Vaccination of Healthy Volunteers with Human Papillomavirus Type 16 L2E7E6 Fusion Protein Induces Serum Antibody that Neutralizes across Papillomavirus Species

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Abstract

Oncogenic human papillomavirus (HPV) infection is a necessary cause of cervical cancer. Therefore, vaccination to prevent or eliminate HPV infection could reduce the incidence of cervical cancer. A fusion protein comprising HPV16 L2, E6, and E7 is a candidate combination preventive and therapeutic HPV vaccine. The L1- and L2-specific and neutralizing serum antibody titers and peripheral blood mononucleocyte antigen–specific proliferative responses generated by vaccination thrice at monthly intervals with HPV16 L2E7E6 were compared in two studies: a phase I randomized double-blind placebo controlled dose escalation trial in 40 healthy volunteers and a phase II trial of HPV16 L2E7E6 at the maximum dose in 29 women with high-grade anogenital intraepithelial neoplasia (AGIN). Vaccination of healthy volunteers induced L2-specific serum antibodies that were detected 1 month after the final vaccination (P < 0.001). There was a significant trend to seroconversion for HPV16 and HPV18 neutralizing antibodies with increasing vaccine dose (P = 0.006 and P = 0.03, respectively). Seroconversion for HPV18 neutralizing antibodies showed a significant positive trend with increasing dose (P = 0.03) and was associated with seroconversion for HPV16 neutralizing antibodies (P = 0.04). The antigen-specific proliferative response of vaccinated healthy volunteers also showed a significant trend with increasing vaccine dose (P = 0.04). However, AGIN patients responded less effectively to vaccination than healthy patients for induction of HPV16 L2–specific antibody (P < 0.001) and proliferative responses (P < 0.001). Vaccination of healthy volunteers thrice with 533-μg HPV16 L2E7E6 at monthly intervals induced L2-specific serum antibodies that neutralized across papillomavirus species. Responses in AGIN patients were infrequent.

Introduction

Oncogenic human papillomavirus (HPV) types are etiologic agents of cervical cancer and 5.2% of all cancers worldwide in 2002 (1). HPV16 and HPV18 occur in 20% of cervical cancer cases, respectively, although there are at least 15 oncogenic genotypes. Vaccination of uninfected women with virus-like particles (VLP) comprising the L1 major capsid protein of HPV16 is highly effective in preventing acquisition of persistent HPV16 infection and HPV16-related cervical intraepithelial neoplasia (CIN; the precursor lesion of cervical cancer; ref. 2, 3). However, an equivalent number of CIN related to other HPV types occurred in the placebo and vaccine groups, suggesting that protection is type restricted (3, 4). Thus, broad protection against the oncogenic HPVs may require a highly multivalent L1 VLP vaccine formulation or the identification of a single broadly protective antigen.

Because ~80% of cervical cancer cases occur in low resource countries, development of a simple, inexpensive but broad-spectrum HPV vaccine is critical. Like L1 VLP produced in yeast or insect cells, vaccination with the minor papillomavirus capsid protein, L2, produced in E. coli is also protective in animal models (5–7). Protection via L2 peptide vaccination is mediated by neutralizing antibody and even low titers are protective (8). Importantly, antibody responses to L2 are broadly cross-neutralizing (9, 10). Therefore, L2 could potentially provide protection against a broad spectrum of oncogenic HPVs.

Unfortunately, evidence suggests limited therapeutic benefit of L1 VLP vaccines (2), which probably reflects the absence of capsid gene expression in infected basal epithelia and HPV-related cancers. Thus, the effect of such preventive vaccination on the incidence of cervical cancer will likely be delayed by two decades. By contrast, E6 and E7 are expressed in all HPV-infected cells and are required for maintenance of a transformed phenotype. Vaccination of mice with HPV16 L2E7E6 fusion protein in adjuvant induces the rejection of a murine model of cervical cancer (11). Vaccination of healthy volunteers and patients with anogenital intraepithelial neoplasia (AGIN) with HPV16 L2E7E6 in the absence of adjuvant was well tolerated and induced E6- and E7-specific cellular immune responses (12–15). However, the potential of the HPV16 L2E7E6 as a preventive vaccine has not been examined. Herein we examine the ability of HPV16 L2E7E6 vaccination of patients to induce L2-specific antibodies that neutralize both homologous and heterologous type HPV infections.

Materials and Methods

Human sera. Studies were done with the permission of the Johns Hopkins University Internal Research Board. The study sera were obtained in two HPV16 L2E7E6 vaccination trials from 40 healthy volunteers (30 men, ages 19-55 years, and 10 postmenopausal women, ages 43-55 years, with a negative Pap smear at entry) at weeks 0 and 12 in a phase I trial (12) and from 27 women, ages 22 to 61 years, all with noncervical high-grade AGIN (2 of 27 with VIN3 and 25 of 27 with VIN3, all but two being HPV16+) in a phase II trial (14, 15). Sufficient sera from 23 of 27 AGIN patients were available for evaluation.

Proliferation assays. The proliferation assays are described in refs. 12, 14.
ELISA. Microwell plates coated with Nickel-NTA (Sigma, St. Louis, MO) were reacted with 50 ng of purified 6His-HPV16 L2 protein (10) in PBS overnight at 4°C. Wells were then blocked with 1% bovine serum albumin (BSA)-PBS for 1 hour at room temperature and incubated with 2-fold dilutions of patient sera for 1 hour at room temperature. Following washing with PBS-T, peroxidase-labeled goat anti-human immunoglobulin G (KPL, Inc., Gaithersburg, MD) diluted 1:5,000 in 1% BSA-PBS was added for 1 hour. The plates were then washed and developed with TMB turbo reagent (Pierce, Rockford, IL) for 10 minutes. The HPV16 and HPV18 L1 VLP ELISAs were done as described in ref. 16 with a cutoff of $A_{405} < 0.05$ at 1:100.

Neutralization assays. The HPV16 and HPV18 pseudoviron in vitro neutralization assays were done as described earlier (17) but the secreted alkaline phosphatase content in the clarified supernatant was determined using the p-nitrophenyl phosphate tablets (Sigma) dissolved in diethanolamine and absorbance was measured at 405 nm. Titers were defined as the reciprocal of the highest dilution that caused a 50% reduction in $A_{405}$, and a titer <50 was not considered significant.

Statistical analysis. We tested the significance of the proportion of seroconverters in specified groups by testing the null hypothesis of a 5% seroconversion rate (i.e., vaccine failure/background seroconversion) using exact binomial probability tests. Dose response to vaccine was tested for trend using a nonparametric extension of the Wilcoxon rank sum test. Differences in seroconversion between groups were tested using Fisher's exact tests. Results were considered statistically significant at $P < 0.05$. All analyses were done using STATTA 9.0 (College Station, TX).

Results

Healthy volunteers were vaccinated thrice at monthly intervals with either HPV16 L2E7E6 fusion protein or placebo (12). We tested sera from this study for in vitro neutralization of HPV16 and ELISA reactivity to either HPV16 L1 VLPs or HPV16 L2 (Fig. 1). Before vaccination, 25 of 40 healthy volunteers tested exhibited no detectable HPV16 neutralization titer. Furthermore, they also lacked reactivity to HPV16 L1 VLP or HPV16 L2. Of the 15 neutralizing baseline sera (median titer, 100; range, 50-200), 10 of 11 evaluated (91%) reacted with HPV16 L1 VLP or HPV16 L2 protein by ELISA (median L2 titer, 50; range, 50-400); 8 patients were baseline seropositive for HPV16 L2 alone, 1 was seropositive for HPV16 L1 VLP alone ($A_{405}$ 0.09), and 1 was seropositive for both antigens ($A_{405}$ 0.42; L2 titer, 400). A prior study suggested that the HPV16 neutralization assay is more sensitive but possibly less specific than the HPV16 L1 VLP ELISA (17).

One month after the third vaccination, 65% of the baseline HPV16 L2 seronegative patients (13 of 20; three patients were not evaluated) seroconverted to being HPV16 L2 ELISA positive ($P_{binomial} < 0.001$) including all doses of vaccine. All women with baseline HPV16 L2 ELISA seropositivity remained seropositive after vaccination. No placebo recipients seroconverted to HPV16 L2 after vaccination (0 of 7; one patient was not evaluated).

When we examined whether the patients exhibited a dose response to vaccination (Fig. 1A), the trend for increased probability of seroconversion with increasing vaccine dose was statistically significant ($P = 0.008$). At the lowest vaccine dose (26 µg), 1 of 3 (33.3%) baseline HPV16 L2 ELISA seronegative patients seroconverted following vaccination (titer, 800). At the intermediate dose, 5 of 8 (62.5%) baseline HPV16 L2 ELISA seronegative patients seroconverted following vaccination (titer, 100 for all). At the highest vaccine dose (533 µg), 7 of 9 (77.8%) baseline HPV16 L2 ELISA seronegative patients seroconverted following vaccination (median, 400; range, 100-1,600).

We examined whether the nine patients with evidence of prior exposure to HPV (median, 50; range, 50-400) also responded to vaccination. Among the baseline HPV16 L2 ELISA seropositive patients, 7 of 9 (78%; median, 100; range, 50-1,600) exhibited a higher titer after vaccination ($P_{binomial} < 0.001$), 3 of 5 (60%) at the lowest dose and 4 of 4 (100%) at the highest dose ($P = 0.3$).

To examine whether HPV16 L2–specific antibodies are neutralizing, the prevaccination (week 0) and postvaccination (week 12) sera were also tested for in vitro neutralization activity against HPV16 pseudovirions (Fig. 1B). When including all vaccine doses, 68.4% (13 of 19) baseline HPV16 seronegative volunteers seroconverted for neutralization following vaccination (median, 400; range, 100-1,600). All 13 also seroconverted for HPV16 L2 ELISA (median, 400; range, 100-1,600). No placebo recipients (0 of 6) seroconverted for HPV16 neutralization after vaccination.

When stratifying by vaccine dose, 1 of 3 (33.3%) of the baseline seronegative volunteers receiving the lowest vaccine dose seroconverted (titer, 400). At the intermediate dose, 5 of 8 (62.5%) baseline HPV16 seronegative volunteers seroconverted following vaccination (median, 200; range, 100-400). At the highest dose, 7 of 8 (87.5%) baseline HPV16 seronegative volunteers seroconverted following vaccination (median, 400; range, 200-1,600). There was a significant trend for HPV16 neutralization seroconversion with increasing vaccine dose ($P = 0.006$). All 13 baseline HPV16 neutralization seropositive patients (median, 50; range, 50-800) remained so after vaccination; 80% (4 of 5) receiving the lowest dose of vaccine and 87.5% (7 of 8) receiving the highest dose of vaccine exhibited a boost in neutralization titer.

We examined the relationship between antigen-specific T-cell proliferation and the induction of HPV16 neutralizing antibodies after vaccination (Fig. 1B and C). Antigen-specific T-cell proliferation was considered as >3-fold increase in stimulation index, as determined using $[^{3}H]$thymidine incorporation by peripheral blood mononucleocytes after in vitro stimulation with vaccine antigen (HPV16 L2E7E6) versus an irrelevant antigen (HPV16 L2E7) purified under analogous conditions. All volunteers were baseline stimulation index negative by this assay and 51% converted to being stimulation index positive after vaccination (including all doses). The proportion of patients converting to a positive stimulation index after vaccination showed a significant positive trend with increasing dose ($P = 0.04$). Similarly, absolute stimulation index levels increased with increasing dose ($P = 0.07$).

Vaccination of animals with full-length L2 induces serum antibodies that are broadly cross-neutralizing (9, 10). HPV18 is a member of a different papillomavirus species than HPV16 (18). Given this divergence from HPV16, we examined the ability of sera of patients vaccinated with HPV16 L2E7E6 to neutralize HPV18 pseudovirions (Fig. 1D). No (0 of 4) baseline HPV16 neutralization seropositive patients seroconverted after vaccination with placebo. However, 25% (1 of 4; titer, 200) seroconverted after vaccination with the lowest dose of HPV16 L2E7E6, 0% (0 of 7) at the intermediate dose, and 66% (4 of 6) at the highest vaccine dose (range, 100-800). Seroconversion for HPV18 neutralizing antibodies showed a significant positive trend with increasing dose ($P = 0.03$) and was significantly associated with seroconversion for HPV16 neutralizing antibodies ($P_{exact} = 0.04$).

To examine the response to HPV16 L2E7E6 vaccination in patients with preexisting disease, we tested the sera of women with HPV16-positive AGIN before and after vaccination at the highest dose for HPV16 L2–specific antibody (Fig. 2A) and HPV16 neutralization titers (Fig. 2B). Of these patients with active HPV16-related disease, 23 of 24 had detectable levels of HPV16 neutralizing antibodies before vaccination (Fig. 2B; median, 400; range, 50-6,400). However, the AGIN patients lacked antibodies to HPV16 L2 (Fig. 2A), suggesting that all of the
responses were directed to neutralizing epitopes on L1. After vaccination, the AGIN patients exhibited a modestly, but not significantly, higher HPV16 neutralization titer ($P = 0.1$).

The levels of HPV16 L1-specific antibodies did not change significantly after vaccination ($P = 1.0$), and only 5 of 22 (22.7%) seroconverted for HPV16 L2 (median, 400; range, 100-1,600). The frequency of seroconversion for HPV16 L2 antibody among AGIN patients versus healthy volunteers receiving the highest vaccine dose was significantly lower ($P_{\text{exact}} < 0.001$).

Furthermore, when examining the stimulation index for proliferation induced by TA-CIN protein, only 37% (10 of 27) of AGIN patients converted from negative to positive after vaccination at the highest dose, as opposed to 100% (11 of 11) of healthy patients. The frequency of stimulation index conversion after vaccination was significantly lower among the AGIN patients versus the healthy volunteers ($P_{\text{exact}} < 0.001$).

Perhaps healthy subjects with a prior exposure to HPV also respond poorly to vaccination with HPV16 L2E7E6. To examine this possibility, the response of AGIN patients to vaccination with HPV16 L2E7E6 was compared in healthy and AGIN patients with serologic evidence of a prior exposure to HPV16 by the neutralization assay. Of healthy patients who were baseline seropositive for HPV16 neutralization, 7 of 8 showed an increased neutralization titer after vaccination at the highest dose. By contrast, only 9 of 21 AGIN patients who were seropositive for HPV16 neutralization at baseline showed an increased titer after vaccination with the same dose. Similarly, 7 of 10 healthy patients who were baseline seropositive for HPV18 neutralization exhibited an increased neutralization titer after high-dose vaccination. In contrast, only 1 of 10 AGIN patients who were seropositive for HPV18 neutralization at baseline showed an increased titer after high-dose vaccination. Thus, baseline seropositive AGIN patients exhibited a boost in titer after high-dose HPV16 L2E7E6 vaccination less frequently than baseline seropositive healthy patients with respect to both HPV16 neutralization ($P_{\text{exact}} = 0.04$) and HPV18 neutralization ($P_{\text{exact}} = 0.02$).

We examined the possibility of differences in L2-specific or neutralizing antibody (HPV16 or HPV18 specific) or proliferation responses to vaccination observed when AGIN patients were stratified by disease severity. All AGIN trial patients had high-grade...
disease. We found no association or trends when assessing immunologic responses by tertile categorization of symptom severity score, time since diagnosis, and lesion size at study entry.

Discussion

In addition to the previously described induction of E6- and E7-specific cellular immunity (12), vaccination of patients with HPV16 L2E7E6 induces neutralizing antibodies. These antibodies neutralize both homologous type virus HPV16 and a heterologous type HPV8 that is a member of a different phylogenetic species with distinct biological properties (18). However, the induction of neutralizing antibodies to the homologous virus type, HPV16, was slightly more effective than to the heterologous virus HPV18. This finding is consistent with studies in animals vaccinated with full-length L2 proteins (9, 10). It is important to note that vaccination with HPV16 L1 VLPs does not induce antibodies that neutralize HPV18 (9, 17) and likely does not protect against HPV18 infection (2–4). However, these neutralizing antibody responses to HPV16 L2E7E6 were weak as compared with those generated by HPV16 L1 VLPs (17). Nevertheless, similarly low neutralizing antibody titers generated by L2 vaccination were protective in animals against experimental animal papillomavirus challenge (5, 7, 8). Furthermore, the previously described E6- and E7-specific cellular immunity induced by vaccination of patients with HPV16 L2E7E6 could also potentially clear infection and/or prevent the clinical manifestations of infection.

The relatively weak immune responses and failure to seroconvert all vaccinees suggests that the HPV16 L2E7E6 vaccine might be improved by the inclusion of an appropriate adjuvant. Likewise, the HPV16 L2E7E6 was only effective at the highest dose (533 μg), but the use of an adjuvant may permit the use of lower doses. It is noteworthy that all of the protection studies done in animal models used L2 with an adjuvant, such as alum, Bibi, or incomplete Freund’s adjuvant (5–8). Ultimately, HPV16 L2E7E6 vaccination might be effective in treating early HPV16 infection and providing prophylaxis versus multiple HPV genotypes if combined with an adjuvant to boost the induction of both HPV neutralizing antibodies and E6/E7–specific cellular immunity.

Vaccination studies with a similar antigen, HPV6 L2E7, have been done using either alum (19) or AS02A adjuvant (20) in genital wart patients. Neither study showed a benefit for conventional therapy plus HPV6 L2E7 vaccination over conventional therapy alone (19, 20). The reasons for this are not currently clear, but herein we find that women with current HPV16-related genital disease (AGIN) responded significantly less frequently than healthy volunteers to vaccination with the same dose of HPV16 L2E7E6. This finding held for L2-specific antibody responses, the induction of HPV16 neutralizing antibody and vaccine-specific T-cell proliferative responses. Furthermore, when analyzing increases in either HPV16 or HPV18 neutralizing antibody titers, healthy volunteers with serologic evidence of prior exposure responded more effectively to vaccination than women with AGIN. Some caution must be taken in interpreting this finding because the trials were not run concurrently and there was no association between disease severity and immune response among AGIN patients. However, should this phenomenon be reproducible, it may reflect systemic tolerance to HPV antigens in patients with recalcitrant HPV-related premalignant disease. Because E6 and E7 expression, but presumably not L2 expression, is retained by these high-grade lesions, it is possible that much of the tolerance is directed against E6/E7 and antibody responses to L2 might have been greater were it not linked to these early proteins. Alternatively, patients with recalcitrant AGIN selected may have some underlying immune deficit, rendering them less able to clear their infection or respond to HPV16 L2E7E6 vaccination as compared with random healthy volunteers.

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