Salmonella enterica Serovar Typhi Ty21a Expressing Human Papillomavirus Type 16 L1 as a Potential Live Vaccine against Cervical Cancer and Typhoid Fever

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Received 17 April 2007/Returned for modification 6 June 2007/Accepted 29 July 2007

Human papillomavirus (HPV) vaccines based on L1 virus-like particles (VLPs) can prevent HPV-induced genital neoplasias, the precursors of cervical cancer. However, most cervical cancers occur in developing countries, where the implementation of expensive vaccines requiring multiple injections will be difficult. A live Salmonella-based vaccine could be a lower-cost alternative. We previously demonstrated that high HPV type 16 (HPV16)-neutralizing titers are induced after a single oral immunization of mice with attenuated Salmonella enterica serovar Typhimurium strains expressing a codon-optimized version of HPV16 L1 (L1S). To allow the testing of this type of vaccine in women, we constructed a new L1-expressing plasmid, kanL1S, and tested kanL1S recombinants of three Salmonella enterica serovar Typhi vaccine strains shown to be safe in humans, i.e., Ty21a, the actual licensed typhoid vaccine, and two highly immunogenic typhoid vaccine candidates, Ty800 and CVD908-htrA. In an intranasal mouse model of Salmonella serovar Typhi infection, Ty21a kanL1S was unique in inducing HPV16-neutralizing antibodies in serum and genital secretions, while anti-Salmonella responses were similar to those against the parental Ty21a vaccine. Electron microscopy examination of Ty21a kanL1S lysates showed that L1 assembled in capsomers and capsomer aggregates but not well-ordered VLPs. Comparison to the neutralizing antibody response induced by purified HPV16 L1 VLP immunizations in mice suggests that Ty21a kanL1S may be an effective prophylactic HPV vaccine. Ty21a has been widely used against typhoid fever in humans with a remarkable safety record. These findings encourage clinical testing of Ty21a kanL1S as a combined typhoid fever/cervical cancer vaccine with the potential for worldwide applicability.

Human papillomavirus (HPV) infection, most often HPV type 16 (HPV16), is considered to be a necessary factor for the development of cervical cancer, with an estimated worldwide annual mortality of 250,000 (7). Given the high prevalence of HPV infection in women and the lack of antiviral agents against HPV, the development of a prophylactic HPV vaccine has been a long-sought strategy to prevent cervical cancer (28). It has been shown that the major papillomavirus capsid protein L1 has the intrinsic ability to self-assemble into virus-like particles (VLPs) that resemble the HPV virion but are noninfectious because they lack the viral genome. HPV vaccines based on these VLPs have proven to be well tolerated, highly immunogenic, and able to prevent the development of HPV16-induced cervical intraepithelial neoplasia in ongoing clinical trials (reviewed in reference 29). One VLP-based vaccine, Gardasil, has been approved for general use in young women in many countries. However, these expensive vaccines are administered in three intramuscular injections over 6 months, which will make access to these vaccines problematic in developing countries, where most cases of cervical cancer occur (38). It is thus of great importance to develop other strategies that have worldwide applicability.

Live attenuated Salmonella strains may be effective antigen delivery systems, as they are able to express foreign antigens and elicit mucosal as well as systemic immune responses against homologous and heterologous antigens after oral vaccination (reviewed in references 13, 26, and 45). We previously reported the induction of high HPV16-neutralizing titers after a single oral immunization of mice with attenuated Salmonella enterica serovar Typhimurium strains expressing a plasmid harboring an L1 codon optimized for expression in Salmonella, L1S (5). In this study, the Salmonella-based vaccine against HPV16 was further refined to make it appropriate for testing in women. First, the ampicillin selection marker used for plasmid maintenance was replaced by a kanamycin resistance gene, which is more suitable for use in humans given its superior biosafety record and given that it was approved by the FDA in 1994 for use in plants (14). The resulting plasmid was tested in three Salmonella enterica serovar Typhi vaccine strains that have been shown to be safe in humans, i.e., Ty21a (17), the actual licensed typhoid vaccine, as well as two highly immunogenic typhoid vaccine candidate strains, i.e., Ty800 (19) and CVD908-htrA (48). Salmonella enterica serovar Typhi vaccine strains have often been directly tested in human volunteers because they do not infect other hosts by the oral route. However, mice can be transiently infected if high doses of these bacteria are administered by the nasal route (15). In the
present study, this unique animal model was used to compare the immune responses elicited by these three recombinant *Salmonella enterica* serovar Typhii vaccine strains against homologous and heterologous antigens.

**MATERIALS AND METHODS**

**Plasmid construction and bacterial strains used.** In plasmid pFS14nsd HPV16-L1S (5), the amplicin resistance coding sequence was replaced by a kanamycin resistance coding sequence as follows. A SacI-XbaI fragment containing the kanamycin coding sequence and promoter was generated by PCR using PET-9a (Novagen) plasmid DNA as template. The primers used were a 25-mer primer located 54 nucleotides upstream from the first ATG of ampicillin and containing a SacI restriction site (underlined), 5'-GGGGCCCCGGGTGTC ATGAAACATAA-3', and a 28-mer primer containing an XbaI restriction site (underlined), 5'-GGGGCTAGAAGCTGTCAAACATGAGAAT-3'. Another large SacI-XbaI fragment containing the entire pFS14nsd HPV16-L1S plasmid sequence, without the ampicillin resistance gene, was generated by inverse PCR with Expand High Fidelity PCR (Roche Molecular Biochemicals) with the following primers: a 26-mer primer located 92 nucleotides upstream from the ATG of ampicillin and containing a SacI site (underlined), 5'-GGGGCCCCGGGTGTC TAGAAACCCGAAGG-3', and a 28-mer primer containing a XbaI site (underlined) and the stop codon of ampicillin (boldface type), 5'-GGGGCTAGATCTAATGCAAAACGTGAAG-3'. These two SacI-XbaI fragments were ligated together to generate plasmid pFS14nsd-kan3-HPV16-L1S. A correct nucleotide sequence was confirmed by sequencing the full plasmid. The new plasmid was introduced by electroporation (46) into the attenuated *Salmonella enterica* serovar Typhimurium PhoP (CS022) (32), PhoP (CS015) (31) (both kindly provided by John Mekalanos, Boston, MA), and AaroA (SL7207) (20) (kindly provided by Irene Cortes-Théaul, Lausanne, Switzerland) strains and into the attenuated *Salmonella enterica* serovar Typhi Ty2800 (19) (kindly provided by the Virus Research Institute, Cambridge, MA), CVD908-intei (48) (kindly provided by Myron Levine, Baltimore, MD), and Ty21a (17) (Berna Biotech, Switzerland) strains. The original pFS14nsd HPV16-L1S plasmid was also introduced into the three attenuated *Salmonella enterica* serovar Typhi vaccine strains for comparative purposes.

**HPV16 L1 expression and analysis of assembly state.** Expression of L1 in *Salmonella* lysates was analyzed by Western blotting as previously described (35) by using the anti-HPV16 L1 monoclonal antibody CAMVIR-1 (Anawa). The content in HPV16 VLP equivalents was measured by a sandwich enzyme-linked immunosorbent assay (ELISA) as previously described (6) by using two monoclonal antibodies (H16E70 or H161A and H16V5) (kindly provided by N. D. Christensen, Hershey, PA), which recognize conformational epitopes on HPV16 VLPs (11).

The L1 assembly state was analyzed by electron microscopy after OptiPrep separation as follows. Ty21a kanL1S bacteria grown to mid-log phase (optical density at 600 nm [OD600] of ±0.7) were harvested by centrifuging at 3,300 × *g*. The *Salmonella* strain was resuspended in ice-cold B-Per II reagent (Pierce). Halt protease inhibitor (Pierce), EDTA, Plasmid Safe, Benzonase, and MgCl2 were added to the lysates was analyzed by Western blotting as previously described (35) for determination of L1 expression. Purified baculovirus-expressed HPV16 VLPs were produced as described previously (18, 22a) and were administered subcutaneously (s.c.) (1-μg doses) or i.n. in anesthetized mice (5-μg doses) as previously described (4, 34). The latter protocol corresponds to an aerosol-like administration with inhalation of the VLP inoculum into the lung (3, 33). However, while the parenteral protocol is optimal, as three 5-μg s.c. VLP doses induced similarly high anti-HPV16 VLP titers (4, 34), the aerosol-like administration is not optimized, but higher VLP doses cannot be used, and higher anti-HPV16 VLPs responses can be achieved only when mucosal adjuvants are added (4, 34, 42a). Sampling of blood and vaginal washes was performed before and after vaccination throughout a 2- to 10-month period depending on the experiments (see figure legends for detailed schedules).

Sampling of blood and vaginal washes as well as determination of specific antibody end-point titers were performed by ELISA as reported previously (22, 35). Briefly, for determinations of anti-HPV16 VLPs, anti-lipopolysaccharide (LPS), or anti-flagellin antibodies, ELISA plates were coated with 50 ng of HPV16 VLPs in phosphate-buffered saline with *Salmonella* serovar Typhi LPS (Sigma) coupled with methylated bovine serum albumin (Sigma) in carbonate buffer as previously described (37) or with flagellin purified from Ty21a (50 ng/well) in carbonate buffer, respectively. For total immunoglobulin G (IgG) or IgA determinations, plates were coated with 100 ng of sheep anti-mouse Ig (Boehringer) in carbonate buffer. Total or specific IgA or IgG antibodies were detected with biotinylated goat anti-mouse IgA (Kirkegaard & Perry Laborato- ries) or IgG (Amersham Pharmacia) as secondary antibodies, respectively. Endpoint dilutions of all samples were carried out. The specific IgA or IgG titers were expressed as the reciprocal of the highest dilutions that yielded an OD450 value 2× higher than dilution of serum samples. The titers were normalized to the amount of total IgA or IgG in vaginal washes (22).

Recovery of *Salmonella enterica* serovar Typhimurium or *Salmonella enterica* serovar Typhi was determined using organs from euthanized mice by plating onto agar plates with and without antibiotics as previously described (35).

**Neutralization assays.** Neutralization assays were performed with secreted alkaline phosphatase (SEAP) HPV16 pseudoviruses as described in detail previously (41). Briefly, OptiPrep-purified SEAP HPV16 pseudoviruses diluted 3,000-fold were incubated on ice for 1 h with twofold serial serum to detect anti-SEAP activity. Neutralization titers were defined as the reciprocal of the highest serum or vaginal wash dilution that caused at least a 50% reduction in SEAP activity (100% SEAP activity ranging from 30 to 150 relative light units). The lowest serum and vaginal wash dilutions tested were 1:10 and 1:5, respectively, which correspond, when mixed with the pseudovirions, to final dilutions in the assay of 1:20 and 1:10, respectively.

**RESULTS**

Expression of HPV16 L1S and plasmid stability in a kana- mycin-resistant PhoP strain. A kanamycin-resistant plasmid expressing HPV16 L1S was constructed by replacing the ampicillin-resistant gene in the original pFSnsd HPV16 L1S (5) by a kanamycin-selectable gene. An inverse PCR strategy was used to amplify the entire plasmid flanking the ampicillin-resistant gene sequence, and the resulting fragment was ligated into a kanamycin-resistance-encoding sequence (see Materials and Methods for details). The resulting plasmid was designated pFS14nsd-kan3-HPV16 L1S and electroporated into PhoP to yield PhoP kanL1S (Table 1). In vitro expression of
neutralizing antibody-reactive HPV16 L1 in this new strain was similar to that measured in the ampicillin-resistant PhoP+ L1S strain (ca. 10 μg VLPs/10^11 CFU) (5). Similar growth rates were also observed in the two strains, with about 7 h to reach the mid-log phase, and plasmid stability was very high, with almost 100% of the bacteria still harboring the plasmid after four consecutive cultures performed overnight in antibiotic-free medium. In addition, the stability of the kanL1S plasmid was increased in vivo compared to the ampicillin plasmid, with almost 100% of the bacteria still harboring the plasmid after the mid-log phase, and plasmid stability was very high, with some decrease in genital titers were observed, as found previously with PhoP+ L1S (5). HPV16 VLP-specific IgG and IgA were also induced in genital secretions of mice immunized with the PhoP+ kanL1S and AroA kanL1S strains, while none were detected in mice receiving the PhoP- kanL1S strain (Fig. 1B). All serum titers remained stable for at least 6 months, while some decrease in genital titers were observed, as found previously with PhoP+ L1S (5; data not shown). These data obtained in mice using oral immunization suggest that recombinant Salmonella enterica serovar Typhi harboring ara deletions may be more immunogenic than those harboring phoP/phoQ deletions.

### Humoral and cellular immune responses induced by Salmonella enterica serovar Typhi vaccine strains expressing HPV16 L1 in the i.n. murine model

We introduced the kanL1S plasmid into three Salmonella enterica serovar Typhi vaccine strains that were available to us, i.e., Ty21a, the licensed typhoid vaccine strain (ΔgalE with undefined attenuating mutations) (17); Ty800 (ΔphoP/ΔphoQ) (19); and CVD908-htrA (ΔaroC ΔaroD ΔhtrA) (48) (Table 1). Salmonella enterica serovar Typhi strains are not invasive in mice by the oral route. However, mice can be transiently infected if bacteria are administered at high doses by the nasal route, as shown with recombinant CVD908-htrA (ΔaroC ΔaroD ΔhtrA) (40, 42). The immunogenicity of the three recombinant Salmonella enterica serovar Typhi strains was therefore evaluated in BALB/c mice after administration of two i.n. doses (10^6 CFU for Ty21a and CVD908-htrA and 10^7 CFU for Ty800 recombinant strains), with the first dose given on day 1 and the second dose given 1 month later. The 10^6-CFU doses and schedule were chosen according to previously reported experiments performed by other groups using the nasal murine model with Ty21a (16, 23) and CVD908-htrA (27). Administration of Ty800 to mice was not previously reported. A lower dose (10^7 CFU) was used because higher

### Table 1. Salmonella strains used in this study

<table>
<thead>
<tr>
<th>Strain (attenuation)</th>
<th>Plasmid electroporated</th>
<th>Abbreviation</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CS022 (PhoP+ pho-42)</td>
<td>pFS14nsd-HPV16 L1S</td>
<td>PhoP+ L1S</td>
<td>5</td>
</tr>
<tr>
<td>CS022 (PhoP+ pho-42)</td>
<td>pFS14nsd-kan1-HPV16 L1S</td>
<td>PhoP+ kanL1S</td>
<td>This work</td>
</tr>
<tr>
<td>CS015 (PhoP- ΔphoPQ)</td>
<td>pFS14nsd-HPV16 L1S</td>
<td>PhoP- L1</td>
<td>5</td>
</tr>
<tr>
<td>CS015 (PhoP- ΔphoPQ)</td>
<td>pFS14nsd-kan1-HPV16 L1S</td>
<td>PhoP- kanL1S</td>
<td>This work</td>
</tr>
<tr>
<td>SL7207 (ΔaroA)</td>
<td>pFS14nsd-HPV16 L1S</td>
<td>AroA L1S</td>
<td>5</td>
</tr>
<tr>
<td>SL7207 (ΔaroA)</td>
<td>pFS14nsd-kan1-HPV16 L1S</td>
<td>AroA kanL1S</td>
<td>This work</td>
</tr>
</tbody>
</table>

Salmonella enterica serovar Typhi

Ty800 (ΔphoPQ)

Ty800 (ΔphoPQ)

CVD908-htrA (ΔaroC ΔaroD ΔhtrA)

CVD908-htrA (ΔaroC ΔaroD ΔhtrA)

Ty21a

Ty21a

Ty21a

Ty21a

### Table 2. Recovery of Salmonella PhoP+ L1S carrying the ampicillin or kanamycin resistance plasmid 2 weeks after oral immunization

<table>
<thead>
<tr>
<th>Organ analyzed</th>
<th>Salmonella recovered (mean log_{10} CFU/organ ± SEM)</th>
<th>% of bacteria bearing plasmid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PhoP+ kanL1S</td>
<td>PhoP+ L1S</td>
</tr>
<tr>
<td>Peyer’s patches</td>
<td>3.31 ± 0.23</td>
<td>2.38 ± 0.29</td>
</tr>
<tr>
<td>Mesenteric lymph nodes</td>
<td>2.93 ± 0.41</td>
<td>2.67 ± 0.15</td>
</tr>
<tr>
<td>Spleen</td>
<td>2.61 ± 0.13</td>
<td>2.46 ± 0.12</td>
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</table>

* Data were taken from reference 7.
inocula were lethal to the mice. Our observation is in agreement with a previously described higher mortality of such PhoPQ-deleted bacteria after intraperitoneal injection with hog gastric mucin in mice (2). The IgG titers against the heterologous HPV16 VLP antigen, as well as against two homologous antigens, LPS and flagellin, were measured in serum over an 8-week period (Fig. 2A). Interestingly, high anti-VLP IgG titers were induced only after immunization with Ty21a kanL1S, while low or barely detectable titers were induced by the two other strains. In contrast, Ty800 kanL1S and CVD908-htrA kanL1S induced higher anti-LPS and anti-flagellin IgG titers than Ty21a kanL1S, indicating that the two former recombinant strains had successfully infected mice and induced humoral responses against the bacterial antigens (Fig. 2B). However, the anti-LPS and anti-flagellin titers induced by Ty21a kan L1S and the parental Ty21a strain were indistinguishable (Fig. 2C). This result suggests that the parental and recombinant Ty21a strains may have a similar potential to protect against typhoid fever.

CD4+ T-helper cells participate in the generation and maintenance of high antibody titers; therefore, cell-mediated immune responses against flagellin and HPV16 VLPs were also examined. Eight weeks after immunization, mice were sacrificed, and antigen-specific proliferation was measured using CD4+ T cells purified from the corresponding spleen (Fig. 3). HPV16 VLP stimulation of CD4+ T cells was strongly induced after i.n. vaccination with Ty21a kanL1S (P < 0.001) (Fig. 3A), in agreement with the induction of anti-HPV16 VLP antibodies. In contrast, weak CD4+ T-cell proliferation was observed in mice immunized with the other two strains (Ty800 kanL1S and CVD908-htrA kanL1S). Only immunization with Ty800 kanL1S induced significant flagellin-specific stimulation of CD4+ T cells (P < 0.001) (Fig. 3B).

Stability and expression of the kanL1S plasmid in three Salmonella enterica serovar Typhi vaccine strains and L1 assembly status in Ty21a kanL1S. In order to gain insights into the higher immune responses against VLP observed with the Ty21a strain, plasmid stability and expression in these three recombinant Salmonella enterica serovar Typhi strains were compared. The stability of the kanL1S plasmid in the three vaccine strains in the absence of antibiotics was first examined in vitro (Fig. 4). In contrast to the above-described results for Salmonella enterica serovar Typhimurium strains, a lower stability of the kanL1S plasmid was observed in all Salmonella

![Graph A: Serum anti-HPV16 VLP IgG](image)

![Graph B: Vaginal anti-HPV16 VLP](image)

**FIG. 1.** Comparison of serum anti-HPV16 VLP antibody titers after oral vaccination with PhoP kanL1S, PhoP+ kanL1S, and AroA kanL1S. Groups of five BALB/c mice were orally immunized with 10^9 CFU of PhoP kanL1S (black bars), PhoP+ kanL1S (white bars), and AroA kanL1S (striped bars). Anti-HPV16 VLP ELISA titers are shown 8 weeks after immunization in serum (A) and in vaginal washes (B). Data are expressed as the geometric means (log_{10}) of the reciprocal dilutions of specific IgG from individual mice in serum and specific IgG and IgA per microgram of total IgG (IgA) in vaginal washes. Error bars indicate the standard errors of the means (SEM).
enterica serovar Typhi strains tested. A slow loss of plasmid occurred after the second culture performed overnight, but after five consecutive cultures performed overnight, the plasmid was retained in 10, 6, and 20% of the bacteria in Ty21a, Ty800, and CVD908- htrA, respectively. To examine whether this plasmid instability was linked to the Salmonella serovar Typhi bacterial host or to the kanamycin selection marker, the original L1S plasmid was introduced into the three Salmonella enterica serovar Typhi vaccines strains, and its stability was determined in vitro (Fig. 4). After five consecutive cultures performed overnight, the stability of the original L1S plasmid containing the ampicillin selection marker was rather similar to that of the kanL1S plasmid in Ty21a and CVD908- htrA (14% and 8% of bacteria retaining the plasmid, respectively). In contrast, the L1S plasmid was highly unstable in Ty800 (0.0002% of bacteria retaining the plasmid). Therefore, plasmid instability can be linked to either strain or resistance marker and remains unpredictable.

Salmonella enterica serovar Typhi vaccine strains will undergo only limited rounds of replication in humans. To estimate how the plasmid instability observed in vitro may translate in vivo, plasmid stability in Salmonella cells recovered from the lung and spleen of mice was examined 1 week after i.n. immunization with $10^9$ CFU of Ty21a kanL1S and CVD908-
after immunization with the strains harboring the original L1S plasmid also revealed a higher plasmid stability in Ty21a (Table 3). Our data thus indicate that the L1S-expressing plasmids are more stable in Ty21a than in the other Salmonella enterica serovar Typhi vaccine strains tested in the nasal murine model.

In vitro expression levels of neutralizing antibody-reactive HPV16 L1 were similar in the three Salmonella serovar Typhi strains (20 to 30 μg VLP/10^11 CFU), suggesting that anti-VLP immunogenicity did not correlate to in vitro expression levels. The sandwich ELISA used to quantify conformationally correct L1 does not discriminate between L1 capsomers, capsomer aggregates, or VLPs, since all are presumably recognized by H16 E70 and H16 V5, the antibodies used in this assay (11). We therefore further evaluated the L1 assembly state in Ty21a extracts. Exponentially growing cultures were lysed and subjected to centrifugation at 10,000 × g. L1, as assessed by Western blotting with an L1-specific monoclonal antibody, was distributed approximately equally between the pellet and supernatant (data not shown), suggesting that a considerable fraction of L1 was in a high-molecular-weight complex, higher than expected for individual VLPs. The supernatant was subjected to OptiPrep step gradient centrifugation under conditions that separate VLPs from capsomers (8). Western blots revealed L1 in both high- and low-molecular-weight fractions (Fig. 5A). Separate fractions or pools of L1-positive fractions were examined by electron microscopy after negative staining. Structures resembling pentamers and various-sized amorphous aggregates of pentamers were seen, but no well-ordered VLPs were visible in any of the fractions (Fig. 5B). Star-shaped objects (Fig. 5B) were detected in the intermediate-molecular-weight fractions. Examination of these fractions after the addition of purified HPV16 L1 VLPs or pentamers confirmed that these objects were intermediate in size (data not shown), with a diameter of 17 nm, compared to 12 nm and 60 nm for pentamers and VLPs, respectively. However, they are clearly not T = 1 capsids, which can spontaneously assemble in vitro from Escherichia coli-derived N-terminally-deleted HPV16 L1, but are reported to be 30 nm (10). The star-shaped objects remained after rabbit HPV16 L1 VLP antisera-mediated immunodepletion of all Western blot-detectable L1 (data not shown). Consequently, it is unlikely that these structures are L1 derived. We were concerned that the lysis buffer used to disrupt the cells (B-Per II reagent) might have caused a dis-aggregation of preformed VLPs after lysis. However, incubation of purified insect cell-derived HPV16 L1 VLPs with B-Per II reagent did not lead to the disassembly of the VLPs, as assessed by electron microscopic examination of negatively stained preparations (data not shown).

**Induction of anti-HPV16 VLPs and HPV16-neutralizing antibodies in serum and genital secretions of mice immunized with Ty21a kanL1S or the prototype HPV16 VLP vaccine.** In order to evaluate the potential of Ty21a kanL1S as a cervical cancer prophylactic vaccine, we compared its immunogenicity to that of purified HPV16 VLPs. Groups of five mice were immunized with two i.n. doses of Ty21a kanL1S (ca. 10^9 CFU) at weeks 0 and 4 (Fig. 6, squares); three s.c. doses of 1 μg VLPs at weeks 0, 4, and 25 (Fig. 6, triangles); or three i.n. (aerosol-like) doses of 5 μg VLPs at weeks 0, 1, and 2 (Fig. 6, circles). The parenteral and aerosol-like protocols using purified VLPs were chosen according to our previous experience (4, 34) and...
to mimic vaccination of humans (18, 33). A combination of VLPs (two s.c. 1-μg doses at weeks 0 and 4) and Ty21a kanL1S (a single i.n. boost at week 8) was also tested (Fig. 6, diamonds). Anti-HPV16 VLP and HPV16-neutralizing antibody titers were determined in serum and vaginal washes by ELISA and the SEAP HPV16 pseudovirion neutralization assay, respectively, at short term (4 to 5 weeks after the last immunization) (Fig. 6A, C, and E) and at long term (18 to 24 weeks after the last immunization) (Fig. 6B, D, and F). Our data show that immunization with Ty21a kanL1S induced VLP-specific and HPV16-neutralizing antibodies not only in serum (Fig. 6A and B, squares) but also in genital secretions (Fig. 6C, D, E, and F, squares), which may be critical for protection against genital HPV infection. The immune responses at short term (Fig. 6A, C, and E, squares) are representative of three independent experiments.

Immunization with the prototype s.c. VLP vaccine (Fig. 6, triangles) induced significantly higher VLP-specific and HPV16-neutralizing titers in serum than the mucosal vaccines, i.e., aerosol vaccination with Ty21a kanL1S or VLPs (Fig. 6A and B, triangles versus squares and circles). However, this was not exactly reflected in genital secretions, where the differential induction of VLP-specific IgG and IgA by parenteral versus mucosal protocols of vaccination complicated the situation. While at short term, there was no statistical differences in the normalized titers of VLP-specific IgG induced in the four groups (Fig. 6C), at long term, the mice vaccinated with the prototype s.c. VLP vaccine exhibited significantly higher VLP-specific IgG titers than the mucosal vaccines (Fig. 6D). However, the opposite was true when the IgA tiers were examined: both at short term and long term, the prototype s.c. VLP

### Table 3. Recovery from the lung of mice of different *Salmonella enterica* serovar Typhi strains carrying the kanL1S plasmid 1 week after nasal immunization

<table>
<thead>
<tr>
<th><em>Salmonella enterica</em> serovar Typhi strain</th>
<th><em>Salmonella</em> recovered (mean log_{10} CFU/organ ± SEM)</th>
<th>% of bacteria bearing plasmid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ty21a kanL1S</td>
<td>4.25 ± 0.06</td>
<td>100</td>
</tr>
<tr>
<td>CVD908-htrA kanL1S</td>
<td>4.67 ± 0.22</td>
<td>1.7</td>
</tr>
<tr>
<td>Ty800 kanL1S</td>
<td>5.56 ± 0.07</td>
<td>15.8</td>
</tr>
</tbody>
</table>

FIG. 4. In vitro stability of the kanL1S and L1S plasmids in different *Salmonella enterica* serovar Typhi strains. The number of successive cultures at a 1/100 dilution performed overnight (ON) in medium without antibiotic is indicated on the horizontal axis. Each morning, bacteria (plain line, kanL1S; dashed line, L1S) were plated onto agar in the presence or absence of antibiotic. The vertical axis represents the percentage of bacteria that have retained the plasmid. Error bars indicate SEM.

FIG. 5. Electron microscopy analysis of Ty21a kanL1S lysate. (A) Clarified lysate was separated on a 27%, 33%, or 39% OptiPrep step gradient. Sixteen fractions were collected from the bottom of the tube. The first 11 fractions were analyzed by Western blotting with CAMVIR-1 monoclonal antibody, which recognizes denatured 16L1. Molecular mass markers (in kilodaltons) are depicted on the right. The location of full-length monomeric L1 is indicated by the arrow. (B) Fractions that were 16L1 positive in CAMVIR-1 Western blots were pooled and further purified on a 2% agarose column. Sixteen L1 Western blot-positive fractions were pooled and examined by transmission electron microscopy at a magnification of ×26,000. Capsomer aggregates (large arrows), 17-nm-diameter “stars” (small arrows), and 12-nm capsomers (arrowheads) are indicated.
vaccine induced significantly lower VLP-specific IgA titers than the mucosal vaccines (Fig. 6C and D). To have more definitive insights into the protective potential of Ty21a kanL1S compared to the prototype VLP vaccine, HPV16-neutralizing titers of vaginal washes were examined. Our data show that the protective potential of Ty21a kanL1S is not lower than those of VLP vaccines at either short or long term (Fig. 6E and F), despite the fact that only two doses were administered, suggesting that this is a promising vaccine to test in women. Evaluation of the combined VLP-Ty21a kanL1S protocol raised the possibility that the third dose of the s.c. prototype VLP vaccine may be replaced by Ty21a kanL1S, as no statistical differences were observed in serum or genital secretions with the complete s.c. VLP protocols. However, the VLP/Ty21a kanL1s prime-boost strategy generated a lower geometric mean neutralizing titer than the two-dose Ty21a kanL1S vaccination, at least at short term (Fig. 6E).

DISCUSSION

The generation of a prophylactic vaccine against cervical cancer that has improved worldwide applicability has been our
major focus for several years. Here, we report on final improvements in the development of a Salmonella-based HPV vaccine for clinical testing, i.e., an acceptable selection marker and identification of a safe and immunogenic typhoid vaccine strain. The development of recombinant bacterial vaccines has usually required the use of selectable markers. Unfortunately, our attempt to use the aspartate β-semialdehyde dehydrogenase-balanced lethal vector-host system (12) to stabilize L1S expression in PhoP⁺ has failed to induce VLP-specific immune responses (data not shown). We have therefore chosen kanamycin as an antibiotic-selectable marker, since it has an established biosafety record and its use has been approved for recombinant plant applications by the FDA (14). Although this does not guarantee that it may be approved for use in a recombinant vaccine, it should be noted that the kanamycin resistance marker will not give the organisms a selectable advantage outside the laboratory, and this phenotype is already quite ubiquitous in nature. In fact, the widespread bacterial resistance to kanamycin has limited the use of this antibiotic in human medicine, and the high substrate specificity of the inactivating enzyme argues against the development of resistance or interference against modern antibiotic therapies. It is human medicine, and the high substrate specificity of the inactivation of Ty21a kanL1S lysates showed L1 assembled in capsomeres and various-sized capsomer aggregates but no VLPs. Previous studies found that purified E. coli-derived L1 capsomers could induce neutralizing antibody responses after parenteral injection (43). Therefore, it is not surprising that high titers of neutralizing antibodies were induced by the L. recombiant Salmonella strain, despite our inability to detect well-formed VLPs in the lysates. The rare VLPs observed in the original PhoP⁺ L1 lysate (35) may be linked to the higher L1 expression level (50 µg VLP/10¹¹ CFU) in this strain.

After i.n. inoculation, the recombinant Ty21a kanL1S strain was able to induce high anti-HPV16 VLP antibodies in both serum and genital secretions. Indeed, the HPV16-neutralizing potential of vaginal washes of mice vaccinated twice with Ty21a kanL1S was similar to that achieved by three vaccinations with the prototype s.c. VLP vaccine, as determined using a standard SEAP-pseudovirion neutralization assay. However, in serum, the anti-HPV16 VLP IgG and HPV16-neutralizing titers induced by Ty21 kanL1S were lower (ca. 1 log) than those induced by s.c. vaccination with VLPs. This was accompanied by lower VLP-specific, probably serum-transudating, IgG in genital secretions but was compensated for by the induction of more VLP-specific IgA by mucosal vaccination with Ty21a kanL1S. In our samples, HPV16 VLP ELISA titers showed a high correlation with HPV16-neutralizing titers in serum (Pearson r = 0.763; P < 0.0001) but also in genital secretions (Pearson r = 0.847; P < 0.0001 [IgA plus IgG]), suggesting that the neutralization assay is also valid for these types of samples. Interestingly, neutralization correlated better with the VLP-specific IgA content (Pearson r = 0.731; P < 0.0001) than with the VLP-specific IgG content (Pearson r = 0.385; P = 0.02) in genital secretions, emphasizing the importance of IgA induction in the Ty21a kanL1 immune response.

The kanL1S instability in CVD908-htrA and in Ty800 may explain the lower immune responses against HPV16 VLP generated by these bacteria. In contrast, CVD908-htrA and Ty800 induced higher anti-LPS and anti-flagellin antibodies as well as higher flagellin-specific CD4⁺ T cells responses for Ty800 than Ty21a. This finding is in agreement with previous studies where
CVD908-\textit{htaA} and/or Ty800 and Ty21a were compared using the murine nasal model (50) or in human oral delivery trials. Ty21a was moderately immunogenic, requiring three oral doses, administered daily, to confer protection against typhoid fever (24), while CVD908-\textit{htaA} and Ty800 were more immunogenic and/or protective with a single oral dose (19, 48, 49). However, the potential of Ty21a kanL1S as a typhoid vaccine was seemingly not impaired by the expression of HPV16 VLPs, as similar titers of anti-LPS and anti-flagellin antibodies were induced by the parental and L1 recombinant strains in the nasal murine model. There have been limited investigations of the use of Ty21a as a carrier for heterologous proteins. The induction of specific antibody responses by Ty21a expressing surface-displayed viral antigens (23) or hemolysin (16) was reported in the mouse nasal model, while no humoral response but some specific T-cell responses were reported after oral vaccination of human volunteers with Ty21a expressing \textit{H. pylori} urease (9, 30).

In conclusion, in this study, we show that Ty21a kanL1S induces high HPV16-neutralizing titers and VLP-specific CD4+ T-cell responses in the nasal murine model, including HPV16-neutralizing antibodies in genital secretions. The titers were similar to those induced by the prototype s.c. VLP vaccine, which is promising for the potential of Ty21a kanL1S to prevent HPV16 infection. To date, CVD908-\textit{htaA} expressing tetanus toxin fragment C is the only recombinant \textit{Salmonella} serovar Typhi strain that has been tested both in the nasal murine model (40) and after oral administration in humans (47). The induction of protective levels of anti-toxin antibodies in mice was confirmed with human volunteers (47), supporting the hypothesis that data obtained using the nasal murine model may be predictive of immune responses in humans. Further support for the premise that Ty21a kanL1S is an attractive candidate for a combined vaccine against both typhoid fever and cervical cancer will require an evaluation of its safety and immunogenicity in women volunteers.

ACKNOWLEDGMENTS

We thank Hakim Echchannaoui for critical reading of the manuscript.

This work was supported by the Swiss National Science Foundation (grants 631-05769.99 and PP00A-104318 to D.N.-H. and 32-63021.00 to D.B.).

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