It has been shown previously that immunization of animals with recombinant virus-like particles (VLPs) consisting of the viral capsid proteins L1 or L1 plus L2 protected animals against experimental viral challenge. However, none of these experimental models addresses the issue of whether systemic immunization with VLPs elicits a neutralizing antibody response in the genital mucosa. Such a response may be necessary to protect the uterine cervix against infection with genital human papillomavirus (HPV) types. African green monkeys systemically immunized with HPV-11 VLPs expressed in *Saccharomyces cerevisiae* and formulated on aluminum adjuvant elicited high-titered HPV-11 VLP–specific serum antibody responses. Sera from these immunized monkeys neutralized HPV-11 in the athymic mouse xenograft system. Significant levels of HPV-11–neutralizing antibodies also were observed in cervicovaginal secretions. These findings suggest that protection against HPV infection of the uterine cervix may be possible through systemic immunization with HPV VLPs.
HPV-11 and -16 VLPs were purified from yeast lysates by the purification method described by Jansen et al. [10] with slight modifications. The purity of the samples was >95% as assessed by SDS-PAGE and colloidal Coomassie staining. VLPs reacted with a panel of monoclonal antibodies (MAbs) known to recognize only native virions or VLPs [7]. HPV-11 L1 VLPs and HPV-11 virions were quantified by sandwich RIA that used a polyclonal anti–HPV-11 VLP serum raised in goats as VLP capture antibody, the HPV-11 virion–specific MAb 8740 (Chemicon, Temecula, CA), and anti-mouse 125I-labeled IgG (NEN Research Products, Boston) as the detector. HPV-16 VLPs were quantified by a similar assay that used a polyclonal goat anti–HPV-16 VLP serum and the HPV-16 VLP–specific MAb H16.V5 [14]. HPV-11 L1 and -16 VLPs were adsorbed to aluminum hydroxyphosphate adjuvant as described [10].

Immunization with HPV-11 L1 VLPs. Most animals used in this study were colony-bred African green monkeys. Whenever necessary, animals were sedated with ketamine HCl (10 mg/kg intramuscularly; Fort Dodge Laboratories, Fort Dodge, IA). At weeks 0, 8, and 24, groups of 4 animals were immunized with varying amounts of HPV-11 L1 VLPs adsorbed to aluminum adjuvant by intramuscular injections deep into the quadriceps muscle mass (extensor group) of each leg (0.5 mL of vaccine per injection site).

Collection of serum and cervicovaginal secretions. Before each immunization and at 2- to 4-week intervals after immunizations, 5-mL serum samples were collected from each animal and stored frozen at −20°C until analysis. To collect cervicovaginal secretions, 2 mL of sterile PBS was used to wash the vaginal vault by use of a syringe attached to flexible tubing. A sterile syringe case with its tip cut off was used as a speculum. The vaginal lumen was flushed five times before collecting the lavage fluid. All cervicovaginal samples were snap-frozen on dry ice and stored at −20°C until analysis. Cervicovaginal samples were tested to be free of blood contamination by the Hemocult SENSA assay (SmithKline Diagnostics, San Jose, CA). We determined the sensitivity of this assay as being able to detect a 1:10,000 dilution of African green monkey blood as a 1:100,000 dilution was not detectable.

ELISA. The isotype and level of antibodies specific for HPV-11 L1 VLPs in serum and cervicovaginal secretions were determined by capture ELISA [15]. The solid-phase antigens were HPV-11 L1 VLPs expressed in insect cells using recombinant baculoviruses or HPV-16 L1 VLPs expressed in yeast. HPV-11 L1 or HPV-16 L1 VLPs were captured by MAbs 8740 (Chemicon) or H16.V5 [14], respectively, and bound monkey immunoglobulin was detected by anti-human IgG, IgM, or IgA (Pierce, Rockford, IL) or secretory (s) IgA (Nordic, Tilburg, Netherlands) coupled to horseradish peroxidase. HPV VLP–specific titers were determined by end point dilution (3-fold dilutions starting at a 1:300 dilution for serum samples; 2-fold dilutions starting at 1:8 for cervicovaginal samples). To analyze antigen-specific IgA and sIgA in cervicovaginal samples, total IgG was first removed by adsorption to GammaBind G Sepharose (Pharmacia, Piscataway, NJ).

HPV-11 neutralization assay. To assess the capacity to neutralize HPV-11, serial dilutions of preimmune and immune sera or cervicovaginal secretions were incubated with ~10^5 HPV-11 virions and small pieces (~2 × 2 mm) of human foreskin tissue at 37°C for 90 min in MEM (Life Technologies GIBCO BRL, Gaithersburg, MD) containing penicillin, streptomycin, and amphotericin B. The amount of HPV-11 used in each neutralization assay was quantified by the HPV-11 VLP–specific RIA, with use of highly purified, yeast-derived HPV-11 VLPs as standards. Cervicovaginal lavage samples were first sterile-filtered through 0.22 μm MILLEX-GV13 (Millipore, Bedford, MA) before adding the sample into the HPV-11 neutralization assay. Foreskin fragments were then implanted under the renal capsules of athymic mice as described by Kreider and colleagues [5, 6]. The mice were sacrificed 70 days after implantation. The foreskin tissue implants were analyzed for HPV-11 infection by HPV-11 DNA in situ hybridization with biotinylated HPV-11 DNA probes (Digene Diagnostics, Boltsville, MD). Typically, 2 mice (4 implants) were used for each serum dilution or cervicovaginal lavage sample.

To control for HPV-11 infection and tumor growth, 2 mice received human foreskin tissue pieces incubated with virus alone. An assay in which ≥75% of recovered implants were positive for HPV-11 was considered valid, and all implants in the assay were included in the data analysis.

Results

HPV-11 L1 VLP–specific antibody responses in serum of African green monkeys immunized with yeast-expressed HPV-11 L1 VLPs. Groups of female African green monkeys were immunized by deep intramuscular injection with three doses of purified yeast-derived HPV-11 L1 VLPs adsorbed to aluminum adjuvant. The dose levels varied for each group from 10 to 700 μg/dose. Each monkey received the same dose for each immunization. Pre- and postimmunization sera and cervicovaginal samples were assayed for the presence of HPV-11 L1 VLP–specific IgG antibodies by ELISA. Fourteen of 16 monkeys seroconverted after a single immunization with HPV-11 L1 VLPs, and all animals were seropositive after the second dose. Anamnestic responses were evident for all monkey groups after booster immunizations (figure 1). Peak titers were obtained 2 weeks after each immunization. All 4 dose levels elicited similar geometric mean titers, indicating that at the lowest dose of VLPs tested (10 μg/dose), a response plateau had already been reached (figure 1). HPV-11 L1 VLP–specific IgM responses were detectable in most animals, but titers were ~100- to 1000-fold lower than the corresponding IgG titers (data not shown). Significant HPV-11 L1 VLP–specific IgG serum titers were maintained in all animals for 6 months after the last booster immunization (figure 1). To determine whether the responses seen were unique to HPV-11 L1 VLPs, 1 group of African green monkeys was immunized with 50 μg/dose HPV-16 L1 VLPs expressed in yeast. As shown in figure 2, the immune response paralleled the responses seen in the HPV-11 groups, indicating that HPV-16 L1 VLPs were equally immunogenic. Furthermore, immunization of Rhesus monkeys with yeast-derived HPV-11 L1 VLPs gave very similar immune response curves, indicating that the responses observed are not limited to African green monkeys (data not shown).

HPV-11 L1 VLP–specific antibody responses in cervicovaginal secretions of African green monkeys immunized with yeast-
Figure 1. Geometric mean HPV-11 L1 VLP–specific titers in sera (solid symbols) and cervicovaginal (open symbols) lavage samples from female African green monkeys immunized with HPV-11 L1 VLPs formulated on aluminum adjuvant. Dose levels were 700 (●), 350 (■), 40 (▲) and 10 (▲) µg/dose. Titers were determined by end point dilution with VLP-specific capture ELISA and were considered positive if L1-specific absorbance exceeded 1.5 times mean absorbance readings of monkey preimmune sera. Only cervicovaginal samples free of blood contamination were included.

expressed HPV-11 L1 VLPs. To test whether systemic immunization elicits HPV-11 VLP–specific IgG in the genital mucosa, cervicovaginal lavage fluids were collected from the immunized monkeys and analyzed by the HPV-11 L1 VLP–specific ELISA. After one dose, all animal groups had detectable HPV-11 L1 VLP–specific IgG antibodies in their cervicovaginal lavage samples (figure 1). The cervicovaginal antibody titers paralleled the responses in serum and were still present 6 months after the last booster immunization (figure 1). Geometric mean titers from cervicovaginal wash samples were calculated only from samples that were shown to be free of blood contamination. The depicted cervicovaginal lavage titers were not corrected for the dilution factor associated with the sampling method. HPV-11 L1 VLP–specific IgA and sIgA in the samples were below the detection limits of the assays. HPV-11 L1 VLP–specific IgM responses were not determined because of the low levels detected in the corresponding serum samples.

Correlation of HPV-11 L1 VLP–specific IgG titers and neutralization of infectious HPV-11 virions. To determine whether the antibody response elicited after immunization with HPV-11 VLPs was neutralizing, serial dilutions of immune and preimmune African green monkey sera were tested in the athymic mouse xenograft system. About 10⁹ virions were used in each neutralization assay. Previous experiments (Brown DR, unpublished data) indicated that monkey preimmune serum samples at dilutions ≤1:10 were partially neutralizing. For this reason, the lowest serum dilution tested in each neutralization assay was 1:40. As shown in figure 3, serum samples with titers ≥500 were found to be completely neutralizing. Serum samples with IgG titers below 500 did not fully neutralize the input virus.

The ability of HPV-11 L1 VLP immunization to successfully protect the female genital tract may require the presence of virus-neutralizing antibodies in the genital mucosa. Cervicovaginal lavage samples from immunized monkeys were therefore also tested in the neutralization assay. Cervicovaginal lavage samples were shown to be partially neutralizing (figure 3). Consistent with the low HPV-11 VLP–specific IgG titers in cervicovaginal lavage samples, a lower percentage of foreskin

Figure 2. Geometric mean HPV-16 L1 VLP–specific titers in sera from African green monkeys immunized with 50 µg of purified yeast-derived HPV-16 L1 VLPs. Titers were determined by end point dilution with VLP-specific capture ELISA and were considered positive if L1-specific absorbance exceeded 1.5 times mean absorbance readings of monkey preimmune sera.
16 L1 VLPs elicited very similar responses in the animals. During the sampling procedure, the amounts present in the VLP-specific antibodies in serum and detectable antibody titers are needed, however, to assess whether complete neutralization with yeast-derived HPV-11 L1 VLPs elicited an immunologic response capable of mucosal protection.

All monkeys immunized with HPV-11 L1 VLPs formulated on aluminum adjuvant developed high-titered HPV-11 L1 VLP-specific antibodies in serum and detectable antibody titers in their cervicovaginal secretions. HPV-11 L1 and HPV-16 L1 VLPs elicited very similar responses in the animals. Although firm evidence is lacking, it is presumed that HPVs infect genital epithelium by adhering to the surface of basal cells, which become exposed by microtrauma. In the athymic xenograft system of HPV-11 infection, antibodies to conformational epitopes of HPV-11 L1 can prevent infection of human foreskin tissue, even when addition of antibodies is delayed for several hours [17], suggesting that antibodies could protect the genital epithelium from infection. The importance of neutralizing antibodies to protect animals from papillomavirus infection has been also demonstrated by passive transfer of neutralizing antiserum [9, 11, 18]. To be fully protective against genital tract HPV infection, antibodies will need to be present at the time of exposure. Our data show that HPV-11 L1 VLP-specific IgG present in the cervicovaginal secretions of monkeys parenterally immunized with HPV-11 L1 VLPs is sufficient to neutralize HPV-11 in the athymic mouse xenograft system.

Strong HPV-11 L1 VLP-specific antibody responses were elicited after a single dose of vaccine. The HPV-11 L1 VLP-specific antibodies were predominantly of the IgG isotype; HPV-11 L1 VLP-specific IgA and sIgA in cervicovaginal secretions were not present in detectable amounts. The absence of sIgA in the cervicovaginal lavage samples suggests that protection from HPV-11 infection may be possible in the absence of mucosal immunity. It is not known at this time whether the antigen-specific IgG detected in the cervicovaginal secretions originated from transudated serum IgG or was locally produced. However, since the cervicovaginal antibody responses closely paralleled the serum responses, it is more likely that the HPV-11 L1 VLP-specific IgG originated from the serum. The HPV-11 L1 VLP-specific antibody response persisted in serum and the cervicovaginal mucosa for >6 months after the last booster immunization.

Parenteral immunization against genital HPVs might overcome several potential obstacles associated with generating a local immune response in the genital tract. First, protein antigens are generally not very immunogenic when administered locally, necessitating frequent booster immunizations, which could potentially induce tolerance [19]. In addition, local responses against protein antigens have been generally short-lived and do not effectively induce local immune memory. On the other hand, parenterual immunization strategies have been successful in protecting from experimental rotavirus [20] and simian immunodeficiency virus challenges [21]. Circumstantial evidence also suggests that mucosal IgG antibodies can protect against viruses replicating at mucosal surfaces [22].

Despite being low-titered compared to the serum samples, the cervicovaginal lavage samples tested were also efficiently neutralizing, albeit not completely. Considering that the HPV-11 athymic mouse xenograft neutralization assay requires a large amount of HPV-11 virions (~10^6 particles) to ensure a consistent infection of human foreskin tissue (Brown DR, unpublished data) and that the lavage samples were diluted during the sampling procedure, the amounts present in the
genital mucosa might be sufficiently protective by either completely neutralizing HPV or dramatically reducing viral transmission and disease incidence. However, clinical studies are needed to fully address this issue and to determine whether the immune responses described in this study can be replicated in humans.

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References