Recombinant baculovirus-synthesized African horsesickness virus (AHSV) outer-capsid protein VP2 provides protection against virulent AHSV challenge

Polly Roy,1,2,3 David H. L. Bishop,2 Stephen Howard,2 Henry Aitchison4 and Baltus Erasmus4

African horsesickness virus serotype 4 (AHSV-4) outer-capsid proteins VP2 or VP2 and VP5, prepared from single or dual recombinant baculovirus expression vectors grown in Sf9 insect cells, were administered in different amounts to horses and the neutralizing antibody responses were measured. Control and vaccinated horses were challenged with virulent AHSV-4 6 months later and monitored post challenge. The results indicated that two inoculations of extracts containing VP2 and VP5, or VP2 alone, in doses of 5 μg VP2 or more per horse, were sufficient to elicit protection against African horsesickness (AHS) disease. The recombinant VP2 protein is a potential candidate vaccine for AHS in horses.

African horsesickness (AHS) disease is characterized by widespread haemorrhages on mucosal and other membranes as well as by a selective increase in vascular permeability resulting in oedema. Oedema is most commonly found in the lungs, subcutaneous tissues and muscular tissues of the head, neck and trunk of the horse. The mortality rate in horses is very high, while other equids (donkeys and zebras) develop subclinical infections (see review by Mertens, 1994). The causative agent, African horsesickness virus (AHSV), is a member of the family Reoviridae, genus Orbivirus. Like bluetongue virus (BTV), the prototype orbivirus, AHSV is transmitted to susceptible animals by biting midges (Culicoides spp.), which become infected by feeding on animals during the viraemic stages of infection. To date, nine different serotypes of AHSV (AHSV-1, -2, etc.) have been identified in Africa (Howell, 1962), of which only one serotype, AHSV-4, was isolated in Spain and Portugal during recent epidemics (Rodriguez et al., 1992). Outbreaks in countries previously considered to be free from AHS are of major concern, particularly since spread of the disease can only be controlled using a live attenuated AHSV vaccine at present. Polyvalent live attenuated AHSV vaccines are frequently used in epidemic situations to achieve sufficient protection against all nine serotypes. The simultaneous administration of several vaccine strains often results in interference during vaccine virus replication, resulting in incomplete immunity. Moreover, AHSV, like other orbiviruses, has the potential for reassortment of gene segments between various strains and serotypes. Of particular concern for AHSV is the fact that at high doses AHSV live vaccines can cause disease in horses and humans (Taylor et al., 1992; Reid et al., 1992; van der Meyden et al., 1992). The development of an efficacious, non-replicating subunit vaccine is a goal of present studies, particularly for AHSV-4, since this serotype has been the cause of recent epidemics in Spain, Portugal and Morocco.

AHSV particles contain seven structural proteins (VP1–VP7), organized in two capsids, which are comparable to those of BTV (see review by Roy et al., 1994a). The outer capsid of the virion contains two major virion proteins, VP2 and VP5; the inner capsid or core consists of two other major proteins, VP3 and VP7, in addition to three minor proteins, VP1, VP4 and VP6. The core encloses the virus genome, which consists of ten double-stranded RNA segments.

We have recently developed candidate BTV vaccines based on baculovirus-expressed BTV outer-capsid proteins VP2 and VP5, and have demonstrated that recombinant virus-infected insect cells expressing VP2 alone could elicit protective neutralizing antibodies in sheep, although the protection was enhanced in the presence of VP5 (Roy et al., 1990). Therefore, to develop non-replicating vaccines for AHS, we have embarked on similar studies with AHSV proteins. We previously reported the construction of baculovirus expression vectors, based on Autographa californica nuclear polyhedrosis virus, capable of synthesizing AHSV-4 VP2 and VP5 in insect cells, either individually or simultaneously, depending on the...
Table 1. AHSV-neutralizing antibody titres in horses

Horses were vaccinated on day 0 and boosted on day 21. Titres are expressed as the reciprocal of the serum dilution causing a 50% plaque resolution. Neg, Negative (< 20); NT, not tested. In (b), the indicated animals were challenged on day 204.

(a)

<table>
<thead>
<tr>
<th>Horse no.</th>
<th>Day 0 inoculum</th>
<th>Day 21 booster</th>
<th>Pre-challenge titre</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Day 0</td>
</tr>
<tr>
<td>1</td>
<td>VP2 (300 µg)</td>
<td>VP2 (300 µg)</td>
<td>Neg</td>
</tr>
<tr>
<td>2</td>
<td>VP2 (300 µg)</td>
<td>VP2 (300 µg)</td>
<td>Neg</td>
</tr>
<tr>
<td>3</td>
<td>VP2 (30 µg)</td>
<td>VP2 (30 µg)</td>
<td>Neg</td>
</tr>
<tr>
<td>7</td>
<td>Control (saline)</td>
<td>Saline</td>
<td>Neg</td>
</tr>
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</table>

(b)

<table>
<thead>
<tr>
<th>Horse no.</th>
<th>Day 0 inoculum</th>
<th>Day 21 booster</th>
<th>Pre-challenge titre</th>
<th>Challenge (1 ml subcutaneously)</th>
<th>Post-challenge titre</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>VP2 (5 µg)</td>
<td>VP2 (5 µg)</td>
<td>Neg</td>
<td>–</td>
<td>160</td>
</tr>
<tr>
<td>2</td>
<td>VP2 (10 µg)</td>
<td>VP2 (10 µg)</td>
<td>Neg</td>
<td>20</td>
<td>320</td>
</tr>
<tr>
<td>3</td>
<td>VP2 (20 µg)</td>
<td>VP2 (20 µg)</td>
<td>Neg</td>
<td>120</td>
<td>640</td>
</tr>
<tr>
<td>4</td>
<td>VP2 (40 µg)</td>
<td>VP2 (40 µg)</td>
<td>Neg</td>
<td>60</td>
<td>320</td>
</tr>
<tr>
<td>5</td>
<td>VP2 (80 µg)</td>
<td>VP2 (80 µg)</td>
<td>Neg</td>
<td>80</td>
<td>&gt; 640</td>
</tr>
<tr>
<td>6</td>
<td>VP2 (5 µg)</td>
<td>VP2 (5 µg)</td>
<td>Neg</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>7</td>
<td>VP2 (10 µg)</td>
<td>VP2 (10 µg)</td>
<td>Neg</td>
<td>60</td>
<td>640</td>
</tr>
<tr>
<td>8</td>
<td>VP2 (20 µg)</td>
<td>VP2 (20 µg)</td>
<td>Neg</td>
<td>320</td>
<td>640</td>
</tr>
<tr>
<td>9</td>
<td>VP2 (40 µg)</td>
<td>VP2 (40 µg)</td>
<td>Neg</td>
<td>20</td>
<td>320</td>
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<tr>
<td>10</td>
<td>VP2 (80 µg)</td>
<td>VP2 (80 µg)</td>
<td>Neg</td>
<td>480</td>
<td>&gt; 640</td>
</tr>
<tr>
<td>11</td>
<td>Adjuvant and saline</td>
<td>Adjuvant and saline</td>
<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
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</tbody>
</table>

* One horse died from colic.
construction. Further, we demonstrated that antiserum raised against recombinant baculovirus-infected insect cell lysates containing the AHSV-4 VP2 proteins elicited antibodies in mice that neutralized AHSV (Martinez-Torrecuadrada et al., 1994). In this report, we present a dose-related evaluation of the protective properties of the expressed AHSV VP2 and VP5 proteins in horses, and demonstrate that VP2 alone induces immunity that protects the animals against a virulent challenge by the homologous virus strain. The data are the first evidence of the feasibility of a subunit vaccine for AHS.

Materials for vaccination were prepared using recombinant baculovirus-infected Spodoptera frugiperda (SF9) cells grown in SF900 II serum-free medium in a 2 l SGI Cytoflow bioreactor operating at 28 °C, 70% dissolved oxygen, pH 5.9-6.1 and stirred at 75 r.p.m. By removing toxic by-products from the culture medium using a 0.2 μm hollow fibre filter, insect cells were grown to a density of 1-2 x 10^7 cells/ml and infected at an m.o.i. of 5 p.f.u. per cell (Brown & Faulkner, 1977) with either a single gene recombinant virus that expressed AHSV-4 VP2, or a dual recombinant virus that expressed AHSV-4 VP2 and VP5 (Martinez-Torrecuadrada et al., 1994). The infected cells were harvested at 70 h post-infection, recovered by centrifugation and then either lysed by three cycles of freezing and thawing to provide a crude cell lysate, or resuspended in 25 mM-NaHCO_3 at approximately 0.5-1 x 10^8 cells/ml to lyse the cells by hypotonic shock. In each case, the solubilized products containing the majority of the AHSV VP2 and VP5 proteins were separated from cell debris by centrifugation at 5000 g. Immediately after harvesting, the cells were pelleted by low-speed centrifugation and lysed. The presence of VP2 and VP5 was confirmed by Western blot analysis using an AHSV-4 polyclonal antibody (data not shown). The amounts of VP2 and VP5 present in the cell lysate after removing cell debris were estimated by comparing the protein bands to known standards run alongside on a Coomassie blue-stained 10% polyacrylamide gel, and the total protein was measured by the Bradford (1976) assay. The amount of AHSV protein in the VP2 recombinant was estimated to be about 300 μg/ml. The concentrations of VP2 and VP5 in the cell lysate supernatant containing both proteins were estimated to be approximately 80 μg/ml and 5 μg/ml, respectively.

One-to four-year-old horses that lacked antibodies to AHSV were used for the vaccination trials. The absence of AHSV antibody was verified by analysing their sera using an AHSV-specific ELISA. In a preliminary study, three horses were inoculated subcutaneously (on the side of their neck) with either 300 μg or 30 μg AHSV-4 VP2 in the insect cell lysate. Each 2 ml of inoculum contained 1 ml Montanide Incomplete Septic Adjuvant (ISA-50). The vaccinated animals were boosted with the same amounts of proteins and adjuvant 21 days later. A control animal received only saline and adjuvant. In our previous vaccination studies with recombinant BTV proteins, control animals inoculated either with saline or with wild-type baculovirus-infected cell lysates showed no difference in terms of eliciting neutralizing antibodies (none were detected) or in the severe level of clinical reaction upon virulent virus challenge; therefore, in order to minimize wastage of horses, we only used one control in these studies. The neutralizing antibody titres of the animals were determined at weekly intervals for 110 days after the vaccination using standard procedures (Huismans et al., 1987) and were expressed as the reciprocal of the serum dilution causing a 50% plaque resolution. As illustrated in Table 1(a), the vaccinated horses produced only a low level of neutralizing antibodies after the primary vaccination. However, following the boost with similar amounts of the immunogens, neutralizing antibody titres increased rapidly (Table 1a). Three weeks after the booster, the titres reached their highest levels. Both doses (30 μg or 300 μg) elicited a similar level of response.

In a subsequent experiment (Table 1b), the dose response to the AHSV immunogens, starting from as low as 5 μg AHSV VP2 in the crude protein extract, was determined. Ten horses were vaccinated with recombinant virus-infected cell extracts containing different amounts of VP2 (5-80 μg) either alone or in the presence of a trace amount of VP5 (0.5-8 μg). The vaccinated horses were boosted on day 21 with similar amounts of the immunogens and the sera were monitored for neutralizing antibodies for a further 173 days. A control horse received only saline and adjuvant. As shown in Table 1(b), all doses of VP2, or VP2 and VP5, elicited antibodies at various levels that neutralized AHSV infections in vitro. The neutralizing antibody titres for the animals that received only 5 μg doses of crude VP2 were not significantly lower than for horses that received higher amounts of VP2. Horses vaccinated with both outer-capsid proteins gave comparable neutralizing antibody titres. The data clearly indicate that VP2 alone was sufficient for eliciting neutralizing antibodies against AHSV. Whether AHSV VP5 elicited neutralizing antibodies was not determined. As in the previous experiment, the antibody titres persisted throughout the period of observation. The control horse remained sero-negative with respect to AHSV.

To determine whether the AHSV-neutralizing antibodies conferred protection against a virulent virus challenge, at day 204 five of the vaccinated horses were inoculated on the side of the neck with infective horse blood containing South African AHSV-4 at approximately 10^6 TCID_50. Since propagation of AHSV in tissue culture frequently results in the loss or reduction of virulence in horses, and to establish the protective efficiency of the induced antibodies, infected horse blood was used for the challenge experiment. Horse sera were monitored for a further 53 days. As shown in Table 1(b), all the challenged horses showed an anamnestic response, and produced higher levels of neutralizing antibodies. The temperature and other clinical reactions of the challenged animals were examined daily. As shown in Table 2, the horse that received the low dose of VP2 (5 μg) had a slightly elevated temperature from day 6 to 9, but otherwise survived the
baculovirus-expressed AHSV-4 VP2 protein at doses as low as 5 µg, followed by similar booster vaccination, provided protection against death caused by virulent AHSV-4 challenge. These results confirmed previous observations that the outer-capsid protein VP2 is the main determinant of the AHSV neutralization-specific immune response and that it induces protection (Burrage et al., 1993). For bluetongue disease, our previous studies indicated that vaccination of sheep required doses in excess of 50 µg VP2 (per dose) (Roy et al., 1990). For BTV, the second outer-capsid protein, VP5, together with VP2, significantly enhanced the neutralization antibody and protective response against virulent BTV challenge. It was postulated that although BTV VP5 alone does not directly elicit neutralizing antibodies, it may enhance the immune response to VP2, perhaps by affecting the conformation of VP2 and, consequently, its serological properties. In vaccination studies with baculovirus-expressed BTV virus-like particles (VLPs), total protection was afforded by 10 µg VLPs containing only 1–2 µg VP2 (Roy et al., 1992, 1994b). Our present study with AHSV VP2 indicates that in low doses it is sufficient for total protection over at least a 6 month period. While we have yet to determine the duration of the immunity conferred by VP2, the minimum amount of VP2 needed for complete protection, or whether at even lower doses VP5 enhances protection, the data indicate that a cost-effective subvirus vaccine for AHSV based on recombinant AHSV VP2 proteins may be possible in the future.

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References


### Table 2. Temperature reactions (°C) of challenged horses

<table>
<thead>
<tr>
<th>Horse no.</th>
<th>Inoculum protein (µg)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
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<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
<th>14</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>VP2 (5 µg)</td>
<td>38.5</td>
<td>38.6</td>
<td>38.7</td>
<td>38.8</td>
<td>40.2</td>
<td>40.3</td>
<td>39.9</td>
<td>39.8</td>
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<td>37.1</td>
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<td></td>
</tr>
<tr>
<td>3</td>
<td>VP2 (20 µg)</td>
<td>37.8</td>
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<td>38.3</td>
<td>37.5</td>
<td>38.2</td>
<td>38.1</td>
<td>38.3</td>
<td>38.1</td>
<td>38.0</td>
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<td>38.1</td>
<td>37.7</td>
<td>38.1</td>
<td>37.8</td>
</tr>
<tr>
<td>4</td>
<td>VP2 (40 µg)</td>
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<td>38.3</td>
<td>38.4</td>
<td>37.5</td>
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<td>37.0</td>
<td>37.8</td>
<td>37.8</td>
</tr>
<tr>
<td>8</td>
<td>VP2 (20 µg) + VP5 (2 µg)</td>
<td>38.2</td>
<td>38.3</td>
<td>38.4</td>
<td>38.5</td>
<td>38.4</td>
<td>38.3</td>
<td>38.3</td>
<td>38.8</td>
<td>38.0</td>
<td>37.5</td>
<td>37.8</td>
<td>37.0</td>
<td>37.8</td>
<td>37.8</td>
</tr>
<tr>
<td>10</td>
<td>VP2 (80 µg) + VP5 (8 µg)</td>
<td>38.2</td>
<td>38.9</td>
<td>38.5</td>
<td>38.5</td>
<td>38.8</td>
<td>38.7</td>
<td>38.8</td>
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<td>38.0</td>
<td>37.1</td>
<td>37.4</td>
<td>37.8</td>
<td>37.8</td>
</tr>
<tr>
<td>11</td>
<td>(control)</td>
<td>38.6</td>
<td>38.4</td>
<td>38.8</td>
<td>39.0</td>
<td>40.6</td>
<td>40.9</td>
<td>41.1</td>
<td>41.0</td>
<td>Died</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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</table>

In summary, trials in horses have clearly demonstrated that baculovirus-expressed AHSV-4 VP2 protein at doses as low as 5 µg, followed by similar booster vaccination, provided protection against death caused by virulent AHSV-4 challenge. The animals that received higher doses of the immunogens had normal temperatures. None of the vaccinated horses showed any other clinical reaction following the virulent virus challenge. The control horse, as expected, had high fever from day 6 post-challenge and exhibited typical mixed lesions of horsesickness disease, including supraorbital oedema, congestion of oral and conjunctival mucosae and cardiac and respiratory symptoms. The animal died on day 10. On this basis, it was concluded that the amount of infected blood (containing approximately 10^5 TCID<sub>50</sub>) was sufficient to establish the disease and to kill horses. All the other horses survived, indicating that the neutralizing antibodies elicited by the expressed proteins conferred protection against the virulent virus challenge.

To confirm whether virus replication was inhibited in the vaccinated animals, the post-challenge whole-blood samples were collected daily for 12 days. Each sample was injected intravascularly into 10–12-day-old embryonated chicken eggs followed by incubation at 33 °C. The embryos were monitored for 7 days. Dead embryos were harvested and suspended at 10% (w/v) in Eagle's medium and seeded onto monolayers of BHK-21 cells. These monolayers were observed for 7 days for the appearance of cytopathic changes and subsequent recovery of virus. All virus isolates were serotyped to confirm their identity with AHSV-4. None of the vaccinated horses gave any indication of viraemia, including the horse which received only 5 µg VP2 and showed slightly elevated temperatures between days 6 and 9. As expected, the post-challenge blood of the control horse was viraemic from day 6 post-challenge. The recovered virus was identified as AHSV-4.


