A Murine Genital-Challenge Model Is a Sensitive Measure of Protective Antibodies against Human Papillomavirus Infection[▽]

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The available virus-like particle (VLP)-based prophylactic vaccines against specific human papillomavirus (HPV) types afford close to 100% protection against the type-associated lesions and disease. Based on papillomavirus animal models, it is likely that protection against genital lesions in humans is mediated by HPV type-restricted neutralizing antibodies that transudate or exudate at the sites of genital infection. However, a correlate of protection was not established in the clinical trials because few disease cases occurred, and true incident infection could not be reliably distinguished from the emergence or reactivation of prevalent infection. In addition, the current assays for measuring vaccine-induced antibodies, even the gold standard HPV pseudovirion (PsV) *in vitro* neutralization assay, may not be sensitive enough to measure the minimum level of antibodies needed for protection. Here, we characterize the recently developed model of genital challenge with HPV PsV and determine the minimal amounts of VLP-induced neutralizing antibodies that can afford protection from genital infection *in vivo* after transfer into recipient mice. Our data show that serum antibody levels >100-fold lower than those detectable by *in vitro* PsV neutralization assays are sufficient to confer protection against an HPV PsV genital infection in this model. The results clearly demonstrate that, remarkably, the *in vivo* assay is substantially more sensitive than *in vitro* PsV neutralization and thus may be better suited for studies to establish correlates of protection.

Cervical cancer, the second most common cause of cancer death in women worldwide, is associated with high-risk types of human papillomavirus (HPV) infections (27). HPV vaccines based on L1 virus-like particles (VLPs) have been shown to be safe and efficient at preventing infections and precancerous lesions caused by HPV vaccine-related types (26, 33) and now have been commercialized, specifically the HPV6/11/16/18 VLP Gardasil and the HPV16/18 VLP Cervarix vaccines. Neutralizing antibodies (Ab) are thought to be the primary immune mechanism of protection by HPV vaccination, primarily based on preclinical papillomavirus (PV) animal models showing that the passive transfer of immunized sera is protective in naïve rabbits and dogs against skin and oral mucosal challenge, respectively (3, 31). In addition, clinical trials showed that vaccinated individuals developed robust anti-VLP antibody titers in serum (15, 32) and in cervicovaginal secretions (21, 23), and that antibody-mediated cross-type neutralization in *in vitro* assays largely parallels the cross-type protection in the trials. However, these trials did not allow the establishment of antibody concentrations or thresholds that could be correlated to protection, mainly because too few disease cases occurred (26, 33) and because breakthrough infections could not be unambiguously distinguished from the emergence or reactivation of prevalent infection. In addition, the serological assays that were used in the trials (primarily a VLP-based enzyme-linked immunosorbent assay [ELISA] that measures total VLP-bind-

ing antibodies [15] or an antibody competition assay [cLIA] that measures the competition of a type-specific neutralizing monoclonal antibody for VLP binding [25]) may be insufficiently sensitive to measure the minimum level of antibodies needed for protection. A striking illustration of this phenomenon was the finding that one-third of Gardasil vaccinees had become HPV18 seronegative by cLIA about 5 years after vaccination, while they remained completely protected against HPV18-related lesions (19). Interestingly, 97% of the vaccinees turned out to be HPV18 seropositive when tested for total HPV18 VLP-specific IgGs (4). Even *in vitro* pseudovirion (PsV) neutralization assays (28), which currently are considered the gold standard, may be of insufficient sensitivity to provide a correlate with breakthrough infection, since their analytic sensitivity is similar to that of the direct ELISAs (11, 12). Indeed, recent studies of HPV cell entry into the basal epithelial cells *in vivo* provided preliminary results that L1 antibodies neutralize at extremely low concentrations (9). Here we have examined whether a mouse model of genital HPV transmission (30) is a more sensitive measure of infection inhibiting antibodies, in addition to more closely mimicking the infectious process in women. Using the passive transfer of endpoint dilutions of Gardasil-induced murine neutralizing antibodies, we have determined the minimal amounts of antibody necessary to prevent genital infections by HPV16 or HPV18 PsVs transducing a luciferase gene.

MATERIALS AND METHODS

Mice, immunization, and sampling. Female BALB/c mice (Charles River Laboratories, France) were used according to Swiss veterinary authority guidelines and maintained under specific-pathogen-free conditions. Mice were immunized intramuscularly (i.m.) with Gardasil (1/10 of the human dose, i.e., 50 μl

corresponded to 2 μg HPV6 VLPs, 4 μg HPV11 VLPs, 4 μg HPV16 VLPs, and 2 μg HPV18 VLPs; Sanofi Pasteur MSD) at weeks 0, 4, and 8. HPV VLPs were formulated in Gardasil as alum-complexed antigens, which may not be appropriate for mucosal vaccination. Thus, intranasal (i.n.) immunization was performed with baculovirus-derived HPV16 VLPs (5 μg/dose) (29) at weeks 0, 1, and 2 as previously reported (2). Serum and genital secretions were obtained as previously described (17): vaginal washes were performed with 200 μl phosphate-buffered saline (PBS) containing protease inhibitors. Sera from Gardasil-immunized mice collected once a month for 6 months were pooled and used for intraperitoneal (i.p.) transfer into naïve mice (see below).

HPV16 VLP-specific ELISA. The determination of VLP-specific antibody endpoint titers was performed by enzyme-linked immunosorbent assay (ELISA) as reported previously (17, 22). Briefly, wells in ELISA plates each were coated with 50 ng of HPV16 L1 VLPs in PBS, and IgA or IgG antibody was detected with biotinylated goat anti-mouse IgA (Kirkegaard & Perry Laboratories) or IgG (Amersham Pharmacia), respectively, as the secondary antibody. Endpoint dilutions of all samples were carried out starting at a dilution of 1:100 for serum and 1:5 for the secretions. The specific IgA or IgG titers were expressed as the reciprocal of the highest dilutions that yielded an optical density at 492 nm that was four times that of preimmune samples.

HPV PsV and neutralization assays. HPV16 and HPV18 PsV (PsV16 and PsV18, respectively) were produced according to Buck et al. (5) using p16L1h and p16L2h (PsV16; all plasmid maps can be found at http://home.ccr.cancer.gov/LCO/plasmids.asp) or peL1f and peL2hb (PsV18). Neutralization assays were performed with secreted alkaline phosphatase (SEAP) expressing PsV16 and PsV18 (pYSEAP as an encapsidated plasmid) as described in detail previously (28). Briefly, OptiPrep-purified SEAP PsV16 or PsV18 diluted 1,000- or 400-fold, respectively (corresponding to ca. 50 ng L1 protein content), was incubated on ice for 1 h with 2-fold serial serum or vaginal wash dilutions, and the PsV-antibody mixtures were used to infect 293TT cells for 3 days. The SEAP content in 10 μl of clarified cell supernatant was determined using the Great ESCAPE SEAP chemiluminescence kit (BD Clontech). Neutralization titers were defined as the reciprocal of the highest dilution that caused at least a 50% reduction in SEAP activity. The lowest serum dilutions tested were 1:10 for HPV16 and 1:20 for HPV18, which correspond, when mixed with PsV, to final dilutions in the assay of 1:50 and 1:100, respectively. The lowest vaginal wash dilutions were 1:5 for HPV16 and 1:10 for HPV18 neutralization assays (i.e., final dilutions in the assay of 1:25 and 1:50, respectively). Preimmune samples (sera or vaginal washes) at those dilutions did not have detectable HPV neutralizing activity. Vaginal washes of immunized mice that were not neutralizing at these dilutions were attributed an arbitrary value of 5 ($\log_{10} = 0.7$), which corresponded to the lower limit of detection in this assay (13).

Transfer of antibodies in mice and genital HPV PsVluc challenge. Monoclonal antibody H16.V5 (6) (50 mg/ml; kindly provided by N. D. Christensen, Hershey, PA) or pooled sera from Gardasil-immunized mice (here referred to as Gardasil serum) was diluted in a final 100-μl volume with PBS and injected i.p. into mice. *In vivo* vaginal PsV challenges were performed according to the protocol of Roberts et al. (30) using PsV16 and PsV18 with the encapsidated luciferase-expressing plasmid pClucf (designated PsVluc). Endpoint titers of PsVluc were determined on 293TT cells in 96-wells plates. After 2 days of infection, D luciferin (15 μg/well) was added and luminescence was measured (in relative light units [RLU]). The titer was defined as the reciprocal of the highest PsVluc dilution that yielded detectable RLU and was expressed as transducing RLU (TRLU)/ml. For *in vivo* genital challenge, 8 days before PsV challenge mice were synchronized in a diestrus-like status by a subcutaneous (s.c.) injection with 0.1 μg β-estradiol and 24 h later with 2 μg DepoProvera (Pfizer AG, Zurich, Switzerland). Six hours prior to PsVluc challenge, deeply anesthetized mice were intravaginally (ivag) pretreated with 20 μl of 4% nonoxynol-9 (N9, Igepal; Sigma). A volume of 0.7 to 8 μl (corresponding to 7×10^3 to 4.5×10^5 TRLU, depending on the PsV stock) of PsVluc that was thoroughly mixed in a 20-μl solution containing 2% carboxymethylcellulose (CMC; Sigma) was ivag instilled using a positive-displacement pipette. Forty-eight hours after PsVluc challenge, mice were anesthetized and 20 μl of firefly D luciferin (15 mg/ml in PBS, filtered through 0.22-μm pores) was ivag instilled. After 5 min, bioluminescence was measured with a cryogenically cooled Xenogen IVIS camera system (Xenogen/Caliper Life Science) (kindly provided by the cellular imaging facility of UNIL, Lausanne, Switzerland). The acquisition time was for 5 min. Luminescence quantified in photons (photons/s/cm²/sr) in regions of interest was measured using Living Image 2.5 software. For experiments shown in Fig. 4 and 5, 1.6×10^5 and 4.5×10^5 TRLU for PsV16luc and PsV18luc (corresponding to ca. 10 μg of L1 protein content), respectively, were used to induce consistent and reproducible genital infections ($\geq 10^6$ photons/s/cm²/sr). Results of experimental antibody-injected groups (see Fig. 4 and 5) were always compared and normalized to

the respective control noninjected group (i.e., the bioluminescence value for each mouse in an experimental group was divided by the mean bioluminescence of the respective control group). When bioluminescence was not detected, a value of 10^3 photons/s/cm²/sr was attributed, which is 10 times lower than the lowest detectable value of bioluminescence. The percent inhibition of PsV infection was calculated using the following formula: (1 − normalized value of bioluminescence) × 100.

Statistical analysis. Statistical analyses were performed using Prism 5.00 for Windows (GraphPad Software, San Diego, CA) as indicated in the text or in figure legends.

RESULTS

Transudation of antibodies in genital secretions and recovery after i.p. transfer. HPV infection does not occur through an intact epithelium but requires microtrauma or wounds. In the mouse model of HPV transmission, this is mimicked by the ivag instillation of a mild detergent (4% N9) 6 h before PsV infection. Serum neutralizing antibodies are thought to transudate into cervical secretions, a phenomenon that may be enhanced by exudation at the wound site. We therefore first examined how the level of antibodies in genital secretions is affected by the ivag N9 treatment. For this purpose, serum was sampled from a group of mice immunized via the intranasal (i.n.) route with purified HPV16 VLPs 4 months earlier, and vaginal washes were recovered just before the ivag instillation of 4% N9 and 6 h later. VLP-specific IgG in serum and VLP-specific IgG and IgA in vaginal washes were measured by ELISA, as this route of immunization induces both systemic and locally produced (IgA) antibodies in genital secretions (2, 14). Our data (Fig. 1A) indeed show that both VLP-specific IgG and IgA can be detected in vaginal washes before N9 treatment, with titers that are about 3 logs lower than those measured in the serum, as previously reported (14). Note that because of the active hepatobilliary transport of IgA in mice, there is a very small amount of IgA circulating in serum and thus the IgA measured in secretions are mainly secretory IgA (SIgA) and are locally produced. Interestingly, 6 h after N9 treatment, VLP-specific IgG levels were significantly increased by about 30-fold in vaginal washes ($P < 0.05$), while VLP-specific IgA levels were not affected. This suggests that an increased transudation/exudation of serum IgG is induced by the ivag N9 treatment, while VLP-specific IgA produced by B cells located below the basement membrane was not altered. Using the *in vitro* PsV neutralization assay, an increase in genital antibodies after ivag N9 treatment also was measured in genital secretions of mice i.m. immunized with Gardasil (a significant 8-fold increase in HPV16 and HPV18 neutralizing antibody titers, $P < 0.05$ and $P < 0.01$, respectively) (Fig. 1B).

To test known antibody concentrations in the mouse genital HPV transmission model, we first assessed the transferability and stability of VLP-specific antibodies after the i.p. injection of 500 μg of a well-known monoclonal HPV16 neutralizing antibody (H16.V5; here referred to as V5) (6). Our data show that HPV16 neutralizing antibodies in serum and genital secretions appeared to be stable between 24 and 72 h after injection (Fig. 2), with a circulating neutralizing titer of ca. 25,000, corresponding to a V5 concentration of ca. 170 μg/ml. Antibody titers in vaginal washes were about 3 logs lower and often were at the limit of detection of the assay.

PsVluc and genital challenge assays. Genital infection with PsV expressing luciferase is detected by measuring biolumi-
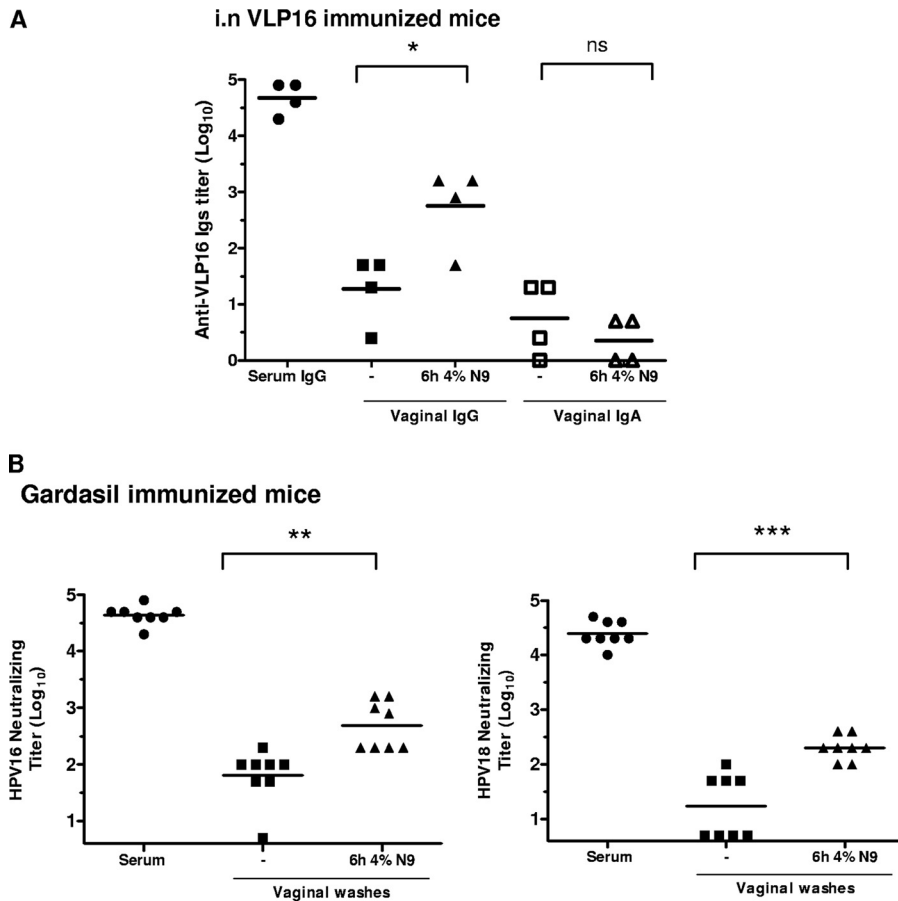
FIG. 1. Effect of ivag N9 treatment in VLP-immunized mice. (A) Groups of four mice i.n. immunized with HPV16 VLPs 6 months earlier were ivag treated with 4% N9 for 6 h. VLP16-specific IgG and/or IgA endpoint titers were measured in serum by ELISA as described in Materials and Methods (black circle, IgG) and in vaginal washes before (black square, IgG; white square, IgA) and after (black triangle, IgG; white triangle, IgA) N9 treatment. (B) A group of eight mice i.m. immunized with Gardasil 8 months earlier were ivag treated with 4% N9 for 6 h. HPV16 and HPV18 neutralizing titers were measured by PsV SEAP neutralization assays as described in Materials and Methods in serum (black circle) and in vaginal washes before (black square) and after (black triangle) N9 treatment. The horizontal bars represent the geometric mean responses. Significant differences are indicated by an asterisk for $P < 0.05$, two asterisks for $P < 0.01$, and three asterisks for $P < 0.001$ by using Student's t test; ns, nonsignificant.
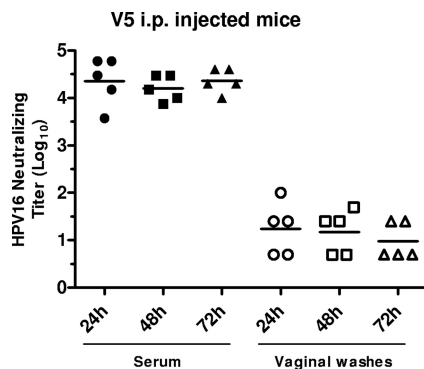

FIG. 2. Recovery of antibodies after i.p. transfer. A group of five mice received i.p. 500 μg of V5. HPV16 neutralizing titers were measured by PsV SEAP neutralization assays in serum (black symbols) and vaginal washes (white symbols) 24 h (circle), 48 h (square), and 72 h (triangle) later. The horizontal bars represent the geometric mean responses.

nescence with the Xenogen IVIS system. To assess the consistency and reproducibility of this detection system, we examined how this may be influenced by the amount of PsVluc used. Different amounts (Fig. 3A) of PsV, corresponding to different TRLU of PsV16 or PsV18 (as measured by endpoint dilutions after the infection of 293TT cells; see Materials and Methods) were assayed. Two days later, bioluminescence measured with the Xenogen IVIS system was plotted against the number of TRLU used. Our data show that there is a correlation between the transduction of 293TT cells *in vitro* and the detection of *in vivo* genital infection by bioluminescence for both PsV16 and PsV18 (Pearson $r = 0.78$ and $r = 0.61$; $P < 0.0001$ and $P < 0.01$, respectively). TRLU of 4×10^4 and 2×10^5 for PsV16luc and PsV18luc, respectively, were sufficient to achieve consistent bioluminescence ($\geq \log_{10} 6$ photons/s/cm^2/sr); however, to increase reproducibility, larger amounts (1.6×10^5 TRLU for PsV16 and 4.5×10^5 TRLU for PsV18) were used in the following experiments. These amounts correspond to approximately 10 μg of L1 for each type. This allowed the detection of genital infection in 96% (52/54) of mice with a biolumines-

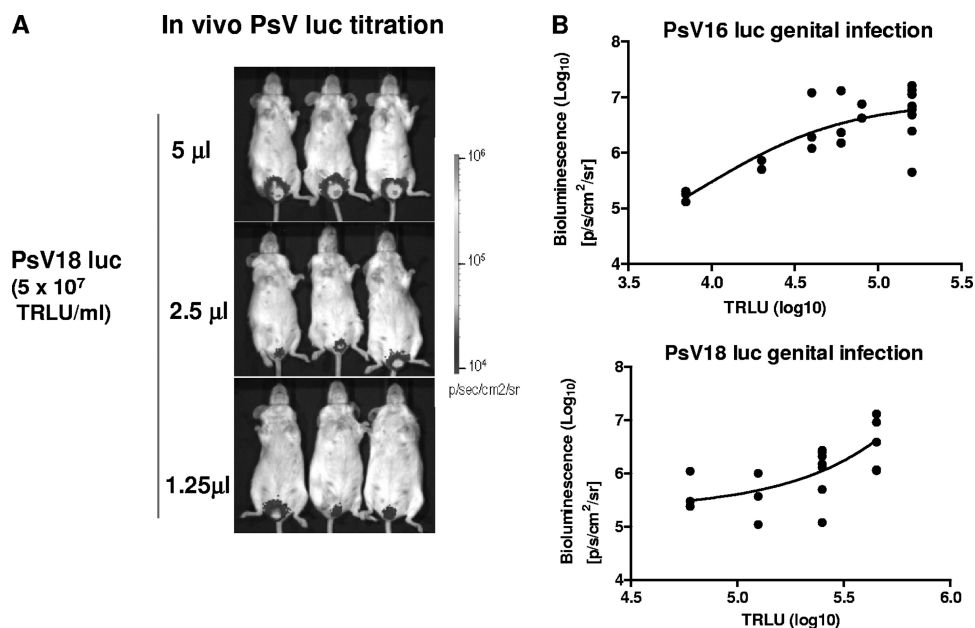**A** **In vivo PsV luc titration**

B

FIG. 3. PsVluc titration in genital challenge assays. Groups of mice ($n = 3$) were ivag challenged with different amounts of PsVluc, and bioluminescence was visualized with a Xenogen camera 2 days later. (A) Representative experiment. (B) Bioluminescence measured (in photons/s/cm²/sr) after PsV16 luc (upper graph) or PsV18 luc (lower graph) genital infection was plotted against the number of TRLU used (see Materials and Methods). Curves were drawn with GraphPad Prism using the standard slope model.

cence (means ± standard deviations) of $\log_{10}$ 6.35 ± 0.87 photons/s/cm²/sr.

V5 antibody titers/amounts conferring protection against *in vivo* genital PsV16 challenge. For practical reasons and to take advantage of the stability of the transferred antibodies, we decided to perform PsVluc genital challenges 24 h after antibody injection, while luciferase expression was monitored 2 days later. Sets of five mice were used for each experimental condition. Experiments always included one group of noninjected control mice and two to three groups of mice injected with different amounts of V5. A representative experiment shows noninjected mice and mice injected with 5×10^{-1} and 5×10^{-4} µg of V5 (Fig. 4A) and the measured bioluminescence (Fig. 4B). Bioluminescence values of the antibody-injected mice were normalized to the mean bioluminescence of the control noninjected group and expressed as the percent inhibition of PsV16 infection (Fig. 4C; see Materials and Methods for calculations). In addition, in all antibody-injected mice, blood was sampled just before or during the ivag N9 treatment preceding genital PsV16luc challenge to determine the circulating HPV16 neutralizing titers and potential correlation with protection. Serum HPV16 neutralizing titers measured in mice injected with 5×10^{-4} to 5×10^{1} µg of V5/ mouse are shown in Fig. 4D. It is noteworthy that the injection of less than 0.5 µg of V5 resulted in recipient serum levels that were undetectable in the neutralization assay. In contrast, the inhibition of PsV16 infection still was observed when less than 0.5 µg V5 was injected. A strong correlation was observed when amounts of injected V5 were plotted against the percent inhibition of infection (Pearson $r = 0.83$; $P < 0.001$). The correlation curve (generated by GraphPad Prism; least square ordinary fit, $R^2 = 0.71$) (Fig. 4E) generated a calculation so that 0.015 µg (95% confidence interval [CI], 0.004 to 0.055 µg)

of injected V5 was required for 50% inhibition of PsV16luc infection, which is ca. 30-fold lower than the minimal amount of V5 detected by *in vitro* PsV neutralization assays.

Protection against *in vivo* genital challenge with PsV16 and PsV18 by serum transferred from Gardasil-immunized mice. Similarly to the previous assays, sets of five mice were used for each experimental condition, always including one group of noninjected control mice and two to three groups of mice injected with different volumes of serum obtained from mice previously i.m. immunized with Gardasil (Fig. 1B; also see Materials and Methods) and pooled, here referred as to Gardasil serum. Serum HPV16 and HPV18 neutralizing titers measured in mice injected with 3×10^{-5} to 3×10^{2} µl of Gardasil serum are shown in Fig. 5A. The injection of less than 3 µl of Gardasil serum was not detectable by either the PsV16 or PsV18 *in vitro* neutralization assays in the recipient mouse serum. In contrast, the inhibition of both PsV16 and PsV18 infections, as assessed in separate experiments, still were observed when less than 3 µl of Gardasil serum was injected. Strong correlations were observed when volumes of injected Gardasil serum were plotted against the percent inhibition of infection (Pearson $r = 0.82$ and 0.83 for PsV16 and PsV18, respectively; $P < 0.0001$ for both). The correlation curves (GraphPad Prism; least square ordinary fit, $R^2 = 0.72$ and 0.70 for PsV16 and PsV18, respectively) (Fig. 5B) indicate that the injected Gardasil serum volume leading to the 50% inhibition of PsV16 and PsV18 infections was 4.2×10^{-3} µl (95% CI, 1.4×10^{-3} to 13.0×10^{-3} µl) and 3.3×10^{-3} µl (95% CI, 1×10^{-3} to 10.3×10^{-3} µl), respectively. These amounts are more than 500-fold lower than the lowest volume of injected Gardasil serum detectable by the PsV neutralization assays in the serum of the recipient mice.
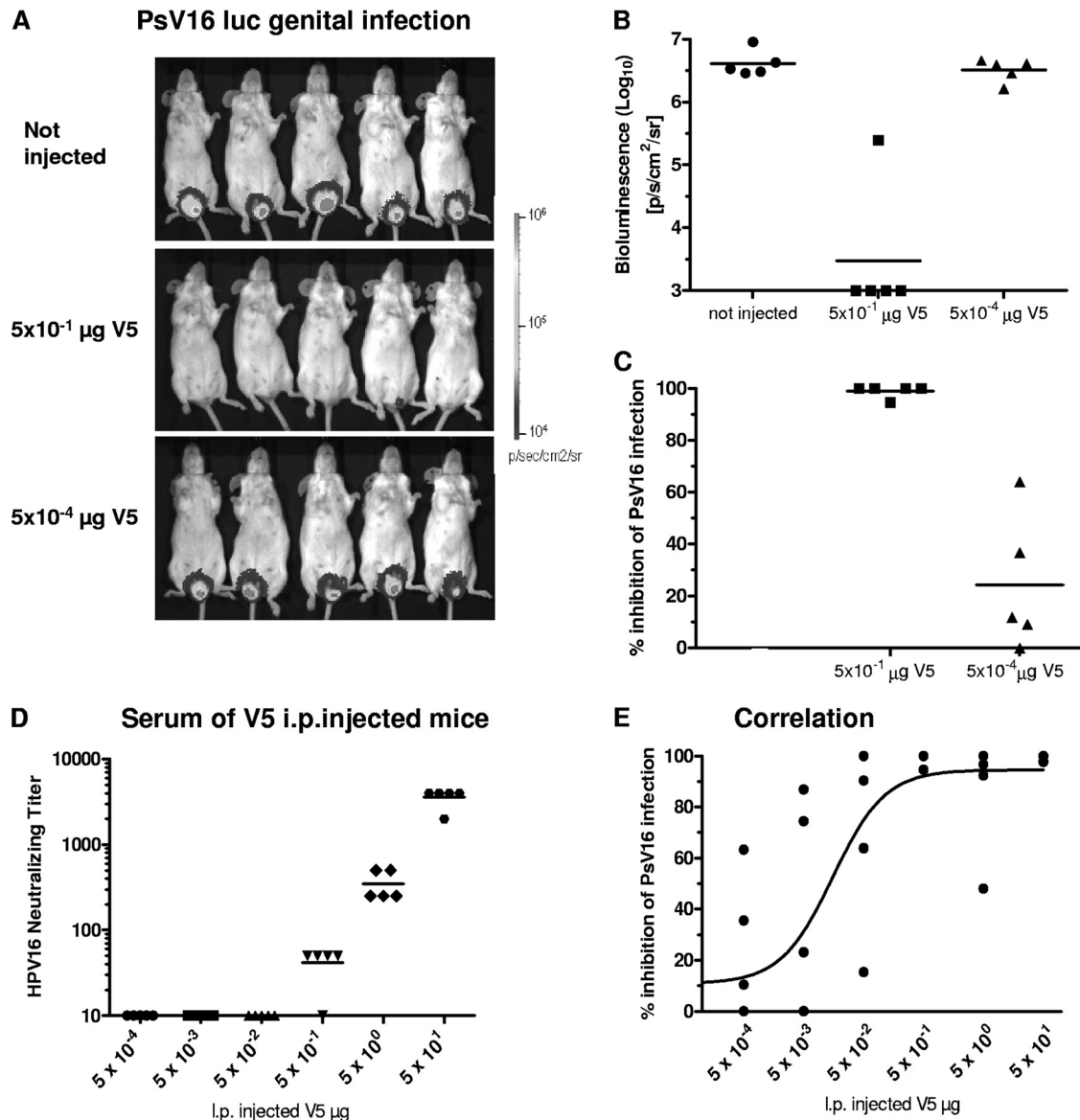
FIG. 4. Transferred V5 antibodies and protection against *in vivo* genital PsV16 challenge. Groups of five mice i.p. injected with 5×10^{-1} μg V5, 5×10^{-4} μg V5, or not injected were ivag challenged 24 h later with PsV16luc. Bioluminescence was visualized with a Xenogen camera 48 h after PsV16luc infection (A) and measured in photons/s/cm²/sr (B). (C) Inhibition of PsV16 infection was calculated as indicated in Materials and Methods. HPV16 neutralizing titers measured by PsV16/SEAP *in vitro* neutralization assay in mouse serum (D) and the inhibition of PsV16luc infection *in vivo* (E) at 24 and 72 h, respectively, after the i.p. transfer of V5 (5 mice/group) were plotted against the quantity of V5 transferred (5×10^{-4} to 5×10^1 μg V5). A correlation curve was drawn with GraphPad Prism using the least-square ordinary fit.

DISCUSSION

Correlates of protection for the HPV vaccines have not yet been determined. Here we show that, surprisingly, *in vivo* genital HPV PsV challenge in mice is more sensitive for detecting protecting antibody levels than the available *in vitro* assays, including the gold standard PsV neutralization assay. *In vivo* HPV PsV challenge in the genital tract, but also on the skin, has been performed mainly in mice previously immunized with HPV vaccines (1, 7, 18, 20). In addition to protection against VLP-induced antibodies, this challenge model also was shown to detect protective antibodies induced by L2-based vaccines (1, 18, 20). Interestingly, in the latter cases, the *in vivo* PsV

challenge assay showed protection against some nonvaccine HPV types, even though cross-neutralizing titers were difficult to detect in *in vitro* neutralization assays, thus suggesting the higher sensitivity of the *in vivo* assay. Of note, such a higher sensitivity is observed here despite ca. 200-fold more PsV particles being used for *in vivo* genital challenge than for the *in vitro* neutralization assay. Our data show that, when using the V5 monoclonal antibody, the sensitivity of the *in vivo* genital challenge assay was only 30-fold higher than that of the *in vitro* neutralization assay, while when using Gardasil serum the difference was close to 500-fold. This may reflect the presence of protecting antibodies in the Gardasil serum that act differently
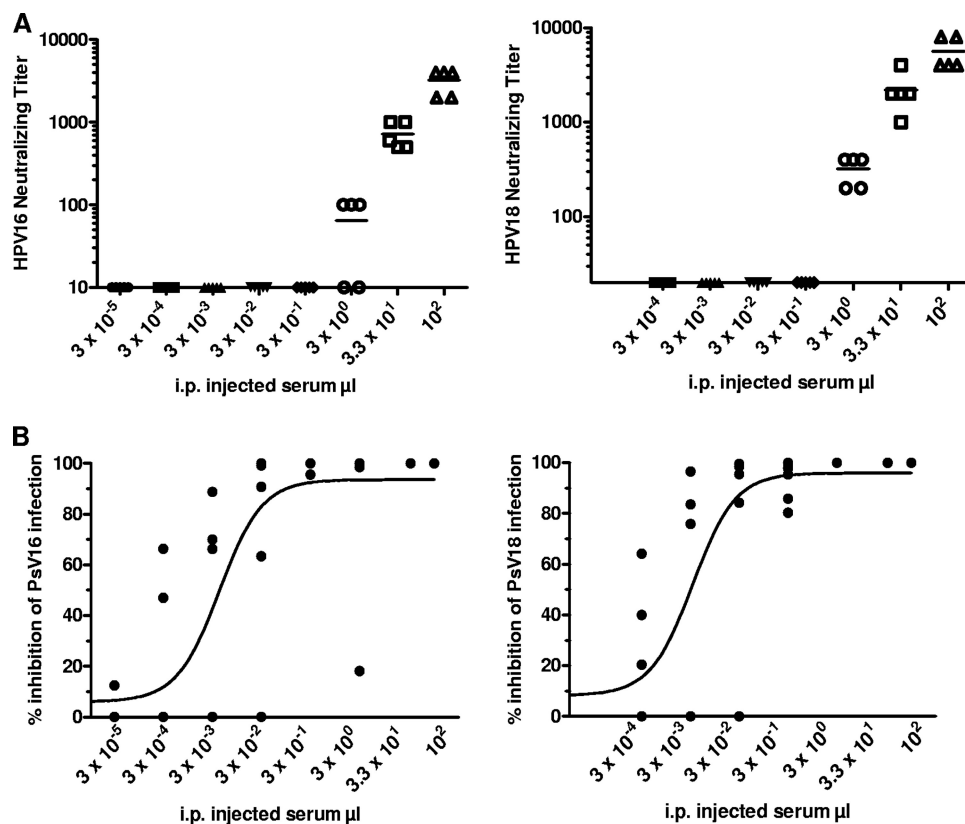
FIG. 5. Amounts of transferred Gardasil serum conferring protection against *in vivo* genital PsV16 or PsV18 challenge. HPV16 or HPV18 neutralizing titers measured by PsV16/SEAP or PsV18/SEAP *in vitro* neutralization assays in mouse serum (A) and inhibition of PsV16luc or PsV18luc infection *in vivo* (B) at 24 and 72 h, respectively, after the i.p. transfer of Gardasil serum (5 mice/group) were plotted against the volume of Gardasil serum transferred (3×10^{-5} to 1×10^2 µl). Correlation curves were drawn with GraphPad Prism using least-square ordinary fit.

from V5 and exert their action at lower concentrations and/or by mechanisms that are not measured in the *in vitro* neutralization assay. Consistently with this conjecture, Day et al. (9) recently reported evidence of a specific mechanism of neutralization when low concentrations of HPV16 VLP-induced rabbit antibodies were transferred in mice and the mice were challenged with PsV16. Low antibody levels did not impede initial PsV binding to the basement membrane or the exposure of L2. Rather, they appeared to inhibit capsid interaction with a secondary keratinocyte-specific receptor (9), a mechanism that may differ from that of V5, which *in vitro* prevents the cell surface conformational change in the HPV capsid that is necessary for anti-L2 antibody binding and/or furin cleavage (8, 10).

Obviously, it is difficult to establish the extent to which genital-challenge murine model mimics infection in women. However, our data show that the mild detergent (4% N9) used to expose the basement membrane for HPV infections induces the transudation/exudation of serum IgG without extensive damage, as indicated by the unaffected local production of anti-VLP IgA. Interestingly, N9 is widely used as a spermicidal by millions of women every year. The approximately 10-fold increase of local transudating/exudating serum antibodies induced by 4% N9 treatment also is probably occurring in the genital mucosa of women at the occurrence of local microtrauma. In mice, it is difficult to estimate the local mucosal concentration of neutralizing antibodies because of the

method of recovery (vaginal washes with ca. 200 µl) leading to a dilution of the original secretion, which may vary between 10 and 200 times (assuming original secretion volumes between 1 and 10 µl). In addition, a gradient of decreasing exudated antibody concentration would be expected with increasing distance from the site of trauma. Thus, it is possible that the concentration of neutralizing antibody at the site of initial virus deposition, the basement membrane, approaches that of circulating antibodies, which are ca. 2 log higher than the measured levels of genital antibodies (Fig. 1 and 2). In women, where the sampling of genital secretions involved absorption to Weck-cell sponges gently applied on the cervix and where dilution factors could be calculated, the concentration of genital antibodies varied between 1 and 90% of the levels measured in serum depending on the individual, period of their hormonal cycle, or use of contraceptive (23). It is noteworthy that the application of Weck-cell sponges at the cervical os, although reported as nontraumatic (16), sometimes was sufficient to induce microtrauma, with visible bleeding occurring in 25% of the cases (23), further supporting the rationale that the exudation of serum antibodies during natural HPV infections readily occurs.

Here we have transferred serum from mice immunized with Gardasil, while rabbit serum was shown to be effective by Day et al. (9). Similar results are anticipated if serum from women vaccinated with the HPV vaccines was used. It will be inter-

esting to determine the minimum circulating levels of passively transferred sera from VLP-vaccinated women that are needed to protect the mice from our high-dose cervicovaginal HPV PsV challenge. One might reasonably expect that similar levels of circulating antibodies in the women would protect them from genital HPV infection, since they are likely to be exposed to lower doses of virus. The testing of serum from vaccinees with breakthrough infections by vaccine-targeted or cross-protected HPV types also could provide a proof-of-principle correlate of infection. However, these types of studies will be confounded by the inability to definitively distinguish incident infection from the reactivation of latent infection or the emergence of prevalent infection.

Establishing correlates of protection of HPV vaccine efficacy is not only important for estimating the durability of protection by currently commercialized vaccines but also is essential for the development of next-generation HPV vaccines for which efficacy trials may be difficult or too expensive to organize. Licensure based on immune correlates is being considered for other vaccines. In the case of anthrax, a lethal toxin neutralization assay likely will be used to correlate the protection offered by new anthrax vaccines in animal models to its immunogenicity in humans (24).

REFERENCES

1. **Alphs, H. H., et al.** 2008. Protection against heterologous human papillomavirus challenge by a synthetic lipopeptide vaccine containing a broadly cross-neutralizing epitope of L2. Proc. Natl. Acad. Sci. USA **105**:5850–5855.
2. **Balmelli, C., et al.** 1998. Nasal immunization of mice with human papillomavirus type 16 virus-like particles elicits neutralizing antibodies in mucosal secretions. J. Virol. **72**:8220–8229.
3. **Breitburd, F., et al.** 1995. Immunization with viruslike particles from cottontail rabbit papillomavirus /CRPV) can protect against experimental CRPV infection. J. Virol. **69**:3959–3963.
4. **Brown, D. R., et al.** 2011. The humoral response to Gardasil over four years as defined by total IgG and competitive luminex immunoassay. Human Vaccines **7**:230–238.
5. **Buck, C., D. V. Pastrana, D. R. Lowy, and J. T. Schiller.** 2004. Efficient intracellular assembly of papillomaviral vectors. J. Virol. **78**:751–757.
6. **Christensen, N. D., et al.** 1996. Surface conformational and linear epitopes on HPV-16 and HPV-18 L1 virus-like particles as defined by monoclonal antibodies. Virology **223**:174–184.
7. **Cuburu, N., et al.** 2009. Sublingual immunization with nonreplicating antigens induces antibody-forming cells and cytotoxic T cells in the female genital tract mucosa and protects against genital papillomavirus infection. J. Immunol. **183**:7851–7859.
8. **Day, P. M., R. Gambhira, R. B. Roden, D. R. Lowy, and J. T. Schiller.** 2008. Mechanisms of human papillomavirus type 16 neutralization by l2 cross-neutralizing and l1 type-specific antibodies. J. Virol. **82**:4638–4646.
9. **Day, P. M., et al.** 2010. In vivo mechanisms of vaccine-induced protection against HPV infection. Cell Host Microbe **8**:260–270.
10. **Day, P. M., et al.** 2007. Neutralization of human papillomavirus with monoclonal antibodies reveals different mechanisms of inhibition. J. Virol. **81**:8784–8792.
11. **Dessy, F. J., et al.** 2008. Correlation between direct ELISA, single epitope-based inhibition ELISA and pseudovirion-based neutralization assay for measuring anti-HPV-16 and anti-HPV-18 antibody response after vaccination with the AS04-adjuvanted HPV-16/18 cervical cancer vaccine. Human Vaccines **4**:425–434.
12. **Einstein, M. H., et al.** 2009. Comparison of the immunogenicity and safety of Cervarix and Gardasil human papillomavirus (HPV) cervical cancer vaccines in healthy women aged 18–45 years. Human Vaccines **5**:705–719.
13. **Fraillery, D., et al.** 2007. Ty21a expressing human papillomavirus type 16 L1 as a potential live Salmonella vaccine against cervical cancer and typhoid fever. Clin. Vaccine Immunol. **14**:1285–1295.
14. **Fraillery, D., N. Zosso, and D. Nardelli-Haefliger.** 2009. Rectal and vaginal immunization of mice with human papillomavirus L1 virus-like particles. Vaccine **27**:2326–2334.
15. **Harper, D. M., et al.** 2004. Efficacy of a bivalent L1 virus-like particle vaccine in prevention of infection with human papillomavirus types 16 and 18 in young women: a randomised controlled trial. Lancet **364**:1757–1765.
16. **Hildesheim, A., et al.** 1999. Cytokine and immunoglobulin concentrations in cervical secretions: reproducibility of the Weck-cel collection instrument and correlates of immune measures. J. Immunol. Methods **225**:131–143.
17. **Hopkins, S., et al.** 1995. A recombinant *Salmonella typhimurium* vaccine induces local immunity by four different routes of immunization. Infect. Immun. **63**:3279–3286.
18. **Jagu, S., et al.** 2009. Concatenated multitype L2 fusion proteins as candidate prophylactic pan-human papillomavirus vaccines. J. Natl. Cancer Inst. **101**:782–792.
19. **Joura, E. A., et al.** 2008. HPV antibody levels and clinical efficacy following administration of a prophylactic quadrivalent HPV vaccine. Vaccine **26**:6844–6851.
20. **Karanam, B., et al.** 2009. Vaccination with HPV16 L2E6E7 fusion protein in GPI-0100 adjuvant elicits protective humoral and cell-mediated immunity. Vaccine **27**:1040–1049.
21. **Kemp, T. J., et al.** 2008. Evaluation of systemic and mucosal anti-HPV16 and anti-HPV18 antibody responses from vaccinated women. Vaccine **26**:3608–3616.
22. **Nardelli-Haefliger, D., et al.** 1997. Human papillomavirus type 16 virus-like particles expressed in attenuated Salmonella typhimurium elicit mucosal and systemic neutralizing antibodies in mice. Infect. Immun. **65**:3328–3336.
23. **Nardelli-Haefliger, D., et al.** 2003. Specific antibody levels at the cervix during the menstrual-cycle of women vaccinated with human papillomavirus 16 virus-like particles. J. Natl. Cancer Inst. **95**:1128–1137.
24. **Omland, K. S., A. Brys, D. Lansky, K. Clement, and F. Lynn.** 2008. Inter-laboratory comparison of results of an anthrax lethal toxin neutralization assay for assessment of functional antibodies in multiple species. Clin. Vaccine Immunol. **15**:946–953.
25. **Opalka, D., et al.** 2003. Simultaneous quantitation of antibodies to neutralizing epitopes on virus-like particles for human papillomavirus types 6, 11, 16, and 18 by a multiplexed luminex assay. Clin. Diagn. Lab. Immunol. **10**:108–115.
26. **Paavonen, J., et al.** 2009. Efficacy of human papillomavirus (HPV)-16/18 AS04-adjuvanted vaccine against cervical infection and precancer caused by oncogenic HPV types (PATRICIA): final analysis of a double-blind, randomised study in young women. Lancet **374**:301–314.
27. **Parkin, D. M.** 2006. The global health burden of infection-associated cancers in the year 2002. Int. J. Cancer **118**:3030–3044.
28. **Pastrana, D. V., et al.** 2004. Reactivity of human sera in a sensitive, high-throughput pseudovirus-based papillomavirus neutralization assay for HPV16 and HPV18. Virology **321**:205–216.
29. **Revaz, V., et al.** 2007. Humoral and cellular immune responses to airway immunization of mice with human papillomavirus type 16 virus-like particles and mucosal adjuvants. Antiviral Res. **76**:75–85.
30. **Roberts, J. N., et al.** 2007. Genital transmission of HPV in a mouse model is potentiated by nonoxynol-9 and inhibited by carrageenan. Nat. Med. **13**:857–861.
31. **Suzich, J. A., et al.** 1995. Systemic immunization with papillomavirus L1 protein completely prevents the development of viral mucosal papillomas. Proc. Natl. Acad. Sci. U. S. A. **92**:11553–11557.
32. **Villa, L. L., et al.** 2005. Prophylactic quadrivalent human papillomavirus (types 6, 11,16 and 18) L1-virus-like particle vaccine in young women: a randomised double-blind placebo-controlled multicentre phase II efficacy trial. Lancet Oncol. **6**:271–278.
33. **Villa, L. L., et al.** 2006. High sustained efficacy of a prophylactic quadrivalent human papillomavirus types 6/11/16/18 L1 virus-like particle vaccine through 5 years of follow-up. Br. J. Cancer **95**:1459–1466.