b). Therefore, it is clear the need of better vaccines and in the last decade naked DNA encoding immunogenic proteins has been introduced for vaccination (Wolff et al., 1990). So far, it has been demonstrated that the BHV-1 glycoproteins gB, gC and gD induce protective immunity in a cattle model, therefore, one can believe that anyone of these glycoproteins is able to induce immune response when delivered and expressed from plasmid DNA (den Hurk et al., 1990). gD is the prime DNA vaccine candidate, indeed, it has been shown that gD subunit vaccine is able to induce the greatest levels of neutralizing antibody and abrogate virus shedding (Babiuk et al., 1987; den Hurk et al., 1993a). The efficacy of DNA vaccination has mainly been tested in experimental model system and in cattle has been shown to decrease respiratory symptoms induced by BHV-1 and also it has been shown to elicit immune responses (Cox et al., 1993; den Hurk et al., 1998). Inoculation of plasmid DNA expression vectors has been protected chickens against avian influenza (Fynan et al., 1993; Robinson et al., 1993) and Newcastle disease (Sakaguchi et al., 1996).

The isolation of BHV-1 from sheep (Lehmkuhl, Cutlip; 1985; Shankar, Yadav, 1987; Clark et al., 1993) and the detection anti BHV-1 antibodies in sheep has been demonstrated (Hasler, Engels, 1986; Goyal et al., 1988; Jetteur et al., 1990; Mayga, Sarr, 1992; Suresh, Suribabu, 1993). Therefore, the sheep is a valid model for experimental research with BHV-1 and this model would be of great benefit for assessing its applicability to BHV-1 vaccination. This paper describes two peroxidase labeled ELISAs using BHV-1.2a and BHV-5 antigens and compares them for its efficacy in detecting serum antibodies in sheep injected with BHV-1.2a genes expressed from cytomegalovirus promoter in the plasmid pCI DNA (Promega Corporation, Madison, WI, USA).

MATERIAL AND METHODS

The BH-83 strain of BHV1-2a and EVI-190 strain of BHV-5 were produced and titrated in Madin-Darby bovine kidney cells grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS). The viruses were clarified from the culture medium by centrifugation at 1,000xg for 15min, and pelleted at 40,000xg for 90min. The pellets were ressuspended in TEN buffer (10mM Tris-Cl, 1mM EDTA, 100mM NaCl, pH 8.0) and centrifuged at 95,000xg through 8.5% to 25% ficoll 400 step gradient for 60min at 4ºC. The virus band at the 8.5-25% ficoll interface was removed, ressuspended in TEN buffer, mixed well and pelleted at 90,000xg for 60min. BH-83 DNA was prepared from the pelleted virus by using sodium dodecyl sulphate and proteinase K lyses, phenol-chloroform extraction and ethanol precipitation.

The 1.3 Kb Mae I fragment (Roche Diagnostics GmbH, Mannheim, Germany) containing the gD coding sequences was filled in with the Klenow fragment of DNA polymerase and inserted into the *Sma I* site of plasmid pCI mammalian expression vector[1]. The resulting plasmid with the insert oriented such that gD was in frame and downstream of the CMV I E promoter was designed pCIgD. In frame cloning and fragment orientation were verified by asymmetric restriction site mapping and DNA sequencing of plasmid-insert junctions. Recombinant and control plasmid were introduced into *E. coli* BL21 and purified by large-scale alkaline lyses method (Birnboim, 1983).

Two groups (A and B) of six lambs of either sex aged four to six months were used and injected intramuscularly in the neck. All animals were reinjected 8 and 16 weeks later. Vaccine formulations were prepared in a volume of 1.0ml/animal with phosphate-buffered saline (PBS: 0.1M NaH_2PO_4/Na_2HPO_4, 0.15M NaCl, pH 7.8) as diluent.

Group A was given doses of 500mg of pCIgD plasmid and group B was given also doses of 500mg of pCI plasmid with no insert. Blood samples were collected at 0 day and days 30, 60, 90, 120, 180 and 360 after the first inoculation.

Serum antibodies for BHV-1.2a (BH-83) and BHV-5 (EVI-190) were determined by ELISA. The antigen for ELISA was obtained ressuspending the virus pellet (BH-83 or EVI-190) in PBS containing 2µM phenylmethylsulphonyl fluoride and sonicated on ice at 20KHz for three cycles of 10sec. This sonicated antigen was centrifuged at 500xg for 10min and the supernatant stored in liquid nitrogen until used as antigen. Control antigen was prepared following the same procedure, using uninfected MBDK cells. Checkerboard titration showed that the antigen

concentration of 15.5µg/ml was the lowest concentration, which demonstrated a positive reaction anti BHV-1 sheep sera. The optimum serum dilution (1:50) was determined based on the lowest dilution of the positive sera tested with negative control antigen, which showed OD values less or equal those presented by negative sera. Polystyrene microtiter plates (Nunc-Immuno Plates PolySorp, VWR Scientific, San Diego, CA, USA) were passively adsorbed with 50µl of ELISA antigen per well diluted in 0.1M carbonate-bicarbonate buffer and incubated overnight at 4ºC. Afterwards, plates were blocked with PBS 0.05% - Tween 20 - 0.5% Casein. Dilutions of sheep sera were prepared in PBS-Tween-Casein and dispensed in 50µl/well. Plates were washed six times with PBS0.05% - Tween 20 and incubated with 1:8000 dilution of mouse anti-sheep IgG peroxidase conjugate for 1 hour at 37ºC. After extensive washing, o-phenylene-diamine dihydrochloride (OPD) and H_2O_2 were added as substrate and absorbance was measured at 492 nm. The ELISA data was analyzed with the aid of a statistical software (Systat 7.0, SPSS Inc., Chicago, IL) and the data was transformed prior to performing the analysis by log transformation and considered for statistical significance the level of 5%.

RESULTS

Transformation of *E. coli* BL21 with recombinant plasmid resulted in generation of pCIgD DNA vaccine. To test the potency of this vaccine one group of six sheep were injected three times with 500 mg of pCIgD and another group of six animals, used as control, also were injected three times with 500 mg of parental pCI vector without the inserted gene. A comparison between the OD means from animals injected with pCIgD and negative controls injected with pCI was used to determine ELISA cut-off that was significantly different ($P< 0.05$). All sera of both groups were negative at the time of first injection. Analysis of sera by ELISA BHV-1.2a and ELISA BHV-5 showed that injection with pCIgD was able to induce specific response and that the response against BHV-1.2a was significantly higher than BHV-5 ($P< 0.05$). The antibody response decreased somewhat over time, but when the animals received the second injection still had higher response ($P< 0.05$) than the control group. As illustrated in Fig.1, the second injection (day 60) leaded to a strong response and the level of antibodies against BHV-5 was lower. One hundred and twenty days after the first injection the animals were inoculated again and at day 150 another decrease in serum antibodies was observed. At that time the antibody levels were also significantly higher in the pCIgD-vaccinated group that compared with the control group. By days 180 and 210, the serum antibody levels decreased and remained in decline ($P< 0.05$) from day 210 to 360. At day 360 all six animals were still positive in ELISA BHV-1.2a but the level of antibodies was not detected in ELISA BHV-5. None of the six sheep injected with parental pCI vector exhibited any ELISA detected antibody to BHV-1.2a/BHV-5 throughout the duration of experiment.

DISCUSSION

It was demonstrated the ability of an injected plasmid, containing the gD gene from BH-83 strain, a BHV-1.2a isolate, to cause specific seroconversion in sheep. By using two ELISAs system it was shown that sera reacted with these two related antigens but response to BH-83, homologous virus strain, was consistently higher than those to EVI-190, a BHV-5 strain. The examination of DNA sequences of both viruses indicated that the two open reading frames of gD extends 1254 base pairs, potentially encoding a protein with a molecular mass of about 417 kilodaltons, including the signal sequence. Therefore, the derived protein sequence of BH-83 is similar to that of open reading frames found in the analogous head-to-head orientation with respect to gD of EVI-190. Comparison of gD sequences of these two isolates revealed 83% homology. Alignment of deduced sequences of polypeptides of BH-83 and EVI-190 exhibited characteristics typical for glycoproteins and exhibited 63% identity. It can be seen in the alignment the two sequences that the greatest homology is located in domains with structural importance such as anchor sequences, N-glycosilation sites and putative signal. Probably because these differences found the antibody levels detected against BH-83 were consistently higher than those to EVI-190.

The presence of ELISA detected antibodies in the pCIgD injected sheep suggests that the expressed protein retained their native antigenic properties. The antigen used in all testes (BHV-1.2a and BHV-5 ELISAs) was obtained from MDBK virus produced rather then from recombinant gD-infected cells lysates and it proved the suitability of such antigen for detection of BHV-1/BHV-5 gD specific antibodies. In this report, it was observed a significant increase in average absorbance values after the second injection booster ($P< 0.05$). A slight decrease in the serological response was observed after the second booster showing that a good serological response can be obtained after just a single or double injection. Then it was demonstrated that IM injection of plasmid encoding gD induces a good antibody production in sheep but the magnitude of the response was not improved by multiples injection. Others have found that animals, which received high dose of recombinant plasmid produced high ELISA titers (Cox et al., 1993). Because of these studies, the same dose of plasmid was delivered per animal. In the present trial, it was selected the neck of the animal because of accessibility and also because in that region one can found the same draining lymphonodes that respond to BHV-1 in case of natural infection. In other studies, partial protection could be achieved with as little as 1-10µg of plasmid DNA per dose, and that increasing the dose to 50m g per injection resulted in seroconversion of 100% of the pigs (Gerds et al., 1997). Chickens were injected with 100µg of NDV F protein (Sakaguchi et al., 1996) or 100-200µg of influenza HA construct (Fynan et al., 1993; Robinson et al., 1993), and mice 50µg of pp89 plasmid (Gonzalez et al., 1996) or 100-200mg of LCMV

nucleoprotein expression vector (Pedroza-Martins et al., 1995; Yokoyama et al., 1995) have been protected from a lethal challenge.

From the results obtained in the present work it was clear that there are some cross reactivity between the serological response against gD of BHV-1.2a and BHV-5. The ELISA reactivity against BHV-5 was always at levels below those attained for BHV-1.2a. Nowadays we are constructing plasmids for gB, gC, gD and gG of both viruses and by mixing these different plasmids it is possible to extend the cross reactivity of the immune response. DNA immunization is though to induce both humoral and cellular immunity, because this kind of immunization is able to provide access of endogenously produced antigens to the MHC I and II-restricted pathways (Raz et al., 1994; Justewicz et al., 1995; Ciernik et al., 1996). In murine models the antibody isotype induced by DNA immunization is predominantly IgG2a with a 10 to 100-fold excess over IgG1 (Coutelier et al., 1987). Compared to IgG1, IgG2a has the advantage that it can activate both the classical and the alternative pathways of complement fixation (Klaus et al., 1979). IgG2a is probably produced after intramuscular immunization, probably because the muscle cells are captured by antigen presenting cells, and how the antigen presentation changed, it is sufficient to alter the type of immune response. Some studies have reported natural occurrence of antibodies against BHV-1 and BHV-5 in sheep (Lindner et al., 1993; Hage et al., 1997) and, in view of that it is probably that sheep is also a natural host for both viruses, and is a valid model for the proposed study. The present work shows that the DNA vaccination has enormous potential and continuing research is needed to well characterize the cell-mediated immune response induced in sheep as well to optimize dose, formulations and delivery of DNA vaccine with a view to enhancing antigen-specific immune response.

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* Autor para correspondência: E-mail: mresende@mono.icb.ufmg.br

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Escola de Veterinária UFMG

Caixa Postal 567
30123-970 Belo Horizonte MG - Brazil
Tel.: (55 31) 3409-2041
Tel.: (55 31) 3409-2042

e-Mail

abmvz.artigo@abmvz.org.br