Serological markers for papillomavirus infection and skin tumour development in the rodent model *Mastomys coucha*

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This study used the rodent *Mastomys coucha* latently infected with *Mastomys natalensis* papillomavirus (MnPv) and *Mastomys coucha* PV2 (McPV2), which induce skin papillomas and anogenital condylomas, respectively, to investigate PV antibody responses as serological markers during pathogenesis. In a case–control study (137 animals), virus and tumour prevalence correlated with the seroresponse against the early E2 and late L1 viral proteins. A prospective study (53 animals) revealed for the first time the course of these antibody responses during all stages of a natural PV infection. Numerous tumour entities were observed on the eyelid and in the oral cavity. DNA analyses indicated that McPV2 was not restricted to condylomas but was also present in these mucosa-associated papillomas. The serological survey using a recently established glutathione S-transferase-capture ELISA detected a strong correlation between MnPV L1-specific antibodies and the presence of papillomas on the skin, eye and ear ($P<0.001$). Notably, extensive antibody responses to MnPV E2 were also detected in these cases. A prospective study revealed that E2 reactivity occurred by the age of 1 month. MnPV L1 antibodies were found at 2.5 months, indicating the initiation of productive viral infection. Thirty-one out of 34 L1-seropositive animals at the age of 4.5 months developed MnPV-associated tumours (positive predictive value = 77%), and none of the seronegative animals developed skin papillomas (negative predictive value = 100%). MnPV E2 and L1 serology thus provides a powerful tool for monitoring early infection and skin tumour progression in *M. coucha*.

**INTRODUCTION**

Papillomaviruses (PVs) are small, non-enveloped DNA viruses that are frequently found in animals, particularly in vertebrates such as mammals and birds (Antonsson & Hansson, 2002). Different virus types infect epidermal or mucosal tissues and may cause diverse epithelial lesions such as papillomas and warts. PVs are represented by a large family of different genera, and specific types can induce cancerous lesions, for instance in rabbits, cattle and horses (Campo, 2002). An oncogenic potential has also been identified for human papillomaviruses (HPVs), which are by far the most heterogeneous group, with more than 100 types identified (de Villiers et al., 2004). Mucosal ‘high-risk’ HPV types have the potential to transform cells and are aetiologically linked to cancers, mostly in the uterine cervix (zur Hausen, 2002). Tumorigenicity and malignant progression are characterized by constitutive expression of the viral oncogenes E6/E7 and are often accompanied by integration of the viral DNA into the host genome. The oncogenic potential arises from their ability to interact with the cellular proliferation machinery, in particular the E6-induced degradation of p53 (Scheffner et al., 1993) and by E7 binding to pRB, releasing the S-phase-promoting factor E2F (Giarré et al., 2001). During normal infection, the viruses replicate and mature in the course of epithelial differentiation, which is tightly regulated by interplay of the early genes E1, E2, E6 and E7. The E2 protein is expressed early in the lower epidermal layers, promoting virus replication, whereby further skin differentiation and virus maturation is ensured (Doorbar, 2006).
In the superficial layers, capsids are released, which can initiate humoral immune responses to the structural protein L1 (Carter & Galloway, 1997). Based on this immunity, a prophylactic vaccine against genital HPV is now available that acts by inducing neutralizing antibodies, thereby preventing primary infections (Lowy & Schiller, 2006; Stanley, 2008).

HPV serology has been employed to monitor the natural history and course of infection. Antibodies against early proteins were detected in the sera of women with cervical cancer and were found to be suitable as diagnostic markers (Meschede et al., 1998). In contrast to genital types, detailed knowledge of viral pathogenesis is lacking for skin HPVs, which in recent years have been suspected to be aetologically linked to the development of non-melanoma skin cancer (Pfister, 2003), especially at UV-exposed areas (Akgül et al., 2006; Nindl et al., 2007). Here, the transforming mechanisms are likely to be different from genital types, and an oncogenic potential of E6 or E7 has not yet been clarified in detail. Genital HPV E2 represses viral oncogene transcription and is frequently disturbed in cervical cancer by viral integration (Schwarz et al., 1985; Choo et al., 1987). In contrast, E2 of cutaneous HPV types seems to have the opposite impact on tumourigenesis. In vitro assays have shown no suppressive effect on cell proliferation for HPV-8 E2 (Oldak et al., 2004) and HPV-8 E2-transgenic mice even spontaneously developed skin tumours (Pfeifferle et al., 2008). Further studies are needed to understand better the aetiopathology of cutaneous HPVs and to identify novel markers or targets against infection. Therefore, appropriate in vivo models are required.

*Mastomys coucha* is an African multimammate mouse and is used as model to investigate PV-associated skin tumourigenesis. The colony maintained at the German Cancer Research Center (DKFZ) is naturally infected with *Mastomys natalensis* papillomavirus (MnPV) and *M. coucha* PV2 (McPV2), which, similar to cutaneous and genital HPV, infect epidermal and mucosal tissues (Müller & Gissmann, 1978; Tan et al., 1994; Nafz et al., 2008). In these mice, there is a high incidence of skin papillomas, which do not regress spontaneously but progress to squamous cell carcinoma after topical application of tumour promoters (Wayss et al., 1981). Furthermore, McPV2 has been found to induce tumours of the anogenital tract, morphologically resembling human condylomas (Nafz et al., 2008). The aim of this study was to investigate the development of tumours in relation to the respective PV infection by analysing the viral DNA status and the host seroresponse. Virus prevalence was investigated in a case–control study where MnPV and McPV2 were found in tumours of different origins. Serological responses against viral proteins were analysed in relation to the tumour type in order to identify potential markers for PV pathogenesis. In addition, a prospective study was performed to monitor the course of antibody production during infection. MnPV and McPV2 serology was measured using a recently established L1-specific capture ELISA with high sensitivity and reproducibility (Schäfer et al., 2010). This method was used for detecting antibodies against the viral proteins E6, E7, E2 and L1 in order to reveal differences in seroreactivity between tumour-free and tumour-bearing animals.

**RESULTS**

**Tumour entities and prevalence of PV DNA**

To investigate the serological responses to cutaneous and mucosal *Mastomys* PV infections, we performed a case–control study with 137 animals (64 males, 73 females; mean age: 12.0 months, range: 0.7–21.4 months), which was designed to detect antibodies against early and late viral proteins. The study comprised 96 cases and 41 controls (Table 1), where cases were selected for the presence of diverse tumour types, whilst controls were tumour-free. All sera were collected at the time point of animals sacrifice.

All lesions observed on the 96 cases were categorized into different tumour entities related to the tissue origin, namely the skin and anogenital mucosa, as well as epithelia of the eyelid, ear canal and oral cavity. The majority of cases (60.4 %) developed different tumours simultaneously. Of the 96 tumour-bearing animals, 15 suffered exclusively from skin papillomas (Fig. 1b). Condylomas (Fig. 1c) were detected in 21 cases; however, only three cases developed this type of lesion exclusively (Table 1). Skin papillomas, irrespective of the presence of other tumours, were found in 52 cases (54.2 %). Seventy-six animals (79.2 %) suffered from papillomas originating from the eye, ear or oral cavity. To correlate these tumours with distinct virus infections, DNA samples were taken from all lesions. Furthermore, to assess the overall virus prevalence, tissue samples of all animals, including tumour-free individuals, were taken from potential infection sites. Fig. 1 shows representative pictures of the appearance of the most prevalent tumour phenotypes observed in the study population, together with the corresponding PCR-based virus detection results. The prevalence of DNA-positive tested animals is shown in Table 1. Eighty-four animals were positive for MnPV and 60 for McPV2 DNA, whilst 48 had tumours associated with both viruses. MnPV DNA was detected in all 52 cases of skin papillomas. In the case of 15 animals that suffered exclusively from skin papillomas, all tumour samples were MnPV positive. Six of these animals also tested positive for this virus in normal skin. McPV2 DNA was not detected in normal skin. With regard to condylomas, 12 cases were MnPV positive and 19 were McPV2 positive. In addition, McPV2 DNA was detected in tumours on the eyelid (Fig. 1a), presumably arising from the conjunctiva (‘papilloma eye’). Fewer tumours were found in the ear canal (‘papilloma ear’), where, out of 12 animals, 11 were
positive for MnPV and six for McPV2. Interestingly, detailed analysis of the oral cavity revealed that the majority of cases with external tumours (73 animals) also developed papillomas on the tongue (48 animals) (Fig. 1d). Eight cases showed these kinds of tumour exclusively without having any other lesions.

**Table 1.** Prevalence of tumours and viral DNA in the case–control study

<table>
<thead>
<tr>
<th>Tumour type</th>
<th>Phenotype (n=137)*</th>
<th>DNA prevalence†</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>MnPV</td>
<td>McPV2</td>
<td>Both</td>
<td></td>
</tr>
<tr>
<td>Tumour-free</td>
<td>41 (29.9)</td>
<td>10 (24.4)</td>
<td>4 (9.8)</td>
<td>1 (2.4)</td>
<td></td>
</tr>
<tr>
<td>Tumour</td>
<td>96 (70.1)</td>
<td>84 (87.5)</td>
<td>60 (62.5)</td>
<td>48 (50.0)</td>
<td></td>
</tr>
<tr>
<td>Skin papilloma total</td>
<td>52 (38.0)</td>
<td>52 (100)</td>
<td>2 (3.8)</td>
<td>2 (3.8)</td>
<td></td>
</tr>
<tr>
<td>Skin papilloma only</td>
<td>15 (10.9)</td>
<td>15 (100)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Condyloma total</td>
<td>21 (15.3)</td>
<td>12 (57.1)</td>
<td>19 (90.5)</td>
<td>10 (47.6)</td>
<td></td>
</tr>
<tr>
<td>Condyloma only</td>
<td>3 (2.2)</td>
<td>2 (66.7)</td>
<td>3 (100)</td>
<td>2 (66.7)</td>
<td></td>
</tr>
<tr>
<td>Papilloma tongue total</td>
<td>56 (40.9)</td>
<td>46 (82.1)</td>
<td>34 (60.7)</td>
<td>25 (44.6)</td>
<td></td>
</tr>
<tr>
<td>Papilloma tongue only</td>
<td>8 (5.8)</td>
<td>6 (75.0)</td>
<td>7 (87.5)</td>
<td>5 (62.5)</td>
<td></td>
</tr>
<tr>
<td>Papilloma eyelid total</td>
<td>35 (25.5)</td>
<td>28 (80)</td>
<td>25 (71.4)</td>
<td>18 (51.4)</td>
<td></td>
</tr>
<tr>
<td>Papilloma eyelid only</td>
<td>12 (8.8)</td>
<td>8 (66.7)</td>
<td>12 (100)</td>
<td>8 (66.7)</td>
<td></td>
</tr>
<tr>
<td>Papilloma ear†</td>
<td>12 (8.8)</td>
<td>11 (91.7)</td>
<td>6 (50)</td>
<td>5 (41.7)</td>
<td></td>
</tr>
</tbody>
</table>

*Number of *Mastomys* with at least one of the respective tumour entities (% of 137 animals).
†Number of *Mastomys* with at least one DNA-positive sample of the respective PV type (% of the respective tumour entity).
‡Papillomas of the ear were observed only in combination with other tumours.

Fig. 1. Detection of MnPV and McPV2 in different tumours. (a–d) Characteristic virus-induced tumours on the eyelid (a), skin (b), anus (c) and tongue (d). (e) DNA-based virus detection is illustrated for five representative animals. PCR analysis of DNA extracted from *Mastomys* tissues was performed to detect MnPV and McPV2 by amplifying an L1 fragment from each virus.
Analysis of the serological response in relation to the tumour phenotype

Seroreactivity against the early proteins E6, E7 and E2 and the late L1 protein was tested for both viruses. In order to investigate whether there was a predominant antibody response for distinct infection-related phenotypes, PV serology was correlated with the different observed tumour entities. First, we concentrated on skin papillomas and anogenital condylomas, which are known to be associated with MnPV and McPV2 infections, respectively. MnPV L1 reactivities of the 15 animals with papillomas only on the skin differed significantly from the controls (P<0.001) (Fig. 2a). A similar phenomenon was observed for animals with condylomas (Fig. 2b), where the L1 reactivities in the McPV2 ELISA were also significantly increased compared with the tumour-free group (P<0.001). Seroresponses to the early proteins E6 and E7 were low compared with the L1 response. In controls, there was no difference between E6 and E7 reactivities for the two viruses (median A450 0.1, respectively). However, a significant correlation with the respective tumours was found for MnPV E7 and McPV2 E6 (P<0.01). The antibody response to E2 was increased for both viruses in tumour-bearing animals. Interestingly, the overall reactivity to MnPV E2 (median A450=1.42) was higher than the L1 response (median A450=0.64). Although the sera of tumour-free animals also reacted with this antigen, there was a significant association of the MnPV E2 response with skin papillomas (P<0.001). To prove that the antibodies were in fact directed against correctly folded protein, we performed immunoprecipitation assays. Recombinant E2 was expressed in mammalian cells and precipitated by reactive Mastomys sera, indicating that the antibodies detected in the glutathione S-transferase (GST)-capture ELISA specifically recognized and bound to conformational E2 epitopes (see Supplementary Fig. S1, available in JGV Online).

To identify the predominant antibody response related to tumours of the eye, ear and tongue, sera of Mastomys that suffered exclusively from such lesions were analysed by MnPV and McPV2 ELISA (Fig. 3). Generally, reactivities to MnPV antigens were higher than to McPV2 antigens in both tumour-bearing animals and controls. This was not seen for E6 (Fig. 3b) or for L1 in the case of tongue papillomas (Fig. 3a), although only eight animals could be observed for the latter. MnPV responses were particularly pronounced for E2 (Fig. 3d). MnPV L1 antibodies correlated strongly with papillomas of the eye and ear (P<0.001) where the highest responses were seen in cases of ear papillomas (median A450=1.38), which occurred exclusively in combination with other tumour types (Fig. 3a). In contrast to E6, the MnPV E7 response was significantly increased in animals with eye and ear papillomas. A similar situation was seen for McPV2 E6. The highest responses to E6 and E7 were observed simultaneously in four animals, which all suffered severely from multiple types of tumour.

To investigate whether DNA sampling detected the respective virus that induced the antibody response, we correlated the serology results with the presence of MnPV and McPV2 in tissue samples of tumour-bearing animals (Fig. 4a). A high concordance was found between MnPV DNA positivity and antibodies against L1 and E2 (P<0.01 and P<0.001, respectively). In the case of McPV2, no significant association was observed. In order to serologically monitor infections in tumour-free Mastomys, the 41 controls were divided into two groups, specified by the presence of viral DNA in the respective non-tumour tissue.

![Fig. 2. Seroreactivity against early and late viral proteins. Antibody responses against the viral capsid protein L1 and the early proteins E6, E7 and E2 were measured by GST-capture ELISA. The sera of animals suffering exclusively from MnPV-induced skin papillomas (a) and McPV2-induced condylomas (b) irrespective of the presence of other tumours were compared with tumour-free controls. *, P<0.01; **, P<0.001.](image-url)
samples. DNA-negative animals revealed antibody reactivities that were indistinguishable from their DNA-positive counterparts (Fig. 4b). In these cases, the infection responsible for the seroresponse was not detected in the randomly taken tissue samples by the PCR protocol.

**Time course of L1 and E2 antibody responses during infection – a prospective study**

The serological response to MnPV and McPV2 was monitored in a prospective study by following 53 *Mastomys* for the duration of their life. This investigation concentrated on L1 and E2 antibodies, as reactivities to E6 and E7 were generally only low (Figs 2 and 3). Over a period of 22 months, 34 animals (64.2%) developed different virus-induced tumours. All had tumours associated with MnPV infections and 22 also developed McPV2-associated lesions. A detailed compilation of the observed phenotypes is listed in Fig. 5(a). Tumours initially occurred on average at the age of 11.5 months. Skin papillomas were the most frequent lesions, followed by papillomas of the eye and tongue. Considering the time course of tumour development, skin and eye papillomas as well as anogenital condylomas were the earliest lesions, occurring at the age of 6 months (Fig. 5b). Papillomas of the ear were the latest appearing tumours on five animals that already suffered from multiple skin lesions. The first incidence of tongue papillomas was not monitored in this study.

Antibody responses against L1 and E2 were determined for MnPV-associated and McPV2-associated tumours (Fig. 6a). The first serum analysis 4 weeks after birth revealed no

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**Fig. 3.** Analysis of antibody responses in relation to the tumour entity. The reactivity of sera against L1 (a), E6 (b), E7 (c) and E2 (d) of animals suffering exclusively from the respective tumour type was compared with tumour-free controls. Ear papillomas occurred only in combination with other lesions. *, P<0.01; **, P<0.001.
L1 reactivity against either virus. In contrast, low reactivities to MnPV E2 were already detectable (mean $A_{450}=0.09$). Significant antibody responses against MnPV L1 were measured at the age of 4.5 months. Thereafter, a remarkable increase in L1 reactivities was observed at 8.5 months that correlated with the initial appearance of skin papillomas (Fig. 5b). The final serum test revealed antibody responses against MnPV L1 and E2 in tumour-bearing animals, which were significantly higher than in tumour-free animals ($P<0.001$ and $P<0.01$, respectively). A similar but overall lower course of antibody responses was observed in the sera of Mastomys suffering from McPV2-associated tumours, although this was not significantly different from the controls. As the sera of 4-week-old animals were reactive to MnPV E2 (Fig. 6a), we analysed whether the early infection correlated with the viral state of the mother. Interestingly, single animals had E2 responses up to $A_{450}=0.4$ at this age; these were not distributed randomly among the litters but were seen in offspring from mating pairs 2 and 6 (Fig. 6b). Both dams had the highest MnPV L1 and E2 responses, reflecting a productive viral infection ($A_{450}=1.17$ and 0.88 for L1, and $A_{450}=0.62$ and 0.38 for E2 for pairs 2 and 6, respectively). The offspring showed increased E2 antibody levels (mean $A_{450}$ of 0.20 and 0.18 for pairs 2 and 6, respectively) compared with offspring from mating pairs with low reactivity of the dams (mean $A_{450}=0.07$), implying that early E2 responses correlated with maternal antibodies.

Fig. 4. MnPV and McPV2 antibody responses in relation to viral DNA. (a) The prevalence of MnPV and McPV2 DNA in all tumour types of the 96 cases was determined by PCR analysis. Animals were classified as DNA positive (+) when viral DNA was detected in at least one sample. The reactivity of sera against L1, E6, E7 and E2 antigens was determined for both viruses and related to the presence of the respective viral DNA. (b) The seroreactivity of animals without tumours was related to the presence of the corresponding viral DNA in non-tumour tissue samples from the skin, anogenital region, tongue and eyelid. *, $P<0.01$; **, $P<0.001$. 
against MnPV. Both parents developed tumours and the offspring also had a high tumour incidence (100 % of pair 2 and 67 % of pair 6). Early infection was not a requirement for tumorigenesis, as animals derived from seronegative parents also developed papillomas. We then analysed whether L1 reactivities, which differed in tumour-bearing and tumour-free animals from the age of 4.5 months, were suitable serological markers to assess future tumour development (Fig. 6c). L1 positivity at that age was found to predict MnPV-associated tumours [positive predictive value (PPV)=77 %]. Moreover, none of the L1-negative animals developed skin papillomas later in life [negative predictive value (NPV)=100 %].

DISCUSSION

Analysis of the serological response against MnPV and McPV2 with the focus on their natural spread within the colony revealed novel insights into viral tropism, the course of infection and the overall tumour variety in the Mastomys model. DNA analysis showed that a large proportion of tumours of the eye, ear and tongue harboured both virus types (Table 1). In fact, multiple infections are frequently observed for PVs (Antonsson et al., 2003; de Koning et al., 2007) and crosstalk during pathology between different viruses is still a matter of debate, as has been discussed for human genital warts (Chan et al., 2009). MnPV has also frequently been found in comparable lesions of the head and neck region, as well as in several condylomas, which is rarely seen for skin wart-associated HPVs. Apart from a few exceptions such as HPV-2 and -57 (Van Ranst et al., 1992; Chan et al., 1997), the presence of skin wart-inducing PVs in genital tissues has also been observed for other animal papillomaviruses, e.g. bovine papillomavirus type 2 in urothelial tumours (Borzacchiello & Roperto, 2008). Taken together, both viruses showed a broad distribution regarding diverse epithelial lesions, although the skin seemed to be restricted to MnPV infections.

The serological study revealed antibody responses to early proteins and also included a response against the viral capsid. Strong L1 responses were especially pronounced in animals with skin papillomas (Fig. 2) and are most likely due to the plethora of virions in these lesions (Nafz et al., 2007), as is also seen for wart-associated PVs such as HPV-1 (Grußendorf, 1980). Our study detected, for the first time, abundant antibody reactivities against the early protein E2, especially for MnPV, suggesting that it is strongly expressed and presented to the immune system. The authenticity of these responses was confirmed by immunoprecipitating native E2 protein using ELISA-reactive Mastomys sera (Supplementary Fig. S1). Although most tumour-free animals had MnPV E2 antibodies, the magnitude of response still correlated with the presence of papillomas.
Fig. 6. (a) Time course of L1 and E2 antibody responses. L1 and E2 serum responses in the prospective study were measured over a period of 22 months at seven time points at intervals of at least 2 weeks. MnPV reactivities were correlated with animals with MnPV-associated tumours and tumour-free individuals (left panels). The same comparison was performed for McPV2 with sera of animals with McPV2-associated tumours (right panels) *, P < 0.01; **, P < 0.001. (b) MnPV E2 antibodies in 4-week-old animals. The antibody responses to MnPV L1 and E2 of the dams in each mating pair (dark grey) were measured 4 weeks after the birth of the litter. Mean seroreactivities of the corresponding offspring at the same date are represented in light grey. (c) Early L1 antibodies as a predictor for tumour development. Antibodies (abs) against MnPV L1 at the age of 4.5 months correlated with the subsequent development of tumours. PPV/NPV, Positive/negative predictive value; Sens., sensitivity; Spec., specificity.
E2 expression occurs early during the manifestation of infection, ensuring virus replication in basal epidermal layers (Doorbar, 2006). In contrast to data on the structural components of the virus, insufficient data are available about the immunological features or cellular quantities of this protein. In early studies with genital HPV types, HPV-6 E2 expression was detected in condylomas (Li et al., 1988) but only a few serum samples contained antibodies against E2 of HPV-6 (Jenison et al., 1988) or HPV-16/-18 (Wikström et al., 1995). In the Mastomys model, the strong E2 seroreactivity seems to be a peculiarity for the PV skin type and might be reflected in the amount of antigen produced, provided there is a proportionality to the number of viral copies in MnPV-associated tumours (Naß et al., 2007). Given the high immunogenicity, E2 may be considered a target to defeat viral infection in the early or even late stages of disease. A therapeutic vaccine might be effective by inducing E2-specific cytotoxic T cells, as has been tested in the cottontail rabbit PV model by antagonizing experimentally induced viral tumours (Leachman et al., 2002; Brandsma et al., 2007).

Further insights into the initial virus infection and tumour progression were obtained by our prospective study, in which L1 and E2 seroreactivity correlated with the outcome of disease. The E2 response was detectable shortly after birth, and it seems that these antibodies mark the early stages of infection. The observed reactivities might also have been due to maternal transmission via the placenta (Jauniaux et al., 1995). However, this was not seen for L1, although the antibody response of the dams to this protein was even higher at the time of blood withdrawal (Fig. 6b). It is probable that, at the birth of the offspring, the 3.5-month-old mothers had predominantly E2 antibodies, and elevated L1 responses may have occurred during lactation. Accordingly, elevated MnPV L1 reactivities of the offspring occurred at a corresponding age of approximately 4 months (Fig. 6a). Viral DNA, however, can be detected by 4 weeks after birth (Naß et al., 2007), indicating that there is presumably a time span between primary infection and L1 seroconversion of about 3–4 months. During this phase of infection, the virus appears to persist in a latent state, producing insufficient amounts of viral particles to induce a proper B-cell response. The dramatic increase in L1 reactivities at the age of 8.5 months correlated with the initial appearance of skin papillomas (Fig. 5b) and can be explained by high proliferation rates in conjunction with massive virus production within the tumours (Naß et al., 2007), giving rise to the induction of a strong B-cell stimulation. L1 seroconversion may thus reflect the actual event of productive infection, occurring approximately 3 months before the first tumours were observed. The preceding period is apparently characterized by viral latency where antibody levels are low in healthy as well as in tumour-prone animals. In the latter case, however, there is an increase in L1 antibodies at the age of about 4 months (Fig. 6a). Here, for still unknown reasons, an escape from latency occurs and production of mature viruses is initiated. Such an increase was not observed in tumour-free animals. Consequently, L1 antibody reactivity at that age emerged as a potent predictor for the development of MnPV-induced tumours (Fig. 6c). In addition, the strength of the L1 response correlated with the severity of the disease. The highest reactivities were seen in animals with papillomas of the ear (Fig. 3a) that only appeared late during pathogenesis, when multiple tumours had already developed.

Combining the data of the case–control and the prospective studies, it is clear that serology provides a reliable tool to monitor virus infection and future vaccination studies in M. coucha, which was validated by the presence of viral DNA (Fig. 4). Furthermore, taking seroreactivity as indicative of an existent or past virus exposure, it was possible to determine infections that were not detected by DNA analyses. MnPV E2 antibodies occurred long before the pathological phenotype and thus may represent a suitable marker for infection, detecting early and latent virus stages. Similar serological features have been observed with McPV2, albeit with overall lower reactivities. The differences are probably based on the fact that McPV2 generally exists at much lower copy numbers than MnPV (Naß et al., 2008). A clear association between McPV2 L1 antibodies and the presence of tumours was found only in the case of condylomas (Fig. 2b).

In conclusion, this report reflects the variety of infection sites and tumour entities emanating from at least two distinct PV types and their impact on the humoral immune system, a process that can be utilized to investigate the principal course of infection. Detection methods such as genomic or serological analyses have been used routinely for papillomavirus diagnosis in clinics and in epidemiological studies. So far, no studies have been performed with E2 antibodies to detect and investigate cutaneous HPV infection in humans. Epidemiological approaches utilizing L1 serology have revealed different seroprevalence patterns for distinct cutaneous HPV types, arising from early childhood for types of the mu and nu genera to the accumulation of beta and gamma skin PVs later in life (Michael et al., 2008). As L1 serology is related to a productive infection, our data suggest that it is also important to investigate the E2 response in humans in order to elucidate the history of infection or overall prevalence within populations.

**METHODS**

**Animals.** M. coucha from the breeding colony at the DKFZ were kept under conventional conditions (21–24 °C, 55 % relative humidity with 12–16 air changes h⁻¹, mouse breeding diet, water ad libitum). When tumours reached a size of 2 cm for papillomas or 0.5 cm for condylomas, the animals were sacrificed and blood was taken by cardiac puncture. The 53 animals of the follow-up study were derived from eight mating pairs and each litter was kept in a singular cage. When tumours reached a size of 2 cm for papillomas or 0.5 cm for condylomas, the animals were sacrificed and blood was taken by cardiac puncture. The 53 animals of the follow-up study were derived from eight mating pairs and each litter was kept in a singular cage until 4 weeks after birth. Parental animals were mated at an age of 2.5 months. Twenty-one days later, the offspring were born and, in week 4, the first blood samples were taken from the submandibular vein.
During the whole period, the animals were routinely inspected for tumour formation at intervals of 1 month until they had to be sacrificed for reasons of tumour development or decrepitude. *M. coucha* at the DKFZ are maintained in compliance with German and European statutes and all animal experiments were undertaken with the approval of the responsible Animal Ethics Committee (Regional Council of Karlsruhe, Germany).

**DNA isolation from dissected tissues.** Animals were sacrificed with CO2 and dissected tissue samples were frozen at −80°C. To avoid cross-contamination among different tissues, surgical instruments were changed after every sample dissection. The DNA was extracted as described elsewhere (Sambrook *et al.*, 1989). Briefly, the tissue was lysed overnight at 55°C in DNA lysis buffer [50 mM Tris/ HCl (pH 8.0), 50 mM EDTA, 0.5% SDS, 100 µg proteinase K ml−1, 25 mM DTT] and the DNA was extracted twice with phenol: chloroform: isooamyl alcohol (25:24:1). The DNA was then precipitated by adding 0.1 vols 3 M sodium acetate (pH 5.2) and 1 vol. 2-propanol, washed with 70% ethanol and resuspended in 10 mM Tris/EDTA (pH 8.0).

**MnPv and McPv2 detection.** DNA samples from various tissues were screened for the presence for MnPv and McPv2 genomes by specific amplification of fragments of the viral L1 gene in a PCR. DNA quality and input were controlled by amplifying part of the β-actin gene. Total extracted DNA (250 ng) was used as input for the PCR in a mix containing 3 mM MgCl2, 200 µM dNTPs, 0.4 µM each primer, 1 x PCR buffer and 1 U recombinant *Taq* polymerase (Invitrogen). PCR was performed in a thermal cycler for 25 (L1) or 30 (E6) cycles. The PCR protocol for all amplifications was based on a primary denaturation step at 94°C for 5 min, followed by cycles of 30 s at 94°C, 45 s at 58°C and 60 s at 72°C, with a final extension step of 10 min at 72°C. For detection of MnPv, a 139 bp L1 fragment was amplified (forward primer: 5’-TCTACACGGGCTATTGTCCA-3’; reverse primer: 5’-GCCACGAGGTATCTCCACTC-3’); for McPv2, an 817 bp L1 fragment was amplified (forward primer: 5’-TCCCCAAGTGTTCGCAATC-3’; reverse primer: 5’-CAACACGGTATTGCTCCACA-3’); and for β-actin, a 599 bp fragment was amplified (forward primer: 5’-ACCCACACTGTGCCC-3’; reverse primer: 5’-CTTGTGTGATCCACATCTCGT-GGA-3’).

**Expression of recombinant proteins.** Early and late genes of MnPv and McPv2 were derived from pUC19 plasmids containing the whole viral genomes (Schafer *et al.*, 2010) and cloned into a modified pGEX4T3 expression vector (Sehr *et al.*, 2001), which contains the coding region for a C-terminal tag peptide of the simian virus 40 (SV40)-tag, amino acid sequence: KKPPPTPEPET) between the 5’ and 3’ NotI sites. Viral sequences were amplified by PCR, introducing NotI/SalI (MnPv E6, E7 and L1, and McPv2 E6, E2 and L1) or EcoRI/SalI (MnPv E2 and McPv2 E7) ends and inserted into the pGEX4T3-SV40-tag vector. *Escherichia coli* BL21 bacteria transformed with expression vectors containing the early genes as well as the empty vector (GST–SV40-tag) were grown at 37°C in Luria–Bertani medium supplemented with 100 µl ampicillin ml−1. The late gene vectors were transformed into *E. coli* BL21 Rosetta and grown under the same conditions in medium containing 100 µl ampicillin ml−1 and 20 µg chloramphenicol ml−1. Recombinant protein expression at 25°C was induced by adding 0.25 M IPTG at an optical density of 0.5 at 600 nm. After 6 h, the bacteria were pelleted by centrifugation and resuspended in lysis buffer [PBS for early genes; 40 mM Tris/HCl (pH 8), 200 mM NaCl, 1 mM EDTA for L1] containing 2 mM DTT and Protease Inhibitor Cocktail (Roche). The pellets were homogenized by sonification and, after centrifugation (30 min, 30000 g, 4°C), the supernatants were stored as 50% (v/v) glycerol solutions at −20°C. In the case of L1, the sonification step was followed by incubating the lysate, prior to centrifugation, with 2 mM ATP (Sigma) and 5 mM MgCl2 for 1 h at room temperature in order to facilitate bacterial chaperone dissociation.

**GST-capture ELISA.** The reactivity of *Mastomys* sera was determined by a GST-capture ELISA based on a glutathione-containing capture protein, which was produced by chemically cross-linking glutathione to casein (Sigma) as described previously (Sehr *et al.*, 2001). ELISAs were performed as described in detail elsewhere (Schafer *et al.*, 2010). Briefly, 96-well plates were coated with 200 ng glutathione–casein in 50 mM carbonate buffer (pH 9.6) per well and incubated with 100 µg per well of cleared lysates from *E. coli* expressing the GST–SV40-tag fused viral antigens. After incubation with *Mastomys* sera, bound antibodies were detected by an HRP-conjugated goat anti-mouse IgG antibody (Promega). Each antigen was quantified by titration to determine the total amount of protein necessary to completely saturate the ELISA (data not shown), where the fusion proteins were detected by a mouse mAb directed against the SV40-tag (MacArthur & Walter, 1984). To block any reactivity with contaminating bacterial proteins, the sera to be assayed were diluted in blocking buffer, containing total lysate protein (0.25 mg ml−1) from *E. coli* expressing the GST–SV40-tag fusion protein. In addition, replicate plates coated with the GST–SV40-tag were included for each serum to detect residual binding to these epitopes. These background values were subtracted from the observed absorbance value (A450) with the respective viral antigen. All sera throughout the study were collected and analysed concurrently in duplicate at the end of the project.

**Statistical analyses.** Box plots were generated using SigmaPlot software. A box spanned the interquartile range and contained the median absorbance as a horizontal line. Outliers were depicted outside the 10th and 90th percentile. Statistical significance was calculated by Student’s t-test. PPV and NPV were calculated using a cut-off of A450=0.35.

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**REFERENCES**


