Evaluation of a prototype sub-unit vaccine against equine arteritis virus comprising the entire ectodomain of the virus large envelope glycoprotein (GL): induction of virus-neutralizing antibody and assessment of protection in ponies

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An Escherichia coli-expressed recombinant protein (6hisGLecto) comprising the entire ectodomain (aa 18–122) of equine arteritis virus (EAV) glycoprotein GL, the immunodominant viral antigen, induced higher neutralizing antibody titres than other GL-derived polypeptides when compared in an immunization study in ponies. The potential of the recombinant GL ectodomain to act as a sub-unit vaccine against EAV was evaluated further in three groups of four ponies vaccinated with doses of 35, 70 or 140 µg of protein. All vaccinated animals developed a virus-neutralizing antibody (VNAb) response with peak titres 1–2 weeks after the administration of a booster on week 5 (VNAb titres of 1–8–3–1), 13 (VNAb titres of 1–4–2–9) or 53 (VNAb titres of 1–2–2–3). Vaccinated and unvaccinated control ponies were infected with EAV at different times post-vaccination to obtain information about the degree of protection relative to the levels of pre-challenge VNAb. Vaccination conferred varying levels of protection, as indicated by reduced or absent pyrexia, viraemia and virus excretion from the nasopharynx. The degree of protection correlated well with the levels of pre-challenge VNAb and, in particular, with levels of virus excretion. These results provide the first evidence that a sub-unit vaccine protects horses against EAV. The use of the sub-unit vaccine in combination with a differential diagnostic test based on other EAV antigens would enable serological discrimination between naturally infected and vaccinated equines.

Introduction

Equine viral arteritis is a systemic infection of horses and donkeys caused by equine arteritis virus (EAV), the prototype member of the family Arteriviridae, a group of small enveloped, single-stranded RNA viruses. Other members of this family are lactate dehydrogenase-elevating virus, simian haemorrhagic fever virus and porcine reproductive and respiratory syndrome virus (Snijder & Meulenberg, 1998). EAV replicates primarily in the endothelium of small arteries and in macrophages (McCullum et al., 1971). Secondary sites of virus replication include the epithelia of adrenals, kidney, liver, seminiferous tubules and mesothelium (Lopez et al., 1996; Timoney & McCullum, 1993). The result of infection is variable and
depends on the virus strain, the age of the host and environmental factors (Lopez et al., 1996; Timoney & McCollum, 1993). Thus, affected animals may or may not develop a syndrome that is typically characterized by one or a combination of the following clinical signs: pyrexia, depression, conjunctivitis, ocular and nasal discharges, oedema of the periorbital region or ventral parts of the body, urticarial skin rash and lymph node swelling. The clinical signs presented and their severity vary widely between outbreaks (Timoney & McCollum, 1990). Furthermore, on many occasions, EAV causes sub-clinical infections (McCollum et al., 1973; McCue et al., 1991). Of particular significance for the equine industry is the capacity of EAV to cause abortion in pregnant mares and to establish persistent infection in stallions. Infection is transmitted by nasal secretions during the acute phase of the disease and venereally through semen of chronically infected stallions. The latter animals are asymptomatic virus carriers and can introduce the disease to naive equine populations, as demonstrated in the 1993 outbreak in the UK (Wood et al., 1995).

The EAV icosahedral nucleocapsid contains a 12.7 kb polyadenylated infectious RNA molecule surrounded by a lipid bilayer envelope. The genome contains nine recognized open reading frames (ORFs 1a, 1b, 2a, 2b and 3–7), of which the two largest (1a and 1b) encode the viral replicase. The latter two ORFs are translated as two alternative gene products, derived from ORF 1a or 1a+1b, depending upon whether termination or frameshifting occurs at the 1a/1b frameshift site (den Boon et al., 1991). Five structural proteins have been characterized, namely the nucleocapsid protein (N, encoded by ORF 7), two glycosylated envelope proteins (G1 and G2, encoded by ORFs 5 and 2b, respectively) and two non-glycosylated envelope proteins (M and E, encoded by ORFs 6 and 2a, respectively) (de Vries et al., 1992, 1993; Iwashita & Harasawa, 1987; Snijder et al., 1999). Proteins N, M and G1 are the major antigens of EAV (Balasuriya et al., 1993, 1995; Chirnside et al., 1995; Deregt et al., 1994; MacLachlan et al., 1998). Also, it has been reported that ORF 3 of EAV encodes an immunogenic protein (Hedges et al., 1999). However, all hitherto known neutralizing epitopes are contained within the putative ectodomain of G1 (Balasuriya et al., 1997).

Animals that recover from EAV infection develop a long-lasting immunity against the disease (Gerber et al., 1978), although not always against reinfection (McCollum, 1969). This immunity is believed to be mediated mainly by virus-neutralizing antibodies (VNAb), since their appearance in serum, usually within a week of infection, coincides with the elimination of virus from circulation (Fukunaga et al., 1981; McCollum, 1969). Furthermore, passive transfer of colostrum antibodies from immune mares to foals was found to moderate or prevent equine viral arteritis (McCollum, 1976). In chronically infected stallions, EAV replication, which is restricted to cells of the accessory sex glands, persists for several months or years, despite high levels of circulating VNAb. The importance of cell-mediated immune clearance mechanisms during acute or chronic infections has yet to be determined.

Tissue culture-adapted and formalin-inactivated whole virus vaccines have been used to control EAV infection (Fukunaga et al., 1996, 1997; McCollum, 1986; Timoney et al., 1988). The live vaccine was attenuated by serial passages in horse kidney (HK), rabbit kidney (RK) and equine dermal (ED) cells. Despite occasional isolation of the attenuated virus after vaccination (Fukunaga et al., 1982; Timoney et al., 1988), no reversion to virulence was observed after five sequential passages of the vaccine virus in horses (Doll et al., 1968; McCollum, 1969). However, vaccination of pregnant mares is not recommended. The commercial live virus vaccine Arvac (passage history HK-131/RK-80/ED-24) and its less-attenuated earlier versions have been shown to be effective for clinical protection (McCollum, 1969, 1986; Timoney & McCollum, 1988). These animals also demonstrated a reduction in both duration of virus excretion from nasal secretions and duration of viraemia. A non-attenuated formalin-inactivated virus vaccine was shown to stimulate high levels of VNAb after repeated inoculations with high titre virus preparations (Fukunaga et al., 1990, 1991, 1994, 1996). Clinical trials with this vaccine showed a reduction in clinical signs, nasal excretion of virus and viraemia in vaccinated horses, which appear to correlate with the VNAb titre at the time of challenge. In 1993, an adjuvanted killed virus vaccine was licensed in the UK under the Animal Test Certificate and has been widely used since then.

Widespread administration of currently available vaccines in non-endemic areas would make it difficult to monitor EAV infection within the horse population, since whole virus vaccines do not permit serological discrimination between naturally infected and vaccinated equines. The development of a sub-unit vaccine for EAV would enable such a differentiation. The determination of immunodominant proteins of EAV was undertaken by Chirnside et al. (1995a). Bacterially expressed glutathione S-transferase (GST) fusion proteins of ORFs 2b–7 were screened by ELISA with EAV-positive equine sera, demonstrating that G1 is the most immunoreactive polypeptide. A fusion protein, FP5RsA1 (aa 55–98), and a synthetic peptide, SP25 (aa 75–97), both derived from G1, induced neutralizing antibodies when injected into rabbits and ponies, thus demonstrating the potential of the sub-unit approach. In the present study, we report the testing of additional G1-based antigens for their ability to stimulate VNAb in ponies. It was found that a recombinant protein comprising the N-terminal ectodomain of G1 (aa 18–122) induced strong neutralizing antibody responses and could confer protection in ponies against experimental infection.

Methods

Synthetic peptides and recombinant proteins. All synthetic peptides and recombinant proteins used as immunogens in this study
Analysis of GL recombinant proteins.

For each dose, the antigen stock solution was diluted in sodium phosphate, pH 7.5, distilled water and resuspended in 6 M guanidine hydrochloride, 20 mM sodium pyrophosphate, pH 9.5, 50 µg/ml lysozyme digestion in the presence of protease inhibitors and treated with Tryptic Proteinase K. The resulting TR-Peptide was split into Pep1 (aa 75–97) and Pep2 (aa 98–111), which were synthesized (Chiron Mimotopes). These immunogens were designed to include an extra cysteine residue at the C terminus in order to allow coupling to KLH via the maleimide cross-linking moiety of sulfo-SMCC [N-succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate]. The Immobilized Antigen Conjugation kit (Pierce) was used for this purpose.

The 6hisG_ecto protein is an N-terminally polyhistidine-tagged recombinant protein encompassing the entire G_ecto domain (aa 18–122). Cloning, expression and antigenic analysis of 6hisG_ecto are described elsewhere (Nugent et al., 2000). Briefly, the sequence encoding the predicted N-terminal ectodomain of ORF 5 (i.e. without the predicted signal sequence) was cloned into the pQE9 expression vector (Qiagen), thereby fusing six histidine residues to the N-terminal G_ecto domain. *Escherichia coli* strain M15, transformed with both pREP-4 and pQE9/G_ecto, was cultured in Terrific broth containing 100 µg/ml ampicillin and 50 µg/ml kanamycin. Protein expression was induced by the addition of IPTG to a final concentration of 2 mM after 1 h of incubation at 37 °C. Following a 5 h period of induction, bacteria were pelleted, subjected to lysozyme digestion in the presence of protease inhibitors and treated with both DNase and RNase. Insoluble protein was then pelleted by centrifugation at 6000 g for 15 min at 4 °C. The pellet was washed in distilled water and resuspended in 6 M guanidine hydrochloride, 20 mM sodium phosphate, pH 7.8, and 500 mM NaCl. The solubilized protein was then applied to an Ni^{2+}-charged affinity column (ProBond column, Invitrogen) and subsequently eluted with 8 M urea, 250 mM imidazole, 0.1 M NaH_{2}PO_{4} and 0.01 M Tris, pH 6.0. The protein solution was stored in the dark at 4 °C until use.

All vaccines were prepared in 2 ml doses and administered by the intramuscular route. For each dose, the antigen stock solution was diluted to the required concentration in 1 ml PBS. Then, 1 ml of proprietary copolymer adjuvant (Solvay-Duphar) was added.

- **Analysis of G_recombinant proteins.** Purified 6hisG_ecto and FP5Sa1 proteins were diluted in Laemmli sample buffer and heated at 95 °C for 4 min. The pre-stained SDS–PAGE molecular mass markers were used according to the manufacturer’s instructions (Bio-Rad). Protein samples were analysed by 12.5 % SDS–PAGE and stained with Coomasie blue.

- **Measurement of antibody responses.** Serum samples were collected regularly from the ponies and VNAb titres were determined according to Senne et al. (1985). Briefly, serial twofold dilutions of heat-treated serum (56 °C for 30 min) were carried out in microtitre plates (25 µl per well) from a starting dilution of 1/2. The diluent was Eagle’s minimum essential medium (EMEM) supplemented with antibiotics, 5 % foetal calf serum and 200 mM HEPES. Subsequently, 25 µl of a virus suspension (100 TCID_{50}) in medium containing 10 % guinea pig complement was added to each well. Bucyrus CVL, the virus used in this study, is a derivative of the Bucyrus strain obtained from the OIE Reference Laboratory, Weybridge, UK. This virus, a standard reagent for the virus neutralization (VN) test, has been evaluated in trials and found to give high sensitivity (Edwards et al., 1998). Three control equine sera and a titration of the virus dose were included in each test. After 1 h of incubation at 37 °C in 5 % CO_{2}, RK-13 cells (100 µl per well, 1:2 x 10^{5} cells/ml) were added to the plates, which were then sealed. Results were recorded after a 48 h period of incubation at 37 °C in 5 % CO_{2}. Wells showing more than 50 % cytopathic effect (CPE) were recorded as positive. Titres were calculated using the Karber formula (Karber, 1931) and expressed as log_{10} VN_{50}.

Anti-G_{e} specific antibody responses were measured using the Peptide-OVA diagnostic ELISA test as described by Nugent et al. (2000).

- **Experimental infections.** Bucyrus LP3A is a large-plaque variant derived from a pleural fluid isolate collected from a horse that died after being infected with the 14th horse passage of the original Bucyrus strain (McCollum et al., 1971). Derivation and characterization of the Bucyrus LP3A strain will be described in more detail elsewhere. Plaque purification and growth of virus stocks were carried out in primary equine embryonic lung cells. All challenges were performed in a controlled environment building. Two vaccines and an unvaccinated control pony were used in each challenge. Ponies entered the building 1 week before challenge in order to acclimatize to the new environment and remained in contact thereafter for 21 days. Ponies were examined twice a day every day while within the building. To infect the ponies with EAV, 1 ml virus suspension containing 10^{6} TCID_{50} was placed into the ponies’ nasopharynx using a flexible tube with a spray attachment. Symptoms and rectal temperatures were recorded daily, from the day of challenge to day 14. Nasopharyngeal swabs collected over the 14 day period were stored in standard virus transport medium and frozen at −70 °C. Heparinized blood samples were processed immediately for virus detection.

- **Virus isolation.** Swabs collected from experimentally infected ponies were thawed, squeezed and the resulting extract used for virus isolation. White blood cells from 8 ml of plasma collected from unclotted blood samples were pelleted by low-speed centrifugation and resuspended in 2 ml EMEM. Procedures to isolate EAV were performed essentially as described previously (Paweska et al., 1996). Volumes of 0.5 ml of nasal swab extract or white blood cell suspension were inoculated into 25 cm² flasks of confluent monolayers of RK-13 cells. Flasks were incubated for 1 h at 37 °C in 5 % CO_{2} and overlaid with 5 ml EMEM supplemented with 2 % bovine serum. The following day, old medium was discarded and fresh medium was added. Flasks were then incubated for 5–6 days at 37 °C in 5 % CO_{2}. Confirmation of isolated virus was made by indirect immunofluorescence using a mixture of two monoclonal antibodies to the nucleocapsid protein, kindly supplied by Paolo Cordioli (Istituto Zooprofilattico Sperimentale, Brescia, Italy). Samples were recorded as negative after failure to isolate EAV on a second passage. To titrate the virus present in nasal swab extracts, serial dilutions (0.5 log_{10}) were made in quadruplicate in 96-well plates using a volume of 150 µl. Then, 100 µl of 2 % EMEM containing 10^{4} RK-13 cells was added to each well and plates were incubated for 4 days at 37 °C in 5 % CO_{2}. Wells presenting CPE were recorded as positive and virus concentration was calculated according to the Karber formula and expressed as TCID_{50}/ml of swab extract.

- **Statistical analysis.** Six variables have been used for the statistical analysis of the data: (i) duration of pyrexia (rectal temperature > 38.5 °C), (ii) maximum temperature, (iii) duration of viraemia (number of days virus was isolated from blood samples), (iv) duration of nasal excretion of virus, (v) maximum virus excretion in a single day, and (vi) total virus excretion, calculated as the arithmetic sum of virus excretion values over the first 10 days post-infection. The study was a standard ‘Randomized Block’ experimental design (with challenge groups as blocks), with the vaccine treatment applied to two ponies and the control treatment applied to one pony in each group. For this experimental design, the appropriate analysis of variance (ANOVA) model is:

\[ Y_{ij} = \mu + \tau_i + \beta_j + e_{ij} \]
μ + Gᵢ + Tⱼ + eᵢⱼ, where Xᵢⱼ is the observed variable value for the pony in the i-th group receiving the j-th treatment, μ is the overall mean value, Gᵢ is the i-th group effect, Tⱼ is the j-th treatment effect and eᵢⱼ is the random error term, which is assumed to have normal distribution (mean = 0) and constant variance. For each variable studied, the mean ± SD was calculated as well as the ANOVA in order to test the null hypothesis of no difference in the effects of the two experimental treatments (vaccination versus no vaccination) on the variable concerned. The test for the null hypothesis is \( T = T_{fl} \), where \( T_{fl} \) is the control sample and \( T_{fl} \) is the vaccine sample; the standard variance ratio F test with 1 and 11 degrees of freedom was used. Details of the randomized block experimental design and analysis may be found in the following textbooks: Cochran & Cox (1957), Scheffe (1959) and Steel & Torrie (1980). The effect of pre-challenge VNAb titres upon subsequent virus shedding by the ponies was assessed using both correlation and regression relationships between pre-challenge titre values and total virus excretion.

**Results**

Previous studies demonstrated the induction of serum VNAb in ponies after intramuscular injection of Carbomer-PD-adjuvanted vaccines containing SP25–KLH and FP5Rsa1 (Chirnside et al., 1995a). We have extended these initial studies by testing the additional G₁-derived immunogens, in comparison with the original antigens, for their capacity to induce VNAb following two intramuscular inoculations administered 4 weeks apart. The additional antigens tested were Pep1–KLH (aa 81–106), which included a neutralizing antigenic site absent from polypeptides tested previously (aa 99–106), Pep2–KLH (aa 81–98) and a bacterially expressed polyhistidine-tagged protein, 6hisG₁ecto, which includes all regions previously identified as targets for neutralizing antibodies (Balasuriya et al., 1997; Chirnside et al., 1995a). A Coomassie-stained gel analysing the purified protein preparations for each of the bacterially expressed recombinant antigens is shown in Fig. 1. All antigens induced a strong antibody response, as measured by G₁-specific ELISA (data not shown). However, only ponies vaccinated with the recombinant proteins (160 µg purified recombinant protein used in each vaccination) induced detectable VNAb; the highest titres of VNAb were detected when 6hisG₁ecto was used as the antigen (data not shown).

In view of these initial results, we investigated further the ability of the 6hisG₁ecto antigen to serve as a sub-unit vaccine. Specifically, we aimed to determine whether a strong antibody response could be induced using a range of antigen doses and whether vaccinated ponies were protected against experimental challenge with EAV.

**Antibody response to the 6hisG₁ecto antigen**

The ability of different doses of the prototype sub-unit vaccine to elicit VNAb was evaluated in three groups of Welsh Mountain ponies, each group comprising one yearling colt and three 2-year-old geldings. Groups A (p1–4), B (p5–8) and C (p9–12) were vaccinated initially (V1) with 6hisG₁ecto doses of 140, 70 and 35 µg, respectively (administered intra-muscularly with the adjuvant). Results show that after the priming vaccination, a weak neutralizing antibody response was observed in groups B and C, but not in group A (Fig. 2). A second dose (V2) on week 5 (140 µg for group A, 70 µg for group B and 35 µg for group C) boosted the antibody response in all ponies, with the highest titres observed for ponies from group B (VNAb titres of 2.3–3.1). Five weeks after V2, two ponies from group A and an unvaccinated control pony were infected with EAV (challenge 1, described in more detail below). Protection was observed despite low VNAb titres for both vaccinates. A third vaccine dose (V3) was administered to all ponies on week 13 (140 µg for group A, 70 µg for group B and 35 µg for group C). This induced another peak in the antibody response, after which the two remaining ponies from group A were challenged on week 15 (challenge 2, described below). In this case, both vaccinates displayed a high degree of protection. The remaining ponies (groups B and C) were sampled over a period of 40 weeks to determine the duration of the antibody response. VNAb declined gradually over time and was still detectable in all ponies on week 27, but was undetectable by week 53. The kinetics of the anti-G₁ antibody response measured by ELISA were broadly in line with the observed VNAb response (data not shown). It should be noted, however, that the ELISA test was significantly more sensitive than the VN test. Thus, all ponies were seropositive by ELISA following V1; all samples at week 53 were also positive by ELISA, despite being below the threshold for detection of VNAb. Following the determination of the kinetics of VNAb induction and decline, the remaining ponies received a fourth vaccination (V4) comprising 35 µg 6hisG₁ecto on week 53. Following V4, VNAb responses were monitored and a series of EAV challenge infections were carried out to establish the protective efficacy of vaccination across a range of VNAb titres. The results from these challenges (Ch3–6), in addition to the previous challenges (Ch1 and 2) are described below.
Prototype sub-unit vaccine against EAV

Fig. 2. Neutralizing antibody responses in groups A (a), B (b) and C (c) after vaccination with 6hisG\textsubscript{ecto}. Vaccinations (open arrows) were performed on weeks 0, 5, 13 and 53. EAV challenges (closed arrows) were performed on weeks 10 (Ch1), 15 (Ch2), 55 (Ch3), 59 (Ch4), 65 (Ch5) and 72 (Ch6).
Protection against experimental infection

Previous challenge infections in naive ponies with Bucyrus LP3A indicated that intranasal administration of this virus (10^{6} TCID_{50}) resulted in reproducible clinical signs, virus shedding from the nasopharynx and cell-associated viraemia (D. Hannant, D. Westcott and J. Castillo-Olivares, unpublished observations).

The results of the six experimental challenge infections of the present study are summarized in Table 1. For each challenge, two vaccinates and one control pony were used. In all challenges, the control ponies became infected and presented a syndrome characterized by pyrexia (detected for 6–11 days with maximum temperatures between 40 and 40.5 °C), viraemia (detected for 6 or 7 days) and virus shedding from nasal secretions (detected for 8–11 days). All control ponies seroconverted to EAV by the VN test (seroconversion is defined as a fourfold increase in VNAb titre). Depression, mild conjunctivitis, sub-mandibular lymph node swelling, nasal discharge and diarrhoea were observed in some ponies.

Preliminary analysis of the challenge data indicated that vaccinated ponies showed a range of clinical and virological outcomes. It was clear that for the second challenge, vaccinated ponies were strongly protected against infection, with negligible pyrexia, viraemia or virus shedding in comparison to the control pony. Partial protection was observed even for the vaccines with undetectable VNAb (challenge 6, 19 weeks after V4), as judged by reduced severity and duration of pyrexia and substantially reduced virus shedding.

Protection against infection in vaccinated ponies was quantitatively evaluated according to objective and measurable variables consistently reproduced in the control ponies, namely the intensity and duration of pyrexia, duration of viraemia, duration of virus excretion, maximum virus excretion, total virus excretion [nasal swab samples with virus concentration below the detection limit (10^{1.75})] were assigned a value of

### Table 1. Clinical and virological results of experimental EAV infections performed in unvaccinated and 6hisG_{ecto}-vaccinated ponies

<table>
<thead>
<tr>
<th>Challenge group</th>
<th>Pony</th>
<th>VNAb titre</th>
<th>Pyrexia</th>
<th>Virus shedding nasal swabs</th>
<th>Clinical signs</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CONJ ND LNS DEP Other</td>
</tr>
<tr>
<td>1</td>
<td>p2</td>
<td>1:30</td>
<td>2:30</td>
<td>+</td>
<td>&lt; 1:75</td>
</tr>
<tr>
<td></td>
<td>p3</td>
<td>1:10</td>
<td>2:60</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
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<td>1:90</td>
<td>8</td>
<td>40:18</td>
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<tr>
<td></td>
<td>p4</td>
<td>2:25</td>
<td>3:33</td>
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</tr>
<tr>
<td>2</td>
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<td>2:10</td>
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<tr>
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<td>6</td>
<td>40:44</td>
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</tr>
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<td>3:15</td>
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<tr>
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<td>c6</td>
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Table 2. Summary of statistical analysis

Statistical analysis results comparing the effects of no vaccination (control, n = 6) and vaccination (vaccine, n = 12) for several variables indicative of severity of infection and disease following EAV infection. The mean ± SD for each of the variables is shown. Statistical significance was assessed using ANOVA. The F ratio (1 and 11 degrees of freedom) used to test the null hypothesis (no difference between control and vaccine treatments) and the significance probability assessed by this test are also shown.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Mean ± SD</th>
<th>Significance</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Vaccine</td>
</tr>
<tr>
<td>Pyrexia (days)</td>
<td>7.50 ± 1.87</td>
<td>2.33 ± 2.42</td>
</tr>
<tr>
<td>Pyrexia (maximum temperature °C)</td>
<td>40.35 ± 0.20</td>
<td>39.13 ± 0.78</td>
</tr>
<tr>
<td>Viraemia (days)</td>
<td>6.50 ± 0.55</td>
<td>1.92 ± 1.83</td>
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<tr>
<td>Virus nasal excretion (days)</td>
<td>8.83 ± 0.75</td>
<td>3.42 ± 3.00</td>
</tr>
<tr>
<td>Virus nasal excretion (maximum)</td>
<td>3.80 ± 0.86</td>
<td>1.78 ± 1.14</td>
</tr>
<tr>
<td>Virus nasal excretion (total)</td>
<td>4.24 ± 0.79</td>
<td>1.93 ± 1.19</td>
</tr>
</tbody>
</table>

In order to determine the significance of protection against each of these variables, the results of challenge experiments were compared between the 12 vaccinates and the six control ponies and the ANOVA was performed, as described in Methods (Table 2). It was found that the 6hisG_{1}ecto sub-unit vaccine provided significant protection (P < 0.01) against EAV infection, as judged by a reduction in pyrexia, cell-associated viraemia and virus shedding from the nasopharynx. Even those vaccinates with negligible VNAb titres at the time of challenge (19 weeks after V4) were partially protected, with a substantial reduction in virus shedding (over 100-fold) compared with the corresponding control pony (Table 1).

The relationship between VNAb titre at the time of challenge and total virus excretion is illustrated in Fig. 3. The
strong correlation displayed is consistent with the induction of VNAb as being the major determinant of protection.

Discussion

Previous studies have demonstrated the potential of G\textsubscript{1L}-derived antigens to elicit VNAb in ponies after vaccination (Chirnside et al., 1995a). In the present study, we initially compared the strength of VNAb responses elicited by either synthetic peptides derived from a previously identified immunodominant domain of G\textsubscript{1L} or bacterially expressed recombinant polypeptides comprising the majority or all of the predicted N-terminal ectodomain of G\textsubscript{1L}. We found that, although all the antigens tested were effective at inducing anti-G\textsubscript{1L} responses (as assessed by a G\textsubscript{1L}-specific ELISA), the virus-neutralizing capacity of the antibodies induced by the synthetic peptides was poor compared with the VNAb titres induced by bacterially expressed antigens, of which the 6hisG\textsubscript{1L} antigen induced the highest titres. Analysis of the nucleotide sequences of the ORF 5 of a wide range of EAV isolates and of mutant viruses escaping neutralization by G\textsubscript{1L}-specific monoclonal antibodies identified four regions in the G\textsubscript{1L} protein where neutralizing epitopes occur (Balasuriya et al., 1997). Of the antigens tested in this study, the 6hisG\textsubscript{1L} recombinant protein includes all four of these antigenic sites. Previous studies have indicated that both linear and conformational epitopes are recognized by neutralizing antibodies (Deregt et al., 1994; Balasuriya et al., 1997). It is uncertain whether the antibodies induced by the 6hisG\textsubscript{1L} antigen are restricted to recognition of linear rather than conformational epitopes. It is possible that the use of protein expression procedures in eukaryotic systems to produce glycosylated 6hisG\textsubscript{1L}ecto, combined with less stringent purification methods, might improve the induction of neutralizing antibodies. A recent report (Balasuriya et al., 2000) indicated that when G\textsubscript{1L} is expressed in eukaryotic cells, co-expression of M is required for the induction of neutralizing antibodies in mice. It is clear from our studies that the recombinant G\textsubscript{1L} ectodomain alone, expressed in bacteria, does form the epitopes that are required for the induction of neutralizing antibodies. It would be interesting to determine whether the G\textsubscript{1L}ectodomain expressed in eukaryotic systems is similarly able to elicit VNAb.

Having identified the 6hisG\textsubscript{1L}ecto protein as the antigen of choice, we conducted an extensive vaccination study using 18 ponies. Three doses of the antigen were compared to provide an indication of optimum antigen dose. We found that the medium (70 µg) and low (35 µg) doses performed similarly and, unlike the high dose (140 µg), induced detectable VNAb titres after the first vaccination. All animals developed VNAb after subsequent booster vaccinations, peaking 1–2 weeks after each vaccination and reaching titres of > 1:8 at their peak. The medium dose induced the highest VNAb titres following V2, with three ponies exhibiting titres of > 2:5. Two preliminary challenge infections were carried out, using ponies from the high-dose vaccination group, to establish whether the VNAb responses induced by vaccination were protective (described below). The results of the challenge experiments indicated that vaccination was protective, justifying continuation of the study to monitor the duration of VNAb responses and to determine the levels of protection in the remaining ponies.

Following three vaccinations, the VNAb titres in the medium- and low-dose groups were monitored for 40 weeks. For both groups, titres peaked 2–3 weeks after V3, but then declined gradually until week 53 when neutralizing activity was not detected in all vaccinates. However, anti-G\textsubscript{1L} antibody was still present, as assessed by ELISA (data not shown). The kinetics of VNAb development in horses vaccinated with formalin-inactivated virus (Fukunaga et al., 1984, 1991) are similar to those observed during this study. High titres were achieved 2 weeks after two vaccine doses were administered 4 weeks apart, which then progressively declined. A third dose boosted antibody levels, which then persisted for 6 months. Similarly, studies performed with the modified live vaccine showed that after a single dose of vaccine, the VNAb levels were relatively low and transient (Fukunaga et al., 1982; McKinnon et al., 1986; Timoney et al., 1988), but supplementary vaccine administration resulted in a good anamnestic response, with titres maintained for 9–12 months.

In order to evaluate the protective capacity of the immune responses generated by the 6hisG\textsubscript{1L}ecto protein, we challenged vaccinated and control ponies with Bucyrus LP3A, derived from the virulent Bucyrus strain. The challenge virus was found to consistently reproduce clinical signs and virus shedding/viraemia in EAV naive ponies (as described in this paper and unpublished observations). With the exception of pyrexia, the clinical signs observed in the control ponies were mild. Relatively mild symptoms were not entirely unexpected, since the virulent Bucyrus strain has been shown to lose its virulence rapidly after passage in tissue culture (McCollum, 1970; McCollum et al., 1961).

At present, there are no methods to evaluate cellular immunity against EAV, but serum VNAb are considered to be a good correlation of immunity (Fukunaga et al., 1990, 1991; McCollum, 1976). In this study, we have experimentally infected 18 ponies, each with different levels of VNAb at the time of challenge, and observed a correlation between the level of protection and the pre-challenge VNAb titre. The three ponies with VNAb titres of > 1:95 showed maximum protection. None of these animals seroconverted and all were negative for viraemia. No significant virus shedding was detected (nasal swab titres were < 10\textsuperscript{3.75} TCID\textsubscript{50}/ml) and, although two of the ponies were positive for virus shedding, this occurred on a single day in each case. It is, therefore, unlikely that an EAV infection had been established. These results are similar to those reported previously in horses vaccinated with formalin-inactivated virus, inducing VNAb titres of > 1:320 (equivalent to 2:3) (Fukunaga et al., 1990), although in this case, seroconversion to EAV after challenge...
was observed despite the lack of viraemia or clinical signs. Varying levels of partial protection were observed in the rest of the 6hisG\textsubscript{L} ecto vaccinates. All ponies with intermediate and low antibody levels, even those vaccinates with undetectable serum VNAb at the time of challenge, showed a reduction in pyrexia, nasal virus shedding and viraemia in comparison to the control ponies infected at the same time. Similar results have been described by Fukunaga et al. (1991) in their evaluation of the efficacy of a whole virus killed vaccine, where vaccinated horses with VNAb titres of $>1$ : $80$ (equivalent to $1$ : $9$) showed complete clinical protection against EAV. It was interesting to observe that after challenge, pony p5 became febrile by day 5 and virus began to be isolated from nasal swabs and peripheral blood samples collected after that day. This apparent increase in the incubation period may indicate that this animal resisted the initial challenge but, subsequently, became infected as a consequence of exposure to virus shed by the control pony. ‘In contact’ transmission of EAV from experimentally infected to susceptible seronegative mares has been reported previously (McCollum et al., 1987). This phenomenon raises the question of whether protection would have been better if controls and vaccinates had been kept separate after challenge. In our study, all unvaccinated ponies excreted large amounts of virus after challenge every day for 7–10 days.

In this study, we have assessed the protection afforded by the experimental vaccine against intranasal challenge. By analogy with previous studies, we anticipate that this vaccine has the potential to protect against venereal challenge, since efficacy studies performed with an inactivated whole virus vaccine showed similar results, independent of the route chosen to challenge the vaccinated animals (Fukunaga et al., 1990, 1997). In the case of ponies with VNAb titres of $>1$ : $95$ (the threshold for complete protection), viraemia was generally observed. Whether, under such conditions, there will be protection against abortion or the establishment of persistent infection in stallions is uncertain. However, the significant reduction in the duration and titre of virus shedding observed in vaccinates, even when VNAb titres were no longer detectable, suggests that sub-unit vaccination could provide significant protection against the respiratory spread of infection for at least 5 months following a boost.

We have used in the present study three viruses derived from the prototype Bucyrus strain: Bucyrus Utrecht was used for the preparation of recombinant antigen, Bucyrus CVL was used for the VN test and Bucyrus LP3A was used for challenge experiments. Plasmids for expression of recombinant G\textsubscript{L} were derived from cDNA from the Bucyrus Utrecht strain, which had been used to determine the complete genomic sequence of EAV (den Boon et al., 1991). The Bucyrus CVL strain has been developed as a standard reagent for EAV VN tests and was therefore utilized for the determination of VNAb titres in this study. Both of the above strains have been passaged in non-equine cells and were not considered suitable for use in experimental challenges. Hence, the third strain, Bucyrus LP3A, which is derived from the original Bucyrus isolate by in vivo passage (McCollum et al., 1971) followed by plaque purification and passage exclusively on equine cells (D. Westcott, unpublished data), was used as the challenge virus. As noted previously, Bucyrus LP3A had been observed to produce consistent virus shedding, viraemia and pyrexia upon infection of naive ponies. Alignment of the G\textsubscript{L} amino acid sequences of the three viruses used is shown in Fig. 4. Within the ectodomain region of the vaccine, two amino acid differences are observed between the vaccine sequence and the challenge virus and five amino acid changes between the vaccine sequence and the VN test virus. Notably, Bucyrus LP3A has preserved the potential N-linked glycosylation site (aa 81–83) of the original Bucyrus isolate (also found in the majority of field isolates; Stadejek et al., 1999), which is lost (N\textsubscript{asi} $\rightarrow$ D) in the other two isolates. Despite these changes, equine sera raised against the G\textsubscript{L} ectodomain of the Bucyrus Utrecht isolate effectively neutralized the Bucyrus CVL isolate in vitro and the Bucyrus LP3A isolate in vivo. Thus, any antigenic differences that may exist between the G\textsubscript{L} of the vaccine and the viruses used both to detect VNAb and for challenge experiments did not prevent
virus neutralization. A single serotype of EAV is recognized and previous studies (Balasuriya et al., 1997) demonstrated that four different post-infection equine sera were able to neutralize a wide range of different EAV isolates, although at varying titres. In view of the observed sequence variation of G1, further studies are required to evaluate the potential of G1 vaccination to protect against heterologous isolates of EAV representative of naturally occurring field isolates.

The combined use of the G1-based sub-unit vaccine and a differential diagnostic test against EAV would allow the discrimination of vaccinated from naturally infected horses. It has been reported that other EAV antigens, such as N and M, are frequently recognized by sera from EAV-infected horses and initial steps to develop ELISA diagnostic tests based on these antigens have been taken (Chirnside et al., 1995b; Hedges et al., 1998; Kheyar et al., 1997; MacLachlan et al., 1998). Thus, a G1-based sub-unit vaccine could be generally administered to horse populations without compromising disease surveillance by serological screening. This would be very useful in those areas of low EAV prevalence (such as the UK) or for the control of outbreaks of EAV by enabling the protection of susceptible populations by vaccination and, at the same time, permitting the surveillance of disease progression by serological testing.

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