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L1-Specific Protection from Tumor Challenge Elicited by HPV16 Virus-like Particles

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A single injection of HPV16 L1 virus-like particles induced potent CD8-mediated protection from tumor challenge by C3 cells, a line derived from embryonic mouse cells transfected with the HPV16 genome. L1 RNA, but not protein, was detected biochemically in C3 cells. These results indicate that low-level expression of HPV16 L1 can occur in proliferating cells and serve as a tumor vaccine target. Although L1 expression is generally thought to be restricted to terminally differentiated epithelial cells, these results suggest that additional analysis for low-level L1 expression in proliferating cells of HPV-induced lesions is warranted and might help in predicting the clinical potential of HPV L1 virus-like particle-based vaccines.

INTRODUCTION

The strong association between cervical cancer and HPV16 infection has generated considerable interest in developing a safe and effective therapeutic vaccine to eliminate infection by this virus. Because E6 and E7 are selectively maintained and expressed during malignant progression, most vaccine strategies have focused on these two viral targets (reviewed in Kast *et al*., 1996; Schiller and Okun, 1996). In contrast, the virion capsid proteins L1 and L2 are expressed in terminally differentiated squamous epithelial cells but are not thought to be expressed in proliferating cells (Taichman and LaPorta, 1987). We recently developed chimeric HPV16 virus-like particles (VLPs) in which the full-length HPV16 E7 protein is incorporated into L1/L2 VLPs via fusion to the carboxyl terminus of L2. In addition to eliciting the production of high-titer neutralizing antibodies, immunization with these chimeric VLPs (but not the parental L1/L2 VLPs), in the absence of adjuvant, protected mice from tumor challenge with the TC-1 cell line (Greenstone *et al.,* 1998). This cell line expresses HPV16 E7 but not HPV16 L1 (Lin *et al.,* 1996).

Another established HPV16 tumor vaccine model is based on the C3 tumorigenic cell line, which was generated from full-length HPV16 plus EJ*ras* transformed C57Bl/6 (B6) mouse embryo cells (Feltkamp *et al.,* 1993). C3 has previously been used extensively to examine antitumor immune responses to HPV16 E6 and E7 peptides (Feltkamp *et al.,* 1993, 1995). In an attempt to demonstrate E7-specific antitumor responses against C3 cells after vaccination with the E7 chimeric VLPs, we obtained unexpected results indicating that these proliferating tumor cells express sufficient L1 to serve as a target for protective antitumor immune responses.

RESULTS

In an attempt to demonstrate E7-specific antitumor immune responses to E7 chimeric VLPs using an HPV16 transformed cell line, we challenged B6 mice subcutaneously with C3 cells 2 weeks after subcutaneous vaccination with a single 50-μg dose of HPV16 L1/L2-E7 chimeric VLPs in incomplete Freund's adjuvant (IFA). Protection from tumor challenge was observed in these mice, unlike control mice vaccinated with IFA alone or mice vaccinated with 10 μg of an irrelevant peptide antigen (SGPSNTPPEI) (Kast *et al.,* 1989) in IFA (Table 1, Exp. 1). The protection induced by the chimeric VLPs was comparable to that obtained with the positive control antigen, 10 μg of the E7 peptide RAHYNIVTF in IFA (Feltkamp *et al.,* 1993). Unexpectedly, a single 50-μg dose of the parental HPV16 L1/L2 VLPs (composed of HPV16 L1 plus HPV16 L2) (Kirnbauer *et al.,* 1993) also gave complete protection in this experiment. In contrast to RAHYNIVTF, the VLPs induced a protective response when a single 50-μg (Exp. 2) or 30-μg (Exp. 3) dose was administered in the absence of adjuvant. There was no significant difference in the degree of protection elicited by HPV16 L1 VLPs compared with HPV16 L1/L2 VLPs

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TABLE 1

Protection from Challenge with C3 Tumorigenic Cell Line

	Antigen	Dosage	Adjuvant	Tumors	*P* value
Exp. 1	PBS	—	IFA	5/9	Ref.
	SGPSNTPPEI	10 μg × 1	IFA	4/7	1.000
	RAHYNIVTF	10 μg × 1	IFA	0/8	0.029
	16 L1/L2	50 μg × 1	IFA	0/8	0.029
	16 L1/L2-E7	50 μg × 1	IFA	0/8	0.029
Exp. 2	PBS	—	IFA	10/12	Ref.
	RAHYNIVTF	10 μg × 1	IFA	3/10	0.027
	16 L1/L2	50 μg × 1	IFA	3/8	0.062
	B L1/L2	50 μg × 1	IFA	6/8	0.999
	RAHYNIVTF	10 μg × 1	PBS	8/8	Ref.
	16 L1/L2	50 μg × 1	PBS	2/6	0.015[a]
	B L1/L2	50 μg × 1	PBS	6/6	0.999[a]
Exp. 3	PBS	—	PBS	8/10	Ref.
	RAHYNIVTF	10 μg × 2	IFA	4/10	0.170
	16 L1	30 μg × 2	PBS	1/10	0.003
	16 L1/L2	30 μg × 2	PBS	0/10	$<$0.001
Exp. 4	PBS	—	PBS	8/10	Ref.
	16 L1/L2	20 μg × 2	PBS	2/10	0.023
	16 L1/L2	20 ng × 2	PBS	8/10	1.000
	B L1/L2	20 μg × 2	PBS	8/10	1.000
	B L1/L2	20 ng × 2	PBS	10/10	0.474

Note. Mice were immunized with VLPs as indicated (16 = HPV16; B = BPV1; L1, L1/L2, and L1/L2-E7 refer to VLPs containing the respective proteins). × 1 indicates single immunization; × 2, two immunizations 2 weeks apart; IFA, incomplete Freund's adjuvant; PBS, phosphate-buffered saline; Ref., reference. Tumor take was assessed on day 93 after challenge for Exp. 1, day 67 after challenge for Exp. 2, day 73 after challenge for Exp. 3, and day 46 after challenge for Exp. 4.

[a] Compared with mice immunized with RAHYNIVTF in PBS.

(using a 30-μg dose, Exp. 3), indicating that a response to L1 was sufficient for protection. Protection also was seen after two subcutaneous vaccinations (2 weeks apart) with 20 μg of HPV16 L1/L2 VLPs but not after two vaccinations with 20 ng of VLPs (Exp. 4).

In contrast to the HPV16 VLPs, BPV1 L1/L2 VLPs that are 49% identical in L1 amino acid sequence with HPV16 (Kirnbauer *et al.,* 1992) did not protect against C3 challenge after a single vaccination with 50 μg in either the presence or absence of adjuvant (Exp. 2). Similarly, no protection was observed after two sequential vaccinations with 20 μg or 20 ng of BPV VLPs (Exp. 4). These results indicated that the protective immune response was specific for HPV16 L1.

The specificity of the tumor protection induced by HPV16 VLPs was examined further in experiments using the AR6 tumorigenic cell line. As with C3, AR6 was derived from B6 mouse embryo cells containing E*Jras,* except that it was generated after transfection with human adenovirus type 5 E1A rather than with the HPV16 genome (Toes *et al.,* 1995). HPV16 L1/L2 VLP vaccination did not protect against challenge with AR6 tumor cells (five of eight mice were tumor positive), whereas the positive control antigen, inactivated SAMB7 cells (Schoenberger *et al.,* 1998) presenting E1A peptides, was protective (none of six mice were tumor positive). The lack of protection from HPV16 L1/L2 VLP immunization was significant when compared to immunization with the positive control SAMB7 cells ($P = 0.031$). Therefore, protection after HPV16 VLP vaccination was specific to the HPV16 transfected embryo cells.

To examine the immunological mechanism responsible for the protection elicited by the VLPs, B6 mice were depleted of $CD4^+$ or $CD8^+$ T cells by injection of a CD4-specific monoclonal antibody (GK1.5) or a CD8-specific monoclonal antibody (2.43), respectively (Dialynas *et al.,* 1983; Sarmiento *et al*., 1980). FACS analysis of peripheral blood mononuclear cells indicated that effective depletion ($>$99%) of $CD4^+$ or $CD8^+$ T cells by GK1.5 and 2.43, respectively, had occurred (data not shown). No protection was observed when the CD8-depleted mice were vaccinated with HPV16 L1/L2 VLPs and challenged with C3 cells (Fig. 1). In contrast, VLP vaccination completely protected the CD4-depleted mice from tumor challenge (Fig. 1). Therefore, we conclude that the antitumor response induced by the VLPs is mediated by $CD8^+$ CTLs.

Our finding that HPV16 VLPs could induce an L1-specific protective immune response against C3 challenge was unexpected because L1 is generally thought to be expressed only in terminally differentiated epithelial cells (Stoler *et al.,* 1992; Taichman and LaPorta, 1987), and C3 is a transformed embryo-derived cell line. However, the fact that C3 was generated after transfection of

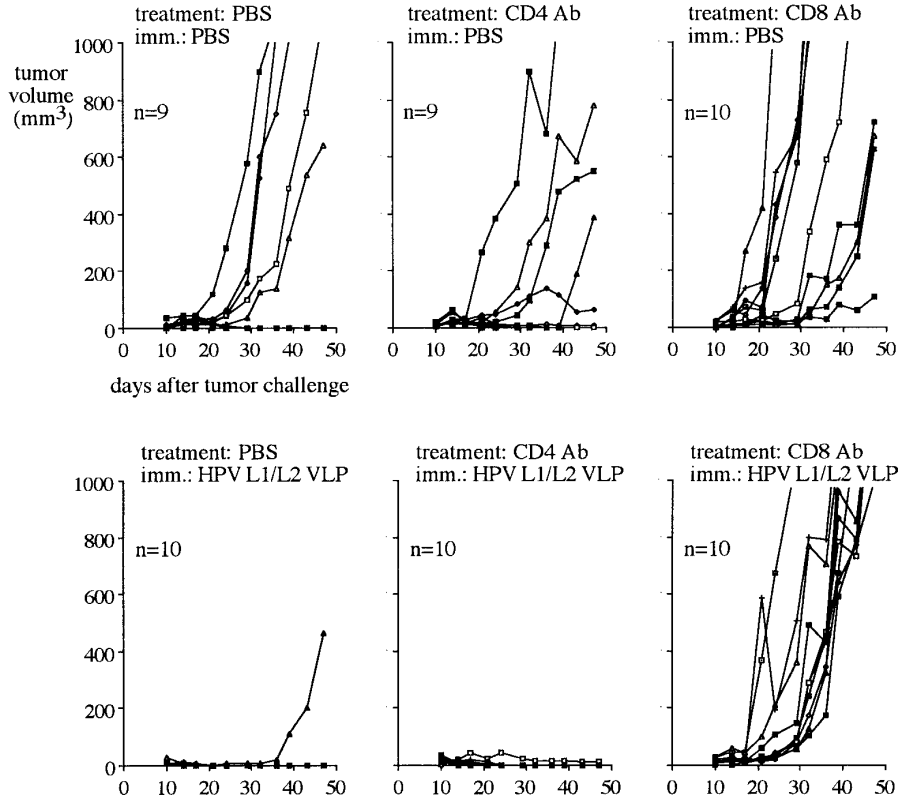

FIG. 1. Protection from C3 challenge after depletion of $CD4^+$ or $CD8^+$ T cells. $CD4^+$ and $CD8^+$ T cells were depleted by injection of monoclonal antibody GK1.5 and monoclonal antibody 2.34, respectively (Dialynas *et al.,* 1983; Sarmiento *et al*., 1980). Depleted and control mice were vaccinated with 50 μg of HPV16 L1/L2 VLPs. Imm. indicates immunogen; n, number of animals per group.

the full-length HPV16 genome raised the possibility that HPV16 L1 polypeptides were expressed, processed, and presented in the context of MHC class I molecules in C3 cells. We therefore sought evidence for L1 expression in this line, using a standard RT-PCR protocol. Amplified products of the predicted length were detected for a 5′ segment (300 bp), a 3′ segment (300 bp), and, to a lesser extent, the almost full-length transcript (1489 of 1518 bp) (Fig. 2). Because no PCR products were detected when the reverse transcriptase was omitted from the reactions, we conclude that L1 RNA rather than L1 DNA was the initial template for amplification.

Having detected L1 RNA, we sought direct evidence for L1 protein in C3 cells. In initial studies, L1 protein was not detected in immunofluorescent staining of fixed C3 cells or in immunoblots of C3 lysates using L1-specific monoclonal or polyclonal sera that recognize either native or denatured L1 (data not shown). However, because these assays measure steady-state levels of protein, they did not address the possibility that substantial amounts of L1 were synthesized but rapidly degraded. Because the peptides required for class I presentation are usually generated by proteolysis in proteasome complexes (Brown *et al*., 1991; Glynne *et al.,* 1991), rapid degradation would be compatible with, and possibly even beneficial for, class I presentation. In an attempt to detect short-lived L1 protein species, we conducted L1 immunoprecipitations of C3 extracts that had been radiolabeled with ^{35}S-cysteine and ^{35}S-methionine for short periods (15 and 60 min). To maximize sensitivity, combined rabbit antisera raised against native HPV16 VLPs and denatured HPV16 VLPs or pooled monoclonal antibodies recognizing both native and denatured L1 were used in the immunoprecipitation reactions. However, neither full-length nor truncated L1 protein was detected after subjecting the precipitates to SDS–PAGE followed by autoradiography, even after exposure of the autoradiographs for several weeks (data not shown). We suspect that the L1 protein is expressed at low levels in C3 cells and too rapidly degraded to be detected by standard immunochemical methods.

DISCUSSION

In this study, we determined that VLPs can induce a CD8-restricted antitumor response specific for HPV16 L1. Consistent with this finding is a recent report demonstrating an increase in the relative number of splenic $CD8^+$ T cells in mice immunized with HPV16 L1 VLPs (Dupuy *et al.,* 1997). Evidence for MHC class I restriction was also found in our recent study of HPV16 L1/L2-HPV16E7 chimeric VLPs, where immunization with the

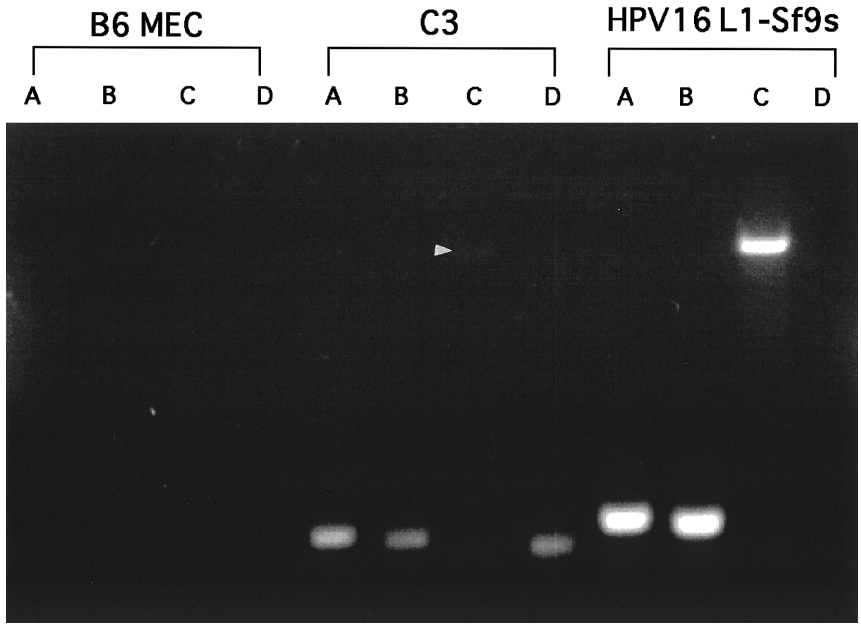

FIG. 2. Detection of HPV16 L1 transcripts in C3 cells. mRNAs from C57B1/6 mouse embryo cells (B6 MEC), C3 tumor cells, or HPV16 L1 recombinant baculovirus-infected Sf9 cells (HPV16 L1-Sf9s) were purified using the Micro-FastTract Kit (InVitrogen Corp.). To remove any contaminating genomic DNA, 130 U of RNase-free DNase (GIBCO BRL, Gaithersburg, MD) was added before RNA precipitation. Then, 100 ng of each mRNA was cycled twice using the cDNA Cycle Kit (Invitrogen Corp.). The cDNAs were PCR amplified with HPV16 L1-specific primers. The reaction products shown in lanes A, B, C, and D were generated using primers specific for the 5′ end of HPV16 L1, the 3′ end of HPV16 L1, the full-length HPV16 L1 (arrow), and the full-length HPV16 E7, respectively.

chimeric VLPs (but not L1 or L1/L2 VLPs) provided E7-specific tumor protection from TC-1 cell challenge in class II knockout but not in β_2-microglobulin or perforin knockout mice (Greenstone *et al.,* 1998). Although CD4-restricted responses were not required for tumor protection in either the C3 or TC-1 assays, the preferential induction of a Th1 type T-helper response to VLP vaccination is likely to promote proliferation of VLP-specific $CD8^+$ T cells under normal conditions (Dupuy *et al.,* 1997).

The precise mechanism by which papillomavirus VLPs were able to induce CD8-restricted responses remains uncertain. MHC class I-mediated cytotoxic T lymphocyte (CTL) responses are usually induced by endogenously, rather than exogenously, presented antigens or by using viral vectors like vaccinia virus expressing HPV16 L1 DNA (Zhou *et al*., 1991). However, enhancement of class I-restricted responses to an exogenous antigen by presentation in a particulate form has been documented, although the smallest particles investigated were 10-fold larger than HPV VLPs (Kovacsovics-Bankowski *et al.,* 1993). In addition, VLPs may mimic authentic virions in their ability to escape endocytic vesicles and enter the cytoplasm after uptake. This would allow for degradation of the L1 protein by the cytoplasmic proteasome complexes with subsequent presentation by the normal TAP-dependent mechanism (Brown *et al*., 1991; Glynne *et al.,* 1991).

Our results indicate that HPV16 L1 can, unexpectedly, be expressed at sufficient levels in proliferating cells transformed by the full-length HPV16 genome to serve as a target of CTL. Furthermore, they demonstrate that VLPs can efficiently induce a protective antitumor response after a single low-dose injection in the absence of adjuvant, even if there is a very low steady-state level of L1 in the proliferating cells. Although the relevance of our *in vitro* cell culture system to *in vivo* infection is unclear, our findings should prompt a more comprehensive examination of L1 expression in infected basal cells of productive lesions and the proliferating cells of more progressed lesions, perhaps using sensitive and specific *in situ* RT-PCR assays. This might aid in predicting the circumstances under which an L1 VLP vaccine might have therapeutic effects in humans. Our findings further support the possibility that VLPs incorporating E7 peptides or other antigens (Greenstone *et al.,* 1998; Muller *et al.,* 1997) may be effective vehicles for generating antitumor or antimicrobial CTL responses against other targets.

MATERIALS AND METHODS

Tumorigenicity assays

VLP vaccination and protection from C3 tumor challenge assays were performed essentially as described previously (Feltkamp *et al.,* 1993, 1995; Greenstone *et al.,* 1998). Mice were immunized subcutaneously with VLPs or control peptide and challenged 14 days later with 0.5×10^6 C3 cells injected subcutaneously.

T cell depletion

B6 mice were depleted of $CD4^+$ or $CD8^+$ T cells by injection of a CD4-specific monoclonal antibody (GK1.5) or a CD8-specific monoclonal antibody (2.43), respectively (Dialynas *et al.,* 1983; Sarmiento *et al.*, 1980). To deplete $CD4^+$ or $CD8^+$ T cells *in vivo*, mice were injected intraperitoneally on multiple days with 100 μg of the appropriate monoclonal antibody. Relative to the first immunization, the antibodies were administered on the following days: -2, 1, 5, 11, and 27. Immunizations were performed on days 0 and 12, and a challenge with 0.5×10^6 C3 cells was given on day 22.

RT-PCR

RT-PCRs were performed according to the manufacturer's protocol (Invitrogen Corp., San Diego, CA). cDNAs from L1 transcripts were amplified using primer pairs specific for the 5′ end (forward primer: 5′-GGCTGCCTAGTGAGGCCACT-3′, reverse primer: 5′-ACCTCAACACCTACACAGGC-3′), the 3′ end (forward primer: 5′-GACTGGAATTTTGGTCTACA-3′, reverse primer: 5′-GCGTTTAGCAGTTGTAGAGG-3′), or the full-length L1 gene (forward primer: 5′-GGCTGCCTAGTGAGGCCACT-3′, reverse primer: 5′-GCGTTTAGCAGTTGTAGAGG-3′) using AmpliTaq polymerase (Roche Molecular Systems, Branchburg, NJ) under the recommended conditions.

Immunochemical assays for L1

Immunoprecipitation reactions were conducted as previously described (Greenstone *et al.,* 1998) using combined rabbit antisera raised against native HPV16 VLPs and denatured HPV16 VLPs (No. 5085 and No. 5166, respectively) (Roden *et al.,* 1996) or pooled monoclonal antibodies recognizing both native and denatured L1 (H16.E70, H16.U4, H16.V5, H16.L4, H16.S1, H16.H5, and H16.J4) (Christensen *et al.,* 1996).

Statistical analysis

P values were determined using Fisher's Exact Test (two-tailed); $P < 0.05$ was deemed statistically significant.

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