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Papillomavirus Virus-like Particles Can Deliver Defined CTL Epitopes to the MHC Class I Pathway

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To evaluate an antigen delivery system in which exogenous antigen can target the major histocompatibility complex (MHC) class I pathway, a single human papillomavirus (HPV) 16 E7 cytotoxic T lymphocyte (CTL) epitope and a single HIV gp160 CTL epitope were separately fused to the C-terminus of bovine papillomavirus 1 (BPV1) L1 sequence to form hybrid BPV1L1 VLPs. Mice immunized with these hybrid VLPs mounted strong CTL responses against the relevant target cells in the absence of any adjuvants. In addition, the CTL responses induced by immunization with BPV1L1/HPV16E7CTL VLPs protected mice against challenge with E7-transformed tumor cells. Furthermore, a high titer-specific antibody response against BPV1L1 VLPs was also induced, and this antiserum could inhibit papillomavirus-induced agglutination of mouse erythrocytes, suggesting that the antibody may recognize conformational determinates relevant to virus neutralization. These data demonstrate that hybrid BPV1L1 VLPs can be used as carriers to target antigenic epitopes to both the MHC class I and class II pathways, providing a promising strategy for the design of vaccines to prevent virus infection, with the potential to elicit therapeutic virus-specific CTL responses.

INTRODUCTION

Papillomaviruses (PV) are small DNA viruses. Certain types of human papillomaviruses (HPV), such as HPV 16 and 18, are strongly associated with cervical cancer (zur Hausen, 1991). Generally, viral infections induce both humoral and cellular immune responses. Induction of neutralizing antibody by a prophylactic vaccination has been the aim of efforts to prevent viral infection. However, a vaccine which aims to prime for $CD8^+$ CTL responses, in the context of MHC class I, is necessary for the elimination of viruses replicating in cells and of virus protein-transformed tumor cells (Tindle and Frazer, 1994). The study of immune responses mounted against papillomavirus infections has been hampered by the lack of a conventional cell culture system for the virus. It has been found that PV major capsid protein L1 (alone or with minor capsid protein L2) can self-assemble into virus-like particles (VLPs) when expressed in eukaryotic systems (Kirnbauer *et al.,* 1992; Zhou *et al.,* 1991). Immunization with VLPs leads to production of PV neutralizing antibodies (Roden *et al.,* 1994; Christensen *et al.,* 1996; Kirnbauer *et al.,* 1992) with high efficiency. A single immunization with 1 ng VLPs without any adjuvants can protect a rabbit from cottontail rabbit PV (CRPV) infection, with the protection lasting at least 12 months (Christensen *et al.,* 1996). Although the role of cell-mediated immunity in the control of PV infection and PV-related epithelial dysplasia is not clear, several clinical and experimental studies indicate that cell-mediated immune responses influence both susceptibility to and regression of HPV infections. In patients with decreased cellular immunity caused by disease such as malignancy, renal allograft, HIV infection, regression of anogenital warts correlated with an active cell-mediated immune response (Coleman *et al.,* 1994; Frazer *et al.,* 1986; Alloub *et al.,* 1989). PV early proteins E7 and E6 have transforming ability and have been implicated in the tumorigenicity of HPV: their consistent expression appears necessary to maintain a neoplastic state (Crook *et al.,* 1989) and in transgenic mouse systems, expression of these genes leads to tumor formation (Lambert *et al.,* 1993), suggesting the importance of E7 and E6 in cervical carcinogenesis. Murine and human HPV 16 E7 CTL epitopes have been identified (Ressing *et al.,* 1995; Feltkamp *et al.,* 1993), and immunization of mice with the CTL epitopes, including peptide, resulted in the prevention of tumor growth (Feltkamp *et al.,* 1993; Meneguzzi *et al.,* 1991), suggesting that the induction of CTLs responses is necessary for the prevention of HPV-related carcinogenesis.

T lymphocytes sense the virus infection of a cell by recognizing viral peptides that are displayed at the cell surface in association with MHC class I molecules. MHC class I-restricted antigen presentation involves a processing of endogenous intracellular proteins into short peptides, predominantly degraded in the cytosol (Goldberg and Rock, 1992; Germain and Margulies, 1993).

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These peptides are then transported by a family of transporters associated with antigen processing (TAP1 and TAP2) into the lumen of the endoplasmic reticulum (ER), where they bind to newly synthesized MHC class I molecules (Townsend and Trowsdale, 1993; Braciale *et al.,* 1987; Germain and Margulies, 1993). Exogenous soluble proteins are usually unable to target this pathway and fail to activate CTL responses. Therefore, a number of antigen presentation systems have been developed to deliver exogenous antigens into the cytosolic pathway. Many of these strategies are designed to present the antigen as a polyvalent particulate structure. Antigen presented in association with appropriate adjuvants could efficiently stimulate CTL responses (Tindle *et al.,* 1995; Schulz *et al.,* 1991; Ke *et al.,* 1995; Zhu *et al.,* 1995), but most of them are not available for human use. Recombinant live vectors have also been shown to induce CTL responses but their safety is still uncertain (Londono *et al.,* 1996; Winter *et al.,* 1995; Zhu *et al.,* 1995). Also, a Ty-VLPs has been developed as a antigen carrier system in which antigens are genetically fused to the *TYA* gene of the yeast retrotransposon Ty. The *TYA* gene encodes a particle-forming protein which normally packages the RNA molecule that is the intermediate in Ty transposition (Layton *et al.,* 1993; Adams *et al.,* 1987). But this system demands the carrier protein be neutral in antigenicity as far as vaccine is concerned. Recently, hybrid VLPs have emerged as a new antigen delivery system to induce both CTL responses and antibody production (Tindle *et al.,* 1994; Schlienger *et al.,* 1992; Clarke *et al.,* 1987; Jenkins *et al.,* 1990; Muller *et al.,* 1997; Sedlik *et al.,* 1997; Griffiths *et al.,* 1993; Evans *et al.,* 1989). Immunization with these hybrid VLPs has been shown to be able to provide complete protection against virus challenge (Sedlik *et al.,* 1997).

We have demonstrated previously that the C-terminus of bovine papillomavirus 1 (BPV) L1 sequence can be deleted without affecting the ability of L1 to assemble into VLPs when expressed in insect cells by recombinant baculovirus (Paintsil *et al.,* 1996). Up to 60 amino acids can be fused into this region and maintain the protein's ability to form VLPs (Muller *et al.,* 1997). In the present study, we fused two different defined CTL epitopes, HPV 16 E7 CTL epitope (aa 49–57) (Feltkamp *et al.,* 1993) and HIV IIIB gp 160 p18-I10 (aa 318–327) (Takahashi *et al.,* 1988) to the C-terminus of BPV 1 L1 sequence, and expressed the fusions in insect cells by recombinant baculoviruses. We demonstrated that these recombinant BPV1 L1 proteins could spontaneously self-assemble into VLPs with a morphology very similar to the wild-type L1 VLPs. Immunization of mice with these hybrid VLPs efficiently stimulated MHC class I-restricted CTL responses, and neutralizing antibodies against BPV1 L1 VLPs were also induced.

MATERIALS AND METHODS

Mice, cell lines, and peptides

Female C57BL/6J and BALB/c mice, 8–10 weeks old, were purchased from Animal Resource Center (ARC, Australia) and kept in a specific pathogen-free room. C2 (HPV 16E7 transfected EL-4 cells) (Tindle *et al.,* 1995) and EL4 cell lines were maintained in complete RPMI-1640 medium plus 10% fetal bovine serum (FBS, CSL, Australia). P815 cell line was cultured in complete DMEM medium plus 10% FBS, and all cell lines were passaged every 3 days. The peptide RAHYNIVTF, corresponding to an H-2^b-restricted CTL epitope of residues 49–57 from the HPV 16 E7 protein, and the peptide RGPGRAFVTI, corresponding to an H-2^d-restricted CTL epitope of residues 318–327 from the HIVIIIB gp160 protein, were synthesised by Chiron Technologies Pty Ltd (Australia).

Construction of recombinant baculovirus transfer vectors

Primers 5′-CCGGGATCCATGGCGTTGTGGCAACAAGGCCAGAAGC-3′ and 5′-CCGGAATTCTTATTTTCCTATTGTAACAAATGCTCTCCCTGGTCCTCTTCCTGCCCCTTGCTGTGCTAAAAATCTTCTTCC-3′ were designed to fuse HIV IIIB gp160 P18-I10 CTL epitope (aa 318–327) to the C-terminus of BPV1 L1 sequence, deleting nucleotides from 7625 downstream. The primers also allowed insertion of a stop codon, and one flanking *Bam*HI site at the 5′ and a flanking *Eco*RI site at the 3′. The primers 5′-CCGGAATCCATGGCGTTGTGGCAACAAGGCCAGAAGC-3′ and 5′-CCGGAATTCTTATTTTTTAAAGGTTACAATATTGTAATGGGCTCTTCCTGCCCCTTGCTGTGCTAAAAATCTTCTTCC-3′ were designed to fuse HPV 16 E7 CTL epitope (aa 49–57) to the C-terminus of BPV1L1 sequence, in which the same part of C-terminus sequence of BPV1L1 was deleted, and a stop codon and enzyme sites were added as above. The PCR products were digested with *Bam*HI and *Eco*RI, and named BPV1L1/HIVp18-I10 and BPV1L1/HPV16E7CTL, respectively. These fragments were then inserted into baculovirus transfer vector pVL1393 (Pharmingen) at the *Bam*HI and *Eco*RI sites and transformed into *Escherichia coli* DH α-5 cells. The correct recombinant clones were confirmed by the existence of *Bam*HI and *Eco*RI sites and also were sequenced by dideoxynucleotide procedures to determine the orientation and integrity of the inserted sequences. The recombinants containing either HIV gp160 p18-I10 CTL epitope or HPV 16 E7 CTL epitope were named pVLBPV1L1/HIVp18-I10 and pVLBPV1L1/HPV16E7CTL, respectively. (See Fig. 1)

Production of recombinant baculoviruses

Recombinant baculoviruses were produced according to Pharmingen's BaculoGold transfection kit. Briefly, *Spodoptera frugiperda* 9 (Sf9) insect cells were cotrans-

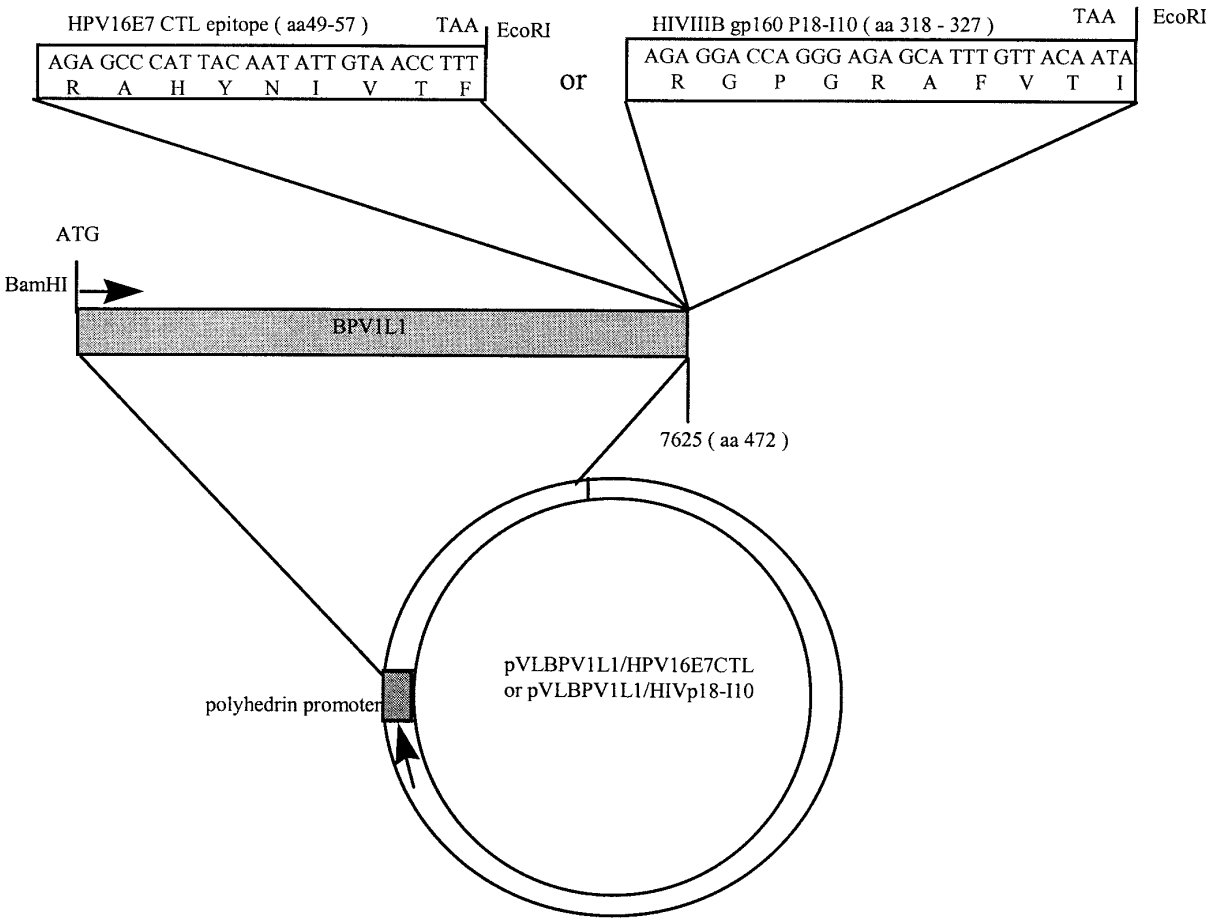

FIG. 1. Construction of pVLBPV1L1/HPV16E7CTL and pVLBPV1L1/HIVP18-I10 transfer vector. Either HPV16E7 CTL epitope (aa 49–57) or HIVIIIB gp160 CTL epitope P18-I10 (aa 318–327) was fused to the C-terminus of BPV1L1 sequence at the position of 7625 by PCR, and then the chimeric fragments were inserted into baculovirus transfer vector pVL1393 at the restriction enzyme sites of *Bam*HI and *Eco*RI to produce the recombinant transfer vector. The integrity of inserted CTL epitopes were confirmed by DNA sequence analysis.

fected with BaculoGold lineralized DNA and the recombinant transfer vectors. Although this procedure produced nearly 100% recombinant efficiencies, plaque-purification was used to ensure no blue plaques (wild-type) could be detected. High-titer ($> 10^8$/ml virus particles) stocks of recombinant baculoviruses AcBPV1L1/HIVp18-I10 and AcBPV1L1/HPV16E7CTL were then prepared by two rounds of amplification.

Production and purification of VLPs

The production and purification procedures were described in detail previously (Qi *et al.,* 1996). In general, Sf-9 insect cells were infected either with recombinant baculovirus AcBPV1L1/HIVp18-I10 or AcBPV1L1/HPV-16E7CTL at a multiplicity of infection of 10 plaque-forming unit (PFU) per cell, and incubated at 27°C for 72 h. The cells were centrifuged, washed with PBS once, and resuspended in an appropriate amount of PBS in the presence of 2 mM PMSF. The cell suspension was homogenized with a dounce homogenizer (tight pestle) by 50 strokes on ice and then centrifuged at 3000 rpm for 10 min at 4°C to separate the nuclear fraction. The nuclear pellet was resuspended in an appropriate resuspension buffer and sonicated for 45 s in ice. The nuclei suspension were then loaded onto a 20% sucrose cushion and centrifuged at 26,000 rpm in Beckman SW-26 rotor at 4°C for 2 h. The pellets were resuspended with resuspension buffer and sonicated again for another 45 s. This resuspension was then mixed with CsCl and centrifuged at 21°C with Beckman SW 41 rotor for 20 h. A band at the CsCl density of 1.30 g/ml was collected and dialyzed extensively against PBS. The samples were then used for Western immunoblotting and for transmission electron microscopic analysis.

Western immunoblot analysis

Protein samples were diluted in SDS–PAGE sample buffer, boiled at 100°C for 10 min, and then electrophoresed through a 10% SDS–PAGE gel and transferred to nitrocellulose membrane. The membrane was blocked with 5% skim milk in PBS and probed with the anti-L1 monoclonal antibody MC15 (Kulski *et al.,* 1997) at a

dilution of 1:2000. Bound antibody was detected by incubation of the membrane with horseradish peroxidase-conjugated sheep anti-mouse antibody (Silenus, Australia) at a dilution of 1:1000 and visualized using enhanced chemiluminescence (ECL) (Amersham).

Transmission electron microscopy

CsCl gradient-purified and dialyzed samples were mounted onto carbon-coated grids, stained with 2% ammonium molybdate, pH 6.2, and examined with an Hitachi H-800 electron microscope.

Antibody production

Groups of 4 BALB/c mice were injected subcutaneously with 20 μg hybrid BPV1L1/HIVp18-I10 VLPs or BPV1L1/HPV16E7CTL VLPs without adjuvant. Following the priming immunization, the mice were boosted twice at 2-week intervals with the same doses. 10 days after the second boost, the mice were test bled. The sera were used for capture ELISA assay and hemagglutination assay.

Capture ELISA

A capture enzyme-linked immunosorbent assay (ELISA) was developed to measure the antibodies against intact BPV1L1 VLPs. A rabbit anti-BPV1 virion polyclonal antibody, which specifically recognized BPV1 virus particle's conformational epitopes (Liu *et al.,* 1997) was used to coat 96-well Maxisorp ELISA plates (Nunc) and incubated at 4°C overnight. After blocking with 5% skim milk in PBS, 50 μl of CsCl gradient-purified BPV1L1 VLPs, diluted in PBS to 10 μg/ml, were added to each well and incubated at 37°C for 1.5 h. Sera from mice immunized with BPV1L1/HPV16E7CTL VLPs or BPV1L1/HIVP18I10 VLPs were tested by using twofold serial dilution in 5% skim milk in PBS. A monoclonal antibody which specifically recognized a BPV1 L1VLP conformational epitope (5B6) (Roden *et al.,* 1994) was used as positive control, and preimmunization mouse serum was used as a negative control. Bound antibody was detected with horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin (Silenus, Australia) at a dilution of 1:1000, followed by incubation with ABTS in the presence of hydrogen peroxide. The reaction was stopped with 1% SDS, and the absorbances read at 414 nm.

Hemagglutination inhibition assay

The method used here was described previously (Roden *et al.,* 1995). Basically, 1 ml of fresh C57BL/6J mouse blood was taken in the presence of 100 μl heparin (1000 units/ml) and was resuspended with 9 ml of PBS + 1 mg BSA/ml and centrifuged at 1000 *g* at 4°C for 5 min. The supernatant and the top white layer of cells were aspirated, and the red blood cells (RBC) were resuspended in 10 ml of PBS + 1 mg BSA/ml and centrifuged at 1000 *g* at 4°C for 5 min. This wash procedure was repeated three times, and the final RBC pellet was diluted to 1% v/v in PBS + 1 mg/ml BSA. One hundred microliters of this RBC suspension was mixed with the same volume of BPV1L1VLPs and added to a 96-well round-bottom plate, held at 4°C without disturbance for 3 h, and then photographed. For the antibody inhibition assay, the sera were incubated with 4 vol of a 25% v/v suspension of mouse erythrocytes overnight at 4°C to reduce nonspecific binding. The antisera then was cleared by centrifugation at 1000 *g* at 4°C and was heat inactivated at 56°C for 30 min. Various dilutions of the antibodies were preincubated with BPV1L1VLPs at room temperature for 2 h with gentle rocking before mixing with mouse RBCs.

Cytotoxic T lymphocyte assay

Eight- to ten-week-old female C57BL/6J or BALB/c mice were immunized with 20 μg of BPV1L1/HPV16E7CTL VLPs or BPV1L1/HIVP18I10 VLPs by footpad injection, either with or without (in PBS) adjuvant (Quil A) (Dalsgaard, 1974). Control mice were immunized with BPV1 L1 VLP. Four days after immunization, the draining popliteal and periaortic lymph nodes were harvested and placed in complete RPMI 1640 medium on ice. A single cell suspension was made using nylon mesh and resuspended in complete RPMI 1640 medium plus 10% FCS, 20 units/ml recombinant IL-2 (Sigma). The 2-ml cells (3.0×10^6 cells/ml) were added to each well of 24-well plates and incubated at 37°C, 5% CO_2 for 4 days. Target cells used for BPV1L1/HPV16E7 CTL were HPV16 E7-transfected EL-4 (C2) (*H-2*^{b}) (Tindle *et al.,* 1995) and EL-4; for BPV1L1/HIVP18I10 CTL assay, HIV-P18-I10 peptide pulsed P815 (H-2^d) and P815 were used. Target cells were pelleted, washed once with serum-free complete RPMI, and 1×10^7 cells were resuspended in 100 μl serum-free medium. For the peptide-pulsed target cell lines, the corresponding peptides were added at a concentration of 10 μg/ml. ^{51}Cr (100 μCi) was added to the cells and incubated at 37°C, 5% CO_2 (with loose lid) for 60–90 min, with occasional agitation. The excess ^{51}Cr was removed using the FCS underlay technique. Effectors and labeled target cells were plated into 96-well round-bottom plates at various effector/target ratios and incubated at 37°C, 5% CO_2 for 5 h. 100 μl of supernatant was collected from each well for counting in a gamma counter. The percentage of specific lysis was calculated according to the following formula: specific lysis = (sample release − spontaneous release)/(maximum release − spontaneous release) × 100. Maximum release was generated by adding 100 μl of 10% SDS to 100 μl of target cells, and spontaneous release was obtained from 100 μl of target cells incubated with 100 μl of medium. All assays were performed in triplicate wells, and spontaneous ^{51}Cr release from the various targets did not exceed 15%.

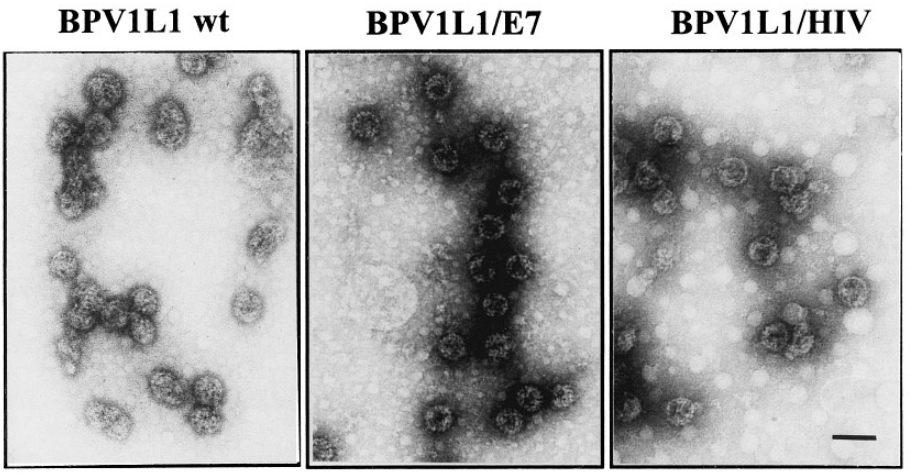

FIG. 2. Electron micrography of purified wild-type BPV1L1 VLPs, hybrid BPV1L1/HPV16E7CTL VLPs, and hybrid BPV1L1/HIVP18-I10 VLPs. Recombinant baculovirus-expressed VLPs were purified as described under Materials and Methods. The bar in BPV1L1/HIV VLPs represents 100 nm, and magnification of all pictures is the same.

Tumor protection assay

Eight- to ten-week-old female C57BL/6J mice were immunized with either BPV1L1/HPV16E7CTL VLPs or BPV1L1 VLPs, or PBS only, by subcutaneous injection, and boosted once 3 weeks after priming. Two weeks later, 2×10^6 HPV 16 E7-transfected C2 cells were injected under the neck scuff, and 10–14 days later, the mice were sacrificed, and the growing tumors were harvested and weighed.

RESULTS

Production of hybrid BPV1L1/HPV16E7CTL and BPV1L1/HIVP18I10 VLPs

The recombinant baculovirus expression system was chosen to express BPV1L1 VLPs which contained either a HPV16E7 or a HIVIIIB gp160 p18-I10 CTL epitope within the L1 sequence (hybrid VLPs). It has been shown by us previously that deletion of part of the BPV1L1 C-terminal sequence did not interfere with L1 self-assembly into VLPs; in contrast, the yield was increased by an unknown mechanism (Paintsil *et al.,* 1996). When either the HPV16E7 or HIVIIIB gp160 p18-I10 CTL epitope was cloned into the C-terminus of the L1 sequence and expressed by recombinant baculoviruses in Sf-9 insect cells, a visible band of around 55-kDa size was detected by Coomassie blue-staining of density gradient-purified VLPs separated by SDS–PAGE. The identity of this band was confirmed by Western immunoblot using L1-specific MAb 15 (Fig. 3). Compared with wild-type BPV1L1, the apparent molecular weight of the hybrid BPV1L1s was lighter as expected. Electron microscopic analysis of purified BPV1L1/HPV16E7CTL or BPV1L1/HIVP18I10 confirmed a 50-nm particle structure (Fig. 2); no structural difference was observed when compared with the wild-type BPV1L1 VLPs.

Antibody responses to hybrid VLPs

To test the ability of hybrid BPV1L1 VLPs to induce anti-VLP antibody, groups of mice were primed with either BPV1L1/HPV16E7CTL or BPV1L1/HIVP18I10 VLPs without adjuvant, followed by two boosts at 2-week intervals. Anti-BPVL1 VLP antibodies were measured by capture ELISA, an ELISA capable of maintaining VLPs structure. Mice produced a strong anti-BPV1L1VLP antibody response (Fig. 4). No significant difference of antibody titer to L1 VLPs was found between the two hybrid VLPs. These data demonstrated that the hybrid BPV1L1 VLPs maintain strong BPV1L1 antigenicity, and also suggest that different foreign antigens fused to the

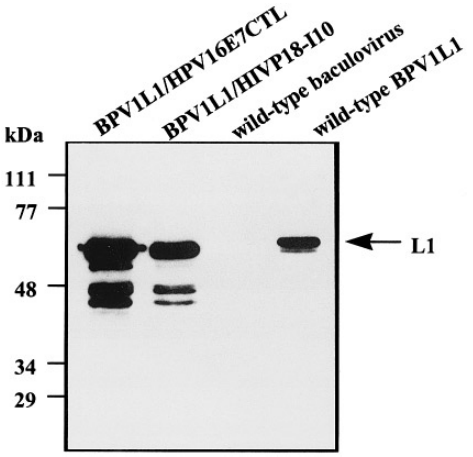

FIG. 3. Western immunoblot detection of BPV1L1 protein. CsCl gradient-purified wild-type BPV1L1 VLPs, hybrid VLPs, and wild-type baculovirus were separated by SDS–PAGE, blotted onto nitrocellulose membrane, and probed with MAb MC15 and detected by enhanced chemiluminescence. Protein markers are shown on the left. The position of the L1 protein (55 kDa) is indicated by the arrow on the right. Lane 1, BPV1L1/HPV16E7CTL; lane 2, BPV1L1/HIVP18-I10; lane 3, wild-type baculovirus; lane 4, wild-type BPV1L1

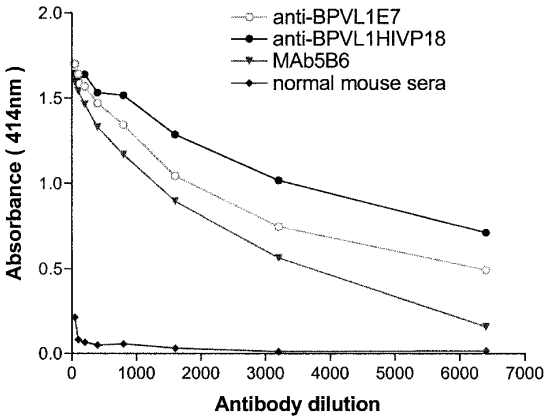

FIG. 4. Mouse anti-BPV1L1 VLP antibody responses induced by immunization with either purified hybrid BPV1L1/HPV16E7CTL or purified BPV1L1/HIVP18-I10 VLPs without adjuvant. The specific antibody was detected by a capture ELISA against intact wild-type BPV1L1 VLPs. No significant differences were found among the individual mouse in the group. The graph represents the results from one mouse.

C-terminus had no significant impact on the integrity of L1 VLPs. To confirm that immunization produced antibody to conformational determinant of VLPs, sera from immunized animals were examined in a hemagglutination inhibition assay. All sera from the two groups of mice were able to prevent BPV1L1VLP-induced agglutination of mouse erythrocytes (Fig. 5). These results were not completely consistent with the capture ELISA titer, but these data suggest that the antisera induced by hybrid VLPs include potentially virus neutralizing antibody to conformational epitopes.

In vivo induction of CTL responses by hybrid BPV1L1 VLPs

Hybrid BPV1L1 VLPs carrying either the *H-2*^{b} CTL epitope of residues 49–57 from HPV 16 E7 protein, or the *H-2*^{d} CTL epitope of residues 318–327 from HIVIIIB gp160 protein, were analyzed for their ability to stimulate *in vivo* cytotoxic responses against the inserted epitopes. C57BL/6J (*H-2*^{b}) mice were immunized once in their foot pads with 20 μg of purified BPV1L1/HPV16E7CTL VLPs plus 10 μg Quil A adjuvant, or with PBS, or with 20 μg of purified BPV1L1 VLPs in PBS as a control. BALB/c mice were injected once in their foot pads with 20 μg of purified BPV1L1/HIVP18I10 VLPs plus 10 μg Quil A, or with PBS, or with 20 μg of purified BPV1L1VLPs in PBS as a control. Four days later, draining popliteal and periaortic lymph nodes were harvested, and T lymphocytes were grown *in vitro* for 4 days in the presence of IL-2. Target CTL activity of the effector T cells was tested on the C2 cell line or HIV P18I10 peptide-pulsed P815 cells by a standard ^{51}Cr release assay. A strong CTL response was induced in both hybrid VLP groups (Fig. 6), and no significant differences were found between the effector cells primed with hybrid VLPs plus adjuvant and those cells primed without adjuvant, demonstrating the strong immunogenicity of these hybrid VLPs in the absence of any adjuvants. Effector cells from both groups of hybrid VLP-primed mice did not lyse EL-4 cells, or P815 cells, confirming the specificity of the CTL responses. Furthermore, in mice immunized with BPVL1 VLP, no CTL response was seen against either C2 cells or P18-I10 peptide-pulsed P815 cells. These results therefore demonstrated that these hybrid VLPs can elicit strong antigen-specific CTL responses using the CTL epitopes of two different proteins in two different MHC class I backgrounds.

Immunization with hybrid VLPs carrying a CTL epitope of a tumor antigen can protect mice against challenge with tumor cells

To test the persistence of the CTL response induced by hybrid VLPs carrying CTL epitopes, and also

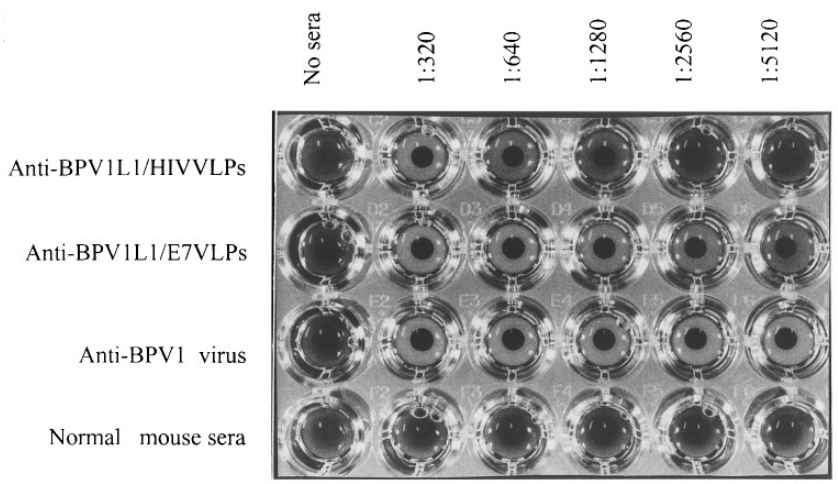

FIG. 5. Mouse anti-hybrid BPV1L1 VLP antibodies inhibit agglutination of mouse erythrocytes by wild-type BPV1L1 VLPs. The assay was performed as described under Materials and Methods section. All tested sera from VLPs immunised mice inhibited agglutination. The figure represents one mouse serum from each group. Rabbit anti-BPV1 virus antibody was used as a positive control and preimmune mouse serum as negative control.

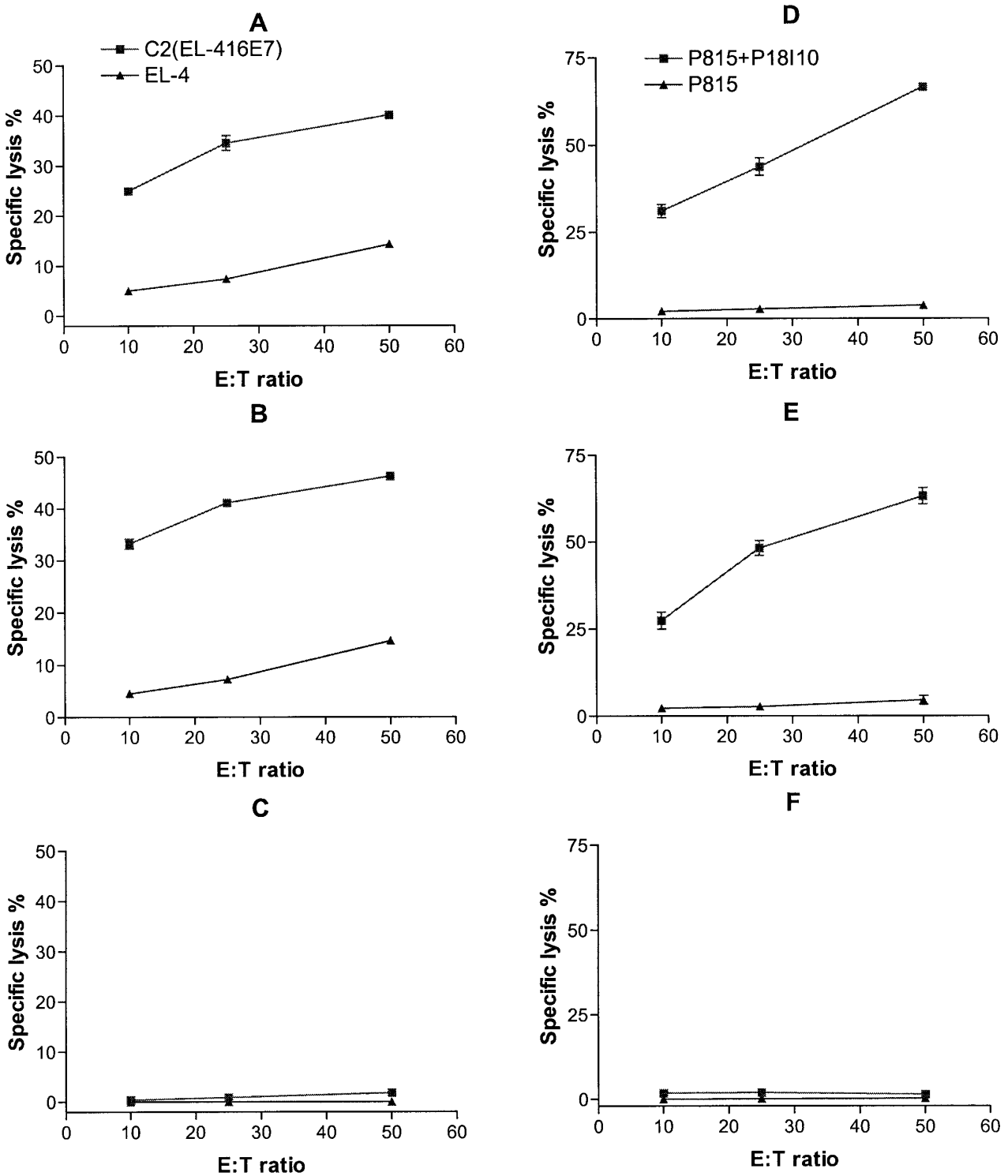

FIG. 6. Specific recognition of HPV16 E7-transformed EL-4 cell line (C2) by CTL bulk cultures generated from hybrid BPV1L1/HPV16E7CTL VLP-immunized mice and of HIVIIIB P18-I10 peptide-pulsed P815 cells by CTLs from hybrid BPV1L1/HIVP18-I10 VLP-immunized mice. T lymphocytes were pooled from the draining popliteal and periaortic lymph nodes from: (A) C57BL/6J mice immunized with BPV1L1/HPV16E7CTL VLPs and Quil A; (B) BPV1L1/HPV16E7CTL VLPs and PBS; (C) C57BL/6J mice immunized with wild-type BPV1L1 VLPs and PBS; (D) BALB/c mice immunized with BPV1L1/HIVP18-I10 VLPs and Quil A; (E) BPV1L1/HIVP18-I10 VLPs and PBS; (F) BALB/c mice immunized with wild-type BPV1L1 VLPs and PBS. Pooled T cells were stimulated *in vitro* with IL-2. After 4 days, the CTL bulk cultures generated were used as effector cells in a standard ^{51}Cr release assay. Target cells for groups A, B, and C were C2 cells and EL-4 cells; target cells for groups D, E, and F were HIVIIIB gp160 P18-I10 peptide-pulsed P815 cells and P815 cells.

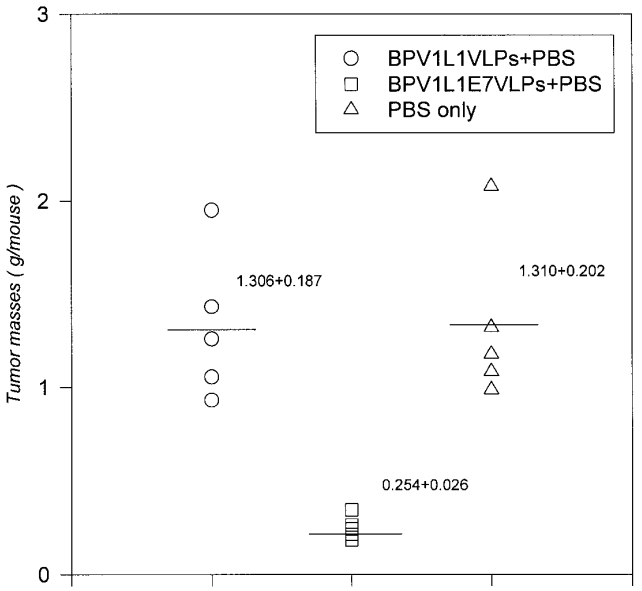

FIG. 7. Protection of C57BL/6J mice against a tumor challenge with HPV16E7-transformed C2 cells after immunization with hybrid BPV1L1/HPV16E7CTL VLPs without adjuvant. C57BL/6J mice were immunized subcutaneously with 20 μg/mouse of hybrid BPV1L1/HPV16E7CTL VLPs without adjuvant, or with wild-type BPV1L1VLPs, or with PBS only, and boosted after 3 weeks. All mice were challenged subcutaneously with 2×10^6 viable C2 cells 2 weeks after the boost. Tumor masses were measured at day 12 after challenge. Tumor growth in mice immunized with BPV1L1/HPV16E7CTL was significantly inhibited compared to mice immunized with wild-type BPV1L1 VLPs ($t = 5.582$, $P = 0.0005 < 0.001$) and to mice immunized with PBS only ($t = 5176$, $P = 0.0008 < 0.001$). No significant difference was found between groups immunized with wild-type BPV1L1 VLPs or PBS ($t = 0.012$, $P = 0.99 > 0.05$).

whether these hybrid VLPs could be used as a therapeutic vaccine candidate to treat virus-associated cancer, a tumor protection assay was performed. It has been shown previously that HPV 16E7 protein can function as a tumor antigen eliciting CTL-mediated tumor rejection (Meneguzzi *et al.,* 1991; Chen *et al.,* 1991), so we immunized C57BL/6J mice subcutaneously with 20 μg purified BPV1L1/HPV16E7CTL VLPs in PBS, at day 0, and then boosted at day 21. Two weeks after this, 2×10^6 HPV 16E7-transfected EL-4 cells/mouse were injected under the neck scuff, and the mice were sacrificed 12 days later to check tumor growth. As a control, another two groups of mice were immunized with either BPVL1 VLPs or with PBS only. As shown in Fig. 7, tumor growth in the hybrid VLP-primed group was significantly inhibited compared to the groups immunized with BPV1L1 VLPs or PBS only, strongly suggesting that the CTL responses induced by the hybrid VLPs were biologically relevant, and that this tumor protection required hybrid VLPs carrying the specific HPV 16 E7 CTL epitope. These data demonstrated that hybrid VLPs could be used as a therapeutic vaccine for the control of virus-induced tumors.

DISCUSSION

In this study, we have shown that VLPs can present antigen to the host immune system in such a way as to induce CTL specific for epitopes carried by the VLPs, and also to induce tumor protective immune responses in a model system in which HPV16E7-specific CTL are both necessary and sufficient to induce host protective immunity (Fernando *et al.,* 1997). CTL responses have been shown to play a critical role in the elimination of virus-infected cells and tumor cells, and so any vaccine against a virus infection or a tumor must induce a specific CTL response. Unlike MHC class II-restricted $CD4^+$ T cell priming, in which antigen presenting cells (APCs) pick up antigens in the extracellular compartment and carry them to the draining lymph nodes where they are processed and presented to T cells (Rock, 1996; Braciale *et al.,* 1987; Germain and Margulies, 1993), the classical MHC class I-restricted antigen presentation pathway requires that the ligand peptide be expressed endogenously in the cytosol in which proteins are hydrolysed by proteasomes or possibly by other proteinases; it has been suggested that this MHC class I-restricted pathway is exclusively concerned with monitoring endogenously synthesized cellular proteins (Rock, 1996), as exogenous proteins or killed pathogens fail to induce MHC class I-restricted CTL response *in vitro* (Braciale *et al.,* 1987). In general, exogenous antigens are not efficiently presented to $CD8^+$ T cells by MHC class I molecules because the antigens are unable to gain access to the cytosolic compartment (Moore *et al.,* 1988), and it has been suggested that this segregation of exogenous antigens from the MHC class I-restricted pathway is being used to prevent CTL response from killing self normal cells that have been exposed to foreign antigens but are not infected by pathogens (Rock, 1996). Vaccines against viruses which are based on viral proteins or CTL epitopes, therefore need to be coupled with certain adjuvants to target this pathway inducing effective CTL responses (Tindle *et al.,* 1995; Schulz *et al.,* 1991; Ke *et al.,* 1995; Zhu *et al.,* 1995), but Alum, the only adjuvant available for human use, is not an efficient inducer of CTLs. Although live vectors have also been used to induce CTL responses against HPV 16 E7 protein (Zhu *et al.,* 1995), they are not the best candidates due to their potential risk.

In the present study, we have demonstrated that PV virus particles incorporating CTL epitopes efficiently elicit CTLs and tumor antigen-specific tumor protection when delivered to mice without adjuvants. Comparing the immunogenicity of the RAHYNIVTF peptide comprising the minimal H-2b restricted CTL epitope of HPV16E7 delivered without adjuvant with that of E7 chimeric VLPs, the VLPs were on a molar basis at least 2000 times more effective at inducing a CTL response in C57BL/6 mice (data not shown). A number of other carrier systems

using viral proteins have been used to induce CTL responses. Hybrid HIV Gag particles induced a strong anti-V3 loop CTL response (Griffiths *et al.,* 1993), and chimeric hepatitis B surface antigen particles containing HIV-1 determinants have also been shown to stimulate specific CTL responses (Michel *et al.,* 1993). More recently, recombinant parvovirus-like particles harboring a CTL epitope from lymphocytic choriomeningitis virus (LCMV) nucleoprotein induced strong CTL responses against both peptide-coated and virus-infected target cells and were able to induce complete protection of mice against a normally lethal LCMV infection (Sedlik *et al.,* 1997). All this evidence, in conjunction with our results, strongly suggests that viral proteins that form VLPs act as an effective antigen delivery system to present CTL epitopes by an as yet undefined mechanism to the MHC class I-restricted pathway.

The classical MHC class I-restricted pathway requires that the newly synthesized MHC class I molecules only bind to peptides derived from endogenous antigens in the lumen of ER, and presumably hybrid VLPs follow the same pathway. It has been shown previously that soluble proteins administered in liposomes can induce CTL responses *in vivo* (Zhou *et al.,* 1992), and hybrid VLPs may possibly use the same mechanism to gain access to the cytosol, but evidence from the present study, and also from recombinant parvovirus-like particles data (Sedlik *et al.,* 1997), has demonstrated that hybrid VLPs can induce CTL responses in the absence of any adjuvants. Therefore it is unlikely that hybrid BPV1L1 VLPs use the same mechanism which liposomes used by fusing with the cell lipid membrane. Exogenous proteins have recently been shown to prime CTL responses *in vivo* via a novel antigen-presenting pathway. It has been postulated that this novel pathway is operative in a special subset of antigen-presenting cells (APCs) *in vivo* (Bevan, 1987), presenting exogenous antigens through a cytosolic pathway in which antigens in the extracellular fluids are internalized by phagocytosis, macropinocytosis, or other endocytic mechanisms, and the class I-presented peptides are generated in an extralysosomal compartment and then transferred from phagosomes or macropinosomes into the cytosol (Norbury *et al.,* 1995; Kovacsovics Bankowski and Rock, 1995). Alternatively, particulate antigens are taken up and digested in phagolysosomes with production of large amounts of peptides that bind directly to MHC class I molecules, thus bypassing the classical cytosolic pathway (Lanzavecchia, 1996); these peptides may be regurgitated and bind the cell surface class I molecules (Pfeifer *et al.,* 1993) or they may bind inside the phagosome to class I molecules derived from the plasma membrane during the process of phagocytosis or specifically targeted from the biosynthetic pathway (Sugita and Brenner, 1995; De Bruijn *et al.,* 1995). However, the efficiency of this process is unknown in nonphagocytic cells *in vivo* where there is no specific mechanism for targeting the protein for entry into the cytosol (Harding and Pfeifer, 1994). It has been shown previously that hybrid HIV Gag particles (Griffiths *et al.,* 1993), native hepatitis B surface antigen particles (Schlienger *et al.,* 1992), and hybrid parvovirus-like particles (Sedlik *et al.,* 1997) display high-efficiency induction of MHC class I-restricted CTL responses *in vivo.* Previous data demonstrated that the presentation of soluble antigens to MHC class I is inefficient, but can be boosted by triggering macropinocytsis (Norbury *et al.,* 1995), or using insolubilizing antigen on a particle (Kovacsovics Bankowski *et al.,* 1993; Falo *et al.,* 1995), or offering inert particles to be cophagocytosed together with soluble antigen (Kovacsovics Bankowski *et al.,* 1993). This suggests that the high efficiency at which hybrid BPV1L1 VLPs reach MHC class I pathway is probably due to its particulate form, although it remains to be established whether there is a specific mechanism of transfer based on virus–cell recognition, or whether phagosomes are simply fragile structures that occasionally burst, thus releasing a large amount of antigen into the cytosol (Lanzavecchia, 1996). In addition to being particulate, the hybrid VLPs also have the advantage of being able to carry multiple repeats of a CTL epitope (360 copies per particle). PV infection is restricted to epithelial surfaces. PVs enter mucosal cells after binding to the $\alpha 6\beta 4$ integrin receptor (Evander *et al.,* 1997) and then enter the cytosol en route to the cell nucleus (Zhou *et al.,* 1995), and therefore might be available to the MHC class I antigen for presentation, although it is unlikely that such a mechanism of uptake would apply for professional APCs which lack $\alpha 6\beta 4$ integrin. The exact pathway which is targeted by hybrid BPV1L1 VLPs is currently under investigation.

In this study, a high titer antibody response against wild-type BPV1L1 VLPs was also induced, demonstrating that the intact antigenicity of the wild-type BPV1L1 VLPs has been maintained by these hybrid BPV1L1 VLPs. These antibodies were able to inhibit agglutination of mouse erythrocytes by wild-type BPV1L1 VLPs, suggesting that they recognize conformational epitopes and might therefore be neutralizing. Immunization with a purified antigen on avidly phagocytized particles primes CTLs which protect animals from subsequent challenge with tumors expressing the antigen (Falo *et al.,* 1995), which suggests that hybrid VLPs could be a delivery system for a combined prophylactic and therapeutic vaccine, aiming to prevent both new virus infection and eradicate existing virus infection.

Both HPV and HIV are sexually transmitted viruses and are difficult to vaccinate against. The present study has coupled the papillomavirus L1 VLP and the HIV gp160 CTL epitope, which is in the V3 loop recognized by mice (Takahashi *et al.,* 1988) and humans (Clerici *et al.,* 1991). The strong CTL response against this epitope induced by hybrid BPV1L1 VLPs suggests that it is possible to

design a vaccine against more than one sexually transmitted virus. The present study has clearly demonstrated that hybrid PV VLPs can be used as an efficient antigen delivery system in which both MHC class II and class I pathways are targeted and provides a novel, safe, and efficient strategy for virus vaccine design. Since up to 60 amino acids can be fused to the C-terminus of PV L1 sequence, it may be possible to insert more than one CTL epitope so that a multivalent vaccine could be designed to control several virus infections on several MHC backgrounds with one immunization.

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REFERENCES

Adams, S. E., Dawson, K. M., Gull, K., Kingsman, S. M., and Kingsman, A. J. (1987). The expression of hybrid HIV:Ty virus-like particles in yeast. *Nature* 329, 68–70.

Alloub, M. I., Barr, B. B., McLaren, K. M., Smith, I. W., Bunney, M. H., and Smart, G. E. (1989). Human papillomavirus infection and cervical intraepithelial neoplasia in women with renal allografts. *Br. Med. J.* 298, 153–156.

Bevan, M. J. (1987). Antigen recognition. Class discrimination in the world of immunology [news]. *Nature* 325, 192–194.

Braciale, T. J., Morrison, L. A., Sweetser, M. T., Sambrook, J., Gething, M. J., and Braciale, V. L. (1987). Antigen presentation pathways to class I and class II MHC-restricted T lymphocytes. *Immunol. Rev.* 98, 95–114.

Chen, L. P., Thomas, E. K., Hu, S. L., Hellstrom, I., and Hellstrom, K. E. (1991). Human papillomavirus type 16 nucleoprotein E7 is a tumor rejection antigen. *Proc. Natl. Acad. Sci. USA* 88, 110–114.

Christensen, N. D., Reed, C. A., Cladel, N. M., Han, R., and Kreider, J. W. (1996). Immunization with viruslike particles induces long-term protection of rabbits against challenge with cottontail rabbit papillomavirus. *J. Virol.* 70, 960–965.

Clarke, B. E., Newton, S. E., Carroll, A. R., Francis, M. J., Appleyard, G., Syred, A. D., Highfield, P. E., Rowlands, D. J., and Brown, F. (1987). Improved immunogenicity of a peptide epitope after fusion to hepatitis B core protein. *Nature* 330, 381–384.

Clerici, M., Lucey, D. R., Zajac, R. A., Boswell, R. N., Gebel, H. M., Takahashi, H., Berzofsky, J. A., and Shearer, G. M. (1991). Detection of cytotoxic T lymphocytes specific for synthetic peptides of gp160 in HIV-seropositive individuals. *J. Immunol.* 146, 2214–2219.

Coleman, N., Birley, H. D., Renton, A. M., Hanna, N. F., Ryait, B. K., Byrne, M., Taylor Robinson, D., and Stanley, M. A. (1994). Immunological events in regressing genital warts. *Am. J. Clin. Pathol.* 102, 768–774.

Crook, T., Morgenstern, J. P., Crawford, L., and Banks, L. (1989). Continued expression of HPV-16 E7 protein is required for maintenance of the transformed phenotype of cells co-transformed by HPV-16 plus EJ-ras. *EMBO J.* 8, 513–519.

Dalsgaard, K. (1974). Saponin adjuvants. 3. Isolation of a substance from Quillaja saponaria Molina with adjuvant activity in food-and-mouth disease vaccines. *Arch. Gesamte. Virusforsch.* 44, 243–254.

De Bruijn, M. L., Jackson, M. R., and Peterson, P. A. (1995). Phagocyte-induced antigen-specific activation of unprimed CD8+ T cells in vitro. *Eur. J. Immunol.* 25, 1274–1285.

Evander, M., Frazer, I. H., Payne, E., Qi, Y. M., Hengst, K., and McMillan, N. A. (1997). Identification of the alpha6 integrin as a candidate receptor for papillomaviruses. *J. Virol.* 71, 2449–2456.

Evans, D. J., McKeating, J., Meredith, J. M., Burke, K. L., Katrak, K., John, A., Ferguson, M., Minor, P. D., Weiss, R. A., and Almond, J. W. (1989). An engineered poliovirus chimaera elicits broadly reactive HIV-1 neutralizing antibodies. *Nature* 339, 385–8,340.

Falo, L. D., Jr., Kovacsovics Bankowski, M., Thompson, K., and Rock, K. L. (1995). Targeting antigen into the phagocytic pathway in vivo induces protective tumour immunity. *Nat. Med.* 1, 649–653.

Feltkamp, M. C., Smits, H. L., Vierboom, M. P., Minnaar, R. P., de Jongh, B. M., Drijfhout, J. W., ter Schegget, J., Melief, C. J., and Kast, W. M. (1993). Vaccination with cytotoxic T lymphocyte epitope-containing peptide protects against a tumor induced by human papillomavirus type 16-transformed cells. *Eur. J. Immunol.* 23, 2242–2249.

Fernando, G. J., Stewart, T., Tindle, R. W., and Frazer, I. H. (1997). Induction of tumour antigen specific CTL mediated tumour protection is not altered by prior induction of a TH2 type response specific for the same tumour antigen. Submitted.

Frazer, I. H., Medley, G., Crapper, R. M., Brown, T. C., and Mackay, I. R. (1986). Association between anorectal dysplasia, human papillomavirus, and human immunodeficiency virus infection in homosexual men. *Lancet* 2, 657–660.

Germain, R. N., and Margulies, D. H. (1993). The biochemistry and cell biology of antigen processing and presentation. *Annu. Rev. Immunol.* 11, 403–450.

Goldberg, A. L., and Rock, K. L. (1992). Proteolysis, proteasomes and antigen presentation. *Nature* 357, 375–379.

Griffiths, J. C., Harris, S. J., Layton, G. T., Berrie, E. L., French, T. J., Burns, N. R., Adams, S. E., and Kingsman, A. J. (1993). Hybrid human immunodeficiency virus Gag particles as an antigen carrier system: Induction of cytotoxic T-cell and humoral responses by a Gag:V3 fusion. *J. Virol.* 67, 3191–3198.

Harding, C. V., and Pfeifer, J. D. (1994). Antigen expressed by Salmonella typhimurium is processed for class I major histocompatibility complex presentation by macrophages but not infected epithelial cells. *Immunology* 83, 670–674.

Jenkins, O., Cason, J., Burke, K. L., Lunney, D., Gillen, A., Patel, D., McCance, D. J., and Almond, J. W. (1990). An antigen chimera of poliovirus induces antibodies against human papillomavirus type 16. *J. Virol.* 64, 1201–1206.

Ke, Y., Li, Y., and Kapp, J. A. (1995). Ovalbumin injected with complete Freund's adjuvant stimulates cytolytic responses. *Eur. J. Immunol.* 25, 549–553.

Kirnbauer, R., Booy, F., Cheng, N., Lowy, D. R., and Schiller, J. T. (1992). Papillomavirus L1 major capsid protein self-assembles into virus-like particles that are highly immunogenic. *Proc. Natl. Acad. Sci. USA* 89, 12180–12184.

Kovacsovics Bankowski, M., Clark, K., Benacerraf, B., and Rock, K. L. (1993). Efficient major histocompatibility complex class I presentation of exogenous antigen upon phagocytosis by macrophages. *Proc. Natl. Acad. Sci. USA* 90, 4942–4946.

Kovacsovics Bankowski, M., and Rock, K. L. (1995). A phagosome-to-cytosol pathway for exogenous antigens presented on MHC class I molecules. *Science* 267, 243–246.

Kulski, J. K., Sadleir, J. W., Kelsall, S. R., Cicchini, M. S., Shellam, G., Peng, S. W., Qi, Y. M., Zhou, J., and Frazer, I. H. Type-specific and genotype cross reactive B epitopes of the L1 protein of HPV16 defined by a panel of monoclonal antibodies. Submitted.

Lambert, P. F., Pan, H., Pitot, H. C., Liem, A., Jackson, M., and Griep, A. E. (1993). Epidermal cancer associated with expression of human papillomavirus type 16 E6 and E7 oncogenes in the skin of transgenic mice. *Proc. Natl. Acad. Sci. USA* 90, 5583–5587.

Lanzavecchia, A. (1996). Mechanisms of antigen uptake for presentation. *Curr. Opin. Immunol.* 8, 348–354.

Layton, G. T., Harris, S. J., Gearing, A. J., Hill Perkins, M., Cole, J. S., Griffiths, J. C., Burns, N. R., Kingsman, A. J., and Adams, S. E. (1993).

Induction of HIV-specific cytotoxic T lymphocytes in vivo with hybrid HIV-1 V3:Ty-virus-like particles. *J. Immunol.* 151, 1097–1107.

Liu, W. J., Gissmann, L., Sun, X. Y., Kanjanahaluethai, A., Muller, M., Doorbar, J., and Zhou, J. (1997). Sequence close to the N-terminus of L2 protein is displayed on the surface of bovine papillomavirus type 1 virions. *Virology* 227, 474–483.

Londono, L. P., Chatfield, S., Tindle, R. W., Herd, K., Gao, X. M., Frazer, I., and Dougan, G. (1996). Immunisation of mice using Salmonella typhimurium expressing human papillomavirus type 16 E7 epitopes inserted into hepatitis B virus core antigen. *Vaccine* 14, 545–552.

Meneguzzi, G., Cerni, C., Kieny, M. P., and Lathe, R. (1991). Immunization against human papillomavirus type 16 tumor cells with recombinant vaccinia viruses expressing E6 and E7. *Virology* 181, 62–69.

Michel, M. L., Mancini, M., Schlienger, K., and Tiollais, P. (1993). Recombinant hepatitis B surface antigen as a carrier of human immunodeficiency virus epitopes. *Res. Virol.* 144, 263–267.

Moore, M. W., Carbone, F. R., and Bevan, M. J. (1988). Introduction of soluble protein into the class I pathway of antigen processing and presentation. *Cell* 54, 777–785.

Muller, M., Zhou, J., Reed, T. D., Rittmuller, C., Burger, A., Gabelsberger, J., Raspenning, J., and Gissmann, L. (1997). Chimeric papillomavirus-like particles. *Virology* 234, 93–111.

Norbury, C. C., Hewlett, L. J., Prescott, A. R., Shastri, N., and Watts, C. (1995). Class I MHC presentation of exogenous soluble antigen via macropinocytosis in bone marrow macrophages. *Immunity* 3, 783–791.

Paintsil, J., Muller, M., Picken, M., Gissmann, L., and Zhou, J. (1996). Carboxyl terminus of bovine papillomavirus type-1 L1 protein is not required for capsid formation. *Virology* 223, 238–244.

Pfeifer, J. D., Wick, M. J., Roberts, R. L., Findlay, K., Normark, S. J., and Harding, C. V. (1993). Phagocytic processing of bacterial antigens for class I MHC presentation to T cells. *Nature* 361, 359–362.

Qi, Y. M., Peng, S. W., Hengst, K., Evander, M., Park, D. S., Zhou, J., and Frazer, I. H. (1996). Epithelial cells display separate receptors for papillomavirus VLPs and for soluble L1 capsid protein. *Virology* 216, 35–45.

Ressing, M. E., Sette, A., Brandt, R. M., Ruppert, J., Wentworth, P. A., Hartman, M., Oseroff, C., Grey, H. M., Melief, C. J., and Kast, W. M. (1995). Human CTL epitopes encoded by human papillomavirus type 16 E6 and E7 identified through in vivo and in vitro immunogenicity studies of HLA-A*0201-binding peptides. *J. Immunol.* 154, 5934–5943.

Rock, K. L. (1996). A new foreign policy: MHC class I molecules monitor the outside world. *Immunol. Today* 17, 131–137.

Roden, R. B., Weissinger, E. M., Henderson, D. W., Booy, F., Kirnbauer, R., Mushinski, J. F., Lowy, D. R., and Schiller, J. T. (1994). Neutralization of bovine papillomavirus by antibodies to L1 and L2 capsid proteins. *J. Virol.* 68, 7570–7574.

Roden, R. B., Hubbert, N. L., Kirnbauer, R., Breitburd, F., Lowy, D. R., and Schiller, J. T. (1995). Papillomavirus L1 capsids agglutinate mouse erythrocytes through a proteinaceous receptor. *J. Virol.* 69, 5147–5151.

Schlienger, K., Mancini, M., Riviere, Y., Dormont, D., Tiollais, P., and Michel, M. L. (1992). Human immunodeficiency virus type 1 major neutralizing determinant exposed on hepatitis B surface antigen particles is highly immunogenic in primates. *J. Virol.* 66, 2570–2576.

Schulz, M., Zinkernagel, R. M., and Hengartner, H. (1991). Peptide-induced antiviral protection by cytotoxic T cells. *Proc. Natl. Acad. Sci. USA* 88, 991–993.

Sedlik, C., Saron, M. F., Sarraseca, J., Casal, I., and Leclerc, C. (1997). Recombinant parvovirus-like particles as an antigen carrier: A novel nonreplicative exogenous antigen to elicit protective antiviral cytotoxic T cells. *Proc. Natl. Acad. Sci. USA* 94, 7503–7508.

Sugita, M., and Brenner, M. B. (1995). Association of the invariant chain with major histocompatibility complex class I molecules directs trafficking to endocytic compartments. *J. Biol. Chem.* 270, 1443–1448.

Takahashi, H., Cohen, J., Hosmalin, A., Cease, K. B., Houghten, R., Cornette, J. L., DeLisi, C., Moss, B., Germain, R. N., and Berzofsky, J. A. (1988). An immunodominant epitope of the human immunodeficiency virus envelope glycoprotein gp160 recognized by class I major histocompatibility complex molecule-restricted murine cytotoxic T lymphocytes. *Proc. Natl. Acad. Sci. USA* 85, 3105–3109.

Tindle, R. W., Herd, K., Londono, P., Fernando, G. J., Chatfield, S. N., Malcolm, K., and Dougan, G. (1994). Chimeric hepatitis B core antigen particles containing B- and Th-epitopes of human papillomavirus type 16 E7 protein induce specific antibody and T-helper responses in immunised mice. *Virology* 200, 547–557.

Tindle, R. W., Croft, S., Herd, K., Malcolm, K., Geczy, A. F., Stewart, T., and Fernando, G. J. (1995). A vaccine conjugate of 'ISCAR' immunocarrier and peptide epitopes of the E7 cervical cancer-associated protein of human papillomavirus type 16 elicits specific Th1- and Th2-type responses in immunized mice in the absence of oil-based adjuvants. *Clin. Exp. Immunol.* 101, 265–271.

Tindle, R. W., and Frazer, I. H. (1994). Immune response to human papillomaviruses and the prospects for human papillomavirus-specific immunisation. *Curr. Top. Microbiol. Immunol.* 186, 217–253.

Townsend, A., and Trowsdale, J. (1993). The transporters associated with antigen presentation. *Semin. Cell Biol.* 4, 53–61.

Winter, N., Lagranderie, M., Gangloff, S., Leclerc, C., Gheorghiu, M., and Gicquel, B. (1995). Recombinant BCG strains expressing the SIVmac251nef gene induce proliferative and CTL responses against nef synthetic peptides in mice. *Vaccine* 13, 471–478.

Zhou, F., Rouse, B. T., and Huang, L. (1992). Induction of cytotoxic T lymphocytes in vivo with protein antigen entrapped in membranous vehicles. *J. Immunol.* 149, 1599–1604.

Zhou, J., Sun, X. Y., Stenzel, D. J., and Frazer, I. H. (1991). Expression of vaccinia recombinant HPV 16 L1 and L2 ORF proteins in epithelial cells is sufficient for assembly of HPV virion-like particles. *Virology* 185, 251–257.

Zhou, J., Gissmann, L., Zentgraf, H., Muller, H., Picken, M., and Muller, M. (1995). Early phase in the infection of cultured cells with papillomavirus virions. *Virology* 214, 167–176.

Zhu, X., Tommasino, M., Vousden, K., Sadovnikava, E., Rappuoli, R., Crawford, L., Kast, M., Melief, C. J., Beverley, P. C., and Stauss, H. J. (1995). Both immunization with protein and recombinant vaccinia virus can stimulate CTL specific for the E7 protein of human papilloma virus 16 in H-2d mice. *Scand. J. Immunol.* 42, 557–563.

zur Hausen, H. (1991). Human papillomaviruses in the pathogenesis of anogenital cancer. *Virology* 184, 9–13.