Eq. 1

**INTRODUCTION**

Papillomaviruses (PV) comprise a heterogeneous group of viral agents with a double stranded DNA genome of approximately 8 kb (Lowy & Howley, 2002). In addition to the more than 100 PV types found in humans, there is a large number of PV types infecting a broad variety of vertebrates including turtles, whales, cattle and horses (de Villiers et al., 2004). In humans, certain PV types are causatively associated with the development of malignant disease, in particular cancer of the cervix uteri. Other HPV types cause benign hyperproliferations of the skin or of mucosal tissues (Lowy & Howley, 2002). Similarly, PV infection of animals can induce mucosal or skin warts, some of which can subsequently progress into malignant disease (Breitbart et al., 1997; Campo, 1997; Chambers et al., 2003; Goodrich et al., 1998; Nicholls & Stanley, 2000). Infection of horses by bovine PV (BPV) can lead to the induction of equine sarcoid, which is the most common skin tumour in horses (Lazary et al., 1985; Müller, 1991). DNA of BPV 1 or BPV 2 in episomal form can be detected in most of equine sarcoïds (Amtmann et al., 1980; Campo, 2002; Chambers et al., 2003; Goodrich et al., 1998; Lancaster et al., 1977; Müller, 1991; Yuan et al., 2007). In addition, viral mRNA or viral proteins have been detected (Carr et al., 2001; Chambers et al., 2003; Nasir & Reid, 1999; Nixon et al., 2005). Although BPV is widely accepted as the causative agent for equine sarcoid, the biology and epidemiology of the infection of horses by BPV remain unknown. No viral late gene products or infectious virions have been detected in equine sarcoïds, leading to the assumption that horses are non-permissive hosts for BPV. This may also be reflected by the semi-malignant characteristics of the tumours and their frequent relapse after surgical removal.

Because of its frequency, equine sarcoid presents a significant problem for horse owners and breeders.

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**Immunotherapy of equine sarcoid: dose-escalation trial for the use of chimeric papillomavirus-like particles**

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Spontaneous regression of equine sarcoïds is a very rare event. Currently, there is no effective therapy of equine sarcoïd available, although a wide range of methods is used for the treatment including surgical excision, autologous vaccination, injection of Bacille Calmette–Guérin (BCG), chemotherapy, homeopathy and others. However, after surgical excision, half of the tumours recur within three years, most of these already within six months (Ragland & Spencer, 1970).

In the past years, we and others have shown that so-called ‘chimeric virus-like particles’ (CVLPs) have a great potential for the immunotherapy of PV-associated tumours (Greenstone et al., 1998; Kaufmann et al., 2001; Müller et al., 1997; Nieland et al., 1999; Rudolf et al., 2001; Schäfer et al., 1999). The major viral structural protein L1 has the intrinsic ability to assemble into virus-like particles (VLPs) in a wide range of experimental systems. VLPs resemble infectious virions by inducing virus-neutralizing antibodies and by their ability to bind to the cellular receptor for PV, followed by penetration of cells in vitro and in vivo by receptor-mediated endocytosis (Garcea & Gissmann, 2004; Gissmann et al., 2001; Kirnbauer, 1996; Lowy & Frazer, 2003; Schiller & Lowy, 2001; Schreckenberger & Kaufmann, 2004). Furthermore, it has been demonstrated that VLPs interact efficiently with dendritic cells and that this interaction leads to the activation of these antigen-presenting cells (Rudolf et al., 2001). Consequently, VLPs are able to induce a cytolytic T-cell response against the L1 protein (Ohlschlager et al., 2003). CVLPs consist of modified VLPs and are generated by fusion of a truncated L1 protein to a tumour antigen. Similar to VLPs, CVLPs are able to mount a strong cytolytic T-cell response against the L1 protein, but in addition also against the fused tumour antigen. In a mouse tumour model, CVLPs have successfully been used for the prevention and treatment of experimentally induced tumours (Schäfer et al., 1999). Safety and immunogenicity of HPV 16 L1–E7 CVLPs were shown in a phase I/II clinical trial, but no information about efficacy was obtained (Kaufmann et al., 2007).

In analogy to the HPV 16 CVLPs, we developed and produced a therapeutic vaccine consisting of BPV 1 L1–E7 CVLPs. This vaccine offers the opportunity to test the concept of therapeutic vaccination on naturally occurring PV-associated tumours. In a phase I clinical trial (not placebo controlled) we investigated whether vaccination with CVLPs is tolerated by horses and whether a humoral immune response is induced by CVLPs. In addition, effects of the vaccine on tumour growth were monitored.

**METHODS**

**Animals.** The experiments described in this report comply with the current legislation covering the protection of animals and have been approved by the independent Ethical Committee for Animal Experiments of the Regierungspräsidium Leipzig, Az. 74-9168.11-100/0.

Horses from various breeds and suffering from sarcoïds were recruited in the area of Leipzig, Germany, and were brought to the Large Animal Clinic for Surgery, Faculty of Veterinary Medicine, University of Leipzig.

**CVLPs.** Three different fragments of the BPV 1 E7 gene (aa 1–54/nt 1–638; aa 46–100/nt 614–779; aa 72–127/nt 691–862) were inserted into a single EcoRV restriction site of the cloned BPV 1 L1 gene encoding a truncated version of the BPV 1 major capsid protein which lacks the 26 C-terminal amino acids (Müller et al., 1997). The E7 fragments were amplified from the cloned BPV 1 genome (GenBank accession no. NC_001522) using primers complementary to the corresponding fragment and carrying EcoRV restriction sites for cloning purposes. The three L1–E7 fusion genes were inserted into the baculovirus transfer vector pVL1393 (Invitrogen). Recombinant baculoviruses were generated by cotransfection of the transfer plasmid together with linearized baculovirus DNA (Novagen BacVector 3000) into S9 insect cells. CVLPs were purified from baculovirus-infected insect cells as described earlier, except that cells were extracted using a pressurized Dharma press (Dianova) was added at a 1 : 4000 dilution. After 1 h at 37°C, Plates were washed again five times and a goat-anti-mouse IgG antibody conjugated with horseradish peroxidase (Dianova) was added at a 1 : 4000 dilution. After 1 h at 37°C, Plates were washed and stained with ABTS (2,2’-azino-bis-3-ethylbenzthiazol-6-sulfonic acid) substrate solution (1 mg ml−1 containing 0.015 % H2O2). Absorbance at 405 nm was measured after 20 min in a Titrak automated plate reader.

**ELISA.** Antigen-capture ELISA (AC-ELISA) for the detection of BPV 1 L1 (CVLP)-specific antibodies: microtitre plates (Becton Dickinson) were coated with an anti-L1-specific monoclonal antibody (Becton Dickinson) overnight at 4°C. Plates were washed three times (PBS, 0.05 % Tween 20) and incubated with BPV 1 CVLPs (50 µl well per well, containing 0.5 µg CVLPs in PBS) for 1 h at 37°C. After three washings, 50 µl sera (diluted in PBS 1 : 5 000) were added and plates were washed again five times and a goat-anti-horse IgG antibody conjugated with horseradish peroxidase (Dianova) was added at a 1 : 4000 dilution. After 1 h at 37°C, Plates were washed and stained with ABTS (2,2’-azino-bis-3-ethylbenzthiazol-6-sulfonic acid) substrate solution (1 mg ml−1 containing 0.015 % H2O2). Absorbance at 405 nm was measured after 20 min in a Titrak automated plate reader.

**Glutathione S-transferase (GST)-capture ELISA for the detection of anti-E7 antibodies.** Rosetta bacteria transformed with expression constructs (pGEX vector system, Invitrogen) coding for GST–BPV 1 E7 or GST alone were grown in 500 ml Luria–Bertani (LB) medium containing 40 µg ampicillin ml−1 to an optical density of 0.9 at 37°C, 120 r.p.m. After cooling down to approximately 9°C, IPTG (100 µM) was added to induce expression of the fusion proteins and bacteria were incubated overnight at 12°C and 120 r.p.m.

Bacteria were harvested by centrifugation (3500 g, 4°C, 10 min) and pellets were resuspended in 50 ml extraction buffer containing 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). Afterwards, bacteria were lysed using a high pressure homogenizer (French press; Avestin) and subsequently bacterial lysates were cleared from insoluble components and cell debris by centrifugation (10 000 g, 4°C, 60 min). For storage at −20°C glycerol was added to a final concentration of 50 % to the supernatants. Total protein amount was determined by colorimetric means using the Bradford assay.

To determine antibody reactivity against the BPV 1 E7 protein, sera from BPV 1 L1–E7 CVLP-immunized horses were analysed by GST-capture ELISA. Briefly, 96-well ELISA plates were coated overnight at 4°C with 200 ng per well of glutathione-casein in 50 mM carbonate buffer.
buffer, pH 9.6. After washing the plates three times with PBS containing 0.3 % (v/v) Tween 20 (washing buffer), the wells were blocked for 1 h at 37 °C with 0.2 % (w/v) casein in PBS containing 0.3 % (v/v) Tween 20 (blocking buffer). Subsequently, the blocking buffer was discarded and cleared lysates from Rosetta bacteria overexpressing either the GST–BPV 1 E7 fusion protein or the unfused GST alone were diluted to 0.25 μg μl−1 total protein in blocking buffer and 50 μl was added to the wells. After incubation for 1 h at 37 °C, the wells were washed three times using washing buffer. Thereafter, 50 μl of horse sera diluted 1 : 100 in blocking buffer was added to the wells and incubated for 1 h at 37 °C. Following washing steps as mentioned above, 50 μl secondary anti-horse IgG antibody (Dianova) diluted 1 : 3000 was added to each well. After 1 h incubation at 37 °C, plates were washed seven times to completely remove unbound antibody to avoid unspecific reactions during substrate addition. Finally, 100 μl substrate (0.5 mM 3,3’-diaminobenzidine (DAB) and 0.01 % (v/v) H2O2 in 50 mM sodium acetate, 50 mM NaH2PO4 pH 4.2) was added to each well and colour development was measured at 405 nm after 10–20 min using a multi-label plate reader (PerkinElmer).

**Immunization.** The study was designed as a non-placebo controlled phase 1 clinical trial including a dose-escalation setting. To this end, the horses were randomly divided into four dosage groups (40, 80, 200 and 400 μg CVLPs per injection), three horses per group.

**Detection of BPV DNA in equine sarcoids.** For the detection of BPV DNA a 100–150 mm3 tumour biopsy was sampled and boiled in 500 μl PBS for 10 min. One μl of the supernatant was used in a PCR reaction. BPV 1 and BPV 2 DNA was amplified using two primers (5’-GACAGGGCCCCGTGTTGGACAT-3’ and 5’-COCCAGTTCGGACGCAGACACGAC-3’) located in the E6 and E7 region of the BPV genome (derived from international patent no. WO 98/10639). Alternatively, E2-specific primer pairs were used to detect BPV DNA (primers 5’-TTTGTATATCATGAGAAGCATGGCAAGCTTTAC-3’ and 5’-TTTAAAGCTTGAAGTCAACGCTGCGTGAACCGG-3’). PCR was carried out under standard conditions with Taq polymerase, as suggested by the manufacturer. Samples were denatured for 3 min at 95 °C followed by 35 cycles of 1 min at 95 °C, 1 min at 57 °C and 1 min at 72 °C. As positive control, a reaction containing 0.1 ng BPV 1 or BPV 2 DNA was carried out. As negative control, the reaction was performed by adding PBS. To discriminate BPV 1 and BPV 2 DNA, the PCR products derived from the E6/E7 region were digested with BsaI (cleaving only the BPV 1 DNA) or BglII (cleaving only the BPV 2 DNA).

**Neutralization assay.** To determine the neutralizing properties of horse sera immunized with BPV 1 L1–E7 CVLPs, sera were analysed using a pseudovirion neutralization assay. BPV 1 pseudovirions used in this assay were produced as described elsewhere (Buck et al., 2005) by cotransfection of 293TT cells with expression plasmids encoding BPV 1 L1, BPV 1 L2 and secreted alkaline phosphatase (SEAP) as reporter enzyme. For the neutralization assay 1 × 106 293TT cells per well were seeded on a 96-well cell culture plate one day prior to the assay. BPV 1 pseudovirions were pre-diluted 1 : 5000 in complete Dulbecco’s modified Eagles Medium (DMEM) and subsequently horse sera (pre-blood and after last immunization) were diluted 1 : 1000 in pseudovirion pre-dilution and incubated for 10 min at room temperature. An anti-BPV 1 L1 VLP rabbit serum produced in our laboratory (#10; unpublished data) was diluted 1 : 1000 in pseudovirion pre-dilution as positive control for neutralization. After discarding the supernatant from the 96-well plates, 50 μl of serum dilutions were added to the cells and incubated for 5 days (37 °C, 5 % CO2). Wells incubated with either BPV 1 pseudovirions or medium only were included as controls. Finally, 15 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 multi-label plate counter (Victor3, PerkinElmer). Neutralization for the rabbit serum #10 (positive control) was set as 100 % and used as reference. Sera showing a reduction in SEAP activity of 75 % were considered to be neutralizing.

**RESULTS**

**Construction and purification of CVLPs**

One aim of the study was to evoke a tumour-specific cytolytic T-cell response against BPV 1 antigens. There is, however, no information available about equine T-cell epitopes present on the viral proteins. Based on our previous work (Müller et al., 1997) and the observation that E7 is a tumour rejection antigen in cattle (Campo, 1994), we decided to use the BPV 1 E7 protein as target in therapeutic vaccination. We generated three different chimeric L1 proteins harbouring overlapping regions of the 127 amino acid long E7 protein: BPV 1 L1–E71–54, L1–E746–100 and L1–E772–127. From our previous studies, we reasoned that insertions of more than 60 aa into the L1 protein would interfere with the assembly of CVLPs. In fact, two of the constructs failed to produce reasonable amounts of CVLPs while one construct (L1–E71–54) was suitable to produce the large quantities of particles required for the subsequent vaccination trial (see Fig. 1). In fact, the particle yield was comparable to that of wild-type BPV 1 L1 VLPs (data not shown). The CVLPs were produced in H5 insect cells using recombinant baculoviruses. Purification was performed as described earlier by a combination of sedimentation and equilibrium density centrifugation. The CVLPs were purified to about 95 % homogeneity as judged by SDS-PAGE (Fig. 1). Analysis by mass spectrometry confirmed the identity of the expected fusion protein (data not shown). Assembly into VLPs was also confirmed by sucrose sedimentation. This method allows separation of different forms of L1, i.e. monomers or capsomeres from capsids, and confirmed that virtually all of the purified L1–E7 fusion protein sediments with the capsid fraction (data not shown).

**Clinical trial: immunization protocol, biopsy, clinical status**

A total of 12 horses of different breeds from the area around Leipzig in Germany were included in the study (Table 1). Exclusion criteria were: impaired health condition, pregnancy or other sarcoid-related therapeutic treatments shortly before or during the trial. The animals were between 3 and 12 years old and carried from 2 to more than 50 sarcoids, some of them for as long as 6 years. The average number of sarcoids at the time of recruitment was 22. All horses had previously undergone numerous sarcoid-directed therapy using different treatment methods such as cryosurgery, conventional surgery and others. After entering the clinical trial, the animals were followed for 61 days. On days 0, 21, 42 and 63 serum was collected.
In addition, on day 0 a tumour biopsy was taken. All tumours were documented macroscopically and the animals were immunized according to their respective dosage group (40, 80, 200 and 400 mg CVLPs per injection). The vaccine was applied intramuscularly without adjuvants on days 0 and 21. The animals were monitored for 24 h post-vaccination to record any side effects. Eight of the twelve horses were available after day 63 and a third vaccination was carried out (between days 145 and 280 after initiation of the trial).

Side effects
During a total of 32 injections with CVLPs no local or systemic side reactions were noticed. The animals were monitored for vital parameters (breathing, heart frequency and temperature) for at least 1 h post-injection. For each of the animals at least one haematological examination of a blood sample collected after vaccination was performed during the study which, however, showed no abnormalities. In one animal there were symptoms of a mild colic on day 21, which was likely due to construction noise in the barn at the time of immunization. Besides this, no local or systemic adverse effects such as swelling, necroses etc. were noticed.

Detection of BPV DNA in tumour biopsies
For 11 out of 12 horses a sarcoid biopsy was available for the detection of BPV DNA. DNA was extracted from the biopsies and tested by PCR for the presence of BPV sequences using E6/E7-specific primer pairs. The primers amplify the corresponding region of BPV 1 and BPV 2. By restriction digest of the amplified fragment it was possible to determine the BPV type present in the sample. In 10 out of the 11 samples BPV 1 DNA was detected. In the biopsy of one of the animals no specific fragment could be amplified using the E6/E7 primer pairs, although in this case we were able to detect BPV DNA by the use of an E2-specific PCR.

Serology: detection of CVLP- and E7-specific antibody responses
Unfortunately, there are only limited options for assaying the induction of cellular immune responses against the PV antigens in horses. To monitor the induction of a humoral immune response against the L1 and E7 components of the CVLP vaccine, sera were collected at days 0, 21, 42 and 63. For some of the animals additional sera were available. ELISAs were used to measure anti-L1 and anti-E7 immune responses and all sera from individual animals were tested simultaneously. For L1, we compared the serum reactivity at day 0 of the horses participating in the study with the reactivity of sera from 12 healthy horses (i.e. animals with no recorded history of equine sarcoid). Results indicate that the animals suffering from sarcoid had no detectable anti-L1 antibody response when entering the trial. Eight animals (A, B, E, F, I, J, K and L) had developed a measurable IgG antibody titre after the first immunization with titres ranging from 1:200 to 1:3200 (see Fig. 3). Twenty-one days after the first booster immunization (day 42), existing titres further increased and a positive reaction was found in 11 out of the 12 animals. In all of these 11 animals L1-specific IgM titres peaked at day 21 (see Fig. 2). One of the horses (animal D) remained negative for both IgG and IgM anti-L1 antibodies throughout the study. At day 63 the titres of the L1 antibody-positive animals had dropped slightly in comparison to the sera collected at day 42. Nine of the horses were available for an additional serum sampling at or after day 196 (sample ‘day I’, see Fig. 3). At this time, the anti-L1 titre of one animal (K) had dropped to background levels while eight animals showed only moderate anti-L1 antibody titres. The second booster immunization led to a significant increase of anti-L1 titres in all of the seven horses (B, C, F, G, H, I and K) from which serum samples were available at time point ‘day II’ (see Fig. 3).

Fig. 1. Purification of BPV 1 L1–E71–54 CVLPs. The BPV 1 L1–E71–54 protein was expressed in insect cells using recombinant baculoviruses. CVLPs were purified by ultracentrifugation and analysed by electron microscopy and SDS-PAGE (Coomassie-blue staining). Lanes 1–3 show the peak fractions of a typical CsCl density gradient which were collected and combined to produce the vaccine. As comparison, BPV 1 virions purified from a cattle wart are shown. The bars represent 100 nm.
To determine whether the antibody titres in the vaccinated horses were dependent on the vaccination dose, sera collected at days 42 and 63 from all 12 animals were analysed simultaneously by ELISA (see Fig. 4). Animal D, which had not developed measurable anti-L1 antibodies during the study, was excluded from this comparison. There were no significant differences in anti-L1 antibody titres in sera from horses of the four different dosage groups among the remaining anti-L1-positive animals at day 42 or day 63, indicating that even the lowest dose of CVLPs (40 \( \mu \)g) was sufficient to induce anti-L1 responses (see Fig. 4).

To measure E7-specific antibodies, we used an antigen-capture ELISA with a GST–E7 protein (data not shown). The antigen was captured by glutathione covalently linked to casein as carrier (Sehr et al., 2001). Sera from all 12 horses from days 0, 21, 42 and 63 (total of 48 sera) were analysed; to discriminate between E7-positive and E7-negative sera we calculated the cut-off as the mean value plus two standard deviations, excluding positive sera from the cut-off calculation (Müller et al., 1992). By this method, 9 of the 48 serum samples from five different animals scored positive. In the serum of one of the horses, we detected E7-specific antibodies already at day 0, indicating an exposure to tumour-derived E7. In three animals, only one serum sample was positive (one from day 21, two from day 63), in two animals three of the four sera were E7-positive. The results confirmed our previous observation that intact CVLPs are poor inducers of E7-specific humoral immune responses (Müller et al., unpublished observations). As the E7 portion is located

![Fig. 2. Detection of anti-L1-specific IgM antibodies in immunized animals. Anti-L1 IgM antibodies were detected in serum samples collected at days 0, 21 and 42 by AC-ELISA for a serum dilution of 1:50. Except for animal D, which remained anti-L1 antibody-negative throughout the study, all animals developed anti-L1 IgM antibodies. In these 11 animals, highest reactivity was detected at day 21.](image_url)
Table 2. Clinical evaluation

<table>
<thead>
<tr>
<th>Horse</th>
<th>Dose group (µg CVLPs)</th>
<th>After day 63</th>
<th>Day 63 until 3rd vaccination</th>
<th>3rd vaccination until follow-up</th>
<th>Evaluation</th>
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<td>B</td>
<td>40</td>
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<td>+</td>
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<tr>
<td>D</td>
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<td>E</td>
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<td>F</td>
<td>80</td>
<td>&lt; (1); +</td>
<td>Status post-surgery</td>
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-, Condition unchanged, all sarcoids present; < (x), reduction in sarcoid number; > (x), increase in sarcoid number; +, growth of existing sarcoids; r (x), relapse of sarcoids; +, sarcoiids appear dried-out; ND, animal not available at time of checkup.

Neutralizing antibody response

The L1–E7 antigen used for immunization was assembled into capsids as confirmed by electron microscopy and sucrose gradient sedimentation. These CVLPs display conformational epitopes as determined by ELISA using monoclonal antibodies (data not shown). Therefore, we wanted to determine whether the immunized animals have developed a virus-neutralizing response. To this end, we used BPV 1 pseudovirions, which have been described previously as being surrogates for infectious PV virions (Buck et al., 2005; Pastrana et al., 2004). The pseudovirions are BPV L1- and L2-containing VLPs, which have packaged a recombinant plasmid carrying a reporter gene. Infection of cells in culture by pseudovirions can be blocked by specific antibodies. We analysed pre- and post-vaccination sera of all 12 animals in the study. None of the animals had detectable neutralizing antibodies at day 0 (see Fig. 5). Eleven of the animals developed a strong neutralizing response 21 days after the second immunization, with titres ranging above 1:1000. One animal (horse D), negative for L1-specific antibodies by ELISA, also failed to develop a neutralizing antibody response. These data suggest that immunization with CVLPs induces a BPV-neutralizing, possibly protective, humoral immune response.

Effects on tumours

The tumour status of all 12 horses was monitored until day 63 by examination and by the horse owners responding to a questionnaire. The observed effects on tumour growth are summarized in Fig. 6 and in Table 2. In five of the animals the number of tumours did not change during the observation period. In one animal the number of tumours increased from 14 to 16. In the remaining five horses a reduction in tumour number (1–5 tumours per animal) was observed. Independently of the change in the number of tumours, we observed growth of existing tumours in four animals and shrinkage of tumours in two animals.

Eight of the animals were available beyond day 63. At that time, one animal had undergone surgery. There were no obvious changes in one (G) of these seven animals. In a second animal (H), there was a recurrent tumour on one site and no changes on the other tumours. Three of the seven animals presented with a loss of tumours, although one of these animals had also grown new tumours. The remaining tumours of this animal appeared to have shrunk. In the remaining two animals growth of additional tumours was observed.

Eight of the seven animals were vaccinated for a third time. Eighty-four days after this vaccination four of the horses showed no change in tumour number. In two animals a loss of tumours was recorded.

In summary, during the full observation period two of the twelve horses showed no alteration in tumour number or tumour size. In three animals we observed tumour regression, in one of these animals however some of these tumours recurred. Two other animals lost the existing tumours but developed additional tumours. Three animals showed regression of some tumours but growth of other existing tumours. In one animal we observed growth of existing tumours and in one growth of additional tumours.

DISCUSSION

Equine sarcoid is the most common tumour in Equidae. Although considered semi-malignant (they do not metas-
tasise) there is currently no efficient treatment available. BPV 1 and 2 are considered the aetiological agents of equine sarcoid and, similar to the situation of the human tumours induced by some of the high-risk HPV types (Zur Hausen, 2006), the tumour cells provide a non-permissive...
Fig. 3 Detection of anti-L1-specific IgG antibodies in immunized animals. Serum samples were titrated on an antigen-capture ELISA for the presence of anti-L1-specific antibodies. All animals were immunized on days 0 and 21; seven animals received a second booster immunization after day 259 (I). Serum samples were collected on days 0, 21, 42, 63 and for nine animals once after day 259 (I) and for seven animals at least 84 days after the second booster (II; day 343 or later). All horses with the exception of animal D developed anti-L1 responses. In some animals, the anti-L1 titres had dropped at day 63, for all animals followed up a significant drop in titre was recorded when sample I (on or after day 259) was analysed. All seven animals that had received a second booster developed a strong anti-L1 recall response (tested on or after day 343). ■, Day 0; ▲, day 42; ●, day 21; □, day I; ●, day 63; ○, day II.
environment for the viruses. Infection of horses with BPV is the only known example for a natural cross-species infection by a PV. In contrast to early BPV genes, which are probably essential for the maintenance of the transformed phenotype, late PV genes are not expressed in the tumour cells, again similar to what is observed in human tumours caused by HPV.

In human tumours the viral antigens E6 and E7 are considered tumour antigens as they are consistently expressed. In the case of equine sarcoid, expression of the BPV E5 protein and E6 and E7 mRNA has been detected (Carr et al., 2001; Nasir & Reid, 1999). In the present study, we used chimeric virus-like particles for immunotherapy of equine sarcoid with the aim to target the E7 protein as potential tumour antigen. In a number of studies using mice and human subjects it has been demonstrated that PV VLPs and CVLPs are able to efficiently induce cytotoxic T-cell responses. VLPs and CVLPs are able to enter and activate antigen-presenting cells leading to major histocompatibility complex (MHC)-I restricted presentation of L1 and, in case of CVLPs, other PV- and non-PV-derived antigens. In addition, VLPs and CVLPs are able to induce high-titre capsid-specific neutralizing antibody responses. A VLP-based vaccine has been recently licensed for the prophylaxis of HPV-induced malignancies.

In the present dose-escalation study, we used BPV 1 L1–E7 CVLPs for the treatment of 12 horses suffering from equine sarcoid. The vaccination was not accompanied by local or systemic side effects and was therefore well tolerated. In the absence of available assay systems, we monitored the development of a humoral immune response directed anti-L1 and anti-E7. All but one of the animals developed anti-L1 responses (IgM and IgG) after the first immunization and this response was further increased by a single booster immunization. The response remained stable for the initial observation period of 63 days, but significantly declined after about 200 days in all of the 9 animals that were followed for that time period. A second boost, however, had a strong recall effect, indicating a robust memory response.

**Fig. 4.** Anti-L1 antibody responses by dose group. Sera collected at days 42 and 63 were titrated by AC-ELISA for the presence of anti-L1 IgG antibodies. Animal D of dose group 2, which failed to develop anti-L1 responses, was not included in this comparison. Each bar represents the mean value of absorption in ELISA for each group for a given serum dilution. The ‘error bars’ show the lowest and highest reactivity measured in each group. No significant difference in ELISA reactivity was observed in the different dose groups. Similarly, all dose groups showed a comparable decline in reactivity at day 63.

**Fig. 5.** Detection of anti-BPV 1 neutralizing antibodies in sera from immunized horses. Sera collected at day 42 were analysed for the ability to neutralize infectious BPV 1 L1/L2 pseudovirions, transducing the SEAP reporter gene, in vitro. Bars represent SEAP activity (RLU, relative light units) from the supernatant of transduced cells. The error bars show the standard deviation of duplicate assays. Sera collected pre- and post-vaccination (days 0 and 42) were used at a 1:1000 dilution. As controls, pseudovirions were untreated or neutralized using a hyperimmune anti-VLP antiserum (#10). All sera post-vaccination, except from animal D, reduced the RLU by 75% or more, which was considered neutralization-positive. None of the pre-immune sera showed significant (above 75%) neutralizing activity.
response. One of the animals failed completely to develop anti-L1 antibodies. We do not have any explanation for this as this horse showed normal serum IgG levels. As expected, there was little anti-E7 response as the E7 portion is located on the inside of intact particles. We observed similar results after immunizing mice and rabbits with CVLPs; E7-specific responses are usually induced only when denatured particles are injected (Müller et al., unpublished). In the 11 horses that developed a humoral immune response we did not observe a dose dependency in respect to antibody titre or maintenance of the antibody response, indicating that the lowest dose of 40 μg CVLPs is already sufficient. In fact, we did not observe that the humoral immune responses were significantly different between the four dose groups, indicating a high immunogenicity of the CVLP vaccines. In smaller animals such as mice and rabbits we observed that as little as 1–50 ng VLPs are sufficient to mount detectable anti-L1 responses (Thönes and Müller, 2007). All anti-L1 (anti-CVLP) responses were also neutralizing BPV 1 pseudovirions in vitro. This fact is important in respect to the use of CVLP vaccination for the prevention of equine sarcoid. However, it would be difficult to demonstrate efficacy of neutralizing antibodies in vivo without an experimental virus challenge, because this would require a rather large study population as the annual incidence rate of equine sarcoid is about 0.7–1 %.

Although the prime aim of this study was to evaluate toxicity and dose effects of CVLP vaccination in horses, we monitored tumour development throughout the trial. Historical data suggest that the spontaneous regression rate of equine sarcoid is zero or extremely low and this is consistent with the information provided by the owners of the animals. In four of the animals we recorded an improvement of the status, i.e. the total number of sarcoids was reduced. One of four animals lost 16 tumours during the study. In one other animal, although there was no reduction in tumour number, we observed a change in the morphological appearance of the sarcoids as they seemed to have dried up. In six animals, the tumour status remained stable during the study. In total, ten animals either improved or remained stable, which was judged an improvement by the horse owners, based on their previous observation over many years regarding the sarcoid status.

Nevertheless, our immunization resulted in only partial response. In the absence of T-cell markers it is unclear whether we actually achieved induction of an anti-E7 cellular immune response. It also remains to be determined whether E7 is an appropriate tumour antigen of equine sarcoid and whether the sarcoid cells have developed strategies for immune escape. The relatively poor clinical response can in part be explained by the composition of the cohort. Most of the animals had a large number of tumours for many years and had undergone several treatment attempts before entering the trial. In fact, we made the observation that better response to the immune therapy can be expected in animals with few and small tumours. Currently, we are determining whether CVLP immunization can be used as adjunct treatment to surgical removal of sarcoids with the aim to lower the frequency of tumour recurrence.

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REFERENCES


