Vaccination of sarcoid-bearing donkeys with chimeric virus-like particles of bovine papillomavirus type 1

G. H. Ashrafi,1† K. Piuko,2 F. Burden,3 Z. Yuan,1 E. A. Gault,1 M. Müller,2 A. Trawford,3 S. W. J. Reid,4 L. Nasir1 and M. S.Campo1

1Division of Pathological Sciences, Institute of Comparative Medicine, University of Glasgow, Glasgow G61 1QH, UK
2Deutsches Krebsforschungszentrum, Forschungsschwerpunkt Angewandte Tumorvirologie, Im Neuenheimer Feld 242, Heidelberg, Germany
3The Donkey Sanctuary, Veterinary Department, Salcombe Regis, Sidmouth, UK
4Comparative Epidemiology and Informatics, Institute of Comparative Medicine, University of Glasgow, Glasgow G61 1QH, UK

Equine sarcoids are fibroblastic skin tumours affecting equids worldwide. While the pathogenesis is not entirely understood, infection with bovine papillomavirus (BPV) type 1 (and less commonly type 2) has been implicated as a major factor in the disease process. Sarcoids very seldom regress and in fact often recrudesce following therapy. Nothing is known about the immune response of the equine host to BPV. Given that the viral genes are expressed in sarcoids, it is reasonable to assume that vaccination of animals against the expressed viral proteins would lead to the induction of an immune response against the antigens and possible tumour rejection. To this end we vaccinated sarcoid-bearing donkeys in a placebo-controlled trial using chimeric virus-like particles (CVLPs) comprising BPV-1 L1 and E7 proteins. The results show a tendency towards enhanced tumour regression and reduced progression in the vaccinated group compared to control animals. Although promising, further studies are required with larger animal groups to definitely conclude that vaccination with CVLPs is a potential therapy for the induction of sarcoid regression.

INTRODUCTION

Equine sarcoids are locally invasive, fibroblastic skin tumours and represent the most common skin tumour in equids worldwide (Jackson, 1936; Ragland et al., 1970; Pascoe & Summers, 1981). Sarcoids may exist as single or multiple lesions and six clinical types are recognized: occult, verrucous, nodular, fibroblastic, mixed and malignant (Knottenbelt, 2005). Currently there is no effective therapy available for sarcoids (Marti et al., 1993).

Bovine papillomavirus (BPV) types 1 and 2 infection is causally associated with the pathogenesis of the sarcoid. Many studies have reported the presence and expression of viral DNA in sarcoids supporting active papillomavirus infection (reviewed by Chambers et al., 2003; Nasir & Reid, 2006). In contrast, BPV is not found in normal skin or in non-sarcoid tumours such as melanomas, papillomas and squamous cell carcinomas (Chambers et al., 2003).

The papillomaviruses (PVs) belong to a large family of animal and human PV that normally infect epithelial cells causing benign hyperproliferative lesions (warts, papillomas, fibropapillomas) which can progress to cancer (Campo, 2006). Although PVs are normally strictly species-specific, sarcoids represent the only documented case of natural cross-species PV infection (Lancaster et al., 1979; Gorman, 1985).

However, there are numerous differences between BPV-1/2 infection in cattle and in equids. BPV-1/2 infection in cattle results in benign cutaneous fibropapillomas, productive for infectious progeny virus, which regress in response to a cell-mediated immune response (Okabayashi et al., 1991; Coleman et al., 1994; Frazer, 1996; Knowles et al., 1996). In contrast with the normal course of BPV disease in cattle, infection with BPV-1/2 in horses can result in sarcoids, lesions which are non-permissive for virus production (Gorman, 1985), locally aggressive and seldom regressing (Ragland et al., 1970; Knottenbelt, 2005). The equine sarcoid has more similarities with BPV-2-induced cancers of the urinary bladder in cattle. In both cancers, no viral
particles have been detected, the viral DNA is present in variable multiple episomal copies (but maintained at a significantly higher genome copy number in sarcoids than in cattle bladder cancers) and many viral genes are expressed, including E5, E6 and E7 (Yuan et al., 2006; Mattil-Fritz et al., 2007; our unpublished observations). Like bovine urinary bladder cancers, sarcoids rarely regress.

Little is known about the immune response to BPV antigens in sarcoid-bearing equids. Early BPV-based vaccines in the therapy of sarcoids proved equivocal (Ragland et al. 1970; Roberts, 1970). However, an extensive anti-BPV vaccination programme in cattle showed that the capsid proteins of the virus are effective prophylactic vaccines, both as proteins or peptides (Jarrett et al., 1991; Campo et al., 1993, 1997; Chandrachud et al., 1995) or assembled in virus-like particles (VLPs) (Kirnbauer et al., 1996), and that the viral protein E7 is effective in promoting early tumour regression (Campo et al., 1993). Chimeric VLPs (CVLPs), comprising L1 and E7 (Müller et al., 1991; Campo et al., 1997), have been used successfully for the prevention and treatment of experimentally induced tumours (Giroglou et al., 2001). In this and in the accompanying paper (Mattil-Fritz et al., 2007) we show that vaccination with CVLPs of horses and donkeys bearing BPV-1-associated sarcoids resulted in a partial beneficial response.

**METHODS**

**Selection of donkeys.** Seventeen donkeys with sarcoids were selected for the trial, comprising seven mares and ten geldings with a mean age of 18 years (range 7–31 years old) (Table 1). The donkeys were housed in one location at Slade House Farm in Devon, a site owned and managed by the Donkey Sanctuary. All donkeys were examined by a veterinary surgeon before entering the trial and blood samples were analysed to minimize the possibility of underlying disease. The selected donkeys had not undergone any previous medical treatment for sarcoid.

**Experimental design.** The donkeys were allocated into either a treatment (group 1, eight animals) or control group (group 2, nine animals) using random numbers. Only the coordinator and veterinary surgeon responsible for treatment were aware of the group the animals were allocated to.

**Generation of BPV-1 L1–E7 CVLPs.** BPV-1 L1–E7 CVLPs were purified as described in the accompanying paper (Mattil-Fritz et al., 2008). Briefly, TN-High Five cells (Invitrogen) were infected with recombinant baculovirus encoding the BPV1 L1–E7 fusion gene at a m.o.i. of 5 (Müller et al., 1997). Cells were harvested by centrifugation (15 min, 4 °C, 3500 g) and resuspended in extraction buffer (5 mM MgCl₂, 5 mM CaCl₂, 150 mM NaCl, 0.01 % Triton X-100, 20 mM HEPES, pH 7.4) containing 200 μl 0.1 M PMSF and protease inhibitor cocktail (Calbiochem) to a final volume of 20 ml. Cells were lysed and supernatant was cleared by centrifugation (30 min, 4 °C, 11 000 g).

Supernatant was loaded onto a two-step gradient consisting of 8 ml CaCl₂ solution (4.7 g CaCl₂ in 8 ml extraction buffer) above 7 ml sucrose solution (40 %) and centrifuged for 2 h, 10 °C, 100 000 g. The CVLP-containing interface between sucrose and CaCl₂ was centrifuged for 16 h at 20 °C, 50000 r.p.m. 500 μl fractions were collected and analysed by ELISA, Western blot and electron microscopy for the presence of particles. Pooled fractions were dialysed (PBS, 0.5M NaCl) overnight at 4 °C.

**Vaccination protocol.** The CVLP vaccine was delivered in doses of 80 μg CVLPs in PBS at 2 or 3 week intervals. The placebo consisted of PBS.

**Table 1. Vaccination of sarcoid-bearing donkeys with CVLPs**

The experimental number, age, sex and status of the animals are indicated. The cumulative volume of the lesions was calculated as described in Methods. Tumour regression is highlighted in bold.

<table>
<thead>
<tr>
<th>Number</th>
<th>Age</th>
<th>Sex</th>
<th>Site</th>
<th>Treatment</th>
<th>Cumulative lesion volume in cm³</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>At day 0</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>At EoT</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>23</td>
<td>Gelding</td>
<td>Thigh</td>
<td>Vaccine</td>
<td>2.2</td>
<td>Stationary</td>
</tr>
<tr>
<td>7</td>
<td>13</td>
<td>Gelding</td>
<td>Sheath</td>
<td>Vaccine</td>
<td>4.32 + 3.52 + 6.25</td>
<td>163.5 Progression</td>
</tr>
<tr>
<td>9</td>
<td>24</td>
<td>Female</td>
<td>Eye lid</td>
<td>Vaccine</td>
<td>0.12</td>
<td>0.087 Stationary</td>
</tr>
<tr>
<td>10</td>
<td>7</td>
<td>Gelding</td>
<td>Lip</td>
<td>Vaccine</td>
<td>5</td>
<td>1.95 Regression</td>
</tr>
<tr>
<td>11</td>
<td>22</td>
<td>Gelding</td>
<td>Lip</td>
<td>Vaccine</td>
<td>131</td>
<td>10.8 + 3.4 + 1.56 + 1.48 Regression</td>
</tr>
<tr>
<td>12</td>
<td>11</td>
<td>Female</td>
<td>Ear</td>
<td>Vaccine</td>
<td>1.44</td>
<td>0.084 Regression</td>
</tr>
<tr>
<td>14</td>
<td>21</td>
<td>Female</td>
<td>Brisket</td>
<td>Vaccine</td>
<td>60 + 0.5 + 1 + 1 + 4</td>
<td>61 + 0.5 + 1 + 1 + 4.1 Stationary</td>
</tr>
<tr>
<td>17</td>
<td>9</td>
<td>Female</td>
<td>Thigh</td>
<td>Vaccine</td>
<td>0.48</td>
<td>0.16 Stationary</td>
</tr>
<tr>
<td>3</td>
<td>23</td>
<td>Gelding</td>
<td>Eye</td>
<td>Control</td>
<td>0.77</td>
<td>0.6 Stationary</td>
</tr>
<tr>
<td>4</td>
<td>13</td>
<td>Female</td>
<td>Navel</td>
<td>Control</td>
<td>4.32</td>
<td>2.25 Stationary</td>
</tr>
<tr>
<td>5</td>
<td>31</td>
<td>Female</td>
<td>Nasal septum</td>
<td>Control</td>
<td>0.51</td>
<td>0.24 Regression</td>
</tr>
<tr>
<td>6</td>
<td>31</td>
<td>Gelding</td>
<td>Sheath</td>
<td>Control</td>
<td>12.8</td>
<td>23.6 Progression</td>
</tr>
<tr>
<td>8</td>
<td>20</td>
<td>Gelding</td>
<td>Vestigial teats</td>
<td>Control</td>
<td>15.6 + 5.83</td>
<td>18.75 + 26.25 Progression</td>
</tr>
<tr>
<td>13</td>
<td>12</td>
<td>Female</td>
<td>Udder</td>
<td>Control</td>
<td>181.4</td>
<td>145.6 Stationary</td>
</tr>
<tr>
<td>15</td>
<td>29</td>
<td>Gelding</td>
<td>Eye lid</td>
<td>Control</td>
<td>0.08 + 1.32 + 6.9 + 1.4</td>
<td>1 Regression</td>
</tr>
<tr>
<td>16</td>
<td>13</td>
<td>Gelding</td>
<td>Eye</td>
<td>Control</td>
<td>10.2</td>
<td>15.3 Progression</td>
</tr>
<tr>
<td>18</td>
<td>13</td>
<td>Gelding</td>
<td>Sheath</td>
<td>Control</td>
<td>9.2</td>
<td>4.8 Stationary</td>
</tr>
</tbody>
</table>
Donkeys received vaccine or placebo at days 0, 14, 35, 49, 70, 95 and 112 by intramuscular injection. The veterinarian administering the vaccine was assisted by the trial coordinator; the treatment administered to each individual animal was unknown to all others involved in the project. The trial was carried out over 5 months in full accordance with the Directives of the Home Office of Great Britain.

Clinical examination and measurements of sarcoids. All donkeys in the trial were monitored by an independent veterinary surgeon (not aware of the treatments) to ensure that any change in the sarcoids had no impact on the welfare of the trial animals. At the start of the trial (day 0), sarcoids were photographed and sarcoid characteristics were recorded, including number of tumours per animal, tumour size, body location, physical appearance and mobility. Sarcoids were measured at the end of the trial (EoT; day 132). The cumulative volume of the lesions was measured in cm³ (width × length × depth). A change in volume between day 0 and EoT of more than 50 % was taken as indicative of tumour progression or regression, while a difference of less than 50 % was indicative of a stationary tumour.

During the trial one donkey (donkey 11) was euthanised on day 114 for reasons unrelated to the trial. During the post-mortem examination, measurements and assessments of the sarcoids were made. Sarcoid tissue was collected and stored below −50 °C for reasons unrelated to the trial. During the post-mortem examination, measurements and assessments of the sarcoids were made. Sarcoid tissue was collected and stored below −50 °C for reasons unrelated to the trial.

Statistical analysis. Statistical analysis was performed using Fisher’s exact test because of the small animal numbers. Sample size calculations were performed on the basis of the results of this study. Assuming that the responses observed here would be the same as in other experiments, power was set at the 80 % level and statistical significance at the 5 % level.

Sarcoid cell collection and measurement of viral DNA load. To monitor the load of viral DNA before and after vaccination, the surface of the sarcoids was swabbed with cotton buds at day 0 and at EoT. DNA was extracted from the swabs using the Qiagen QIAamp kit as described in the Qiagen DNA Micro Handbook. Before quantitative PCR (Q-PCR), the DNA samples were tested for viral DNA by amplifying the viral E7 gene by PCR using the primers previously described (Yuan et al., 2007). All samples were positive and were subsequently processed through Q-PCR, using a BPV E7 primer/probe set as described previously (Yuan et al., 2006, 2007). Briefly, Q-PCR reaction mixtures contained 0.2 μM forward and reverse primers, 0.1 μM probe, 25 μl of 2 x Platinum Quantitative PCR SuperMix-UDG (Invitrogen), 1 μl ROX Reference Dye (Invitrogen) and water to a final volume of 45 μl. Five μl of 0.01–0.1 μg sarcom DNA was added, and reactions were performed in a spectrophotometric thermo-cycler (7500 Real-Time PCR System; Applied Biosystems). 2 min at 50 °C, 2 min at 95 °C, and 60 cycles of 15 s at 95 °C and 45 s at 60 °C. Serial dilutions of a BPV-1 plasmid were used to generate a standard curve and an equine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene primer/probe set as an internal control for normalization of input DNA (GAPDH_F: gggtgagccaaagggctat; GAPDH_R: ttcacgcccatcaacaat; GAPDH_P: FAM-actctctgctccttgctatgcccc-TAMRA). Q-PCR of each sample was carried out in triplicate. BPV viral loads were calculated as number of BPV genome copies per cell genome equivalent.

Enzyme-Linked ImmunoSorbent Assay (ELISA)

CVLP. Sera from control and vaccinated donkeys were collected at day 0 and 2–3 weeks after each vaccination and analysed for the presence of antibodies to CVLPs. Micro-ELISA plates (96-well plate, C-bottom, Immulon 4HBX; Fisher Scientific, UK) were coated with 50 μl of monoclonal antibody (mAb 3.6.7.2; M. Müller, unpublished data) for BPV-1 L1–E7 CVLP capture at a dilution of 1:300 in PBS. After overnight incubation at 4 °C, plates were washed three times with 0.3 % Tween 20 in PBS (T-PBS) and blocked with 100 μl blocking solution (3 % skimmed milk, in T-PBS) for 1 h at 37 °C. A 1:100 dilution of BPV-1 CVLPs in blocking solution was added to each well (12.5 μg per well) and incubated for 1 h at 37 °C. Sera from control and vaccinated donkeys (50 μl) were added at a 1:500 dilution in blocking solution and incubated at 37 °C for 1 h. The plates were washed and incubated with a 1:20 000 dilution of horseradish peroxidase (HRP)-labelled goat polyclonal anti-donkey IgG H&L (HRP) (ab6988, Abcam) for 1 h at 37 °C in the dark. After incubating and washing, 100 μl of substrate solution (ABTS Peroxidase substrate system (2-component); KPL) was added and the reaction was stopped after 30–40 min by adding an equal volume of ABTS Peroxidase stop solution (KPL). The plates were analysed at 405 nm using an ELISA plate reader and Ascent software (Thermo Scientific).

Preparation of GST proteins. Rosetta bacteria transformed with expression constructs for GST–BPV-1 L1, GST–BPV-1 E7 or GST alone (pGEX vector system, Invitrogen) were grown in 500 ml LB medium containing 1 mM ampicillin to an optical density of 0.9 at 595 nm at 37 °C. After cooling to 9 °C, 100 μM IPTG was added to induce expression of the fusion proteins and bacteria were incubated overnight (12 °C, 120 r.p.m.).

Bacteria were harvested by centrifugation (3500 g, 4 °C, 10 min) and pellets were resuspended in 50 ml extraction buffer (20 mM HEPES, pH 7.2; 200 mM potassium acetate; 2 mM magnesium acetate; 0.5 % Tween 20, 1 mM DTT) and protease inhibitor cocktail (Calbiochem). Bacteria were lysed using a high pressure homogenizer (Avestin) and lysates were cleared by centrifugation (173 000 g, 4 °C, 60 min). For storage at −20 °C, glycerol was added to a final concentration of 50 %. Total protein amount was determined by colorimetric means using the Bradford assay.

GST-capture ELISA. To determine antibody reactivity against L1 or E7 proteins, sera from CVLP-immunized and control donkeys were analysed by GST-capture ELISA. Briefly, 96-well ELISA plates were coated overnight at 4 °C with 200 ng well−1 of glutathione-casein in 50 mM carbonate buffer, pH 9.6. After washing the plates with T-PBS (washing buffer), wells were blocked for 1 h at 37 °C with 0.2 % (w/v) casein in T-PBS (blocking buffer). Blocking buffer was discarded and cleared lysates from bacteria expressing GST–L1, GST–E7 fusion proteins or GST alone were diluted to 0.25 μg μl−1 total protein in blocking buffer and 50 μl was added to wells. After incubation for 1 h at 37 °C, wells were washed with washing buffer. Donkey sera diluted 1:100 in blocking buffer (50 μl) were added to wells containing GST–L1, GST–E7 or GST alone and incubated for 1 h at 37 °C. Following washing as above, 50 μl of secondary anti-horse IgG antibody (cross-reactive with donkey IgG; Dianova) diluted 1:3000 was added to each well. After 1 h incubation at 37 °C plates were washed to remove unbound antibody. Finally, 100 μl of substrate [0.5 ml ABTS (stock 1 mg in 50 ml H2O) plus 4 μl 50 % H2O2 solution in 10 ml 100 mM sodium acetate; 50 mM NaH2PO4 pH 4.2] was added to each well and colour development was measured at 405 nm after 10 min using a multilabel plate reader (Perkin Elmer).

Neutralization assay. To determine the neutralizing properties of donkey sera immunized with CVLPs, sera were analysed using a pseudovirus neutralization assay. BPV-1 pseudovirions used in this assay were produced as described elsewhere (Mejia et al., 2006) by co-transfection of 293TT cells with expression plasmids encoding BPV-1 L1 and L2 and secreted alkaline phosphatase (SEAP) as reporter enzyme. For the neutralization assay 1 × 105 293TT cells per well were seeded in a 96-well plate. BPV-1 pseudovirions were pre-diluted 1:5000 in complete Dulbecco’s modified Eagle’s Medium (D-MEM) and subsequently donkey sera (pre-blood and at day 111) were diluted 1:500 in pseudovirus pre-dilution and incubated for 10 min
at room temperature. After discarding the supernatant 50 μl of serum-dilutions was added to the cells and incubated for 5 days (37 °C, 5% CO₂). Finally, 50 μl of cell supernatant was analysed for SEAP expression using a SEAP-reporter assay (Roche), following the manufacturer’s instructions. Light emission was analysed using a multilabel plate counter (Victor3, PerkinElmer). Wells with medium only were included as controls; as positive control, a neutralizing anti-BPV-1 L1 VLP rabbit serum (#10, 1:1000; Mattil-Fritz et al., 2007) was used; neutralization by this serum was set at 100% and used as reference. Sera showing a reduction in SEAP activity of 75% or more were considered to be neutralizing.

RESULTS

The crude incidence of sarcoid in donkeys at the Donkey Sanctuary is 0.6 cases animal⁻¹ year⁻¹ (Reid et al., 1994). Seventeen sarcoid-bearing donkeys were divided at random into two groups, one group of eight donkeys (group 1; numbers 1,7,9,10,11,12,14,17; age range 7–24 year old, average 16.2, median 13) and one group of nine donkeys (group 2; numbers 3,4,5,6,8,13,15,16,18; age range 12–31, average 20.5, median 20). Sarcoid size ranged from 0.12 cm³ to 60 cm³ in group 1 (average 15.77, median 2.86) and from 0.51 cm³ to 181.4 cm³ in group 2 (average 23.8, median 5.61). Two animals in group 1 and one animal in group 2 had multiple sarcoids (Table 1). The sarcoids covered the spectrum of clinical types (Knottenbelt, 2005), with the exception of type six, malignant (data not shown). Animals in group 1 were vaccinated; animals in group 2 were treated with a placebo.

Vaccination

The vaccine consisted of CVLPs in PBS at a concentration of 0.44 μg μl⁻¹. Each vaccine dose consisted of 80 μg of CVLPs made up to 1 ml in PBS. Animals in group 1 received six doses at approximately 3 week intervals by intramuscular injection. Animals in group 2 were injected with 1 ml PBS per dose. Sarcoid size was measured at the beginning of the trial (day 0) and five months later at the end of the trial (EoT). Tumour volume was calculated in cm³ by multiplying width by depth by length. Sarcoid development was monitored periodically to ensure the animals were not undergoing progressive disease that would necessitate removal from the trial.

Development of sarcoids

Animals were defined as progressors, regressors or stationary according to whether the size of their sarcoids increased or decreased by more than 50% of the total volume, or varied by less than 50% of the total volume. The 50% cut-off was chosen because of the inherent difficulty in measuring the tumours, particularly when they are deep in the derma or in awkward sites.

Group 1. Animal 1 had a single non-ulcerated sarcoid of 2.2 cm³. At EoT, the sarcoid measured 1.77 cm³. No significant change had taken place in either the shape or the morphology of the tumour, and the disease was considered stationary.

Animal 7 had one large sarcoid with three lobes of 4.32, 3.52 and 6.25 cm³. At EoT, the tumours had coalesced into a single non-ulcerated large mass of 163.5 cm³. There was definite disease progression.

Animal 9 had a small soft mobile sarcoid of 0.12 cm³. This sarcoid had almost disappeared at EoT, leaving a very small lesion, difficult to measure, of approximately 0.087 cm³. Although the sarcoid appeared to have regressed clinically, the change in volume was less than 50% and the tumour was considered stationary.

Animal 10 had a slight ulcerated sarcoid of 5 cm³. At EoT the sarcoid was non-ulcerated and measured only 1.95 cm³. Regression had taken place.

Animal 11 had a very large partly ulcerated sarcoid measuring 131 cm³. At EoT, this large mass has broken into four non-ulcerated smaller tumours, the largest of which was 10.8 cm³, and the other three measured 3.4, 1.56 and 1.48 cm³. Breakage of the large tumour mass into much smaller tumours suggested that a degree of regression had occurred.

Animal 12 had a hairless flaky tumour of 1.44 cm³. At EoT the tumour had almost disappeared and only a small lesion of 0.084 cm³ was left. This animal was diagnosed as a regressor.

Animal 14 had five sarcoids at different sites, the largest of which was ulcerated and measured 60 cm³; a second crusty non-ulcerated sarcoid measured 0.5 cm³; the third non ulcerated tumour measured 1 cm³; the fourth and fifth sarcoids were non-ulcerated and measured 1 cm³ and 4 cm³, respectively. At EoT the tumour measurements did not differ and the animal was diagnosed as stationary.

Animal 17 had a round, crusty and ulcerated sarcoid measuring 0.48 cm³. At EoT the tumour was no longer crusty, was shallower, smooth and non-ulcerated; it measured 0.16 cm³. This sarcoid had definitely undergone regression.

Thus in group 1, four of the eight animals (50%) had undergone different degrees of regression, in one animal the tumours progressed and in three animals the tumours were stationary.

Group 2. Animal 3 had a smooth, non-ulcerated, hard, mobile sarcoid measuring 0.77 cm³. At EoT, the sarcoid was hard, hairless in places, not ulcerated and measured 0.6 cm³. This tumour was deemed to be stationary.

Animal 4 (female, 13 years old) had a soft, round sarcoid, measuring 4.32 cm³. At EoT this tumour had undergone very little morphological change and measured 2.25 cm³. The change in size of this tumour was close to, but less than 50% and hence was regarded as stationary.

Animal 5 had a flaky but non-ulcerated sarcoid, measuring 0.51 cm³. This sarcoid measured 0.24 cm³ at EoT
and, although borderline, it was diagnosed as having regressed.

Animal 6 had a soft, mobile, smooth, pink, slightly ulcerated sarcoid measuring 12.8 cm$^3$. At EoT, the tumour had progressed: it was mushroom-like, covered in flaky skin and very soft; it measured 23.6 cm$^3$.

Animal 8 had two round, soft and smooth sarcoids, the left one measuring 5.8 cm$^3$ and the right one 11.1 cm$^3$. At EoT the two sarcoids had definitely progressed and grown in size, the left sarcoid measuring 18.75 cm$^3$ and the right one 26.25 cm$^3$.

Animal 13 had four hard non ulcerated sarcoids coalescing in a very large volume of 181.4 cm$^3$. At EoT, the sarcoids had grown in a grape-like bunch, very deep in the skin, non mobile and very hard. The tumour mass measured 145.6 cm$^3$ and the disease was defined as stationary.

Animal 15 had four sarcoids, the first of which was verrucous, spongy and slightly ulcerated and measured 0.08 cm$^3$; the second and third sarcoids were hard and measured 1.32 and 6.9 cm$^3$; the fourth sarcoid was hairless and mobile and measured 1.4 cm$^3$. At EoT, only one sarcoid was left, of 1 cm$^3$. The tumours had regressed.

Animal 16 had three adjacent hairless ulcerated sarcoids, spongy in the lower side and hard on the upper side, the whole tumour mass measuring 10.2 cm$^3$. At EoT, the sarcoids were very hard, hairless, nodular and non-mobile. The whole area measured 15.3 cm$^3$. The tumours in this animal had progressed.

Animal 18 had a soft, mobile and smooth sarcoid measuring 9.2 cm$^3$. At EoT the sarcoid had not changed in its appearance and measured 4.8 cm$^3$. The change in size of this tumour was close to, but less than 50% and hence was regarded as stationary.

Thus in group 2, the sarcoids in two out of nine animals (22%) had regressed, in four (44%) remained stationary, and in three (30%) progressed.

When the reduction in tumour mass is considered, the difference in the responses to the vaccination in these two small groups was not significant at the 5% level ($P \geq 0.05$; Fisher’s exact test) between day 0 and EoT in either group. Nevertheless, there is a tendency for the vaccinated animals to be more likely to regress, as likely to be stationary and less likely to progress than the animals in the control group. Assuming regression would occur in 22% of non-vaccinated animals and in 50% of vaccinees, sample size calculations suggest that around 44 animals in each group would be required for this effect size to be statistically significant. The results are presented in Table 1 and Fig. 1.

**Viruses DNA load**

To ascertain whether sarcoid regression was accompanied by a decrease in viral DNA load, the sarcoids were swabbed before vaccination and at EoT to collect tumour cells from which DNA was extracted. The load of BPV-1 DNA was measured by quantitative PCR (Q-PCR). As reported (Yuan et al., 2006), there was a wide variation in viral DNA load per cell among the animals. At the beginning of the trial, the viral load in group 1 ranged from 0.04 genome equivalents (g.e.) to 15.9 g.e. (average: 4.7 g.e.), and at EoT from 0.39 g.e. to 84 g.e. (average: 10.5 g.e.). In group 2, at day 0 the viral load ranged from undetectable to 36 g.e. (average: 7.7 g.e.), and at EoT from undetectable to 10.8 g.e. (average: 1.7 g.e.) (data not shown). The viral load varied markedly between sarcoids and between day 0 and EoT; there was no correlation between pre- and post-vaccine viral loads, or between regressed, stationary or progressive tumours (data not shown).

**Humoral response to CVLPs and their components**

Sera from vaccinated and control animals were tested for the production of antibodies to the vaccine CVLPs and to their components L1 and E7 by ELISA.

**Humoral response to CVLPs.** Sera were analysed at day 0 and at each time point during vaccination. At day 0 most of the sera from control animals gave no response, apart from animals 6 and 8 which gave a weak response. Although the response varied during the course of the trial, it remained very low (Fig. 2, Table 3). In contrast and as expected, in the vaccinated group the response was higher in all the animals and, although somewhat variable, was sustained throughout the trial (Fig. 2, Table 2).
Humoral response to L1. Sera were analysed for antibody response to the L1 component of the vaccine at day 0 and at EoT. The response to L1 was poor at day 0 in most animals, although some animals, including animal 13 in the control group, had a measurable response, possibly due to residual antibodies to the original infection (Fig. 3a, Table 2). At EoT, the response had increased somewhat in the vaccinated animals, with the highest amplitude detected in animal 10 (Fig. 3a, Table 2).

Thus, both the antibody response to CVLPs and that to L1 was dependent on vaccination, but there appeared to be no relationship between amplitude of response and tumour regression. The generally lower response to L1 compared to the vaccinated animals, with the highest amplitude detected in animal 10 (Fig. 3a, Table 2).

### Table 2. Humoral response to CVLPs, L1 and E7 in vaccinated and control animals at day 0 and at EoT

<table>
<thead>
<tr>
<th>Number</th>
<th>Treatment</th>
<th>Anti-CVLP Ab</th>
<th>Anti-L1 Ab</th>
<th>Anti-E7 Ab</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 0</td>
<td>EoT</td>
<td>Day 0</td>
</tr>
<tr>
<td>1</td>
<td>Vaccine</td>
<td>0.200</td>
<td>1.565</td>
<td>0.1</td>
</tr>
<tr>
<td>7</td>
<td>Vaccine</td>
<td>0.000</td>
<td>0.473</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>Vaccine</td>
<td>0.324</td>
<td>0.710</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>Vaccine</td>
<td>0.000</td>
<td>0.791</td>
<td>0.15</td>
</tr>
<tr>
<td>11</td>
<td>Vaccine</td>
<td>0.129</td>
<td>0.617</td>
<td>0.05</td>
</tr>
<tr>
<td>12</td>
<td>Vaccine</td>
<td>0.139</td>
<td>0.558</td>
<td>0.06</td>
</tr>
<tr>
<td>14</td>
<td>Vaccine</td>
<td>0.053</td>
<td>0.031</td>
<td>0.3</td>
</tr>
<tr>
<td>17</td>
<td>Vaccine</td>
<td>0.114</td>
<td>0.150</td>
<td>0.25</td>
</tr>
<tr>
<td>3</td>
<td>Control</td>
<td>0.000</td>
<td>0.000</td>
<td>0.1</td>
</tr>
<tr>
<td>4</td>
<td>Control</td>
<td>0.000</td>
<td>0.000</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>Control</td>
<td>0.000</td>
<td>0.045</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>Control</td>
<td>0.174</td>
<td>0.071</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>Control</td>
<td>0.068</td>
<td>0.000</td>
<td>0.2</td>
</tr>
<tr>
<td>13</td>
<td>Control</td>
<td>0.000</td>
<td>0.310</td>
<td>0.7</td>
</tr>
<tr>
<td>15</td>
<td>Control</td>
<td>0.000</td>
<td>0.220</td>
<td>0</td>
</tr>
<tr>
<td>16</td>
<td>Control</td>
<td>0.000</td>
<td>0.046</td>
<td>0</td>
</tr>
<tr>
<td>18</td>
<td>Control</td>
<td>0.000</td>
<td>0.005</td>
<td>0</td>
</tr>
</tbody>
</table>
CVLPs is in agreement with the induction of conformational antibodies by VLPs rather than antibodies against the linear epitopes present in L1.

**Humoral response to E7.** Sera were analysed for antibody response to the E7 component of the vaccine at day 0 and at EoT. Some animals in both groups had detectable antibodies to E7 at day 0, possibly due to a response to the E7 protein produced in the tumours (Fig. 3b, Table 2). Animal 14 in the vaccinated group and animal 13 in the control group showed the highest response, but neither in these nor in the majority of animals did the response increase significantly after vaccination or at EoT. There were no major differences between the vaccinated and the control animals, confirming the hypothesis that the response to E7 was due to E7 present in the sarcoids more than to E7 present in the vaccine.

As for CVLPs and L1, there was no correlation between antibody response to E7 and the outcome of the vaccination.

**Virus neutralization**

Finally, we established the presence of neutralizing antibodies in the donkey sera by monitoring the neutralization of BPV-1 pseudovirus at day 0 and at day 111 as described in the accompanying paper (Mattil-Fritz et al., 2007). Sera inducing a reduction in SEAP activity of 75% were considered to be neutralizing. According to this cut-off, only animal 12 in the vaccinated group had serum neutralizing antibodies at day 0. At day 111, all the vaccinated donkeys had developed neutralizing antibodies (Fig. 4, Table 3). Surprisingly, the serum of donkey 13 in the control group developed neutralizing activity at day 111 (Fig. 4, Table 3), although it showed little or no anti-CVLP antibodies (Fig. 2). The experiment has been repeated four times with the same result, so an artefact has to be excluded. We cannot explain this observation.

**DISCUSSION**

No effective therapy is available for equine sarcoids. BPV is one of the causes of this tumour and the expression of viral antigens in sarcoids (Nasir & Reid, 2006) presents the attractive possibility of vaccination against virus and/or virus-harboursing sarcoideal cells.

An extensive vaccination programme against BPV in cattle has shown that infection can be prevented by, among...
others, VLP vaccines and that pre-malignant lesions can be induced to regress by vaccination with E7 (Campos, 2006). A possible therapeutic effect of VLPs was suggested by the relatively early regression of pre-malignant lesions in VLP-vaccinated calves compared to control animals (Kirnbauer et al., 1996).

Similar antigens have been used for vaccines against other animal papillomaviruses and against human papillomavirus. A tetravalent prophylactic vaccine made up of VLPs of HPV-16, -18, -6 and -11 is now available (European Medicines Agency, 2006; Saslow et al., 2007) while therapeutic vaccines based on HPV-16 and HPV-18 E7 are currently at different stages of clinical trial (Govan, 2005; Wu, 2007).

Chimeric VLPs composed of L1 and E7 have been shown to induce cytotoxic T-cell responses in pre-clinical models. Like VLPs, CVLPs are able to enter and activate antigen-presenting cells leading to major histocompatibility complex (MHC) class I restricted presentation of L1 and E7. In addition, like VLPs, CVLPs are able to induce high titre capsid-specific neutralizing antibody responses (Roden & Viscidi, 2006).

**Vaccination with CVLPs induces partial sarcoid regression in donkeys**

The placebo-controlled CVLP vaccination trial in donkeys was encouraging. However, definitive work will require larger sample sizes, which, on the basis of the observed regression rates, would be in the order of 44 animals per group. Given the annual sarcoid incidence rate of approximately 0.6% (Reid et al., 1994), studies of this scale are prohibitive.

Although the results were not statistically significant, a tendency could be discerned. In the vaccinated group regression was observed in four out of eight animals, sarcoids were stationary in three animals and in only one animal the sarcoids progressed. Furthermore, in two out of three donkeys, with very heavy and increasing tumour burden, which were vaccinated on humanitarian grounds and were not included in the trial, the sarcoids have remained stable for the first time since the animals have been under observation (data not shown).

Sarcoïd regression was not age or sex dependent: the youngest donkey undergoing regression was a seven-year-old gelding (animal 10) in the vaccine group and the oldest donkey was a 31-year-old female (animal 5) in the control group. Additionally, regression did not correlate with any particular tumour type or with site of lesion. However, with one exception (animal 11), regression was observed in animals with small single tumours, suggesting that therapy vaccination is more effective in the early stages of sarcoid disease.

In the control group, regression was observed in two out of nine animals, progression in three animals and stationary tumours in four. However, two of the animals with stationary tumours were borderline, precluding a definite conclusion about regression rates in this group. Sarcoïd regression in the control group was surprising as regression of this tumour is rare (Ragland et al., 1970; Knottenbelt, 2005).

Contrary to expectations, there was no clear relationship between tumour regression and viral DNA load. Viral DNA load was very variable between animals in both groups, in agreement with our previous observations (Yuan et al., 2006). Variability in viral DNA load was still observable at EoT and there was no correlation between regression or progression and viral DNA load. Among the seven donkeys with regressing sarcoids (five in the vaccinated group and two in the placebo group), lower viral DNA loads were observed in only three animals. These results may be due in part to sampling variability. Because of experimental restrictions, the tumours were not biopsied but their surface was swabbed. Not all sarcoïd cells harbour viral DNA and therefore the collection of superficial cells will affect the calculations of viral DNA load.

**Vaccination with CVLPs induces virus-neutralizing antibodies in sarcoïd-bearing donkeys**

The vaccinated animals developed antibodies against CVLPs. As found previously in cattle (Chandrachud et al., 1994; Kirnbauer et al., 1996), there was no correlation between amplitude of the response and vaccine outcome. The anti-CVLP antibodies were virus-neutralizing in all vaccinated animals, and one animal (number 12) had developed neutralizing antibodies before vaccination. This is surprising as it is believed that no viral structural
proteins are produced in sarcoids. It is however in agreement with our finding that L1 RNA is present in some sarcoids (Nasir & Reid, 1999) and virions can occasionally be detected (S.W.J. Reid, unpublished observations).

Measurable anti-CVLP antibodies were found also in the sera of the control animals, but these were scarcely above background. The serum of one control donkey (number 13) was also virus-neutralizing which is surprising as this animal had low levels of antibodies against CVLPs, and furthermore did not undergo regression.

It is noticeable that the amplitude of the humoral response to CVLPs in donkeys was lower than in horses (Mattil-Fritz et al., 2008, accompanying paper). There are several possible explanations for this observation: one, horses and donkeys can respond differently to the same pathogen; as it has been shown for equine influenza virus (Newton & Mumford, 2004), African horse sickness virus (Cotzer & Guthrie, 2004) and equine encephalosis virus (Howell et al., 2004); two, the CVLP-vaccinated horses had been previously treated for sarcoids, while the donkeys had not, and previous treatment may have already exposed the horses to viral antigens; three, the CVLP vaccinated horses were on average ten years younger than the donkeys and perhaps more immunologically responsive.

Most of the vaccinated animals, and a few of the control animals, developed antibodies against linear epitopes of L1, but again there was no correlation with the outcome of vaccination. The only observed correlation was the presence of anti-L1 antibodies and neutralizing antibodies in control animal 13.

There was very little response to E7 in the vaccinated animals. Detectable anti-E7 antibodies pre-existing vaccination and did not increase after the vaccine. A similar level and frequency of anti-E7 antibodies was found in the control group. This observation is in agreement with the location of E7 internal to the VLPs, and therefore not exposed, and indicates that antibodies to E7 had been raised against E7 expressed in the tumours (Nasir & Reid, 1999). This result is at variance with the humoral response to E7 found in bovine papillomas where anti-E7 antibodies were detected only at late stages of infection before the onset of regression (Chandrachud et al., 1994), and it is yet another difference between BPV-induced tumours in cattle and equids. Interestingly, animal 13 in the control group again had the highest levels of anti-E7 antibodies.

**Conclusions**

Therapeutic vaccination of sarcoid-bearing donkeys with CVLPs is encouraging despite the partial outcome. Sarcoid regression was observed in the majority of vaccinated animals (4/8), more frequently than in placebo-treated animals (2/9). Conversely, tumour progression was observed in only one vaccinated donkey. In the accompanying paper (Mattil-Fritz et al., 2008) sarcoid-bearing horses were vaccinated with an identical CVLP vaccine. In that work, the trial was not placebo-controlled and the vaccine was tested for tolerability and safety rather than efficacy. Nevertheless, sarcoid regression was also observed in this trial and many sarcoids remained stationary. Taken together, our data suggest that CVLPs are a potential therapeutic vaccine.

One obstacle to a more successful therapeutic vaccine is likely to be presented by the viral oncoprotein E5. E5 is expressed virtually in every sarcoid (Nasir & Reid, 1999; Carr et al., 2001; Chambers et al., 2003; Yuan et al., 2007) and, as in bovine papillomas (Ashrafi et al., 2002; Marchetti et al., 2002, 2006; Araibi et al., 2004), is responsible for the downregulation of MHC class I (our unpublished observations). MHC class I presents antigenic peptides to the cytotoxic T cells and its downregulation leads to evasion of the immune response. It is possible that the CVLP vaccine would be more successful if accompanied by anti-E5 intervention.

**ACKNOWLEDGEMENTS**

This work was supported by The Donkey Sanctuary, Sidmouth, UK. M. S. C. is a Fellow of Cancer Research UK.

**REFERENCES**


cattle with the N-terminus of L2 is necessary and sufficient for preventing infection by bovine papillomavirus-4. *Virology* **211**, 204–208.


