Journal of General Virology (1999), **80**, 2471–2475. Printed in Great Britain SHORT COMMUNICATION

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 these 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 Th_1 and Th_2 cytokines, and afforded protection against virus challenge from recombinant VV expressing HPV-16 L1 ($VVL1_R$-16). Protection was demonstrated by a 4·6 $\log_{10}$ reduction in ovarian titres of $VVL1_R$-16 in vaccinated BALB/c mice and a 2·3 $\log_{10}$ 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 (Kirnbauer *et al.*, 1992; Hagensee *et al.*, 1993; Rose *et al.*, 1993) are emerging as the immunogen of choice for prophylactic HPV vaccines (Kirnbauer, 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.*, 1994*a*; 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 Th_1-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 impaired 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

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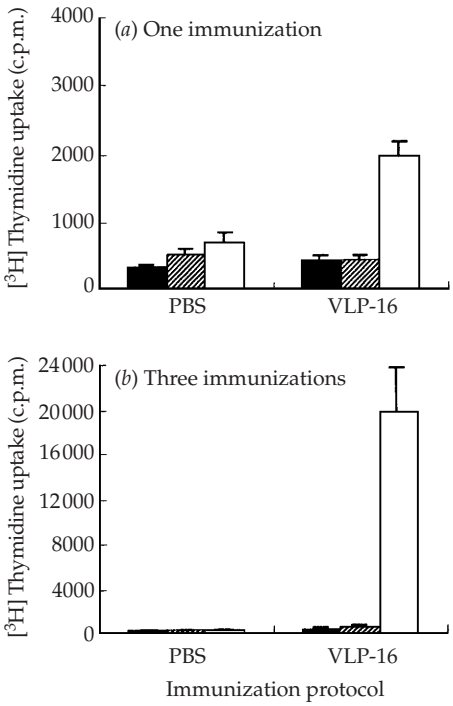

Fig. 1. Lymphoproliferative responses of HPV-16 VLP-primed splenocytes re-stimulated *in vitro* with VLP-16 (unfilled bars; 15 μg/ml) or baculovirus insect cell extract (hatched bars; 10%, v/v) or unstimulated (solid bars). (*a*) Mice were given a single i.p. immunization with VLP-16 (20 μg per mouse) or PBS (control). (*b*) Mice were immunized weekly for three consecutive weeks with VLP-16 (10 μg per mouse) or PBS (control). Proliferation was determined by [^{3}H]thymidine incorporation. Each bar represents the mean proliferative response (c.p.m. ± SD) of triplicate wells.

shows that immunization with VLP-16 elicits T cell proliferative responses characterized by the production of both Th_1 and Th_2 cytokines and is capable of protecting mice against challenge with recombinant HPV-16 L1–VV ($VVL1_R$-16).

VLP-16 were produced in a baculovirus expression system (Rose *et al.*, 1994 *b*). 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 2×10^6 cells/ml in RPMI-1640 medium supplemented with 10% foetal calf serum. For the lymphoproliferation assay, splenocytes (2×10^5 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% CO_2 atmosphere in the presence of purified VLP-16 (15 μg/ml) or baculovirus–insect cell extract (10% v/v). [^{3}H]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 (2×10^5 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 (Th_1-type) and interleukin (IL)-4 (Th_2-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. 1*a*). When mice were given three consecutive weekly booster doses, proliferative responses to VLP-16 were increased 12-fold (Fig. 1*b*). 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.

	IFN-γ (pg/ml)		IL-4 (pg/ml)	
Immunization protocol	No antigen	VLP-16	No antigen	VLP-16
Single immunization				
PBS	< 47	< 47	< 15	< 15
HPV-16 L1	< 47	215	< 15	< 15
Three weekly boosters				
PBS	< 47	< 47	< 15	< 15
HPV-16 L1	< 47	1349	< 15	128

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 Th_1-type (IFN-γ) or Th_2-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*., 1998), had been cloned as described by Rose *et al*. (1993, 1994*b*) and White *et al*. (1998). This gene was cloned into the *Sma*I site of pMos blue and then further subcloned into the *Sal*I/*Sma*I sites of the VV shuttle vector pSC65 downstream of the synthetic early/late promoter (PE/L), generating pSC65L1-16_R. This vector introduces the L1 gene into the thymidine kinase (TK) site of VV and also contains the β-galactosidase gene for the visual selection of blue recombinant plaques (Chakrabarti *et al*., 1985). VV (strain WR) was propagated in eggs and purified as described by Stannard *et al*. (1998). CV-1 and human TK^- 143 cells were grown in DMEM with 10% foetal bovine serum. CV-1 cells were infected with VV at an m.o.i. of 5. The virus was removed 1 h later and the cells were transfected with plasmid DNA by using DOTAP (Boehringer) according to the manufacturer's instructions. For blue plaque identification, staining with X-Gal (35 μg/ml) was performed in agarose overlay. TK^- recombinants were isolated by plaque assay in the presence of 5-bromo-2′-deoxyuridine (0·5 mg/ml) and designated $VVL1_R$-16.

Two groups of four female BALB/c mice (8–12 weeks old) were compared. Group A was vaccinated with 10 μg VLP-16 (Bachmann *et al*., 1994) and group B was not vaccinated. After 13 days, groups A and B were challenged with $VVL1_R$-16. Five days after challenge, the ovaries were harvested. The ovaries were chopped finely, placed in McIlvain's buffer (4 mM citric acid, 0·2 M $Na_2HPO_4.12H_2O$) (100 mg ovary/ml) and homogenized by using a ten Broeck grinder (30 strokes). This was followed by three cycles of freeze–thawing and then preparations were centrifuged at 2000 r.p.m. for 5 min. The supernatant fluid was titrated in CV-1 cells and the $VVL1_R$-16 titre per mouse was calculated.

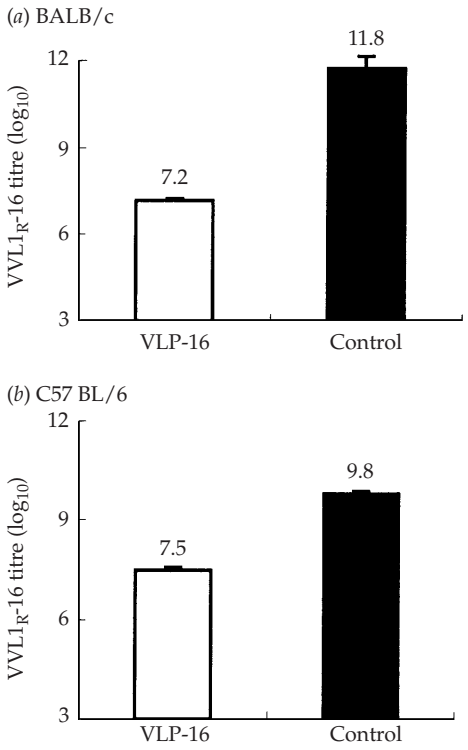

Fig. 2. VLP-16 immunization protects mice against challenge with L1-16-expressing VV. Unvaccinated control (solid bars) or VLP-16-immunized (open bars) BALB/c (*a*) and C57 BL/6 (*b*) mouse ovarian titres were measured 5 days after challenge with $VVL1_R$-16. Ovarian titres per mouse are expressed as $\log_{10}$ p.f.u. Each bar represents the mean $\log_{10}$ VVL1-16 p.f.u. (±SD). In (*a*), the difference is significant ($P < 0{\cdot}05$; Student's *t*-test).

VLP-16-immunized BALB/c mice were better able to control $VVL1_R$-16 infection following challenge than the unvaccinated control mice (4·6 $\log_{10}$ protection, $P < 0{\cdot}05$; Fig. 2*a*). Protection was VLP-16-specific, as challenge with wild-type VV only resulted in a 1 $\log_{10}$ reduction in vaccinated mouse ovarian virus titre ($2{\cdot}4 \times 10^7$ p.f.u.) compared with unvaccinated mice ($2{\cdot}3 \times 10^8$ p.f.u.). X-Gal staining of titration plates containing virus from mouse ovaries confirmed that uncleared virus in vaccinated mice was recombinant $VVL1_R$-16. A similar experiment with VLP-16-immunized C57 BL/6 mice confirmed that they were also protected from $VVL1_R$-16 challenge compared with their unvaccinated littermates (2·3 $\log_{10}$ 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 $VVL1_R$-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 Th_1 and Th_2 cytokines and affords protection against challenge from $VVL1_R$-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.

We thank Dr John Williamson (St Mary's Hospital, London, UK) for provision of the vaccinia virus WR strain and Dr B. Moss (NIH) for providing the vaccinia shuttle vector, pSC65. We acknowledge discussions with Professor Keith Dumbell and Professor Bernard Ryffel, which helped stimulate the ideas for this research. This research was funded by the Cancer Association of South Africa (CANSA) and THRIP.

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Received 30 March 1999; Accepted 19 May 1999