

Cellular Immune Responses to Human Papillomavirus (HPV)-16 L1 in Healthy Volunteers Immunized with Recombinant HPV-16 L1 Virus-Like Particles

Ligia A. Pinto,¹ Jessica Edwards,² Philip E. Castle,² Clayton D. Harro,⁴ Douglas R. Lowy,³ John T. Schiller,³ Dora Wallace,¹ William Kopp,¹ Joseph W. Adelsberger,¹ Michael W. Baseler,¹ Jay A. Berzofsky,³ and Allan Hildesheim²

¹SAIC-Frederick, Inc./NCI-Frederick, Frederick, ²Division of Cancer Epidemiology and Genetics and ³Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, and ⁴The Johns Hopkins University, Baltimore, Maryland

The causal association between papillomavirus (HPV) infection and cervical cancer has been demonstrated; the development of a prophylactic vaccine to protect against HPV infection may therefore reduce the incidence of this cancer worldwide. Noninfectious HPV-like particles (VLPs), composed of the L1 major capsid protein, are current candidate vaccines for prevention of HPV infection and cervical neoplasia. Although neutralizing antibodies have a pivotal role in the prevention of initial infection, cellular immune responses to HPV antigens may have an important role in viral clearance. A phase II trial was conducted to further evaluate the immunogenicity of a recombinant HPV-16 L1 VLP vaccine administered intramuscularly, without adjuvant, at 0, 1, and 6 months. Cell-mediated immune responses (lymphoproliferation and cytokine production) to HPV-16 L1 VLPs were evaluated in peripheral blood mononuclear cells (PBMCs) from 43 individuals receiving the L1 VLP vaccine and from 10 individuals receiving placebo. Vaccination resulted, at months 2 and 7 (i.e., 1 month after the second immunization and 1 month after third immunization, respectively), in increases in T cell–proliferative response to HPV-16 L1 VLPs ($P < .001$). In addition, significant increases in cytokine (interferon- γ , interleukin [IL]-5 and IL-10) responses to L1 VLPs were observed after vaccination ($P < .001$). The strongest cytokine responses at month 7 were observed in individuals with high antibody titers at month 2, suggesting that neutralizing antibodies generated by initial vaccination may augment T cell responses to subsequent booster vaccinations. No significant increases in lymphoproliferative or cytokine responses to L1 VLPs were observed in individuals receiving placebo. In summary, the HPV-16 L1 vaccine induces not only robust B cell responses but also L1-specific T cell responses detectable by proliferation of both CD4⁺ and CD8⁺ T cells and in vitro production of both Th1- and Th2-type cytokines. Future efficacy studies are needed to evaluate whether and/or how VLP vaccines confer protection against genital HPV infection and associated disease.

Cervical cancer is the second leading cause of cancer-related death in women and is the most common cancer among women in developing countries, with an estimated 500,000 cases diagnosed each year and >200,000

cervical cancer–related deaths occurring annually [1]. Genital human papillomavirus (HPV) infections are the most common viral sexually transmitted diseases worldwide and, in epidemiological and experimental studies, have been found to be the central etiological risk factor for cervical cancer [2–5]. HPV-16 is the most frequently occurring type, present in 50% of cases, and together with types 18, 45, and 31, constitutes 80% of cervical cancers [3]. Papanicolaou screening programs have been effective at reducing the incidence of and the mortality from cervical cancer in countries where organized screening programs have been implemented, but they have been so at very high cost [6]. An effec-

Received 26 September 2002; accepted 11 February 2003; electronically published 9 July 2003.

Financial support: National Cancer Institute, National Institutes of Health (grant N01-CO-12400).

Reprints or correspondence: Dr. Ligia A. Pinto, NCI-Frederick, Bldg. 469, Rm. 120, Frederick, MD 21702 (lpinto@ncifcrf.gov).

The Journal of Infectious Diseases 2003;188:327–38

© 2003 by the Infectious Diseases Society of America. All rights reserved.
0022-1899/2003/18802-0019\$15.00

tive HPV vaccine that targets HPV-16 and possibly other known oncogenic HPV types is therefore needed to decrease the incidence of cervical cancer and its associated screening and treatment costs. Two main types of vaccine are currently being developed: (1) prophylactic vaccines to prevent HPV infection and the concomitant cervical neoplasia and (2) therapeutic vaccines to induce viral clearance and regression of precancerous lesions.

A promising prophylactic papillomavirus vaccine candidate currently in clinical trials is composed of the viral L1 capsid protein that is synthesized in eukaryotic or prokaryotic systems and purified in the form of virus-like particles (VLPs) [7]. VLPs are morphologically and immunologically similar to authentic virions, except that they do not contain the papillomavirus genome and are therefore noninfectious. VLP vaccines have been highly successful in the prevention and treatment of papillomavirus infection in animal models of cottontail rabbit virus (CRPV), bovine virus, and canine oral papilloma virus (COPV) [4]. In each model, the animals were protected from experimental challenge with high doses of homologous virus [8–11]. Passive transfer of serum or IgG from vaccinated to naïve animals in the CRPV and COPV models indicated that the protection afforded by the VLPs was mediated by neutralizing antibodies [8, 11].

In phase I and phase II clinical trials, L1 VLPs have been found to safely induce a strong humoral response even in the absence of adjuvant, with type-specific, high-titer neutralizing antibodies against conformationally dependent L1 epitopes [12]. Thus, VLPs represent attractive candidates for HPV prophylactic subunit vaccines with the long-term goal of eliciting protection against HPV-induced diseases, including cervical cancer, by prevention of virus infection. Our previous phase I, double-blind, randomized, placebo-controlled dose-escalation study of 72 healthy young women and men receiving either 10- or 50- μ g doses of VLPs intramuscularly (im), with or without adjuvant, demonstrated that the group vaccinated with 50 μ g without adjuvant yielded the highest geometric-mean neutralizing-antibody titers [12]. On the basis of these observations, the 50- μ g dose without adjuvant was selected for subsequent testing in the phase II clinical trials of this vaccine.

Immune correlates of protection against HPV infection and HPV-associated disease progression have not been established. Although the focus of early prophylactic trials has naturally been on the generation of neutralizing antibodies, cell-mediated immunity (CMI) responses to HPV proteins are also important for an HPV prophylactic vaccine and may participate in early defense against HPV. CMI responses, in particular T helper responses, participate in the generation and maintenance of protective B cell responses and therefore may have an important role in the achievement of high neutralizing-antibody titers [13]. Cytotoxic T lymphocyte (CTL) responses to L1 may also be important for maximization of prophylactic efficacy, by eliminating or limiting the number of HPV-infected cells that

have escaped antibody neutralization. Although HPV L1 proteins are detectably expressed only in the more superficial layers of the epithelium, evidence suggests that cells expressing levels of L1 protein that are below detectability by present-day assays can still be targeted by CTLs induced by vaccination with VLPs [14]. Furthermore, in at least 1 human trial, there is preliminary evidence to suggest that CMI induced by vaccination with VLPs might have a therapeutic effect against genital warts [15]. Finally, antiviral cytokines released by antigen-specific T cells, such as interferon (IFN)- γ and tumor necrosis factor (TNF)- α , also may contribute to inhibition and control of infection [16].

This study analyzes CMI responses induced by a recombinant HPV-16 L1 vaccine in healthy adult women enrolled in a double-blind, randomized, placebo-controlled phase II trial. Lymphoproliferative and cytokine responses (IFN- γ , IL-10, and IL-5) to L1 VLPs from peripheral blood leukocytes were examined *in vitro* before and after im vaccination.

SUBJECTS, MATERIALS, AND METHODS

Study design. A double-blind, randomized, placebo-controlled phase II trial was initiated to further examine the safety and immunogenicity of 3 injections of 50 μ g of the HPV-16 L1 VLP vaccine, without adjuvant, in a sample of 220 healthy, HIV-seronegative adult female volunteers 18–25 years of age. Subjects were enrolled at The John Hopkins University Center for Immunization Research (Baltimore). Subjects were determined, on the basis of sexual history, to be at low risk for HPV-16 exposure. Individuals were not eligible if they had a history of >4 sexual partners. Women were randomly assigned to 1 of 2 groups to receive either 50 μ g of HPV-16 L1 vaccine ($n = 180$) or placebo ($n = 40$), 0.5 mL of sterile saline solution. Subjects received vaccine or placebo, injected im into the deltoid, at 0, 1, and 6 months. Subjects were evaluated clinically, and blood specimens were collected for immunologic assays before the initial vaccination (i.e., at month 0) and 1 month after each of the 2 subsequent vaccinations (i.e., at months 2 and 7). All vaccine recipients were monitored for clinical signs and symptoms for 7 days after each vaccination. The vaccine was well tolerated and induced high levels of antibodies, as reported elsewhere [12]. The protocol for this study was approved by The John Hopkins University Institutional Review Board. The blood specimens earmarked for CMI assays were shipped fresh to the monitoring laboratory, where peripheral blood mononuclear cells (PBMCs) were separated by density centrifugation over a ficoll-hypaque gradient [17]. For the present study, 53 subjects were selected and were tested immediately. PBMCs from the remaining trial participants were cryopreserved for future testing. In addition to the 53 subjects (43 vaccine recipients and 10 placebo recipients) selected for fresh testing, cryopreserved PBMCs from 36 participants (29 vaccine

recipients and 7 placebo recipients) were selected for additional experiments, performed to determine the specificity of the observed responses to VLPs (see below).

HPV-16 L1 VLP vaccine. Recombinant HPV-16 L1 virus-like particles (VLPs) expressed in the baculovirus system were used to investigate cellular immune responses to vaccination with VLPs. HPV-16 L1 VLPs were expressed in baculovirus-infected Sf9 insect cells (Novavax). Production of clinical lots of recombinant HPV-16 L1 VLP vaccines was performed in accordance with GMP guidelines at the Vaccine Production Facility of Novavax, Inc., as reported elsewhere [12]. Formulated L1 VLPs from a single lot were dispensed aseptically into sterile vials (3.0-mL size, type 1 borosilicate glass, silanized, depyrogenated; Wheaton Glass) as a single-unit dose and were designated the final container vials. Vials containing the L1 VLPs were stored at -20°C and were thawed immediately before being administered. Filling of vials from the single manufacturing lot used for our trial occurred at 3 separate times, denoted as fills "A," "B," and "C." Subsequent to our study, fills B and C were noted to have used vials that had been inadequately silanized, and L1 VLPs were shown to have adhered to the walls of the unsilanized vials. Among participants in the present study, 11 volunteers received all 3 doses from fill A, whereas the remaining 32 volunteers received 2 doses, at months 0 and 1, from fill A and received the third dose, at month 6, from either fill B or fill C. Since the dose of antigen delivered to these 32 individuals at month 6 is unknown, CMI data obtained from these individuals at month 7 were not included in our final analysis.

Lymphoproliferation assays. Assays of lymphoproliferation were performed on fresh PBMCs collected before the initial vaccination (i.e., at month 0) and after the 2 subsequent vaccinations (i.e. at months 2 and 7), from a total of 43 vaccine recipients and 10 placebo recipients. PBMCs were plated in quadruplicate, at 2×10^5 cells/well, in 96-well round-bottom plates (Costar) in RPMI-1640 (Gibco, Invitrogen Life Technologies) supplemented with penicillin-streptomycin (100 $\mu\text{g}/\text{mL}$ –100 U/mL; Gibco), glutamine (2 mM) and HEPES buffer (10 mM), and 10% fetal calf serum (R-10; Gibco). Cells were cultured in the presence or absence of HPV-16 L1 VLPs (10, 2.5, 1, and 0.25 $\mu\text{g}/\text{mL}$) diluted in R-10 media. Stocks of L1 VLP preparations were provided from the vaccine manufacturer to the laboratory, at 0.8 mg/mL and 1.0 mg/mL. The purity of the HPV-16 L1 VLPs was $>96\%$, as determined by SDS-PAGE. Phytohemagglutinin (PHA) (1:100; Sigma) and influenza virus (1:100; ATCC) were used as controls for the assay. Cultures containing mitogens or antigens were pulsed with 1 μCi of [^3H]-thymidine (Perkin-Elmer) for 18 h after either 48 or 96 h of culture, respectively. Cultures were harvested and counted in an automated scintillation counter (Microbeta; Perkin-Elmer). Results were expressed as cpm or stimulation indices (SIs), cal-

culated as cpm of cultures in the presence of antigen or mitogens divided by cpm of cultures in the presence of media alone.

Because the L1 VLPs were purified from a baculovirus expression system, an Sf-9/baculovirus insect-cell lysate (0.1 $\mu\text{g}/\text{mL}$) was used as control antigen for the system of production of the L1 VLPs in experiments performed to determine specificity of the responses to L1 VLPs. These experiments were performed with cryopreserved PBMCs from a total of 29 vaccine recipients and 7 placebo recipients.

Bromodeoxyuridine (BrdUrd) labeling and flow-cytometric analysis. The following assay was performed as described elsewhere [18]. BrdUrd incorporation into CD4 $^+$ and CD8 $^+$ T cells from a subset of 4 vaccine recipients and 2 placebo recipients was determined before the initial immunization (i.e., at month 0) and after the second immunization (i.e., at month 2). PBMCs cultured for 5 days at 37°C in the presence of L1 VLPs (2.5 $\mu\text{g}/\text{mL}$), control baculovirus lysate, influenza, and control media were incubated with 10 μM 5-BrdUrd (BrdUrd; Sigma) for the final 4.5 h of culture at 37°C , in 5% CO_2 . Cell-surface staining was performed with either anti-human CD3 phycoerythrin PE (Becton Dickinson), anti-human CD4 PC5 (Beckman Coulter), or anti-human CD8 ECD (Beckman Coulter) antibodies. The stained cells were treated with OptiLyse C lysing solution (Immunotech) for 10 min at room temperature, followed by incubation, for 15 min at 37°C , with 1% paraformaldehyde and 1% Tween-20 in PBS, to fix and permeabilize the cells. Cellular DNA in the permeabilized cells was partially digested, for 30 min at 37°C , with 100 Kunitz units of DNase-I (Boehringer-Mannheim) in DNase buffer (PBS with 4.2 mM MgCl_2 , pH 5) and then was stained, for 30 min, with anti-BrdUrd FITC (Becton Dickinson) antibody in $1 \times$ PBS containing 1% bovine serum albumin and 0.5% Tween-20. Cells were washed twice before flow-cytometric analysis. A total of 100,000–150,000 CD3 $^+$ T cells were collected. Samples were stained and analyzed in parallel with unlabeled cells (without BrdUrd) from the same individual, and this value was subtracted from the value obtained for BrdUrd-labeled cells. Data are presented as the percentage of cells in the specific lymphocyte pool that are BrdUrd $^+$. The high sensitivity ($<0.01\%$ BrdUrd $^+$ cells) of this assay derives from analysis of large numbers of events (50,000–100,000), strong anti-BrdUrd-antibody staining of labeled cells, and low background binding of anti-BrdUrd antibody to unlabeled cells [18].

Cytokine induction assays. PBMCs (at a final concentration of $1.5 \times 10^6/\text{mL}$) were incubated, for 3 days at 37°C , in the absence or presence of PHA-M (1:100), influenza virus (1:100), HPV-16 L1 VLPs (10, 2.5, 1, and 0.25 $\mu\text{g}/\text{mL}$), and 6% CO_2 , in R-10 media. Cell-free supernatants were harvested and were frozen at -20°C . As described above for the assays of lymphoproliferation, an Sf-9/baculovirus insect-cell lysate (0.1 $\mu\text{g}/\text{mL}$) was

Table 1. Immune responses (lymphoproliferation and cytokine production) to human papillomavirus–like particles (VLPs), in vaccine recipients and placebo recipients.

Response type, L1 VLP concentration	Time (months)	Vaccine		Placebo	
		No. tested	Immune response, mean ± SE (range)	No. tested	Immune response, mean ± SE (range)
Lymphoproliferation, SI ^a					
L1 VLP concentration 10 µg/mL	0	43	4.2 ± 0.6 (0.9–24.1)	10	4.1 ± 1.5 (0.7–17.0)
	2	39	6.0 ± 0.6 (1.9–19.7)	10	2.6 ± 0.3 (1.3–3.7)
	7 ^b	11	7.3 ± 2.3 (0.9–28.4)	10	2.8 ± 0.3 (1.7–4.8)
L1 VLP concentration 2.5 µg/mL	0	43	2.8 ± 0.3 (0.8–11.0)	10	2.9 ± 0.6 (0.7–6.7)
	2	39	5.4 ± 0.4 (2.2–13.6)	10	2.0 ± 0.2 (1.3–3.0)
	7 ^b	11	5.8 ± 1.2 (1.0–14.9)	10	2.0 ± 0.3 (0.8–4.2)
Interferon-γ, pg/mL ^c					
L1 VLP concentration 10 µg/mL	0	36	94.9 ± 11.8 (7.8–326.6)	9	62.3 ± 16.1 (7.8–129.5)
	2	39	347.7 ± 37.5 (36.3–939.2)	10	81.7 ± 36.5 (7.8–382.7)
	7 ^b	11	321.9 ± 80.4 (1832.4–782.2)	9	62.0 ± 15.1 (7.8–144.2)
L1 VLP concentration 2.5 µg/mL	0	17	34.5 ± 8.0 (7.8–102.7)	4	13.9 ± 3.5 (7.8–20.5)
	2	23	162.7 ± 30.6 (7.8–579.4)	7	13.1 ± 3.8 (7.8–34.3)
	7 ^b	11	214.6 ± 72.0 (18.8–709.0)	9	19.1 ± 4.7 (7.8–43.2)
Interleukin-10, pg/mL ^c					
L1 VLP concentration 10 µg/mL	0	36	44.8 ± 5.1 (4.7–154.4)	9	37.8 ± 7.1 (4.7–66.6)
	2	39	191.3 ± 26.5 (31.7–600.0)	10	33.3 ± 6.8 (10.1–67.6)
	7 ^b	11	225.1 ± 49.7 (46.9–600.0)	9	22.2 ± 5.7 (10.5–65.5)
L1 VLP concentration 2.5 µg/mL	0	17	14.4 ± 2.2 (4.7–35.2)	4	13.0 ± 3.0 (4.7–18.2)
	2	23	62.9 ± 6.5 (13.8–136.3)	7	10.4 ± 3.6 (4.7–30.2)
	7 ^b	11	145.4 ± 40.3 (33.4–491.5)	9	18.3 ± 13.1 (4.7–122.9)
Interleukin-5, pg/mL ^c					
L1 VLP concentration 10 µg/mL	0	36	6.0 ± 0.9 (3.2–19.5)	9	6.6 ± 1.5 (3.2–14.1)
	2	39	52.6 ± 8.3 (3.2–183.8)	10	5.9 ± 1.2 (3.15–12.7)
	7 ^b	11	62.7 ± 22.9 (8.5–263.5)	9	6.7 ± 1.5 (3.15–13.7)
L1 VLP concentration 2.5 µg/mL	0	17	4.9 ± 1.1 (3.2–19.0)	4	7.6 ± 4.4 (3.15–20.8)
	2	23	56.2 ± 11.7 (3.2–169.3)	7	3.2 ± 0 (3.15–3.15)
	7 ^b	11	46.4 ± 19.8 (3.2–230.7)	9	4.4 ± 2.0 (3.15–8.2)

NOTE. *P* < .001 for all values at months 2 and 7, compared with values at month 0, in vaccine recipients. SI, stimulation index.

^a Calculated as described in Subjects, Materials, and Methods.

^b Includes only individuals that received lot A at the third immunization.

^c Determined by ELISA.

used as a control antigen in experiments performed to evaluate specificity of the responses to L1 VLPs.

Cytokine determinations. Supernatants from the cytokine-induction assay were thawed and were tested, in duplicate wells, for IFN-γ, IL-10, and IL-5, by ELISA (Endogen), according to the manufacturers' instructions. The lower levels of detection for IFN-γ, IL-10, and IL-5 were 15.6, 9.3, and 6.3 pg/mL, respectively. Levels lower than the lowest detection levels were arbitrarily considered to be one-half of the lower detection level (i.e., 7.8, 4.7, and 3.2 pg/mL, respectively).

HPV-16 serology. Presence of antibodies to HPV-16 L1 VLPs was assessed by ELISA, as described elsewhere [12]. The

antibody titers were expressed as the reciprocals of the highest dilution showing positive reactivity in each assay. Since our interest was to evaluate whether antibody titers induced by vaccination affect T cell responses to subsequent vaccination, serum samples from the volunteers who received the third dose of vaccine from fill A were examined (*n* = 11).

Statistical analysis. The nonparametric Kruskal-Wallis or Mann-Whitney test was used to determine statistical differences, over time, within a group, and a nonparametric test for trend [19] was used to test time trends. For categorized outcomes, Pearson χ^2 tests and χ^2 tests for trend were used. To determine the relationships between different immune markers, we used

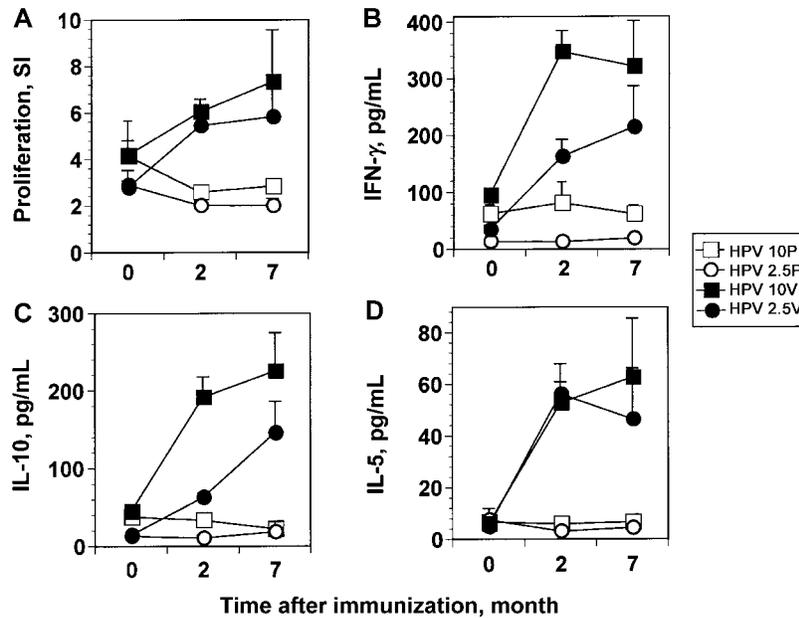


Figure 1. Lymphoproliferative (A) and cytokine (B–D) responses to human papillomavirus (HPV)–16 L1 HPV-like particles (VLPs), in vaccine (V) recipients (black symbols) and placebo (P) recipients (white symbols), with 2 concentrations (10 $\mu\text{g}/\text{mL}$ [squares] and 2.5 $\mu\text{g}/\text{mL}$ [circles]) of HPV-16 L1 VLPs in vitro. Results are expressed as mean \pm SE. Lymphoproliferative responses were evaluated by use of peripheral blood mononuclear cells (PBMCs) stimulated with L1 VLPs (10 $\mu\text{g}/\text{mL}$ and 2.5 $\mu\text{g}/\text{mL}$) for 5 days; results are presented as stimulation indices. Cytokine responses were determined as described in Subjects, Materials, and Methods, by use of supernatants from cultures stimulated for 3 days with L1 VLPs; cytokine levels were determined by ELISA and are expressed as picograms per milliliter. At month 0, cytokine levels for unstimulated cultures were 10.1 ± 2.0 pg/mL ($n = 39$) for interferon- γ , 11.3 ± 1.1 pg/mL ($n = 39$) for interleukin (IL)–10, and 3.7 ± 2.2 pg/mL ($n = 39$) for IL-5; similarly low levels were detected in unstimulated supernatants from PBMCs collected at months 2 and 7.

both Spearman rank correlation, for continuous values, and exact agreement, for categorized values (0–25 percentile, 25–75 percentile, and 75–100 percentile). $P < .05$ was considered significant.

RESULTS

Lymphoproliferative responses to HPV-16 L1 VLPs after immunization. Freshly isolated PBMCs were tested, in vitro, for lymphoproliferative responses to L1 VLPs (10 and 2.5 $\mu\text{g}/\text{mL}$) before the initial injection (i.e., at month 0), 1 month after the second injection, and 1 month after the third injection (i.e., at months 2 and 7, respectively). Table 1 summarizes these responses in vaccine recipients and placebo recipients. For lymphoproliferative responses to L1 VLPs, an SI >3 was seen, before immunization, in a sizeable proportion of individuals (49% and 30% for L1 VLPs at concentrations of 10 $\mu\text{g}/\text{mL}$ and 2.5 $\mu\text{g}/\text{mL}$, respectively; SI, 4.2 ± 0.6 and 2.8 ± 0.3 , respectively; $P < .001$). A statistically significant increase in the lymphoproliferative responses to L1 VLPs was seen after vaccination regardless of the L1 VLP concentration used in the in vitro assay ($P_{\text{trend}} < .001$). The peak lymphoproliferative responses were seen at month 7 (SI, 7.3 ± 2.3 and 5.8 ± 1.2 , for HPV L1 VLPs at concentrations of 10 $\mu\text{g}/\text{mL}$ and 2.5 $\mu\text{g}/\text{mL}$, respec-

tively; $P < .002$). After in vitro stimulation with 10 $\mu\text{g}/\text{mL}$, the proportion of individuals with an SI >3.0 was 87% (34/39) at month 2 and 91% (10/11) at month 7, compared with 49% at month 0 ($P_{\text{trend}} < .001$). In contrast, no significant increases in lymphoproliferative responses to L1 VLPs were observed among the placebo recipients at months 2 and 7, compared with month 0 (figure 1A, and table 1) (SIs at months 2 and 7 were 2.6 ± 0.3 [range, 1.3–3.7] and 2.8 ± 0.3 [range, 1.7–4.8], respectively, relative to an SI of 4.1 ± 1.5 [range, 0.7–17.0] at month 0; $n = 10$; $P = .93$). Similar trends for lymphoproliferative responses were observed when L1 VLPs were used in vitro at a concentration of 2.5 $\mu\text{g}/\text{mL}$ (figure 1A), although the level of response at this concentration of L1 VLPs was lower than those seen at higher concentrations of L1 VLPs. Increases in lymphoproliferative responses to L1 VLPs after immunization were still detected when L1 VLPs were tested, in vitro, at 1 $\mu\text{g}/\text{mL}$ and 0.25 $\mu\text{g}/\text{mL}$ (data not shown). Similar patterns were seen in analysis restricted to individuals who received all 3 vaccine doses from fill A (data not shown).

When we examined before-and-after-vaccination changes in lymphoproliferative responses at the individual level rather than by comparing the means, similar patterns were observed (figure 2). In 40% (15/38) of vaccine recipients at month 2, >2 -fold

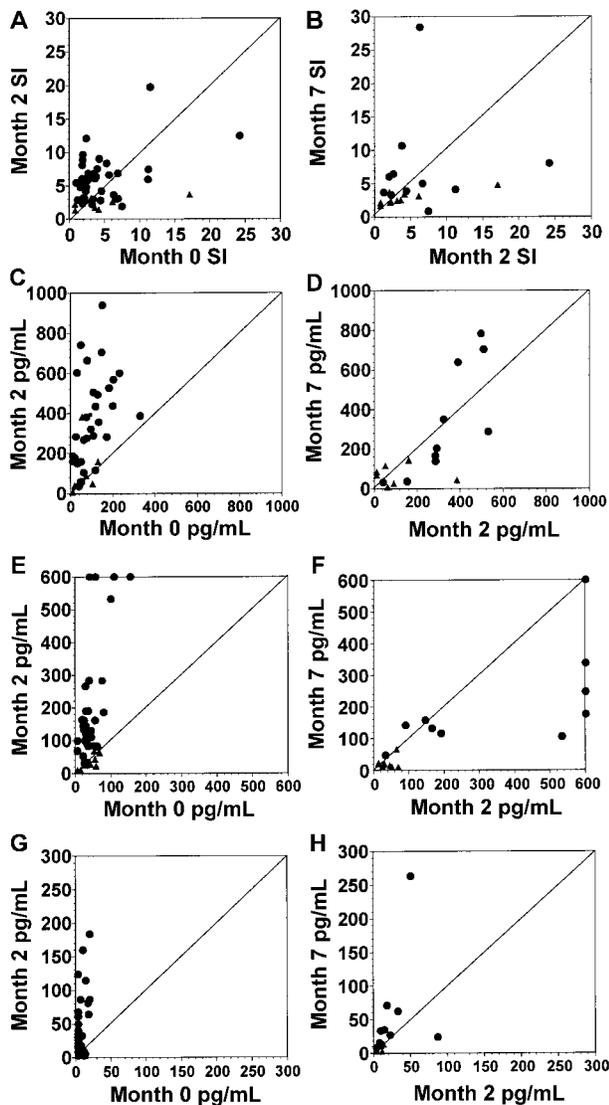


Figure 2. Lymphoproliferative (A and B) and cytokine (C–H) responses to human papillomavirus (HPV)–16 L1 HPV-like particles (VLPs); comparison between months 0 and 2 (A, C, E, and G) and months 2 and 7 (B, D, F, and H), in vaccine recipients (circles) and placebo recipients (triangles). Lymphoproliferative responses were evaluated by use of peripheral blood mononuclear cells stimulated with L1 VLPs (10 $\mu\text{g}/\text{mL}$) for 5 days; results are presented as stimulation indices (SIs) of cultures performed in quadruplicate and determined as described in Subjects, Materials, and Methods. Cytokine responses also were determined as described in Subjects, Materials, and Methods, by use of supernatants from cultures stimulated for 3 days with L1 VLPs (10 $\mu\text{g}/\text{mL}$); cytokine levels were determined by ELISA and are expressed as picograms per milliliter. Each cytokine value represents the mean of duplicate cultures.

increases in lymphoproliferative responses to L1 VLPs in vitro at a concentration of 10 $\mu\text{g}/\text{mL}$ were observed; similarly, 55% (21/38) of vaccine recipients at month 2 showed >2-fold increases in lymphoproliferative responses to L1 VLPs in vitro at a concentration of 2.5 $\mu\text{g}/\text{mL}$. None of the 10 placebo recipients

showed >2.0-fold increases in lymphoproliferative responses to L1 VLPs. Further increases in the lymphoproliferative responses to L1 VLPs (10 $\mu\text{g}/\text{mL}$) were seen at month 7, relative to month 2, for 46% (5/11) of vaccinated individuals, but only 9% (1/11) had increases at month 7 that were >2-fold higher than their responses at month 2.

In contrast to the increases in responses to L1 VLPs after vaccination, a slight trend of decreasing SIs, over time, for both vaccine and placebo, was observed in the lymphoproliferative responses to the positive control mitogens PHA ($P_{\text{trend}} < .01$) and the positive control antigen (influenza virus; $P_{\text{trend}} < .01$) during the course of the study.

To determine the relative contribution of CD4⁺ and CD8⁺ T cells in the lymphoproliferative responses to L1 VLPs detected after immunization, incorporation of the thymidine analog BrdUrd was used to measure, before and after immunization, the relative numbers of CD4⁺ and CD8⁺ lymphocytes progressing through S phase of the cell cycle after in vitro stimulation of PBMCs by L1 VLPs. BrdUrd incorporation into the DNA of dividing lymphocyte subsets was quantified by flow cytometry. Figure 3 shows profiles of BrdUrd incorporation into the DNA of dividing peripheral CD4⁺ (figure 3A and C) and CD8⁺ (figure 3B and D) T cells from 1 of the vaccine recipients tested before (figure 3A and B) and after immunization (figure 3C and D). After immunization, increased levels of proliferating CD4⁺ and CD8⁺ T cells were observed in the 4 vaccine recipients but in neither of the 2 placebo recipients tested. The level of lymphoproliferative responses to L1 VLPs was higher among CD4⁺ cells than among CD8⁺ T cells. The mean relative levels of proliferating CD4⁺ and CD8⁺ T cells in response to the L1 VLPs and control antigens, in 4 vaccine recipients and 2 placebo recipients, is shown in table 2. The results of the labeling experiments are in agreement with the data on lymphoproliferation.

Cytokine responses to HPV-16 L1 VLPs after immunization. To evaluate the pattern of cytokine release by PBMCs from vaccine recipients and placebo recipients, cytokines characteristic of a Th1 (IFN- γ) and Th2 type response (IL-10 and IL-5) were measured in supernatants from cultures stimulated with HPV-16 L1 VLPs in vitro. The mean cytokine levels produced in response to L1 VLPs at concentrations of 10 $\mu\text{g}/\text{mL}$ and 2.5 $\mu\text{g}/\text{mL}$ are presented in figure 1B–D and table 1. Statistically significant increases in the mean levels of all three cytokines were seen after vaccination, regardless of the L1 VLP concentration used in the in vitro assay ($P_{\text{trend}} < .001$; figure 1). Similar increases were also detected when L1 VLPs were tested, in vitro, at concentrations of 1 $\mu\text{g}/\text{mL}$ and 0.25 $\mu\text{g}/\text{mL}$ and when analysis was restricted to individuals who received all 3 vaccine doses from fill A (data not shown). The highest increment in cytokine responses was observed at month 2 (i.e., after the second vaccination). Further increases were seen after the

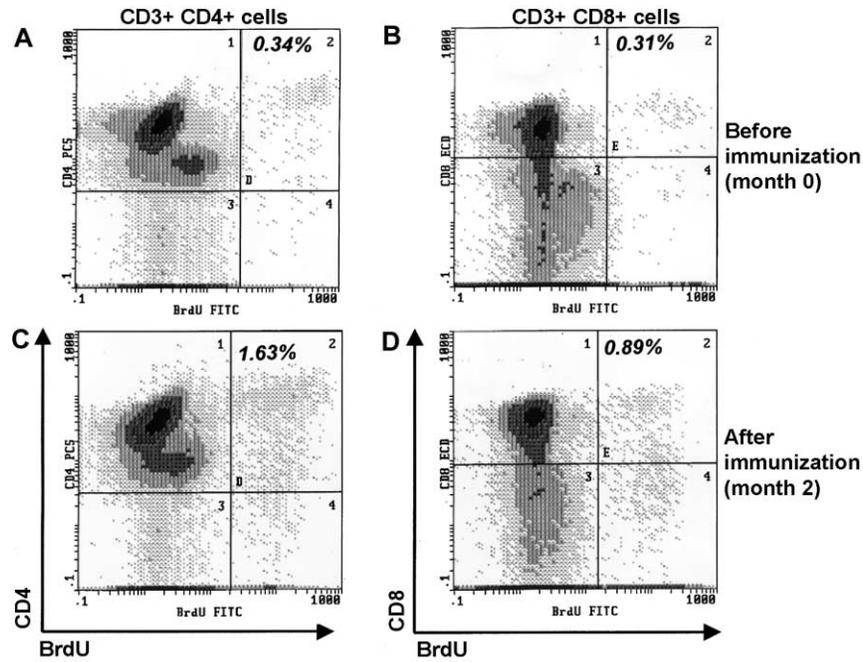


Figure 3. Detection of BrdUrd-labeled CD4⁺ (A and C) and CD8⁺ (B and D) T cells in a vaccine recipient, after in vitro stimulation of peripheral blood mononuclear cells (PBMCs) with L1 VLPs (2.5 $\mu\text{g}/\text{mL}$), before the initial immunization (i.e., at month 0) (A and B) and after immunization (i.e., at month 2) (C and D). PBMCs cultured for 5 days at 37°C in the absence or presence of either L1 VLPs (2.5 $\mu\text{g}/\text{mL}$) or control agents were incubated with and without BrdUrd for 4.5 h at 37°C. Cell staining and flow-cytometric analysis of labeled cells were performed as described in Subjects, Materials, and Methods. Values in the right quadrants of the histograms are percentages of BrdUrd⁺ cells within corresponding subsets of CD3⁺ cells. A total of >140,000 CD3⁺ T cell events were analyzed.

third immunization, but these increases were, for the most part, small and nonsignificant (figure 2B–D and table 1). The one exception was IL-10, for which a significant increase in response to L1 VLPs (at a concentration of 2.5 $\mu\text{g}/\text{mL}$) was observed at month 7, compared with month 2 ($P = .008$). In the placebo recipients, no significant increases in the mean cytokine levels produced in response to L1 VLPs were observed at months 2 and 7, compared with month 0 (figure 1B–D) (IFN- γ , $P = .94$; IL-10, $P = .19$; and IL-5, $P = .89$) (figure 1B–D).

When before-and-after-vaccination changes in cytokine responses were examined at the individual level rather than by comparison of the means, similar patterns were observed (figure 2). At month 2, the vast majority of vaccine recipients showed increases in cytokine responses to L1 VLPs, relative to prevaccination (month 0) levels; 75% to 85% of vaccine recipients showed >2-fold increases in cytokine production at month 2. For IFN- γ , a further increase was observed in 36% (4/11) of vaccine recipients at month 7, but these increases were <2-fold more than those at month 2 (figure 2D). Similarly, for IL-10, a further increase was observed in 18% (2/11) of vaccine recipients at month 7, but the increases observed were negligible (figure 2F). The largest observed increases at month 7 were for IL-5 (figure 2H); increases over the levels at month

2 were seen in 82% (9/11) of the vaccine recipients, and for 55% (6/11) of them the increase was >2-fold. During the course of the study, no significant increases were observed in the cytokine responses to the positive control mitogens and antigens (PHA and influenza virus), in either the vaccine recipients or the placebo recipients (data not shown).

Specificity of L1-induced immune responses. To address the specificity of the immune responses to HPV-16 L1 VLPs, cryopreserved PBMCs from a subset of vaccine recipients and from a subset of placebo recipients were tested, in parallel, for lymphoproliferative (29 vaccine recipients and 7 placebo recipients) and cytokine (22 vaccine recipients and 5 placebo recipients) responses to HPV-16 L1 VLPs and to a lysate of wild-type baculovirus-infected Sf-9 insect cells, similar to that used to produce the L1 VLPs. Results are summarized in table 3. Fresh and cryopreserved PBMCs showed similar patterns of immune responses to the L1 VLPs, although the cryopreserved cells had, in general, lower levels than did the fresh cells (data not shown). Although significant immune responses to L1 VLPs were mounted at months 2 and 7 ($P_{\text{trend}} < .05$), there were no similar increases in the lymphoproliferative responses to the insect-cell/baculovirus control lysate ($P_{\text{trend}} < .80$ (table 3). Similarly, there were no significant increases in the cytokine (IFN- γ , IL-10, or

Table 2. Percentage of proliferating (BrdUrd⁺) cells within CD4 and CD8, CD3 lymphocytes to L1 human papillomavirus (HIV)-like particles (VLPs) before the initial immunization (i.e., at month 0) and after the second immunization (i.e., at month 2) with HPV-16 L1 VLPs, and corresponding lymphoproliferative responses determined by incorporation of tritiated thymidine.

Treatment, time, conditions ^a	CD3 ⁺ CD4 ⁺ BrdUrd ⁺ , mean ± SD, % ^b	CD3 ⁺ CD8 ⁺ BrdUrd ⁺ , mean ± SD, % ^b	Lymphoproliferation, mean ± SD, cpm ^b
Vaccine (n = 4)			
0 months			
Media	0.03 ± 0.02	0.02 ± 0.01	1495 ± 899
L1 VLP concentration 2.5 µg/mL	0.17 ± 0.16	0.14 ± 0.12	3939 ± 1600
Control lysate	0.016 ± 0.01	0.03 ± 0.02	1897 ± 2453
Influenza virus	6.13 ± 3.47	6.07 ± 4.17	44,304 ± 15,712
2 months			
Media	0.05 ± 0.02	0.08 ± 0.05	1880 ± 628
L1 VLP concentration 2.5 µg/mL	0.80 ± 0.62	0.45 ± 0.31	29,521 ± 17,886
Control lysate	0.13 ± 0.11	0.09 ± 0.09	3301 ± 4165
Influenza	5.05 ± 1.14	4.32 ± 2.56	41,303 ± 25,342
Placebo (n = 2)			
0 months			
Media	0.01 ± 0.01	0.03 ± 0.03	1892 ± 61
L1 VLP concentration 2.5 µg/mL	0.01 ± 0.00	0.01 ± 0.01	3801 ± 1682
Control lysate	0.00 ± 0.00	0.01 ± 0.00	1172 ± 405
Influenza	2.29 ± 1.49	2.49 ± 3.09	29,209 ± 20,303
2 months			
Media	0.01 ± 0.02	0.01 ± 0.02	749 ± 35
L1 VLP concentration 2.5 µg/mL	0.01 ± 0.00	0.01 ± 0.00	1997 ± 1794
Control lysate	0.01 ± 0.01	0.01 ± 0.01	900 ± 412
Influenza virus	1.99 ± 0.26	1.87 ± 1.77	21,821 ± 8000

^a Control lysate is baculovirus-infected Sf-9 insect-cell lysate.

^b Determined as described in Subjects, Materials, and Methods.

IL-5) responses to the control lysate after vaccination (table 3). In addition, no increases in lymphoproliferative or cytokine responses to either the L1 VLPs ($P_{\text{trend}} \geq .55$ for all measurements) or the control lysate ($P_{\text{trend}} \geq .21$ for all measurements) were observed, over time, in placebo recipients.

Correlations between CMI parameters. We evaluated the correlation between the multiple measures of CMI responses to L1 VLPs used in this study (i.e., lymphoproliferation, IFN- γ , IL-10, and IL-5). For this analysis, responses at months 2 and 7 were combined. When L1 VLPs were tested at a concentration of 2.5 µg/mL, significant positive correlations were observed for all combinations of measures except for IL-10 and proliferation; at this concentration, the Spearman values ranged from 0.34 (for IL-5:IL-10; $P < .05$) to 0.66 (for IL-10:IFN- γ ; $P < .05$). Percentage-agreement levels ranged from 44% (for IFN- γ :lymphoproliferation) to 65% (for IL-5:IL-10).

Relationship between antibody and cellular immune responses. It has recently been reported that neutralizing antibodies elicited in mice by L1 vaccination with VLPs can block subsequent vaccinations' boosting of CD8-mediated anti-tu-

mor responses [20]; however, T cell proliferative responses and cytokine release, which can be largely dependent on CD4⁺ T helper response, were not examined. To examine the potential effect that neutralizing antibodies could have on cellular responses, we evaluated the relationship between antibody titers to L1 VLPs at month 2 (i.e., 1 month after the second immunization) and CMI responses to L1 VLPs (i.e., lymphoproliferation, IFN- γ , IL-10 and IL-5 responses) at month 7 (i.e., 1 month after the third immunization), in vaccine recipients whose third dose was from fill A ($n = 10$). Figure 4 shows fold increases in lymphoproliferative and cytokine responses to the L1 VLPs at month 7, compared with month 2, in individuals with titers of anti-L1 VLP antibody that were either ≤ 2560 or >2560 at month 2. There was a direct positive correlation between the level of anti-L1 VLP antibodies after the second immunization (i.e., at month 2) and the level of cytokine responses to L1 VLPs (at a concentration of either 10 µg/mL or 2.5 µg/mL) after the third immunization. Individuals with the highest antibody titers (>2560) at month 2 had the highest increments in IFN- γ , IL-10, and IL-5 responses at month 7,

compared with month 2. Antibody titers at month 2 correlated positively with IFN- γ ($P = .03$, $\rho = 0.815$) levels induced by L1 VLPs *in vitro* at month 7. Positive correlations were observed for the other two cytokines examined but did not reach statistical significance (for IL-10, $P = .06$, $\rho = 0.714$; for IL-5, $P = .1$, $\rho = 0.583$). No significant correlation between antibody titers after the second immunization and lymphoproliferative responses to L1 VLPs after the third immunization was observed ($P = .86$, $\rho = 0.06$).

Consistent with these findings, a comparison between the pattern of cellular immune responses and antibody responses induced by L1 vaccination with VLPs indicates that they follow a similar pattern over a 7-month period (figure 5A and B).

DISCUSSION

In the present study, we have addressed whether parenteral immunization with a recombinant HPV-16 L1 VLP vaccine, in the absence of adjuvant, induces systemic CMI responses—including lymphoproliferative and cytokine responses—to the vaccine. Our results indicate that, in healthy young women, parenteral vaccination with L1 VLPs induces, 1 month after the second and third doses (i.e., at months 2 and 7, respectively), significant systemic lymphoproliferative and cytokine (both Th1 and Th2 type) responses.

This study presents the first results regarding CMI responses

in recipients of a recombinant HPV-16 L1 VLP vaccine administered in the absence of adjuvant. A recent report from a phase I trial of an HPV-11 VLP vaccine administered with alum has indicated similar patterns of lymphoproliferative and cytokine (IFN- γ and IL-5) responses after immunization [21]. Mouse studies that preceded human trials also have demonstrated that L1 VLPs are capable of priming a CMI response associated with production of Th1-type cytokines and cytotoxic CD8⁺ T cell responses [22, 23]. Furthermore, results from animal-model studies have indicated that inoculation of mice with HPV-16 L1 VLPs results in HPV-specific T cell-proliferative responses and production of both Th1 and Th2 cytokines and that vaccinated animals are protected against virus challenge with recombinant vaccinia expressing HPV-16 L1 [24].

Although peak responses were often seen after the third immunization (i.e., at month 7), the highest increments in both lymphoproliferative and cytokine responses were seen after the second immunization (i.e., at month 2). Increments over the levels seen at month 2 were found in some, but not all, individuals at month 7, suggesting that boosting of CMI response is possible even after high levels of HPV-specific neutralizing antibodies have been generated. This finding is consistent with our observation that titers of anti-L1 VLP antibody after the second vaccination are positively rather than negatively correlated with CMI response observed after the third vaccination. So, although anti-L1 VLP antibodies appear to strongly an-

Table 3. Immune responses to L1 human papillomavirus-like particles (VLPs) and control antigens, by cryopreserved peripheral blood mononuclear cells (PBMCs) from vaccine recipients, at months 0, 2, and 7.

Response type, time	Control lysate, mean \pm SE	L1 VLP concentration, mean \pm SE	
		10 μ g/mL	2.5 μ g/mL
Lymphoproliferation, SI			
0 months	1.1 \pm 0.2 ($n = 29$)	2.0 \pm 0.2 ($n = 29$)	1.8 \pm 0.2 ($n = 29$)
2 months	1.3 \pm 0.1 ($n = 28$)	5.4 \pm 0.5 ^a ($n = 29$)	4.1 \pm 0.4 ^a ($n = 29$)
7 months	1.2 \pm 0.2 ($n = 9$)	5.6 \pm 0.9 ^a ($n = 9$)	4.7 \pm 0.7 ^a ($n = 9$)
IFN- γ , pg/mL			
0 months	7.8 \pm 0 ($n = 22$)	11.0 \pm 1.5 ($n = 22$)	7.8 \pm 0 ($n = 22$)
2 months	7.8 \pm 0 ($n = 18$)	90.4 \pm 24.1 ^a ($n = 18$)	41 \pm 9.0 ^a ($n = 18$)
7 months	9.5 \pm 0.5 ($n = 6$)	206.8 \pm 94.2 ^a ($n = 6$)	107.9 \pm 50.2 ^a ($n = 6$)
IL-10, pg/mL			
0 months	5.8 \pm 0.7 ($n = 22$)	15.3 \pm 6.4 ($n = 22$)	6.1 \pm 0.6 ($n = 22$)
2 months	6.0 \pm 0.8 ($n = 18$)	38.4 \pm 6.6 ^a ($n = 18$)	18.8 \pm 3.1 ^a ($n = 18$)
7 months	6.2 \pm 1.6 ($n = 6$)	61.7 \pm 25.2 ^a ($n = 6$)	30.0 \pm 8.6 ^a ($n = 6$)
IL-5, pg/mL			
0 months	3.2 \pm 0 ($n = 22$)	3.2 \pm 0 ($n = 22$)	3.2 \pm 0 ($n = 22$)
2 months	3.4 \pm 0.2 ($n = 18$)	16.0 \pm 3.4 ^a ($n = 18$)	11.7 \pm 2.4 ^a ($n = 18$)
7 months	7.9 \pm 4.8 ($n = 6$)	17.5 \pm 6.1 ^a ($n = 6$)	22.9 \pm 15.3 ^a ($n = 6$)

NOTE. Results were obtained by use of cryopreserved peripheral blood mononuclear cells. SI, stimulation index.

^a $P_{\text{trend}} < .05$.

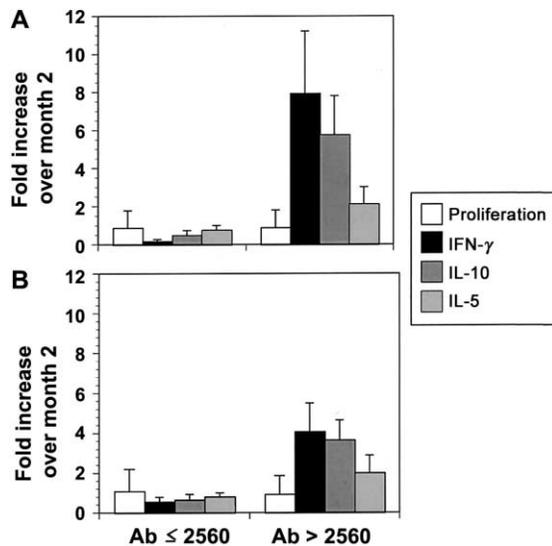


Figure 4. Lymphoproliferative and cytokine responses, at month 7, to HPV-16 L1 VLPs at concentrations of 10 µg/mL (A) and 2.5 µg/mL (B), in vaccine recipients whose antibody titers, at month 2, were either ≤2560 or >2560. Lymphoproliferative responses and cytokine responses are expressed as fold increase over month-2 levels.

tagonize major histocompatibility complex (MHC) class I presentation [20], they may promote increased MHC class II antigen presentation to T helper cells, as a result of opsonization of the L1 VLPs [20]. Cytokines released by primed T helper cells may then have a synergistic effect on the action of memory B cells and may stimulate the development of cytotoxic T cells and/or natural killer cells, which might be able to directly eliminate infected cells [13, 16].

In this phase II trial, a considerable number of individuals had, prior to vaccination, lymphoproliferative responses >3.0 for the L1 VLPs, consistent with the results of a recent report of a phase I trial of an HPV-11 VLP vaccine [21]. These responses could result from the fact that some of these individuals had previous HPV exposures that resulted in priming to HPV epitopes. Alternatively, the responses could be due to lymphocyte cross-reactivity [25] to antigens with homology with HPV-16 L1, to contaminants from the L1 VLP preparation, or to the fact that L1 VLPs are highly immunogenic and can stimulate production of multiple cytokines, particularly from antigen-presenting cells [26, 27], in a memory independent fashion.

The lymphoproliferative and cytokine responses to L1 VLPs after vaccination appeared, for the most part, to be L1 VLP specific and not due to contaminants in the L1 VLP preparation, since similarly processed lysates of Sf9 insect cells infected with baculovirus did not induce significant levels of responses in PBMCs from the vaccine recipients. In addition, in both vaccine recipients and placebo recipients, either no significant increases or some slight decreases in the immune responses (lymphopro-

liferation and cytokine production) to the PHA and influenza-virus controls were observed at either month 2 or month 7. This finding, together with the fact that placebo recipients did not show any significant increases in immune responses to HPV-16 L1 VLPs in vitro at either month 2 or month 7, compared with month 0, is consistent with the induction of an L1 VLP memory response in the vaccine recipients. Experiments using L1 VLPs from heterologous HPV virus are underway, to determine whether the responses elicited by this vaccine are HPV-16-type specific.

The results of the BrdUrd labeling experiments indicate that the lymphoproliferative response seen after immunization with L1 VLPs includes proliferation of both CD4⁺ and CD8⁺ T cells, suggesting that both subsets of lymphocytes proliferate in response to the vaccine; however, in the small group of vaccine recipients tested, the relative levels of lymphoproliferative response to L1 VLPs appears to be higher among CD4⁺ T cells than among CD8⁺ T cells. The impact that the L1 VLP vaccine has on CD4⁺ and CD8⁺ T cell effector function remains to be addressed.

The cellular immune responses (lymphoproliferation and cytokine levels) after healthy individuals are immunized with L1 VLP vaccine are consistently higher than those previously reported in the context of natural infection [28]. In fact, T cell responses to HPV antigens in natural HPV infection have been difficult to measure [29]. Similarly, the levels of neutralizing antibodies elicited by vaccination with L1 VLPs have been shown to be ~40-fold higher than that seen in natural infection [12]. Although the high levels of systemic antibody and T-cell

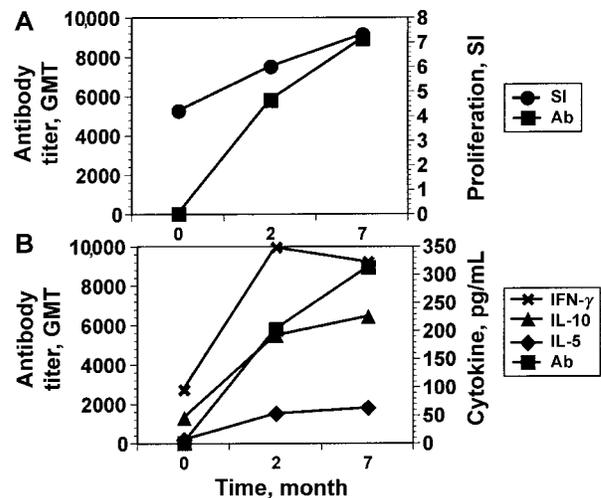


Figure 5. Relationship between anti-L1 VLP antibodies and lymphoproliferative (A) or cytokine (B) responses in the vaccine recipients. Antibodies were determined by ELISA and are expressed as geometric mean titers (GMTs). Lymphoproliferative and cytokine responses are expressed as stimulation indices (SIs) and picograms per milliliter, respectively.

responses observed after vaccination with L1 VLPs are reassuring, protection from infection and from disease progression will have to occur at the genital tract, where infection occurs. Little is known about the levels of antibody responses or T-cell responses in the genital tract, although recent evidence suggests that HPV-specific antibodies are consistently detected at the cervix after vaccination with HPV L1 VLPs (C. D. Harro and D. R. Lowy, unpublished observations). Also, cervical cytokine responses to HPV-16 L1 have been reported in women infected with HPV-16 [30]. Cervical T cell responses in vaccine recipients were not considered in the current study but deserve future attention, in light of their putative relevance in protection against infection.

Although our findings suggest that HPV-16 vaccination with L1 VLPs induces a robust CMI response to the vaccine, the in vivo role of these responses in both prophylactic and therapeutic vaccination settings has not yet been defined. Issues that remain to be addressed include (1) the determination of what proportion of the measured cytokine responses are mediated by CD4⁺ and CD8⁺T cells, (2) the identification of specific epitopes involved in T cell response to vaccination with HPV-16 L1 VLPs, (3) the determination of whether vaccination with L1 VLPs induces cytotoxic T cell responses targeted against HPV-16-infected cells, and (4) the determination of whether the responses elicited by the vaccine may cross-react with heterologous HPV types. Most important, larger trials are necessary to evaluate whether CMI responses are important modulators of both prophylactic and therapeutic effects of vaccination with L1 VLPs.

Acknowledgments

We thank Marcus Williams (NCI-Frederick/SAIC-Frederick, Inc.) for technical assistance.

References

1. Parkin DM, Pisani P, Ferlay J. Global cancer statistics. *CA Cancer J Clin* **1999**; 49:33–64.
2. Schiffman MH, Bauer HM, Hoover RN, et al. Epidemiologic evidence showing that human papillomavirus infection causes most cervical intraepithelial neoplasia. *J Natl Cancer Inst* **1993**; 85:958–64.
3. Bosch FX, Manos MM, Munoz N, et al. Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. International Biological Study on Cervical Cancer (IBSCC) Study Group. *J Natl Cancer Inst* **1995**; 87:796–802.
4. Breitburd F, Coursaget P. Human papillomavirus vaccines. *Semin Cancer Biol* **1999**; 9:431–44.
5. Walboomers JM, Jacobs MV, Manos MM, et al. Human papillomavirus is a necessary cause of invasive cervical cancer worldwide. *J Pathol* **1999**; 189:12–9.
6. Kurman RJ, Henson DE, Herbst AL, Noller KL, Schiffman MH. Interim guidelines for management of abnormal cervical cytology. The 1992 National Cancer Institute Workshop. *JAMA* **1994**; 271:1866–9.

7. Lowy DR, Schiller JT. Papillomaviruses: prophylactic vaccine prospects. *Biochim Biophys Acta* **1999**; 1423:M1–8.
8. Breitburd F, Kirnbauer R, Hubbert NL, et al. Immunization with viruslike particles from cottontail rabbit papillomavirus (CRPV) can protect against experimental CRPV infection. *J Virol* **1995**; 69:3959–63.
9. Christensen ND, Reed CA, Cladel NM, Han R, Kreider JW. Immunization with viruslike particles induces long-term protection of rabbits against challenge with cottontail rabbit papillomavirus. *J Virol* **1996**; 70:960–5.
10. Kirnbauer R. Papillomavirus-like particles for serology and vaccine development. *Intervirology* **1996**; 39:54–61.
11. Suzich JA, Ghim SJ, Palmer-Hill FJ, et al. Systemic immunization with papillomavirus L1 protein completely prevents the development of viral mucosal papillomas. *Proc Natl Acad Sci USA* **1995**; 92:11553–7.
12. Harro CD, Pang YY, Roden RB, et al. Safety and immunogenicity trial in adult volunteers of a human papillomavirus 16 L1 virus-like particle vaccine. *J Natl Cancer Inst* **2001**; 93:284–92.
13. Zinkernagel RM, Bachmann MF, Kundig TM, Oehen S, Pirchet H, Hengartner H. On immunological memory. *Annu Rev Immunol* **1996**; 14: 333–67.
14. De Bruijn ML, Greenstone HL, Vermeulen H, et al. L1-specific protection from tumor challenge elicited by HPV16 virus-like particles. *Virology* **1998**; 250:371–6.
15. Zhang LF, Zhou J, Chen S, et al. HPV6b virus like particles are potent immunogens without adjuvant in man. *Vaccine* **2000**; 18:1051–8.
16. Guidotti LG, Chisari FV. Noncytolytic control of viral infections by the innate and adaptive immune response. *Annu Rev Immunol* **2001**; 19:65–91.
17. Boyum A. Isolation of mononuclear cells and granulocytes from human blood: isolation of mononuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g. *Scand J Clin Lab Invest Suppl* **1968**; 97:77–89.
18. Lempicki RA, Kovacs JA, Baseler MW, et al. Impact of HIV-1 infection and highly active antiretroviral therapy on the kinetics of CD4⁺ and CD8⁺ T cell turnover in HIV-infected patients. *Proc Natl Acad Sci USA* **2000**; 97:13778–83.
19. Cuzick J. A Wilcoxon-type test for trend. *Stat Med* **1985**; 4:87–90.
20. Da Silva DM, Pastrana DV, Schiller JT, Kast WM. Effect of preexisting neutralizing antibodies on the anti-tumor immune response induced by chimeric human papillomavirus virus-like particle vaccines. *Virology* **2001**; 290:350–60.
21. Evans TG, Bonnez W, Rose RC, et al. A phase 1 study of a recombinant viruslike particle vaccine against human papillomavirus type 11 in healthy adult volunteers. *J Infect Dis* **2001**; 183:1485–93.
22. Peng S, Frazer IH, Fernando GJ, Zhou J. Papillomavirus virus-like particles can deliver defined CTL epitopes to the MHC class I pathway. *Virology* **1998**; 240:147–57.
23. Dupuy C, Buzoni-Gatel D, Touze A, Le Cann P, Bout D, Coursaget P. Cell mediated immunity induced in mice by HPV 16 L1 virus-like particles. *Microb Pathog* **1997**; 22:219–25.
24. Marais D, Passmore JA, Maclean J, Rose R, Williamson AL. A recombinant human papillomavirus (HPV) type 16 L1-vaccinia virus murine challenge model demonstrates cell-mediated immunity against HPV virus-like particles. *J Gen Virol* **1999**; 80 (pt 9):2471–5.
25. Welsh RM, Selin LK. No one is naive: the significance of heterologous T-cell immunity. *Nat Rev Immunol* **2002**; 2:417–26.
26. Lenz P, Day PM, Pang YY, et al. Papillomavirus-like particles induce acute activation of dendritic cells. *J Immunol* **2001**; 166:5346–55.
27. Rudolf MP, Fausch SC, Da Silva DM, Kast WM. Human dendritic cells are activated by chimeric human papillomavirus type-16 virus-like particles and induce epitope-specific human T cell responses in vitro. *J Immunol* **2001**; 166:5917–24.

28. Luxton JC, Rose RC, Coletart T, Wilson B, Shepherd PS. Serological and T-helper cell responses to human papillomavirus type 16 L1 in women with cervical dysplasia or cervical carcinoma and in healthy controls. *J Gen Virol* **1997**; 78(pt 4):917–23.
29. Da Silva DM, Eiben GL, Fausch SC, et al. Cervical cancer vaccines: emerging concepts and developments. *J Cell Physiol* **2001**; 186:169–82.
30. Passmore JA, Burch VC, Shephard EG, et al. single-cell cytokine analysis allows detection of cervical T-cell responses against human papillomavirus type 16 L1 in women infected with genital HPV. *J Med Virol* **2002**; 67:234–40.