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Vaccine Delivery by Polymeric Vehicles in the Mouse Reproductive Tract Induces Sustained Local and Systemic Immunity

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Abstract: Design of easily administered vaccines to protect the female reproductive tract against STIs such as HIV, HPV and HSV is a major step in improving world health standards. However, the effect of immunization routes and regimens (prime/boost) on immune response is not well-understood. Here, we present a systematic study of vaccine delivery by different routes and prime/boosting regimens to produce a robust humoral immune response in the reproductive tract. A model antigen, ovalbumin (OVA), was delivered orally or intranasally via polymer particles, and intravaginally via polymer disks to female mice. Repeated prime/boost at a single site result in high OVA-specific antibody levels in the serum for mice immunized orally (IgA) and invaginally (IgA and IgG) after 3 months. Vaginal antibody titers were the highest for mice immunized by intravaginal routes. Vaginal boosting following intranasal or oral priming did not appear to offer similar advantages to those primed intravaginally. Systemic immunization with OVA in Freund's adjuvant produced robust serum IgG levels, but little serum IgA or antibodies in the vaginal washings. All immunization schemes produced a significant level of IgG in the intestinal mucosa, with the exception of nasal priming followed by intravaginal boost with slow-releasing disks. In contrast, only immunization by nasal priming and intravaginal boost with fast-releasing disks was able to achieve significantly high intestinal IgA titers.

Keywords: OVA; vaccine delivery; mucosal immunization; antibodies; polymer

Introduction

The mucosal tissue is the body's first line of defense against constant challenge by bacterial and viral infectious agents. The physical barriers (mucus gel) and cellular components (macrophages, submucosal dendritic cells, network of draining lymph nodes) of the mucosal tissue are complex systems, armed to respond at a hint of invasion of the host immune system. Delivery of vaccine, which consists of some form of the antigen without the virulence factor, to the mucosal tissue can induce robust and lasting local (mucosal) and systemic immune responses.[1,2] This is an important consideration in the development of prophylactic vaccines to protect the female reproductive mucosa, the target site for sexually transmissible infections (STIs) such as HIV, the herpes simplex virus (HSV), the human papilloma virus (HPV) and others. However, it remains that there are few prophylactic vaccines commercially available to protect individuals against these infections. The high number of fatalities, the morbidity, the exponential increase in new infections per year that impact millions of the world's

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(1) Iwasaki, A. Mucosal dendritic cells. *Annu. Rev. Immunol.* **2007**, *25*, 381–418.

(2) Walker, R. I. New strategies for using mucosal vaccination to achieve more effective immunization. *Vaccine* **1994**, *12* (5), 387–400.

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population, and the chronic drain on the world healthcare resources posed by these prevalent STIs are a strong motivation to develop an effective vaccination scheme to curb their escalating growth.[3−7]

To effectively protect the cervicovaginal tissue, it is imperative that the host immune system produce a high antigen-specific antibody level at the mucosal site to neutralize the invading infectious agent. At the same time, a large number of macrophages and lymphocytes must be recruited to the mucosal site to quickly dispose of the antigen and infected cells. However, vaccine delivery in the female reproductive organ is faced with several obstacles, such as the low residence time for soluble agents due to presence of degradative enzymes and frequent shedding of mucus,[8] the high immunotolerance (low sensitivity to antigen),[9,10] and the change of tissue environment and microbiota due to fluctuating hormone cycle.[11] Delivery of vaccine to mucosal tissues requires a vehicle that can lend protection to vaccine constituents against degradation, increase residence time at the local site, and facilitate controlled release of the therapeutic payload over time. To this end, polymer carriers that deliver the desired payload can be formulated from degradable and biocompatible poly(lactic-*co*-glycolic) acid (PLGA) or nondegradable ethylene-vinyl acetate copolymer (EVAc). The encapsulation and release of vaccine constituents from these polymeric vehicles can be controlled using well-established approaches; including carrier geometry, the polymer content, or the ratio of polymer to payload.[12−16] Our early work has demonstrated the potential for long-releasing polymer disks to efficiently carry antibodies[17,18] or plasmid DNA[19] and mediate their release in the reproductive mucosa over an extended period of time.

Mucosal immunization to produce antibody response in local and systemic sites has been demonstrated for vaccines delivered via intranasal,[20−22] oral,[23−26] intravaginal and

(3) UNAIDS Global summary of the AIDS epidemic. In *AIDS Epidemic Update*; World Health Organization: 2007.

(4) Looker, K. J.; Garnett, G. P.; Schmid, G. P. An estimate of the global prevalence and incidence of herpes simplex virus type 2 infection. *Bull. World Health Org.* **2008**, *86* (10), 805–812.

(5) Harper, D. M. Preliminary HPV vaccine results for women older than 25 years. *Lancet* **2009**, *373* (9679), 1921–1922.

(6) de Sanjose, S.; Diaz, M.; Castellsague, X.; Clifford, G.; Bruni, L.; Munoz, N.; Bosch, F. X. Worldwide prevalence and genotype distribution of cervical human papillomavirus DNA in women with normal cytology: a meta-analysis. *Lancet Infect. Dis.* **2007**, *7* (7), 453–459.

(7) Bosch, F. X.; de Sanjose, S. The epidemiology of human papillomavirus infection and cervical cancer. *Dis. Markers* **2007**, *23* (4), 213–227.

(8) Cone, R. A. Barrier properties of mucus. *Adv. Drug Delivery Rev.* **2009**, *61* (2), 75–85.

(9) Mestecky, J.; Moldoveanu, Z.; Russell, M. W. Immunologic uniqueness of the genital tract: challenge for vaccine development. *Am. J. Reprod. Immunol.* **2005**, *53* (5), 208–214.

(10) Mestecky, J.; Russell, M. W.; Elson, C. O. Perspectives on mucosal vaccines: is mucosal tolerance a barrier? *J. Immunol.* **2007**, *179* (9), 5633–5638.

(11) Parr, M. B.; Parr, E. L. In *Handbook of Mucosal Immunology*; Ogra, P. L., Mestecky, J., Lamm, M. E., Strober, W., McGee, J. R., Bienenstock, J., Eds.; Academic Press, Inc.: San Diego, 1994; pp 677−689.

(12) Bhavsar, M. D.; Amiji, M. M. Polymeric nano- and microparticle technologies for oral gene delivery. *Expert Opin. Drug Delivery* **2007**, *4* (3), 197–213.

(13) Mundargi, R. C.; Babu, V. R.; Rangaswamy, V.; Patel, P.; Aminabhavi, T. M. Nano/micro technologies for delivering macromolecular therapeutics using poly(D, L-lactide-co-glycolide) and its derivatives. *J. Controlled Release* **2008**, *125* (3), 193–209.

(14) Shive, M. S.; Anderson, J. M. Biodegradation and biocompatibility of PLA and PLGA microspheres. *Adv. Drug Delivery Rev.* **1997**, *28* (1), 5–24.

(15) Perez, C.; Castellanos, I. J.; Costantino, H. R.; Al-Azzam, W.; Griebenow, K. Recent trends in stabilizing protein structure upon encapsulation and release from bioerodible polymers. *J. Pharm. Pharmacol.* **2002**, *54* (3), 301–313.

(16) Di Colo, G. Controlled drug release from implantable matrices based on hydrophobic polymers. *Biomaterials* **1992**, *13* (12), 850–856.

(17) Kuo, P. Y.; Sherwood, J. K.; Saltzman, W. M. Topical antibody delivery systems produce sustained levels in mucosal tissue and blood. *Nat. Biotechnol.* **1998**, *16* (2), 163–167.

(18) Saltzman, W. M.; Sherwood, J. K.; Adams, D. R.; Castle, P.; Haller, P. Long-term vaginal antibody delivery: delivery systems and biodistribution. *Biotechnol. Bioeng.* **2000**, *67* (3), 253–264.

(19) Shen, H.; Goldberg, E.; Saltzman, W. M. Gene expression and mucosal immune responses after vaginal DNA immunization in mice using a controlled delivery matrix. *J. Controlled Release* **2003**, *86* (2−3), 339–348.

(20) Dumais, N.; Patrick, A.; Moss, R. B.; Davis, H. L.; Rosenthal, K. L. Mucosal immunization with inactivated human immunodeficiency virus plus CpG oligodeoxynucleotides induces genital immune responses and protection against intravaginal challenge. *J. Infect. Dis.* **2002**, *186* (8), 1098–1105.

(21) Gallichan, W. S.; Rosenthal, K. L. Long-term immunity and protection against herpes simplex virus type 2 in the murine female genital tract after mucosal but not systemic immunization. *J. Infect. Dis.* **1998**, *177* (5), 1155–1161.

(22) Gallichan, W. S.; Woolstencroft, R. N.; Guarasci, T.; McCluskie, M. J.; Davis, H. L.; Rosenthal, K. L. Intranasal immunization with CpG oligodeoxynucleotides as an adjuvant dramatically increases IgA and protection against herpes simplex virus-2 in the genital tract. *J. Immunol.* **2001**, *166* (5), 3451–3457.

(23) Allaoui-Attarki, K.; Fattal, E.; Pecquet, S.; Trolle, S.; Chachaty, E.; Couvreur, P.; Andremont, A. Mucosal immunogenicity elicited in mice by oral vaccination with phosphorylcholine encapsulated in poly (D, L-lactide-co-glycolide) microspheres. *Vaccine* **1998**, *16* (7), 685–691.

(24) Challacombe, S. J.; Rahman, D.; O'Hagan, D. T. Salivary, gut, vaginal and nasal antibody responses after oral immunization with biodegradable microparticles. *Vaccine* **1997**, *15* (2), 169–175.

(25) Chattaraj, S. C.; Rathinavelu, A.; Das, S. K. Biodegradable microparticles of influenza viral vaccine: comparison of the effects of routes of administration on the in vivo immune response in mice. *J. Controlled Release* **1999**, *58* (2), 223–232.

(26) He, X. W.; Wang, F.; Jiang, L.; Li, J.; Liu, S. K.; Xiao, Z. Y.; Jin, X. Q.; Zhang, Y. N.; He, Y.; Li, K.; Guo, Y. J.; Sun, S. H. Induction of mucosal and systemic immune response by single-dose oral immunization with biodegradable microparticles containing DNA encoding HBsAg. *J. Gen. Virol.* **2005**, *86* (Part 3), 601–610.

intrarectal routes.[27−31] Particularly in the latter cases, gel and long-releasing polymer devices can be used to deliver vaccine directly to the mucosa with efficacy.[31−33] Previous studies have also demonstrated that greater and long-lasting antibody titers can be achieved in animals immunized by mucosal routes over those immunized by systemic routes (i.e., subcutaneous, intramuscular, or intraperitoneal).[21,27] Induction of an immune response in the reproductive tract can also be achieved by way of the common mucosal immune system. Vaccines may be delivered to the nasal or gastrointestinal tracts, the surfaces of which are better equipped to respond against antigen presentation via specialized lymphoid tissues (i.e., NALT, GALT) than the relatively more immunotolerant reproductive mucosa. This effectively produces an immune response in all mucosal sites, including the reproductive mucosa, that may be more robust than immunization in the reproductive tract alone.[34−36] Comparisons of multiple mucosal vaccination routes, and the humoral responses due to single priming or combined prime and boosting in different tissues, also indicate a site-specific immune response.[25,31,33,37−41] Though much progress has been made, the complexity of the biological system still presents a great challenge and must be explored with further systematic studies.

Table 1. Immunization Schedule for Mice Receiving OVA in the Following Delivery Routes and Vehicles

immunization			
primary	secondary	*N*	abbrev
microspheres (po)	microspheres (po)	5	O/O
microspheres (po)	disk: fast-release (vag)	5	O/V_f
microspheres (po)	disk: slow-release (vag)	4	O/(V_s)
microspheres (i.n.)	microspheres (i.n.)	4	N/N
microspheres (i.n.)	disk: fast-release (vag)	4	N/V_f
microspheres (i.n.)	disk: slow-release (vag)	5	N/(V_s)
disk: fast-release (vag)	disk: fast-release (vag)	5	V_f/V_f
disk: slow-release (vag)	disk: fast-release (vag)	4	V_s/V_f
in FCA (sc)	in FIA (sc)	3	FCA/FIA
soluble form (po)	soluble form (po)	4	LIQ/LIQ

In the current report, we immunized female mice by different routes: nasal and oral delivery was achieved using particles, and intravaginal delivery was achieved using EVAc disks, both encapsulating OVA as a model antigen. These mucosal immunization schemes were compared to systemic immunization, achieved by subcutaneous injection of OVA with Freund's adjuvant, and oral delivery of soluble OVA (Table 1). Production of IgA and IgG antibody isotypes was monitored in mucosal secretions (vaginal and intestinal) and serum over 12 weeks. Particular consideration is given to the level of antibody secretion in the mouse vaginal washes, with the goal to identify an effective vaccination method that can induce high antibody titers both systematically and at the surface of the female reproductive tract.

Materials and Methods

Preparation of Particles. OVA-loaded PLGA particles were made by a double emulsion and solvent evaporation

(27) Livingston, J. B.; Lu, S.; Robinson, H.; Anderson, D. J. Immunization of the female genital tract with a DNA-based vaccine. *Infect. Immun.* **1998**, *66* (1), 322–329.
(28) Luci, C.; Hervouet, C.; Rousseau, D.; Holmgren, J.; Czerkinsky, C.; Anjuere, F. Dendritic cell-mediated induction of mucosal cytotoxic responses following intravaginal immunization with the nontoxic B subunit of cholera toxin. *J. Immunol.* **2006**, *176* (5), 2749–2757.
(29) Kwant, A.; Rosenthal, K. L. Intravaginal immunization with viral subunit protein plus CpG oligodeoxynucleotides induces protective immunity against HSV-2. *Vaccine* **2004**, *22* (23−24), 3098–3104.
(30) Kato, H.; Bukawa, H.; Hagiwara, E.; Xin, K. Q.; Hamajima, K.; Kawamoto, S.; Sugiyama, M.; Sugiyama, M.; Noda, E.; Nishizaki, M.; Okuda, K. Rectal and vaginal immunization with a macromolecular multicomponent peptide vaccine candidate for HIV-1 infection induces HIV-specific protective immune responses. *Vaccine* **2000**, *18* (13), 1151–1160.
(31) Wyatt, T. L.; Whaley, K. J.; Cone, R. A.; Saltzman, W. M. Antigen-releasing polymer rings and microspheres stimulate mucosal immunity in the vagina. *J. Controlled Release* **1998**, *50* (1−3), 93–102.
(32) Tengvall, S.; O'Hagan, D.; Harandi, A. M. Rectal immunization generates protective immunity in the female genital tract against herpes simplex virus type 2 infection: relative importance of myeloid differentiation factor 88. *Antiviral Res.* **2008**, *78* (3), 202–214.
(33) Park, J. S.; Oh, Y. K.; Kang, M. J.; Kim, C. K. Enhanced mucosal and systemic immune responses following intravaginal immunization with human papillomavirus 16 L1 virus-like particle vaccine in thermosensitive mucoadhesive delivery systems. *J. Med. Virol.* **2003**, *70* (4), 633–641.
(34) Mestecky, J.; Abraham, R.; Ogra, P. L. *Handbook of Mucosal Immunology*; Ogra, P. L., Mestecky, J., Lamm, M. E., Strober, W., McGee, J. R., Eds.; Academic Press, Inc.: New York, 1994; pp 357−372.
(35) Scicchitano, R. A. Stanisz, P. Ernst, P. Bienenstock, J. In *Migration and Homing of Lymphoid Cells, Vol. II*; Husband, A., Ed.; CRC Press: Boca Raton, 1988; pp 1−34.
(36) Phillips-Quagliata, J. M.; Lamm, M. E. In *Migration and Homing of Lymphoid Cells, Vol. II*; CRC Press: Husband, A., Ed.; Boca Raton, 1988; pp 53−75.
(37) Akagi, T.; Kawamura, M.; Ueno, M.; Hiraishi, K.; Adachi, M.; Serizawa, T.; Akashi, M.; Baba, M. Mucosal immunization with inactivated HIV-1-capturing nanospheres induces a significant HIV-1-specific vaginal antibody response in mice. *J. Med. Virol.* **2003**, *69* (2), 163–172.
(38) Akagi, T.; Ueno, M.; Hiraishi, K.; Baba, M.; Akashi, M. AIDS vaccine: Intranasal immunization using inactivated HIV-1-capturing core-corona type polymeric nanospheres. *J. Controlled Release* **2005**, *109* (1−3), 49–61.
(39) Barnett, S. W.; Srivastava, I. K.; Kan, E.; Zhou, F.; Goodsell, A.; Cristillo, A. D.; Ferrai, M. G.; Weiss, D. E.; Letvin, N. L.; Montefiori, D.; Pal, R.; Vajdy, M. Protection of macaques against vaginal SHIV challenge by systemic or mucosal and systemic vaccinations with HIV-envelope. *Aids* **2008**, *22* (3), 339–348.
(40) Kozlowski, P. A.; Cu-Uvin, S.; Neutra, M. R.; Flanigan, T. P. Mucosal vaccination strategies for women. *J. Infect. Dis.* **1999**, *179 Suppl. 3*, S493–498.
(41) Parr, E. L.; Parr, M. B. Immune responses and protection against vaginal infection after nasal or vaginal immunization with attenuated herpes simplex virus type-2. *Immunology* **1999**, *98* (4), 639–645.

process. PLGA polymers (75/25, Birmingham) were dissolved in dichloromethane (DCM) at 25% w/v. OVA (150 μL of 100 mg/mL, Sigma) was added dropwise to the polymer solution and emulsified by microprobe sonicator (Tekmar) for 10 s at 40% amplitude. The mixture was added to 80 mL of well-stirred 3% (w/v) PVA with 5% NaCl (Polysciences) to form the second emulsion. Solvent evaporation was carried out for 3 h at room temperature, after which the particles were pelleted at 2,000 rpm for 10 min at 4 °C. The supernatant was discarded and particles were resuspended in DI water. This wash was repeated 3× in DI water, followed by drying of the particles by lyophilization overnight.

Loading Efficiency of Particles. Particles were dissolved in 2 mL of DCM, and OVA was extracted out of the organic phase with the addition of 2 mL of DI water. Three extractions were conducted per sample, and aqueous supernatant was pooled and quantified with Coomassie protein assay (Pierce); number of samples (n) = 4. The encapsulation efficiency was calculated by dividing the total amount of OVA extracted from microspheres after encapsulation over the maximum theoretical value.

Sizing and Morphology of Particles. Particles were first deposited as a monolayer on double-sided carbon tape, mounted on an aluminum stub. The sample was sputter-coated with gold and palladium (60:40, Denton Vacuum, Inc.) and viewed under the SEM with electron beam at 20 keV (Stereoscan 440, Leica Cambridge, Ltd.). Particle size was evaluated from images of samples suspended in DI water taken by a light microscope at 40× magnification, n = 210.

Preparation of EVAc Disks. OVA was dissolved with Ficoll 400 (MW = 426 kDa, Pharmacia Biotech) at a 10:76 w/w ratio in DI water and lyophilized for 24 h. The mixture was ground through a wire mesh to obtain particles between 80 and 170 μm in size. Ground particles were mixed with 10% (w/v) EVAc (Dupont) in methylene chloride, 50% w/w (particle/EVAc) for fast-releasing and 30% for slow-releasing disks. The mixture was poured into a prechilled mold at −80 °C, then transferred to −20 °C for 48 h, and lyophilized for an additional 48 h. Disks were made by cutting with a 4 mm diameter cork borer. Resulting disks were ∼1.08−1.14 mm thick.

In Vitro Controlled Release of Particles and EVAc Disks. Particles (40 mg, n = 4) were suspended in 0.5 mL of PBS at 37 °C by gentle horizontal shaking. At each time point, the samples were pelleted by centrifugation at 2,000 rpm for 10 min and the supernatant was collected to be assayed by Coomassie protein assay and reconstituted with the same volume of fresh PBS buffer. Cored EVAc polymer discs (n = 3) were placed in 0.75 mL of PBS at 37 °C in horizontal shaker. At each time point, PBS was collected and similarly assayed by Coomassie protein assay. A fresh volume of PBS was used to reconstitute the volume.

Immunization Schedule and Protocols. Female Balb/c mice (8−10 wk, Harlan Sprague−Dawley, Inc.) were administered OVA by various delivery routes and vehicles (Table 1). Mice were administered a booster dose of OVA 4 weeks following primary vaccination.

Subcutaneous Injection. Each mouse was injected subcutaneously at the back of the neck with 400 μg of OVA emulsified in 50 μL of Freund's complete adjuvant (FCA, Sigma). After 4 weeks, 400 μg of OVA emulsified in 50 μL of Freund's incomplete adjuvant (FIA, Sigma) was similarly administered.

Oral Administration. PLGA particles (16.3 mg, total dose 400 μg of OVA) suspended in 100 μL of bicarbonate buffer with 5 μg of cholera toxin was drawn into a 1 mL syringe attached to an oral feeding needle (Samuel Perkins Company). The particle suspension was injected slowly into the esophagus of each mouse, which was held upright, and care was taken to avoid injecting into the pharyngeal passageway.

Intranasal Administration. A similar dose of PLGA particles (16.3 mg, total dose 400 μg of OVA) was suspended in 100 μL of PBS and administered on one of the mouse external nares. The animal's breathing pattern was closely observed to prevent suffocation while the particles were inhaled.

Intravaginal Administration. One week prior to treatment, mice were injected subcutaneously with 2 mg of Depo-Provera (Pharmacia & Upjohn) to induce a diestrus-like state in the vaginal tissue. Cut out disk (fast-, slow-OVA releasing, or blank EVAc) was inserted into the animal's lower reproductive tract and secured with a suture. As a control, blank EVAc were inserted, and PBS or 400 μg of soluble OVA, in a total volume of 20 μL, was subsequently administered.

Hyperimmunization with OVA. Female BALB/c mice (8−10 weeks, Harlan Sprague−Dawley, Indianapolis, IN) were hyperimmunized with OVA for the production of OVA-specific serum antibodies. This hyperimmunization protocol was adopted from O'Hagan et al.[42] Briefly, mice were anesthetized with Metofane, and injected intraperitoneally with OVA emulsified in FCA. Two weeks and four weeks later, the mice were injected with OVA emulsified in FIA. Seven days after the final injection, blood was collected by cardiac puncture. Blood was pooled from hyperimmunized mice, and was allowed to clot at room temperature for 30 min and transferred to 4 °C overnight. Then, the blood was centrifuged at 3400 rpm for 10 min at 4 °C, and the serum was aliquotted into 1 mL cryogenic vials and stored at −20 °C until use.

Collection of Samples. Blood was collected from the tail artery every 2 weeks, except for the final collection at week 12, which was by cardiac puncture. Blood was allowed to clot at room temperature for 30 min and then overnight at 4 °C. The samples were centrifuged at 3,400 rpm for 10 min at 4 °C, and the sera (supernatant) transferred to a fresh tube and frozen until analysis.

(42) O'Hagan, D. T.; Rahman, D.; McGee, J. P.; Jeffery, H.; Davies, M. C.; Williams, P.; Davis, S. S.; Challacombe, S. J. Biodegradable microparticles as controlled release antigen delivery systems. *Immunology* **1991**, *73* (2), 239–242.

Vaginal secretions were also collected every 2 weeks. Vaginal lavages were collected by gentle pipetting of 20 μL of PBS × 4 for a total volume of 80 μL. The lavages were centrifuged at 10,000 rpm for 15 min, and supernatant was frozen until analysis. Samples were not collected for mice immunized intravaginally at week 2, due to the presence of disks.

Intestinal secretions were collected at week 12 as adopted from Elson et al.[43] Each mouse was given 0.5 mL of intestinal lavage solution (25 mM NaCl, 40 mM Na_2SO_4, 10 M KCl, 20 mM $NaHCO_3$, 48.5 mM polyethylene glycol) in 2 doses at 15 min intervals. After 30 min of the second dose, each mouse was given 0.1 mg of pilocarpine (Sigma) intraperitoneally. The intestinal discharge was collected into a Petri dish with 0.1 mg/mL soybean trypsin inhibitor in 50 mM EDTA (Sigma). The sample was centrifuged at 650*g* for 10 min at room temperature, and 30 μL of 100 mM phenylmethylsulfonyl fluoride (PMSF, Sigma) in 95% ethanol was added to the supernatant. The solution was the centrifuged at 27000*g* for 20 min at 4 °C, and 20 μL of 100 mM PMSF in 95% ethanol and 20 μL of 1% sodium azide were added to the supernatant. 100 μL of fetal bovine serum (FBS, Life Technologies) was added to the supernatant after 15 min. Samples were stored at −20 °C until analysis.

ELISA Assay for OVA-Specific Antibodies. A 96-well plate (Dynex) was coated at 4 °C overnight with OVA (50 μg/mL, grade V, Sigma) in 0.05 M carbonate−bicarbonate buffer at pH 9.6, to probe for OVA-specific antibodies. To probe for total antibodies, goat-anti-mouse IgA or IgG (Sigma, 5 μg/mL for IgA, 10 μg/mL for IgG) was used. The plates were blocked with 0.5% BSA and 0.05% Tween 20 in PBS for 1 h at room temperature, and then washed 5× with washing buffer (0.05% Tween 20 in PBS). Unknown samples were serially diluted in 0.05% BSA and PBS, starting at 1:200 for serum, 1:10 for vaginal lavages and 1:2 for intestinal lavages, and added to each well. Plates were covered and stored overnight at 4 °C. After five washes, alkaline-phosphatase-conjugated goat-anti-mouse IgA or IgG (Zymed Laboratories) diluted 1:200 in PBS (with 0.05% BSA and Tween 20, 5 μg of antibody/mL PBS) was added to each well and incubated 3 h at room temperature. Another five washes followed, and substrate solution of *p*-nitrophenyl phosphate (pNPP, Sigma) was added. After 10 min incubation at room temperature and an additional 20 min at 37 °C, the reaction was quenched with 3 M NaOH. Absorbance at 405 nm was read per well with a microplate reader (ThermoMax). Data were reported as an average of duplicate measurements.

ELISA Data Analysis. Standard curves from the hyperimmune serum (see the section Hyperimmunization with OVA) was expressed in arbitrary antibody values of 1×10^5 IgA antibody units/mL and 2×10^6 IgG antibody units/mL similar to the method reported by Challacombe et al.[44] Duplicates of unknown samples were averaged and compared to the hyperimmune serum standard curves (Supplementary Figure 1 in the Supporting Information). Then data from the unknown samples were transformed logarithmically ($\log_{10}$).

Statistical Analysis. One-way analyses of variance (ANOVA) and Tukey's multiple-comparison (W) procedure were performed on ELISA data obtained from weeks 4−12. Statistical significance was evaluated at $\alpha = 0.05$ and compared to the reference group by Tukey's W procedure.[45] Data for OVA-specific serum IgA at week 12 were compared to the average preimmune serum IgA level of all the animals to determine statistical significance, instead of those from the reference group, which received soluble OVA orally, at this time point which was aberrantly low. Tukey's W procedure was similarly performed to determine differences among treatment groups.

Results

Female mice were immunized with OVA by various routes (subcutaneous, oral, intranasal, and intravaginal) using different delivery methods. PLGA particles encapsulating OVA were formulated for oral and intranasal delivery, and OVA-loaded EVAc disks for placement in the lower reproductive tract. The PLGA particles were small (77% of particle population <5 μm and 87% <10 μm in diameter), were spherical, and displayed a smooth surface morphology (Figure 1A). The OVA encapsulation efficiency was 82 ± 6% of maximum input, or 3.7 μg of OVA/mg of PLGA. The particles display a biphasic in vitro release profile, starting with an initial burst of ~25% encapsulated OVA in the first 2 days, and followed by a more gradual release that was sustained up to 4 weeks (Figure 1B). EVAc polymer disks loaded with specific ratio of OVA to codispersant (50% and 30% w/w OVA:Ficoll 400) released OVA either fast or slow, where 50% loading resulted in faster releasing disks (V_f) and 30% in slower releasing disks (V_s). The cumulative amount of OVA released from each disk for both formulations was similar after 2 weeks, at ~400 μg of OVA/disk (Figure 2).

Primary and secondary immunizations were performed in a combination of routes (Table 1). OVA-specific IgG and IgA levels, sampled every 2 weeks from serum and vaginal washes, indicate an overall higher response after receiving a boost at week 4 (Figure 3). OVA-specific IgG serum levels at week 0 for all animals were similar, at 3.39 ± 0.61 IgG units/mL. Mice immunized subcutaneously with OVA and Freund's adjuvant (FCA/FIA, positive control) produced the highest OVA-specific serum IgG response, that is, about 3

(43) Elson, C. O.; Ealding, W.; Lefkowitz, J. A lavage technique allowing repeated measurement of IgA antibody in mouse intestinal secretions. *J. Immunol. Methods* **1984**, *67*, 101–108.

(44) Challacombe, S. J.; Rahman, D.; Jeffery, H.; Davis, S. S.; O'Hagan, D. T. Enhanced secretory IgA and systemic IgG antibody responses after oral immunization with biodegradable microparticles containing antigen. *Immunology* **1992**, *76* (1), 164–168.

(45) Ott, R. L. *An Introduction to Statistical Methods and Data Analysis*; Wadsworth Publishing Company: Belmont, 1993.

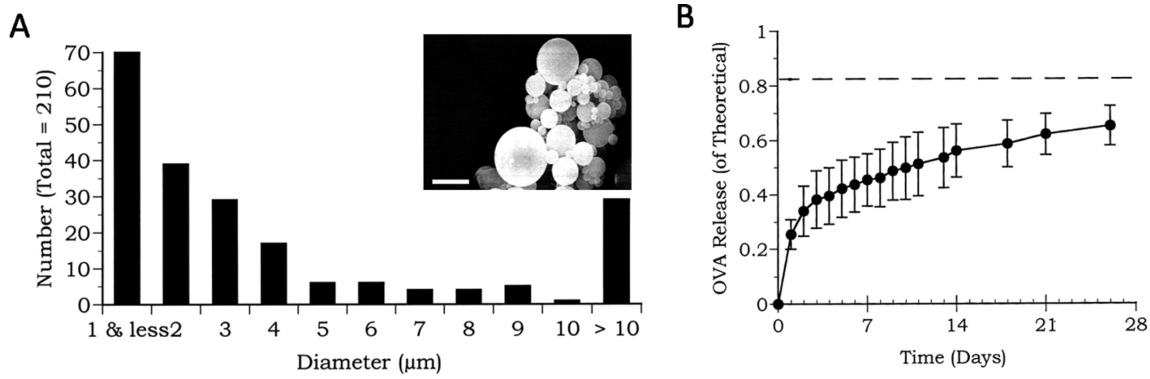

Figure 1. PLGA particles encapsulating OVA. The size distribution of particles was largely small, with 77% of the population measured <5 μm in diameter (A). Particles appeared spherical with smooth surface morphology (inset, scale bar = 10 μm). Release of OVA from PLGA particles measured as fraction of theoretical loading showed a biphasic release profile with a rapid burst during the first 3 days and a more gradual release over the period of 4 weeks (B). The dashed line indicates that the OVA loading efficiency was 82 ± 6% of theoretical maximum, which corresponded to 3.7 μg of OVA/mg of PLGA.

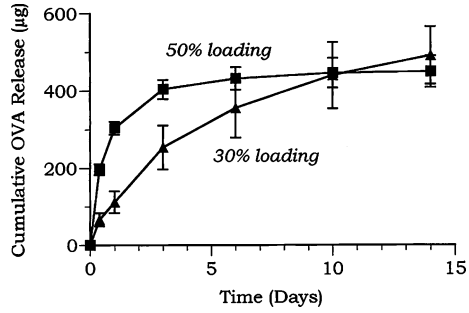

Figure 2. EVAc disks encapsulating OVA at different OVA:codispersant ratios. Two formulations of OVA-releasing disks, with 50% and 30% (w/w) OVA:co-dispersant in EVAc polymer, released proteins at distinct rates: fast-releasing (V_f) disks release ~2× greater OVA during the initial burst (3 days) than slow-releasing disks (V_s). The cumulative OVA released over a period of 2 weeks for both formulations was similar, at 400 μg of OVA/disk.

orders of magnitude above the reference titer, which was selected as mice immunized orally with soluble OVA (LIQ/LIQ). The second highest responses were observed in mice immunized with either combination of OVA-loaded EVAc disks (V_f/V_f, V_s/V_f), at 2 orders of magnitude above the reference titer. Similar to the positive control, the high IgG responses for these mice were sustained for the duration of the study, and significantly different from reference titer during sampling points at 6−12 weeks (Figure 3A). Primary immunization and boosting strictly by oral or intranasal routes produced serum IgG levels that were on average higher, but often not statistically significant, from the reference titer with exception for those primed and boosted orally (O/O) at weeks 4, 8 and 12.

OVA-specific serum IgA, shown in Figure 3B, was 3.45 ± 0.38 IgA units/mL at week 0 for all animals. Serum IgA, which is closely associated with mucosal response, was highest for mice orally immunized with OVA-loaded particles (statistically significant for weeks 4−12 above reference titer). Mice with fast-releasing EVAc disks, V_f/V_f, produced significantly higher response at week 6. All other samples, with the exception of those immunized by intranasal route (N/N), produced a significantly higher serum IgA titer than the reference at week 12.

OVA-specific antibody concentration measured in the vaginal washes of mice immunized by oral or intranasal routes was roughly similar to the reference titer. Interestingly, mice immunized with vaginal disks (V_f/V_f, V_s/V_f) produced significantly higher mucosal IgG than the reference at weeks 6−12. In addition, these mice also produced comparable mucosal IgG as those immunized with Freund's adjuvant subcutaneously (FCA/FIA). Antibody productions for this population were significantly higher than reference at 4−12 weeks. Oral and nasal immunization produced modest IgG in vaginal washes that was only significantly higher than the reference titer at week 12 (Figure 3C). The same trend was shown in OVA-specific IgA collected from vaginal washes from reproductive mucosal surface, where mice immunized with EVAc disks were observed to have significantly higher titer at weeks 4 and 6 but decreased after week 8. However, all other samples, including the subcutaneous positive control, did not produce IgA that was higher than the reference level throughout the duration of the experiment (Figure 3D).

In comparing mice initially immunized by different routes (oral, intranasal, intravaginal) and subsequently boosted vaginally with fast or slow OVA releasing disks, an increased response in OVA-specific serum IgG and IgA was found in all populations after boost, which remained higher than the reference titer (LIQ/LIQ) (Figure 4). Populations immunized and boosted intravaginally by combinations of fast and slow releasing disks (V_f/V_f and V_s/V_f) produced the most robust serum IgG response that was significantly higher than reference from week 6 to 12 (Figure 4A). The responses between these groups, however, were not statistically different. Serum IgG responses for mice immunized orally or intranasally, and boosted with slow-releasing disks, peaked at week 8 but did not remain significantly high at the end of

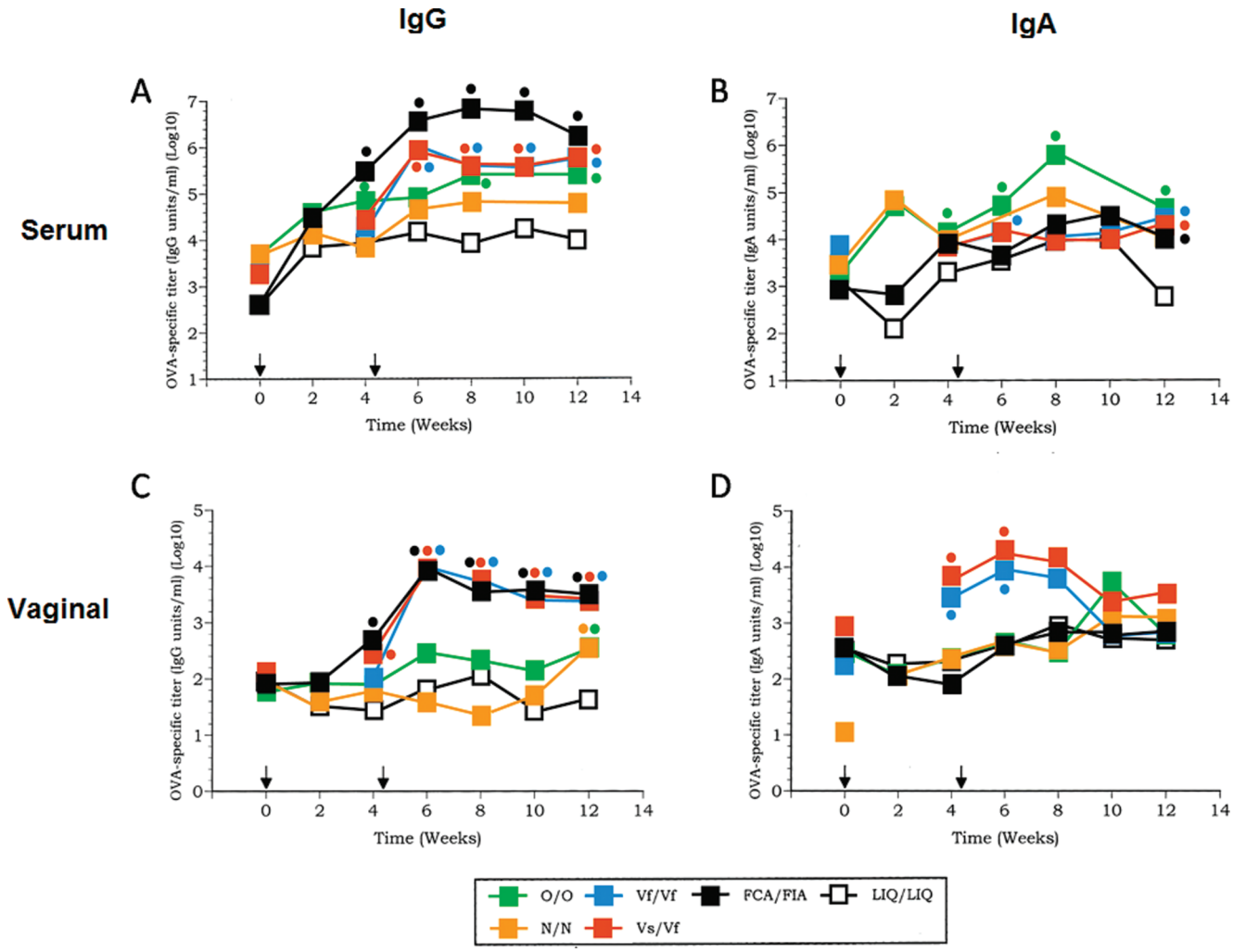

Figure 3. Antibody production in response to immunization via similar prime/boost sites. OVA-specific IgG (A, C) and IgA (B, D) as measured in serum and vaginal washes in mice with primary and secondary immunizations by identical routes: oral (O/O), nasal (N/N), intravaginal (V/V), oral soluble OVA (LIQ/LIQ), and subcutaneous with Freund's adjuvant (FCA/FIA). The nomenclature scheme was primary/secondary treatment, as summarized in Table 1. Arrows indicate the times of immunization at weeks 0 and 4, and filled circles denote statistically significant data from reference sample (LIQ/LIQ) as analyzed by Tukey's multiple-comparison procedure, confidence level = 0.05, $n = 3$.

12 weeks. OVA-specific serum IgA for the same samples reveal a robust response in mice primed orally and boosted with V_s or V_f (Figure 4B). These produced statistically higher titers at weeks 4 (O/V_f only), 8 and 12. Similar to IgG levels, the serum IgA titer peaks at week 8, with the highest by O/V_f and O/V_s at second. At week 12, all OVA immunized populations produced high IgA titers over reference, with the exception of O/V_s and N/V_s. However, we note that the reference level was particularly low at week 12.

In vaginal washes, OVA-specific IgG were highest for mice immunized intravaginally, starting from week 4 (V_s/V_f), and significantly higher than all other samples by 2 orders of magnitude during weeks 6−12 (V_f/V_f and V_s/V_f). Mice immunized systemically, orally, or intranasally did not produce significant mucosal IgG in the reproductive tract, except for those immunized intranasally and boosted with V_s (N/V_s) at week 10, and N/V_f at week 12 (Figure 4C). OVA-specific IgA was highest for mice receiving prime and boost immunization intravaginally, but not for other formulations (Figure 4D). In addition, this production of mucosal IgA dropped sharply after week 8.

OVA-specific IgG and IgA were obtained from intestinal washes at week 12. Mean intestinal IgG levels for all groups, except for N/V_s, were significantly higher than those for the reference titer. In Figure 5A, hatch backgrounds indicate group means that were similar. Of the three groups, N/N, O/V_s, O/O, N/V_f, and V_s/V_f fall into the first group, O/V_f, FCA/FIA, V_f/V_f in the second, and last N/V_s and LIQ/LIQ, in order of highest titer to lowest. OVA-specific intestinal IgA titers after 12 weeks treatment, while overall higher than IgG titers, yielded only one group that measured significantly higher than reference titer (N/V_s). In addition, group means

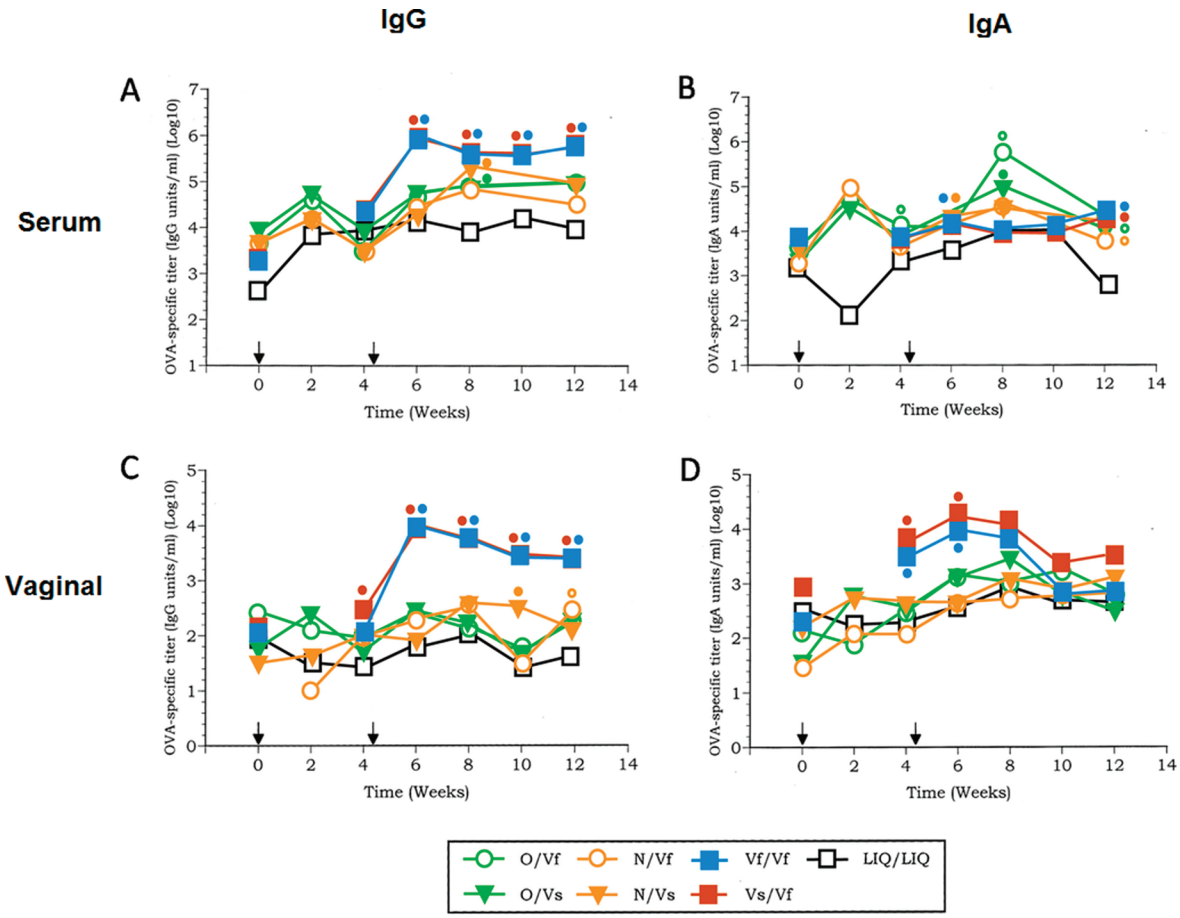

Figure 4. Antibody production in response to immunization via different priming sites followed by vaginal boosting. Similar OVA-specific IgG (A, C) and IgA (B, D) levels from serum and vaginal washes of mice immunized by oral, intranasal, or intravaginal routes but received secondary immunization via fast (V_f) or slow (V_s) OVA-releasing disks. Arrows denote the times of primary and secondary immunization at weeks 0 and 4, and filled circles indicate statistically significant data from reference sample (LIQ/LIQ) as analyzed by Tukey's multiple-comparison procedure, confidence level = 0.05, $n = 3$.

within the gray background (all sample groups except for reference group) were not significantly different from each other (Figure 5B).

Discussion and Conclusions

The efficacy of vaccine delivery to mucosal tissues, such as nasal, oral, or intravaginal sites, can be enhanced through use of polymer vehicles, which protect the payload and facilitate its gradual release over time. PLGA particles formulated in this study for nasal and oral delivery were small and encapsulated OVA at a high efficiency. With regard to uptake of particles in the mucosal epithelium, previous studies have shown that particle uptake is size-dependent in Peyer's patches, and those with 11−15 μm in diameter are not internalized.[46] In our formulation we assumed that the larger particles, which make up a small percentage of the population (~13%), are also not internalized. Rather, they remain on the gastric mucosa to release OVA in the soluble form that is then taken up by epithelial cells. Thus, our total dose of OVA delivered by particles includes encapsulated OVA delivered into cells—which was released intracellularly from internalized particles, and soluble OVA released from uninternalized particles on the cell surface. However, compared to the negative control which is entirely soluble OVA, mice administered with encapsulated OVA produce higher antigen-specific antibody production at the mucosa, which is likely attributable to uptake of encapsulated antigen.

For the controlled release disks used in this study, a solvent-casting method was used to prepare the EVAc disks. Mechanisms of protein release from EVAc disks are well-described in the literature.[8,31,47] In this method, solid particles of OVA and carbohydrate (Ficoll 400) were dispersed in the polymer and dissolved in an organic solvent, and the mixture was quickly frozen before the particles could sediment. After

(46) Tabata, Y.; Inoue, Y.; Ikada, Y. Size effect on systemic and mucosal immune responses induced by oral administration of biodegradable microspheres. *Vaccine* **1996**, *14* (17−18), 1677–1685.

(47) Saltzman, W. M.; Langer, R. Transport rates of proteins in porous materials with known microgeometry. *Biophys. J.* **1989**, *55* (1), 163–171.

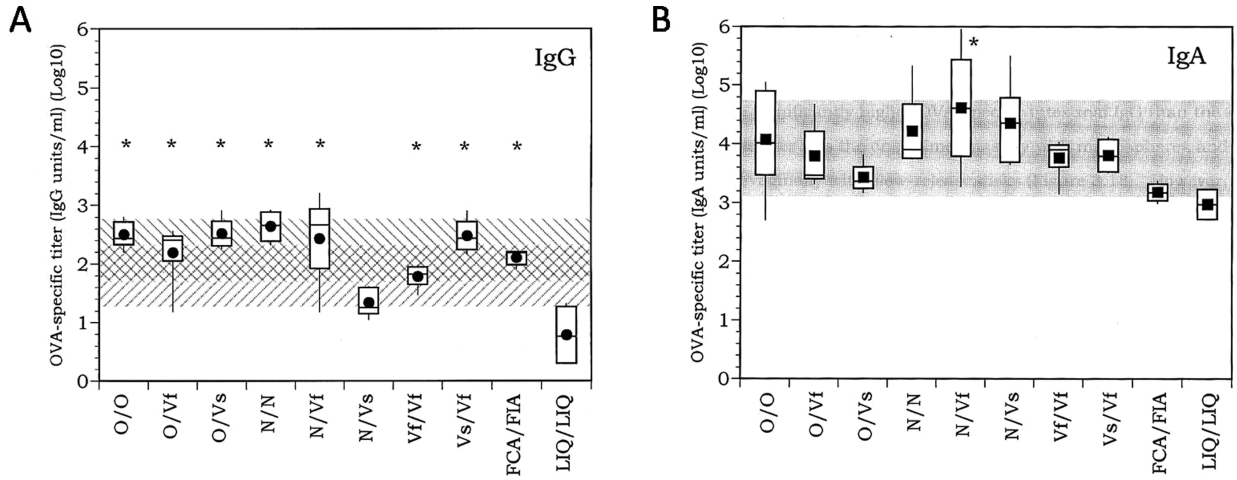

Figure 5. OVA-specific intestinal IgG (A) and IgA (B) levels for mice immunized with various treatment regimes at week 12. Data analysis is based on Tukey's multiple-comparison procedure with confidence level = 0.05. IgG levels for all treatment groups, except that N/(V_s), indicated with asterisks, are significantly higher than the reference (LIQ).

solvent evaporation, the encapsulated OVA:Ficoll particles are interconnected by a tortuous network within the nonpermeable polymer. Release of OVA occurs through dissolution of the OVA:Ficoll particle by the surrounding aqueous environment, and diffusion of the protein through the network of empty pores created as a result of this dissolution. OVA:Ficoll particles that are not connected to the network of pores are entrapped inside the polymer and not released. The degree of interconnection governs the percentage of the encapsulated bioactive molecules that are released.

Factors that influence the release profile from a nonbiodegradable polymeric device include loading, size of the encapsulated particle, degree of tortuosity of the porous network, molecular weight of the polymer, and size and shape of the device. Intrinsic properties of the protein, such as molecular weight, dissolution constant, and diffusion coefficient, also influence the release profile.[47] Two different formulations of EVAc disks were formulated for intravaginal delivery; these disks released OVA at different rates. Slow- and fast-releasing disks (V_s, V_f) were made by varying the amount of OVA:Ficoll. Although the initial release rate in vitro varied between disk formulations, all of these vehicles released similar cumulative dose of OVA (400 μg) after 2 weeks.

Mouse mucosal IgA and IgG productions vary with the estrous cycle.[11,48] In this study, Depo-Provera, a long-lasting progestin, was used to synchronize the cycle in female mice prior to delivery (at diestrus). In this experiment, OVA-specific vaginal IgA titers were slightly higher than OVA-specific vaginal IgG titers for most of the animals immunized mucosally with controlled release devices. This observation was reported in another study where mice[49] and rats[27] were treated with Depo-Provera and immunized mucosally with a DNA-based vaccine.

Mice immunized by delivery to mucosal tissues showed some levels of immune response at the mucosal tissue and serum, subcutaneous immunization did not result in significant mucosal titers of IgG or IgA. Remarkably, EVAc disks produce high IgG and IgA in mucosal fluids and serum, with higher antibody titers in vaginal washes than in serum. This suggests that local production of antibodies occurs at the vaginal mucosa; mucosal antibody production is not due to serum transudation. However, we did not find this trend between intestinal and serum antibody titers. Intestinal antibody levels were only measured at a single time point (12 weeks). This is a terminal procedure and was carried out at the final time point after all antibody monitoring experiments (i.e., mucosal and serum antibody collection) were completed. It is possible that, after this time, the levels of secreted OVA-specific antibodies are low overall in the intestinal tract. In intestinal secretions, the IgA titer measured was 10-fold greater than IgG, which may be attributed to the dominance of IgA-producing immunocytes in the intestines of mice.[50]

For most animal groups that mounted a mucosal antibody response, IgA levels were slightly higher than IgG. This is in line with findings previously reported by Livingston et al.[27] Release kinetics of different disk formulations (V_s, V_f) produced similar levels of OVA-specific antibody. In an early study, Challacombe et al.[24] showed that only 100 μg of OVA is sufficient to elicit an OVA-specific response. However, we note that this dose may not result in equivalent responses

(48) Kutteh, W. H. In *Mucosal Immunology*; Ogra, P. L.; Mestecky, J.; Lamm, M. E.; Strober, W.; Bienenstock, J. McGee, J. R., Eds.; Academic Press: New York, 1999; pp 1423−1434.

(49) Kanazawa, T.; Takashima, Y.; Hirayama, S.; Okada, H. Effects of menstrual cycle on gene transfection through mouse vagina for DNA vaccine. *Int. J. Pharm.* **2008**, *360* (1−2), 164–170.

(50) Aicher, W. K.; Fujihashi, K.; Yamamoto, M.; Kiyono, H.; Pitts, A. M.; McGhee, J. R. Effects of the lpr/lpr mutation on T and B cell populations in the lamina propria of the small intestine, a mucosal effector site. *Int. Immunol.* **1992**, *4* (9), 959–968.

when delivered via other mucosal routes. Thus, both OVA-releasing disks used here may have released sufficient antigen to achieve a high (maximum) response in the experimental time frame. It is also important to note that the reference group was immunized orally with soluble OVA and not saline. This may explain the elevated OVA-specific intestinal IgA titer in these animals compared to a true negative control that is not previously exposed to OVA. Furthermore, this may have led to preferential return of IgA-committed plasma cell precursors to the small intestinal mucosa.[51]

In our results, IgA and IgG in vaginal washes are low for both N/N and O/O groups, which may be evidence that lymphoid cells do not stay resident in the cervicovaginal tract. The mucosal immune system is subcompartmentalized, where inductive sites may serve as a preferential, but not exclusive, source of precursor cells for certain effector sites.[48] Immunization within mucosal tissues can lead to an immune response at other mucosal sites, via the common mucosal immune system. However, activated lymphocytes circulate selectively, and their location is regulated by homing mechanisms via surface receptors. For example, lymphoid cells in Peyer's patches are known to preferentially return to small intestinal mucosal, and nasal immunization also stimulates lymphoid cells that return to lacrimal, nasal and salivary glands.[51]

In this study, female mice were immunized by systemic and mucosal routes. We compared the production of mucosal and serum OVA-specific IgA and IgG antibodies after immunization. Priming and boosting vaginally with OVA delivered via controlled release disks was shown to be the most effective regimen at inducing a high OVA-specific vaginal antibody response. Kozlowski et al.[40] and Livingston et al.[27] reported similar findings, in which intravaginal immunization induced higher local antibody responses than immunization by other routes against recombinant cholera toxin B or human growth hormone, respectively. In other studies, vaccines delivered intravaginally performed as effectively as[41,52] if not worse than[37,38] vaccines delivered via a different mucosal site. For information on findings and immunization strategies to produce immune protection in the female reproductive tract, see a review by Russell et al.[53] In our experiments, it is possible that the delivery of antigens by polymer disks helps to sustain the presence of antigens at the local mucosal surface, which is needed to induce a high immune response. This delivery method may convey an advantage over intranasal or orally delivered doses in our experiments, where polymer particles have to overcome the harsh environment, mucus gel and shorter residence time within the digestive or nasal tract. Furthermore, the high local antibody production may also be attributed to subcompartmentalization of the mucosal immune system.

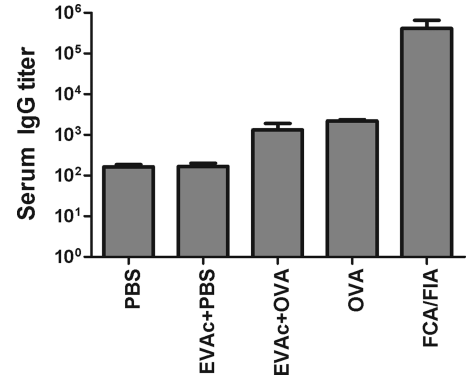

Figure 6. Serum IgG titers of mice immunized intravaginally with saline (PBS), EVAc disk and saline (EVAc + PBS), EVAc disk and OVA (EVAc + OVA), soluble OVA (OVA) compared to subcutaneously immunized mice in Freund's adjuvant (FCA/FIA). While immunization with OVA resulted in elevated serum IgG titers over untreated samples, there was no difference between those with and without intravaginal EVAc disk.

We did not expect low titers of OVA-specific IgA in serum for vaginally primed/boosted animals (V/V). However, vaginally immunized mice produced high serum IgG titers. It is possible that mice were immunized both systemically and locally when implanted with disks, due to "leakage" into the suture that was used to hold disks in place. To test for the effect of prolonged release on immune response and any adjuvant-effect of the physical presence of EVAc disk, we introduced a control group that received a single bolus dose of soluble OVA (400 μg) with and without the presence of EVAc disks. These control animals produced antibodies in serum and vaginal washes that was slightly higher than the reference level, but are not different from each other. Here we report the serum IgG as a representative of the data trend (Figure 6). In our test groups, high responders typically display peak antibody production before the boost. While the control groups here did not receive a booster dose, it is unlikely that the antibody production would deviate from this trend (high antibody production at week 4, before boost). Thus, we conclude that the prolonged release at the mucosal surface is critical in producing the elevated antibody production in the vaginal mucosa.

To fully evaluate the kinetics of antibody production due to differences in the release rate of the vaginally administered primary immunization, frequent measurements of the antibody appearance in various mucosal secretions and the blood may be necessary. Collecting mucosal secretions and blood every 3 or 4 days for four weeks can capture subtleties in the course of the antibody response that may be missed when sampling weekly or every two weeks. As a result, a better

(51) Brandtzaeg, P.; Farstad, I. N.; Johansen, F. E.; Morton, H. C.; Norderhaug, I. N.; Yamanaka, T. The B-cell system of human mucosae and exocrine glands. *Immunol. Rev.* **1999**, *171*, 45–87.
(52) De, F.; Bernardis, M.; Boccanera, D; Adriani, A.; Girolamo, A. Cassone, Intravaginal and intranasal immunizations are equally effective in inducing vaginal antibodies and conferring protection against vaginal candidiasis. *Infect. Immun.* **2002**, *70* (5), 2725–2729.
(53) Russell, M. W.; Mestecky, J. Humoral immune responses to microbial infections in the genital tract. *Microbes Infect.* **2002**, *4* (6), 667–677.

correlation may be made between antibody production and the antigen release rate into the vagina.

In this study, we assessed the efficacy of immunization schemes by measuring the level of antibody response, which is necessary to protect the female reproductive tract from viral infection. The presence of antibodies specific to viruses, especially those secreted at mucosal surface, can reduce the occurrence of infection by binding to viral particles and impeding transport into the epithelium; antibodies can also mark the particles for elimination by macrophages or dendritic cells. We have shown here that vaccine delivery in the reproductive tract using local polymeric release devices can achieve high antibody response systemically and in the local tissue; antibody levels are higher and longer-lasting than obtained by immunization by other mucosal routes. This is a promising approach for delivering vaccines and other therapeutics to protect the female reproductive tract against prevalent STIs.

Abbreviations Used

PLGA, poly (lactic-*co*-glycolic) acid; EVAc, poly(ethylene-*co*-vinyl acetate); OVA, ovalbumin; IgG, immunoglobulin G; IgA, immunoglobulin A; FCA, Freund's complete adjuvant; FIA, Freund's incomplete adjuvant; PBS, phosphate buffered saline; PVA, poly(vinyl alcohol); DCM, methylene chloride or dichloromethane; ELISA, enzyme-linked immunosorbent assay; po, peroral; i.n., intranasal; vag, intravaginal; sc, subcutaneous.

Acknowledgment. This work was supported by NIH Grant EB000487.

Supporting Information Available: Standard curve from ELISA measuring OVA-specific IgA and OVA-specific IgG. This material is available free of charge via the Internet at http://pubs.acs.org.

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