RESEARCH COMMUNICATION

Immune responses in a horse inoculated with the VP2 gene of African horsesickness virus

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ABSTRACT


The ability of a DNA vaccine to elicit an immune response in a horse was evaluated. The outer capsid protein VP2 of African horsesickness virus is known to elicit protective immunity in horses. Reverse transcribed DNA of the gene encoding VP2 was placed under the transcriptional control of the cytomegalovirus immediate-early enhancer/promoter and was injected on several occasions intramuscularly into a horse. Low antibody levels could be detected by ELISA. Antibodies directed against VP2 alone were shown by Western blot while low levels of neutralizing antibodies were detected by a 50% plaque reduction assay. In contrast to a relatively poor humoral response, a significant lymphoproliferative response in the presence of whole virus proteins, as well as a cytotoxic cellular reaction against virus-infected syngeneic target cells was shown.

Keywords: African horsesickness virus, DNA vaccines, VP2 gene

African horsesickness virus (AHSV), a double-stranded RNA virus (Orbivirus group, family Reoviridae), is responsible for serious disease in horses. The virus, of which nine serotypes have been described, is transmitted by Culicoides biting midges (Burrage & Laegreid 1994; Roy, Mertens & Casal 1994). Currently, horses in South Africa are immunized yearly with two polyvalent attenuated AHSV vaccine combinations, each containing four viral serotypes. It has been shown that VP2, one of the two proteins comprising the outer capsid layer of the virus, possesses important neutralization epitopes (Ranz, Miguet, Anaya, Venteo, Cortes, Vela & Sanz 1992; Burrage, Trevejo, Stone-Marschat & Laegreid 1993; Martinez-Torrecuadrada, Iwata, Venteo, Casal & Roy 1994; Martinez-Torrecuadrada & Casal 1995).

For this reason, recombinant VP2 has been tested as a potential subunit vaccine in horses (Roy, Bishop, Howard, Aitchison & Erasmus 1996; Martinez-Torrecuadrada, Diaz-Laviada, Roy, Sanchez, Vela, Sanchez-Vizcaino & Casal 1996; Stone-Marschat, Moss, Burrage, Barber, Roy & Laegreid 1996). DNA vaccines have been intensively investigated recently and their applications and advantages have been described in several reviews (Waine & McMahon 1995; Kalinna 1997; Ramsay, Ramshaw & Ada 1997; Beard & Mason 1998; Kucerova 1998; Tighe, Corr, Roman & Raz 1998). This approach has, however, not yet been applied to AHSV. As a step towards evaluating the efficacy of DNA immunization in horses, this communication describes some responses resulting from inoculating a single horse with a DNA vaccine consisting of a DNA copy of the double-stranded VP2 gene of AHSV. For this study, a recombinant mammalian cell expression plasmid (pCl-VP2) was constructed by inserting cDNA of the African horsesickness virus serotype 3 (AHSV 3) VP2 gene (Vreede & Huismans 1994) downstream of the CMV immediate-early enhancer/promoter in

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the pCI plasmid vector (Promega). The resulting construct was introduced into Escherichia coli DH5α by calcium chloride transformation (Sambrook, Fritsch & Maniatis 1989). Large scale plasmid purification was by anion exchange column chromatography (Qiagen). In order to establish whether the VP2 gene could be expressed in vitro in animal cells, purified plasmid was introduced into CER cells by electroporation as described (Liljestrom & Garoff 1991) and protein expression evaluated by F(ab')2 indirect sandwich ELISA (Du Plessis, Van Wyngaardt & Bremer 1990).

An unvaccinated adult mare obtained from an AHSV-virus-free CER cell lysate, and recombinant CER cells transfected with pCI-VP2 were used as antigen and virus-free CER cell lysate, respectively (Boehringer Mannheim) or without either antigen or mitogen. After incubation for 24 h, cells were washed and placed into the wells of a 96-well plate at 2 x 10⁴ cells/well. Virus-stimulated effector cells were loaded in triplicate onto target cells at several different effector to target ratios. After an incubation period of 8 h, release of ¹¹Cr into the medium following cell lysis in vitro was determined by β-scintillography. Percent specific lysis was then determined as described (Coligan et al. 1991; Andrew et al. 1995; Suradhat, Yoo, Babiku, Griebel & Baca-Estrada 1997).

In addition, EDF cells, transfected the previous day with plasmid DNA (pCI-VP2) using FuGENE™ 6 (Boehringer Mannheim) in serum-free medium according to the manufacturer's instructions, were similarly labelled. After an incubation period of 1 h, target cells were washed and placed into the wells of a 96-well plate at 2 x 10⁴ cells/well. Virus-stimulated effector cells were loaded in triplicate onto target cells at several different effector to target ratios. After an incubation period of 8 h, release of ¹¹Cr into the medium following cell lysis in vitro was determined by β-scintillography. Percent specific lysis was then determined as described (Coligan et al. 1991; Andrew et al. 1995; Suradhat, Yoo, Babiku, Griebel & Baca-Estrada 1997).

The recombinant plasmid was first tested for its ability to express VP2 in cell culture. In a sandwich ELISA, signals at least ten times higher than those produced by cell lysates which did not receive the plasmid or those that received plasmid without insert were obtained (Fig. 1a), confirming that the DNA copy of the VP2 gene was functional once introduced into a cell. The plasmid was then injected into the gluteal muscle of a horse. Serum tested in ELISA indicated that low levels of antibodies that recognized recombinant VP2 were present after four inoculations (Fig. 1b). Additional DNA vaccinations did not enhance this response (not shown). Neutralizing antibodies are primarily responsible for protection against AHSV in horses (Alexander 1935; Blackburn & Swanepoel 1986; Burrage et al. 1993). Plaque reduction assays using serum from the DNA vaccinated horse revealed, however, that titres of less than 1:10 had been attained, marginally more than at the onset of the trial when no neutralization was detectable. This is in accordance with findings showing that antibody responses are often not significant following IM vaccination with DNA (Cox, Zamb & Babiku 1993; Suradhat et al. 1997).

The low neutralization titres notwithstanding, a Western blot with serum obtained after four inoculations indicated that VP2-specific antibodies were indeed
FIG. 1a Absorbance readings in an anti-AHSV F(ab')_{2} sandwich ELISA used to detect VP2 expression. F(ab')_{2} coated wells were loaded with CER cell lysate (1), lysate of CER cells transfected with non-recombinant plasmid, pCI (2) and VP2-recombinant plasmid, pCI-VP2 (3). Hyper-immune anti-AHSV3 rabbit serum was used as detecting antibody.

FIG. 1b Absorbance readings in an anti-AHSV3 F(ab')_{2} sandwich ELISA used to detect horse antibody against VP2. Anti-AHSV3 F(ab')_{2} coated wells were loaded with VP2-expressing cell lysate, followed by horse sera (1:100) obtained before DNA immunization (1) and 7 d after four DNA immunizations (2).

FIG. 2a SDS-PAGE polyacrylamide gel showing protein profiles of Rainbow™ (Amersham) molecular mass markers (lane 1), blue-tongue virus VP2 and VP7 (lane 2), CER cell lysate (lane 3) and AHSV3 (lane 4).

FIG. 2b Immunoblot illustrating a VP2-specific band (lane 1) obtained from serum from the DNA vaccinated horse. Lanes were loaded with AHSV3 (lane 1), CER cell lysate (lane 2) and Rainbow™ molecular mass markers (lane 3). The predicted Mr of VP2 is 123 078 (Vrede & Huismans 1994).

present (Fig. 2b). No signs of antibody binding to other viral proteins such as VP5 or VP7 were evident, suggesting that exposure to virus in the field during the course of the experiment had not occurred. The reason for the pronounced VP2-specific band on Western blot in the absence of significant levels of neutralizing antibodies could be an indication that neutralization epitopes were not presented to the horse's immune system in an acceptable form. For instance, they may have been incorrectly folded or alternatively, degraded.

In the light of a marginal humoral immune response, a lymphocyte proliferation assay was carried out to further confirm that a specific response had indeed been elicited. Incorporation of BrdU by PBMCs incubated with AHSV3 or recombinant VP2 (Fig. 3) indicated that cellular proliferation had occurred, thus confirming that the immune system of the horse had been exposed to VP2. It is conceivable that cell-mediated immunity plays a role in diminishing the severity of disease and facilitating recovery from AHSV infection, but no evidence has been available regarding its role in immunity (Burrage & Laegreid 1994). A ^{51}Cr cytotoxicity assay was therefore done as an indicator of effector T cell function. PBMC effector cells primed in vivo and further stimulated in vitro induced cytolysis of AHSV infected (or VP2-plasmid transfected) syngeneic target cells (Fig. 4). Infected VERO cells, on the other hand, did not show appreciable signs of cytolysis, suggesting that MHC class I restricted presentation was important and that the effector cells most likely consisted of specifically activated cytotoxic CD8+ T cells. In a related orbivirus, Andrew et al. (1995) demonstrated that VP2 of BTV is one of the major immunogens for CTLs in BTV-immunized sheep. In addition, it has also been shown that MHC class I restricted (CD8+) cytotoxic
T lymphocytes (CTLs) are important in recovery from infection with BTV (Jeggo, Wardley & Brownlie 1984; Ellis, Luedke, Davis, Wechsler, Mecham, Pratt & Elliott 1990) and that heterologous protection could be elicited by CTLs that cross-react with other BTV serotypes (Jeggo et al. 1984).

Despite an outbreak of AHSV among horses in the region during the rainy season that followed the demonstration of humoral and cellular immune responses, the horse vaccinated with DNA did not succumb, despite several deaths amongst neighbouring horses. It seems unlikely that any field challenge would have been exclusively from AHSV 3. Cross-reactive CTL responses may therefore have reduced the severity of infection and allowed for survival.

Several other DNA vaccines have in fact been shown to induce broad CTL-based protection against heterologous virus strains (Ulmer, Donnelly, Parker, Rhodes, Felgner, Dwarki, Gromkowski, Deck, De Witt, Friedman, Leander, Martinez, Perry, Shiver, Montgomery & Liu 1993; Ramsay, Leong & Ramshaw 1997; Seo, Wang, Smith & Collisson 1997). Moreover, in sheep infected with BTV, low levels of viraemia are not always associated with high neutralizing antibody titres, suggesting that mechanisms of protection other than neutralization are involved with orbiviruses (Stott, Barber & Osburn 1985).

With DNA vaccines, induction of an appropriate immune response in mice appears to be considerably easier than in most other species (Beard & Mason 1998; Van Drunen Littel-van den Hurk, Braun, Lewis, Karvonen, Baca-Estrada, Snider, Mccartney, Watts & Babiuk 1998). For instance, in cattle, IM injection of plasmid DNA encoding the bovine herpesvirus-1 gD glycoprotein elicited antibody responses and partially protective immunity against bovine herpesvirus (Cox et al. 1993) while neutralizing antibody titres remained low despite repeated inoculations.

Nonetheless, the immune response observed in a single horse following immunization with pCI-VP2 was characterized by AHSV VP2-specific antibodies, low levels of neutralizing antibodies as well as lymphoproliferation and cytotoxic T cell activity. The horse also survived an outbreak of the disease. DNA vaccination in combination with subunit vaccines (Rothel, Waterkeyn, Strugnell, Wood, Seow, Vadolas & Lightowlers 1997), recombinant poxvirus vectors (Hanke, Blanchard, Schneider, Hannan, Becker, Gilbert, Hill, Smith & McMichael 1998), or alternatively administered via a different route (Van Drunen Littel-van den Hurk et al. 1998; Van Rooij, Haagmans, De Visser, De Bruin, Boersma & Bianchi 1998) may, however, be necessary to enhance levels of neutralizing antibodies to acceptable levels before attempting an experimental challenge.

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