Vaccination of cattle with bovine papillomavirus type 4 L2 elicits the production of virus-neutralizing antibodies

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Introduction

Infection of epithelia by papillomavirus results in papillomas, benign lesions which are eventually cleared by an immune response mounted by the host. In a small minority of cases, papillomas persist and in the presence of co-factors can provide a focus for malignant transformation. Although the overall frequency of neoplastic progression of papillomas is low, papillomavirus is implicated in the overwhelming majority of ano-genital cancers in the human population (zur Hausen, 1991). It follows that prevention or cure of papillomavirus infection would reduce the incidence of one of the commonest forms of human cancer.

We have been studying bovine papillomavirus type 4 (BPV-4) as a model for the human mucosotropic papillomaviruses. BPV-4 infects the mucosal epithelium of the upper alimentary canal of cattle and the resulting papillomas can progress to squamous cell carcinomas in animals grazing on bracken fern (Campo et al., 1994). We have developed both prophylactic and therapeutic vaccines against BPV-4 which prevent viral infection and induce early regression of papillomas respectively (Campo, 1994). Prevention is achieved by vaccination with the minor capsid protein L2 (Campo et al., 1993) and the N terminus of L2 (L2a), comprising amino acids (aa) 11–200, is necessary and sufficient for protection against infection (Chandrachud et al., 1995). Here we show that vaccination with L2 or L2a elicits the production of serum neutralizing antibodies and that the neutralizing activity of immune sera can be abrogated by pre-absorption with L2, thus demonstrating that anti-L2 antibodies are responsible for virus neutralization.

Methods

Vaccines. The L2mix vaccine consisted of a mixture of glutathione S-transferase fusion proteins: GST–L2w, GST–L2a, GST–L2b and GST–L2c, corresponding respectively to the whole L2 protein (aa 8–524), the N terminus (aa 11–200), the middle portion (aa 201–326) and the C terminus (aa 327–524), in aluminium gel as adjuvant; the ratio of the four GST-fusion proteins was 1:5:5:5. The L2a vaccine consisted of GST–L2a in aluminium gel.

A full description of the construction and production of the fusion peptides and of the composition of the vaccines has already been presented (Campo et al., 1993; Chandrachud et al., 1995).

Animals. Calves no. 87 and 93 were vaccinated with two doses each of 1 mg GST–L2mix 4 weeks apart; calf 259 was vaccinated with two doses each of 330 µg GST–L2a; calves 110 and 114 were not vaccinated. Two weeks after the second vaccine dose, all calves were inoculated in the soft palate with 1011 particles of BPV-4 in ten sites.

The vaccinated animals were free of papillomas whereas the non-vaccinated animals developed papillomas at all the injection sites (Table 1). Blood samples were taken from each animal before vaccination (P.I.), after vaccination but before challenge [V2 + 1w and V2 + 2w(C)], and then at intervals of 3–4 weeks after challenge (C + 4w, etc.).

Full vaccination, bleeding protocols and comprehensive results have been published previously (Campo et al., 1993; Chandrachud et al., 1995).
Table 1. Absorption of sera with L2 removes anti-L2 antibodies

Sera from animals vaccinated with L2mix, L2a or non-vaccinated were tested in ELISA against L2mix or L2a respectively, before and after vaccination, and before and after serum absorption with L2 or L2a. Serum dilutions are all I:4. Calves 87, 93, 110 and 114 are calves 3, 8, 28 and 32 respectively of Fig. 2(b) in McGarvie et al. (1994); calf 259 is calf 27 in Chandrachud et al. (1995). NA, Not applicable.

<table>
<thead>
<tr>
<th>Calf no.</th>
<th>Vaccine</th>
<th>No. of papillomas</th>
<th>ELISA absorbance reading</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Pre-immune</td>
</tr>
<tr>
<td>110</td>
<td>None</td>
<td>12</td>
<td>0.237</td>
</tr>
<tr>
<td>114</td>
<td>None</td>
<td>12</td>
<td>0.185</td>
</tr>
<tr>
<td>87</td>
<td>L2mix</td>
<td>0</td>
<td>0.167</td>
</tr>
<tr>
<td>93</td>
<td>L2mix</td>
<td>0</td>
<td>0.183</td>
</tr>
<tr>
<td>259</td>
<td>L2a</td>
<td>0</td>
<td>0.299</td>
</tr>
</tbody>
</table>

Nude mouse xenografts. Athymic female nude mice were obtained from Harlan-Olac. Bovine fetal palate tissue was cut into approximately 1 mm chips which were then incubated overnight with \(10^{11}\) particles of BPV-4. Individual chips were then implanted subcutaneously in the anterior left flank of anaesthetized mice. The implants were allowed to grow for 24 weeks, by which time papilloma development can be unequivocally established, and were then removed for histological analysis and detection of BPV-4 DNA by in situ hybridization. Full protocols for the preparation, infection and implantation of the bovine xenograft have been published before (Gaukroger et al., 1989, 1993).

Calves and mice were maintained in complete accordance with the regulations stipulated by the Home Office of Great Britain.

Virus neutralization assay in nude mouse xenografts. Pre-immune and immune sera from animals 87 and 93, which had been vaccinated with GST–L2mix, and non-immune sera from animals 110 and 114, which had not been vaccinated, were incubated at 56 °C for 30 min to inactivate complement. Immune sera (0.3 ml) were absorbed with 140 µg each of GST–L2a, –L2b and –L2c at room temperature for 5 h and then at 4 °C overnight. The immune precipitates were removed by filtering the sera through a Nalge filter of 0.2 µm pore size. This procedure removed anti-L2 antibodies from the sera as determined by ELISA (Table 1). All the serum samples were diluted to give a final concentration of 1:5 and virus stock was diluted to a final concentration of \(3 \times 10^{11}\) particles/ml. Similar serum dilutions had been found to be effective in neutralizing virus in studies with BPV-2 (Jarrett et al., 1990). The mixtures of virus and serum were incubated overnight at 4 °C and then added to the palate tissue chips as described above.

Virus neutralization assay in cattle. Pre-immune and immune serum samples from animal 259, vaccinated with GST–L2a, and immune serum from animal 87, vaccinated with GST–L2mix (see above), were used. Immune serum from animal 259 was absorbed with 500 µg of GST–L2a and bovine IgG–L2a complexes were removed as described above. Any possible residual excess GST–L2a, which might have induced immunity if injected into calves with the challenge virus, was removed by incubating the serum with 125 µg of a polyclonal antibody against GST overnight at 4 °C and filtering the serum as above. The absence of any GST–L2a from the final serum preparation was confirmed by running a sample on a 10% polyacrylamide gel (data not shown). Complete absorption of anti-L2a antibodies was confirmed by the lack of reactivity of the serum against GST–L2a in ELISA (Table 1). The serum samples were incubated with virus as above and the virus–serum mixtures were then injected into 10–12 sites of the soft palate of each animal by intradermal inoculation.

Histology. The nude mouse xenografts were fixed in buffered formalin and embedded in paraflin for standard histology prior to sectioning and staining with haematoxylin and eosin.

In situ hybridization. Cloned BPV-4 DNA was labelled with digoxigenin-dUTP (Boehringer Mannheim) by the random primer method. Paraffin sections of xenografts were de-waxed, incubated with labelled BPV-4 DNA, washed, stained and mounted exactly as previously described (Gaukroger et al., 1991).

PCR analysis. DNA was prepared from palate tissue removed from euthanased calves. PCR analysis was carried out using primers designed to amplify the E7 region of BPV-4 as previously described (Gaukroger et al., 1991). The primers were synthesized by Cruachem, Glasgow, UK.

Results

BPV-4 L2 vaccination prevents viral infection in calves and induces the production of anti-L2 antibodies

The calves used as the source of serum samples were part of a large experiment on L2 vaccination, previously described (Campo et al., 1993). Calves 87 and 93 were vaccinated with two doses of 1 mg of GST–L2mix each. Serum samples were obtained from both animals before vaccination (pre-immune) and 2 weeks after the second vaccine dose (V2 +2w) but before virus challenge (immune). Antibodies to L2 were detected in the immune sera but not in the pre-immune sera (Table 1). Calves 110 and 114 were not vaccinated. Serum samples were obtained before virus challenge (non-immune) at the same time as the vaccinated animals. No antibodies to L2 were detected in these sera (Table 1). Seven weeks after virus challenge, the control non-vaccinated animals had mature...
Table 2. Vaccination of cattle with BPV-4 L2 elicits production of virus neutralizing antibodies

Virus was preincubated with sera before infection of tissue chips from bovine fetal palate. The chips were grafted subcutaneously in the flank of nude mice. The implants were histologically examined for the presence of papillomas 24 weeks later. Calf numbers are as in Table 1.

<table>
<thead>
<tr>
<th>Serum</th>
<th>Papillomas/implants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fetal calf serum</td>
<td>7/8</td>
</tr>
<tr>
<td>Control calves</td>
<td></td>
</tr>
<tr>
<td>110 Non-immune</td>
<td>7/8</td>
</tr>
<tr>
<td>114 Non-immune</td>
<td>4/7</td>
</tr>
<tr>
<td>L2 vaccinated calves</td>
<td></td>
</tr>
<tr>
<td>87 Pre-immune</td>
<td>5/6</td>
</tr>
<tr>
<td>87 Immune</td>
<td>1/9</td>
</tr>
<tr>
<td>87 L2 absorbed</td>
<td>5/6</td>
</tr>
<tr>
<td>93 Pre-immune</td>
<td>7/9</td>
</tr>
<tr>
<td>93 Immune</td>
<td>1/9</td>
</tr>
<tr>
<td>93 L2 absorbed</td>
<td>4/9</td>
</tr>
</tbody>
</table>

Papillomas, while the GST–L2mix-vaccinated calves were completely free of papillomas (Table 1). The correlation between vaccination with GST–L2mix and absence of papillomas was absolute (Campo et al., 1993).

Incubation of BPV-4 with L2mix immune serum neutralizes virus infectivity in the xenograft system

BPV-4 was incubated with commercial fetal calf serum, non-immune serum from calves 110 and 114, pre-immune and immune serum from calves 87 and 93 and the mixture of virus and serum was then used to infect fetal bovine palate tissue chips. The chips were implanted into nude mice and allowed to develop for 24 weeks. They were removed and analysed histologically for the presence of papillomatous growth (Gaukroger et al., 1989). Although not all the implants developed papillomas, there were no significant differences in the number of papillomas in implants infected with virus incubated with fetal calf serum, non-immune sera or pre-immune sera (Table 2). This indicated that viral infectivity had not appreciably changed in the presence of the different sera. In contrast, the number of papillomas in implants infected with virus incubated with immune sera decreased dramatically and significantly (P < 0.001, Fisher’s Exact Test) (Table 2). Therefore, incubation of BPV-4 with immune sera reduced virus infectivity to a very large extent.

Removal of anti-L2 antibodies restores BPV-4 infectivity

The immune sera from calves 87 and 93 were incubated with 140 μg each of GST–L2a, –L2b and –L2c, to remove the anti-L2 antibodies. After removal of the antigen–antibody immune complexes by filtration, the sera were devoid of detectable anti-L2 antibodies as judged by ELISA (Table 1). BPV-4 was incubated with the L2-absorbed sera and used to infect bovine tissue chips. The infectivity of the virus was restored and the number of papillomas was either the same or approaching that obtained with virus incubated with pre-immune sera (P < 0.001) (Table 2). This showed that anti-L2 antibodies are responsible for reduced virus infectivity.

Antibodies to the N terminus of L2 (L2a) neutralize virus infectivity in the cattle system

Vaccination with the N terminus of L2 (GST–L2a) prevented infection by BPV-4 (Chandrachud et al., 1995). Animal 259 was part of the L2a vaccination experiment and was completely protected from experimental challenge with BPV-4 (Table 1).

To ascertain whether antibodies to L2a were capable of neutralizing BPV-4, a sample of immune serum from V2 + 1w was incubated with 500 μg of GST–L2a and the immune complexes were removed as described in Methods. The L2a-absorbed serum sample was devoid of detectable antibodies to L2a (Table 1). To ensure that no GST–L2a was left in the absorbed serum, which might have caused an immune response if injected into the calves, the serum was tested in ELISA against GST and was proved to be negative (data not shown). BPV-4 was incubated with pre-immune, immune and L2a-absorbed serum samples from animal 259 and with immune...
Fig. 2. In situ hybridization of bovine xenografts in nude mice. (a) Section of bovine papilloma induced by BPV-4 incubated with pre-immune serum from animal 87, hybridized in situ to labelled BPV-4 DNA. (b) Section of a bovine xenograft which had been incubated with L2mix immune serum from animal 87, hybridized in situ as above; note the absence of papilloma and viral DNA positive cells. (c) Section of bovine papilloma induced by BPV-4 incubated with L2mix immune serum from animal 87 pre-absorbed with GST-L2, hybridized as above. Magnification is 50 x in all cases.
serum from animal 87 as control. Each serum-incubated virus batch was injected into the soft palate of seven calves and the calves were examined 7 weeks after infection. Both the total papilloma number and the mean papilloma number were significantly smaller ($P = 0.031$) in the group infected with BPV-4 incubated with L2a-immune serum (group 2) than in the group infected with virus incubated with pre-immune serum (group 1) (Fig. 1). In addition, the papillomas in group 2 were predominantly at a very early stage of development, whereas the majority of tumours in group 1 were mature papillomas (data not shown). Also, in the group infected with BPV-4 incubated with L2mix immune serum (group 3) the papillomas were fewer and smaller (Fig. 1). No statistics were conducted for group 3, as the immune serum used originated from a different animal. These results confirm that immune serum from L2mix-vaccinated animal 87 greatly reduced virus infectivity in calves and showed that the immune serum from animal 259, vaccinated with GST-L2a, had similar, albeit lower, virus neutralizing activity. The number and the stage of papillomas in the group infected with BPV-4 incubated with L2a-absorbed serum (group 4) were very close to the number of papillomas in group 1 (Fig. 1) and significantly ($P = 0.017$) higher than the number of papillomas in group 2. Therefore, removal of anti-L2a antibodies abrogated the virus neutralizing activity of the immune serum.

**Neutralization of BPV-4 prevents virus DNA replication but not virus entry into the cell**

Histological sections of nude mouse xenografts were analysed for the presence of BPV-4 DNA by *in situ* hybridization. The implants with papillomas induced by virus incubated with pre-immune sera were heavily positive for BPV-4 DNA by *in situ* hybridization (Fig. 2a); in contrast, papilloma-negative implants derived from infection with virus which had been incubated with immune sera were completely negative (Fig. 2b); removal of anti-L2 antibodies re-established virus infectivity and viral DNA replication (Fig. 2c). The absence of viral DNA detectable by *in situ* hybridization in papilloma-negative implants showed that no vegetative viral DNA replication had taken place.

As *in situ* hybridization does not detect small amounts of viral DNA, the presence of any latent non-replicating BPV-4 genome would go undetected. PCR analysis was therefore employed in the analysis of bovine palate tissue. Three different palate tissue samples were examined: from a calf infected with virus pre-incubated with pre-immune serum before the onset of papillomas; from a calf infected with virus pre-incubated with L2a-immune serum that was papilloma-free, and from a non-experimental non-infected calf. The expected PCR product of 170 bp was obtained from DNA from the first two calves but not from the third, non-experimental calf, even in conditions that could amplify 0.001 viral genome copies/cell (data not shown). The PCR products were sequenced and this confirmed that BPV-4 DNA had been amplified (data not shown). The detection of BPV-4 DNA by PCR in a calf infected with 'neutralized' virus suggested that L2a neutralization prevented disease but not viral DNA entry into the cell.

**Discussion**

We report here that prophylactic immunity against BPV-4 infection in cattle vaccinated with the virion minor capsid protein L2 is due to the induction of serum neutralizing antibodies. This conclusion is based on the observation that incubation of BPV-4 with immune sera from vaccinated animals greatly reduces virus infectivity both in nude mouse xenografts and in cattle. In both systems, infection with virus incubated with immune sera results in the production of fewer and smaller papillomas.

The antibodies responsible for neutralization of BPV-4 are anti-L2 antibodies. Removal of these antibodies by incubation of sera with the L2 antigen removes the neutralizing activity of the sera and restores virus infectivity. This is definitive proof that L2 encodes neutralizing epitopes and that antibodies directed against these epitopes can neutralize virus infectivity. This is true also for antibodies against the N terminus of L2; their removal by incubation with L2a abrogates virus neutralization.

Induction of neutralizing antibodies by immunization with L2 has been reported also for cottontail rabbit papillomavirus (CRPV; Christensen *et al.*, 1991; Lin *et al.*, 1992). In those studies, removal of anti-L2 antibodies was not attempted and the neutralizing activity was mapped to the C-terminal half of CRPV L2 (Lin *et al.*, 1992). Antibodies raised against BPV-1 L2 in rabbits prevent transformation of C127 cells by BPV-1 although they do not prevent virus binding to the cell surface (Roden *et al.*, 1994). In these studies the neutralizing activity was mapped to the N terminus of L2 (Roden *et al.*, 1994) in agreement with our conclusions.

As BPV-4 DNA was detected by PCR in tissue inoculated with neutralized non-infectious virus, BPV-4 neutralization by anti-L2 antibodies would appear not to inhibit cell attachment, as already shown for BPV-1 (Roden *et al.*, 1994a), or subsequent DNA entry into the cell. The absence of detectable BPV-4 DNA in papilloma-negative nude mouse cysts by *in situ* hybridization shows that no vegetative viral DNA replication has taken place, but does not clarify which step in early infection between viral DNA entry and replication has been blocked by neutralization. The absence of latent infection was demonstrated in rabbits vaccinated with CRPV L1 (Lin *et al.*, 1993). It appears that antibodies against L1 or L2 neutralize virus by different mechanisms, but this is still to be determined.

We are grateful to the stockmen of the Department of Veterinary Pathology for their expert handling of the calves and to A. Bradley for his invaluable help with the nude mice. We thank Professor Wyke for critical reading of the manuscript. G.K. is supported by Cantab Pharmaceuticals, Cambridge. The work was supported by the Cancer Research Campaign.
References


Received 25 January 1996; Accepted 1 March 1996