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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

Nicolas Çuburu,†‡ Mi-Na Kweon,* Catherine Hervouet,† Hye-Ran Cha,* Yuk-Ying S. Pang,‡ Jan Holmgren,§ Konrad Stadler,* John T. Schiller,‡ Fabienne Anjüere,† and Cecil Czerkinsky2*†

We have recently reported that the sublingual (s.l.) mucosa is an efficient site for inducing systemic and mucosal immune responses. In this study, the potential of s.l. immunization to induce remote Ab responses and CD8+ cytotoxic responses in the female genital tract was examined in mice by using a nonreplicating Ag, OVA, and cholera toxin (CT) as an adjuvant. Sublingual administration of OVA and CT induced Ag-specific IgA and IgG Abs in blood and in cervicovaginal secretions. These responses were associated with large numbers of IgA Ab-secreting cells (ASCs) in the genital mucosa. Genital ASC responses were similar in magnitude and isotype distribution after s.l., intranasal, or vaginal immunization and were superior to those seen after intragastric immunization. Genital, but not blood or spleen, IgA ASC responses were inhibited by treatment with anti-CCL28 Abs, suggesting that the chemokine CCL28 plays a major role in the migration of IgA ASC progenitors to the reproductive tract mucosa. Furthermore, s.l. immunization with OVA induced OVA-specific effector CD8+ cytolytic T cells in the genital mucosa, and these responses required coadministration of the CT adjuvant. Furthermore, s.l. administration of human papillomavirus virus-like particles with or without the CT adjuvant conferred protection against genital challenge with human papillomavirus pseudovirions. Taken together, these findings underscore the potential of s.l. immunization as an efficient vaccination strategy for inducing genital immune responses and should impact on the development of vaccines against sexually transmitted diseases. The Journal of Immunology, 2009, 183: 7851–7859.

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2 Address correspondence and reprint requests to Dr. Cecil Czerkinsky, International Vaccine Institute, Seoul National University Research Park, Kwanak-Gu, Seoul, Republic of Korea 151-818. E-mail address: cczerkinsky@ivi.int

Abbreviations used in this paper: ASC, Ab-secreting cell; CT, cholera toxin; HPV, human papillomavirus; i.vag., intravaginal; i.n., intranasal; ILN, ileosacral lymph node; i.v., intravenous; SEAP, secreted alkaline phosphatase; s.l., sublingual; SMLN, submandibular lymph node; VLP, virus-like particle.

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lower doses of Ag to induce genital Ab responses of the same magnitude and is theoretically less sensitive to the host hormonal status (18). However, the use of the i.n. route has raised certain concerns associated to the potential redirection of live-attenuated organisms or toxin-based adjuvants to the CNS (21–24).

We have recently reported that the sublingual (s.l.) mucosa is an efficient site for inducing broad spectrum immune responses, including secretory and systemic Ab responses and mucosal as well as systemic CTLs (25, 26). In this study, we present evidence that s.l. immunization of female mice with a nonreplicating Ag, OVA, and cholera toxin (CT) adjuvant induces remote mucosal immune responses associated with the migration of Ag-specific IgA Abs secreting cells (ASCs) and CTLs in the genital tract mucosa. Furthermore, we show that HPV virus-like particles (VLPs) administered sublingually (s.l.) induced HPV-neutralizing Abs in serum, virus-specific Abs in genital tissues, and protection against genital challenge with HPV even in the absence of CT.

Materials and Methods

Mice

Female BALB/c and C57BL/6 mice were used at 6–8 wk of age. All mice were bred and maintained under the American Association for Accreditation of Laboratory Animal Care (AAALAC)-accredited animal care facilities of Nice Medical School (Nice, France), the International Vaccine Institute (Seoul, South Korea), and the U.S. National Cancer Institute (Bethesda, MD) in accordance with national and international guidelines, and all experiments described in this article were approved by the respective ethical committees for animal experimentation.

Immunizations

Progestosterone (Depo-Provera)-treated mice were used in all experiments. Grade V chicken egg OVA (Sigma-Aldrich) and CT (List Biological Laboratories) were diluted in isotonic, pyrogen-free saline. Unless otherwise indicated, OVA (200 μg) alone or admixed with CT (2 μg) was administered on days 0, 7 and 21, and mice were sacrificed 7 days after the last immunization. These doses were selected for inducing the most consistent serum Ab responses to OVA (25). For s.l. and i.n. immunization, mice were heavily anesthetized with ketamine/xylazine and a total volume of 5 μl of OVA solution with/without CT in saline was topically administered onto the s.l. mucosa or split into each nostril, respectively. Animals were maintained with the head placed in an anteflexion position for 30 min to keep the Ag solutions on the s.l. mucosa. Sham-treated control animals were fed and maintained under the same pathogen-free conditions in the animal care facilities of Nice Medical School (Nice, France), the International Vaccine Institute (Seoul, South Korea), and the U.S. National Cancer Institute (Bethesda, MD) in accordance with national and international guidelines, and all experiments described in this article were approved by the respective ethical committees for animal experimentation.

Preparation of cell suspensions

For HPV vaccination, mice were immunized with HPV16 L1 VLPs on days 0, 7, and 21. HPV16 L1 VLPs were produced as described previously (27, 28). Anesthetized mice were s.l. administered 10 μl of saline containing either 5 μg of HPV16 L1 VLPs alone or admixed with 2 μg of CT. A separate group of mice received an i.m. injection of 50 μl of HPV16 L1 VLPs adsorbed with alum (Pierce).

Chemotaxis assay

Lymph node cells were isolated from the SMLNs and ILNs of mice immunized s.l. or i.vag., respectively. Cells were suspended in complete medium and allowed to rest for 1 h at 37°C with 5% CO2. Rested cells were placed in the upper chamber of a 8-μm Transwell plate (Corning Costar) at 106 cells per well. In some wells, 105 cells were placed directly in the lower chamber as a positive control for maximum migration. The lower chamber contained medium alone or medium containing cytokines at concentrations determined to be optimal (250 nM for CCL2, 300 nM for CCL25, and 100 nM for CXCL12). After incubation at 37°C with 5% CO2 for 4 h, cells that had migrated were harvested from the lower chamber.

In vivo Ab-mediated inhibition of ASC migration

Groups of five female BALB/c mice were primed s.l. with 5 μg of CT. One month later, mice were boosted s.l. with 5 μg of CT. To neutralize CCL28 activity, mice were injected i.p. on day s.l. 3, and 5 after a boost with 100 μg of rat monoclonal anti-CCL28 Ab or rat Ig2b isotype control (R&D Systems). On day 7 after the boost, mice were sacrificed and blood, genital tract, SMLNs, and spleen were harvested. The frequency of Ag-specific ASCs in these organs was determined by ELISPOT.

Cytokine assays

Triplicate cultures of SMLN or spleen cells were seeded at 4 × 104 cells per flat-bottom well of 96-well culture plates (Falcon; BD Biosciences) together with 1 × 103 accessory cells. Accessory cells were prepared by depleting naive syngeneic spleen cell suspensions with magnetic beads coated with anti-CD3 (clone KT3-1.1) and anti-rat Ig following the manufacturer’s (Dynal Biotech) protocol. After incubation at 37°C with 5% CO2 for 72 h in the presence or absence of 2 ng/ml OVA, culture supernatants were assayed for IFN-γ, IL-4, and IL-10 contents by means of calibrated ELISAs as described previously (33).

Flow cytometry detection of tissue CTLs

An in vivo CTL assay was performed essentially as described (34) with minor modifications. Spleen cell suspensions were prepared from C57BL/6
mice and split into two fractions. One fraction was labeled with 4 μM CFSE (nominal CFSE<sup>high</sup> cells) for 5 min at room temperature and pulsed for 45 min with 1 μM OVA<sub>257–264</sub> SIINFEKL peptide (Proimmune) at 37°C with 5% CO<sub>2</sub>. The other cell fraction consisted of cells labeled with 0.4 μM CFSE for 5 min at room temperature (nominal CFSE<sup>low</sup> cells) and without peptide pulse. A total of 15 × 10<sup>5</sup> cells comprising equal numbers of CFSE<sup>high</sup> and CFSE<sup>low</sup> cells in 200 μl of saline were injected i.v. into sham (control) mice and into mice that had been previously immunized with CT (2 μg) and/or OVA (200 μg). For detection of genital CTLs, 2 × 10<sup>6</sup> target cells were injected directly into the vaginal mucosa of anesthetized mice using an insulin syringe (35). Single cell suspensions from ILN, spleen, and genital tract mucosa were prepared 24 h after cell transfer and analyzed for differential CFSE staining by flow cytometry. The uterus and vagina were excised and digested for 45 min at 37°C with dispase II (1:2 U/ml in RPMI 1640) (Roche Diagnostics) before analysis. Specific killing activity was calculated according to the formula 1 – (ratio of CFSE<sup>low</sup>/CFSE<sup>high</sup> cells in control mice/ratio of CFSE<sup>low</sup>/CFSE<sup>high</sup> cells in immunized mice) × 100 and expressed as percentage of specific lysis.

Production of HPV16 pseudovirus

HPV16 pseudovirions containing the reporter plasmid pCLucf or pYSEAP, which encodes luciferase and GFP or a secreted form of alkaline phosphatase, respectively, was produced as described previously (27, 28). Detailed protocols and plasmid maps are accessible on the website of the Laboratory of Cellular Oncology, National Cancer Institute, National Institutes of Health (Bethesda, MD; home.ccr.cancer.gov/Leco/). Plasmid purity and capsid content were determined on 10% SDS-Tris-glycine gels (Bio-Rad). For HPV16 pseudovirions carrying the pCLucf plasmid, the infectious titer was determined by flow cytometry on 293TT cells 48 h postinfection. For HPV16 pseudovirions carrying the pYSEAP plasmid, infectivity was determined on 293TT cells by SEAP detection 24 h postinfection.

Mouse model of vaginal HPV16 infection

All mice were challenged for HPV genital infection 2 wk after the third immunization. The protocol for in vivo genital infection has been previously described in detail (36). Two weeks after the third immunization, mice were injected s.c. with 3 mg of medroxyprogesterone acetate (Depo-Provera) for 4 days before challenge with HPV pseudovirions. Mice were pretreated i.v. with 50 μl of nonoxynol-9 (Conceptrol) to permeabilize the epithelium and challenged 5 h later with an i.v. instillation of 2 × 10<sup>7</sup> IU of HPV pseudovirions diluted in carbocryethyl cellulose 2% (w/v).

HPV infection was monitored by measuring luciferase expression in the genital tract on day 2 postchallenge. Anesthetized mice were instilled i.v. with 20 μl of luciferine (Sigma-Aldrich) and imaged 3 min later during a 1-min exposure using a Xenogen IVIS in vivo imager (Caliper Science).

Statistical analyses

The corrected (Bonferroni) nonparametric Mann-Whitney U test was used for pair wise multiple comparisons between experimental groups. A value of p < 0.05 was considered significant.

Results

Sublingual administration of CT and OVA induces Ag-specific Abs in the cervicovaginal secretions

Groups of 8–15 mice were immunized s.l. with different doses of OVA (10, 50, and 200 μg) given together with a CT (2 μg) adjuvant, and the presence of specific Abs was examined in genital secretions 1 wk after the last of three consecutive immunizations (Fig. 1A). Genital IgA Ab responses to OVA were readily detected in mice immunized with 10 μg of OVA plus CT adjuvant, and these responses were comparable between animals immunized by the s.l. and the i.n. routes. Increased doses (50 and 200 μg) of OVA yielded more pronounced and more consistent responses (Fig. 1A). Genital Ab titers induced by s.l. immunization were comparable to those observed after i.vag. immunization of mice treated with progesterone but higher than those of untreated mice (Fig. 1B and data not shown). Furthermore, s.l. administration of OVA alone induced significant IgA and IgG Ab titers that were enlarged when OVA was coadministered with CT (Fig. 1B). Overall, s.l. immunization induced IgA Abs in genital secretions at levels comparable to those seen after i.n. or i.vag. immunization, but markedly higher than after i.g. immunization (Fig. 1B).

Whereas OVA-specific Ab responses in blood were predominantly of the IgG class (Fig. 1C), genital secretions from animals immunized by the s.l. route with OVA and CT comprised comparable proportions of IgA and IgG Abs to OVA (Fig. 1B). Similar findings were obtained in animals immunized by either the i.n. or the i.vag. routes (Fig. 1, B and C). In contrast, i.g. immunization with OVA plus CT induced weak or negligible Ab responses in genital secretions (Fig. 1B).

Consistent with our previous study (23), s.l. immunization with CT and OVA gave rise to specific Ab responses in blood, and these responses were comparable to those observed after i.n. or i.vag. immunization, but significantly stronger than after oral immunization (Fig. 1C). Sublingual immunization with CT also elicited high anti-CT IgA and IgG Ab titers in serum and genital secretions (data not shown).

Sublingual administration of CT and OVA induces Ag-specific ASCs in the genital tract mucosa but not in the ILN

To further determine to which extent genital Ab responses observed after s.l. immunization result from local Ab formation, ELISPOT analyses of enzymatically dispersed genital tissue specimens were conducted 7 days after the last of three immunizations with OVA plus CT. As shown in Fig. 2A, s.l. immunization induced large numbers of OVA-specific and CT-specific ASCs in the
These responses were markedly dominated by IgA ASCs and were comparable to or even higher than those seen after i.n. and i.vag. immunization (Fig. 2A). ILNs were devoid of ASCs after either s.l. or i.n. immunization with CT plus OVA but contained large numbers of IgG ASCs in animals immunized by the vaginal route (Fig. 2B). Splenic ASC responses to CT induced by s.l. immunization were comparable in magnitude and isotype distribution to the corresponding responses induced by i.n. or i.vag. immunization, being comprised of larger numbers of IgG ASCs, especially after i.vag. immunization (Fig. 2C). Surprisingly, spleen ASC responses to OVA were predominantly of the IgA isotype and were larger after i.n. and s.l. immunization than after i.vag. immunization. The i.g. administration of OVA plus CT failed to induce appreciable genital ASC responses and very weak splenic ASC responses.

These findings suggest that genital IgA Ab responses induced by s.l. immunization are to a large extent contributed by local ASCs, whereas IgG responses appear to be derived from an extragenital source, probably from blood and peripheral lymphoid tissues such as the spleen. Furthermore, these results indicate that genital IgA ASCs induced by s.l. and i.vag. immunization do not originate from draining ILNs, contrary to IgA ASCs induced by local genital immunization.

Sublingual immunization induces CCL28-dependent selective migration of IgA ASCs to the genital mucosa

The observation that IgA and IgG ASCs induced by s.l. immunization markedly differ with respect to their anatomic distribution suggested that IgA and IgG ASCs induced in lymph nodes draining the site of immunization, i.e., SMLNs, comprise ASC populations with different migratory properties. To address this issue, in vitro migration experiments were performed with SMLN cells collected after s.l. immunization with CT and subjected to various chemokine gradients. As seen in Fig. 3A, CT-specific IgA ASCs isolated from SMLNs migrated to CCL28, a common mucosa-associated epithelial chemokine known to selectively attract IgA plasmablasts (37). These ASCs failed to respond to CCL25, a chemokine mediating extravasation of IgA ASCs into the small intestine (38). In striking contrast, SMLN IgG ASCs failed to respond to CCL28 and CCL25 (Fig. 3A). In addition, IgG and IgA ASCs did not differ with regard to their potential to migrate against CXCL12. Because both IgA and IgG ASCs to CT could be induced in ILN by local i.vag. immunization (Fig. 2B), similar experiments were performed with ILN cells from i.vag. immunized mice. Fig. 3B

FIGURE 2. Sublingual immunization with CT and OVA induces systemic and genital Ab-secreting cells. Mice were immunized on days 0, 7, and 21 with saline by the s.l. route (sham) or with 200 μg of OVA together with 2 μg of CT given in saline by the s.l., i.n. i.vag., or i.g. routes and assayed 1 wk after the last immunization for OVA-specific IgA and IgG ASCs in the genital tract (A), ILN (B), and spleen (C). Data are expressed as mean ASC numbers per million cells ± S.D. (vertical bars) determined from groups of five to eight mice and are representative of three separate experiments. Asterisks denote significant differences between immunized mice and sham-immunized control mice (*, p < 0.05; Mann-Whitney U test).

FIGURE 3. Differential chemotactic responses of IgG and IgA ASCs after s.l. (A) or i.vag. (B) immunization. Mice were immunized on days 0, 7, and 21 with 2 μg of CT in saline by the s.l. or i.vag. route. Mononuclear cells isolated from draining SMLNs (A) and ILNs (B) were migrated to optimal concentrations of CCL28, CCL25, or CXCL12 chemokines. The numbers of migrated and input CT-specific IgG and IgA ASCs were determined by ELISPOT, and data are expressed as arithmetic mean percentages of migrated IgG (clear bar) and IgA (black bar) ASCs ± SD, determined in triplicate Transwells and pooled from two separate experiments.
Sublingual immunization with CT and OVA promotes Th1/Th2 cytokine responses

<table>
<thead>
<tr>
<th>Immunization</th>
<th>SMLN Cells</th>
<th>Spleen Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IFN-γ</td>
<td>IL-10</td>
</tr>
<tr>
<td>Sham (s.l.)</td>
<td>&lt;10^6</td>
<td>10^6</td>
</tr>
<tr>
<td>OVA (s.l.)</td>
<td>&lt;10^6</td>
<td>24 ± 15</td>
</tr>
<tr>
<td>CT + OVA (s.l.)</td>
<td>6919 ± 1603</td>
<td>813 ± 255</td>
</tr>
</tbody>
</table>

* Mice were immunized on days 0, 7, and 21. One week after the last immunization, mononuclear cells from the SMLN or spleen were stimulated with OVA in vitro and 72-h supernatants were assayed for cytokine contents.

Sublingual vaccination with HPV VLPs induces serum neutralizing Abs and genital Ab responses

Prior studies have shown that HPV VLPs are not only potent immunogens for systemic immunization but are also strongly immunogenic when administered i.n. or in the lungs (39, 40). Mice were immunized on days 0 and 7 and s.l. with 5 μg of HPV16 VLP with or without CT or i.m. with 5 μg of HPV16 L1 VLP adsorbed with alum. On day 21, serum samples were collected and assayed for HPV16-neutralizing activity. Sera from mice immunized s.l. with HPV16 L1 VLP alone or in combination with CT had respectively moderate (geometric mean titer, 1114; range, 640–2560) to strong neutralization titers (geometric mean titer, 31041; range, 15136–640–2560) to strong neutralization titers (geometric mean titer, 31041; range, 15136–640–2560). Of note, i.m. immunization with HPV16 L1 VLP plus alum induced significantly higher neutralizing titer (geometric mean titer, 31041; range, 15136–640–2560). These data indicate that s.l. immunization can induce effector CTL responses in genital tissues.

Sublingual immunization with CT and OVA induces remote mucosal CTLs

We next examined whether s.l. immunization could induce remote CD8+ T cells and cytolytic activity in the genital tract mucosa. A modified in vivo cytolytic assay in which labeled target spleen cells pulsed with a peptide entailing a prominent CTL epitope on OVA (OVA257–264), were directly injected into the vaginal wall was established for measuring putative genital CTL responses. In parallel, CTL responses were measured in spleen and ILN suspensions after i.v. injection of target cells. As shown in Fig. 5A, in vivo specific cytotoxic activity was readily detected in the genital tracts and ILNs of mice immunized s.l. with CT and OVA, and these responses were comparable to those seen after local i.vag. immunization and somewhat lower than after i.n. immunization (Fig. 5B). The s.l. immunization with CT and OVA also induced specific lysis in the SMLN (data not shown) and in the spleen (Fig. 5B). These data indicate that s.l. immunization can induce effector CTL responses in genital tissues.
Sublingual vaccination with HPV VLPs protects mice against genital challenge with HPV pseudovirions

A model mouse model of genital HPV infection based on the use of HPV pseudovirions carrying a luciferase reporter gene has been developed recently and recapitulates the early steps of HPV natural infection (36, 41). Mice were immunized on days 0, 7, and 21 and challenged i.vag. 2 wk after the last immunization with HPV16 pseudovirions carrying a luciferase gene. On day 2 after challenge the intensity of infection was monitored by measuring luciferase activity (expressed in photon/s (p/s)) in the genital area using a Xenogen IVIS imager. As illustrated in Fig. 7B, s.l. immunization with HPV16 VLPs given alone conferred complete protection against HPV16 genital infection compared with sham treated mice (Fig. 7A). Similar results were obtained in mice given s.l. VLP with CT adjuvant or i.m. VLP with alum. Thus, despite marked differences in the levels of serum and genital IgA and IgG Ab responses, s.l. immunization with VLPs even in the absence of CT adjuvant conferred protection as good as that of i.m. administration of the same dose of alum-adjuvanted VLPs (Fig. 7B).

Discussion

In this study, we show for the first time that s.l. immunization can induce secretory Ab responses and CTLs in the reproductive tract mucosa. We have previously shown that secretory Ab responses and bona fide mucosal CTLs can be induced in the respiratory mucosa after s.l. immunization (25). The present study further expands on these initial findings and demonstrates that, much in the same way as i.n. immunization, this route of vaccine administration is exceptionally potent for inducing disseminated mucosal effector B and T cell responses.

The magnitude and isotype distribution of genital Ab responses in animals immunized s.l., i.n., or i.vag. were comparable. The fact that IgA Ab responses dominated in genital secretions whereas IgG Abs were predominant in blood indicates that a proportion of these Abs are formed locally and not only result from the mere transudation of serum Abs through the genital epithelium. This
was immunized i.m. with 5 μg of HPV16 L1 VLP alone, or admixed with 2 μg of CT only, 5 μg of HPV16 L1 VLP alone or together with 2 μg of CT. A group of mice was immunized i.m. with 5 μg of HPV16 L1 VLP in alum. Vaginal washes were collected 2 wk after the second immunization and assayed for neutralization activity.

IgA ASCs, and to a lesser extent IgG ASCs, in genital tissues from s.l. (and for that matter also i.n. and i.vag.) immunized animals. It is also noteworthy that IgA ASCs predominated the genital ASC responses after s.l. immunization whereas IgG responses were predominant in serum. This suggests that genital IgA responses were contributed mainly by local ASCs, whereas the IgG Abs detected in genital secretions were to a large extent contributed by blood-derived Abs.

The fact that large numbers of specific ASCs could be detected in genital lymph nodes after local i.vag. immunization, confirming previous reports (13, 20), but not after s.l. (and i.m.) immunization suggests that genital ASCs induced by s.l. immunization originate from remote inductive site(s), presumably SMLN draining the sublingual mucosa. The finding that IgA ASCs predominated the genital response whereas the responses in SMLN comprised comparable numbers of IgA and IgG ASCs (23) suggests that IgA ASC precursors induced by s.l. immunization have unique migratory properties. The latter hypothesis is supported by the finding that SMLN IgA ASCs, but not IgG ASCs from s.l. immunized mice, migrated in vitro in response to CCL28, a major chemokine involved in the selective migration of IgA plasmablasts to mucosal tissues (37, 42, 43). Furthermore, in vivo treatment of mice with anti-CCL28 Ab markedly inhibited genital IgA ASC responses after s.l. immunization but had negligible if any effect on IgG and IgA ASC responses in the blood or spleen. Taken together, these results indicate that s.l. vaccination induces the migration of Ag-specific IgA ASCs into the vaginal mucosa in a CCL28-dependent manner. The finding that the female genital mucosa is an enriched source of CCL28 (H.-R. Cha and M. Kweon, manuscript in preparation) is compatible with this interpretation and further supports the notion that CCL28 plays an important role in the selective migration of IgA immunoblasts into genital tissues (44). The observation that local vaginal immunization with CT induced comparable IgA and IgG ASC responses in the genital tract mucosa and draining lymph nodes tends to indicate that CT modifies the genital microenvironment by inducing chemoattractive signals for both IgA and IgG ASCs. Experiments are ongoing to address this issue.

Given the marked degree of compartmentalization of the mucosal immune system (3, 5), the choice of the most appropriate route for immunization is of critical importance to develop effective vaccination strategies against genital infections. To date, the vaginal and nasal routes have proven to be effective for inducing genital immune responses, albeit the effectiveness of i.vag. immunization appears to depend on the host hormonal status (18, 19). Several studies have documented that i.n. immunization can induce genital immune responses characterized by the presence of ASCs and memory CD8+ T cells in the genital mucosa (9, 13, 18, 44, 45). We previously reported that, similarly as i.n. immunization, s.l. immunization with a nonreplicating Ag combined with CT as an adjuvant induced mucosal Abs and CTLs in the systemic compartment and in the upper aerodigestive tract mucosa (25).

Table II. Sublingual immunization with HPV VLP elicits neutralizing Abs in cervicovaginal secretions

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Neutering Ab Titer in Genital Secretionsb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Geometric mean</td>
</tr>
<tr>
<td>Sham</td>
<td>&lt;20</td>
</tr>
<tr>
<td>CT</td>
<td>&lt;20</td>
</tr>
<tr>
<td>HPV16 L1 VLP (s.l.)</td>
<td>46</td>
</tr>
<tr>
<td>HPV16 L1 VLP + CT (s.l.)</td>
<td>61</td>
</tr>
<tr>
<td>HPV16 L1 VLP + alum (i.m.)</td>
<td>32</td>
</tr>
</tbody>
</table>

a Mice were immunized on days 0 and 7 by the s.l. route with saline (sham), 2 μg of CT only, 5 μg of HPV16 L1 VLP alone or together with 2 μg of CT. A group of mice was immunized i.m. with 5 μg of HPV16 L1 VLP in alum. Vaginal washes were collected 2 wk after the second immunization and assayed for neutralization activity.

b Determined on groups of five mice.

c Not applicable.

FIGURE 7. Sublingual immunization with HPV16 L1 VLP protects mice against genital challenge with HPV16 pseudovirions. Groups of 7–10 mice were immunized on days 0, 7, and 21 by the s.l. route with saline (sham), 2 μg of CT alone, 5 μg of HPV16 L1 VLP (16VLP) alone, or admixed with 2 μg of CT adjuvant. A group of 10 mice was immunized i.m. with 5 μg of HPV16 L1 VLP adsorbed with 2 mg of alum (Pierce) adjuvant. Two weeks after the last immunization, mice were challenged i.vag. with 2 × 107 IU of HPV16 pseudovirions carrying a luciferase gene. Luciferase expression was measured 24 h after genital challenge using an in vivo imager (IVIS). A, Visualization of luciferase expression is shown for sham-treated mice (top) and for mice immunized s.l. with VLP alone (bottom). B, Arithmetic mean numbers of infectious units (extrapolated from levels of luciferase activity) ± SD are depicted and have been pooled from two experiments. Asterisks denote significant differences between groups of immunized vs sham-treated control animals (*, p < 0.01; Mann-Whitney U test).
major finding of this study was that s.l. administration of a non-replicating protein Ag was also able to induce systemic (splenic) and local genital expansion of effector CTLs. These responses, detected by means of an in vivo cytology assay, were also induced by i.n. and i.vag. immunizations and also required the coadministration of a CT adjuvant. This finding builds on our previous study and is consistent with early reports showing that CT can act as adjuvant for enhancing CTL responses to coadministered protein Ags (46–48). In addition, the proportion and avidity of Ag-specific CD8+ T cells in the gut have been shown to be compartmentalized to tissues proximal to the sites of immunization (46, 47). The question of whether CTLs induced by remote mucosal immunization may display functional properties different from those induced by local (i.vag.) immunization remains to be addressed.

The vaginal mucosa is under the control of sex hormones that can modify the ability of this tissue to serve as a site of induction and/or expression of immune responses (19, 49–51). In our hands, progesterone pretreatment of female mice enhanced the genital immune responses induced by i.vag. immunization but had little if any effect on immune responses induced by s.l. or i.n. immunization (H.-R. Cha and M. Kweon, manuscript in preparation), which is in line with data reported after i.n. and i.vag. immunization in humans (50, 51). Furthermore, the s.l. route of administration does not carry the risk of retrograde transport of Ags, including replicating and nonreplicating Ags or adjuvants, including CT or lymphotoxin, into the brain as has been reported for certain Ags administered i.n. (24). Together, these features should confer a safety advantage for the s.l. route to deliver vaccine against genital pathogens.

Two injectable VLP-based HPV vaccines (Gardasil and Cervarix) have recently been shown to confer protection against HPV-associated cervical intraepithelial neoplasia and adenocarcinoma (1, 2). IgG Abs transuding from blood into genital tissues and secretions are considered the main effector mechanism conferring protection. In the present study, s.l. vaccination with HPV VLPs, given either alone or together with CT adjuvant, induced HPV-specific IgG Abs in serum and IgA Abs in vaginal secretions. In addition, HPV-neutralizing activity was demonstrated in both serum and genital secretions after s.l. immunization. These data are reminiscent of earlier studies showing that the delivery of HPV VLPs into the lower respiratory tract induced HPV-neutralizing Abs in serum and in genital secretions (39, 40).

The observation that serum IgG Ab and neutralizing responses to HPV were lower after s.l. vaccination compared with i.m. vaccination, whereas genital IgA Ab responses were higher after s.l. immunization is intriguing, given the fact that both types of immunization induced complete protection against genital HPV challenge. A plausible explanation is that the threshold level of neutralizing Abs required to achieve complete protection was induced by either route. Another explanation is that genital IgA Abs, which were induced mainly after s.l. immunization, could provide an additional and perhaps distinct protective mechanism against genital HPV infection by interfering with the attachment and subsequent entry of HPV peudovirions into the cervicovaginal epithelium. In this regard, secretory IgA Abs have been shown to prevent the entry of viruses into mucosal epithelial cells, interfere with transcytosis across epithelial cells, and even neutralize virus replication within epithelial cells (52–56).

The finding that s.l. administration of HPV VLPs evoked unusually strong mucosal immune responses in the absence of any added adjuvant is also particularly surprising and underscores the exceptional immunogenicity of such VLPs. Such immunogenic properties could be related to their remarkable ability to bind several types of professional APCs, including B cells, dendritic cells, and macrophages (57, 58), induce the production of proinflammatory cytokines (59), and activate innate immune responses (58, 60). These properties may explain why the HPV vaccine has been so efficient and why s.l. vaccination with VLPs in the absence of any added adjuvant is sufficient to induce systemic and genital Ab responses.

This first demonstration that s.l. vaccination with nonreplicating Ags can induce simultaneous systemic and genital Ab and CTL responses provides a foundation for further evaluation of this alternative form of vaccination against genital pathogens. Clinical studies are ongoing to assess the applicability of this concept in humans.

Disclosures

The authors have no financial conflict of interest.

References


