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Effect of Preexisting Neutralizing Antibodies on the Anti-tumor Immune Response Induced by Chimeric Human Papillomavirus Virus-like Particle Vaccines

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Chimeric human papillomavirus virus-like particles (HPV cVLPs) carrying HPV16 E7 protein are potent vaccines for inducing cell-mediated immunity (CMI) against HPV-induced tumors in animal models. We tested the hypothesis that virion-neutralizing antibodies generated during an initial vaccination might prevent effective boosting of CMI to the cVLPs. Mice with circulating HPV16-neutralizing antibodies, generated by direct immunization with wild-type VLPs or by passive transfer of hyperimmune anti-HPV16 VLP mouse sera, were subsequently vaccinated with HPV16 E7-containing cVLPs. Mice with preexisting neutralizing antibodies were not protected from HPV16 E7-positive TC-1 tumor challenge, compared to the protection seen in mice lacking these antibodies. Antibody-coated VLPs bound very inefficiently to receptor-positive cell lines, suggesting that one of the mechanisms of antibody interference is blocking of VLP binding to its receptor and thereby uptake of VLPs by antigen-presenting cells. Our results suggest that repetitive vaccination with a cVLP for induction of cellular immune responses to an incorporated antigen may be of limited effectiveness due to the presence of neutralizing antibodies against the capsid proteins induced after the first application. This limitation could potentially be overcome by boosting with cVLPs containing the same target antigen incorporated into other papillomavirus-type VLPs. © 2001 Academic Press

Key Words: virus-like particle; vaccine; neutralization; papillomavirus; HPV16; pseudovirion.

INTRODUCTION

Both cervical dysplasia and cervical cancer in humans are highly associated with infection by certain high-risk human papillomaviruses (HPV), with HPV16 being found in 50–60% of HPV-positive biopsies (Bosch *et al.,* 1995; Walboomers *et al.,* 1999). Papillomaviruses are members of the papovavirus family of viruses and consist of a 55-nm, nonenveloped, icosahedral-shaped virion. The 7.9-kb circular, double-stranded DNA viral genome contains open reading frames for early proteins that are responsible for viral DNA replication and transcription and cellular transformation and late proteins that make up the virus capsid. Infectious HPV virions cannot be isolated from naturally occurring lesions, nor can they be propagated *in vitro* in cell culture or in animal systems in large enough quantities to be used as a prophylactic vaccine. *In vitro,* however, the papillomavirus L1 major and L2 minor capsid proteins are able to self-assemble into virus-like particles (VLPs) which are highly immunogenic (Kirnbauer *et al.,* 1992, 1993). VLPs mimic HPV virions in structure and morphology (Hagensee *et al.,* 1994) and in their ability to induce high titers of virus-neutralizing antibody, even in the absence of adjuvant (Kirnbauer *et al.,* 1992; Christensen *et al.,* 1994). Additionally, VLPs can be modified as vaccine delivery vehicles to carry nonviral plasmid DNA (i.e., pseudovirions) (Touze and Coursaget, 1998) or polypeptides (i.e., chimeric VLPs; cVLPs), which upon vaccination elicit a CTL response against tumor cells expressing the appropriate tumor rejection antigen (Greenstone *et al.,* 1998; Peng *et al.,* 1998).

Infection with papillomaviruses is strictly species-specific, therefore the use of various animal papillomaviruses has allowed researchers to study the protective effects of papillomavirus VLP-based vaccines. Immunization of rabbits, cattle, or dogs with cottontail rabbit papillomavirus VLPs, bovine papillomavirus (BPV) VLPs, or canine oral papillomavirus VLPs, respectively, protects animals against infection with the homologous animal papillomavirus (Breitburd *et al.,* 1995; Christensen *et al.,* 1996c; Kirnbauer *et al.,* 1996; Suzich *et al.,* 1995). Passive transfer of serum containing antibodies generated against VLPs to naïve animals similarly protects animals from experimental virus challenge, suggesting that the protective effect is mediated by neutralizing antibodies (Breitburd *et al.,* 1995; Suzich *et al.,* 1995).

Chimeric HPV VLPs are being developed for an anti-HPV vaccine that has the combined potential of directing

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the immune response toward prevention of virus infection and elimination of existing HPV-infected cells (Da Silva *et al.,* 1999). E6 and E7 are expressed in cervical carcinoma cells and their expression is required for maintenance of the transformed phenotype (Von Knebel Doeberitz *et al.,* 1994), which makes them unique and attractive targets for cellular immunotherapy. Chimeric HPV16 VLPs comprising HPV16 L1 and an L2–E7 fusion protein (HPV16 L1/L2-E7 cVLPs) have been shown to deliver the tumor rejection antigen to the immune system, prime E7-specific CTLs, and protect mice against challenge with E7-transformed tumor cells (Greenstone *et al.,* 1998). The cVLPs also retain the ability to induce high titers of virion neutralizing antibodies. In theory, when used in humans, the capsid component of a chimeric VLP would induce a protective humoral immune response, while the protein components would be delivered to and processed by antigen-presenting cells (APCs) and would elicit a CTL response against HPV-induced tumor cells.

While cVLPs are able to induce both a humoral and a cellular immune response in naïve animals, it is unclear whether the VLPs can induce a cellular immune response (i.e., CTLs) in the presence of neutralizing antibodies against HPV. Existing antibodies could arise by natural infection with HPV, or when an individual is first vaccinated with wild-type (wt) HPV VLPs or cVLPs. We hypothesized that the immunogenicity of VLP-based vaccines would be influenced by antibodies generated by previous exposure to the antigen. This possibility is of potential clinical relevance since chimeric VLP-based vaccines designed for therapeutic use will be given to women who have already encountered HPV and will likely need to be administered multiple times in order to show positive effects. In the present study, we examined the ability of HPV16 L1/L2-E7 cVLPs to elicit a cellular immune response in the presence of existing neutralizing antibodies generated by preimmunization with wt VLPs. The induction of effective cellular immunity was determined by protection from E7-expressing tumor cell challenge and quantification of antigen-specific $CD8^+$ T cells following immunization.

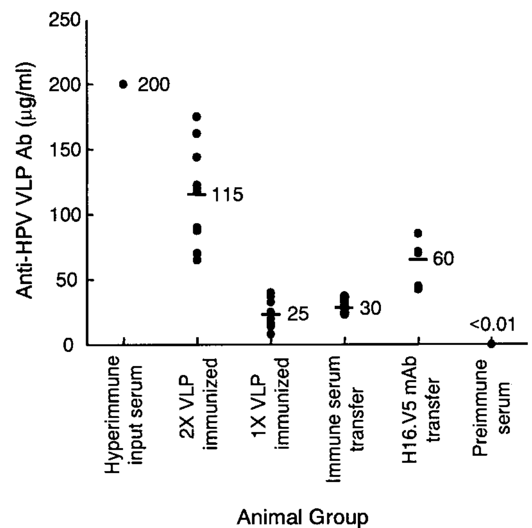

FIG. 1. Anti-HPV16 VLP IgG antibody titers in mice immunized directly with wt VLP or given antibodies by passive immunization. 6- to 8-week-old C57Bl/6 mice (10 animals/group) were immunized once or twice directly with wt HPV16 L1/L2 VLPs prior to HPV16 L1/L2-E7 cVLP immunization. Mice receiving passive antibody (hyperimmune serum or H16.V5 monoclonal antibody) were injected ip 24 h prior to chimeric VLP immunization. Input serum was used as the source of antibody for mice that received hyperimmune serum. Serum samples were collected 1 h prior to cVLP immunization to determine the levels of VLP-specific IgG antibodies by ELISA. Each point represents the concentration of anti-VLP antibody in the serum for each mouse. Numerical values represent the mean serum concentration for each group of animals.

RESULTS

Generation of preexistent neutralizing antibody conditions in mice

Mice were preimmunized with wt HPV16 L1/L2 VLPs either once or twice in order to generate a high-titered anti-VLP antibody response prior to HPV16 L1/L2-E7 cVLP immunization. Another group of mice received hyperimmune anti-VLP serum isolated from mice immunized with wt VLPs by passive transfer. This was done to exclude other possible effects of immunization with wt VLPs, such as vaccine-induced changes in lymphokine levels or cellular immune responses against the capsid proteins. Another group of mice was treated with a defined neutralizing monoclonal antibody, H16.V5, that recognizes a conformational epitope on HPV16 L1 VLPs (Christensen *et al.,* 1996a) and neutralizes infectious HPV16 virions and pseudovirions (White *et al.,* 1999; Roden *et al.,* 1997). In all groups of mice, the anti-VLP IgG antibody titer was determined by ELISA prior to mice being immunized with HPV16 L1/L2-E7 cVLPs (Fig. 1). Mice that were immunized once with wt VLPs and mice that received passive transfer of hyperimmune input serum had approximately equal average titers of antibody (25 μg/ml vs 30 μg/ml). Mice that were boosted with a second dose of wt VLP had a higher average anti-VLP concentration of antibody and mice that received the H16.V5 antibody had an intermediate final concentration of anti-VLP antibody relative to the other experimental groups (Fig. 1).

To confirm that immunization with our wt VLP preparation generated neutralizing antibodies, serum samples from the mice were tested in an *in vitro* pseudovirion neutralization assay (Roden *et al.,* 1996a). In this assay, the HPV16 capsid proteins are used to package the genome of BPV1 and infectivity is measured *in vitro* by focal transformation of mouse fibroblasts. The ability of antiserum from a random sample of mice immunized once or twice with wt VLPs and from the hyperimmune

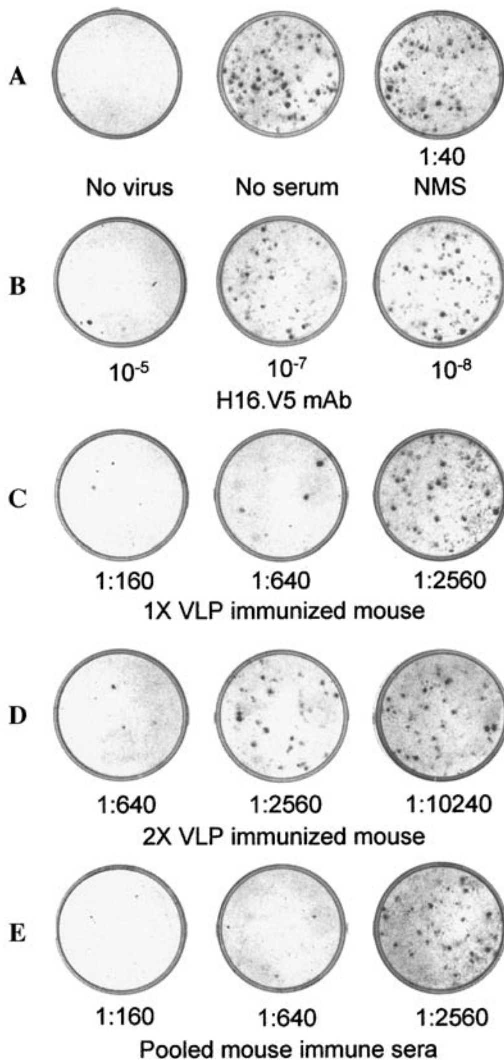

FIG. 2. Neutralization of pseudovirion infection by mouse immune serum. HPV16 L1/L2 recombinant SFV stock was used to infect BPHE-1 cells harboring the BPV1 genome to generate infectious HPV16 L1/L2{BPV1} pseudovirions. Pseudovirions were incubated with no serum, control normal mouse preimmune serum (NMS), H16.V5 antibody (row B), or fourfold dilutions, starting with 1:40, of serum collected from mice directly immunized with wt VLPs (rows C and D) or serum used for passive transfer (row E). The serum–pseudovirus mixtures were placed onto monolayers of mouse C127 fibroblasts. The cells were washed after 1 h and maintained for 3 weeks, and the monolayers were stained for visualization of transformed foci. For rows C–E, the middle plate represents the dilution of serum that resulted in a reduction of foci formation by at least 50% compared to the no-serum control. One representative from two randomly chosen mice/group tested in this assay is shown. Control plates were done in triplicate.

input serum to neutralize the pseudovirion preparation was determined (Fig. 2). No transformation foci were detected in the absence of HPV16 pseudovirions, whereas in the presence of pseudovirions an average of 76 foci was detected (Fig. 2). The monoclonal antibody H16.V5 inhibited focus formation by at least 50% at a titer of 10^{-5}. Fourfold dilutions of serum from mice immunized directly with wt VLPs or from the pooled hyperimmune serum showed neutralization titers ranging from 640 to 2560 (Fig. 2). As expected, no neutralization of pseudovirions was observed with the preimmune normal mouse serum control, since there were no detectable anti-VLP antibodies without exposure to antigen (Figs. 1 and 2). These results confirm that virus-neutralizing antibodies were present and readily detectable at the time of chimeric VLP vaccination.

Presence of neutralizing antibody blocks chimeric VLP immunization

In the presence of the neutralizing antibodies described above, mice were immunized with a single dose of HPV16 L1/L2-E7 cVLPs and 10 days later were challenged with E7-expressing TC-1 tumor cells to examine induction of E7-specific anti-tumor immunity. Because TC-1 cells express only HPV16 E6 and E7, and not the late capsid proteins, L1 and L2, only immunization with chimeric E7-containing VLPs (and not wt L1L2 VLPs) protects mice from tumor challenge (Greenstone *et al.*, 1998). In this study, all mice that were directly preimmunized with wt VLPs once prior to cVLP immunization developed progressively growing tumors and 9/10 mice immunized twice with wt VLPs developed tumors (Fig. 3). All mice that were given passive anti-VLP antibody, by means of hyperimmune serum or the neutralizing monoclonal H16.V5 antibody, also developed progressively growing tumors, similar to naïve PBS-immunized mice

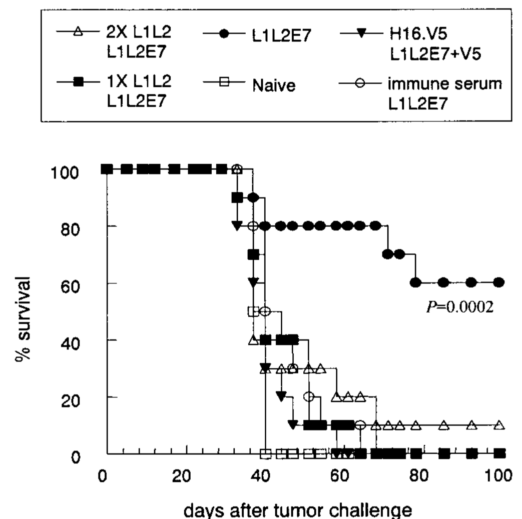

FIG. 3. Long-term survival of mice immunized with HPV16 L1/L2-E7 cVLPs in the presence or absence of neutralizing antiserum after TC-1 tumor challenge. C57Bl/6 mice (10 animals/group) were preimmunized sc 1× or 2× with wt VLP, were injected ip with hyperimmune anti-VLP serum or monoclonal H16.V5 antibody, or were left untreated. Mice were immunized sc in the left flank with HPV16 L1/L2-E7 cVLPs and 10 days later challenged sc in right flank with TC-1 tumor cells. Tumor occurrence and volume were assessed two times per week. Mice were sacrificed when tumor volume reached >1500 mm^3 or when tumor broke through skin surface.

TABLE 1

Tumor-Free Animals after Immunization with HPV16 L1/L2-E7 Chimeric VLPs in the Presence of Neutralizing Antibodies[a]

Vaccine group	Expt 1	Expt 2	Expt 3	*P* value[b]
PBS	1/7	0/10	0/10	Ref.
L1L2E7	5/7	5/10	6/10	0.0001
2× L1L2				
L1L2E7	2/7	nd[c]	1/10	0.282
1× L1L2				
L1L2E7	2/7	0/10	0/10	1.0
Immune sera				
L1L2E7	nd	nd	0/10	1.0
H16.V5				
L1L2E7	nd	nd	0/10	1.0

[a] Neutralizing antibodies were generated *in vivo* by direct immunization with wt L1/L2 VLPs or were given passively by ip injection prior to immunization with chimeric HPV16 L1/L2-E7 VLPs.

[b] *P* values determined by a two-tailed Fisher's exact test comparing tumor incidence in PBS-vaccinated control animals to each vaccine group using the sum of the results of three experiments.

[c] nd, not determined.

(Fig. 3). In contrast, 6/10 mice immunized with HPV16 L1/L2-E7 cVLPs were completely protected from tumor challenge. Of the 4 mice that did develop tumors, 2 had tumors in which the growth was inhibited, resulting in a significant survival advantage of the whole group compared to all other groups in the study ($P = 0.0002$) (Fig. 3). Similar results were obtained in two additional sets of experiments (Table 1). Significant protection from tumor challenge was observed only when there were no neutralizing antibodies present at the time of cVLP immunization ($P = 0.0001$) (Table 1). Immunization with wt L1/L2 VLPs did not protect mice from TC-1 tumor challenge, confirming previously published findings (data not shown). H16.V5 antibody and mouse anti-VLP antibodies were not able to bind to TC-1 tumor cells by flow cytometry, excluding the possibility of any direct effects of the anti-VLP antibodies on TC-1 tumor growth (data not shown).

In order to evaluate $CD8^+$ class I-restricted T cell induction in the presence of neutralizing antibodies, mice were immunized once with wt L1/L2 VLPs prior to immunization with HPV16 L1/L2-E7 cVLPs or with HPV16 L1/L2-E7 cVLPs alone. Splenocytes from vaccinated and control mice were tested in an ELISPOT assay for specific IFNγ release upon stimulation with an H-2D^b-binding $E7_{(49-57)}$ peptide, RAHYNIVTF (Feltkamp *et al.,* 1993), and for binding of E7-specific T cells to H2-D^b-RAHYNIVTF tetramer complexes (Table 2). Specific IFNγ-secreting cells were detected in HPV16 L1/L2-E7 cVLP-immunized mice, whereas mice that had been preimmunized with wt VLP had on average a 9-fold lower number of secreting cells in response to stimulation with the E7 peptide (Table 2). Double immunofluorescent staining and flow cytometric analysis of 5-day *in vitro*-stimulated splenocytes with anti-CD8 antibody and H-2D^b-RAHYNIVTF tetramers showed an average 1.8-fold decrease in tetramer-positive $CD8^+$ cells from mice that had preexisting antibodies prior to cVLP immunization (Table 2). Taken together, the *in vivo* and *in vitro* data indicate that the induction of E7-specific anti-tumor cell-mediated immune (CMI) response is severely inhibited in the presence of virus-neutralizing antibodies.

Neutralizing antibodies block VLP binding to its receptors

Neutralizing antibodies can have different effects on different pathogens. In the case of nonenveloped viruses, neutralizing antibodies can inhibit virus binding to its cellular receptor and/or inhibit virus uptake and uncoating. In order to determine what effects neutralizing antibodies have on the VLPs used in this study, we analyzed both the structural integrity of antibody-coated cVLPs and the ability of antibody-coated wt VLPs to bind to cells that express an HPV receptor(s). HPV16 L1/L2-E7 cVLPs that had been incubated with H16.V5 antibody for 30 min at room temperature were examined by electron microscopy. The structure of the antibody-coated VLPs looked identical to uncoated cVLPs (Fig. 4A). The structure of the cVLPs was very similar to that of wt HPV16 L1/L2 VLPs, with a capsid size of approximately 55 nm. The data indicate that binding of a neutralizing antibody to the VLP does not result in the destruction of capsid structure.

Antibody-coated VLPs were tested for binding to four different cell lines that express a papillomavirus receptor and have been used to characterize VLP–cellular interactions (Roden *et al.,* 1994; Müller *et al.,* 1995; Volpers *et al.,* 1995; Zhou *et al.,* 1995). The addition of the H16.V5 antibody to the VLPs completely blocked VLP binding to

TABLE 2

Analysis of $E7_{(49-57)}$ CTL Frequencies by ELISPOT and H-2D^b-RAHYNIVTF Tetramer Staining

Vaccine group	Mouse	ELISPOT[a]	Tetramer staining (%)[b]
PBS	1	6 ± 4	0.7
	2	13 ± 7	1.3
	3	10 ± 2	0.9
L1L2E7	4	**240** ± 24	**2.5**
	5	**275** ± 8	**2.7**
	6	**431** ± 10	**4.2**
L1L2/L1L2E7	7	**55** ± 7	**2.1**
	8	18 ± 6	1.5
	9	**32** ± 17	1.6

[a] Shown are the numbers of spots per 10^6 splenocytes following stimulation with RAHYNIVTF peptide in freshly isolated splenocytes cultured in triplicate. Figures in bold represent positive responses.

[b] Percentage of H-2D^b-RAHYNIVTF tetramer-positive $CD8^+$ T cells in 5-day-stimulated splenocyte cultures. Figures in bold represent positive responses.

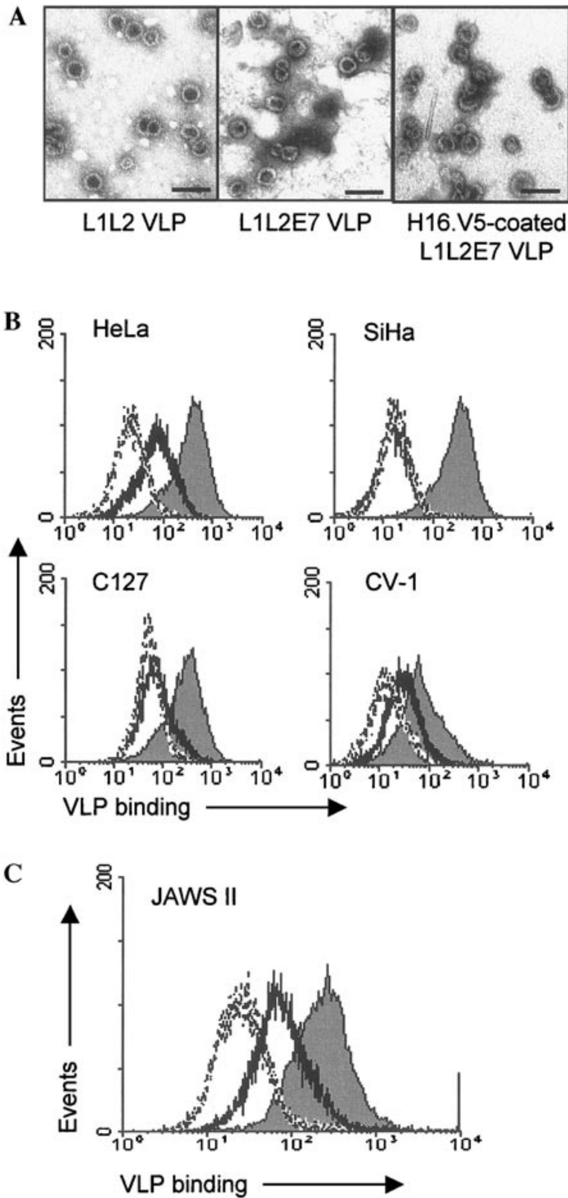

FIG. 4. Structural analysis and binding activity of antibody-coated HPV VLPs. (A) HPV16 L1/L2 VLPs, HPV16 L1/L2-E7 cVLPs, and H16.V5-coated HPV16 L1/L2-E7 cVLPs were examined by electron microscopy for retention of 55-nm diameter structure. H16.V5 antibody was incubated with VLPs for 30 min prior to coating grids and negative staining. Grids were analyzed at 40,000× magnification. Bar, 100 nm. (B) Binding of antibody-coated VLPs to receptor-positive cell lines detected by flow cytometry. HeLa, SiHa, C127, and CV-1 cell lines were incubated with biotinylated VLPs (shaded histograms), V5-coated biotinylated VLPs (solid line), or biotinylated VLPs in the presence of 50× excess of unlabeled VLPs (dashed line) to determine nonspecific background binding, followed by the addition of streptavidin–allophycocyanin. (C) Binding of antibody-coated VLPs to immature dendritic cells. JAWS II cells were incubated with Fc Block prior to the addition of antibody-coated VLPs. All other binding was performed as described in (B). For each sample, 15,000 viable cells were collected after gating on forward and side scatter and exclusion of propidium iodide. Shown are representative results of one of three independent experiments.

SiHa cervical cancer cells and C127 mouse fibroblasts (Fig. 4B). Greater than 80% blocking of VLP binding to HeLa cervical cancer cells and CV-1 monkey kidney epithelial cells was similarly observed by the addition of H16.V5 antibody (HeLa plus VLP mean fluorescence intensity (MFI), 460; HeLa plus V5-VLP MFI, 99; HeLa control MFI, 30; CV-1 plus VLP MFI, 125; CV-1 plus V5-VLP MFI, 45; CV-1 control MFI, 28) (Fig. 4B). VLP binding was also tested on the mouse immature dendritic cell (DC) line, JAWS II, to determine whether neutralizing antibodies could block VLP binding to professional APCs. Fc Block was used to prevent nonspecific VLP–immune complex binding to Fc receptors expressed on DCs. In the absence of immune complex binding, V5-VLP binding to DCs was inhibited up to 70% by the presence of neutralizing antibodies compared to normal VLP binding to DCs (JAWS plus VLP MFI, 318; JAWS control MFI, 99; JAWS plus V5-VLP MFI, 162) (Fig. 4C). These data suggest that a primary mechanism for neutralizing antibody-mediated inhibition of cVLP-induced CMI response may be inhibition of VLP binding to a cellular receptor(s), ultimately leading to an inhibition of particle uptake and processing by APCs.

DISCUSSION

In this study we have determined that preexisting systemic humoral immunity against HPV VLPs severely interferes with anti-tumor $CD8^+$ T cell induction by E7-containing HPV cVLPs in a murine tumor model system. The abrogation of protective responses seen after wt VLP preimmunization or after passive transfer of serum containing antibodies generated against wt VLPs or a defined neutralizing monoclonal antibody indicates that neutralizing antibodies are the primary mediators of the inhibition. Quantification of E7-specific T cells in mice immunized with HPV16 L1/L2-E7 VLPs in the presence or absence of preexisting antibodies revealed significantly reduced numbers of tumor-specific T cells elicited in mice that had preexisting humoral immunity compared to mice without preexisting antibodies.

There are several studies that demonstrate that one immunization with VLPs or cVLPs is sufficient to induce antigen-specific effector $CD8^+$ T cells. Nieland *et al.* demonstrated that one immunization with HPV16 L1-P1A cVLPs is sufficient to generate P1A-specific CTLs leading to rejection of $P1A^+$ P815 tumor cells after subcutaneous injection (Nieland *et al.,* 1999). One injection of HPV16 L1-$E7_{(1–60)}$ cVLPs was shown to also induce E7-specific CTLs that were able to kill E7-expressing tumor cells (Schafer *et al.,* 1999). There are also studies in which two immunizations of cVLPs containing an E7 epitope or the E7 protein induced good protection against challenge with $E7^+$ tumor cells (Peng *et al.,* 1998; Greenstone *et al.,* 1998). However, there are no studies to date that demonstrate that a second immunization is

required for protection or even increases the CMI response to VLP-based vaccines, since one immunization can induce protection similar to that of two immunizations (Greenstone *et al.,* 1998; De Bruijn *et al.,* 1998; Revaz *et al.,* 2001).

In this study, either one or two preimmunizations with wt VLPs resulted in the same inhibitory effect on cVLP immunization even though the neutralizing titer was higher in twice immunized mice. No dose-dependent inhibition was detected because all but one mouse that were preimmunized twice and all mice preimmunized once with wt VLPs prior to cVLP immunization developed progressively growing tumors. However, this study does not address the question of whether the levels of virion antibodies elicited by natural genital tract infection by HPV, which is approximately 40-fold lower than after VLP vaccination (Harro *et al.,* 2001), would also inhibit cVLP immunization.

The data on the inhibition of antibody-coated VLP binding to receptor-positive cell lines, including a dendritic cell line, suggest that a significant mechanism by which the antibodies inhibit CMI responses is by preventing binding and uptake of the VLP by APCs in a manner that occurs in the absence of antibody. Fc receptor (FcR)-mediated uptake of immune complexes (i.e., antigen–IgG complexes) by murine DCs has been reported to induce DC maturation and promote efficient MHC class I- and class II-restricted peptide presentation (Regnault *et al.,* 1999). Surprisingly, if any FcR-mediated uptake of the cVLP immune complexes did occur *in vivo,* those events did not lead to efficient protective CTL induction against the E7 antigen. In the case of HPV VLPs, antibody-coated VLPs may be targeted to less efficient APCs such as macrophages, which express higher levels of FcR than DCs, in addition to being shuttled more to the exogenous MHC class II pathway of antigen presentation.

Consistent with the idea that VLPs might normally be targeted to professional APCs directly after immunization, we have recently shown the direct *in vitro* binding of VLPs to APCs with high affinity (Da Silva *et al.,* 2001). Avid VLP binding to immature DCs induces their phenotypic maturation and secretion of T-cell-inducing cytokines (Rudolf *et al.,* 2001; Lenz *et al.,* 2001). Therefore, neutralizing antibodies could inhibit DC binding and activation by VLPs, thus inhibiting uptake and presentation of the linked antigens and priming of T cell responses in the efficient manner that seems to normally occur. That antibodies preventing virus infection can also inhibit antigen presentation supports the idea that access to the cytosol of APCs, where the contents can be processed as endogenous antigens to activate MHC class I-dependent T cells, is an important mechanism by which VLPs deliver antigens for class I presentation. In the presence of neutralizing antibodies, the cVLPs are most likely destroyed by opsonization, targeted to a different processing pathway, or targeted to a different cell type, all of which could explain the lack of an efficient anti-tumor response observed in this study.

Although a single cVLP immunization can induce good protection from outgrowth of injected tumor cells in mice, it is likely that boosting of CMI responses will be required to see therapeutic effects against existing tumors and long-term prevention of tumor recurrence in the case of human cervical neoplasias. Therefore a challenge for the future will be to develop strategies to effectively boost CMI to cVLP-based vaccines by preventing or bypassing the effects of virion-neutralizing antibodies. A similar problem is faced by developers of viral vectors for gene transfer. Inhibition of gene transfer and subsequent gene expression by virus-specific neutralizing antibodies have been described for recombinant vaccinia virus vectors, recombinant adenovirus vectors, and recombinant adeno-associated virus vectors (Rooney *et al.,* 1988; Dong *et al.,* 1996; Halbert *et al.,* 1997). Various strategies have been used to overcome previous immunological exposure to these traditional viral vectors, including the use of alternative serotypes of human adenovirus (Kass-Eisler *et al.,* 1996; Halbert *et al.,* 2000) and the use of nonhuman adenovirus vectors, such as ovine adenoviruses and bovine adenoviruses (Hofmann *et al.,* 1999; Moffatt *et al.,* 2000). Recently, it has been shown that altering the route of vector administration, from systemic immunization to mucosal immunization, can also bypass the effect of systemic neutralizing antibodies (Belyakov *et al.,* 1999). Some of these strategies may prove to be useful for overcoming natural and vaccine-induced humoral immunity against HPV in repeated vaccinations with papillomavirus cVLP-based vaccines.

Multiple studies have shown that mucosal immunization with wt VLPs or cVLPs results in the generation of L1-specific antibodies in mucosal secretions and also specific T cells with cytotoxic effector function (Liu *et al.,* 1998; Balmelli *et al.,* 1998; Dupuy *et al.,* 1999; Rose *et al.,* 1999; Revaz *et al.,* 2001). If the mucosa does not have significant amounts of antibody after systemic immunization, then the mucosal route of immunization remains naïve to the virus vector and can be used for the induction of immune responses in the presence of systemic preexisting humoral immunity. Whether mucosal immunization can be performed with cVLPs to boost the immune system in the presence of systemic neutralizing antibodies is currently under investigation.

In vivo and *in vitro* data suggest that neutralizing antibodies raised against VLPs are predominantly type specific (Christensen and Kreider, 1990; Roden *et al.,* 1995, 1996a; White *et al.,* 1999). Notable exceptions are some limited cross-neutralization seen between very closely related HPV types such as HPV16 and 33, HPV18 and 45, and HPV6 and 11 (White *et al.,* 1999; Roden *et al.,* 1996b; Christensen *et al.,* 1996b). Multiple cVLPs, containing the same tumor rejection antigen, could be constructed from

the L1 and L2 proteins of different HPV types or nonhuman papillomaviruses. After the first immunization with one type of cVLP, the host could be boosted with a different cVLP, as the immune system would still be naïve to the capsid of the second VLP (Greenstone *et al.,* 1998).

The potential effects of preexisting immunity must be considered in anticipation of clinical trials using cVLPs to treat existing tumors or residual disease. Since neutralizing antibodies prevent repeated vaccination with the same cVLP in our mouse model, and human vaccinees develop similar antibody titers after VLP vaccination (Harro *et al.,* 2001), alternative strategies to deliver tumor rejection antigens may need to be developed. These will likely be in the forms discussed above such as altering the routes of immunization and altering the capsid proteins used to deliver the encapsidated antigens. If studies in animal models show that these strategies will not work for papillomavirus VLPs as they have worked for traditional virus vectors, there is still the possibility that chimeric VLPs can be effectively used in immunization strategies in which the method to prime an immune response is different from the method used to boost the immune response. Heterologous prime/boost regimens have been shown to induce immune responses that are stronger than boosting with the same antigen since the immune system is naive to each new delivery method (Hanke *et al.,* 1998; Chen *et al.,* 2000; van der Burg *et al.,* 2001). Therefore, the use of cVLPs in heterologous prime/boost regimens would overcome the neutralizing antibody response and could result in a more potent anti-tumor immune response than cVLPs alone.

MATERIALS AND METHODS

Cell lines and antibodies

Cell lines were purchased from American Type Culture Collection (Manassas, VA) unless otherwise noted. HeLa (ATCC CCL-2), SiHa (ATCC HTB-35), and CV-1 (ATCC CCL-70) cell lines were cultured in Iscove's modified Dulbecco's medium (Biowhittaker, Walkersville, MD) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 μg/ml kanamycin, and 50 mM 2-mercaptoethanol. C127 clone C cells were obtained from W. Vass (National Institutes of Health, Bethesda, MD). TC-1 tumor cells were a gift from T. C. Wu (Johns Hopkins Medical Institutions, Baltimore, MD). TC-1 cells are mouse lung epithelial cells transformed by HPV16 E6/E7 plus activated c-Ha-*ras* (Lin *et al.,* 1996). TC-1 cells were cultured in RPMI medium (Biowhittaker) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 μg/ml kanamycin, 50 mM 2-mercaptoethanol, 1 mM sodium pyruvate, 2 mM nonessential amino acids, 0.4 mg/ml G418, and 0.2 mg/ml hygromycin. The JAWS II cell line (ATCC CRL-11904) was cultured in RPMI medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 μg/ml kanamycin, 50 mM 2-mercaptoethanol, 1 mM sodium pyruvate, 2 mM nonessential amino acids, and 5 ng/ml murine GM-CSF. All cell lines were grown at 37°C with 5% CO_2. Antibodies H16.V5 (mouse IgG2b) and H16.E70 (mouse IgG2b) were a generous gift from Dr. Neil Christensen (Penn State University, Hershey, PA).

Animals

C57Bl/6 (H-2^b) female mice 6–8 weeks of age were purchased from Taconic (Germantown, NY). All mice were approximately 12–14 weeks of age at the time of tumor challenge. Mice were housed under specific pathogen-free conditions.

Virus-like particles

HPV16 L1/L2 VLPs were produced in *Trichoplusia ni* (Hi Five) cells by infection with HPV16 L1/L2 recombinant baculovirus. HPV16 L1/L2-E7 cVLPs were produced by infection of Hi Five cells with recombinant baculovirus containing the HPV16 L1 gene and the HPV16 L2–E7 chimeric fusion gene (Greenstone *et al.,* 1998). VLPs were purified from cells by sucrose and CsCl gradient centrifugation, quantitated, and examined by electron microscopy, Western blot, and ELISA as previously described (Müller *et al.,* 1997; Greenstone *et al.,* 1998).

Electron microscopy

Transmission electron microscopy was performed by adsorbing 20 μl VLP samples to carbon-coated copper grids and staining with 2% uranyl acetate. Specimens were examined with a Zeiss EM 900 electron microscope at 75 kV. For antibody-coated VLPs, 1 μg of H16.V5 antibody was incubated with 1 μg HPV16 L1/L2-E7 cVLPs (10^{10} particles) for 30 min at room temperature prior to coating grid.

Production of hyperimmune mouse anti-VLP antiserum

Twenty mice were immunized subcutaneously (sc) with 30 μg HPV16 L1/L2 VLPs. Three weeks later, mice were boosted with 15 μg HPV16 L1/L2 VLPs and boosted again 3 weeks later. Two weeks after the last immunization, mice were anesthetized, total blood was collected by heart puncture, and mice were sacrificed by cervical dislocation. Blood was transferred into Microtainer serum separator tubes (Becton–Dickinson, Franklin Lakes, NJ) and centrifuged for 2 min at 15,000 *g*. Serum was collected, pooled, and stored at −20°C until further use.

VLP ELISA

Purified HPV16 L1/L2 VLPs (500 ng/well) were used to coat 96-well Maxisorp ELISA plates (Nunc, Naperville, IL). Mice sera were added to wells at dilutions from 1:100 to 1:100,000. After washing, bound antibody was detected by the addition of peroxidase-labeled goat anti-

mouse IgG antibody (Biosource, Camarillo, CA), followed by addition of *o*-phenylenediamine substrate in 0.05 M phosphate citrate buffer (Sigma, St. Louis, MO). Concentration of anti-VLP antibody in the sera was determined by extrapolation of OD_{490nm} values to standard curves made by using known concentrations of H16.V5 antibody or H16.E70 antibody in the same ELISA.

Generation of HPV16 pseudovirions

HPV16 pseudovirions were generated as previously described (Roden *et al.,* 1996a). Briefly, infectious virions composed of the HPV16 L1 and L2 capsid proteins and the BPV1 genome were generated by infection of BPHE-1 hamster cells, which contain autonomously replicating BPV genomes, with replication-defective Semliki Forest virus (SFV) vectors expressing HPV16 L1 and L2 genes. BPHE-1 cells were grown in complete Dulbecco's minimal essential medium (DMEM) containing 10% FBS and penicillin/streptomycin. L1 and L2 SFV stocks were mixed together, activated with chymotrypsin (10 mg/ml; Boehringer Mannheim, Indianapolis, IN), and used to co-infect 10 plates (150 mm in diameter) each with 14 million BPHE-1 cells for 2 h at 37°C. Complete DMEM replaced the infectious material, and cells were incubated for 24 h, scraped, pelleted by centrifugation, and kept at −80°C until used.

Neutralization assay

Individual infections by the HPV16 pseudovirions were detected as transformed foci in a monolayer of mouse C127 cells (Roden *et al.,* 1996a). The stored pellets from BPHE-1 cells that had been co-infected with recombinant SFV that express HPV16 variant 114K L1 and L2 were sonicated in 11 ml PBS just prior to addition of sera and infection of C127 cells. Aliquots of the sonicated pseudovirus solution were mixed for 1 h at 4°C with PBS or with fourfold serial dilutions of serum starting with a 1:40 dilution to assay for inhibition of focal transformation. The neutralizing monoclonal antibody H16.V5 was used as a positive control for the assay. The pseudovirus/PBS or pseudovirus/serum combination was added to the C127 cell monolayer for 1 h at 37°C and then replaced with DMEM and 10% fetal bovine serum. The cells were fed twice weekly for 14–18 days, then stained with 2.5% methylene blue, 0.5% carbol fuchsin (w/v) in methanol for visualization of foci. The neutralization titer was defined as the reciprocal of the highest serum dilution that was able to reduce the number of foci induced by the pseudovirions in the positive control plate by at least 50%.

Biotinylation of VLPs

VLPs were biotinylated with Sulfo-NHS-biotin (Pierce, Rockford, IL). One milligram of HPV16 L1/L2 VLPs was incubated with biotin at a final concentration of 0.5 μg/ml at 4°C for 30 min. Biotinylation was quenched by the addition of serum-containing medium. Excess biotin was removed by dialysis against two changes of 0.5 M NaCl/PBS overnight, particles were aliquoted and stored at −80°C for future use. An ELISA and electron microscopy analysis were performed to ensure that biotinylation did not interfere with the display of antibody-neutralizing epitopes and there was no disruption of capsid morphology.

VLP binding assay and FACS analysis

VLP binding as detected by flow cytometry was performed as previously described (Da Silva *et al.,* 2001). Briefly, HeLa, SiHa, CV-1, C127, and JAWS II cells were detached from cell culture flasks with 200 mM EDTA for 15 min at 37°C, washed once with cell culture medium, and then washed twice with FACS buffer (PBS/0.5% BSA/0.01% NaN_3). Cells (10^6) were incubated with 1 μg of biotinylated HPV16 L1/L2 VLPs for 1 h on ice. After extensive washing, streptavidin–allophycocyanin (Pharmingen, San Diego, CA) was added. Fluorescence was analyzed by flow cytometry on a Becton–Dickinson FacsCalibur using CellQuest software (Becton–Dickinson, San Jose, CA). For antibody blocking experiments, biotinylated VLPs were preincubated with H16.V5 antibody for 1 h at 4°C at a concentration of 1 μg/10^{10} VLPs. For detection of background VLP binding, cells were preincubated with a 50× excess of unlabeled VLPs prior to the addition of biotinylated VLPs. For detection of antibody-coated VLP binding to JAWS II cells, cells were first incubated with anti-FcγRII/III antibody (clone 2.4G2, Fc Block; Pharmingen) to block binding of immune complexes to Fc receptors. Percentage of blocking was calculated using the following equation: [(MFI of VLP binding) − (MFI of V5-coated VLP binding)]/[(MFI of VLP binding) − (MFI of VLP background binding)] × 100.

ELISPOT assay

The number of peptide-specific IFNγ-producing cells was measured as follows. Multiscreen HA plates (Millipore, Bedford, MA) were coated with 5 μg/ml anti-mouse IFNγ antibody (Pharmingen) in PBS at 4°C overnight. Plates were washed with PBS/0.5% Tween 20 and blocked with culture medium. Freshly isolated splenocytes were added at 1×10^6 cells per well in triplicate followed by three twofold dilutions in medium containing 25 IU IL-2 and 10 μg/ml HPV16 $E7_{(49-57)}$ peptide. After 24 h incubation at 37°C and 5% CO_2, plates were washed with PBS–Tween and incubated for 2 h at room temperature with 2.5 μg/ml biotinylated anti-mouse IFNγ antibody (Pharmingen). After extensive washing, 1.25 μg/ml avidin–alkaline–phosphatase (Sigma, St. Louis, MO) was added to the wells for 2 h. Plates were developed by the addition of BCIP/NBT substrate (Promega, Madison, WI) for 15 min. The reaction was stopped by the addition of

tap water and plates were dried overnight. Individual spots were counted with a dissecting microscope. A positive response was defined as a greater than the average of PBS-immunized mice ± 3 (SD).

Tetramer analysis

H2-D^b tetramers labeled with phycoerythrin and containing the HPV16 $E7_{(49-57)}$ peptide RAHYNIVTF were obtained from the NIAID Tetramer Facility (Atlanta, GA). Splenocytes from immunized and control mice were cultured for 5 days in 24-well plates with 5 μg/ml RAHYNIVTF peptide. $CD8^+$ T cells were enriched using MACS cell sorting (Miltenyi Biotec, Auburn, CA) prior to tetramer staining. One million $CD8^+$-enriched splenocytes were incubated for 1 h with 20 μl 1:100 diluted tetramer and 2 μg FITC-labeled anti-mouse CD8 antibody (Pharmingen) in PBS/0.5% BSA/2 mM EDTA. Cells were washed four times and analyzed by flow cytometry for the percentage of cells that stained with both CD8 and the tetramer. A positive response was defined as a greater than the average of PBS-immunized mice ± 3 (SD).

Chimeric VLP immunization, antibody transfer, and tumor protection

Mice preimmunized with HPV16 L1/L2 VLPs were injected sc with 30 μg VLP 3 weeks (for 1× VLP immunized) or 3 and 6 weeks (for 2× VLP immunized) prior to sc immunization with 50 μg HPV16 L1/L2-E7 cVLPs. Mice receiving passive transfer of immune sera or monoclonal antibody H16.V5 received 0.5 ml hyperimmune input sera or 0.2 mg H16.V6 antibody intraperitoneally 24 h prior to cVLP immunization. HPV16 L1/L2-E7 cVLPs used to immunize mice given H16.V5 antibody ip were additionally coated with H16.V5 antibody directly (1 μg/10^{10} VLPs) 30 min prior to sc injection. One hundred microliters of blood was collected from the tail vein of mice prior to chimeric VLP immunization to determine ELISA and neutralization titers. Ten days after immunization on the day of tumor challenge, TC-1 cells were harvested and washed twice with Hanks' balanced salt solution (HBSS) (Sigma). Tumor challenge was performed by injecting 6×10^4 TC-1 cells sc suspended in 100 μl HBSS. Tumor diameter was measured two times a week along three axes, and tumor volume was calculated as (diameter 1 × diameter 2 × diameter 3). Mice were sacrificed when tumor burden reached >1500 mm^3 or when tumors broke through the skin surface.

Statistical analysis

Survival data were subjected to Kaplan–Meier survival analysis comparing curves with the log-rank test. Tumor incidence data were analyzed in 2 × 2 contingency tables by a two-tailed Fisher's exact test. In both cases, a P value < 0.05 was considered statistically significant.

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