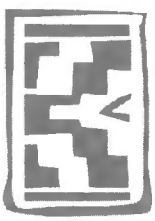

RESEARCH COMMUNICATION

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

M. ROMITO[1], D.H. DU PLESSIS[1] and G.J. VILJOEN[2]

ABSTRACT

ROMITO, M., DU PLESSIS, D.H. & VILJOEN, G.J. 1999. Immune responses in a horse inoculated with the VP2 gene of African horsesickness virus. *Onderstepoort Journal of Veterinary Research*, 66:139–144

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; Martínez-Torrecuadrada, Iwata, Venteo, Casal & Roy 1994; Martínez-Torrecuadrada & Casal 1995). For this reason, recombinant VP2 has been tested as a potential subunit vaccine in horses (Roy, Bishop, Howard, Aitchison & Erasmus 1996; Martínez-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 & McManus 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 (pCI-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

[1] Immunology Division, Onderstepoort Veterinary Institute (OVI), Private Bag X5, Onderstepoort, 0110 South Africa

[2] Applied Biotechnology Division, Onderstepoort Veterinary Institute, Private Bag X5, Onderstepoort, 0110 South Africa

Accepted for publication 20 April 1999—Editor

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 (Liljeström & 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-free area was injected with 500 mg of pCI-VP2 in a volume of 5 mℓ phosphate buffered saline (PBS) into the major gluteal muscle. This initial injection was followed by a series of intramuscular (IM) boosters at varying intervals. Blood was collected by jugular venipuncture approximately 7–10 d after each inoculation. The $F(ab')_2$ indirect sandwich ELISA was used to determine antibody levels; captured VP2 expressed by transfected CER cells was used as an antigen and the secondary antibody was detected using peroxidase-labelled protein G (Pierce). Neutralizing antibody was determined by means of a 50% plaque reduction neutralization assay (Huismans & Erasmus 1981) using VERO cells incubated with AHSV 3.

For use in immunoblotting, AHSV 3 was cultured in CER cells and purified as described for the related orbivirus, bluetongue virus (BTV) (Huismans, Van der Walt, Cloete & Erasmus 1987). Viral proteins were separated electrophoretically in 10% SDS polyacrylamide gels (Laemmli 1970) under reducing conditions. Separated proteins were transferred to a polyvinylidene fluoride (Immobilon™-P; Millipore) transfer membrane without methanol (Towbin, Staehelin & Gordon 1979; Harlow & Lane 1988). Serum used for the blot was obtained approximately 10 d after the fourth inoculation. Peroxidase-conjugated protein G and 4-chloro-1-napthol (Sigma) were used to detect bound antibodies.

Lymphocyte proliferation assays were performed using peripheral blood mononuclear cells (PBMCs) isolated by Histopaque®-1077 (Sigma) gradient centrifugation (Coligan, Kruisbeek, Margulies, Shevach & Strober 1991). PBMCs were incubated in the presence of AHSV inoculum (CER cell lysate) and recombinant baculovirus-expressed VP2 (kindly supplied by Melinda du Plessis, Onderstepoort Veterinary Institute). Controls included PBMCs incubated with virus-free CER cell lysate, 20 units/mℓ of the lymphoblastogenic cytokine human IL-2 (hIL-2; Boehringer-Mannheim) or without either antigen or mitogen. After 4 d incubation, cells were labelled with 5-bromodeoxyuridine (BrdU) as instructed by the kit manufacturer (Boehringer Mannheim BrdU colorimetric immunoassay). Two days later, cells were fixed and denatured by the supplied proprietary reagent. Peroxidase-labelled BrdU antibody and a substrate resulting in a colour reaction were added to determine levels of BrdU incorporation.

Cellular cytotoxicity assays and the establishment of syngeneic target cells from skin biopsies (equine dermal fibroblasts, EDF) were essentially as described by Andrew, Whitley, Janardhana, Lobato, Gould & Coupar (1995) with minor modifications. Peripheral blood mononuclear cells (effector cells) were infected with AHSV 3 at approximately two plaque forming units (pfu)/cell and incubated for 5 d. Target cells (both EDF and VERO) were infected with AHSV 3 at about 10 pfu/cell and labelled with chromium-51 (^{51}Cr; 10 mCi/mℓ; Amersham). Uninfected cells were labelled for use as controls.

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 2×10^4 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 ^{51}Cr 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, Babiuk, 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. Sera 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 1988; 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 & Babiuk 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

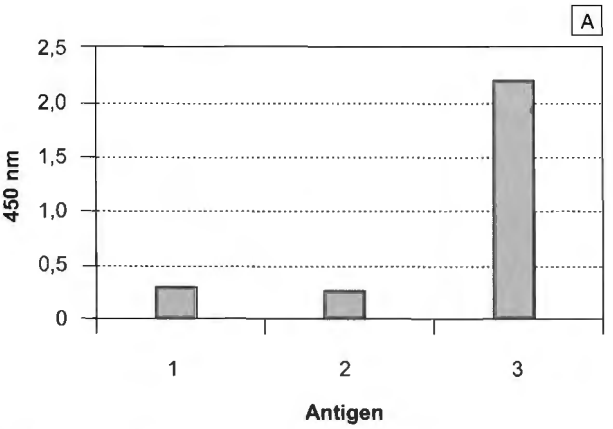

FIG. 1a Absorbance readings in an anti-AHSV $F(ab')_2$ sandwich ELISA used to detect VP2 expression. $F(ab')_2$ coated wells were loaded with CER cell lysate (1), lysate of CER cells transfected with non-recombinant plasmid, pCI (2) and VP2-recombinant plasmid, pCI-VP2 (3). Hyperimmune anti-AHSV3 rabbit serum was used as detecting antibody

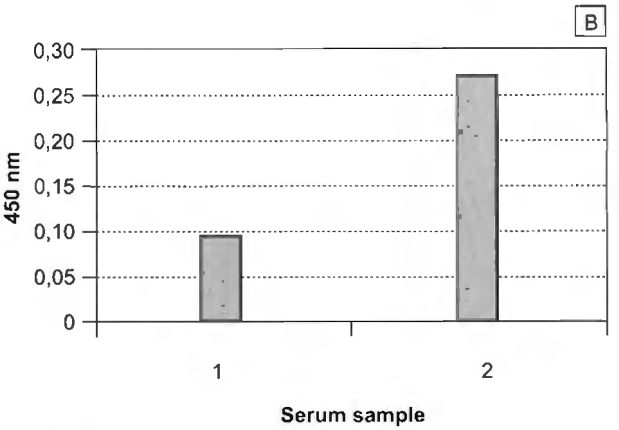

FIG. 1b Absorbance readings in an anti-AHSV3 $F(ab')_2$ sandwich ELISA used to detect horse antibody against VP2. Anti-AHSV3 $F(ab')_2$ coated wells were loaded with VP2-expressing cell lysate, followed by horse sera (1:100) obtained before DNA immunization (1) and 7 d after four DNA immunizations (2)

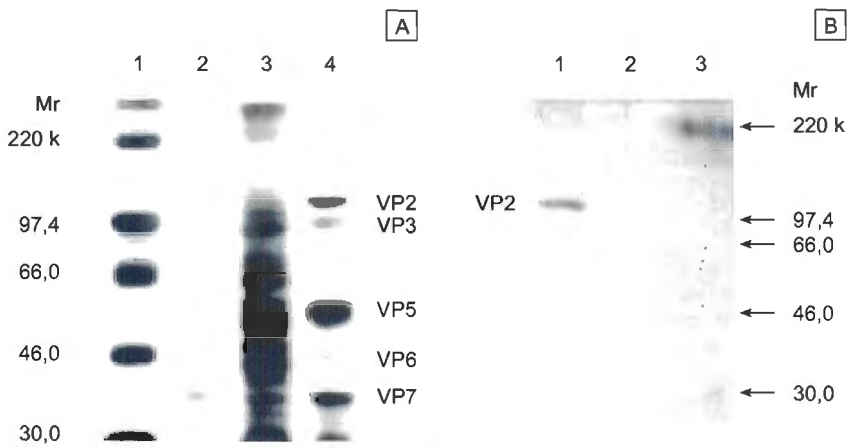

FIG. 2a SDS-PAGE polyacrylamide gel showing protein profiles of Rainbow™ (Amersham) molecular mass markers (lane 1), bluetongue virus VP2 and VP7 (lane 2), CER cell lysate (lane 3) and AHSV3 (lane 4)

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

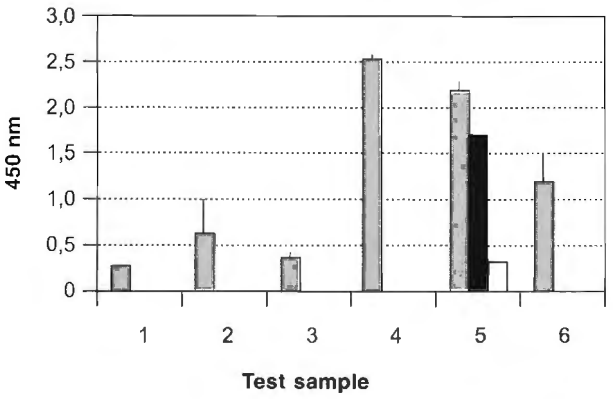

FIG. 3 ELISA absorbance readings resulting from a non-radioactive lymphocyte proliferation assay. Horse PBMCs were either incubated in the presence of virus (AHSV3) at 10, 1 or 0.1 pfu/cell (5), or with recombinant VP2 synthesized by recombinant baculovirus in Sf cells (6). Controls consisted of medium without cells (1), PBMCs alone (2), or PBMCs with CER cell lysate (3). PBMCs cultured in the presence of 20 units hIL-2 (4) were used as positive control

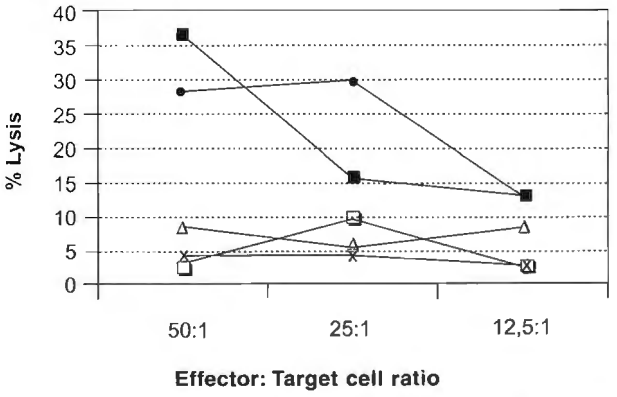

FIG. 4 Percentage target cell lysis in a cytotoxic T cell assay. Peripheral blood mononuclear cells stimulated with whole virus *in vitro*, were layered onto target cells at several effector to target ratios. Equine dermal fibroblasts were infected with AHSV (■) and labeled with ^{51}Cr. Fibroblasts transfected with plasmid encoding VP2 (●) were also used. Controls included fibroblasts not infected with virus (□) and VERO cells uninfected (x) or infected (Δ) with AHSV. ^{51}Cr released from lysed target cells into medium was monitored by scintillography

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, Donnely, 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.

ACKNOWLEDGEMENTS

We acknowledge the assistance given by Mrs A. van Wyk, Miss A-M. Bosman, Mr L. Taljaard (OVI), and Dr B.J. Erasmus and co-workers (Onderstepoort Biological Products) in supplying and stabling the horse. Dr Marion Andrew (CSIRO, Geelong) is thanked for invaluable advice in establishing the CTL assay.

REFERENCES

ALEXANDER, R.A. 1935. Studies on the neurotropic virus of horsesickness III. The intracerebral protection test and its application to the study of immunity. *Onderstepoort Journal of Veterinary Research,* 4:349–377.

ANDREW, M., WHITLEY, P., JANARDHANA, V., LOBATO, Z. GOULD, A. & COUPAR, B. 1995. Antigen specificity of the ovine cytotoxic T lymphocyte response to bluetongue virus. *Veterinary Immunology and Immunopathology,* 47:311–322.

BEARD, C.W. & MASON, P.W. 1998. Out on the farm with DNA vaccines. *Nature Biotechnology,* 16:1325–1328.

BLACKBURN, N.K. & SWANEPOEL, R. 1988. Observation on antibody levels associated with active and passive immunity to African horsesickness. *Tropical Animal Health and Production,* 20:203–210.

BURRAGE, T.G. & LAEGREID, W.W. 1994. African horsesickness: pathogenesis and immunity. *Comparative Immunology, Microbiology and Infectious Diseases,* 17:275–285.

BURRAGE, T.G., TREVEJO, R., STONE-MARSCHAT, M. & LAEGREID W.W. 1993. Neutralizing epitopes of African horsesickness virus serotype 4 are located on VP2. *Virology,* 196:799–803.

COLIGAN, J.E., KRUISBEEK, A.M., MARGULIES, D.H., SHEVACH, E.M. & STROBER, W. (Eds.) 1991. *Current protocols in immunology.* New York: John Wiley & Sons.

COX G.J., ZAMB T.J. & BABIUK L.A. 1993. Bovine herpesvirus I: immune responses in mice and cattle injected with plasmid DNA. *Journal of Virology,* 67:5664–5667.

DU PLESSIS, D.H., VAN WYNGAARDT, W. & BREMER, W.C. 1990. An indirect sandwich ELISA utilising $F(ab')_2$ fragments for the detection of African horsesickness virus. *Journal of Virological Methods,* 29:279–290.

ELLIS, J.A., LUEDKE, A.J., DAVIS, W.C., WECHSLER, S.J., MECHAM, J.O., PRATT, D.L. & ELLIOTT, J.D. 1990. T lymphocyte subset alterations following bluetongue virus infection in sheep and goats. *Veterinary Immunology and Immunopathology,* 24:49–67.

HANKE, T., BLANCHARD, T.J., SCHNEIDER, J., HANNAN, C.M., BECKER, M., GILBERT, S.C., HILL, A.V., SMITH, G.L. & MCMICHAEL, A. 1998. Enhancement of MHC class I-restricted peptide-specific T cell induction by a DNA prime/MVA boost vaccination regime. *Vaccine,* 16:439–445.

HARLOW, E. & LANE, D. 1988. *Antibodies. A laboratory manual.* New York: Cold Spring Harbor Laboratory Press.

HUISMANS, H. & ERASMUS, B.J. 1981. Identification of the serotype-specific and group-specific antigens of bluetongue virus. *Onderstepoort Journal of Veterinary Research,* 41:51–58.

HUISMANS, H., VAN DER WALT, N.T., CLOETE, M. & ERASMUS, B.J. 1987. Isolation of a capsid protein of bluetongue virus that induces a protective immune response in sheep. *Virology,* 157:172–179.

JEGGO, M.H., WARDLEY, R.C. & BROWNLIE, J. 1984. A study of the role of cell-mediated immunity in bluetongue virus infection in sheep, using cellular adoptive transfer techniques. *Immunology,* 52:403–410.

KALINNA, B.H. 1997. DNA vaccines for parasitic infections. *Immunology and Cell Biology,* 75:370–375.

KUCEROVA, L. 1998. DNA/genetic vaccination. *Viral Immunology* 11:55–63.

LAEMMLI, U.K. 1970. Cleavage of the structural proteins during assembly of the head of bacteriophage T4. *Nature,* 227:608–658.

LILJESTRÖM, P. & GAROFF, H. 1991. A new generation of animal cell expression vectors based on the Semliki Forest virus replicon. *Biotechnology,* 9:1356–61.

MARTÍNEZ-TORRECUADRADA, J.L., IWATA, H. VENTEO, A., CASAL, J.I. & ROY, P. 1994. Expression and characterization of the two outer capsid proteins of African horsesickness virus: the role of VP2 in virus neutralization. *Virology,* 202:348–359.

MARTÍNEZ-TORRECUADRADA, J.L. & CASAL, I. 1995. Identification of a linear neutralization domain in the protein VP2 of African horsesickness virus. *Virology,* 210:391–399.

MARTÍNEZ-TORRECUADRADA, J.L., DIAZ-LAVIADA, M., ROY, P., SANCHEZ, C., VELA, C., SANCHEZ-VIZCAINO, J.M. & CASAL, J.I. 1996. Full protection against African horsesickness (AHS) in horses induced by baculovirus-derived AHS virus serotype 4 VP2, VP5 and VP7. *Journal of General Virology,* 77:1211–1221.

MARTÍNEZ-TORRECUADRADA, J.L., DIAZ-LAVIADA, M., ROY, P., SANCHEZ, C., VELA, C., SANCHEZ-VIZCAINO, J.M. & CASAL, J.I. 1997. Serological markers in early stages of African horsesickness virus infection. *Journal of Clinical Microbiology,* 35:531–535.

RAMSAY, A.J., LEONG, K.H.,& RAMSHAW, I.A. 1997. DNA vaccination against virus infection and enhancement of antiviral immunity following consecutive immunization with DNA and viral vectors. *Immunology and Cell Biology,* 75:382–8.

RAMSAY, A.J., RAMSHAW, I.A. & ADA, G.L. 1997. DNA immunization. *Immunology and Cell Biology,* 75:360–363.

RANZ, A.I., MIGUET, J.G., ANAYA, C., VENTEO, A., CORTES, E., VELA, C. & SANZ, A. 1992. Diagnostic methods for African horsesickness virus using monoclonal antibodies to structural and nonstructural proteins. *Veterinary Microbiology* 33: 143–153.

ROTHEL, J.S., WATERKEYN, J.G., STRUGNELL, R.A., WOOD, P.R., SEOW, H-F, VADOLAS, J. & LIGHTOWLERS, M.W. 1997. Nucleic acid vaccination of sheep: Use in combination with a conventional adjuvanted vaccine against *Taenia ovis. Immunology and Cell Biology,* 75:41–46.

ROY, P., BISHOP, D.H., HOWARD, S., AITCHISON, H. & ERASMUS, B. 1996. Recombinant baculovirus-synthesized African horsesickness virus (AHSV) outer-capsid protein VP2 provides protection against virulent AHSV challenge. *Journal of General Virology,* 77:2053–2057.

ROY, P., MERTENS, P.C. & CASAL, I. 1994. African horsesickness virus structure. *Comparative Immunology, Microbiology and Infectious Diseases* 17: 243–273.

SAMBROOK, J., FRITSCH, E.F. & MANIATIS, T. 1989. *Molecular cloning. A laboratory manual,* 2nd ed. New York: Cold Spring Harbor Laboratory Press.

SEO, H.S., WANG, L., SMITH, R. & COLLISSON, E.W. 1997. The carboxyl-terminal 120-residue polypeptide of infectious bronchitis virus nucleocapsid induces cytotoxic T lymphocytes and protects chickens from acute infection. *Journal of Virology,* 71: 7889–7894.

STONE-MARSCHAT, M.A., MOSS, S.R., BURRAGE, T.G., BARBER, M.L., ROY, P. & LAEGREID, W.W. 1996. Immunization with VP2 is sufficient for protection against lethal challenge with African horsesickness virus type 4. *Virology,* 220:219–222.

STOTT, J.L., BARBER, T.L. & OSBURN, B.I. 1985. Immunological response of sheep to inactivated and virulent bluetongue virus. *American Journal of Veterinary Research* 46:1043–1049.

SURADHAT, S., YOO, D., BABIUK, L., GRIEBEL, P. & BACA-ESTRADA 1997. DNA immunization with a bovine rotavirus VP4 gene induces a Th1-like immune response in mice. *Viral Immunology,* 10:117–127.

TIGHE, H., CORR, M., ROMAN, M. & RAZ, E. 1998. Gene vaccination: plasmid DNA is more than just a blueprint. *Immunology Today,* 19:89–96.

TOWBIN J., STAEHELIN T. & GORDON J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. Procedure and some applications. *Proceedings of the National Acadamy of Science U.S.A.,* 76:4350–4354.

ULMER J.B., DONNELY J.J., PARKER S.E., RHODES G.H., FELGNER P.L., DWARKI V.J., GROMKOWSKI S.H., DECK R.R., DE WITT C.M., FRIEDMAN A., LEANDER K.R., MARTINEZ D., PERRY H.C., SHIVER J.W., MONTGOMERY D.L. & LIU M.A. 1993. Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science,* 259:1745–1749.

VAN DRUNEN LITTEL-VAN DEN HURK, S., BRAUN, R.P., LEWIS, P.J., KARVONEN, B.C., BACA-ESTRADA, M.E., SNIDER, M., MCCARTNEY, D., WATTS, T. & BABIUK, L.A. 1998. Intradermal immunization with a bovine herpesvirus-1 DNA vaccine induces protective immunity in cattle. *Journal of General Virology,* 79:831–839.

VAN ROOIJ, E.M.A., HAAGMANS, B.L., DE VISSER, Y.E., DE BRUIN, M.G.M., BOERSMA, W. & BIANCHI, A.T.J. 1998. Effect of vaccination route and composition of DNA vaccine on the induction of protective immunity agaisnt pseudorabies infection in pigs. *Veterinary Immunology and Immunopathology,* 66:113–126.

VREEDE F.T. & HUISMANS H. 1994. Cloning and characterization of the gene that encodes the major neutralization-specific antigen of African horsesickness virus serotype 3. *Journal of General Virology,* 75:3629–3633.

WAINE, G.J. & MCMANUS (1995). Nucleic acids: vaccines of the future. *Parasitology Today,* 11:113–115.