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Human Papillomavirus Type 16 Virus-Like Particles Expressed in Attenuated *Salmonella typhimurium* Elicit Mucosal and Systemic Neutralizing Antibodies in Mice

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Attenuated strains of *Salmonella* are attractive live vaccine candidates for eliciting mucosal as well as systemic immune responses. The ability to induce immune responses in the reproductive tract may be critical for the effectiveness of a prophylactic vaccine against genital human papillomaviruses (HPV), which are important etiologic agents in the development of cervical cancer. To examine the potential of a live *Salmonella*-based vaccine to prevent genital HPV infection, the L1 major capsid protein from HPV type 16 (HPV16) was constitutively expressed in the PhoP^c strain of *Salmonella typhimurium*. As demonstrated by electron microscopy, the L1 protein expressed in these bacteria assembled into virus-like particles (VLPs) that resemble authentic papillomavirus virions. This is the first demonstration that papillomavirus VLPs can self-assemble in prokaryotes. BALB/c mice were immunized with the HPV16 L1 recombinant PhoP^c strain by the oral and nasal routes. Despite a low stability of the L1-expressing plasmid in vivo, a double nasal immunization was effective in inducing L1-specific serum antibodies that recognized mainly native, but not disassembled, VLPs. These antibodies effectively neutralized HPV16 pseudotyped virions in an in vitro infectivity assay. Conformationally dependent anti-VLP immunoglobulin A (IgA) and IgG were also detected in oral and vaginal secretions, indicating that potentially protective antibody responses were elicited at mucosal sites. Recombinant attenuated *Salmonella* expressing HPV capsids may represent a promising vaccine candidate against genital HPV infection.

Strains of *Salmonella* that are attenuated, yet invasive, are effective vehicles for delivering heterologous antigens to the mucosal and systemic immune systems. The antigen is delivered by the live *Salmonella* to the mucosal inductive sites where, after priming, antigen-specific B and T cells migrate from the site of induction and mature into effector cells. The migrating immunoglobulin A (IgA)-expressing B cells home to mucosal sites, both local and distant, where they differentiate into IgA-secreting plasma cells (24).

When expressed in the *Salmonella typhimurium* strain PhoP^c, which is attenuated for macrophage survival, the hepatitis B virus core antigen (HBc) spontaneously assembled into core particles. Immunization of mice with the live recombinant bacteria via the nasal or oral route resulted in anti-HBc IgA in oral, rectal, and vaginal secretions. In addition, high levels of systemic anti-HBc IgG were elicited (10). Similarly, mucosal immunization with a live recombinant attenuated strain of *Salmonella typhi* has recently been shown to elicit antibody responses in the genital mucosa of humans (28). Therefore, it might be possible to use attenuated salmonellae to develop single-dose vaccines to control infection by human mucosal pathogens.

Considerable interest has recently been focused on the development of a prophylactic vaccine to prevent genital infection by certain mucosa-tropic human papillomavirus (HPV) types. This interest has been prompted by the determination that sexually transmitted infections with these viruses, most commonly HPV type 16 (HPV16), are etiologically linked to over 90% of cervical cancers (2). Cervical cancer is the second leading cause of cancer deaths in women worldwide. Development of vaccines against the oncogenic genital HPV types have been hampered by the inability to propagate the virus in culture, the lack of animal models for human types, and the paucity of virions in clinical lesions (reviewed in reference 23).

High-level expression of the papillomavirus major virion capsid protein, L1, via heterologous promoters in eukaryotic cells has previously been shown to generate virus-like particles (VLPs) (7, 8, 12, 14, 34, 36). The VLPs are morphologically indistinguishable from the authentic naked icosahedron virions, except that they lack the potentially oncogenic viral genome. These VLPs have elicited high titers of systemic neutralizing antibodies and protection from experimental challenge with infectious virus in animal papillomavirus models (4, 12, 13, 34, 44). Denaturation of the VLPs prior to vaccination abrogated the protective response, indicating that neutralizing antibodies primarily recognize conformational L1 epitopes displayed on intact VLPs or virions (4, 12). VLPs purified from *Saccharomyces cerevisiae* or recombinant baculovirus-infected insect cells might serve as the basis for vaccines to prevent genital HPV infection. However, genital mucosal antibodies were not detected after the vaccination of mice with purified HPV VLPs (6). This class of antibodies may be critical to the effectiveness of a prophylactic vaccine against

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the oncogenic HPVs, since the viruses normally infect the genital mucosa and the benign productive and premalignant precursor lesions induced are confined to that tissue.

In contrast to the VLPs generated in eukaryotic systems, the L1 proteins produced in bacteria were not effective in eliciting neutralizing antibodies or protecting from experimental challenge (31). However, the L1 was isolated in insoluble inclusion bodies and so presumably was denatured and therefore unable to present the critical conformational epitopes displayed on intact virions or VLPs. These experiments suggested that L1 might not have the intrinsic capacity to fold properly or to self-assemble in prokaryotic cells. However, since L1 was truncated or fused to peptides of bacterial proteins in these earlier experiments, the ability of authentic L1 to self-assemble into VLPs in bacteria has not been critically evaluated (1, 11, 15, 31).

The fact that HBc was able to self-assemble and elicit a potent mucosal as well as a systemic immune response after expression in *S. typhimurium* prompted us to investigate whether the L1 of an oncogenic HPV had similar characteristics. Therefore, we have expressed wild-type (14) HPV16 L1 in the PhoP^c strain and examined the lysates for the presence of VLPs. In addition, the production of conformationally dependent and neutralizing antibodies in serum and genital secretions was assessed after nasal or oral immunization of mice with the live recombinant bacteria.

MATERIALS AND METHODS

Plasmid construction and bacterial strains used. Plasmid pFS14nsd HPV16-L1 was constructed by exchanging in the plasmid pFS14 NSD (kindly provided by Florian Schödel, Hôpital Herriot, Lyon, France) (41) the hepatitis B nucleocapsid gene (HBcAg; *Nco*I-*Hin*dIII fragment) with an *Nco*I-*Hin*dIII fragment encoding the HPV16-L1 open reading frame. The HPV16-L1 *Nco*I-*Hin*dIII fragment was generated by PCR with the baculovirus expression plasmid pSynwtVI$^-$ HPV16 114/B-L1+L2 (14) as a template with a 28-mer containing an *Nco*I site, 5′-GGG*CCATGG*CTCTTTGGCTGCCTAGTGA-3′, and a 27-mer containing a *Hin*dIII site, 5′-GGG*AAGCTT*CAATACTTACAGCTTACG-3′. The final construct, containing the Tac promoter, places the HPV16-L1 **ATG** at position +8 relative to the Shine-Dalgarno sequence and introduces a change in the second amino acid, which becomes an alanine instead of the serine encoded by the original sequence. Sequencing of the entire L1 open reading frame was carried out on an automatic sequencer, and no further nucleotide changes were observed. Plasmid pFS14nsd HPV16-L1 was amplified in *Escherichia coli* JM105 and then electroporated as described previously (37) into *S. typhimurium* CS022. This strain was kindly provided by John Mekalanos (Harvard Medical School, Boston, Mass.); it is derived from the ATCC 14028 strain, into which the pho-24 mutation was introduced by P22 transduction, resulting in attenuation in both virulence and survival within macrophages in vitro (PhoP^c) (26). The resultant recombinant strain is called PhoP^c/HPV16 hereafter.

Expression of HPV16 L1 in *Salmonella* and VLP purification. After overnight growth at 37°C the recombinant bacteria were lysed by boiling in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis buffer containing 5% SDS. The lysates were separated on SDS–10% polyacrylamide gel electrophoresis gels, and expression of L1 was analyzed by Western blot with HPV16 L1 monoclonal antibody (MAb) CAMVIR-1 (25) (kindly provided by Jian Zhou, University of Queensland, Woolloongabba, Australia) as the primary antibody, an alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma) as the secondary antibody, and BCIP (5-bromo-4-chloro-3-indolylphosphate)-NBT (nitroblue tetrazolium chloride) (Boehringer) as the substrate.

To prepare VLPs, bacteria were lysed by sonication and the lysate was fractionated on a 10 to 40% sucrose gradient in phosphate-buffered saline (PBS) containing 1 M NaCl for 1 h at 40,000 rpm with a TST41.14 rotor. Fractions of the gradient were then analyzed for the presence of the L1 protein by Western blotting. The high-sedimentation fractions containing the L1 protein were pooled and dialyzed against PBS–0.5 M NaCl. VLPs were pelleted for 1 h at 50,000 rpm with a TST65.1 rotor, adsorbed to carbon-coated grids, negatively stained with phosphotungstic acid, and examined with a Philips electron microscope.

Purification of HPV16 VLPs expressed in insect cells from a recombinant baculovirus. The transfer vector pSynwtVI$^-$ HPV16 114/B-L1+L2 (14) was cotransfected with the linearized genome of baculovirus (Baculo-Gold; Pharmingen) by the calcium-phosphate method into SF9 cells. The recombinant baculoviruses were plaque purified and propagated by standard methods (30). Baculovirus-derived HPV16 VLPs were purified as described previously (14).

Immunization and sampling of mice. For each immunization, a single colony of PhoP^c/HPV16 was grown for 18 h at 37°C in 50 ml of Luria-Bertani broth with ampicillin (100 μg/ml). After 10 min of centrifugation at 6,000 × *g*, the bacterial pellet was resuspended in 1 ml of PBS. The bacterial suspension contained 0.5×10^{11} to 5×10^{11} CFU, as estimated by plating dilutions. A 20-μl portion of the bacterial suspension was used for oral immunization. For nasal immunization, the bacterial suspension was diluted at 1/20 in PBS and a 20-μl portion was used.

Six-week-old female BALB/c mice were used in all experiments. For oral immunization, the mice were fed 30 μl of 10% sodium bicarbonate to neutralize stomach acidity 5 min prior to immunization and then were given 20 μl of inoculum. For nasal immunization, mice were anesthetized by intraperitoneal injection with 100 μl (per 10 g of body weight) of both 0.2% xylazine hydrochloride (Rompun; Bayer) in PBS and 10 mg of ketamine hydrochloride (Ketavet; Parke-Davis) per ml. The inoculum (20 μl) was introduced dropwise into one nostril. Blood, saliva, and genital samples were taken as described previously (10). All samples were stored at −70°C.

Recovery of *S. typhimurium* from immunized mice. Mice were killed 2 weeks after immunization by inhalation of CO_2. Lungs, spleens, Peyer's patches, and mesenteric and cervical lymph nodes were excised, rinsed in PBS, and either homogenized in a Dounce homogenizer (lungs and spleens) or dissociated between two glass slides into 2 ml of 15% sucrose in PBS. One hundred microliters of the lysed cell suspension was plated onto Luria-Bertani agar plates containing BCIP (50 μg/ml; Sigma) with and without ampicillin (100 μg/ml). BCIP in the medium was used to identify PhoP^c salmonellae, which form dark-blue colonies due to their high phosphatase activity. Only the PhoP^c salmonellae bearing the L1-expressing plasmid are ampicillin resistant and will form dark-blue colonies on BCIP plus ampicillin plates. For each survival experiment, three mice were used and the geometric means with their standard errors of the means are shown (see Fig. 5). The minimal amount of salmonellae detectable is 20 CFU/organ (one colony per plate); therefore, when no colony was identified in a plate the results are shown as $<\log_{10} 1.3$.

ELISA. The amount of total IgA, anti-lipopolysaccharide (LPS) IgA, and IgG antibodies in samples was determined by enzyme-linked immunosorbent assay (ELISA) as described previously (10). For the measurement of total IgG, the microtiter plates were coated with 100 ng of rabbit anti-mouse Ig (Boehringer) in carbonate buffer (pH 9.6) per well, a biotinylated rabbit anti-mouse IgG (Amersham) diluted 1:500 was used as the second antibody, and mouse IgG (1 mg/ml; Sigma) was used as the standard to calculate the amount of total IgG in the samples. For the anti-HPV16 VLPs, ELISA plates were coated with 10 ng of a preparation of baculovirus-derived HPV16 VLPs in PBS (total protein content was determined with a Bio-Rad protein assay with bovine serum albumin as the standard). This amount of VLPs was saturating in our ELISA test. End point dilutions of samples were carried out. The specific IgA or IgG amounts are expressed as the reciprocal of the highest dilution that yielded an optical density at 492 nm (OD_{492}) four times that of preimmune samples. These reciprocal dilutions were normalized to the amount of total IgA or IgG in saliva and genital washes (10). ELISA plates were also coated with 10 ng of baculovirus-derived HPV16 VLPs in 0.2 M carbonate buffer, pH 9.5, to determine the titer of antibodies recognizing unfolded VLPs (6). The preimmune samples always yielded an OD_{492} lower than 0.010. Secretions from mice immunized with a recombinant PhoP^c strain expressing an unrelated antigen (HBc) yielded no detectable cross-reactivity with the HPV antigen, while sera from these mice yielded a low cross-reactivity with the HPV antigen (a titer of 50 to 100 on VLP-PBS- and VLP-carbonate-coated plates). This low background is probably due to the high titers of anti-HBc IgG found in those sera (about 10^6) (10).

In vitro HPV16 neutralization assay. Infectious pseudotyped virions consisting of the HPV16 capsid, made of L1 and L2, surrounding the bovine papillomavirus type 1 (BPV1) genome, designated HPV16{BPV1}, were generated as previously described (33). Briefly, BPHE-1 hamster cells harboring autonomously replicating BPV1 genomes were coinfected with defective recombinant Semliki Forest viruses that expressed the L1 and L2 capsid genes of HPV16. Infectious pseudotype HPV16 in cell extracts was quantitated by the induction of transformed foci in monolayers of mouse C127 cells. Neutralizing activity was measured after preincubation of the cell extracts with mouse sera diluted 1:50 (final volume, 1.0 ml) in culture medium. Preimmune serum (see Fig. 6E) and week 5 sera from three mice that had received a single nasal immunization with the PhoP^c/HBc strain (anti-HBc IgG titer of 800,000) were used as negative controls. Mouse MAbs H16.E70 and B1.A1 (a gift of N. Christensen, Milton S. Hershey Medical Center, Hershey, Pa.) were generated against recombinant baculovirus-expressed HPV16 L1 VLPs and BPV1 virions, respectively, and used at a 1:100 dilution. H16.E70 and B1.A1 served as positive and negative controls for HPV16 {BPV1} neutralization, respectively.

RESULTS

HPV16 L1 expressed in PhoP^c assembles into VLPs. The open reading frame encoding the major protein, L1, of HPV16 was cloned in place of the HBc open reading frame in the plasmid pFS14 NSD (40). L1, under the control of the Tac promoter, is constitutively expressed in *S. typhimurium*. A 57-

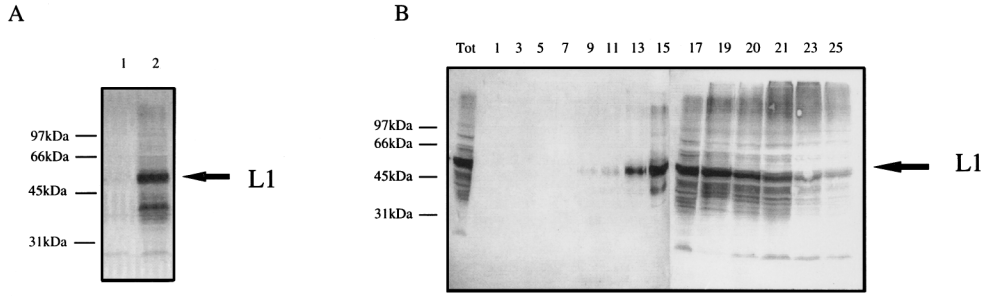

FIG. 1. HPV16 L1 expression in the PhoP^c/HPV16 strain. *Salmonella* were grown overnight and prepared as indicated in Materials and Methods. Immunoblots with anti-HPV16 L1 MAb of a PhoP^c (lane 1) or PhoP^c/HPV16 (lane 2) lysate (A) and of the total (Tot) and fractionated (lanes 1 to 25) PhoP^c/HPV16 lysate (B) are shown. Fractionation was performed through a 10 to 40% sucrose gradient with the heavier fraction of the gradient in lane 1. The 57-kDa protein band identified as L1 is indicated by the arrows.

kDa protein detected in the lysate of PhoP^c/HPV16 overnight cultures was identified as HPV16 L1 by Western immunoblotting with an anti-HPV16 L1 MAb (CAMVIR-1 [25]) (Fig. 1). Lower- and higher-molecular-mass minor bands were often observed. Some might be degradation products or aggregates of HPV16 L1, since the CAMVIR1 MAb did not recognize such bands in a total lysate of PhoP^c (Fig. 1A). To determine whether the L1 protein expressed by PhoP^c/HPV16 assembled into VLPs, the bacterial lysate was fractionated through a 10 to 40% sucrose gradient and the heavier fractions, containing the L1 protein (Fig. 1B), were analyzed by electron microscopy. Typical spherical particles were recovered from the bacterial preparation (Fig. 2A). However, the bacterial VLPs appeared less homogeneous in size, with diameters ranging from 40 to 55 nm (Fig. 2A), than the ~55-nm particles generated in insect cells (Fig. 2B).

Two nasal immunizations with the PhoP^c/HPV16 strain induce systemic and mucosal antibody responses. Since nasal immunization with recombinant *S. typhimurium* was shown to elicit strong vaginal secretory IgA responses against the expressed foreign antigen (10), we first immunized three mice nasally with the PhoP^c/HPV16 strain (5×10^7 CFU). Samples of blood, saliva, and vaginal washes were taken 0, 2, 4, and 6 weeks after immunization. The immune responses against both the carrier, i.e., anti-LPS, and the carried, i.e., anti-HPV16 VLP, antigens were determined, and the geometric mean titers are shown in Fig. 3. Serum HPV16 VLP-specific IgGs were detected after 2 weeks in one mouse and after 4 weeks in all mice. The antibody response peaked after 6 weeks at relatively low titers and persisted at least until week 14. At that time, no HPV16 VLP-specific antibodies were detected in vaginal secretions, although one mouse had low titers of IgA in the saliva. The systemic and the mucosal immune responses against LPS were relatively low (Fig. 3) but similar to those elicited by the PhoP^c/HBc strain in a previous study (10), suggesting a normal infection of the mice by *S. typhimurium* PhoP^c/HPV16. The low anti-LPS response observed after nasal immunization prompted us to perform a booster immunization. Thus, a second nasal immunization was performed at week 14 with 5×10^8 CFU, and samples were taken 5 and 10 weeks later (weeks 19 and 24, respectively). The mice were sacrificed and bled at week 27. The second immunization induced, 5 weeks later (week 19), a 15-fold increase of anti-HPV16 VLP IgG in the sera, as well as anti-HPV16 VLP IgA in the vaginal washes (Fig. 3), from the three mice. Anti-HPV16 VLP IgG was also found in vaginal washes, but only in two mice at week 19, and titers were again almost undetectable at week 24 (Fig. 3). Anti-HPV16 VLP IgA and IgG were also found in the saliva of the three mice in amounts comparable to or slightly higher than those found in the vaginal washes.

Oral versus nasal immunization with PhoP^c/HPV16. Since only a double nasal immunization efficiently induced HPV16 VLP-specific antibodies in the previous experiment, we compared the oral and nasal routes of immunization with two doses of inoculum. Three and four mice were immunized by the oral and the nasal route, respectively, at week 0 (2.2×10^9 CFU oral and 1.1×10^8 CFU nasal) and at week 2 (4×10^9 CFU oral and 2×10^8 CFU nasal). Higher doses were used for the oral route, as we have previously shown with the PhoP^c/HBc strain that doses of 10^9 CFU were effective by the oral route while they were lethal by the nasal route (10). A double nasal immunization again induced anti-HPV16 VLP IgG in serum and IgA in secretions, while oral immunization was less effective (Fig. 4). The geometric means are shown in Fig. 4. It should be noted that only one mouse, out of three orally immunized, exhibited HPV16 VLP-specific IgG in the serum and IgA in the saliva and in genital secretions. It had titers similar to those induced by nasal immunization. The three orally immunized mice had detectable anti-LPS antibodies in serum and secretions, although only two exhibited high titers. This variability in the immune response against both LPS and the foreign antigen was previously observed for oral immunization with PhoP^c/HBc (10).

Plasmid stability in vivo. Overall, the antibody titers against the foreign antigen induced by PhoP^c/HPV16, compared to those induced by PhoP^c/HBc, were about 10-fold lower (10). Assuming similar sensitivities of the ELISAs, this could reflect differences in antigenicity between the two viral antigens (38) or, more likely, differences in plasmid stability. We examined the latter possibility by investigating the survival of *S. typhimurium* after immunization. For this purpose, we analyzed relevant mouse organs 2 weeks after immunization by the oral or nasal route. These organs were chosen to be representative of immediate-to-late sites of *Salmonella* invasion and persistence. Specifically, Peyer's patches, mesenteric lymph nodes, and spleens were tested after oral immunization and lungs, cervical lymph nodes, and spleens were tested after nasal immunization. Peyer's patches were also examined after nasal immunization because part of the inoculum is usually ingested with this type of immunization (10). We chose to do the analysis two weeks after immunization because we had previously determined, with the PhoP^c/HBc strain, that that time corresponded to a peak titer of persisting attenuated salmonellae in the different organs (unpublished results). Overall, the total numbers of salmonellae persisting in the different organs after each type of immunization with PhoP^c/HPV16 were similar to

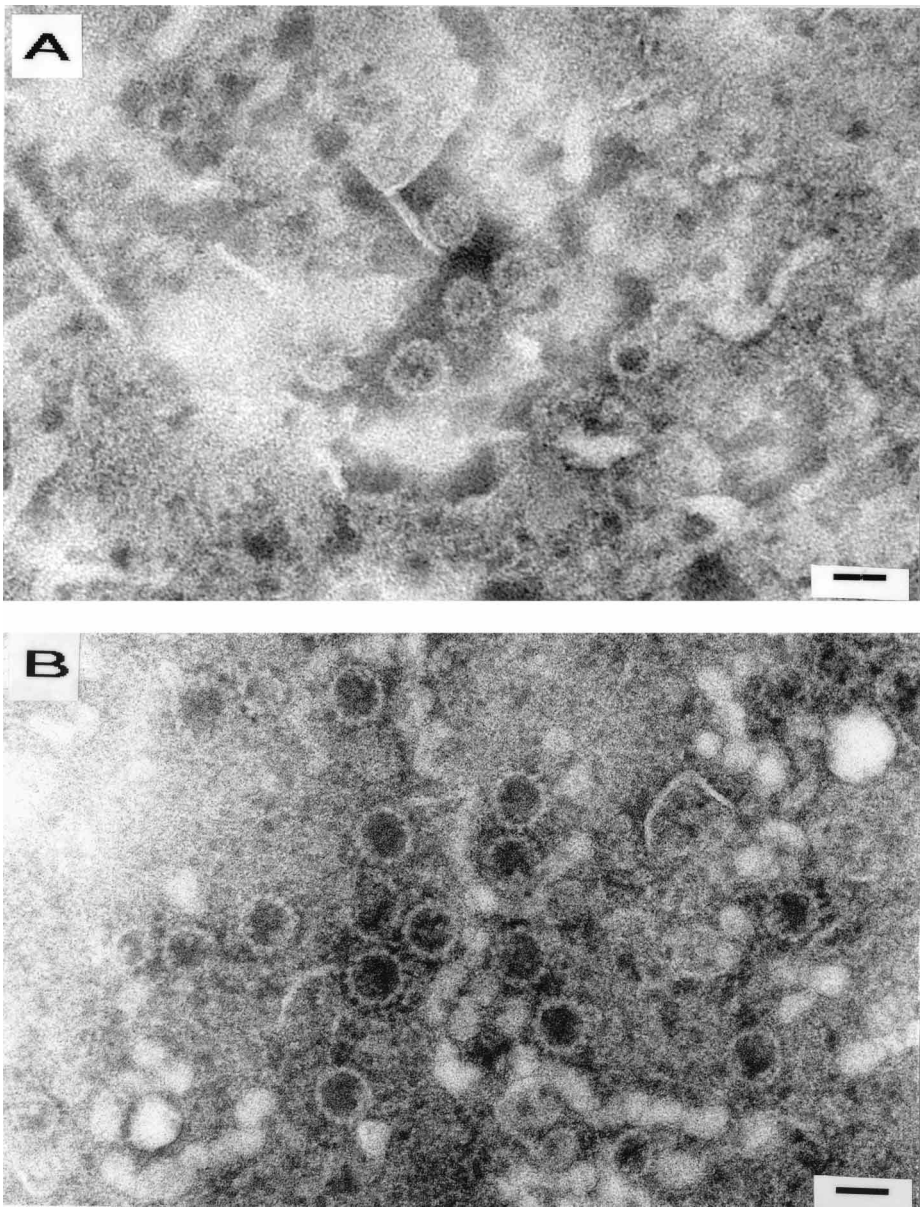

FIG. 2. HPV16 L1 assembles into VLPs. Electron micrographs of PhoP^c/HPV16 VLPs (A) and baculovirus-derived HPV16 VLPs (B). Fractions 7 to 13 of PhoP^c/HPV16 lysate (Fig. 1) are pooled in panel A. The samples were negatively stained with phosphotungstic acid. Bars, 53 nm.

those previously found with the PhoP^c/HBc strain. Unexpectedly, and in contrast to the HBc recombinant plasmid, the plasmid carrying the HPV16 L1 gene was unstable in vivo. Two weeks after nasal immunization, only a few percent (3% in the lungs, 10% in cervical lymph nodes, 6% in Peyer's patches, and 0.3% in the spleens) of the persisting salmonellae still harbored the L1-expressing plasmid (Fig. 5A). Even worse, 2 weeks after oral immunization, no *Salmonella* harboring the plasmid was detected in any organs (Fig. 5B).

Anti-HPV16 VLP antibodies predominantly recognize conformational epitopes. In order to determine whether the immune responses induced by the PhoP^c/HPV16 strain predominantly generated antibodies directed primarily against native or denatured VLPs, we measured by ELISA (Table 1) the binding of antibodies from the nasally immunized mice to baculovirus-derived VLPs in PBS (native VLPs) or in carbonate buffer (pH 9.5) (disassembled VLPs) (6). The specific IgG or IgA elicited by the PhoP^c/HPV16 strain very poorly recognized denatured VLPs, suggesting that the majority of L1 proteins were folded into highly ordered structures when expressed in PhoP^c/HPV16.

In vitro neutralizing activity of the immune sera. In previous studies of baculovirus-derived VLPs, neutralizing activity and protection from experimental infection generally correlated with ELISA reactivity to native VLPs. We therefore wished to determine if the conformationally dependent anti-VLP antibodies elicited by the live *Salmonella* vaccine were also neutralizing. Although no infectivity assay or source of virus currently exists for authentic HPV16, it has recently been demonstrated that the HPV16 capsid proteins can encapsidate autonomously replicating BPV1 genomes, resulting in HPV16 {BPV1} pseudotyped virions whose infectivity can be moni-

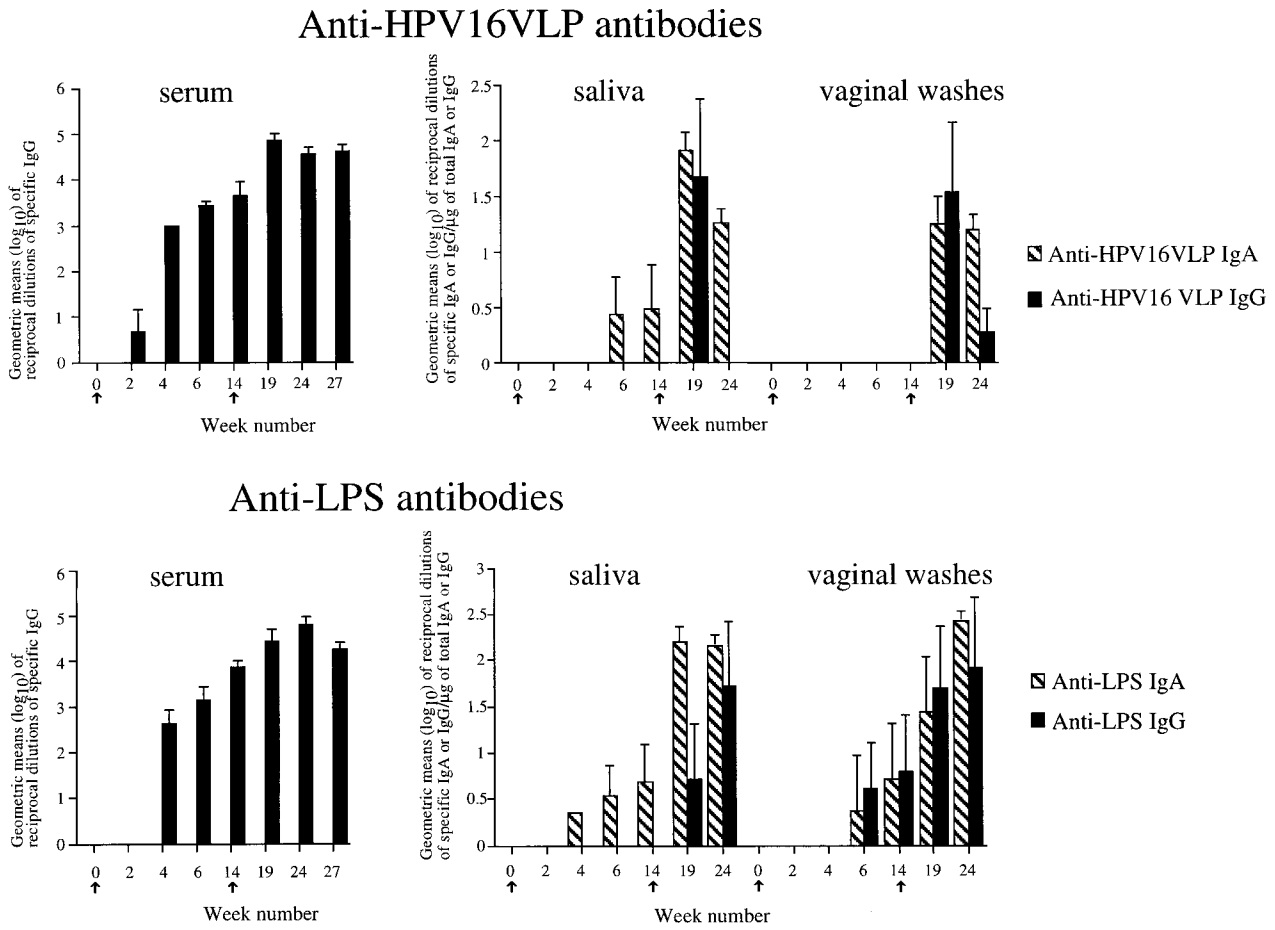

FIG. 3. Anti-HPV16 VLP and anti-LPS systemic and mucosal antibody responses after nasal immunization with the PhoP^c/HPV16 strain. Three 6-week-old BALB/c female mice were immunized with 5×10^7 CFU at week 0 and 5×10^8 CFU at week 14 (indicated by arrows). Data are expressed as the geometric means (log_{10}) of the reciprocal dilutions of specific IgG in serum and specific IgA per microgram of total IgA or IgG per microgram of total IgG in secretions. Error bars indicate the standard errors of the means.

tored by focal transformation of cultured mouse fibroblasts (33). We therefore used the HPV16{BPV1} infectivity assay to examine the neutralizing activity of the mouse sera generated after nasal immunization. Each of the three immune sera displayed strong neutralizing activity against HPV16{BPV1} (Fig. 6) but did not neutralize BPV1 virions (data not shown). Neither the preimmune sera (Fig. 6E; 70 transformed foci) nor the sera from PhoP^c/HBc-immunized mice (72 transformed foci) (data not shown) exhibited neutralizing activity. The neutralizing activities of the immune sera appeared to correlate with the titers measured in the native VLP ELISA (Table 1), although the sera were only tested at a single dilution (1:50).

DISCUSSION

Prophylactic vaccines based on injection of purified papillomavirus VLPs have been shown to induce type-specific protection from experimental infection by cottontail rabbit papillomavirus or canine oral papillomavirus in domestic rabbits or dogs, respectively (4, 44). Passive-transfer experiments indicated that protection was due to the production of virion-neutralizing antibodies. Since HPV16 VLP-specific antibodies raised in rabbits neutralized HPV16{BPV1} pseudovirions in vitro, one would expect that HPV16 VLP-specific antibodies in cervical secretions could likewise prevent sexually transmitted infection by HPV16 in women. However, it is difficult to predict whether secretory IgA produced by mucosal lymphoid tissues, transuded serum IgG, or a combination of the two will be most effective in preventing genital HPV infection (3). Therefore, induction of both local and systemic antibody responses may be desirable in a prophylactic genital HPV vaccine.

Recombinant and attenuated enteropathogenic bacteria, such as *Salmonella*, may represent ideal antigen delivery systems, since they efficiently cross all mucosal surfaces to gain access to both mucosa-associated lymphoid tissue and draining lymph nodes (32). They exploit the two basic sampling systems mediating uptake of mucosally administered antigens, M cells in simple epithelia and dendritic cells in both simple and stratified epithelia (29). Two recent studies have analyzed the immune response to HPV16 E6 and E7 after vaccination with live *S. typhimurium* expressing these oncoproteins (16, 22).

We have previously used an *S. typhimurium* strain attenuated for macrophage survival, because long-lasting antibody responses were elicited by a single nasal, oral, rectal, or vaginal administration of recombinant bacteria expressing HBc as a foreign antigen (10). In that study, the best genital responses were obtained after nasal immunization. In the airways, antigen uptake occurs through M cells found in the nasopharyngeal lymphoid tissue (17) and the bronchus-associated lym-

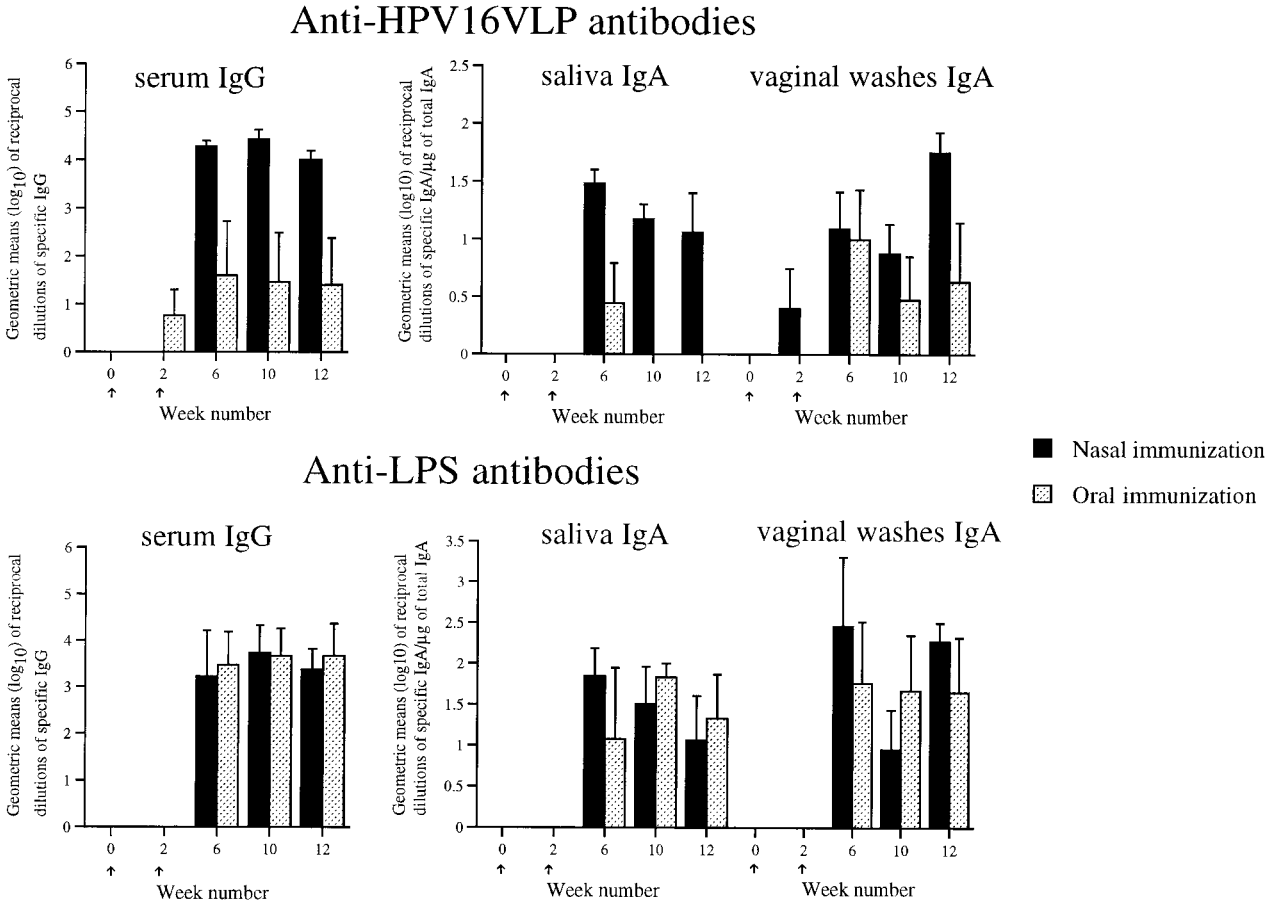

FIG. 4. Anti-HPV16 VLP and anti-LPS systemic IgG and mucosal IgA after oral and nasal immunization with the PhoP^c/HPV16 strain. Six-week-old BALB/c female mice were immunized at week 0 and at week 2 (indicated by arrows) as described in Materials and Methods. Data are expressed as the geometric means (log_{10}) of reciprocal dilutions of specific IgG in serum and specific IgA per microgram of total IgA in secretions. Error bars indicate the standard errors of the means.

phoid tissue (42). The primed IgA-expressing lymphocytes then migrate into cervical and uterine tissues, where they produce polymeric IgA antibodies, which are transported across the epithelium by the polymeric Ig receptor (18–20). Intraepithelial dendritic cells in the bronchial epithelium also play a major role in antigen presentation by taking up the antigens in the respiratory epithelium and carrying them to distant draining lymph nodes, where priming occurs (9). This probably explains why nasal immunization is so efficient in triggering both local and systemic antibody responses. Furthermore, after oral immunization the salmonellae have to survive the gastric environment before reaching their site of invasion, i.e., the Peyer's patches in the intestine. This might explain the relatively high dose threshold below which oral immunization with PhoP^c/HBc is not effective (10^8 CFU) (10) and the variability observed among the inoculated animals. In this study, we have shown that the L1-expressing plasmid was unstable in the absence of selective pressure in vivo. This suggests that the L1 protein is toxic for the bacteria, although it is unclear why such a structural protein should be deleterious. The fact that no *Salmonella* harboring the plasmid was recovered two weeks after oral immunization together with the high dose required for that route of immunization might explain the sporadic success of oral immunization, with one out of three mice exhibiting anti-HPV16 VLP antibodies. To increase the stability of the plasmid, we are currently recloning the L1 gene in β-aspartate semialdehyde dehydrogenase-based vectors, which maintain plasmid selection in vivo (27, 43).

Previous data suggested that to produce HPV-neutralizing antibodies with recombinant *Salmonella* it would be essential

TABLE 1. Titers of IgG (in serum) or IgA (in vaginal washes) against native and unfolded HPV16 VLP in mice immunized with PhoP^c/HPV

Sample	Titer against[a]:	
	HPV16 VLP	Unfolded HPV16 VLP[b]
No. 4 serum (week 27)	60,000	100
No. 5 serum (week 27)	80,000	200
No. 6 serum (week 27)	20,000	100
CAMVIR-1[c]	16,000	80,000
No. 4 vaginal washes (week 19)	40	<1
No. 5 vaginal washes (week 19)	20	<1
No. 6 vaginal washes (week 19)	40	1

[a] Titers are expressed as the reciprocal of the highest sample dilution that yielded an OD_{492} four times that of the preimmune sample.
[b] ELISA plates were coated with VLP in carbonate buffer, pH 9.5.
[c] Anti-HPV16 L1 monoclonal IgG (35 μg/ml) (25) which was used as a positive control.

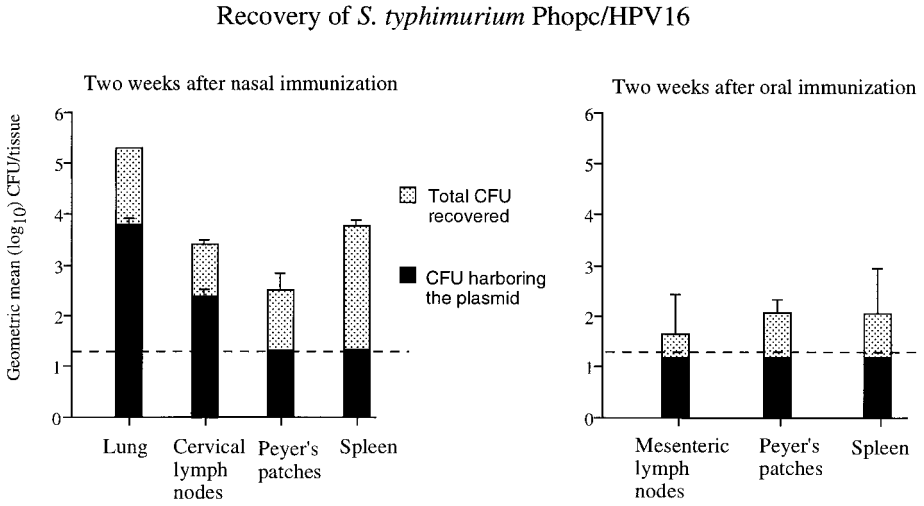

FIG. 5. Recovery of *S. typhimurium* PhoP^c/HPV16 two weeks after nasal and oral immunization. Six-week-old BALB/c mice were immunized with 2×10^9 CFU by the oral route and with 5×10^7 CFU by the nasal route. The different organs were prepared and plated on BCIP plates with (black column) and without (shaded column) ampicillin as described in Materials and Methods. Three mice were used for each experiment, and data are expressed as the geometric means ($\log_{10}$) of the CFU/organ. The level of detection ($\log_{10}$ 1.3) is indicated by a horizontal dashed line, and values below the level of detection are shown below that line (see text for details). Error bars indicate the standard errors of the means.

that the expressed L1 protein assume its native conformation and assemble into VLPs (4, 12). Although papillomavirus VLPs have been shown to assemble in diverse eukaryotic cell types (7, 8, 12, 14, 34, 36), assembly has not previously been demonstrated in prokaryotes. L1 fusion proteins have mainly been expressed in bacteria (1, 11, 15, 31), and when bona fide L1 proteins were expressed, VLP assembly was not examined (5). In this study, we have demonstrated that HPV16 VLPs do assemble in *Salmonella*, probably because of the high-level expression of wild-type L1 and because neither glycosylation of L1 nor eukaryotic chaperons are required for capsid assembly (46). Capsid production in bacteria has also been reported for other viruses, such as the nucleocapsid of hepatitis B virus (39). In addition, polyomavirus VP1 major capsid protein forms capsomers when expressed in *E. coli*, which subsequently self-assemble into VLPs in vitro (21, 35).

Nasal immunization with PhoP^c/HPV16 induced systemic and mucosal antibodies that mostly or exclusively recognized native HPV16 VLPs. In contrast, HPV16 VLPs isolated from recombinant baculovirus- and vaccinia-expressing cells elicit substantial titers of serum antibodies to both native and denatured VLPs (45). This may reflect defects in protein folding that eventually result from the cytopathic effects of these recombinant viruses, the extremely high levels of protein expression, or partial denaturation of L1 during purification of the VLPs. Furthermore, recombinant vaccinia expressing HPV1 capsid protein also triggered serum antibodies recognizing both folded and unfolded VLPs, probably reflecting different

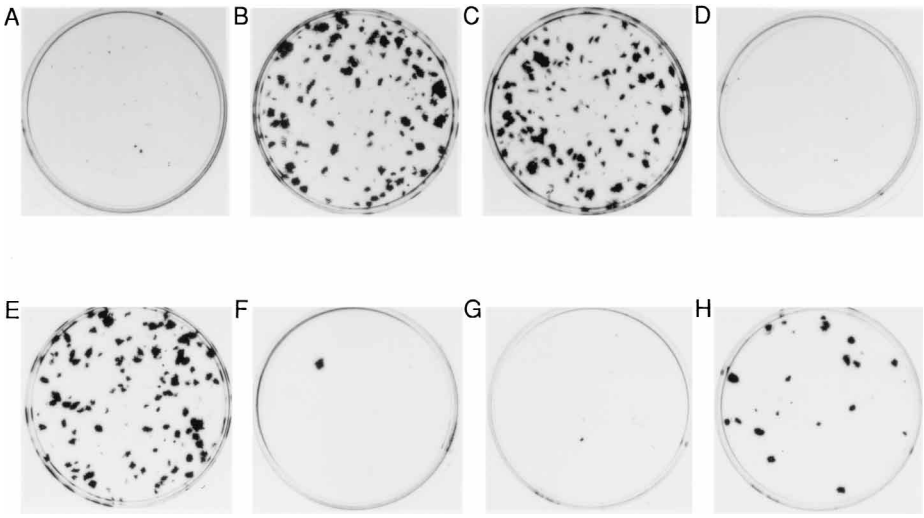

FIG. 6. In vitro neutralization of HPV16 pseudotype virus infection of mouse C127 cells by serum from PhoP^c/HPV16-immunized mice. (A) No virus added. (B to H) Equal aliquots of an HPV16{BPV1} pseudotype virus containing extract were added. The aliquots were preincubated with no antibodies (B), BPV1-neutralizing MAb B1.A1 (C), HPV16{BPV1}-neutralizing MAb H16.E70 (D), mouse preimmune serum (E), mouse no. 4 immune serum (week 27) (F), mouse no. 5 immune serum (week 27) (G), and mouse no. 6 immune serum (week 27) (H). Sera no. 4, 5, and 6 had anti-HPV16 VLP IgG titers of 60,000, 80,000, and 20,000, respectively.

modes of viral protein expression, and low HPV-specific genital IgA antibody titers (6), as expected with a nonmucosal route of immunization.

In summary, our results provide evidence that an attenuated *Salmonella* strain expressing the major capsid protein of HPV16 represents a promising vaccine candidate against HPV16 infection. Conformationally dependent VLP-specific antibodies, which correlate with protection from experimental challenge in animal virus models, were generated both systemically and locally at genital and oral mucosal sites. Consistent with the idea that the L1-specific antibodies generated after vaccination with live recombinant *Salmonella* are potentially protective was the finding that serum from the vaccinated animals effectively neutralized HPV16 pseudovirus infection in vitro. Attenuated strains of *S. typhi* are under development for use in humans. If safety concerns can be adequately addressed, one of these strains might be an ideal vehicle for delivery of a genital HPV vaccine, since the vaccine could be cheaply produced and could potentially induce long-lasting protection after a single inoculation. These characteristics are particularly desirable in a vaccine targeted for use in developing countries, where cervical cancer is the leading cause of cancer-related deaths in women.

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