African horsesickness virus VP7 sub-unit vaccine protects mice against a lethal, heterologous serotype challenge

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An established mouse model was used to evaluate the effectiveness of the major outer core protein of African horsesickness virus (AHSV), VP7, as a sub-unit vaccine. Adult female BALB/c mice were immunized with VP7 crystals purified from BHK cells infected with AHSV serotype 9 (AHSV-9), using three inoculations in Freund’s adjuvant. Eighty to one hundred per cent of the immunized mice were protected against a heterologous challenge with a known lethal dose of AHSV-7. The protected immunized mice did not develop any clinical signs characteristic of virulent AHSV infection in this model during the study. In contrast, 80–100% mortality was observed in the non-immunized mice that received the same challenge virus. Subsequent studies indicated that a single inoculation of 1.5 μg purified AHSV VP7 in Freund’s complete adjuvant was sufficient to protect at least 90% of mice from AHSV-7 challenge. If the antigen was presented in the absence of Freund’s complete adjuvant, 70% of the mice were still protected by one inoculation of VP7 crystals. Titres of circulating antibody against AHSV VP7, determined by competitive ELISA, did not appear to correlate with protection and passive antibody transfer from immunized BALB/c mice failed to protect syngeneic recipients from AHSV-7 challenge. Therefore, the observed protection is unlikely to be due to an antibody-mediated immune response. The number of viraemic mice and the duration of viraemia post-challenge was significantly reduced in vaccinated mice compared to non-vaccinated controls. However, the levels of viraemia were similar.

Introduction

African horsesickness (AHS) is an infectious, but non-contagious arthropod-borne disease, which affects all species of the Equidae. The causative agent, African horsesickness virus (AHSV), is a dsRNA virus classified as a distinct species in the genus Orbivirus, within the family Reoviridae. AHSV is closely related to bluetongue virus (BTV), the prototype member of the orbiviruses (Borden et al., 1971; Holmes et al., 1995). Nine different AHSV serotypes have currently been identified (Howell, 1962). The disease is enzootic in a large area of sub-Saharan Africa and in certain African territories may cause up to 95% mortality in susceptible horses. Mules, donkeys and zebras are less susceptible, the death rate being considerably lower than 50%. Periodic wide-scale epizootics have caused devastating outbreaks of the disease outside the African continent, ranging eastwards as far as India and Pakistan and westward into Spain, Portugal and Morocco (Lubroth, 1988; Mellor, 1993). The development of effective vaccination regimes against AHSV has been complicated by the necessity to protect equines against multiple serotypes of the virus. Vaccination with a single serotype of AHSV does not generate significant levels of neutralizing antibody to, or protect animals from infection with, heterologous serotypes (McIntosh, 1958). Prophylactic immunization in South Africa with a single serotype is considered largely ineffective since it is likely that more than one serotype will be circulating in the field during an epizootic. One of the vaccines currently in use in South Africa contains eight attenuated AHSV serotypes, given as two separate quadruvalent inoculations (Taylor et al., 1992). Outbreaks that have occurred in areas other than the African continent have always been due to one AHSV serotype, but because current vaccines are not cross-protective, susceptible animals must be vaccinated with the relevant serotype. The serotype responsible for the epizootic must therefore be typed before vaccination regimes can be adopted.

A similar problem exists with the prototype member of the genus Orbivirus, BTV, of which there are 24 serotypes (Huismans et al., 1987; Holmes et al., 1995). Although the use

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of several different BTV serotypes may generate a broad protective immune response, even to virus serotypes not previously encountered (Jeggo et al., 1986), the use of live, attenuated, multivalent vaccines introduces several potential hazards (Erasmus, 1963, 1978). Even limited replication of AHSV and BTV attenuated viruses in vivo is likely to result in the production of virus-specific antibodies which complicates the distinction between vaccinated and infected animals for import/export purposes (Laviada et al., 1995; Anderson et al., 1993). A major potential problem in the use of live, attenuated, multivalent vaccines is that reassembly between serotypes can result in the generation of progeny viruses with novel phenotypes, in terms of serological and virulence characteristics (Cowley & Gorman, 1989; Nuttall et al., 1992; O’Hara, 1994). An additional problem associated with vaccine production is that there have been reported cases of laboratory workers being infected during large-scale preparation of live, attenuated AHSV vaccines. This has resulted in the withdrawal of certain neurotropic vaccine strains from vaccine preparations (van der Meyden et al., 1992). There is, therefore, clearly the requirement for the development of a safe, economical vaccine which is effective against multiple serotypes of AHSV.

VP7 of BTV, the major inner capsid protein and group-specific antigen (Gumm & Newman, 1982), has been shown to contain immunodominant, serotype-cross-reactive T-cell epitopes (Angove, 1995), but does not generate neutralizing antibodies to intact virus particles (Mertens et al., 1996). The stimulation of a cross-reactive, cell-mediated immune response may also stimulate cross-reactive protection in the same animal. This has been demonstrated by the use of adoptive transfer techniques (Jeggo et al., 1984) which indicate that cell-mediated immunity may play an important role in the development of a protective immune response to BTV in the absence of neutralizing antibodies. This paper investigates the protective role of AHSV VP7 (equivalent to the VP7 of BTV), a highly conserved, serogroup-specific antigen. The VP7 antigen used in these studies was obtained from two sources, either as a bacterial glutathione S-transferase (GST) fusion protein or as crystals purified from BHK cells infected with African horsesickness virus serotype 9 (AHSV-9).

Methods

Synthesis of bacterial GST–VP7 fusion protein. The GST–AHSV VP7 fusion protein (GST–VP7) was engineered, expressed and inclusion bodies were purified as described by Wade-Evans et al. (1993).

Purification of AHSV VP7 crystals. Crystals of AHSV VP7 were purified from BHK cells infected with AHSV-9 according to the method described by Burroughs et al. (1994). Samples of the crystals were analysed by SDS–PAGE according to the method described by Laemmli (1970), together with a known quantity of molecular mass standards. The amount of VP7 present in each sample was estimated by densitometric analysis of Coomassie blue-stained gels. The crystals were heat-treated prior to use by incubation at 56 °C for 1 h.

<table>
<thead>
<tr>
<th>Group</th>
<th>Antigen inoculated</th>
<th>Antigen concentration (µg/100 µl)</th>
<th>Day 1†</th>
<th>Day 21†</th>
<th>Day 42†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>GST</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>GST–VP7</td>
<td>1.5</td>
<td>FIA</td>
<td>FIA</td>
<td>FIA</td>
</tr>
<tr>
<td>4</td>
<td>VP7 crystals, boiled</td>
<td>1.5</td>
<td>FIA</td>
<td>FIA</td>
<td>FIA</td>
</tr>
<tr>
<td>5</td>
<td>VP7 crystals</td>
<td>1.0</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>0.5</td>
<td>FCA</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td>0.25</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>8</td>
<td></td>
<td>1.5</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>9</td>
<td>AHSV-9</td>
<td>200 µl of 2 × 10⁶ TCID₅₀/ml</td>
<td>No adjuvant</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td></td>
<td>No FIA</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>11</td>
<td></td>
<td></td>
<td>No FIA</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>12</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* FCA, inoculum prepared in 50% Freund’s complete adjuvant.
† FIA, inoculum prepared in 50% Freund’s incomplete adjuvant.

Analysis of virulence of three AHSV serotypes in an established, adult mouse model for AHS. Groups of five adult female BALB/c mice, 8 weeks old were inoculated intraperitoneally or intravenously with various doses of individual AHSV serotypes (see Table 2) as described by O’Hara (1994). The mice were closely observed for 21 days following challenge and any mice that showed signs of distress, i.e. shivering, fever, paralysis or wasting, were euthanized immediately.

Titration of AHSV stocks. AHSV-7, isolated 29/6/60 and designated Karen, was originally supplied by B. J. Erasmus (Onderstepoort, South Africa) as a high-mouse-brain-passage-number isolate and has been further passaged three times in BHK cells. AHSV-9 (South African virus isolated 2/5/73, designated 90/61, MB3, BHK6) and AHSV-4 (South African virus isolated 21/4/89, designated Ricardo, MB1, BHK6) were also supplied by B. J. Erasmus. The AHSV-9 isolate was identical to that used for the VP7 crystal preparations. All virus isolates were grown in BHK cells of low passage number (approx. 30). Virus titres (TCID₅₀) were calculated by the method of Karber (1931). The same AHSV-7 virus stock was used in all the challenge inoculations. The stock was re-titrated prior to use to confirm that there was no loss in infectivity during storage at 4 °C.

Immunization of mice with VP7 sub-unit vaccine. Groups of ten female BALB/c mice (6 weeks old) were inoculated subcutaneously as summarized in Table 1. Group 1 was kept as the non-immunized control group. Groups 2, 3, 4 and 5 were immunized three times with 1.5 µg (100 µl volume purified GST, GST–VP7, boiled VP7 crystals (100 °C for 30 min) or VP7 crystals, respectively, on days 1 [in 50% Freund’s complete adjuvant (FCA)], 21 and 42 [in 50% Freund’s incomplete
Protection by AHSV VP7 sub-unit vaccine

Results

Of the three virus isolates tested (AHSV-4, AHSV-7 and AHSV-9), AHSV-7 was the only virus isolate to reproducibly cause mortality in adult female BALB/c mice (see Table 2), but only using the intravenous route of inoculation. \( 3 \times 10^8 \text{TCID}_{50} \) (200 µl of \( 1 \times 10^7 \text{TCID}_{50} / \text{ml} \)) of this AHSV-7 isolate were used in all the challenge experiments described in this paper as detailed in the Methods section.

The percentage mortalities observed in control and vaccinated groups of mice following a lethal dose of AHSV-7 are summarized in Fig. 1. Seventy to eighty per cent mortality was observed in mice that had received a triple inoculation of bacterial GST–VP7 (group 3), which was not significantly different \((P = 0.534)\) to the levels observed in the non-immunized controls (80–100%, group 1). In groups of mice that had been inoculated three times with \( 1 \mu g \) of the bacterial fusion protein GST–VP7 (group 3), mortality rates ranged from 30 to 50% following AHSV-7 challenge (significantly different from controls; \( P = 0.0028 \)). A similar level of mortality (50%) was observed in mice that had received a triple inoculation of purified VP7 that had been heated to 100 °C for 30 min prior to inoculation (group 4), which was not significantly different from the mortality observed in group 3 \((P = 0.796), \) but was significantly different from the controls \((P = 0.048)\).

However, significantly lower levels of mortality were observed in the groups of mice that had been inoculated with purified VP7 crystals: 0–20% in those groups which had received three inoculations of \( 1 \mu g \) (4.5 µg in total, group 5); 10% in the groups that had only two inoculations of \( 1 \mu g \) (3 µg in total, group 6); and 0–10% in the mice that had a single inoculation of \( 1 \mu g \) of antigen (group 7). The results obtained from these three groups (groups 5, 6 and 7) were not significantly different from each other \((P = 0.612), \) but were significantly different from the controls \((P < 0.0001) \) in each case. All these inoculations contained FCA or FIA (see Table 1), but even in the absence of any adjuvant only 30% mortality was observed with this quantity \((1.5 \mu g)\) of protein (group 11). Group 11 was significantly different from Group 7 \((P = 0.012)\) and from the controls \((P < 0.001)\).

The minimum quantity of VP7 required in a single inoculation to significantly reduce mortality rates following a lethal dose of AHSV-7 was analysed by using a range of concentrations of purified VP7 antigen in the inoculum (1.5, 1.0, 0.5 or 0.25 µg). Zero to ten per cent mortality was observed with an inoculum of \( 1.5 \mu g \) (group 7) and 40% with \( 1.0 \mu g \) (group 8). The groups receiving an inoculum of less than \( 1.0 \mu g \) had mortality rates comparable to the controls (group 9, \( P = 0.492; \) group 10, \( P = 1.00)\).

Although no other AHSV proteins were detectable in the purified VP7 crystal preparations using SDS–PAGE (Burroughs et al., 1994; and unpublished data), it was considered possible that a very low level of contamination of the crystal
Table 2. Total percentage mortality observed over 21 days following intravenous (i.v.) or intraperitoneal (i.p.) inoculation of different virus isolates in groups of five adult female BALB/c mice

<table>
<thead>
<tr>
<th>Virus</th>
<th>Titre (TCID₅₀/ml)</th>
<th>i.v. inoculum (µl)</th>
<th>i.p. inoculum (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AHSV-4</td>
<td>1.4 × 10⁷</td>
<td>0 0 0 0</td>
<td>0 0 0 0</td>
</tr>
<tr>
<td>AHSV-7</td>
<td>1.2 × 10⁷</td>
<td>20 40 40 80</td>
<td>0 0 0 0</td>
</tr>
<tr>
<td>AHSV-9</td>
<td>2.0 × 10⁷</td>
<td>0 0 0 0</td>
<td>0 0 0 0</td>
</tr>
</tbody>
</table>

![Graph showing mortality (%) over immunization groups](Fig. 1)

Fig. 1. Percentage mortality observed in vaccinated and non-vaccinated mice challenged with AHSV-7. Each group consisted of ten female BALB/c mice (8 weeks old), which had been immunized and challenged as shown in Table 1. The mice were observed for a further 21 days and euthanized if they developed any of the disease symptoms described in Methods. Percentage mortalities were calculated from the total number of deaths recorded over the 21 days p.c. The minimum and maximum values from triplicate studies are shown.

preparation with AHSV-9 particles could have been responsible for the protection observed. Consequently, a group of ten mice were inoculated intravenously with 200 µl of 2 × 10⁷ TCID₅₀/ml of AHSV-9 49 days prior to challenge with AHSV-7. The AHSV-9 isolate used did not cause any signs of disease in the mouse model. Fifty to sixty per cent mortality was observed in these mice by 21 days p.c. with AHSV-7 (see Fig. 1). The partial protection observed in these animals (40–50%) was not significantly different from the controls but only marginally (P = 0.095) and was comparable to that generated using the GST–VP7 fusion protein. No significant levels of cross-neutralization have previously been detected between AHSV serotypes 7 and 9 (McIntosh, 1958; Howell, 1962). The inoculation of mice with relatively large amounts of AHSV-9 particles failed to duplicate the high levels of protection observed with the VP7 crystals (statistical comparison of groups 7 and 12 resulted in P < 0.0001). These results indicate that the presence of very low levels of intact AHSV-9 virions in the VP7 crystal preparations could not adequately explain the protection generated against AHSV-7.

The time period over which deaths occurred in each of the groups was similar. The titres of anti-VP7 antibodies, determined by competitive ELISA, following immunization but prior to challenge, varied considerably from log₁₀ 0.5–log₁₀ 3.25. Mice that survived subsequent challenge with AHSV-7 had log₁₀ titres of 0.5–3.25. Mice that died following AHSV-7 challenge had antibody titres ranging from log₁₀ 2–log₁₀ 3.25 prior to challenge. These results indicate that anti-
VP7 antibody titres did not correlate with protection against AHSV-7 challenge. Mice with an anti-AHSV antibody titre close to log<sub>10</sub> 3·0 died following virus challenge, whereas other mice with log<sub>10</sub> titres less than 1·0 were protected.

The levels of viraemia in non-vaccinated mice ranged from log<sub>10</sub> 1·66 to log<sub>10</sub> 3·66 TCID<sub>50</sub>/ml and in vaccinated mice from log<sub>10</sub> 2·5 to log<sub>10</sub> 3·5 TCID<sub>50</sub>/ml. No virus was detected in the vaccinated group on days 12 and 14, and the remaining mice in the control group had already died from disease by day 10 p.c. There did not appear to be a significant reduction in levels of viraemia in the immunized mice. The duration of viraemia observed in the non-vaccinated controls was nine days (days 2–10 p.c.), but was significantly shorter in the immunized animals, being only detectable over three days (days 4–6 p.c.). The vaccinated mice that had titres of virus higher than log<sub>10</sub> 3·0 TCID<sub>50</sub>/ml were visibly sick and would have been euthanized as AHSV-positive animals if they had not been selected to be killed in order to measure levels of viraemia. These three mice represented the 10% of the vaccinated group that would be predicted from the results described above to be unprotected from AHSV challenge.

The distribution of virus in the organs of the two groups of mice was similar, with antigen detectable only in the heart and brain tissue. The brain registered the highest accumulation of antigen, as determined by sandwich ELISA (results not shown).

Passive transfer of antibodies, either from mice immunized with VP7 or from mice that had been inoculated with FCA alone, into syngeneic recipients which were then challenged with AHSV-7 resulted in very similar levels of mortality (assessed at day 21 p.c.), 90% and 100%, respectively. The titre of circulating anti-VP7 antibody (as measured by competitive ELISA) in individual mice, prior to challenge, ranged from log<sub>10</sub> 1·48 to log<sub>10</sub> 1·68. This was within the range observed for individual mice vaccinated with VP7 crystals that had been protected against challenge in the previous experiments (log<sub>10</sub> 0·5–log<sub>10</sub> 3·25).

**Discussion**

These studies demonstrate that VP7 is effective as a sub-unit vaccine in this small animal model in the absence of other AHSV proteins, with at least 90% protection being achieved after a single inoculation of purified VP7 crystals. It has also been demonstrated that immunization with AHSV VP7 reduces the length of viraemia observed p.c., although the levels of viraemia were not reduced. This reduction in length of viraemia could have two explanations: (1) a true decrease in the time window over which viraemia occurred, or (2) a reduction in the number of mice with detectable viraemia. Only 7 out of 30 vaccinated mice (23%) had detectable viraemia, in contrast to the control group in which 17 out of 30 were positive. An additional 6 control mice died during the course of the experiment that were also positive (76% in total for the control group). The number of mice predicted to be susceptible to AHSV-7 infection following immunization with VP7 crystals from the other experiments described in this paper was between 0 and 20%, so it is possible that the 23% of the vaccinated mice that had detectable viraemia were those that would have developed disease anyway. If this were the case then the vaccination regime could have resulted in a reduction in the level of viraemia as well as protection against disease.

Immunization with denatured VP7 crystals or the bacterial GST–VP7 fusion protein resulted in lower levels of protection compared to the use of VP7 crystals as the immunogen. The conformation of VP7 therefore appears to be important in providing protection against virus challenge. The crystalline nature of the purified VP7 antigen may also be an important factor since purified AHSV-9 particles, in which VP2 and VP5 are exposed on the surface of the virus particle, but which also contain VP7, were not as effective as VP7 crystals in protecting mice against challenge, although the fact that the virus particle immunization contained significantly less VP7 than the VP7 crystal preparation may be an alternative explanation for the decreased efficiency of protection. This result also rules out the possibility that a very low level of AHSV particle contamination is solely responsible for the observed protection with purified VP7 crystals.

Passive antibody transfer from immunized BALB/c mice failed to protect naive, syngeneic recipients from subsequent AHSV-7 challenge. This suggests that antibody is unlikely to be solely responsible for the protective response. These results confirm those recorded by Jeggo et al. (1984) for BTV, a closely related orbivirus, indicating that cell-mediated immunity may play an important role in the development of a protective immune response to BTV. Further studies are needed to determine whether the protective response observed following vaccination with VP7 is due to a cell-mediated immune response and, if so, which subset of cells is involved.

The results also demonstrate that a single inoculation of AHSV-9 VP7 crystals generated protection against a heterologous virus serotype (AHSV-7). This viral protein, which is regarded as the major serogroup-specific and therefore serotype-cross-reactive antigen, may therefore be effective as a prophylactic sub-unit vaccine against all serotypes of AHSV.

This is the first report of a high level of protection against a heterologous serotype challenge within the *Orbivirus* genus using a single, conserved major core antigen (VP7) of the virus as an exogenously presented, sub-unit vaccine and may represent a significant step forward in the design of sub-unit vaccines. However, since this work was performed using a small animal model, there are restrictions on the interpretation of this data with respect to the natural host (equines). In order to obtain more relevant results on the effectiveness of VP7 as a sub-unit vaccine, a vaccine trial must also be conducted in horses. Other workers have obtained positive results in horses using VP7, but in combination with other AHSV proteins, synthesized by baculovirus recombinants, which could also...
indicate that VP7 may be involved in protecting horses against AHSV (Roy, 1995; Martínez-Torrecuadrada et al., 1996).

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