Protein aggregation complicates the development of baculovirus-expressed African horsesickness virus serotype 5 VP2 subunit vaccines

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ABSTRACT

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This paper describes the expression of a cloned African horsesickness virus (AHSV) serotype 5 VP2-gene by a baculovirus recombinant that was generated by the BAC-TO-BAC™ system. Immunization of horses with crude cell lysates containing recombinant baculovirus-expressed AHSV5 VP2 did induce neutralizing antibodies, but afforded only partial protection against virulent virus challenge. Further analysis of partially protective crude cell lysates revealed that baculovirus-expressed AHSV5 VP2 was predominantly present in the form of insoluble aggregates. Only approximately 10 % of VP2 was present in a soluble form. Immunization of guinea-pigs with aggregated and soluble forms of AHSV5 VP2 established that only soluble VP2 was capable of inducing neutralizing antibodies. This finding adds a new dimension to the development of AHSV VP2s as subunit vaccines. Further investigation is needed to limit formation of insoluble aggregates and optimize conditions for producing VP2 in a form capable of inducing protective immunity.

Keywords: African horsesickness virus, baculovirus, serotype 5 VP2, vaccines

INTRODUCTION

African horsesickness (AHS) is an infectious, non-contagious, arthropod-borne viral disease of equine animals which is endemic in most of sub-Saharan Africa. It occurs sporadically in north Africa, Mediterranean countries and the Middle East and is an OIE "A"-list disease. African horsesickness has a high mortality rate in horses and mules, whereas the African donkey and zebras are resistant (Henning 1956; Erasmus, Young, Pieterse & Boshoff 1978). African horsesickness virus (AHSV) belongs to the Orbivirus genus of the *Reoviridae* family. Blood-sucking *Culicoides* midges transmit AHSV (Meiswinkel, Nevill, & Venter 1994). There are nine serotypes of AHSV. For full protection, horses have to be immune to all nine serotypes. In general, the neutralizing antibody titre of a horse for a particular serotype reflects its immune status to infection with respect to that serotype.

During the 1970s Onderstepoort Biological Products (OBP), the local vaccine factory, formulated a freeze-dried polyvalent AHS vaccine for the South African market consisting of two combinations (doses) each containing four serotypes, namely:

- Combination one: serotypes 1, 3, 4 and 5
- Combination two: serotypes 2, 6, 7 and 8

The serotype 1, 2, 3, 5 and 6 strains in this formulation were neurotropic vaccine strains originally derived after about 100 intracerebral passages in adult mouse brains, while the serotype 4, 7 and 8 strains were fully cell-culture attenuated large plaque strains

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(Erasmus 1963, 1977). This particular vaccine formulation was discontinued in 1990 when it was realized that it caused four cases of non-fatal encephalitis and chorioretinitis in workers of OBP during the 1980s. It is hypothesized that these workers were inadvertently exposed to aerosol infection with the neurotropic AHSV strains, particularly serotypes 1 and 6, in the vaccine-packaging section as a result of accidental breakages of bottles of freeze-dried vaccine. It is important, however, to stress that these infections occurred under specific circumstances with known encephalitogenic strains and there is no reason to suggest that AHSV should ordinarily be considered a human pathogen (Swanepoel, Erasmus, Williams, & Taylor 1991). Nevertheless, all the remaining neurotropic cell culture-produced vaccine strains were subsequently replaced with fully cell culture-attenuated vaccine strains. Ever since, there have been problems with some of the AHSV vaccine strains. In 1990, a newly-attenuated serotype 5 vaccine strain had to be discontinued following safety problems and all subsequent attempts to prepare another AHSV5 vaccine strain by traditional attenuation procedures have not yet been successful. A serious problem encountered with preparing new vaccine strains nowadays is that, over time, passaging of Vero cells seems to have resulted in cells that no longer exhibit the large plaque variant phenomenon that was previously used to select attenuated AHSV strains.

Recombinant DNA technology offers a solution to this dilemma since genes encoding protective antigens from field, virulent, or partially attenuated viruses can be cloned and expressed. In addition, recombinant subunit vaccines in conjunction with new diagnostics will allow differentiation between vaccinated and naturally infected animals and contribute significantly to streamlining and speeding up clearance of horses for safe international movement (Van Dijk 1998, in press).

African horsesickness virions consist of seven structural proteins, VP1–7. They form a double layered capsid which contains ten double-stranded RNA genes. Two structural proteins, VP2 and VP5, form an outer capsid (Bremer 1976; Van Dijk & Huismans 1982; Roy, Mertens & Casal 1994). The feasibility of developing VP2-based subunit vaccines that induce protective, serotype-specific humoral immunity has been established for both AHSV and bluetongue virus (BTV), the latter being the prototype Orbivirus.

In the case of BTV, it was first established that BTV VP2, isolated from virions and subsequently produced by baculovirus recombinants, induces protective, serotype-specific neutralizing antibodies that protect sheep fully against virulent virus challenge (Huismans, Van der Walt, Cloete & Erasmus 1987; Roy, Urakawa, Van Dijk & Erasmus 1990). After the 1987 AHS serotype 4 outbreak in Spain, the AHSV4 VP2 gene was cloned. Baculovirus- and vaccinia-virus recombinants expressing AHSV4 VP2 were constructed and found to be able to induce a protective humoral, serotype-specific immunity 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).

The VP2 gene of AHSV serotype 5 has recently been cloned and was shown to have an intact open reading frame (Vreede, Cloete, Napier, Van Dijk & Viljoen 1998). The aim of the work reported here was to generate a baculovirus recombinant expressing this gene and to investigate the subunit vaccine potential of baculovirus-expressed AHSV5 VP2.

MATERIALS AND METHODS

Cloning of the AHSV serotype 5 VP2 gene

The AHSV5 VP2 gene that was used for this investigation was cloned as described by Vreede *et al.* (1998). For clarity, the main steps are summarized:

An AHSV5 field isolate was plaque purified and propagated in monolayer CER cells (Verwoerd 1969). AHSV5 dsRNA was purified from infected CER cells essentially as described by Sakamoto, Mizukoshi, Apiwatnakorn, Iwata, Tsuchiya, Ueda, Imagawa, Sugiura, Kamada & Fukusho (1994). The AHSV5 VP2 gene was cloned by means of oligonucleotide ligation. A primer was used for cDNA synthesis—PC3: 5'pGGATCCCGGGAATTCGGAAAAAAAAAAAAAAAAA-$NH_2$3'. Plus and minus-strand single-stranded cDNA was annealed, 5'-single-stranded overhangs were filled in with Klenow DNA dependent DNA polymerase (Sambrook, Fritsch & Maniatis 1989) and PCR was performed on annealed DNA using DyNAZyme™II (Finnzymes Oy) in a 100 µℓ reaction volume and PC2: 5'pCCGAATTCCCGGGATCC3' as primer in 30 cycles (95° C for 45 s, 63 °C for 45 s and 72 °C for 210 s—extended to 420 s on the final cycle). The PCR-product was cloned into pMOS*Blue* (Amersham, UK). Northern blot analysis confirmed that the cloned VP2 gene was AHSV5-specific. The presence of an intact open reading was confirmed by *in vitro* transcription and translation.

Construction of a baculovirus recombinant expressing AHSV5 VP2

A prototype BAC-TO-BAC™ Baculovirus Expression System obtained from Life Technologies Inc. (Gaithersburg, Maryland, USA) was used according to the instructions of the manufacturer. This system permits rapid and efficient generation of recombinant baculoviruses by site-specific transposition of a DNA cassette into a baculovirus shuttle vector (bacmid) propagated in *E. coli* (Luckow, Lee, Barry & Olins 1993).

To facilitate sub-cloning of the AHSV5 VP2 gene as a KpnI/HindIII fragment into pFASTBAC1, pFASTBAC1 was first modified by replacing the EcoRI/HindIII fragment from its multiple cloning site with that of pBLUESCRIBE. The modified pFASTBAC1 was called pFBS1. Subsequently, the AHSV5 VP2 gene was excised from pMOS*Blue* as a KpnI/HindIII fragment and cloned into KpnI/HindIII digested pFBS1 following standard procedures (Sambrook *et al.* 1989).

Transposition of the cloned AHSV5 VP2 gene from pFASTBAC1 into the bacmid and isolation of a recombinant bacmid containing the AHSV5 VP2 gene were done following the instructions of the BAC-TO BAC™ Baculovirus Expression System's Draft Manual obtained from Life Technologies Inc. (Gaithersburg, Maryland, USA). The insertion of the AHSV5 VP2 gene in the bacmid was confirmed by SmaI digestion followed by Southern blotting using nick translated ^{32}P-labelled cloned AHSV5 VP2 DNA as a probe.

Spodoptera frugiperda cells (Sf9 cells) were grown in monolayer or suspension cultures at 28 °C in Grace's medium (Highveld Biologicals, South Africa) supplemented with 10% foetal calf serum (FCS) and standard amounts of a commercially formulated penicillin/streptomycin/neomycin mixture (Highveld Biologicals, South Africa).

Transfections of Sf9 cells with AHSV5 VP2 bacmid DNA were done following the manufacturer's instructions. Briefly: 9x10^5 Sf9 cells were seeded per well in a six-well (35 mm) tissue culture plate in 2 mℓ Grace's medium containing antibiotics and allowed to attach for 2 h at 28 °C. Seven microlitres bacmid miniprep DNA was diluted in 100 µℓ Grace's medium without antibiotics. Seven microlitres Lipofectin (Gibco-BRL) was diluted separately in 100 µℓ Grace's medium without antibiotics. The two solutions were combined, mixed gently and incubated at 25 °C for 1 h. The seeded cells were washed once with 2 mℓ Grace's medium without antibiotics. For each transfection 0,8 mℓ Grace's medium without antibiotics was added to the 0,2 mℓ lipid-DNA complexes, mixed gently, overlayed onto the washed Sf9 cells and incubated for 5 h at 28 °C. The transfection mixtures were then removed, 2 mℓ Grace's medium with antibiotics was added and the cells incubated at 28 °C for 72 h. At 72 h the medium was collected and referred to as passage #1 virus stock which was kept at 4 °C.

Preparation of baculovirus-expressed AHSV5 VP2

A working stock of the recombinant virus was prepared by plaque purification of the passage #1 virus stock and propagation in Sf9 cells as described above. Shaking suspension cultures of 80 mℓ containing 1x10^6 Sf9 cells per mℓ or 75 cm^2 tissue culture flasks seeded with 5x10^6 Sf9 cells in 15 mℓ Grace's medium with antibiotics and 10% FCS were infected with an AHSV5 VP2-baculovirus recombinant at a multiplicity of infection (MOI) of 5 and incubated for 48–72 h at 28 °C. After incubation the cells were harvested by centrifugation at 12 000 *g* for 3 min at 10 °C, washed twice with 15 mℓ phosphate buffered saline (PBS) and finally resuspended in PBS (250 µℓ per 75 cm^2 flask; 1000 µℓ per 80 mℓ suspension culture). These crude cell lysate preparations were kept at –70 °C. The amount of baculovirus-expressed AHSV5 VP2 in these crude cell lysates was quantified from SDS-PAGE as described below.

SDS-PAGE

SDS-PAGE was performed as described by Laemmli (1970) using 7 x 8 cm gels composed of a 4% polyacrylamide stacking and 12% polyacrylamide separating part. The proteins were visualized by Coomassie^R brilliant blue G250 (Merck) staining.

Quantification of proteins

The amount of recombinant AHSV5 VP2 in different preparations originating from recombinant baculovirus infected cells was quantified both by visual comparison and image analysis of Coomassie^R brilliant blue stained SDS-polyacrylamide gels. The VP2 protein band was compared to known standards of a commercial molecular mass marker (Rainbow™ of Amersham, UK) run in separate lanes on the same gel by visual inspection as well as by image analysis from white light transillumination of the gel in an imaging device (Lumi-Imager™ F1 Workstation purchased from Boehringer Mannheim) and quantification by means of LumiAnalyst™ image analysis software version 3.0 for Windows NT™.

Immunization of guinea-pigs and horses

Guinea-pigs (Duncan-Hardly cross-breed) were obtained from OBP. The horses originated from an AHS-free area in the high-lying eastern Free State region of South Africa. One- to four-year-old horses that did not have any neutralizing antibodies to AHSV were selected for the experiment.

The inocula were prepared immediately before use as follows:

Crude lysates, prepared as described above from recombinant baculovirus infected Sf9 cultures, were lysed by three cycles of freezing and thawing. Appropriate volumes of this material containing the required amount of recombinant AHSV5 VP2, as estimated by SDS-PAGE, were made up to 1 200 µℓ with PBS. An equal volume of Montanide incomplete Seppic ISA-50 adjuvant was added before the mixtures were emulsified and inoculated. Horses were

inoculated subcutaneously in the side of the neck, while guinea-pigs were inoculated intraperitoneally.

Challenge virus

Challenge material was prepared as follows: an ampoule containing 0,5 mℓ lyophilized 10% suckling mouse brain suspension in 50% phosphate-buffered lactose peptone, prepared years ago by intracranial inoculation of blood from a viraemic horse, was reconstituted and inoculated onto a 175 cm^2 confluent monolayer of Vero cells in a Roux flask in 100 mℓ Eagle's medium. The cell suspension was harvested when it showed full cytopathic effect and kept at 4 °C. Horses were challenged by subcutaneous inoculation in the side of the neck with 2 mℓ of this Vero cell suspension. The horses were monitored daily for clinical signs of AHS for a 21 day period after the challenge. The rectal temperatures of the animals were recorded twice a day and blood was taken daily from which serum was prepared.

Plaque-reduction neutralization assay

The neutralizing antibody titres of sera were determined using a plaque-reduction neutralization assay described by Huismans & Erasmus (1981). The titres are expressed as the reciprocal of the serum dilution causing a 50 % reduction in the number of plaques.

RESULTS

Construction of a baculovirus recombinant expressing AHSV5 VP2

The AHSV5 VP2 gene used in this investigation was cloned as described by Vreede *et al.* (1998) using poly(dA)-oligonucleotide ligation based on the original procedure developed by Lambden, Cooke, Caul & Clarke (1992). A recombinant baculovirus containing this AHSV5 VP2 gene under the control of the baculovirus polyhedron promoter was constructed using the BAC-TO-BAC™ baculovirus expression system Life Technologies Inc. (Gaithersburg, Maryland, USA) as described above.

SDS-PAGE verification of baculovirus expression of AHSV5 VP2

To verify expression of AHSV5 VP2 by the baculovirus recombinant, $3x10^5$ Sf9 cells seeded in a 15 mm 4-well plate (NUNC) were inoculated with the passage #1 virus stock. After 72 h incubation at 28 °C the medium was removed and a crude lysate was prepared. The cells were harvested in PBS, pelleted by centrifugation at 12 000 *g* for 3 min, washed with 1 mℓ PBS, pelleted again by centrifugation at 12000 *g* for 3 min and resuspended in 20 µℓ water. An equal volume of double concentrated protein suspension buffer (Laemmli 1970) was added, the sample was

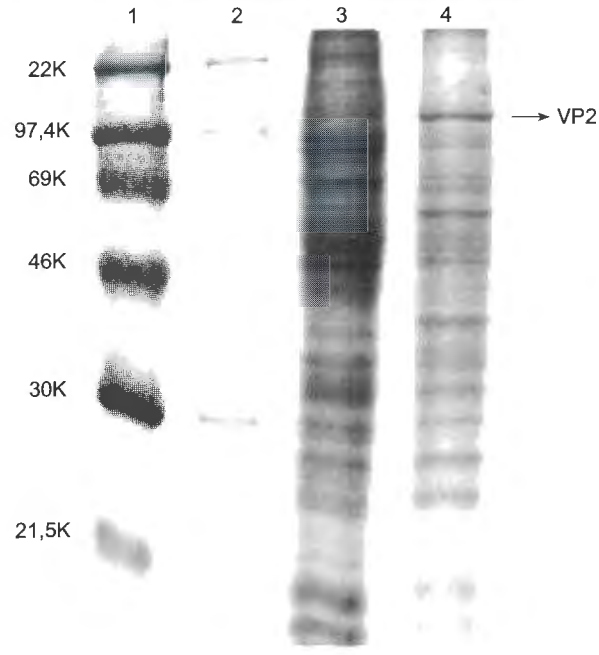

FIG. 1 Coomassie® brilliant blue stained 12% SDS-PAGE analysis of baculovirus-expressed AHSV5 VP2. Lane 1: 7, 0 µg Rainbow™ molecular mass marker proteins; lane 2: 0,7 µg Rainbow™ molecular mass marker proteins; lane 3: crude lysate of uninfected Sf9 cells; lane 4: crude lysate of recombinant AHSV5 VP2 baculovirus-infected Sf9 cells

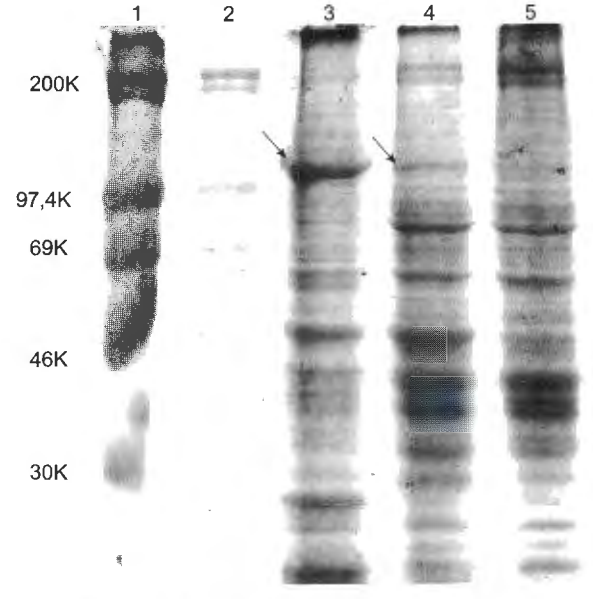

FIG. 2 Coomassie® brilliant blue stained 12% SDS-PAGE analysis of aggregated and soluble baculovirus-expressed AHSV5 VP2. Lane 1: 5,0 µg Rainbow™ molecular mass marker proteins; lane 2: 1,0 µg Rainbow™ molecular mass marker proteins; lane 3: aggregated fraction of crude lysate of recombinant AHSV5 VP2 baculovirus-infected Sf9 cells; lane 4: soluble fraction of crude lysate of recombinant AHSV5 VP2 baculovirus-infected Sf9 cells; lane 5: crude lysate of uninfected Sf9 cells. The position of the recombinant VP2 is indicated by arrows

boiled for 3 min, sonicated for 10 min and subjected to SDS-PAGE (Fig. 1, lane 4). A crude lysate was also prepared in a similar manner from uninfected Sf9 cells (Fig. 1 lane 3), while different amounts of commercially available marker proteins, (Rainbow™ from Amersham) were run in separate lanes for quantification (Fig. 1, lanes 1 and 2). Proteins were visualized by Coomassie® brilliant blue staining. The estimated yield of baculovirus-expressed AHSV5 VP2 in crude cell lysates such as these was in the order of 10 µg per 10^6 Sf9 cells.

Ability of baculovirus-expressed AHSV5 VP2 to elicit neutralizing antibodies and protect horses against challenge

To determine the ability of baculovirus-expressed AHSV5 VP2 to elicit neutralizing antibodies and protect horses against lethal virulent virus challenge, three horses were immunized subcutaneously with lysates of recombinant baculovirus-infected Sf9 insect cells containing an estimated 50 µg baculovirus-expressed AHSV5 VP2 per horse in the presence of Montanide incomplete ISA-50 Seppic adjuvant. One control horse was not immunized. The immunized horses were boosted on day 21 with a 2,4 mℓ emulsion of a cell lysate again containing an estimated 50 µg baculovirus-expressed AHSV5 VP2 per horse and ISA-50 as adjuvant. Since none of the immunized horses developed significant neutralizing antibodies titres after the first booster, all three received a second booster on day 63 with a 2,4 mℓ emulsion of a cell lysate containing an estimated 150 µg baculovirus-expressed AHSV5 VP2 per horse (three times the amount of VP2 in the first booster) and ISA-50 as adjuvant. Following this, all three immunized horses developed high titres of AHSV serotype 5 specific neutralizing antibodies (Table 1).

The neutralizing antibody titre of the horses was monitored over time. It dropped steadily and by four months after the second booster the neutralizing antibody titre of all the immunized horses was very low. At this point, on day 133 after the second booster, all four horses were challenged with a lethal dose of a virulent AHSV serotype 5 strain. The control horse died 7 d after the challenge confirming the efficacy of the challenge dose. All three immunized horses survived the challenge, but they did develop severe clinical AHS signs such as high fever, swollen supra-orbital fossae and petechiae of the ventral aspect of their tongues. This was interpreted as indicating that only partial protection had been achieved (Table 1).

SDS-PAGE analysis of baculovirus-expressed VP2 which did not induce full protection in horses

Baculovirus-expressed AHSV5 VP2 crude cell lysate preparations which failed to induce a fully protective immune response in horses were further investigated. The ratio of aggregated and soluble VP2 in these preparations was determined as follows:

Sf9 insect cells were infected at a MOI of 5 with the recombinant baculovirus expressing AHSV5 VP2, harvested at 48 h post infection, washed three times with PBS, resuspended in PBS at a final concentration of $1x10^8$ cells per mℓ and lysed by three freeze-thaw cycles. Lysates were centrifuged for 15 min at 12 000 *g* at 25 °C and comparable amounts of the supernatant, which contained soluble VP2, and pellet which contained mostly aggregated VP2, were analyzed on a 12 % SDS-polyacrylamide gel. VP2 was found to be present predominantly in the form of insoluble aggregates (Fig. 2, lane 3). Two different amounts of commercially available marker proteins, (Rainbow™ from Amersham) were run in separate lanes for quantification (Fig. 2, lanes 1 and 2). Quantification by visual inspection and image capturing of white light transillumination of the Coomassie® brilliant blue stained gel shows that only about 10 % of the expressed AHSV5 VP2 in the lysate of these recombinant baculovirus-infected Sf9 cells was soluble (Fig. 2, lane 4).

Immunization of guinea-pigs with soluble and aggregated recombinant VP2

In general, the neutralizing antibody titre of a horse reflects its immunity to AHSV infection for a particular serotype. Guinea-pigs serve as an excellent small animal screening model to identify vaccine preparations with a good inherent immunogenicity based on the level of AHSV-specific neutralizing antibodies that they induce (Erasmus 1963). An experiment was therefore done to compare the ability of soluble and aggregated recombinant AHSV VP2 preparations to induce neutralizing antibodies in guinea-pigs. Soluble and aggregated recombinant AHSV5 VP2 was prepared from infected Sf9 cells as described above. Two groups of ten guinea-pigs each were immunized intraperitoneally and boosted twice, on days 28 and 56, with soluble and aggregated VP2 preparations containing approximately 50 µg AHSV5 VP2 respectively and Montanide incomplete ISA-50 Seppic as adjuvant. Animals were bled 14 and 28 d after the second booster to allow for individual variation between animals to reach their peak antibody response. Neutralizing antibody titres were determined using a plaque-reduction neutralization assay. The results are depicted in Table 2.

Seven of the ten guinea-pigs immunized with soluble VP2 developed high neutralizing antibody titres. Of these, five had titres of 620 and higher. Nine of the guinea-pigs injected with aggregated VP2 did not develop any neutralizing antibodies, while only one developed a good neutralizing antibody titre. In general, there was no significant difference in neutralizing antibody titre between sera prepared from blood

TABLE 1 Neutralizing antibody (NAb) response in three horses immunized with baculovirus-expressed AHSV5 VP2 and their reaction to challenge with virulent AHSV5

Horse no.	Highest Nab titre	NAb titre at challenge (day 133 post 150 µg booster)	Highest NAb titre after challenge	Survived challenge
1	640	30	20 480	Yes[1]
2	640	40	10 240	Yes[1]
3	640	40	20 480	Yes[1]
4	Control	NA[2]	< 20	No[1]

[1] Horse did develop clinical signs characteristic of AHS
[2] Not applicable

TABLE 2 Neutralizing antibody titres induced in guinea-pigs by inoculation of soluble and aggregated preparations of baculovirus-expressed AHSV5 VP2

Antigen	Guinea-pig number	Neutralizing antibody titres	
		Day 14 post 2nd booster	Day 28 post 2nd booster
Soluble VP2	1	1280	1280
	2	2560	1280
	3	160	160
	4	80	40
	5	< 20	< 20
	6	5120	5120
	7	< 20	< 20
	8	5120	–[1]
	9	20	80
	10	640	–[1]
Aggregated VP2	11	< 20	< 20
	12	< 20	< 20
	13	< 20	< 20
	14	320	640
	15	< 20	< 20
	16	< 20	< 20
	17	< 20	< 20
	18	< 20	< 20
	19	< 20	< 20
	20	< 20	< 20

[1] Guinea-pig died after day 14 post 2nd booster bleed

taken on day 14 and day 28 after the booster, as is characteristic of sampling during the plateau phase of the antibody response.

DISCUSSION

This paper reports on baculovirus-vectored expression of a cloned AHSV serotype 5 VP2 gene, the cloning of which has been described by Vreede *et al.* (1998). Apart from AHSV serotype 5, the VP2 gene of only three other AHSV serotypes has been cloned, namely AHSV3 (Vreede & Huismans 1994), AHSV4 (Martinez-Torrecuadrada, Iwata, Venteo, Casal & Roy 1994) and AHSV6 (Williams, Inoue, Lucus, Zanoot & Roy 1998). For two of these, AHSV serotypes 3 and 4, baculovirus recombinants expressing the cloned VP2 gene have been constructed using the traditional method where homologous recombination occurs upon transfection of baculovirus infected insect cells (Vreede & Huismans 1994; Martinez-Torrecuadrada *et al.* 1994).

The baculovirus recombinant expressing AHSV5 VP2 reported here is the first AHSV VP2 recombinant generated by means of the BAC-TO-BAC™ baculovirus expression system. This system greatly reduces the time required to identify and purify recombinant viruses (Luckow *et al.* 1993). As with most of the other baculovirus-expression systems, the foreign gene is expressed under the control of the baculovirus polyhedron promoter. Similar to what has been reported for BTV VP2 (Roy *et al.* 1990) and AHSV4 VP2 (Roy *et al.* 1996), the AHSV5 VP2 baculovirus recombinant produced high yields of VP2, namely in the order of 10 µg recombinant AHSV5 VP2 per 10^6 Sf9 cells.

Initial inoculation and boosting of three horses with crude cell lysates containing 50 µg AHSV5 VP2 did

not induce demonstrable serotype-specific neutralizing antibodies. Only after boosting with a crude cell lysate containing 150 µg AHSV5 VP2 did the horses develop a high neutralizing antibody titre. Although all three immunized horses subsequently survived a lethal challenge, they did develop severe clinical signs characteristic of AHS and a huge anamnestic response indicating that this particular immunization schedule induced only partial protection.

The reason for partial protection despite the high neutralizing antibody response is not clear and needs further investigation. Nevertheless, the results presented in this paper demonstrate the subunit vaccine potential of baculovirus-expressed AHSV5 VP2, since, as for AHSV4 (Roy *et al.* 1996; Martinez-Torrecuadrada *et al.* 1996), recombinant AHSV5 VP2 elicited serotype-specific neutralizing antibodies and protected horses, albeit only partial, against a lethal challenge. It remains to be investigated whether full protection can be achieved if the whole immunization schedule is performed with sufficient amounts of recombinant VP2 to induce high neutralizing antibody titres.

Further characterization of baculovirus-expressed AHSV5 VP2 in crude cell lysates which failed to induce a fully protective immune response in horses revealed that VP2 is mainly present in the form of insoluble aggregates. Approximately 10% of AHSV5 VP2 in crude lysates of recombinant baculovirus-infected Sf9 cells was in a soluble form. Only the soluble form of AHSV5 VP2 was able to elicit neutralizing antibodies in guinea-pigs. Taking this ten-fold correction factor retrospectively into consideration, the initial dose of AHSV5 VP2 used for injection and boosting the horses contained only 5 µg soluble VP2 and the second booster only 15 µg soluble VP2. Therefore, the results of the pilot experiment in horses reported here correlate with those published for AHSV4 VP2 where a 5 µg dose also resulted in partial protection, namely protection against death and a high anamnestic response after challenge, while full protection was only achieved with a dose of 40 µg VP2 or more (Roy *et al.* 1996).

Conflicting results relating to the ability of baculovirus-expressed AHSV4 VP2 to induce a protective immune response have been published. Roy *et al.* (1996) reported a high yield of baculovirus-expressed AHSV4 VP2 (produced in Sf9 cells in a 2 ℓ bioreactor using SF900 II serum-free medium from a recombinant generated by the traditional homologous recombination-transfection method) that induced a fully protective immune response in horses and state that the majority of VP2 was present as solubilized products that separated from cell debris by centrifugation at 5 000 *g*. A separate paper by Martinez-Torrecuadrada *et al.* (1996), apparently using the same recombinant as Roy *et al.* (1996) [propagated in Sf9 cells in suspension or monolayer cultures using TNM-FH medium supplemented with 5% FCS], reported that neither crude cell extracts from AHSV4 VP2 recombinant baculovirus-infected cells, nor affinity column-purified VP2 was able to induce neutralizing antibodies or protect horses. However, crude extracts of cells co-infected with AHSV4 VP2 and VP5 or VP2, VP5 and VP7 did induce high titres of neutralizing antibodies and protected horses against challenge. The authors concluded that, as for BTV VP2 (Roy *et al.* 1990), AHSV4 VP2 was more immunogenic when used together with the other outer capsid protein, VP5. Furthermore, based on recognition by a panel of VP2-specific monoclonal antibodies, it was established that the antigenicity of AHSV4 VP2 in recombinant baculovirus-infected cell extracts and affinity column-purified recombinant baculovirus-expressed AHSV4 VP2 differed significantly from that of VP2 on virions. The new data presented here relating to the inability of aggregated baculovirus-expressed AHSV5 VP2 to induce neutralizing antibodies and the efficacy with which soluble VP2 elicits neutralizing antibodies, therefore, correlates with and extends that reported for AHSV4 VP2 (Martinez-Torrecuadrada *et al.* 1996; Roy *et al.* 1996). Inability to elicit a protective immune response with recombinant VP2 has also been encountered with BTV, the prototype and only other Orbivirus for which work has been done on the development of VP2-based subunit vaccines. Martyn, Gould & Yu (1994) found that *Saccharomyces cerevisiae*-expressed BTV1 VP2 failed to protect sheep against virulent virus challenge.

The inability of some baculovirus-expressed AHSV VP2 preparations to induce protective immunity is clearly not serotype-specific. The conclusion from the results presented in this paper together with the data reported for baculovirus-expressed AHSV4 VP2 (Martinez-Torrecuadrada *et al.* 1996; Roy *et al.* 1996), is that the notorious phenomenon of protein aggregation and misfolding, which is regularly encountered in modern biotechnology (Yon 1996), is probably also the underlying cause for the failure of aggregated recombinant AHSV VP2 to stimulate a protective immune response. Many factors, all of which have yet to be investigated, may contribute to aggregation, including the effect of specific propagation conditions of recombinant baculoviruses, the role of different baculovirus expression systems and the best time in the baculovirus replication cycle for expression of VP2, since the production of large amounts of VP2 very late in the replication cycle regulated by the polyhedron promoter might result in saturation of, for example, baculovirus chaperon proteins.

In summary, the finding that only soluble baculovirus-expressed AHSV5 VP2 induces neutralizing antibodies, adds a new dimension to the development of AHSV VP2s as subunit vaccines. Further investigation is needed to keep formation of insoluble VP2

aggregates to a minimum and to find optimum conditions for producing VP2 in a form capable of inducing a protective immune response. Ensuring correct protein folding to induce protective immunity will thus be crucial for the further development and eventual commercial viability of VP2-based recombinant subunit vaccines for AHS. Approaches to achieve this will include improving the protective antigenic conformation of VP2 by co-expression of VP2 with VP5, the other AHSV outer capsid protein, or presenting AHSV VP2s on virus-like particles as has been described for BTV (Roy, Bishop, LeBlois & Erasmus 1994).

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REFERENCES

BREMER, C.W. 1976. A gel electrophoretic study of the protein and nucleic acid components of African horsesickness virus. *Onderstepoort Journal of Veterinary Research*, 43:193–200.

ERASMUS, B.J. 1963. Preliminary observations of the value of the guinea pig in determining the innocuity and antigenicity of neurotropic attenuated horsesickness strains. *Onderstepoort Journal of Veterinary Research*, 30:11–22.

ERASMUS, B.J. 1977. Arbovirus vaccines: Veterinary aspects, in *Medicine in a Tropical Environment. Proceedings of the International Symposium, Pretoria 19–23 July 1976. South Africa*, edited by J.H.S. Gear. A.A. Balkema, Cape Town.

ERASMUS, B.J., YOUNG, E., PIETERSE, L.M. & BOSHOFF, S.J. 1978. The susceptibility of zebra and elephants to African horsesickness virus, in *Equine Infectious Diseases, Proceedings of the Fourth International Conference on Equine Infectious Diseases, 24–27 September 1976 in Lyon, France*, edited by J.T. Bryan & H. Gerber. Princeton, New Jersey: Veterinary Publications, Inc.

HENNING, M.W. 1956. *Animal Diseases of South Africa*, 3rd ed. Pretoria: Central News Agency Ltd.

HUISMANS, H. & ERASMUS, B.J. 1981. Identification of the serotype-specific and group-specific antigens of bluetongue virus. *Onderstepoort Journal of Veterinary Research*, 48:51–58.

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.

LAEMMLI, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227:680–685.

LAMBDEN, P.L., COOKE, S.J., CAUL, E.O. & CLARKE, I.N. 1992. Cloning of non-cultivatable human rotavirus by single primer amplification. *Journal of Virology*, 66:1817–1822.

LUCKOW, V.A., LEE, S.C., BARRY, G.F. & OLINS, P.O. 1993. Efficient generation of infectious recombinant baculoviruses by site-specific transposon-mediated insertion of foreign genes into a baculovirus genome propagated in *Escherichia coli*. *Journal of Virology*, 67:4566–4579.

MARTINEZ-TORRECUADRADA, J.L., IWATA, H., VENTEO, A., CASAL, 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.

MARTINEZ-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.

MARTYN, J.C., GOULD, A.R. & YU, M. 1994. Expression of the outer capsid proteins VP2 and VP5 of bluetongue virus in *Saccharomyces cerevisiae*. *Virus Research*, 33:11–25.

MEISWINKEL, R., NEVILL, E.M. & VENTER, G.J. 1994. Vectors: Culicoides *spp.*, in *Infectious Disease of Livestock*, edited by J.A.W. Coetzer, G.R. Thomson & R.C. Tustin. Cape Town: Oxford University Press.

ROY, P., URAKAWA, T., VAN DIJK, A.A. & ERASMUS, B.J. 1990. Recombinant virus vaccine for bluetongue disease in sheep. *Journal of Virology*, 64:1998–2003.

ROY, P. BISHOP, D.H.L., LEBLOIS, H. & ERASMUS, B.J. 1994. Long-lasting protection of sheep against bluetongue challenge after vaccination with virus-like particles: evidence for homologous and partial heterologous protection. *Vaccine*, 12: 805–811.

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

ROY, P., BISHOP, D.H.L., HOWARD, S. AITCHISON, H. & ERASMUS, B.J. 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.

SAMBROOK, J., FRITSCH, E.F. & MANIATIS, T. 1989. Molecular Cloning — A Laboratory Manual. Cold Spring Harbor, New York, USA: Laboratory Press.

SAKAMOTO, K., MIZUKOSHI, N., APIWATNAKORN, B., IWATA, A., TSUCHIYA, T., UEDA, S., IMAGAWA, H., SUGIURA, T., KAMADA, M. & FUKUSHO, A. 1994. The complete sequences of African horsesickness virus serotype 4 (vaccine strain) RNA 2 and 6 which encode outer capsid protein. *Journal of Veterinary Medical Science*, 56:321–327.

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.

SWANEPOEL, R., ERASMUS, B.J., WILLIAMS, R. & TAYLOR, M.B. 1991. Encephalitis and chorioretinitis associated with neurotropic African horsesickness virus infection in laboratory workers, III. Virological and serological investigations. *South African Medical Journal*, 81:458–461.

VAN DIJK, A.A. African horsesickness vaccine development. *Proceedings of the Eighth International Conference on Equine Infectious Diseases, 23–28 March 1998, Dubai, United Arab Emirates*, edited by J.F. Wade. Newmarket, UK: R&W Publications Ltd (in press).

VAN DIJK, A.A. & HUISMANS, H. 1982. The effect of temperature on the *in vitro* transcriptase reaction of bluetongue virus, epizootic haemorrhagic disease virus and African horsesickness virus. *Onderstepoort Journal of Veterinary Research*, 49:227–232.

VERWOERD, D.W. 1969. Purification and characterization of bluetongue virus. *Virology*, 38:203–212.

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

VREEDE, F.T., CLOETE, M., NAPIER, G.B., VAN DIJK, A.A. & VILJOEN, G.J. 1998. Sequence-independent amplification and cloning of large dsRNA virus genome segments by poly(dA)-oligonucleotide ligation. *Journal of Virological Methods*, 72: 243–247.

WILLIAMS, C.F., INOUE, T., LUCUS, A-M., ZANOTTO, P.M. DE A. & ROY, P. 1998. The complete sequence of four major structural proteins of African horse sickness virus serotype 6: evolutionary relationships within and between orbiviruses. *Virus Research,* 53:53–73.

YON, J.M. 1996. The specificity of protein aggregation. *Nature Biotechnology*, 14:1231.