Prophylactic DNA immunization against multiple papillomavirus types

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Abstract

At least 15 different papillomavirus types are causatively associated with the development of tumors in humans. Since the middle of 2006 a protective, virus-like particle based vaccine against the tumor-related HPV types 16 and 18 is commercially available. We investigated the possibility of applying DNA vaccination to obtain protective antibody responses against multiple papillomavirus types. Our data indicate that low amounts of DNA were sufficient to induce neutralizing antibodies in mice although a DNA dose-dependency in respect to the L1-specific antibody titers was observed. Furthermore, we found that immune responses against different PV types could be induced by simultaneous DNA vaccination with a mixture of expression vectors encoding L1 proteins of different papillomavirus types. However, we observed that there was a strong interference when plasmids encoding different L1 genes were used together. HPV 16 responses were repressed by co-administration of HPV 11 and/or BPV 1 L1 expression constructs. Likewise, BPV 1 responses were repressed by co-administration of HPV 16 or HPV 11 L1 plasmids. This interference could be overcome by administration of the different constructs into different sites of the animals or by sequential immunization. Thus, our results suggest that the mode of repression was due to interference with L1 particle assembly and was not a consequence of immunodominance of certain L1 proteins.

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1. Introduction

The human papillomaviruses comprise a highly heterogeneous group of more than 120 epitheliotropic viruses [1]. Infection by certain types of human papillomavirus is considered to be the main risk factor in the development of cervical cancer [2–4]. Every year, about half a million of women develop cervical cancer of which 99% are HPV positive. The most prevalent HPV type associated with malignancy is HPV 16 which can be found in about 50% of the tumors [5]. Prophylactic vaccination against HPV was shown to prevent infection and cancer precursors and thus most likely the development of malignant disease [6,7]. Protective immunity is achieved by inducing virus-neutralizing antibodies directed against the major capsid protein L1 in the form of virus-like particles or capsomeres. In fact, a commercial vaccine containing L1 VLPs of the cancer-related papillomavirus types 16 and 18 has been licensed by mid-2006 and another one is expected within a few months [6–9]. Although highly efficient, these vaccines induce protective immunity predominantly against the vaccine types. This is mainly due to the fact that each papillomavirus type is considered a separate serotype and that neutralizing antibodies are highly type-restricted [10–13]. Although the L1 capsid protein is the best conserved viral protein, it displays heterogeneous loops on the surface of intact virions which are targets for neutralizing antibodies [14]. The development of second generation vaccines is aimed to provide either broader protection by including more HPV types into a vaccine or to develop antigens that induce cross-protective immunity against multiple papillomavirus types.

A number of studies have shown that as an alternative to protein-based vaccines, protective humoral responses can be achieved by DNA vaccination using codon-modified L1
2. Material and methods

2.1. DNA constructs

DNA encoding for humanized L1 (L1h) antigen of different papillomavirus types (HPV 11, HPV 16, HPV 18 and BPV 1) were subcloned into the mammalian expression vector pUF3 under the control of the cytomegalovirus (CMV) immediate early promoter [23]. The L1h sequences of HPV 11, HPV 16 and BPV 1 [18,24,25] were inserted directly after the CMV promoter at the restriction site NotI, replacing the GFP gene, whereas the HPV 18 L1h DNA sequence was cloned at the restriction site HincII. By subcloning the HPV 18 L1h DNA into the HincII site, the SD/SA intron was disrupted. Consequently, the HPV 18 construct might not be fully comparable to the constructs of the other L1h genes, as the disruption of the SD/SA intron might affect the expression level of the HPV 18 L1h gene. The empty pUF3 vector was created by HincII restriction digest to excise the GFP gene and the neomycin expression cassette, whereas the integrity of the CMV promoter was conserved.

2.2. Immunization

The plasmid DNA used for immunization was isolated from E. coli DH5α by CsCl gradient centrifugation and dissolved in phosphate buffered saline (PBS).

The concentration of DNA was determined visually from agarose gels by comparing the band intensity with the intensity of the marker bands, which contain known amounts of DNA. Upon linearization of plasmid DNA by restriction digest, different amounts of linearized DNA were loaded on the gel (10, 5, 2.5, 1.25 and 0.625 μl). HinIIIIII digested Lambda marker (New England Biolabs) and SM mass (MBI Fermentas) ruler were used as a reference with known DNA amounts in particular bands to produce ng-to-intensity calibration curves after analysis of the gel photo using the ImageQuant TL program.

The mice were immunized intramuscularly in both tibialis anterior muscles with mixtures of either two or four different DNA constructs. For the immunization with two DNA constructs, the DNA mixtures with various ratios of DNA constructs were prepared and a total of 50 μg of DNA (25 μg each muscle) per mouse and per immunization was applied in a total volume of 100 μl. For the immunizations with four DNA constructs, a DNA mixture containing equal amounts of each DNA construct was prepared and a total of 100 μg of DNA (50 μg each muscle) per mouse and per immunization was applied.

In both cases, mice were treated by injection of 100 μl cardiotoxin (Latoxan, 0.062 μg/μl) on day 0, which was followed by three consecutive DNA immunizations on days 5, 19 and 38–42. Blood samples were collected 11–14 days after the third immunization by cardiac puncture.

2.3. ELISA

Mice sera were screened for the presence of anti-HPV and -BPV antibodies by ELISA, whereby the corresponding VLPs diluted in PBS served as antigens. All sera were tested in duplicates.

VLPs used in ELISA were produced by infection of High Five cells (Invitrogen) with recombinant baculovirus and purified by CsCl density gradient centrifugation [20]. The optimal VLP concentration for coating was determined by titration of each VLP sample. The dilution, at which the saturation of the plates with VLPs of particular PV types was reached, was used in further analysis. After coating with VLPs (5 μg/ml in PBS) overnight, the plates were blocked with a 3% blocking solution (3% skim milk, PBS/0.3% Tween-20). Mice sera diluted 1:25 in 1.5% blocking solution were added to the plates for further incubation. A goat-anti-mouse–antibody conjugated with horseradish peroxidase, diluted 1:3000 (Dianova, Germany) in 1.5% blocking solution, was used as secondary antibody. The measurement at 405 nm was carried out 10–20 min after the 100 μl staining solution (100 mM NaAc, 44 mM NaH₂PO₄, pH 4.2, 1 mg/ml ABTS, 0.012% H₂O₂) was added. All the incubation steps were carried out for 1 h at 37°C and followed by washing three times with PBS/0.3% Tween-20.

2.4. Pseudovirion production

Production of pseudovirions was performed by cotransfection of 293TT cells with a plasmid containing the humanized HPV 16 L1 and L2 genes and an SV40 origin (pCDNA4-HPV16L1h-L2h/SV40ori) together with a second plasmid encoding the reporter protein secreted alkaline phosphatase (SEAP) under the control of the CMV promoter (pCMVSEAP) following a procedure described earlier [21]. 7 × 10⁶ 293TT cells were seeded on 10 cm culture dishes in DMEM (Sigma), supplemented with 10% FCS (Gibco, BRL), 1% penicillin/streptomycin (Life Technologies) and
125 μg/ml hygromycin (Roche) 18 h in advance. The cotransfection was performed using METAFECTENE™ (Biontex) according to the manufacturer’s instructions with minor changes. Fifteen micrograms of both plasmids were mixed with 70 μl METAFECTENE™ in 700 μl of medium, incubated for 20 min and added to the cells. After 5 h a medium change was performed and the cells were incubated at 37°C for 3–4 days. For pseudovirion extraction, the cells were harvested by trypsinization, washed once with PBS and resuspended in 1 ml modified PBS (containing 1 mM CaCl₂ and 5.6 mM MgCl₂) per 5 × 10⁷ cells. After addition of 4 μl Benzonase (250 U/ml, Sigma) and 50 μl Brij58 (Sigma), cells were incubated on ice for 5 min followed by the addition of NaCl to a final concentration of 710 mM to induce the maturation of the pseudovirions during a 10 min incubation at 4°C. The cellular lysate was cleared by centrifugation (10 min, 3500 × g) and the pseudovirions were stored at −70°C.

2.5. Neutralization assay

As target cells 293TT cells were seeded at a concentration of 15,000 cells per well on 96-well plates in DMEM (Sigma), supplemented with 10% FCS (Gibco, BRL), 1% penicillin/streptomycin (Life Technologies) and 125 μg/ml hygromycin (Roche). The following day, the pseudovirions were diluted in DMEM (1:5000) and mixed with the sera at different dilutions. After 15 min incubation at room temperature, the medium of the 293TT cells was replaced by 200 μl of the pseudovirion solution. The following controls were included: untreated cells, cells treated with pseudovirions alone, and cells treated with pseudovirions in the presence of a known neutralizing polyclonal antiserum specific for HPV 11, 16, or BPV 1. Detection of SEAP in cell culture supernatant was performed 5 days later with the chemiluminescent SEAP Reporter Gene Assay (Roche) following the manufacturer’s instructions. All sera were tested in duplicates.

2.6. Transfection of 293T cells

Cotransfection experiments of 293T cells with plasmids encoding for the HPV 16 L1, HPV 11 L1 and BPV 1 L1 proteins and with the empty vector were done using the calcium phosphate method [22]. Fifteen micrograms of each DNA were mixed with 50 μl of a 3 M CaCl₂ solution and a total volume of 500 μl was completed with water. The CaCl₂/DNA solution was gently mixed with 500 μl 2× BBS buffer (280 mM NaCl, 50 Mm BES; 0.75 mM Na₂HPO₄, 0.75 mM NaH₂PO₄, pH 7.5) and incubated for 15 min at room temperature. The medium of the cells was removed and after one washing step with PBS fresh medium was added. Afterwards the transfection solution was added drop by drop. The cells were incubated overnight at 35°C, 3% CO₂, and medium was changed after 16–18 h.

2.7. VLP preparation and purification

High Five insect cells (Invitrogen) were grown at 27°C to a density of 2 × 10⁶ cells/ml as 250 ml suspension culture in a spinner flask using Ex-Cell 405 serum-free medium (JRH Biosciences). The cells were infected with baculoviruses recombinant for HPV 16 L1 protein as previously described [20] and collected 3 days after infection. For purification of VLPs, pellets were resuspended in 20 ml of extraction buffer (5 mM MgCl₂, 5 mM CaCl₂, 150 mM NaCl, 0.01% Triton X100, 20 mM Hepes pH 7.4 and 1 mM PMSF). Protein extraction was done using a French Press, and cell lysates were cleared by centrifugation at 11,700 × g for 10 min at 4°C. The supernatant was loaded on top of a two-step gradient consisting of 7 ml 40% sucrose on top of 7 ml of 58% CsCl (w/v) and centrifuged in a SW28 rotor (Beckman Ultracentrifuge) at 96,500 × g at 10°C for 3 h. The interphase between sucrose and CsCl was collected, mixed and transferred to a quick seal tube (Beckman). Centrifugation was done in a Beckman 70 Ti rotor with 184,000 × g at 20°C for 16–18 h. Fractions were collected by puncturing the tubes with a 20 gauge needle and stored at 4°C. Using ELISA and Western blot analysis, VLP-containing fractions were detected.

3. Results

3.1. DNA immunization I: combining two papillomavirus L1 genes

Our objective was to determine whether L1-directed immunity against multiple papillomaviruses can be induced by simultaneous immunization with expression constructs encoding for different L1 proteins. First, we decided to determine the minimal amount of DNA of a given L1 expression construct that is required for the induction of an anti-L1 response in the background of related as well as unrelated DNA. To this end, we immunized six groups of mice (see Table 1A) with seven different ratios (three mice per ratio, I–VII, Table 1A) of HPV 16 L1 vector and either the empty vector, vectors encoding the L1 of other papillomaviruses, or a vector encoding a non-papillomavirus gene (GFP). Mice were immunized with a total of 50 μg DNA containing 0, 0.5, 2.5, 5, 10, 25 or 50 μg of HPV 16 expression construct, respectively. Mice were pre-treated by injection of cardiotoxin on day 0 and then immunized a total of three times on days 5, 19, and 38–42. Eleven to 14 days after the last booster immunization blood was collected and analyzed for the presence of anti-L1 antibodies by VLP-based ELISA.

In the group of mice immunized with mixtures of HPV 16 L1 together with the empty expression vector (group 2, Table 1A) 14 out of 15 (17 out of 18 including the three mice immunized with HPV 16 L1 DNA only; see Table 2 and Fig. 1) that received HPV 16 L1 DNA developed antibodies as determined by VLP-based ELISA (Fig. 2). More important, all mice that were immunized with the lowest dose (i.e.
0.5 μg) of HPV 16 L1 DNA were anti-HPV 16 L1 antibody-positive. In this group the ratio of 16 L1 to unspecific DNA was 1:100 suggesting that at least 100 different L1 expression construct could be applied simultaneously. As expected, titering selected sera (Table 1A, group 5: HPV 16/HPV 11 L1) indicated that the amount of DNA injected influences the strength of the antibody response. The mean titer of the sera from the mice immunized with 50 μg of HPV 16 was 1:1600 whereas in the sera of mice immunized with 25 and 10 μg of HPV 16 L1 DNA the titer was 1:200 (data not shown).

Comparative results were obtained when the HPV 16 L1 expression construct was injected either alone (Table 1A, group 1: 9/15 positive), with the vector encoding GFP (group 3: 10/15 mice receiving HPV 16 DNA were HPV 16 L1 positive) or with HPV 18 (group 6: 15/15 positive). In the presence of the constructs expressing either HPV 18 L1 (group 6) or GFP (group 3) 0.5 μg of the HPV 16 L1 expression construct were again sufficient to induce an anti-HPV 16 response in five out of six mice. When the HPV 16 DNA was administered alone (group 1), this amount did not induce a measurable response (0/3 positive) which indicates that unrelated DNA might contribute to the immune response possible due to tracer effects or due to the fact that larger doses of unmethylated CpG motifs contribute to the induc-
Table 2
Results of DNA immunization I–IV

<table>
<thead>
<tr>
<th>Group no.</th>
<th>Group</th>
<th>HPV 16 positive</th>
<th>PV X positive&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Double positive&lt;sup&gt;a&lt;/sup&gt;</th>
<th>HPV 11/16/18/ BPV 1 positive</th>
<th>16 Neut.</th>
<th>11 Neut.</th>
<th>BPV Neut.</th>
<th>16 + BPV Neut.</th>
<th>16 + 11 Neut.</th>
<th>11 + BPV Neut.</th>
<th>16 + 11 + BPV Neut.</th>
</tr>
</thead>
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</tr>
<tr>
<td>1</td>
<td>16&lt;sup&gt;–b&lt;/sup&gt;</td>
<td>9/15</td>
<td>n.a.</td>
<td>n.a.</td>
<td>9/15</td>
<td>0/15</td>
<td>0/15</td>
<td>0/15</td>
<td>0/15</td>
<td>0/15</td>
<td>0/15</td>
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<tr>
<td>2</td>
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<td>14/15</td>
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<td>n.a.</td>
<td>14/15</td>
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<td>0/15</td>
<td>0/15</td>
<td>0/15</td>
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<td>0/15</td>
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</tr>
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<td>n.a.</td>
<td>n.a.</td>
<td>9/15</td>
<td>4/15</td>
<td>0/15</td>
<td>0/15</td>
<td>4/15</td>
<td>0/15</td>
<td>0/15</td>
<td>0/15</td>
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<tr>
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<td>2/15</td>
<td>14/15</td>
<td>1/15</td>
<td>0/15</td>
<td>6/15</td>
<td>12/15</td>
<td>0/15</td>
<td>4/15</td>
<td>0/15</td>
<td>0/15</td>
<td>0/15</td>
</tr>
<tr>
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<td>16/11&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5/15</td>
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<td>5/15</td>
<td>4/15</td>
<td>14/15</td>
<td>0/15</td>
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<td>0/15</td>
<td>0/15</td>
<td>0/15</td>
</tr>
<tr>
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<td>16/18&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>0/15</td>
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<td>(1–6)</td>
<td>16 (50 µg) mice</td>
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<td>n.a.</td>
<td>16/18</td>
<td>0/18</td>
<td>0/18</td>
<td>0/3</td>
<td>0/18</td>
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<td>0/18</td>
<td>0/18</td>
</tr>
<tr>
<td>(5)</td>
<td>11 (50 µg) mice</td>
<td>n.a.</td>
<td>3/3</td>
<td>n.a.</td>
<td>0/3</td>
<td>3/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
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<tr>
<td>(6)</td>
<td>18 (50 µg) mice</td>
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<td>2/2.</td>
<td>n.a.</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
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<td>0/3</td>
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<tr>
<td>(4)</td>
<td>BPV (50 µg) mice</td>
<td>n.a.</td>
<td>3/3</td>
<td>n.a.</td>
<td>0/3</td>
<td>1/3</td>
<td>3/3</td>
<td>0/3</td>
<td>0/3</td>
<td>1/3</td>
<td>0/3</td>
<td>0/3</td>
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<tr>
<td>(1–3)</td>
<td>Empty/GFP&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0/9</td>
<td>n.a.</td>
<td>n.a.</td>
<td>0/9</td>
<td>0/9</td>
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<td>Immunization B</td>
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</tr>
<tr>
<td>7</td>
<td>11/16/18/GFP</td>
<td>1/8</td>
<td>n.a.</td>
<td>n.a.</td>
<td>8/1/10</td>
<td>1/8</td>
<td>8/8</td>
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<td>0/8</td>
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<tr>
<td>8</td>
<td>11/16/GFP/BPV</td>
<td>1/8</td>
<td>n.a.</td>
<td>n.a.</td>
<td>8/0/4</td>
<td>0/8</td>
<td>7/8</td>
<td>0/8</td>
<td>0/8</td>
<td>2/8</td>
<td>0/8</td>
<td>0/8</td>
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<tr>
<td>9</td>
<td>11/GFP/18/BPV</td>
<td>0/8</td>
<td>n.a.</td>
<td>n.a.</td>
<td>8/0/1/7</td>
<td>0/8</td>
<td>8/8</td>
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<td>0/8</td>
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<tr>
<td>10</td>
<td>11/16/18/BPV</td>
<td>1/8</td>
<td>n.a.</td>
<td>n.a.</td>
<td>8/1/0/3</td>
<td>1/8</td>
<td>7/8</td>
<td>1/8</td>
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<tr>
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<td>GFP/16/18/BPV</td>
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<td>n.a.</td>
<td>n.a.</td>
<td>1/0/0/7</td>
<td>0/8</td>
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<tr>
<td>Immunization C + D</td>
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<tr>
<td>12</td>
<td>16 left 11/BPV right</td>
<td>7/8</td>
<td>n.t.</td>
<td>n.t.</td>
<td>n.a.</td>
<td>7/8</td>
<td>7/8</td>
<td>1/8</td>
<td>1/8</td>
<td>7/8</td>
<td>1/8</td>
<td>1/8</td>
</tr>
<tr>
<td>13</td>
<td>11/16/BPV right</td>
<td>2/8</td>
<td>n.t.</td>
<td>n.t.</td>
<td>n.a.</td>
<td>0/8</td>
<td>7/8</td>
<td>0/8</td>
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<tr>
<td>14</td>
<td>16 week 1 11/BPV week 2</td>
<td>4/4</td>
<td>n.t.</td>
<td>n.t.</td>
<td>n.a.</td>
<td>4/4</td>
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</tr>
<tr>
<td>15</td>
<td>11/BPV week 1 16 week 2</td>
<td>5/8</td>
<td>n.t.</td>
<td>n.t.</td>
<td>n.a.</td>
<td>4/8</td>
<td>6/8</td>
<td>2/8</td>
<td>2/8</td>
<td>2/8</td>
<td>2/8</td>
<td>1/8</td>
</tr>
</tbody>
</table>

The numbers show mice positive for anti-L1 antibodies and for neutralization of PV pseudovirions. PV X means positive for antibodies against L1 of the PV in the particular immunization group (HPV 11, 18 or BPV 1). n.a, non applicable; n.t., not tested. Although for immunization experiment A each set of mice consisted of seven groups with three animals each, results for animals immunized with HPV 16 L1 DNA only or with DNA X only are listed separately.

<sup>a</sup> Only mice immunized with two different L1s.

<sup>b</sup> Excludes the three mice immunized with 50 µg HPV 16 L1 and three mice immunized with DNA X, these are listed separately.

<sup>c</sup> All mice that were not immunized with any of the L1s.
Fig. 1. Summary of immunization experiments A–D. The percentage of HPV 16 L1 antibody-positive sera in the different animal groups are indicated. The Fisher exact test was used to calculate statistical significance of differences between various experimental groups. For details see Table 2.

3.2 Cross-reactivity

While generally no specific reactions were detected against HPV 18 or BPV 1 VLPs in mice that had not received the L1 genes of these PVs, some of the sera from mice not immunized with HPV 11 L1 reacted with the HPV 11 VLPs. We interpret this result by the inhomogeneity and/or partial disruption of the HPV 11 VLPs used for ELISA that thereby expose conserved linear epitopes. On the other hand it seems likely that DNA immunization not only induces conformation-specific antibodies but also antibodies against linear L1 epitopes.

3.3 DNA immunization II: combining four papillomavirus L1 genes

Although the results described above suggested an inhibitory effect of HPV 11 and BPV 1 L1 on the immunogenicity of the HPV 16 L1 expression construct, we wanted to determine whether it is possible to simultaneously apply three or four different L1 vaccines. To this end, we immunized five groups of mice (Table 1B, groups 7–11; 8 mice per group) with different combinations of HPV 11, 16, 18, BPV 1 L1 and GFP expression vectors. In group 10 the mice received the L1 constructs in equal amounts (25 μg each construct), in groups 7, 8, 9, and 11 one of the four L1 constructs was replaced by the GFP construct. The results confirmed our previous finding of pairwise immunizations. All of the 32 mice that were immunized with HPV 11 L1 (groups 7–10) developed an anti-HPV 11 response. There was little or no...
response against HPV 18 L1 (groups 7, 9, 10 and 11) when the reactivity was compared to the group of mice not exposed to HPV 18 L1 DNA. Only 3 of the 32 mice immunized with HPV 16 L1 developed anti-HPV 16 antibodies, confirming that the HPV 16 response can be suppressed by the presence of either HPV 11 or BPV 1 L1 or both. The response to BPV 1 L1 was partially influenced by the presence of other L1 proteins as well. There was a good anti-BPV 1 L1 response in the two groups lacking either HPV 16 L1 (7/8 positive) (group 9) or HPV 11 L1 (7/8 positive) (group 11), but a reduced response in the two groups where HPV 16 and 11 were both present (3/8 and 4/8 positive) (see Figs. 1 and 4 and Table 2).

Of the 40 mice immunized with three or four L1 expression constructs only one was triple positive whereas 15 mice were antibody-positive against two different L1 proteins.

3.4. Neutralizing responses

Next, we wanted to assess whether DNA immunization leads to the induction of neutralizing antibodies, whether protection against multiple PV types can be achieved simultaneously and whether DNA immunization induces cross-neutralizing antibody responses.

To determine whether L1 DNA immunization leads to the induction of neutralizing antibodies, we analyzed the mouse sera in a pseudovirion based neutralization assay [21,24]. All sera were tested for their ability to neutralize HPV 11, 16 and BPV 1 pseudovirions at a 1:50 dilution. In addition the sera were tested for neutralization of HPV 16 pseudovirions at a 1:500 dilution. Since an inhibition of pseudovirions by control sera of up to 55% was observed, we arbitrarily selected a cut off of 75% or greater of pseudovirion inhibition to discriminate between neutralization-positive and -negative sera.
Fig. 3. Detection of anti-L1 antibodies in mice immunized with HPV 16 L1 + BPV 1 L1. (A) Mice were immunized with seven different ratios (50/0 to 0/50 μg) of HPV 16 L1 and BPV 1 L1 expression constructs. Anti-HPV 16 (top) and anti-BPV 1 L1 (bottom) antibodies were detected by VLP-based ELISA and each bar represents one serum at a 1:50 dilution. Fourteen out of 15 mice immunized with both BPV 1 L1 and HPV 16 L1 DNA were BPV 1 L1 sero-positive. In contrast, only two of these mice were anti-HPV 16 L1 positive. (B) Mice were immunized with seven different ratios (50/0 to 0/50 μg) of HPV 16 L1 and HPV 11 L1 expression constructs. Anti-HPV 16 and anti-HPV 11 L1 antibodies were detected by VLP-based ELISA. All of the 15 mice immunized with both HPV 11 L1 and HPV 16 L1 DNA were HPV 11 L1 sero-positive. In contrast, only six of these mice were also anti-HPV 16 L1 positive.

3.5. Correlation of antibodies and neutralizing responses

In general, there was a good correlation of neutralizing activity and reactivity in VLP-based ELISA (Figs. 2 and 5) for all three PV types assayed. Nevertheless, there were ELISA-positive sera that repeatedly failed to neutralize the corresponding HPV type. This was more frequently found for HPV 11 positive sera and might be explained by the fact that DNA immunization induces some responses against non-neutralizing epitopes conserved among different PVs and that these antibodies are detected by ELISA if VLPs become par-

Fig. 4. Detection of anti-L1 antibodies in mice immunized with HPV 16, 11, 18 and BPV 1 L1. A group of eight mice was immunized with a mixture of four different L1 expression constructs. Antibodies against the four L1 proteins (anti-HPV 16, anti-HPV 11, anti-HPV 18, anti-BPV 1) were detected by VLP-based ELISA, each bar represents one serum at a 1:50 dilution. While all sera were positive for anti-HPV 11 antibodies, only one of the sera reacted with HPV 16 L1.
tially denatured. On the other hand, we observed that sera non- or weakly reactive in ELISA were strongly neutralizing. As ELISA-negative/neutralization-positive sera were only observed in groups of mice that had received the corresponding HPV L1 DNA for immunization this observation is likely not an artefact. The correlation of neutralization and ELISA was weaker when sera were used at higher dilutions (1:500) in the neutralization assay which indicates that neutralizing titers for a number of sera was below 1:500.

Eighty-five mice of groups 1–11 were immunized with two or more PV L1 expression constructs, 80 of these were ELISA-positive for any PV L1 and 42 were double ELISA-positive (Table 2). However, only 24 sera of the 85 mice were able to neutralize two different PV types, only 1 out of 40 sera from mice immunized with at least three different PVs was able to neutralize all three PV types tested. This reflects again the fact that double or triple immunization did not induce high titers of antibodies against all L1 equally well and it is therefore difficult to induce multiple neutralizing activities.

3.6. Is there an indication of cross-neutralization?

We also wanted to determine if there was an indication for the induction of PV-cross-neutralizing antibodies. In fact, with no exception, only sera from mice immunized with HPV 16 and/or BPV 1 L1 expression constructs were able to neutralize HPV 16 or BPV 1 pseudovirions, respectively. In contrast, 13 sera obtained from the 107 mice not immunized with HPV 11 L1 (Tables 1 and 2, groups 1–11) were able to efficiently neutralize HPV 11 pseudovirions. Six of these mice had been treated with HPV 16 L1, six other mice with HPV 16 L1 and BPV 1 L1 and one mouse with BPV 1 L1 only. Of the 13 sera 9 were positive for either HPV 16 neutralization (6 sera) or for BPV 1 neutralization (3 sera). The remaining three sera, neutralizing only HPV 11, were all from mice immunized with HPV 16 and BPV 1 L1. Of these, one serum was anti-BPV 1 L1 ELISA-positive and two were ELISA-negative for both, HPV 16 and BPV 1.

3.7. DNA immunization III: topical separation of L1 expression constructs in DNA vaccination

The immunization experiments in which two or more different L1 expression constructs were injected simultaneously as a mixture indicate that there is a strong interference between different L1 proteins on the induction of a VP-specific antibody response. We wanted to determine whether this interference is due to immunodominance of one L1 over another or whether there is interference in particle assembly when two or more L1 genes are expressed in one cell upon DNA vaccination.

To address these questions, we separated the injections of the expression constructs either topically or chronologically. Two groups of mice were immunized according to the scheme in Table 1C. In one group, HPV 16 DNA was injected into the left side (tibialis anterior muscle) while a mixture of the HPV 11 and BPV 1 expression construct was injected into the right side of the mice. In another group, all three expression constructs were injected together into one side. In mice where there was a topical separation of HPV 16 injection from the site where HPV 11 and BPV 1 were injected seven out of eight mice developed a strong anti-HPV 16 response (Fig. 5) all of which were neutralizing. In this group, seven out of eight mice were also positive for HPV 11 L1 neutralizing antibodies but only one of the mice developed anti-BPV 1 L1 neutralizing antibodies. In contrast, in the mice into which the three constructs were co-injected, only two out of the eight mice showed a (weak) anti-HPV 16 L1 antibody response but were negative for neutralization. In this group, again seven out of eight mice were positive for anti-HPV 11 L1 neutralizing antibodies but none of the eight sera was able to neutralize BPV 1 confirming the previous observation that HPV 11 L1 and HPV 16 L1 interfere with the induction of anti-BPV 1 L1 antibodies.

3.8. DNA immunization IV: chronologically separation of injection

Next, we alternated injection of HPV 16 L1 DNA with the injection of HPV 11/BPV 1 L1 DNA. In one group HPV 16 L1 was injected on weeks 1, 3, 5 and HPV 11/BPV 1 L1 was injected on weeks 2, 4, and 6. In another group this scheme was reversed. This immunization protocol led to the induction of anti-HPV 16 L1 antibodies in four out of four and five out of eight mice, respectively (Fig. 5). Both groups showed a robust response against HPV 11 L1 but not against BPV 1 L1.

Taken together, these data indicate that it is possible to induce immune responses against multiple HPV types, but there is a strong interference of different L1s during immunization which can be overcome by spatial or temporal separation of the vaccination.
3.9. Interference in VLP assembly

Topical separation of injection of HPV 16 L1 DNA from the site where HPV 11 and BPV 1 DNA was injected led to the induction of anti-HPV 16 L1 responses which suggests that immunodominance is not the mechanism for the observed interference. To provide evidence for interference on the level of L1 expression or VLP assembly, we transfected 293T cells in vitro with HPV 16 L1 expression constructs either alone or in presence of BPV 1 or HPV 11 L1. Two days after transfection cells were lysed and presence of HPV 16 VLPs in the crude extracts was measured by antigen-capture ELISA. Fig. 6A shows the results of three independent experiments which were carried out on different days. When HPV 16 L1 DNA was cotransfected together with HPV 11 L1 DNA the amount of HPV 16 particles was reduced by about 38%. Cotransfection of HPV 16 L1 with BPV 1 L1 or with HPV 11 L1 plus BPV 1 L1 led to a reduction of HPV 16 particles in both cases by about 78%. Results of the control experiment, in which HPV 16 L1 DNA was cotransfected with the empty pUF3 vector, indicated that the interference observed in above described cotransfections is unlikely due to increased DNA amount in the transfection experiment or due to a titration of transcription factors by the expression constructs. When we analyzed the total amount of either HPV 16 L1 or BPV 1 L1 protein in cells after simultaneous transfection of three different constructs, we observed that BPV 1 L1 levels remained constant, even when the BPV 1 L1 con-
struct was co-expressed with HPV 16 and HPV 11 L1. For HPV 16 we observed, in comparison to the reduced amount of HPV 16 VLPs, only a slight decrease of HPV 16 L1 protein in the presence of BPV 1 L1 or HPV 11 L1 (Fig. 6B). This data suggests that presence of either HPV 11 or BPV 1 L1 inhibits HPV 16 L1 particle formation also in vivo providing a possible explanation for the observed interference in the vaccination experiments.

4. Discussion

Prophylactic papillomavirus vaccines became recently available, the major challenge for future candidate papillomavirus vaccines is to provide cheaper, simpler vaccination that covers more different types. The commercial vaccines developed by Merck and Co. Inc. and GlaxoSmithKline Biologicals each cover the two high risk HPV types 16 and 18 leaving out at least 13 other oncogenic papillomaviruses responsible for almost 30% of all cervical cancer cases. In this study, we analyzed whether co-administration of DNA expression vectors for different PV L1 genes is able to induce immunity against multiple PV types.

In previous studies, we and others demonstrated that high titers of anti-L1 antibodies can be induced with genetic, L1-based vaccines, although, not surprisingly, efficiency lacks behind the usage of the exceptionally immunogenic protein-based vaccination using L1 VLPs [16–19,26–30]. Here we were able to confirm that very low amounts (i.e. 0.5 μg) of DNA are sufficient to induce neutralizing anti-L1 responses, although larger amounts of DNA led to higher L1-specific antibody titers. This provoked us to investigate whether combined administration of different L1 expression constructs would be a simple solution for the generation of vaccines against multiple papillomaviruses. Unfortunately, the short answer to this question is no. In most instances, we observed interference between different L1 vaccines in the induction of antibody responses. In particular HPV 11 and BPV 1 L1 vectors suppressed the anti-HPV 16 and anti-HPV 18 responses. Likewise, HPV 16 and 11 L1 seem to have a negative effect on the anti-BPV 1 L1 response. This suppression can be overcome by separation of immunization either topically or chronologically. Although this does not provide a feasible solution in view to potential prophylactic vaccines in humans, it suggests that the observed interference in simultaneous application of L1 genes is not due to selective preferences of the immune system but rather due to direct effects on production or presentation of correctly folded L1 antigen. This is also consistent with the fact that the commercial vaccines effectively induce responses against two or four different HPV types [9,31], however it would need to be determined whether co-administration of purified VLPs of different HPV types under less favourable conditions such as limiting amount of proteins, would lead to a well-balanced response against all types. Although we do not have an explanation for the interference phenomena, it is thinkable that co-expression of different L1 genes within the same transfected cells leads to the accumulation of misfolded L1 aggregates which are unable to contribute to the humoral response. In a previous study it has, however, been shown that mixed particles consisting of the L1 types of HPV 6 and 16 can be produced in yeast, indicating that different L1s can join in the formation of particles and that these particles induce immunity against both HPV types [32]. This is consistent with our preliminary observation obtained by immunoprecipitation, that upon cotransfection the L1 proteins of BPV 1 L1 and HPV 16 L1 are able to co-assemble. The fact that the interference is asymmetrical, i.e. HPV type-specific, could then be explained by different expression levels of the different L1 genes, e.g. an excess of BPV 1 L1 titrates out HPV 16 L1.

We were also able to demonstrate that DNA immunization induces neutralizing antibodies. This was as expected, as VLP-based ELISA was already generally accepted as surrogate marker for neutralization [33] although this conclusion is largely drawn from studies in which sera from VLP-immunized individuals were analyzed by both assays and these sera were indeed strongly neutralizing. We also observed that ELISA reactivity correlated generally well with neutralizing activity. Nevertheless, a number of sera were ELISA-positive but non-neutralizing. Some of these reactions can be explained by the fact that VLPs are partially denatured in the assay and thereby show PV type cross-reactivity. It is conceivable that DNA immunization per se induces also a significant amount of antibodies that are non-neutralizing. A small fraction of sera showed clear evidence for the presence of cross-neutralizing activity, i.e. immunization with HPV 16 DNA led to neutralization of HPV 11 pseudovirions. Although this observation was found only in very few sera, it might indicate the presence of shared neutralizing epitopes on HPV particles which, however, are under normal circumstances not sufficiently immunogenic. Nevertheless, it needs to be determined whether the observed cross-neutralization is due to the specific nature of DNA immunization, which likely leads to the formation of partial assembly intermediates or whether it can be explained by specific properties of the HPV 11 pseudovirions, as cross-neutralization was exclusively observed for HPV 11.

In conclusion, we observe that DNA immunization against multiple HPV types is yet not feasible as we observed strong interference between different L1 proteins in three different set of experiments. Recently, a number of improvements, among them in vivo electroporation or co-administration of GM-CSF have led DNA vaccines into human trial [34–36]. In addition, use of viral vectors could significantly enhance the effectiveness of genetic vaccines [16,27,37–39], it would have to be re-evaluated whether interference between different L1 genes could be overcome by more potent vaccination strategies.
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