A recombinant human papillomavirus (HPV) type 16
L1–vaccinia virus murine challenge model demonstrates
cell-mediated immunity against HPV virus-like particles

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Human papillomavirus (HPV) virus-like particles (VLP) are emerging as the immunogen of choice for prophylactic vaccines. The inability to infect animals with HPV has prevented the testing of potential vaccines such as those in animal systems. This study describes the development of a recombinant vaccinia virus (VV)–HPV type 16 (HPV-16) VLP challenge model to evaluate the efficacy of the cell-mediated immune response following HPV-16 VLP immunization in mice. Inoculation of BALB/c and C57 BL/6 mice with HPV-16 VLP resulted in HPV VLP-specific T cell proliferative responses characterized by the production of both Th1 and Th2 cytokines, and afforded protection against virus challenge from recombinant VV expressing HPV-16 L1 (VVL1r-16). Protection was demonstrated by a 4–6 log10 reduction in ovarian titres of VVL1r-16 in vaccinated BALB/c mice and a 2–3 log10 reduction in vaccinated C57 BL/6 mice, compared with unvaccinated mice.

Specific types of human papillomavirus (HPV) are known to play a causal role in cervical cancer (NIH, 1996). Cervical cancer is the second most common cancer in women worldwide (Bosch et al., 1995). In developing countries, cervical cancer is the most common cancer, due to the lack of proper screening practices, encouraging the development of an effective prophylactic vaccine. The development of an HPV vaccine has been significantly hindered by the difficulty of HPV propagation in culture (Lowy et al., 1994). However, HPV virus-like particles (VLP) produced by baculovirus expression in insect cells (Krbauer et al., 1992; Hagensee et al., 1993; Rose et al., 1993) are emerging as the immunogen of choice for prophylactic HPV vaccines (Krbauer, 1996). Several studies have shown that papillomavirus VLP elicit high titres of neutralizing antibodies in serum and protection from experimental challenge with infectious virus in animal papillomavirus models (Breitburd et al., 1995; Kirnbauer et al., 1996; Rose et al., 1994a; Suzich et al., 1995). Immunization of cottontail rabbits with VLP composed of the cottontail rabbit papillomavirus (CRPV) L1 major capsid protein has recently been shown to protect rabbits against CRPV challenge (Breitburd et al., 1995; Christensen et al., 1996; Jansen et al., 1995). In a canine oral papillomavirus model, neutralizing antibodies have been shown to confer protection from infection with canine oral papillomavirus (Suzich et al., 1995). A tumour cell challenge model reported by De Bruijn et al. (1998) has demonstrated the therapeutic effect of HPV-16 VLP (VLP-16) immunization. The role of cell-mediated immunity in the control of papillomavirus infection is not well established. Several clinical and experimental studies have shown, however, that cell-mediated immune responses play a role in both susceptibility to and regression of HPV infections (Coleman et al., 1994; Frazer et al., 1986; Alloub et al., 1989). Several recent studies in mice have shown that VLP are also capable of priming a productive cell-mediated immune response. This response is associated with the production of Th1-type cytokines and the delivery of HPV antigens to the HLA class I processing pathway for priming of cytolytic CD8+ T cells (Peng et al., 1998; Dupuy et al., 1997).

HPV vaccine development has been further impeded by the inability to infect laboratory animals with HPV. This has prevented the demonstration of protection against HPV challenge. The present study describes the development and use of a recombinant HPV-16 L1–vaccinia virus (VV) challenge model in mice to demonstrate protection in VLP-16-vaccinated mice and to investigate the importance of cell-mediated immunity induced by VLP-16. The use of recombinant VV expressing the nucleoprotein of vesicular stomatitis virus as a challenge system was first described by Bachmann et al. (1994) to investigate the efficacy of a potential vesicular stomatitis virus vaccine. This technique has also been applied to assess human immunodeficiency virus type 1 vaccine regimes in mice (Belyakov et al., 1998; Kent et al., 1998). The present study...
shows that immunization with VLP-16 elicits T cell proliferative responses characterized by the production of both Th1 and Th2 cytokines and is capable of protecting mice against challenge with recombinant HPV-16 L1–VV (VVL1R-16).

VLP-16 were produced in a baculovirus expression system (Rose et al., 1994b). To examine the T cell response to VLP-16, groups of 6–8-week-old BALB/c mice (Animal Unit, University of Cape Town) were immunized intraperitoneally (i.p.) either with a single dose of 20 μg VLP-16 or with three consecutive weekly doses of 10 μg VLP-16. Two weeks after the last inoculation, splenocytes were isolated by passage through a steel mesh (Sigma) to obtain a single-cell suspension. Contaminating red blood cells were removed by centrifugation over Ficoll-Hypaque density gradients as described previously (Boyum, 1968). Viable cells were resuspended at 2 × 10⁶ cells/ml in RPMI-1640 medium supplemented with 10% foetal calf serum. For the lymphoproliferation assay, splenocytes (2 × 10⁶ cells per well) were seeded in triplicate into round-bottomed 96-well culture plates (Nunc). Splenocytes were incubated for 6 days at 37 °C in a humidified 5% CO₂ atmosphere in the presence of purified VLP-16 (15 μg/ml) or baculovirus–insect cell extract (10% v/v). [³H]Thymidine (1 μCi per well) was added to each well for the last 18 h of the assay. The cells were harvested by using an automated cell harvester (PHD, Cambridge Technology) and the radioactivity was measured by using a liquid scintillation counter (Tricarb-4640). For generation of cytokine-containing supernatants, splenocytes (2 × 10⁶ cells per well) were seeded in quadruplicate into round-bottomed 96-well culture plates (Nunc) in the presence or absence of purified VLP-16 (15 μg/ml). Culture supernatants were collected at 4, 5 and 6 days and stored at −20 °C. Interferon (IFN)-γ (Th1-type) and interleukin (IL)-4 (Th2-type) were assessed by a sandwich ELISA according to the manufacturer’s recommendations (Biotrak, Amersham).

A single i.p. immunization of 20 μg VLP-16 resulted in detectable T cell proliferation after only a single cycle of in vitro stimulation (Fig. 1a). When mice were given three consecutive weekly booster doses, proliferative responses to VLP-16 were increased 12-fold (Fig. 1b). Because the VLP were purified from a baculovirus expression system, insect cell extract was used as

### Table 1. IFN-γ and IL-4 production after HPV-16 L1-VLP immunization

Peak cytokine production was measured in supernatants after 6 days in vitro stimulation. IL-4 production was not detected at earlier time-points. The detection limits of the assays were 47 pg/ml for IFN-γ and 15 pg/ml for IL-4.

<table>
<thead>
<tr>
<th>Immunization protocol</th>
<th>No antigen</th>
<th>VLP-16</th>
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<td>1349</td>
<td>&lt; 15</td>
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a control for non-specific proliferation to residual insect cell proteins. Lymphocytes from VLP-16-primed mice did not proliferate in response to these insect cell proteins, indicating that the response to VLP-16 was antigen-specific. Lymphocytes from control mice, given placebo inoculations with PBS, did not proliferate to HPV antigen or insect cell proteins.

The supernatants from splenocyte culture were examined for the presence of Th1-type (IFN-γ) or Th2-type (IL-4) cytokines (Table 1). Although low levels of IFN-γ (215 pg/ml) were detectable after a single immunization with VLP-16, IFN-γ levels were significantly boosted (6-fold) after the three consecutive immunizations (1349 pg/ml). Low levels of IL-4 were also detected after VLP-16 inoculation (128 pg/ml). Control mice immunized with PBS alone showed no detectable cytokine production in response to VLP-16.

In order to evaluate the protective effects induced by VLP-16-specific cell-mediated immunity, a recombinant VV–HPV-16 L1 challenge model was developed. The use of recombinant VV challenge allows selective evaluation of cell-mediated rather than neutralizing antibody immune mechanisms because VV recombinants do not express the transfected gene products in their envelope in a form that is directly accessible to antibodies (Zinkernagel et al., 1990). Further reports have confirmed that resistance to challenge with recombinant VV was mediated predominantly by CD8+ T cells (Binder & Kundig, 1991). The Western Reserve (WR) strain of VV was used, which causes disseminating infection in mice, with the highest titres of virus being measured in the ovaries (Binder & Kundig, 1991). The L1 gene of HPV-16 (Rochester strain), which was obtained from W. Bonnez (Bonnez et al., 1991) and group B was not vaccinated. After 13 days, groups A and B were challenged with VVL1R-16. Protection was VLP-16-specific, as challenge with wild-type VV only resulted in a 1 log10 reduction in vaccinated mouse ovarian virus titre (2.4 × 107 p.f.u.) compared with unvaccinated mice (2.3 × 108 p.f.u.). X-Gal staining of titration plates containing virus from mouse ovaries confirmed that unclared virus in vaccinated mice was recombinant VVL1R-16. A similar experiment with VLP-16-immunized C57 BL/6 mice confirmed that they were also protected from VVL1R-16 challenge compared with their unvaccinated littermates (2.3 log10 protection) but the level of protection was lower compared with BALB/c mice (Fig. 2 b). This finding is consistent with previous reports that have shown that C57 BL/6 mice are genetically more resistant to pox virus infection.
than the innately susceptible BALB/c strain (O’Neill & Brenan, 1987). These results indicate that BALB/c mice may be a better strain for the VVL1R-16 challenge model.

This study has demonstrated that the immunization of mice with VLP-16 produces a protective T cell response characterized by the production of both Th1 and Th2 cytokines and affords protection against challenge from VVL1R-16. The ability to titrate VV in ovaries offers a quantitative measure of protective immunity induced in mice by potential HPV prophylactic vaccines. Current studies are examining whether there is cross-protection between HPV VLP types and the relative advantages of different routes of VLP vaccine administration and virus challenge. These results confirm others indicating that VLP are efficient candidates for a prophylactic vaccine.

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