

Vaginal Mucosa Serves as an Inductive Site for Tolerance¹

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These data demonstrate that tolerance can be induced by vaginal Ag exposure. In these experiments, mice were given vaginal agarose gel suppositories containing either 5 mg OVA or saline for 6 h. Mice were given suppositories either during the estrous (estrogen dominant) or diestrous (progesterone dominant) stage of the estrous cycle. Mice were restrained during the inoculation period to prevent orovaginal transmission of the Ag. After 1 wk, mice were immunized s.c. with OVA in CFA. After 3 wk, mice were tested for delayed-type hypersensitivity responses by measuring footpad swelling and measuring in vitro proliferation of lymphocytes to Ag. Using ELISA, the magnitude of the serum Ab response was also measured. In some mice, FITC conjugated to OVA was used to track the dissemination of the protein into the systemic tissues. The magnitude of footpad swelling was significantly reduced in mice receiving OVA-containing suppositories during estrus compared with mice receiving saline suppositories. Concomitant decreases in the Ag-specific proliferative response were also observed in lymph node lymphocytes and splenocytes. Conversely, mice inoculated during diestrus did not show a decreased response to Ag by either footpad response or in vitro proliferation. Serum Ab titers in the estrus-inoculated mice did not decrease significantly. These data demonstrate that the reproductive tract can be an inductive site for mucosally induced tolerance. However, unlike other mucosal sites such as the lung and gastrointestinal tract, reproductive tract tolerance induction is hormonally regulated. *The Journal of Immunology*, 2000, 165: 5077–5083.

Feeding innocuous Ag in a single large dose or repeated small doses induces classical oral tolerance (1–3). Cell-mediated immunity (CMI)³ is profoundly decreased with low Ag doses in an Ag-specific manner, and humoral immunity is decreased to a lesser extent (4–6). More recently, immunological tolerance has been induced through the mucosa by inhalation of Ag and placing Ag in the conjunctiva of the eye (7, 8). These data have shown that tolerance is inducible in other sites besides the gastrointestinal tract and that mucosally induced tolerance may potentially be inducible at any site in the common mucosal immune system.

Immunological tolerance in the upper reproductive tract related to pregnancy has been convincingly demonstrated, and the fetus can be considered a semiallograft (9). Tolerance in the lower reproductive tract is tacitly assumed to exist for bacterial commensals, but experimental evidence for tolerance to bacteria in the vagina has been demonstrated only with uropathic *Escherichia coli* in cynomolgus monkeys (10). Presumably, a number of reproductive tract pathologies are due to a breakdown in reproductive tolerance,

specifically sperm and semen allergies, antisperm Ab induction in the female, and some ulcerative conditions in the vagina (11, 12).

The rodent model has proved useful for studying lower reproductive tract immunology. The local immune responses in mice and other animals including humans are profoundly affected by the stage of cycle and sex hormones. Infection models for such diseases as chlamydia, candidiasis and herpes must use exogenous hormones to induce or maintain infection (13–15). In humans, episodes of disease can correspond to the stage of cycle (16, 17). In fact, systemic immunity as well as Ab secretion in the cervix varies over the course of the cycle (18, 19). Estrogen is generally described as attenuating immune responses whereas progesterone either has no effect or increases the magnitude of immune responses systemically (18, 20).

To adapt the mouse model for lower reproductive tract studies, a number of controls must be implemented. First, to control for cycle stage in a rodent model, the Ag must be administered during fairly narrow temporal windows since the estrous cycle is only 4 days. If Ag is administered as a liquid, it leaks out and very little of the Ag is retained in the vaginal vault for any length of time, so the Ag delivery system should minimize loss. Finally, mice must be restrained from grooming the vaginal area to prevent oral Ag inoculation.

Wyatt et al. (21) has shown that vaginal rings can be used for Ag and drug delivery in a rodent model; however, the ring must be surgically implanted and cannot be removed to test responses at different stages of the estrous cycle.

We report an experimental technique that prevents the animals from orovaginal ingestion of Ag and also allows specifically timed, nonsurgical Ag dosing by agarose vaginal suppositories with a minimum amount of trauma. Using this technique, we can demonstrate that tolerance is inducible by the vaginal route. However, induction is under hormonal regulation, which has important implications for reproductive tract vaccine design and delivery.

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³ Abbreviation used in this paper: CMI, cell-mediated immunity.

Materials and Methods

Mice

Female 6- to 8-wk-old specific pathogen-free C3H/He mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed for 1 wk before use. Cycle stage was determined by microscopic examination of wet mounts of a 30- μ l vaginal wash with saline. For estrous phase animals large squamous epithelial cells were present in the wash, whereas in diestrous animals, small neutrophils were present almost exclusively in the wash fluid. Animal's cycles were charted daily for 1 wk before being given the suppository to assure a regular estrous cycle. At the end of the experiments, the animals were humanely sacrificed. All experiments were approved by the Magee-Womens Hospital Institutional Animal Care and Use Committee.

Restraint to prevent orovaginal grooming

Mice were restrained with a collar wrapped around the abdomen from behind the shoulder blades to just above the haunches. When the restraint was properly placed, the animals could walk normally. The collar was formed with household masking tape (2.5 cm wide) and pressure-sensitive labeling tape (2.0 cm wide) (VWR, West Chester, PA) applied to its adherent side. The width of the laboratory tape is slightly less than that of the masking tape, so a thin band of adhesive ran the edge of the tape. This adhesive strip was necessary to prevent the mice from squeezing through the restraint. The restraint was placed and removed under light inhalation anesthesia. Mice showed no stress from having been in the restraint and continued to feed and water normally. Numerous mice were observed, and none was able to bend enough to vaginally groom. Mice were individually caged for the duration of restraint to prevent each other from vaginally grooming or chewing the restraints.

Formulation of suppositories

The agarose suppository method was used for these experiments, since it was impossible to load high protein concentrations in standard industrial Carbowax formulations. Vaginal suppositories were made by mixing OVA (Sigma, St. Louis, MO) dissolved in water or saline alone with electrophoresis grade agarose (Life Technologies, Gaithersburg, MD). Specifically, 5% agarose solution was made with distilled, boiled water and incubated in a 60°C water bath until use. Powered OVA (500 mg) was added to a 15 ml conical centrifuge tube, and 0.5 ml distilled water was overlaid and then vortexed vigorously. The solution was centrifuged at $800 \times g$ for 5 min to remove air bubbles. The OVA solution remained at room temperature for all procedures. Agarose (3 ml) was then poured over the OVA solution or saline solution, mixed briefly with a Pasteur pipette and vortexed vigorously for 5 s. The mixture was then suctioned into prewarmed (60°C) Silastic tubing (1/8-inch inside diameter $\times$ 1/16-inch wall, Fisher, Pittsburgh, PA) with a syringe. The gel was allowed to polymerize at 4°C for 15 min and extruded from the tube with compressed air. The gel "rope" was cut into 0.5 cm sections with a scalpel. This method yields a 3% agarose gel containing 5 mg OVA per suppository. Using a length of 0.5 cm and a cast in diameter of 1/8 inch (0.3175 cm), the gel volume can be calculated by the formula for the area of a cylinder ($\pi r^2 h$). These suppositories yielded a total volume of 39.5 mm³. The suppository contained 5 mg of OVA with an effective concentration of 205 mg/ml OVA, which was the upper limit of the amount that could be dissolved in water without raising the viscosity too high. The gel matrix was stable and highly cross-linked, even at this level of protein loading.

Initially, agarose gel concentrations ranging from 1 to 5% were used to determine the best gel consistency for the suppository formulation. Gel concentrations <2% final were too fragile to be used in the mice. Concentrations exceeding 4% final were too viscous to be properly mixed and drawn into the tubing. The 3% agarose suppositories are sufficiently firm that they can be easily removed from the vaginal vault with forceps. In some instances, if the suppository was difficult to remove, a flush of 30 μ l PBS was used to wet the vaginal walls to assist in extraction.

When the gel was cast, it was critical to keep the OVA below 45°C to prevent polymerization; however, this is below the melting temperature of the agarose. To preserve the OVA in its monomeric state and the agarose as a liquid, the agarose was first precooled to 60°C and then rapidly mixed with the OVA. The agarose remained liquid for $\sim$ 1 min, which was long enough to mix the gel and extrude it into the tubing.

Fluorescent labeling of OVA

In some experiments, FITC-labeled OVA was used. OVA (50 mg in 1 ml 0.05 M boric acid, 0.2 M NaCl, pH 9.6) was incubated with 200 μ l 5 mg/ml FITC (Sigma) dissolved in DMSO for 2 h at room temperature. The sample was then passed over a Sephadex G-10 column to remove unbound FITC.

Samples were dialyzed against PBS and concentrated to 50 mg/ml protein in a vacuum centrifuge. OVA-FITC-containing suppositories were prepared in a manner identical with those containing unconjugated OVA. In some experiments, unconjugated FITC (1 mg/ml) was used instead of OVA-FITC in the formulation.

Determination of Ag diffusion rate

To characterize the rate of release of OVA from the suppositories, diffusion studies were conducted in accordance with the U.S. Pharmacopeia National Formulary standard assay requirements for dissolution studies (22). The murine basal body temperature has a range of 37.1–37.3°C (23). One suppository was incubated in 3 ml PBS at 37°C. Aliquots of the buffer were taken at specific time points and assayed for OVA content using a Coomassie Plus Protein Assay (Pierce, Rockford, IL). Additional studies were performed using FITC-conjugated OVA as described in the previous section. Fluorescence was measured in the buffer at different time points using a Millipore 2350 fluorimeter (Millipore, Bedford, MA).

Immunizations

Animals were inoculated vaginally with OVA-containing suppositories or saline suppositories as a control for 6 h at day 0. One week after vaginal inoculation, all animals were injected in the hind flank with 200 μ g OVA emulsified in 100 μ l CFA (Sigma). On day 39–40, mice were footpad tested against OVA vs saline, and on day 42–43 the mice were sacrificed by cervical dislocation. Blood was collected at various time points using the retroorbital collection method.

Assessment of delayed-type hypersensitivity

To test for delayed-type hypersensitivity responses in the animals, mice were footpad tested with Ag in one foot vs saline in the other foot. Halothane (Halocarbon Laboratories, River's Edge, NJ) anesthetized mice were injected in the right hind footpad with 10 μ l saline containing 10 μ g OVA, and the left footpad was injected with 10 μ l saline only. The thickness of the footpads was measured at 24 and 48 h with a spring micrometer (Starrett, Athol, MA). The net swelling was calculated as the thickness of the Ag-inoculated footpad subtracted from the thickness of the saline-inoculated footpad.

Determination of in vitro Ag stimulation

Spleens and inguinal lymph nodes were removed from the mice at sacrifice and mechanically disaggregated. Splenocytes were incubated in Tris-buffered ammonium chloride to lyse RBC. Lymphocytes were washed in RPMI containing 10% FCS, 25 mM HEPES, 20 mM L-glutamine, 100 U/ml penicillin/100 μ g/ml streptomycin, and 0.05 mM 2-ME. Cells (10^6 /well in quadruplicate) were incubated with 100 μ g/ml OVA or medium alone in a final volume of 200 μ l for 4 days and pulsed with 0.5 μ Ci [³H]thymidine (Amersham, Arlington Heights, IL) for 6 h in flat-bottom 96-well plates. Cells were harvested on glass fiber filters and counted in a scintillation counter. Results are expressed as mean cpm of the Ag culture subtracted from the mean cpm of medium alone $\pm$ the SEM of quadruplicate cultures.

Determination of Ab titer

A modified sandwich ELISA was used to assess Ag-specific serum Ab levels. OVA (100 μ g) in carbonate-bicarbonate buffer (pH 9.2) was used to coat Falcon PVC ELISA plates (Becton Dickinson, Franklin Lakes, NJ) overnight at 4°C. Wells were then blocked with 200 μ l 5% FCS in PBS for 2 h at room temperature. Plates were washed with PBS, and the mouse serum was diluted in PBS + 1% FCS + 0.1% Tween 20 and incubated for 2 h at 37°C. Plates were washed in PBS-Tween, and 1 μ g/ml biotin-conjugated detecting Ab anti-mouse IgG,A,M (Southern Biotechnology Associates, Birmingham, AL) was added. After incubation at 37°C for 1 h, plates were washed in PBS-Tween, and streptavidin peroxidase (Sigma) was added for 30 min at room temperature. Plates were washed with PBS and then developed with 3,3',5,5'-tetramethylbenzidine substrate (Pierce, Rockford, IL). Plates were read at 450 nm/650 nm on a plate reader after the wells were acidified with 150 μ l 1 M H₂SO₄. Titration endpoints are calculated using x-intercept ($y = mx + b$).

Determination of Ag dissemination by histology and flow cytometry

In mice given FITC, the entire reproductive tract was removed including the vagina and uterus. Tissues were embedded in OCT (Sakura Finetek, Torrance, CA) and snap-frozen in liquid nitrogen. Sections of 5 μ m were cut, fixed, mounted, and observed using an upright fluorescent microscope

(Nikon Eclipse E800, Melville, NY). Optimus software (Data Cell, Finchampstead, U.K.) was utilized to collect two sequential images (one differential interference contrast and the other fluorescent FITC), which were then superimposed.

In other experiments with animals receiving FITC-conjugated OVA, the caudal lymph node and the iliac lymph nodes were removed. The nodes were mechanically disaggregated, washed in PBS, stained with Ab in PBS/1% nonimmune murine serum, and fixed in 2% paraformaldehyde for analysis by flow cytometry. The caudal node drains the lower reproductive tract, and the iliac nodes drain the upper reproductive tract in the mouse. Before removal of nodes, the peritoneum was irrigated with 4 ml HBSS, and the recovered cells were washed three times in PBS before fixation in paraformaldehyde for flow cytometric analysis of FITC uptake. Anti-mouse IA^b-PE was used to stain for MHC class II-positive cells (PharMingen, San Diego, CA). Flow cytometry was performed on a Becton Dickinson FACScan.

Statistical analysis

For analysis of significance, an unpaired Mann-Whitney *U* test was used to compare the control group (those receiving saline suppositories) vs the experimental group (those receiving OVA suppositories) for footpad, proliferation, and Ab responses at estrus and diestrus. Significance was defined as $p < 0.05$ in these studies.

Results

OVA rapidly diffuses from agarose gel suppositories

To properly formulate the suppositories, it was important to demonstrate that Ag is not irreversibly bound to the agarose matrix and is capable of free diffusion from the gel. In Fig. 1, the solubility and rapid diffusibility of the Ag from the suppository are shown. A 3% agarose suppository containing OVA conjugated to FITC (5 mg/suppository) was incubated in a 37°C water bath in 5 ml PBS, and the liquid was sampled at various time points to measure fluorescence. Identical experiments with unconjugated OVA and the efflux assayed by ELISA yielded similar results (data not shown). In this experiment, the Ag rapidly diffuses out of the agarose gel. Greater than 50% of the protein was in solution after 1 h. OVA began to efflux from the suppository almost immediately after the suppository was placed in solution. Maximal dissolution was reached by 2 h.

Vaginal OVA administration differentially attenuates CMI

The hallmark of mucosally induced tolerance is a reduction in the cell-mediated immune response (3). Classically, this is measured by diminution of the footpad swelling response in immunized animals after feeding or inhalation of Ag. In these experiments, the tolerizing dose was in the form of an OVA-containing suppository given in either the estrous or diestrous phase of the cycle. After 1 wk, animals were immunized systemically with the Ag in CFA. In Fig. 2 the results are shown for footpad swelling in response to an

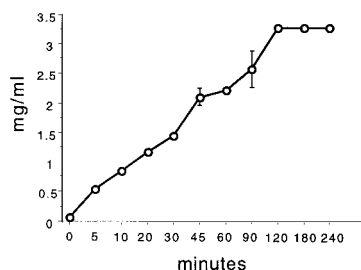


FIGURE 1. Transport kinetics of OVA-FITC from a 3% agarose microsuppository. OVA-containing suppositories (5 mg/suppository) were incubated in 3 ml PBS at 37°C. Samples of 50 μ l were taken at regular time intervals and assayed for fluorescence. Greater than 50% of the OVA had diffused out of the suppository by 1 h, and maximal dissolution was reached by 2 h. These data are representative of three separate experiments.

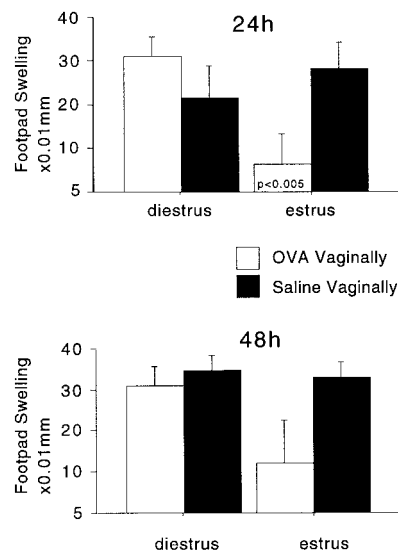


FIGURE 2. Net footpad swelling responses of immunized (40 days postimmunization) with prior inoculation by vaginal OVA or saline suppositories. Net responses as measured in millimeters at 24 and 48 h after footpad injection of OVA are shown. The OVA suppository significantly diminished the footpad response if it was given in the estrous phase of cycle. There was no significant effect in the animals given a suppository during the diestrous phase of the cycle. These data are representative of three separate experiments ($n = 6$ per arm in each experiment). Average footpad swelling response $\pm$ SEM is shown.

OVA challenge after a diestrus- or estrus-administered suppository. The delayed-type hypersensitivity response was significantly diminished in animals that had received suppositories during estrus but not during diestrus ($p < 0.005$). A vaginal inoculation with the OVA suppository alone did not boost immune responses over that of the s.c. inoculated controls. Simply giving an OVA-containing suppository did not induce a detectable immune response as measured by footpad swelling or systemic Ab response before immunization. Therefore, OVA administered vaginally does not induce a detectable immune response (data not shown).

In vitro proliferation of lymphocytes to OVA was diminished in animals receiving suppositories during estrus (Fig. 3). This response was consistent with observed diminution of the footpad swelling response. In these experiments, splenocytes and inguinal node lymphocytes were stimulated with OVA in culture. The results from splenocytes are represented as individual splenocyte cultures from individual animals. However, since far fewer cells were available for the inguinal lymph node, the lymphocytes were pooled from each group to test for proliferation. As in the footpad swelling response, only animals receiving a suppository during estrus had a diminution in the proliferative response. Animals receiving a suppository during diestrus did not have a diminution in this response.

Vaginal OVA administration does not attenuate humoral immunity

The serum Ig response to OVA was also measured in animals receiving suppositories (Fig. 4). However, no significant decrease in the total Ag-specific Ab response was found. These data indicate that humoral immunity is not affected as profoundly as CMI in this model. These data are consistent with other types of mucosally induced tolerance (24). Serum taken from animals before immunization showed no significant reactivity to OVA (endpoint dilution, $<1:100$) (data not shown).

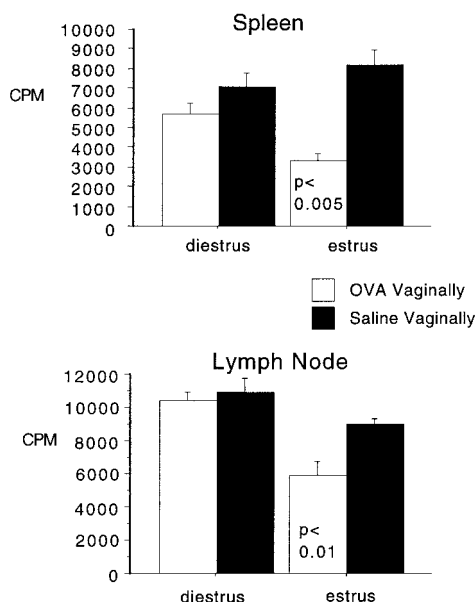


FIGURE 3. OVA-specific lymphocyte-proliferative responses 40 days postimmunization in animals receiving prior saline or OVA vaginal suppositories. Splenocytes and inguinal lymph node cells were isolated from animals and stimulated with 100 μ g OVA for 4 days. Proliferation was measured by [^{3} H]thymidine incorporation. In estrus, a significant diminution in the immune response is observed if the animals receive vaginal OVA before immunization compared with a saline suppository. However, there is no significant effect in animals receiving the OVA suppository during diestrus. These data are representative of two separate experiments ($n = 6$ per arm in each experiment). Average cpm $\pm$ SEM is shown.

Ag traffic into the uterine horns and lymph nodes varies in the estrous cycle

Although a significant immunological effect could be demonstrated, the site of tolerance induction, whether the vagina or uterus, remained unclear. First, it was important to demonstrate that Ag could traffic through the vagina into uterus and peritoneal cavity, since the vaginal epithelium at estrus is too thick to permit rapid Ag transport into the nodes. To examine trafficking, we loaded FITC instead of OVA into the suppository as a visible marker. This allowed direct observation of the depth of penetration of the marker into the tissue. After the FITC suppository had been in the vagina for 6 h, the reproductive tract was excised, and frozen sections were visualized with superimposed fluorescent and differential interference contrast microscopy (Fig. 5).

Fluorescent staining in the vagina where the suppository was inserted was apparent because the FITC adhered to the vaginal lining, but fluorescence was not observed past the superficial squamous epithelial cell layer, indicating that it could not freely diffuse in large amounts into the deeper stromal layers of tissue in either estrus or diestrus in the time frame examined. Cross-sections taken from the uterine horns also showed positive epithelial staining but only during estrus, indicating that the Ag is trafficked from the vagina into the upper reproductive tract but only during specific stages of the estrous cycle. A similar technique using liquid infusion of H33342 vital dye has been previously used to determine Ag trafficking patterns in the rodent vagina (25).

In another set of experiments, animals were given FITC-labeled OVA suppositories in estrus or diestrus 6 h before sacrifice to test whether Ag could be trafficked into the draining lymph nodes. Lymphocytes were isolated from the caudal lymph node which drains the rectum and perineum, the lumbar lymph nodes which

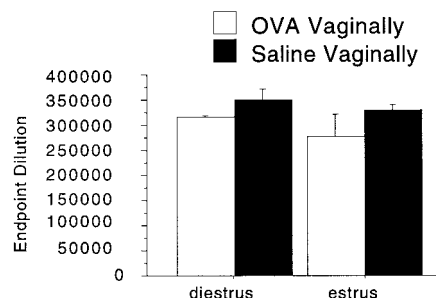


FIGURE 4. Serum anti-OVA responses 40 days postimmunization in animals receiving prior saline or OVA vaginal suppositories. No significant diminution of serum Ab was observed in the animals receiving OVA vaginally compared with saline controls. The stage of cycle did not significantly affect the humoral response. Naive animals had anti-OVA responses with endpoint dilutions of $<1:100$ (data not shown). Results are representative of two separate experiments ($n = 6$ per arm in each experiment). Average endpoint dilution $\pm$ SEM is shown.

drain the uterus and vagina, and peritoneal washes. IA^k-positive cells were measured for concomitant FITC expression by FACS (Fig. 6). Ag-positive FITC-fluorescent cells were observed at 6 h in the lumbar lymph node (Fig. 6, A and B) and but not in the caudal lymph node (not shown) at equal levels. The lumbar nodes were positive, indicating that Ag can be trafficked into these nodes during both diestrus and estrus stages of the cycle.

When IA^{k+} peritoneal exudate cells were examined (Fig. 6, C and D), $>35\%$ of the cells were positive for FITC in estrous phase animals, but no cells were positive in the diestrous phase animals. The FITC-positive cells were entirely class II positive. These data indicate that during estrus, protein and low molecular weight compounds can traffic from the vagina into the uterus and peritoneal

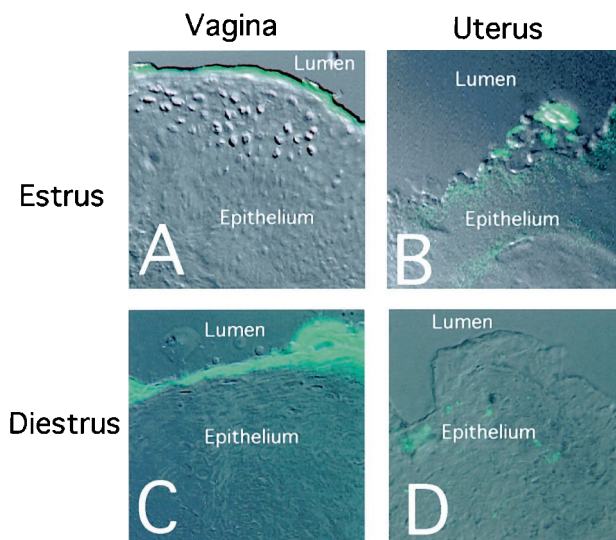


FIGURE 5. Tissue staining after inoculation with vaginal suppositories containing FITC. After the suppository had been in place for 6 h, bright green fluorescence could be detected in the reproductive tract tissues. FITC adhered to the outer vaginal epithelium (A) and the outer uterine epithelium (B) in estrus animals. The dye was present only on the vaginal epithelium of the diestrus animals (C) and not the epithelium of the uterine horns (D). Images are superimposed fluorescence, and differential interference contrast pictures were taken at a magnification of $\times 40$ under oil immersion. A small amount of artifactual green fluorescence occurred in the uterine mucosa of both estrus and diestrus animals (C and D). FITC was not observed directly transiting into the lower mucosa although it could not be ruled out.

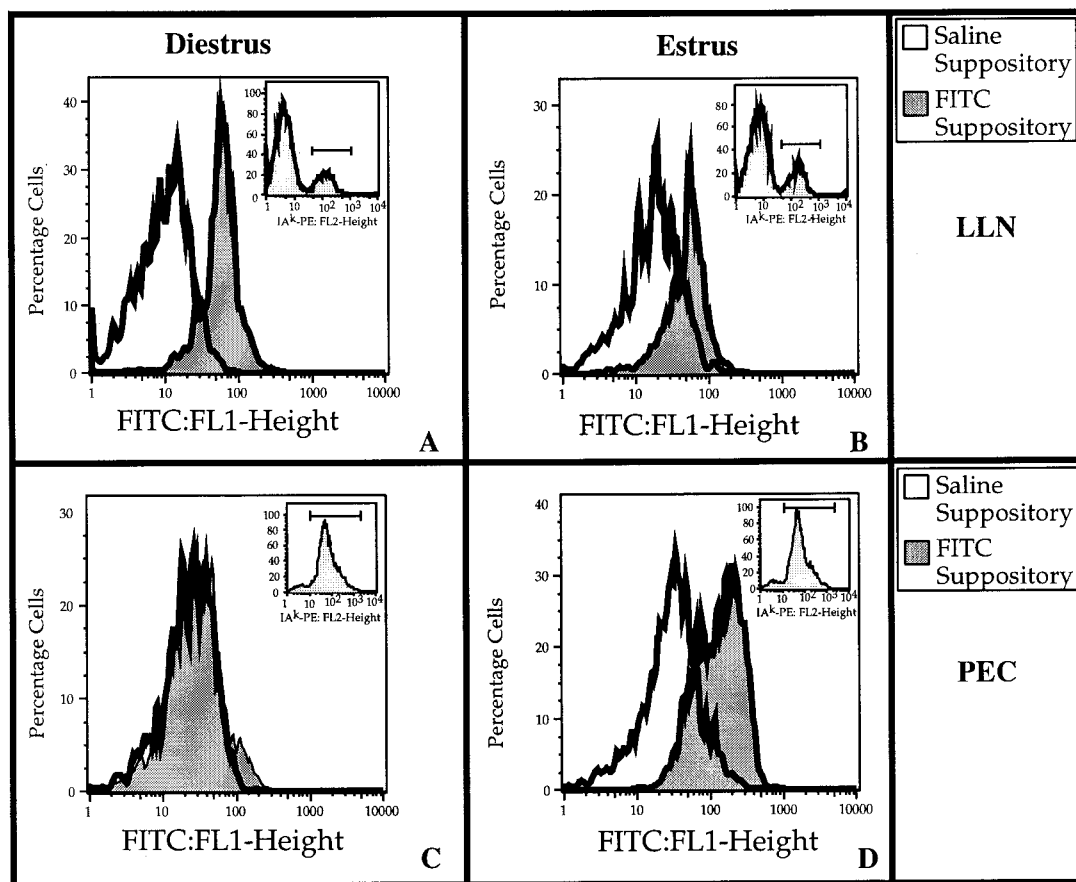


FIGURE 6. Appearance of Ag-positive lymph node and peritoneal cells after vaginal suppository inoculation for 6 h with OVA-FITC. Animals were given either a saline control suppository or an OVA-FITC suppository at estrus or diestrus. When cells were gated for IA^k staining (inset), the corresponding fluorescence (FL1) in the FITC channel was measured. No difference in the number of FITC⁺ cells were seen in the lumbar lymph node (LLN) at diestrus (A) or estrus (B). However, while <1% of peritoneal exudate class II⁺ cells (PEC) were positive for FITC during diestrus (C), >35% were positive in estrus, which indicates that Ag can enter into the peritoneal cavity only during estrus. These data are representative of four separate experiments.

cavity where Ag can then be acquired by APCs. This effect is not seen during diestrus.

Discussion

These data demonstrate the feasibility of using rodents in vaginal vaccination/tolerization. Specifically, this method allows the use of vaginal suppositories that can be easily made at the benchtop and inserted vaginally without using deep anesthesia on the animals. Although the suppositories are inserted completely into the vaginal vault against the cervix, some animals did expel the suppository. This occurred in ~10% of the mice. In this event, the animal was excluded from the study. If the suppository length exceeded 5 mm, then it could be expelled (data not shown). The 5-mm-length suppository was held in the vagina by natural closure by the vaginal sphincter and mucoadherence of the suppository to the vaginal walls.

The suppository design itself is also unique. It is difficult to formulate suppositories in which the active ingredient is incorporated into a dissolving matrix. It is especially difficult to design them for use in the rodent due to the small size of the suppository that is required. A second problem is the use of soluble excipients which may have potentially unintended side effects and adds to the total liquid volume deposited in the vagina, which effectively dilutes the active ingredient. By using agarose, which is insoluble, the suppository does not melt but the Ag is free to diffuse out of the gel matrix (26). Additionally, agarose itself is nontoxic and can be cast in small diameters. The agarose gel suppository method uti-

lizes low temperature formulation that can generally preserve the protein in a native conformation. Protein can be loaded into the gel at the benchtop using common laboratory equipment. We have not tested the upper limit of the m.w. that can diffuse freely from the suppository; however, Pernodet et al. (27) have shown that a 3% agarose gel has a pore diameter of 289 nm, which is approximately the size of a retrovirus. OVA, which has a m.w. of only 45,000, was able to diffuse rapidly from the gel. It also should also be possible to design suppositories using gelatin, polyacrylamide, and other insoluble matrices.

Because vaginal grooming could easily confound the results of vaginal tolerance, the animals had to be isolated and restrained. The restraint mechanism, which is similar to a surgical collar, prevented the mice from bending enough to groom vaginally but did not prevent them from feeding, sleeping, and defecating. The restraint system provided an optimal compromise between restraint and free movement. Animals scratched at the restraint periodically but were never in any undue stress. No animal was able to remove the abdominal band in the 6 h during which they were restrained. Animals were housed separately while they were in restraint to prevent other mice from chewing each other's restraint. The collar can be rapidly removed, and compared with other options such as long term anesthesia or full body restraint, the collar was less stressful. This restraint design would also be useful in preventing anal grooming when using rectal suppositories.

By using microsuppositories, we were able to test the novel hypothesis that the vaginal mucosa is an inductive site for tolerance like the intestine and lung. Certainly, some form of immune modulation is present in the vagina given, that it maintains a commensal flora load that is exceeded only by the gastrointestinal tract. Presumably, the host is exposed to various commensal floral species through breaks in the vaginal/cervical lining and ascension of bacteria into the upper reproductive tract. However, this exposure does not usually result in inflammatory responses. Also important is the down-regulation of the immune response against sperm. Immunosuppressive cytokines have been described in seminal fluid, underscoring the importance of attenuating the immune response in the reproductive tract (28). In some clinical pathologies, breakdown in tolerance against sperm results in infertility. Thus, tolerance to vaginal Ags and sperm may play a crucial role in maintaining fertility. Reproductive tract tolerance could also be exploited by sexually transmitted diseases to evade immune surveillance.

It has been virtually impossible to locally immunize in the uterus or vagina against sperm (29, 30). Vaginal immunization using liquid Ag instilled into the vagina has been attempted; however, the immune responses as, measured particularly by Ig levels have been low compared with the systemic immunization route (31, 32). This has prompted the use of various adjuvants including cholera toxin (33). Our data indicate that one factor that may reduce the effectiveness of vaginal immunizations is induction of tolerance through vaginal inoculation.

To test for vaginal tolerance induction, it is critical to control for cycle-dependant immune responses. The mouse has a 4-day cycle with a estrogen-dominated (estrous) phase lasting 1.5 days and progesterone-dominated (diestrous) phase lasting 2–3 days (In these studies, metestrus, a transitory period, was not examined). The only time that an animal would be exposed to sperm is during the estrogen-dominated phase. Additionally, mice carry significant numbers of bacteria and the titers are highest during estrus (34). The results indicated that it was only during the estrogen-dominated phase of ovulation (estrus) that tolerance could be induced. Presumably, tolerance is being induced to prevent inflammatory responses against sperm and commensal bacteria.

We did not find that diestrus was an immunogenic phase, merely that Ag introduced at this stage was not capable of inducing tolerance. This result could be due to a lack of transport of Ag from the vagina into the putative tolerance inductive site like the uterus or peritoneal cavity, or it could be due to hormone-regulated immunological mechanisms. Because FITC-labeled OVA was found in the peritoneal cells at estrus but not diestrus, we favor the hypothesis that Ag must traffic into the upper reproductive tract to be toleragenic, but an immune mechanism could not be ruled out.

In fact, the deep Ag penetration of OVA into the peritoneal cavity in estrus could mimic classical mucosally induced tolerance. It has been shown that i.p. injection of pure proteins leads to immunological tolerance (35, 36). It is possible that some of the OVA can gain access into the peritoneal cavity from the vagina through the fallopian tubes at estrus. Thus, the vaginal inoculation may act like an i.p. OVA injection. The actual mechanisms of immune tolerance are still being debated, although it has been shown that in oral tolerance a combination of anergy, deletion, and bystander suppression plays a role (37). These data did not determine which mechanism of tolerance was involved. Future studies will examine this aspect and the possible role of hormones in tolerance regulation.

Classically, CMI has been easier to suppress in mucosal tolerance experiments than humoral immunity and CMI suppression requires lower Ag doses (3). One reason for the differential sus-

ceptibility is the rapid recovery of the potency of B cells compared with recovery of T cell responses (38). Mechanistically, two types of gastrointestinal tract-induced tolerance have been described, low dose and high dose tolerance (39). In low dose tolerance, the Ag is repeatedly inoculated or continuously infused into mucosal compartments at a relatively low level. In high dose tolerance, the Ag is given in a large bolus but only once or twice. Both have the effect of inducing tolerance, but a hallmark difference of low dose tolerance is the suppression of CMI but not humoral immunity as opposed to both types of immunity in high dose tolerance (40). The difference in high zone vs low zone tolerance has been related to the generation of Th2 responses (low dose) vs deletion of Th1 and Th2 responses (high dose) (41). We hypothesize that the vaginal Ag dose in the suppository is most analogous to low zone tolerance induction as evidenced by the fact that CMI was down-regulated but not humoral. Despite the high Ag load in the suppository, the actual dose received by the animal is far lower as evidenced by retained Ag in the suppository. In fact, the vaginal vault is generally a poor site for uptake of all but the smallest hydrophobic molecules (42, 43). However, in these experiments the Ag exposure was for a relatively long and continuous time. The suppression of CMI alone after vaginal inoculation is consistent with human experiments in which suppression of CMI but not humoral immunity was observed after feeding innocuous Ag to volunteers (24).

In the vagina, this differential CMI vs humoral response could be protective in that Ab directed against commensals in vaginal fluid will not result in possibly sterilizing inflammation. However, a hyperactive cell-mediated response to local commensals or sperm could easily lead to sterilization of the vagina and uterus with possibly long term scarification and irreversible damage to the reproductive tract. Selection pressure would thus favor individuals capable of down-regulating responses in the reproductive tract to environmental Ags and sperm. However, to prevent sexually transmitted diseases from exploiting this immunological "blindspot" in CMI, Abs may be critical in preventing pathogens from entering through the mucosa.

These data demonstrate a new and potentially very useful modeling system for vaginal delivery of Ag into the rodent vagina and that cycle stage is critical in defining the immune response to vaginally administered Ag. If Ag is present around the time of ovulation or insemination, tolerance can occur, whereas this effect will not occur during diestrus, possibly due to decreased transport into the uterine horns. Using this experimental system, it will be relatively simple to introduce cytokines or other adjuvants into the suppository to test whether mucosally induced tolerance in the vagina can be broken or enhanced.

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