Type-specific L1 virus-like particle-mediated protection of horses from experimental bovine papillomavirus 1-induced pseudo-sarcoïd formation is long-lasting

Julia Harnacker,1 Edmund K. Hainisch,1,2 Saeed Shafti-Keramat,3 Reinhard Kirnbauer3 and Sabine Brandt1,*

Abstract

Equine sarcoïds are common therapy-resistant skin tumours induced by bovine papillomavirus type 1 or 2 (BPV1, BPV2) infection. We have previously shown that prophylactic vaccination with BPV1 L1 virus-like particles (VLPs) efficiently protects horses from experimental BPV1-induced pseudo-sarcoïd development. Here, we assessed BPV1 L1 VLP vaccine-mediated long-term protection from experimental tumour formation in seven horses 5 years after immunization with three different doses of BPV1 L1 VLPs, and three unvaccinated control animals. Horses were challenged by intradermal inoculation with infectious BPV1 virions at 10 sites on the neck (106 virions per injection). In vaccinated horses, BPV1 challenge did not result in any apparent lesions irrespective of vaccine dosage and BPV1-neutralizing antibody titres that had dropped considerably over time and below the detection limit in one individual. Control horses developed pseudo-sarcoïds at all inoculation sites. We conclude that immunization of horses with BPV1 L1 VLPs induces long-lasting protection against experimental BPV1 virion-induced disease.

Papillomaviruses (PVs) constitute a family of small, non-enveloped viruses consisting of an icosahedral capsid harbouring a circular double-stranded DNA genome of up to 8 kbp. Early research in natural animal hosts has revealed the pronounced species-specificity and tropism of PVs for epithelial keratinocytes. Delta-PVs (δ-PVs) are an exception to this rule in that they can also infect dermal fibroblasts. In addition, some δ-PVs, i.e. genetically highly homologous bovine PV types 1 and 2 (BPV1, BPV2) not only infect cattle but also a wide range of other ungulate species including antelopes, giraffes, water buffaloes and tapirs [1–4]. Importantly, BPV1 and 2 are chiefly involved in the onset and maintenance of benign, yet locally aggressive, skin tumours in equids termed sarcoïds [5]. Whilst sarcoïds in Europe usually harbour BPV1, BPV2 is the predominant type infecting equids in western USA [6]. In addition, sarcoïds collected in Brazil were shown to contain DNA of another δ-PV, i.e. BPV type 13, that is genetically more than 90% homologous to BPV1 and 2 [7]. A possible aetiological association of BPV13 with sarcoïd development remains to be determined. BPV1/2 mainly reside in sarcoid fibroblasts as numerous viral episomes that multiply in synchrony with cell division. Accidental or iatrogenic trauma is a factor that contributes to disease onset and progression because wound healing in infected equids is achieved via proliferation of infected cells. Synchronous amplification of BPV1/2 episomes may entail oncogene expression to levels inducing cell transformation [5, 8]. Sarcoïds affect up to 12% of horses and other equids worldwide, are difficult to treat, and tend to recur in a more aggressive, multiple form following ineffective therapy or trauma. Therefore, sarcoïds are still the most common dermatological reason for euthanasia, even though they do not metastasize [9].

In an attempt to establish a vaccine for protection of horses and other equids from BPV1/2 infection-associated sarcoïd development, we have previously generated BPV1 L1 virus-like particles (VLPs) [10] and evaluated their safety and immunogenicity in a dose-escalation trial involving 15 teaching horses at the Equine Clinic, University of Veterinary Medicine, Vienna, Austria, that were assigned to four groups. Groups 1–3 each comprised four horses that received 50, 100 or 150 µg of BPV1 L1 VLPs plus 250 mg Al(OH)3 per dose as an adjuvant by deep intramuscular injection on days 0, 28 and 168 (May 2008). Group 4 consisted of three control horses that only received an alum adjuvant. Sera were collected immediately before and 2 weeks after immunization as well as 1 day, 2 weeks and 1 year after the first vaccination. BPV1-specific antibodies were measured in serum by neutralization assays (NAs) against infectious BPV1 virions at 10 sites on the neck (106 virions per injection). The results showed that the neutralizing antibody titres dropped considerably over time and were below the detection limit in one individual 5 years after the last vaccination. However, no clinical signs or lesions of sarcoïd development were observed in any horses. We conclude that immunization of horses with BPV1 L1 VLPs induces long-lasting protection against experimental BPV1 virion-induced disease.
In this study, we assessed the longevity of BPV1 L1 VLP-induced neutralizing antisera and immune-mediated protection of horses. To this aim, we collected blood from seven still-available BPV1 L1 VLP-immunized horses from the safety and immunogenicity trial in April 2013, i.e. almost 5 years after the third immunization (Table 1) [11]. Serum was used to address the potential of BPV1 L1 VLP vaccination to protect horses from experimental BPV1-induced formation of transient pseudo-sarcoids. Two immunizations of horses with 100 µg of BPV1 L1 VLP and 250 mg Al(OH)₃ 4 weeks apart conferred complete protection from intradermal challenge with BPV1 virions 2 weeks after the boost and related pseudo-sarcoi d development [16].

Where applicable, tumour sizes were recorded using a calliper until pseudo-sarcoids had completely resolved. Heparinized blood (9 ml per horse) was taken on the day of virus challenge and then at weekly intervals for 11 weeks. PBMCs were isolated by routine Ficoll-Paque PLUS (GE Healthcare Life Sciences, Vienna, Austria) density gradient centrifugation and DNA extracted using a DNeasy Blood and Tissue Kit according to the manufacturer’s instructions (Qiagen, Hilden, Germany). Presence of amplifiable PBMC DNA was confirmed by standard β-actin PCR. Then, 40 ng of PBMC DNA per 50 µl reaction mixture was subjected to BPV1 E5 PCR screening as described previously [12, 17]. Sarcoid DNA, PBMC DNA from a healthy horse and sterile water served as positive, negative and no template controls in this experiment. Amplification products (16 µl) were visualized by gel electrophoresis and ethidium bromide staining using a Quantitas 100 bp DNA ladder (Biozym, Vienna, Austria) as a molecular weight marker.

Sera were obtained once more 10 weeks after intradermal challenge from vaccinated and control horses (25 June 2013). Then, BPV1 antibody titres were determined by PsV neutralization assay [11] to test whether inoculation had led to a booster effect in vaccinated horses and to a type-specific antibody response in control horses.

Importantly, seven of seven horses that had been immunized 5 years earlier with 50, 100 or 150 µg BPV1 L1 VLP were completely protected against pseudo-sarcoi d disease induced by intradermal BPV1 inoculation. Protection was irrespective of the vaccine dosage and the individual BPV1-neutralizing antibody titres on the day of challenge (Table 2). BPV1-neutralizing antibody titres had dropped over time from levels ≥3200 in June 2008 to levels ≤400 on the day of intradermal challenge in April 2013. In one case (immunized horse no. 10), antibody titres decreased within a year from 12 800 (June 2008) to undetectable levels (Tables 1 and 2).

The three unvaccinated control horses tested negative for BPV1-neutralizing antibodies on the day of inoculation, as

**Table 1. Temporal course of BPV1-neutralizing antibody titres between third immunization of horses with different doses of BPV1 L1 VLPs and the day of intradermal challenge with BPV1 virions 5 years later**

<table>
<thead>
<tr>
<th>Horse</th>
<th>VLP dose per vaccination</th>
<th>BPV1-neutralizing antibody (Ab) titres</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(4 weeks after third immunization)</td>
<td></td>
</tr>
<tr>
<td>04</td>
<td>IND</td>
<td>50 µg</td>
</tr>
<tr>
<td>05</td>
<td>DUD</td>
<td>50 µg</td>
</tr>
<tr>
<td>08</td>
<td>HAN</td>
<td>100 µg</td>
</tr>
<tr>
<td>09</td>
<td>RAM</td>
<td>100 µg</td>
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<tr>
<td>10</td>
<td>SON</td>
<td>100 µg</td>
</tr>
<tr>
<td>12</td>
<td>VIC</td>
<td>150 µg</td>
</tr>
<tr>
<td>14</td>
<td>BIL</td>
<td>150 µg</td>
</tr>
<tr>
<td>C1</td>
<td>LUX</td>
<td>–</td>
</tr>
<tr>
<td>C2</td>
<td>MIN</td>
<td>–</td>
</tr>
<tr>
<td>C3</td>
<td>RON</td>
<td>–</td>
</tr>
</tbody>
</table>

*As published by Hainisch et al. [11].

**Table 2. Protective efficacy of BPV1 L1 vaccination of horses 5 years after the last immunization and booster effect of intradermal challenge with BPV1 virions**

<table>
<thead>
<tr>
<th>Horse</th>
<th>BPV1 Ab titres April 2013</th>
<th>Σ tumours (10 inoculations per horse)</th>
<th>BPV1 Ab titres 10 weeks post challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>Code</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td>04</td>
<td>IND</td>
<td>400</td>
<td>800</td>
</tr>
<tr>
<td>05</td>
<td>DUD</td>
<td>100</td>
<td>200</td>
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<td>09</td>
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<tr>
<td>10</td>
<td>SON</td>
<td>&lt;50</td>
<td>&lt;50</td>
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<tr>
<td>12</td>
<td>VIC</td>
<td>100</td>
<td>800</td>
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<tr>
<td>14</td>
<td>BIL</td>
<td>200</td>
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<td>C1</td>
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<td>C3</td>
<td>RON</td>
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increased during the first year after the third immunization, i.e. in 2009, and then decreased considerably until the day of virus challenge in April 2013 (Tables 1 and 2). In three of the seven horses that had received 50 (no. 5) or 150 µg VLP (nos 12 and 14), BPV1-neutralizing antibody levels reached a maximum after the third immunization in 2008 and then dropped to low levels (≤200) until the day of virus challenge (Tables 1 and 2). The longevity of vaccination-induced protection of these six immunized horses from experimental BPV1-induced pseudo-sarcom formation was probably mediated by low levels of BPV1-neutralizing antibodies still detectable in sera. The observed moderate increase of antibody titres in response to BPV1 challenge suggests that three immunizations of horses with BPV1 L1 VLPs in 2007–2008 might have elicited a specific memory B cell response. A robust secondary antibody response from memory B cells has been shown to occur in women immunized with high-risk HPV type L1 VLPs [18–20]. The comparatively weak increase of antibody titres observed in horses 10 weeks post challenge could be due to the intradermal route of virion administration. This interpretation is supported by the observation that the immune response of horses and cattle to natural BPV infection of the skin is compromised by passive immune escape [8].

Individual no. 10 mounted a robust antibody response to three immunizations with 100 µg of BPV1 L1 VLP, as reflected by antibody titres of 12 800 4 weeks after the third immunization in 2008. These titres then irrevocably dropped below the detection level within the following year (Table 1). Nonetheless, this horse showed complete protection from challenge-induced tumour formation (Table 2). Since all horses were inoculated with the same batch of virions by the same person on two consecutive days [12, 16], erroneous omission of inoculum as an explanation for this observation is very unlikely. Alternatively, protection may be based on a challenge-induced memory B cell response as observed in the other vaccinated individuals (Table 2). Certainly, even the lowest dilution (1:50) of serum collected 10 weeks post challenge tested negative by BPV1 PsV neutralization assay. However, there is evidence that even low concentrations of anti-L1 antibodies can protect against high doses of infectious virions, as exemplarily shown in rabbits and dogs [21, 22]. In accordance with these early findings, more recent evidence suggests that antibody concentrations 100-fold below the detection limit of the PsV assay are still protective in mice [23]. In humans, HPV18-specific antibody titres induced by immunization with a quadrivalent HPV6/11/16/18 L1 VLP vaccine dropped below the detection level in about 40 % of vaccinees within 4 years. Yet, immunization still proved effective in protecting from HPV18 infection and induced disease [24].

Immunization with HPV L1 VLPs was shown to elicit an antibody response that is 2–4 logs higher than the one induced by natural HPV infections [25]. In cattle, the antibody response to natural BPV infection is very poor [8] and, in sarcoid-affected equids, no anti-BPV1 antibodies are detectable from serum [26]. The high protective efficacy
of PV L1 VLP-based vaccines is mainly based on circum-
vention of the viral epithelial defence line by intramuscular
delivery and the ability of L1 VLPs to induce a strong T
h cell-dependent B cell response. This response entails the
generation of high titres of neutralizing antibodies and the
establishment of B cell memory [27].

Interestingly, several studies suggest that cytotoxic T lympho-
cyte (CTL) responses to L1 VLPs and effector memory T
cells (CD8+ T_EM) likewise contribute to the prophylactic
efficacy of L1 VLPs [28]. In 1998, De Bruijn et al. reported
that a single injection of HPV16 L1 VLPs elicited efficient
and type-specific CTL-mediated protection of C57Bl/6 mice
from challenge with syngeneic HPV16-transformed C3
tumour cells that express L1, but not with L1-deficient TC1
tumour cells [29]. In more recent studies, immunization of
healthy volunteers with HPV16 L1 VLPs was likewise
shown to induce an L1-specific CTL response [30, 31].

In analogy, three-time immunization of horses with BPV1
L1 VLPs in 2007–2008 may have elicited an L1-specific CTL
and CD8+ T_EM response. In a previous study, we have
shown by immunohistochemistry that BPV1-induced pseudo-sarcoïd cells express L1 protein [16]. Similarly, we
detected L1 protein in naturally BPV1-induced equine sar-
coids [32]. On these grounds, it is conceivable that BPV1
L1-specific CD8+ T_EM may have contributed to the longev-
ity of BPV1 L1-mediated protection of the study horses by
migrating into the skin and becoming resident memory T
cells supporting protection from intradermal challenge with
BPV1 [28, 33]. This could also explain complete protection
of individual no. 10 from virus challenge.

This hypothesis is also in agreement with the finding that vac-
cinia virus (VACV) skin infection leads to CD4+ T cell- and
interferon γ-independent recruitment of VACV-specific
CTLs to the skin and the generation of long-lived resident
CD8+ memory T cells (CD8+ T_RM) within the entire integu-
ment that protect efficiently from re-infection [28, 33].

Taken together, BPV1 L1 VLP-mediated protection of seven
horses from experimental BPV1-induced pseudo-sarcoïd
formation is shown to be long-lasting, irrespective of VLP
dosage and individual BPV1-neutralizing antibody titres on
the day of virus challenge. Therefore, we propose that long-
term protection of BPV1 L1 VLP-immunized horses is pro-
vided by a sustained neutralizing antibody response aided
by immune cell memory: plasma cells and memory B, but
also memory T cells. Further in vivo studies are needed to
test this intriguing possibility.

Protective efficacy of BPV1 L1 VLPs against challenge with
wild-type virions and the demonstrated longevity of protec-
tion recommend this vaccine for routine use as sarcoïd pro-
phylaxis in horses and other equids.

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Medicine, Vienna, Austria, for providing the 10 teaching horses for
this study.

Conflicts of interest
None of the authors has any financial or personal relationships that
could inappropriately influence or bias the content of the paper.

Ethical statement
The animal trial was approved by the institutional ethics committee,
the Advisory Committee for Animal Experiments (§12 of Law for Ani-
mal Experiments, Tierversuchsgesetz – TVG) and the Federal Ministry
for Science and Research, Approval No. 68.205/0144-II/3b/2012. All
horses involved continued their work as teaching horses during and
after the trial.

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