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Differential Induction of Mucosal and Systemic Antibody Responses in Women After Nasal, Rectal, or Vaginal Immunization: Influence of the Menstrual Cycle[1]

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A cholera vaccine containing killed vibrios and cholera toxin B subunit (CTB) was used to compare mucosal immunization routes for induction of systemic and mucosal Ab. Four groups of women were given three monthly immunizations by the rectal immunization (R_{imm}) route, nasal immunization (N_{imm}) route, or vaginal immunization route during either the follicular (V-FP_{imm}) or luteal (V-LP_{imm}) menstrual cycle phase. N_{imm} was performed with 10-fold less vaccine to determine if administration of less Ag by this route can, as in rodents, produce mucosal Ab responses comparable to those induced by higher dose R_{imm} or vaginal immunization. Concentrations of Ab induced in sera and secretions were measured by ELISA. None of these routes produced durable salivary Ab responses. N_{imm} induced greatest levels of CTB-specific IgG in sera. R_{imm} failed to generate CTB-specific IgA in genital tract secretions. N_{imm}, V-FP_{imm}, and V-LP_{imm} all produced cervical CTB-specific IgA responses comparable in magnitude and frequency. However, only V-FP_{imm} induced cervical IgA2-restricted Ab to the bacterial LPS vaccine component. V-FP_{imm}, but not V-LP_{imm}, also induced CTB-specific IgA in rectal secretions. N_{imm} was superior to V-FP_{imm} for producing rectal CTB-specific IgA, but the greatest amounts of CTB-specific IgA and LPS-specific IgA, IgG, and IgM Ab were found in rectal secretions of R_{imm} women. These data suggest that in women, N_{imm} alone could induce specific Ab in serum, the genital tract, and rectum. However, induction of genital tract and rectal Ab responses of the magnitude generated by local V-FP_{imm} or R_{imm} will likely require administration of comparably high nasal vaccine dosages. ***The Journal of Immunology,*** **2002, 169: 566–574.**

Generating protective immunity against HIV and other sexually transmitted diseases (STD)[3] could be achieved if vaccines deliver appropriate antigenic epitopes and are administered at sites that induce durable humoral and cellular immune responses in the genital tract, rectum, and systemic compartment. Available data suggest that an HIV vaccination protocol that combines mucosal with parenteral immunization would be superior to parenteral administration alone for induction of antiviral IgA Ab in host secretions, neutralizing IgG Ab in serum, and HIV-specific Th cells and CTL in the genital tract and rectum as well as in the systemic compartment (1–6).

To determine which mucosal vaccination routes might be most effective for induction of immune responses in the female genital tract and rectal mucosa, we previously compared the oral immunization (O_{imm}), rectal immunization (R_{imm}), and vaginal immunization (V_{imm}) routes for their ability to induce cholera toxin B subunit (CTB)-specific IgA Ab in secretions of women who were immunized three times at biweekly intervals with cholera vaccine (7, 8). Results indicated that local R_{imm} was more effective than O_{imm} or V_{imm} for induction of specific IgA in the rectum (7, 8). Conversely, local V_{imm} was found more effective than O_{imm} or R_{imm} for generating mucosal IgA Ab in cervical and vaginal secretions (7, 8). However, it was noted that women in the V_{imm} group developed local Ab responses that differed tremendously in magnitude (8), possibly because vaccine was administered at alternating follicular and luteal phases of the menstrual cycle. It has been reported that biweekly V_{imm} of women on days 10 and 24 of the menstrual cycle is better than random biweekly V_{imm} for induction of specific IgA Ab in cervical secretions (9). However, comparative studies addressing the outcome of V_{imm} in relation to the specific menstrual cycle phases during which Ag is administered have not been performed in humans. Thus, it is currently unclear whether there is an optimal time for administration of vaccines in the female reproductive tract.

In rodents, available data suggest that during diestrus, immune responses may be suppressed in the upper reproductive tract but enhanced in the lower tract, presumably to facilitate fertilization and prevent infection in the event that fertilization occurs (10, 11). The numbers of dendritic cells (DC), MHC class II^+ cells, and the Ag-presenting capability of macrophages and DC have been found to be minimal in the uterus, but maximal in the vagina during the progesterone-associated diestrus phase that follows ovulation in these animals (10–12). In contrast, induction of tolerance was successful in mice when Ag was vaginally administered during the estrogen-dominant ovulatory estrus phase, but not during diestrus (13). Under the influence of progesterone, the number of layers of epithelial cells lining the vagina of rodents decreases dramatically

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[3] Abbreviations used in this paper: STD, sexually transmitted disease; CTB, cholera toxin B subunit; DC, dendritic cells; N_{imm}, nasal immunization; O_{imm}, oral immunization; R_{imm}, rectal immunization; S-IgA, secretory IgA; SC, secretory component; V_{imm}, vaginal immunization; V-FP_{imm}, vaginal follicular phase immunization; V-LP_{imm}, vaginal luteal phase immunization.

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during diestrus, which would be expected to enhance uptake of lumenal Ag. Indeed, uptake of proteins and the ability of V_{imm} to induce specific Ab responses in mice were optimal when preparations were administered during diestrus (14, 15).

The above data suggest that V_{imm} in women might be more effective for induction of immune responses if performed during the progesterone-associated luteal phase of the menstrual cycle. However, the human and rodent reproductive tracts differ in several respects that may influence Ag uptake and induction of immune responses. In humans, the vaginal epithelium may not be more permeable to Ag during the luteal phase because progesterone does not significantly thin the epithelium (16). Both the human and rodent reproductive tracts contain large numbers of DC (12, 17), but the human (and nonhuman primate) reproductive tract contains much greater numbers of $CD4^+$ and $CD8^+$ T cells, macrophages, B lymphocytes, and plasma cells (17–24). In contrast to rodents, the number of these cells in the cervicovaginal mucosa of women and rhesus macaques do not appear to change during the menstrual cycle (17–20). CTL activity in the cervix and vagina of women has also been demonstrated at all menstrual cycle phases (25). These findings suggest that the lower reproductive tract of humans may be immunologically active throughout the entire menstrual cycle. Nevertheless, the possibility remains that the time of the menstrual cycle at which vaccines are vaginally administered could influence the magnitude of immune responses generated.

In this study, we sought to determine whether V_{imm} during the midluteal phase (V-LP_{imm}) of the menstrual cycle might be more effective than V_{imm} during the midfollicular phase (V-FP_{imm}) by administering cholera vaccine at monthly intervals in two groups of women. The vaccine, containing whole-killed vibrios and rCTB, has been used in several previous studies (7–9, 26). CTB- and cholera LPS-specific Ab induced in sera and secretions of these women were also compared to those induced by R_{imm} or nasal immunization (N_{imm}). N_{imm} in humans has been shown to induce specific IgG Ab in serum and specific IgA in saliva, respiratory tract secretions, urine, and female genital tract secretions (9, 26–28). However, N_{imm} has not been tested for its ability to evoke specific IgA Ab responses in the human rectum. If N_{imm} induces both rectal and genital tract mucosal immune responses, it could be a more effective mucosal administration route for STD vaccines, because R_{imm} and V_{imm}, though highly effective for induction of local responses, generate little or no specific IgA at distal mucosal sites (7–9, 29, 30). Studies in mice have also suggested that N_{imm} might be economically advantageous to other mucosal immunization routes because lower Ag doses can generate mucosal Ab responses comparable to those induced by higher doses administered elsewhere (6, 31, 32). To determine whether this may be the case in humans, we administered 10-fold lower cholera vaccine doses in the nasal cavity of women and compared the magnitude of the mucosal and systemic Ab responses generated to those induced by higher doses in women who received V-FP_{imm}, V-LP_{imm}, or R_{imm}. The data indicate that V-FP_{imm} may be superior to V-LP_{imm} for induction of local and distal mucosal Ab responses. In addition, we show that N_{imm} can induce specific secretory IgA (S-IgA) Ab in rectal secretions. However, low-dose N_{imm} induced less rectal IgA Ab when compared to local R_{imm}.

Materials and Methods

Study population

Nonhysterectomized, healthy, premenopausal women with low-risk sexual behavior and no history of cholera infection or vaccination were enrolled for either V-FP_{imm} ($n = 5$), V-LP_{imm} ($n = 5$), N_{imm} ($n = 6$), or R_{imm} ($n = 5$) with cholera vaccine. Enrollment into a V_{imm} group required that the volunteer have a history of menstrual periods at regular 26- to 30-day intervals. The racial composition and ages of women representing each immunization group are presented in Table I. Ethnic background did not appear to influence the magnitude of Ab responses generated in these or previous participants (7). One woman in the R_{imm} group was using Depo-Provera for birth control, and hence did not experience menstrual periods. In the N_{imm} group, two women were taking oral contraceptives; one was immunized during the midfollicular phase, the other during the midluteal phase. None of the other women were using hormonal methods of birth control. Informed consent was received from all volunteers. Study procedures were approved by the Miriam Hospital Clinical Review Board (TMH93-006; Providence, RI).

Vaccine and Immunizations

Women were immunized a total of three times at rough monthly intervals with the WC/rBS cholera vaccine (Swiss Serum and Vaccine Institute, Berne, Switzerland). For R_{imm} or V_{imm}, each 3-ml dose of vaccine contained 1 mg rCTB, and 2.5×10^{10} of each of the following cholera vibrios suspended in PBS: heat-inactivated Classical Inaba, formalin-inactivated El Tor Inaba, and heat- and formalin-inactivated Classical Ogawa. V_{imm} was performed as described (7) by mixing 3 ml vaccine with Eldexomer powder (Perstörp, Uppsala, Sweden), then applying the thickened vaccine to surfaces of the ectocervix and posterior fornix of the vagina. R_{imm} was performed by instilling 3 ml vaccine 6 cm into the rectum with a syringe (7). For each N_{imm}, 0.3 ml vaccine was administered. N_{imm} was performed after seating the subject with her head tilted back slightly. A total of 50 μl vaccine was applied inside the front of each of the nostrils, which were immediately pressed shut and released to ensure coating of nasal surfaces. This procedure was repeated two times such that a total of 150 μl vaccine was administered in each nostril. No adverse side effects were reported after V_{imm}, R_{imm}, or N_{imm}.

V-FP_{imm} and V-LP_{imm} were performed each month 7–12 and 17–23 days after the start (day 1) of the menstrual period, respectively. Phases of the menstrual cycle were not monitored by measurement of hormones, because this could not be done practically at many international STD vaccination sites. Instead, women were asked to keep a menstrual calendar because it has been established that, in women with regular menstrual cycles, the interval from the start of menstruation to ovulation (follicular phase) can vary from 12–17 days, but the interval from ovulation to menstruation (luteal phase) is almost always exactly 14 days (33). Therefore, it was possible to determine with greater certainty that subjects had been immunized during the appropriate phase by recording the interval that had elapsed from the day of each immunization to the onset of the following menstrual period.

Table I. *Characteristics of women in each immunization group*

Group	n^a	Age	Racial Composition[a,b]			Menstrual Cycle Phase[a,c]	
		Mean (range)	L-A	A-A	C	Midfollicular	Midluteal
V-FP	5	32 (19–41)	1	2	2	5	0
V-LP	5	30 (21–35)	0	2	3	0	5
N	6	32 (19–42)	1	1	4	4	2
R[d]	5	32 (18–45)	2	1	2	3	1

[a] Number of subjects.
[b] Latin-American (L-A), Afro-American (A-A), and Caucasian (C).
[c] Phase during which both immunization and sample collection was performed.
[d] One woman using DepoProvera was immunized independently of menstrual cycle phase.

Collection of sera and secretions

Nonheparinized whole blood (for serum), whole salivary, rectal, vaginal, and endocervical secretions were collected immediately before the first and third immunizations, then roughly 1 and 2 mo later. Extra secretions were collected from one V-FP_{imm} and three V-LP_{imm} women 2 wk after the third immunization. Whole salivary and vaginal secretions were collected as described (7) by placing two absorbent Weck-Cel (since renamed Ultra-Cel) Opthamalic Surgical "sponges" (Windsor Biomedical, Newton, NH) in the oral cavity or on surfaces in the vaginal posterior fornix for 5 min. To collect endocervical secretion, the tip of the arrowhead-shaped sponge was held on the cervical os for 5 min. Rectal secretions were collected as described in detail (34) by delivering a sponge 6 cm into the rectum using a tampon applicator-based technique. After absorption, each sponge was placed in a 1.5-ml microcentrifuge tube on ice. The volume of secretion absorbed into each sponge was determined by weighing the microcentrifuge tube plus sponge before and after placement on mucosal surfaces (7). After postweighing, the plastic handle attached to each sponge was clipped off with toenail clippers so the lid of the microcentrifuge tube could be closed. Specimens were then stored at −80°C.

Extraction of secretions from sponges

Secretions were eluted as described previously (34) after placing each sponge in the top compartment of a spin assembly and adding 50 μl PBS containing 0.5% Igepal detergent (Sigma-Aldrich, St. Louis, MO) and the following protease inhibitors: leupeptin, aprotinin (both Sigma-Aldrich), 4-(2-aminoethyl)benzenesulfonyl fluoride, and bestatin (both Calbiochem, La Jolla, CA) at previously described concentrations (7). After a 15-min soaking period, the sponges were centrifuged at 4°C and 20,000 × *g* for 10 min. The sponges were subsequently soaked in PBS with protease inhibitors and 0.25% BSA (Sigma-Aldrich) for 15 min. BSA, rather than Igepal, was used in the second buffer simply because the presence of 0.5% Igepal in secretions interfered with LPS Ab assays. For sponges containing rectal or cervical secretion, 250 μl of the BSA-supplemented buffer was added. Each of the paired salivary and vaginal sponges received 100 μl. Sponges were then centrifuged at 4°C and 20,000 × *g* for 30 min. Secretions eluted into the lower compartment of spin assemblies were placed on ice and combined if paired specimens. Dilution factors introduced into secretions during the elution process were calculated as: (300 μl + microliters secretion)/(microliters secretion). The amount of blood in secretions was determined (34) by measuring hemaglobin with ChemStrips 4 (Boehringer-Mannheim, Indianapolis, IN).

ELISA for total Ig, anti-CTB, and anti-cholera LPS Ab

Concentrations of total IgA1, IgA2, IgG, and IgM in sera and secretions were measured by ELISA as described (34) using the serum Human Immunoglobulin Calibrator (Binding Site, San Diego, CA) as a reference standard. Concentrations of CTB-specific IgA and IgG Ab were measured in a GM-1 ganglioside-based ELISA using previously characterized serum anti-CTB IgA and IgG Ab standards and biotinylated secondary Ab specific for either human IgA or IgG (7, 34). Anti-CTB IgA or IgG Ab concentration was divided by the total IgA (IgA1 + IgA2) or IgG concentration in each serum or secretion to obtain the CTB-specific IgA or IgG activity.

To measure LPS-specific IgA1, IgA2, IgG, and IgM Ab, Immulon I microtiter plates (Dynatech, Chantilly, VA) were coated overnight with 5 μg/ml Vibrio cholerae Clasical Inaba 569B LPS (Sigma-Aldrich) in PBS, pH 7.2. Plates were blocked with 0.5% FBS in PBS for 30 min at room temperature, loaded with 100 μl of 2-fold serial dilutions of samples or standard, and stored overnight at 4°C. Sera obtained from an N_{imm} woman who developed circulating anti-LPS IgA1, IgA2, IgG, and IgM Ab was pooled, arbitrarily assigned units per milliliter of each Ab type, and used as a reference preparation in these assays. Plates were washed and reacted for 1.5 h at room temperature with one of the following biotinylated Ab at the concentrations previously described (34): mAb to human IgA1 or IgA2 (Southern Biotechnology Associates, Birmingham, AL) or goat polyclonal Ab to human IgG or IgM (BioSource International, Camarillo, CA). Plates were developed with avidin-labeled peroxidase and ABTS (Sigma-Aldrich) as described (34). Units per milliliter of anti-LPS Ab in specimens were interpolated from 4-parameter standard curves constructed with SoftMax Pro computer program (Molecular Devices, Sunnyvale, CA).

To measure endpoint titers of CTB-specific Ab having attached secretory component (SC), plates were developed with an anti-human SC mAb (Sigma-Aldrich) that was purified from ascites in the laboratory using protein G-Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ) and biotinylated with EZ link Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL). Endpoint titers were defined as the last reciprocal dilution of sample that produced an absorbance more than or equal to arithmetic mean plus three SD of eight blank wells reacted with sample buffer, but otherwise treated identically to wells reacted with sample or standard. CTB-specific Ig-SC activity was calculated by dividing the reciprocal endpoint titer by the summed total IgA1, IgA2, and IgM in each sample.

Four different samples of saliva and serum collected from five nonimmunized female volunteers were used to establish the normal background variation in these assays. Using these samples, the (mean specific activity + 3 SD)/mean specific activity measured in each CTB-specific, LPS-specific, and total Ig ELISA was first calculated for each subject, then averaged as a group, which produced values ranging from 1.75–1.95. Thus, for simplicity, in vaccinated subjects the fold increases (postimmune/preimmune) in specific activity were considered significant if they were ≥2-fold. However, immunized subjects were not considered significant responders unless 2-fold increases in specific activity were noted at two consecutive monthly time points.

Statistics

The Statview 5 computer program (Abacus Concepts, Berkeley, CA) was used for calculations and statistical comparisons at the 95% confidence level. Data was logarithmically transformed to obtain geometric means; these were used to perform within group comparisons by two-tailed paired *t* test or between group comparisons by ANOVA using Fisher's protected least significant difference, or for correlation analyses by Spearman rank. Results of statistical analyses were not considered significant unless *p* values ≤0.05 were obtained.

Results

Ig concentrations in sera and undiluted secretions

In women with normal menstrual cycles, it has been shown that concentrations of total IgA and IgG in cervical secretions decrease dramatically at ovulation, but then rebound during the midluteal phase so that they are equivalent to levels present during the midfollicular phase (35). In contrast, Ig concentrations in sera, saliva, or rectal secretions of women or nonhuman primates are not dramatically influenced by the menstrual cycle (36, 37). These findings were confirmed in this study by comparing the IgA1, IgA2, IgG, and IgM concentrations measured by ELISA in sera and undiluted secretions collected from all women during either the midfollicular or midluteal menstrual cycle phases (all comparisons $p > 0.05$ by ANOVA). Secretion volumes and levels of blood contamination also did not differ significantly between specimens collected during these two phases or among specimens collected from women segregated according to immunization group. Therefore, geometric means were calculated using all specimens collected at monthly intervals and are presented in Table II. Note that the total IgA in secretions was determined by multiplying original concentrations determined in ELISA by 2.5 to adjust for the underestimation of dimeric IgA that occurs in assays that use a monomeric serum IgA standard (38). This adjustment in total IgA was not made when calculating CTB- or LPS-specific IgA activity (IgA Ab concentration/total IgA concentration) in secretions because serum standards were used for measurement of both total IgA and CTB- and LPS-specific IgA Ab.

N_{imm} with 10-fold less CTB induces greater systemic Ab responses to CTB than V_{imm} or R_{imm}

In all immunization groups, CTB-specific IgG activity in serum of the majority of women were significantly increased 1 mo after the second vaccination (Fig. 1). At this time, the CTB-specific IgG activity in sera did not differ significantly among groups. However, a third N_{imm} considerably boosted CTB-specific IgG in serum, whereas a third V_{imm} or R_{imm} did not (Fig. 1*B*). Thus, at 1 and 2 mo after the third immunization, significantly greater CTB-specific IgG activity was present in the sera of women who had received N_{imm}, even though they received 10-fold less cholera vaccine (all $p < 0.05$ by ANOVA). Considering the mean 9640 μg/ml total IgG (Table II) and percentage of CTB-specific IgG activity in sera

Table II. *Total Ig and blood in undiluted secretions collected with Weck-Cel sponges*[a]

Specimen	Secretion Volume[b]	Blood Contaminant (%)[c]	Ig Concentration (μg/ml)			IgA1:IgA2 Ratio
			IgG	IgM	IgA	
Serum	–[d]	–	9641.2	1933.1	1491.2	79:21
Whole saliva	186.5	<0.001	30.6	19.2	381.8	70:30
Endocervical secretion	56.2	0.008	1210.2	22.5	1267.0	59:41
Vaginal secretion	49.1	<0.001	615.9	0.3	423.0	55:45
Rectal secretion	35.7	0.086	301.4	29.6	3045.7	38:62

[a] Shown are geometric means.
[b] Microliters per sponge.
[c] The number of lysed RBC which would release the amount of Hb measured in the secretion was multiplied by the dilution factor introduced during processing, then compared to the number of RBC present in normal female blood (4,800,000/μl).
[d] Not applicable.

(Fig. 1*B*), it is clear that anti-CTB IgG Ab concentrations in sera of N_{imm} women were very high, ranging from 190–1200 μg/ml, after the third immunization.

Serum IgA Ab responses to CTB were highly correlated with CTB-specific IgG Ab responses in women who received N_{imm} or V_{imm} ($p < 0.0001$). However, R_{imm} generated CTB-specific IgA in sera of only 2 of 5 subjects, despite the induction of CTB-specific IgG Ab in all (Fig. 1). The lack of a serum IgA Ab response to CTB after R_{imm} was also noted after biweekly R_{imm} with cholera vaccine (7, 8). Other findings were also consistent with our previous biweekly cholera vaccination study (7). For example, V_{imm} produced very similar percentages of CTB-specific IgA and IgG activity in serum (Fig. 1). In contrast, N_{imm}, R_{imm}, and O_{imm} typically induced greater percentages of CTB-specific IgG than IgA in serum (Fig. 1; Ref. 7).

No significant anti-LPS IgA1, IgA2, IgG, or IgM Ab responses were detected in postimmunization sera of most study subjects (data not shown). One V-LP_{imm} woman demonstrated 2- to 4-fold increases in LPS-specific IgA1 after the second and third immunization, while one of five R_{imm} and one to six N_{imm} women developed 2- to 3-fold increases of LPS-specific IgM in serum after the third immunization. One N_{imm} volunteer, from whom only sera was collected and pooled for use as a standard in LPS ELISAs, did consistently exhibit brief increases of LPS-specific IgA1, IgA2, IgM, and IgG 7–10 days after each immunization. Therefore, we cannot rule out the possibility that transient LPS Ab responses may have been induced in serum of other participants.

N_{imm}, V-FP_{imm}, and V-LP_{imm} induce comparable CTB-specific IgA and IgG activity in cervical and vaginal secretions

N_{imm}, V-FP_{imm}, and V-LP_{imm} induced similar proportions of CTB-specific IgA activity in cervical secretions collected after the second and third immunizations (Fig. 2*A*). When examined at monthly intervals, four of five V-FP_{imm} and three of four V-LP_{imm} responders demonstrated peak cervical IgA Ab responses 1 mo after the third immunization. However, extra cervical secretions collected 2 wk after the third immunization from three responding women (a V-FP_{imm} woman during the luteal phase and two V-LP_{imm} women during the follicular phase) contained greater CTB-specific IgA (and IgG) activity than secretions collected 1 mo after the third V_{imm} (Fig. 2*A*). This is consistent with previous studies in which peak cervical CTB-specific IgA Ab responses were most often observed 2 wk after a third biweekly V_{imm} (8). Thus, we consider it likely that the majority of the responding V-FP_{imm} and V-LP_{imm} women in this study would have demonstrated peak genital tract IgA Ab responses 2 wk after the third immunization, regardless of the menstrual cycle phase during which vaccination or sample collection was performed.

In contrast to V_{imm} women, the majority (3 of 5) of responding N_{imm} women exhibited peak cervical CTB-specific IgA Ab responses 2 mo after the third immunization. This was the last time point in the study. Therefore, we cannot exclude the possibility that levels of CTB-specific IgA may have continued to increase in cervical secretions of N_{imm} women.

R_{imm} at monthly intervals, as with biweekly intervals (7), failed to induce CTB-specific IgA in cervical secretions (Fig. 2*A*). Most R_{imm} women did demonstrate significant CTB-specific IgG activity in cervical secretions (Fig. 2*B*). However, these anti-CTB IgG Ab may have been derived from serum since cervical CTB-specific IgG activity did not exceed that in serum and the CTB-specific IgG activity in sera and cervical secretions of R_{imm} women was highly correlated ($p = 0.0008$).

N_{imm}, V-FP_{imm}, and V-LP_{imm} also induced comparable amounts of CTB-specific IgG activity in cervical secretions (Fig. 2*B*). In all N_{imm} women, the CTB-specific IgG activity in cervical secretions was highly correlated with that in serum ($p < 0.0001$), and was not significantly greater than that in serum. Cervical CTB-specific IgG activity in V-FP_{imm} and V-LP_{imm} women also highly correlated with that in serum ($p = 0.0002$ and 0.0003, respectively). However, V-FP_{imm} and V-LP_{imm} induced 5.5- and 5.1-fold greater CTB-specific IgG activity in cervical secretions than in serum. This indicates that V-FP_{imm} and V-LP_{imm} both produced local IgG Ab responses to CTB. Nevertheless, it is important to note that N_{imm} with 10-fold less CTB was able to produce proportions of CTB-specific IgA and IgG in cervical secretions comparable to those seen after local V-FP_{imm} or V-LP_{imm}.

In N_{imm}, V-FP_{imm}, and V-LP_{imm} women, the CTB-specific IgA and IgG activity measured in vaginal secretions (data not shown) was highly correlated with that in cervical secretions ($p < 0.0001$), and although proportions were typically 50% (for IgG) and 40% (for IgA) lower than those in cervical secretions, the same women found to have significant cervical CTB-specific IgA or IgG also demonstrated significant vaginal CTB-specific IgA or IgG. One exception was a V-LP_{imm} woman with significant cervical-specific IgA but insignificant vaginal-specific IgA due to 68% lower proportions of CTB-specific IgA activity in vaginal secretions. Thus, only three of five V-LP_{imm} women had significant CTB-specific IgA activity in vaginal secretions.

N_{imm}, V-FP_{imm}, and V-LP_{imm} induce comparable levels of CTB-specific S-IgA Ab in cervical secretions

Because N_{imm} induced very high proportions of CTB-specific IgA in sera (Fig. 1*A*), we considered the possibility that the CTB-specific IgA measured in cervical secretions of N_{imm} women may have originated from serum transudate rather than receptor-mediated transport in the genital tract. The finding that CTB-specific

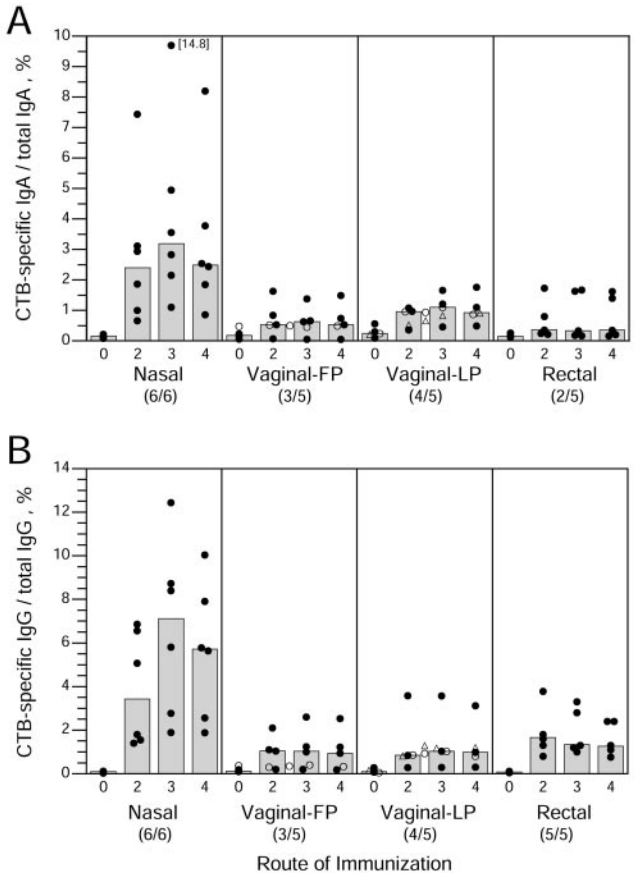

FIGURE 1. Systemic IgA and IgG Ab responses to CTB. Shown are the CTB-specific *A*, IgA and *B*, IgG activity measured by ELISA in serum of each N_{imm}, V-FP_{imm}, V-LP_{imm}, and R_{imm} woman before the first vaccination (time 0), 1 mo after the second vaccination (time 2), and 1 and 2 mo after the third vaccination (times 3 and 4, respectively). Columns denote medians. Open triangles and circles denote the V-FP_{imm} and two V-LP_{imm} women from whom extra samples were collected 2 wk after the third immunization (between times 3 and 4). A third V-LP_{imm} woman sampled at this extra time point is designated by a closed circle, because she completely failed to respond to vaccination. Symbols with adjacent specific activity in brackets represent individuals with values outside the range of the *y*-axis. For each immunization group, the frequency of women with significant specific activity at a minimum of two postimmunization time points is indicated in parenthesis.

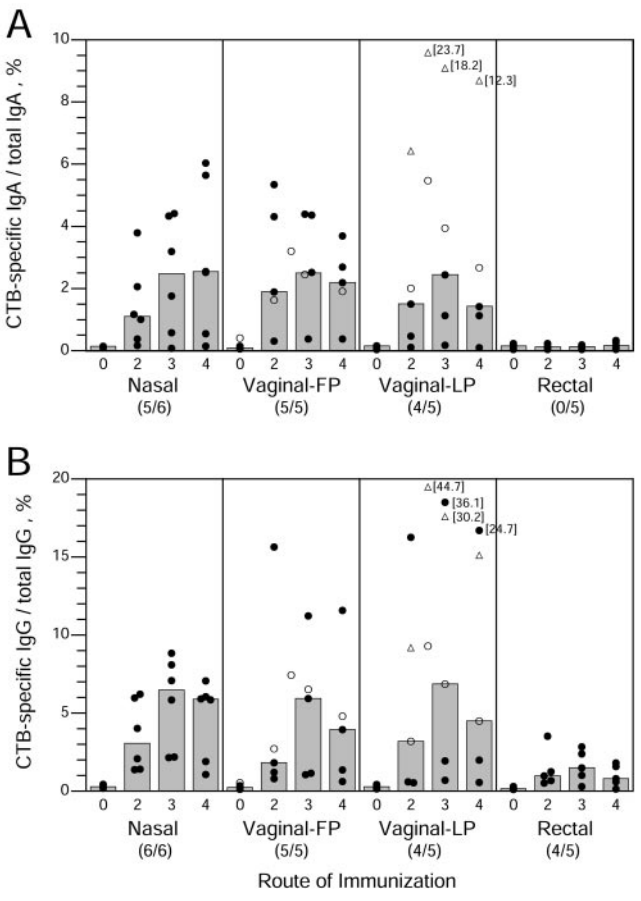

FIGURE 2. CTB-specific Ab in cervical secretions. Shown are the CTB-specific *A*, IgA and *B*, IgG activity in cervical secretions collected before and after vaccination. See Fig. 1 legend for details.

IgA activity in cervical and vaginal secretions of N_{imm} (and V_{imm}) women were not correlated with the CTB-specific IgA activity in sera (all $p > 0.05$) indicated that serum transdate was not the major source of the IgA. If the CTB-specific IgA measured in cervical secretions of N_{imm} women was specifically transported, then it should have attached SC, whereas monomeric serum IgA would not. Therefore, we compared the postimmunization increases in cervical CTB-specific IgA activity to those in cervical CTB-specific SC-associated Ab activity. As shown in Fig. 3, 85 and 86% of the CTB-specific IgA activity in cervical secretions of responding V-FP_{imm} and V-LP_{imm} women was represented by increases in CTB-specific Ig-SC activity. In N_{imm} women, a similar 81% of the CTB-specific IgA activity in cervical secretions was paralleled by increases in CTB-specific Ig-SC activity (Fig. 3). Since CTB-specific IgM was not detected in cervical secretions of study participants (data not shown), it can be concluded that the SC-attached Ab to CTB was S-IgA. Thus, in the reproductive tract of N_{imm} and V_{imm} women, comparable amounts of polymeric CTB-specific IgA Ab were specifically transported into secretions.

V-FP_{imm}, but not V-LP_{imm} or N_{imm}, induces LPS-specific IgA Ab responses in the genital tract

Since CTB is extremely immunogenic, it could be argued that other weaker Ag might produce different results. Thus, we analyzed cervical IgA Ab responses to LPS, a less immunogenic component in the cholera vaccine. As shown in Table III, N_{imm} and V-LP_{imm} were not as effective as V-FP_{imm} for induction of LPS-specific IgA Ab in cervical secretions. Significant LPS-specific Ab responses, primarily of the IgA2 subclass, were generated in the genital tract of 5 of 5 V-FP_{imm} women, but in only one of five V-LP_{imm} and two of six N_{imm} women. With the exception of N_{imm} woman no. 6 and V-FP_{imm} woman no. 8, no LPS-specific IgG or IgM Ab were detected in cervical secretions (Table III). Extra cervical secretions collected 2 wk after the third vaccination from CTB-responsive V-LP_{imm} woman no. 16 (Table III) during the midfollicular phase did not contain any LPS Ab. In addition, cervical secretions were collected during the midfollicular phase from the majority (four of six) of N_{imm} women, yet only one of four demonstrated LPS IgA Ab (Table III). This suggests that the lack of LPS Ab in V-LP_{imm} and N_{imm} women was not related to the phase of the menstrual cycle when secretions were collected. Thus, V_{imm} with Ag less immunogenic than CTB may be more effective for induction of genital tract Ab responses if performed during the midfollicular phase of the menstrual cycle. In addition, these data suggest that N_{imm} with lower doses of Ag may not be able to produce distal genital tract Ab responses of the magnitude induced by local V-FP_{imm}.

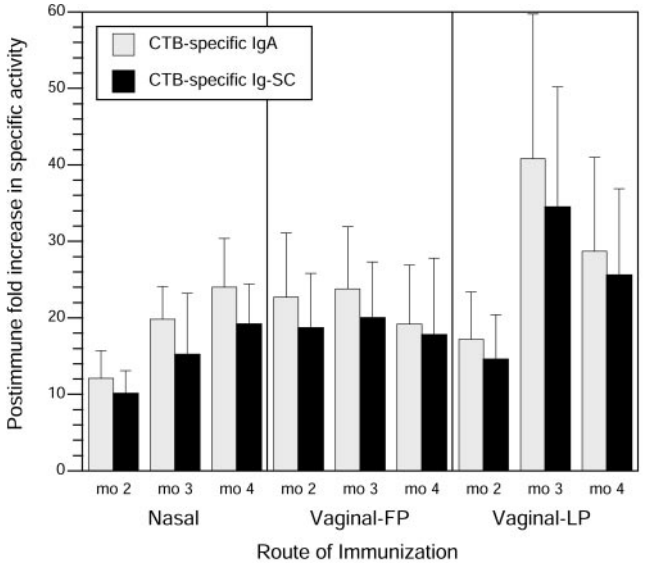

FIGURE 3. Anti-CTB IgA Ab in cervical secretions of N_{imm} and V_{imm} women is associated with SC. Shown are the arithmetic mean fold increases in CTB-specific IgA and Ig-SC activity measured in cervical secretions of the five of six N_{imm}, five of five V-FP_{imm}, and four of five V-LP_{imm} women demonstrating significant cervical CTB-specific IgA activity after vaccination. Fold increases were calculated by dividing the specific activity measured 1 mo after the second vaccination (time 2), 1 mo after the third vaccination (time 3), and 2 mo after the third vaccination (time 4) by that measured in the corresponding cervical secretion collected before the first vaccination. Error bars represent SEM.

N_{imm} with a low dose of cholera vaccine does not induce distal rectal CTB- or LPS-specific Ab responses comparable to those induced by local R_{imm}

CTB-specific IgA Ab in rectal secretions were induced in three of five V-FP_{imm}, but zero of five V-LP_{imm} women (Fig. 4, *A*). N_{imm} induced significantly greater CTB-specific IgG activity in rectal secretions than V-FP_{imm} ($p < 0.006$), and more frequently induced CTB-specific IgA Ab (Fig. 4*A*). The responses detected were of local mucosal origin, since CTB-specific IgA and specific Ig-SC activity were correlated ($p < 0.001$) and increases in CTB-specific Ig-SC represented 87% of the 6- to 11 (median peak)-fold increases in rectal CTB-specific IgA in V-FP_{imm} and N_{imm} women (data not shown). However, R_{imm} induced the greatest CTB-specific IgA activity (Fig. 4*A*) in rectal secretions at all postimmunization time points ($p < 0.04$). R_{imm} also induced the largest postimmunization fold increases in rectal CTB-specific Ig-SC activity (median peak 48-fold), which typically represented 89% of the CTB-specific IgA activity (data not shown).

The CTB-specific IgG activity in rectal secretions of R_{imm} women was also equal to or greater than that in N_{imm} women, although significant differences could only be demonstrated 1 mo after the second immunization ($p < 0.035$). However, the CTB-specific IgG activity in rectal secretions of N_{imm} women was only 42% of that measured in sera, whereas four of five R_{imm} women had, on average, 7.1-fold greater CTB-specific IgG activity in rectal secretions than in sera. Thus, local IgG Ab responses to CTB were induced in the majority of R_{imm} women.

N_{imm} with the lower dose of cholera vaccine was also less effective than local R_{imm} for induction of LPS Ab in rectal secretions. As shown in Table IV, R_{imm} consistently induced LPS-specific IgA1, IgA2, IgG, and surprisingly large amounts of LPS-specific IgM in rectal secretions. N_{imm} failed to induce LPS-specific IgG or IgM in rectal secretions, and only one of six and three of six N_{imm} women developed anti-LPS IgA1 and IgA2 Ab responses, respectively, which were of considerably lower magnitude than those generated by R_{imm} (Table IV). These data suggest that in humans, low-dose vaccination in the nasal cavity will not generate rectal Ab responses analogous to those induced by local R_{imm}. It is also noteworthy that administration of cholera vaccine by the rectal route induced both strong anti-LPS IgA1 and IgA2 Ab responses in the rectum. This is in contrast to observations that LPS Ags tend to induce IgA2-restricted specific IgA Abs in saliva, colostrum (39), and, as shown above, in cervical secretions of humans.

Table III. *Cholera LPS-specific Ab in endocervical secretions*[a]

Subject	Immunization Group	Peak Fold Increase in Specific Activity: LPS IgA1	LPS IgA2	LPS IgM	LPS IgG
1	N-LP	—[b]	—	—	—
2	N-FP	—	—	—	—
3	N-FP	—	2.8	—	—
4	N-FP	—	—	—	—
5	N-FP	—	—	—	—
6	N-LP	2.6	2.1	4.8	—
7	V-FP	—	4.0	—	—
8	V-FP	—	4.3	2.2	2.6
9	V-FP	—	13.1	—	—
10	V-FP	2.3	6.4	—	—
11	V-FP	—	2.7	—	—
12	V-LP	—	—	—	—
13	V-LP	—	—	—	—
14	V-LP	3.6	25.8	—	—
15	V-LP	—	—	—	—
16	V-LP	—	—	—	—

[a] Numbers denote significant LPS-specific activity in secretions collected at monthly intervals after the second immunization and during the same phase of the menstrual cycle that the three immunizations were performed.
[b] No detectable Ab or insignificant (less than 2-fold) LPS-specific acivity.

N_{imm}, V_{imm}, or R_{imm} with cholera vaccine is ineffective for induction of salivary Ab

N_{imm} failed to induce LPS- and CTB-specific IgA in whole saliva (data not shown), and no CTB-specific IgG was detected despite the high concentrations of CTB-specific IgG in serum (Fig. 1*B*). Significant increases in CTB-specific IgA and IgG activity were found in saliva of only two of five V-FP_{imm}, one of five V-LP_{imm}, and two of five R_{imm} women, but the magnitude of these responses (2- to 6-fold) and the CTB-specific IgA and IgG activity (all $<0.7\%$) were unimpressive. The only study subjects who developed LPS IgM, IgA1, and IgA2 Ab in saliva (2- to 7-fold increases) were the two CTB Ab-responsive R_{imm} women (data not shown).

Although the results for saliva were inconclusive, we generally observed more frequent local and distal mucosal anti-CTB IgA Ab responses in V-FP_{imm} women than in V-LP_{imm} women (Table V). This, in addition to the LPS-specific IgA2 Ab detected in cervical secretions of V-FP_{imm} but not V-LP_{imm} women, suggests that uptake of Ag and/or inductive capacity may be more efficient in the female genital tract during the follicular phase of the menstrual cycle.

Discussion

Other investigators have previously reported that N_{imm} of humans with rCTB or the WC/rBS cholera vaccine used in this study can induce CTB-specific IgA in nasal secretions, bronchoaveolar fluids, female genital tract secretions, and urine of men (9, 26, 27).

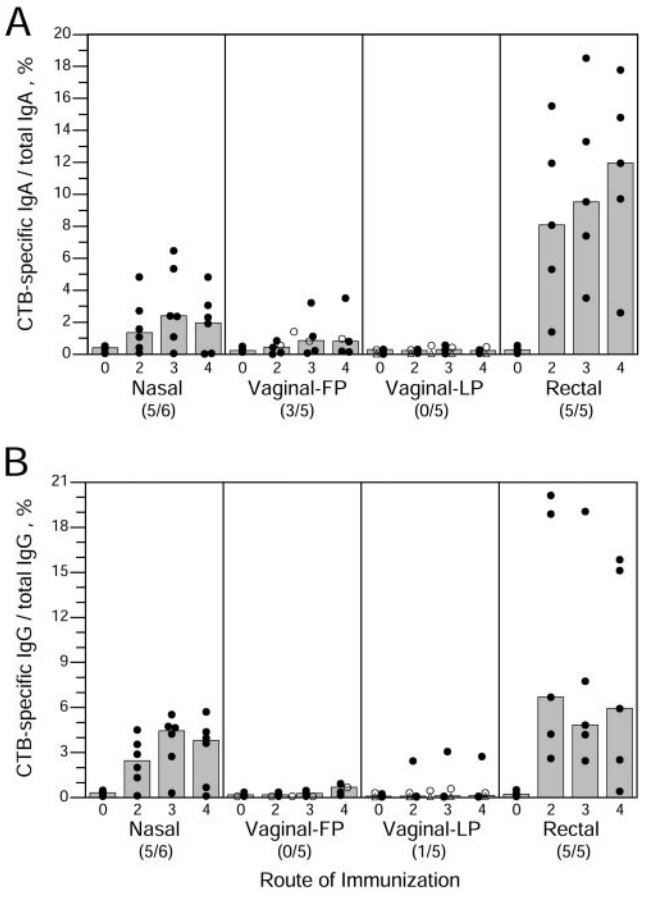

FIGURE 4. Induction of CTB Ab in rectal secretions. Shown are the CTB-specific IgA (*A*) and IgG (*B*) activity measured in rectal secretions before and after vaccination. See Fig. 1 legend for details.

Nasal administration of live attenuated influenza vaccine in humans has also produced specific IgA Ab in saliva (28). We now have extended these findings to show that N_{imm} can also induce distal Ag-specific IgA in rectal secretions of humans. We provide evidence that after N_{imm}, the majority of the CTB-specific IgA in both rectal and cervical secretions was S-IgA; and therefore, entered secretions through local polymeric IgR-mediated transepithelial transport (21). Finally, we showed that the phase of the menstrual cycle at which V_{imm} is done may be important for some Ag, but not for others. Cervical S-IgA responses to CTB were comparable after V_{imm} during either the mid-folicular or -luteal phases, whereas only midfollicular phase V_{imm} induced consistent responses to LPS and distal rectal CTB-specific IgA responses.

Table IV. *Cholera LPS-specific Ab in rectal secretions*[a]

Subject	Immunization Group	Peak Fold Increase in Specific Activity: LPS IgA1	LPS IgA2	LPS IgM	LPS IgG
1	N	—[b]	—	—	—
2	N	—	—	—	—
3	N	2.8	2.6	—	—
4	N	—	3.7	—	—
5	N	—	3.6	—	—
6	N	—	—	—	—
17	R	6.5	37.6	115.3	8.8
18	R	6.0	12.0	165.4	4.6
19	R	3.4	6.5	68.2	2.5
20	R	4.9	4.8	26.0	2.8
21	R	4.8	9.2	4.8	3.4

[a] Numbers denote significant LPS-specific activity in secretions collected at monthly intervals after the second immunization.
[b] No detectable Ab or insignificant (less than 2-fold) LPS-specific activity at any time after immunization.

Table V. *Frequency of cholera vaccine-specific mucosal IgA Ab responses after vaginal immunization during the midfollicular or midluteal menstrual cycle phase*

Secretion	Specific Ab	Number of Women With Significant Ab: $V\text{-}FP_{imm}$ ($n = 5$)	$V\text{-}LP_{imm}$ ($n = 5$)
Endocervical	Anti-CTB IgA	5	4
Endocervical	Anti-LPS IgA2	5	1
Vaginal	Anti-CTB IgA	5	3
Rectal	Anti-CTB IgA	3	0
Salivary	Anti-CTB IgA	2	1

Generating durable protection against sexual transmission of HIV will likely require that vaccination establishes antiviral Th cells, CTL, Ab-secreting plasma cells, and memory lymphocytes in the genital tract and rectal mucosa as well as in the systemic compartment. A growing body of evidence indicates that optimal induction of cellular and humoral immunity in the female genital tract and rectum of humans and nonhuman primates requires local mucosal immunization (2–4, 7, 29, 30). However, the N_{imm} route may be an effective alternative that is more convenient and acceptable. Since N_{imm} with cholera vaccine induced very high concentrations of CTB-specific IgA (as well as IgG) in serum, and serum transudate contributes more Ab to genital tract secretions than other mucosal secretions (21), it could be argued that the CTB-specific IgA we detected in cervical and vaginal secretions of N_{imm} women was derived from transudated serum. However, the vast majority of IgA in serum is monomeric (40) and could not associate with SC, which was detected on roughly 80% of the CTB-specific Ab in cervical secretions of N_{imm} women. In addition, proportions of CTB-specific IgA in sera of N_{imm} women did not correlate with proportions of specific IgA in cervical and vaginal secretions. This latter observation further suggests that insignificant amounts of dimeric CTB-specific IgA in serum were transported into genital tract secretions by the polymeric IgR on endometrial and endocervical epithelium (21). Thus, the majority of the CTB-specific S-IgA detected in cervical and vaginal secretions of N_{imm} women was most likely synthesized by plasma cells residing in the genital tract.

It is striking that N_{imm} with 0.1 mg rCTB generated cervical and vaginal CTB-specific IgA Ab responses comparable to those induced by V_{imm} with a 10-fold greater dose. This might be explained by the recent discovery that rCTB has adjuvant activity in the nasal cavity of mice, though not in the intestine or female genital tract (41, 42). It is not known whether this is true in humans or nonhuman primates. Rhesus macaques immunized by the nasal route with CTB and *Streptococcus mutans* Ag I/II have been shown to develop specific IgA responses to this protein in the female genital tract (43). However, nasal adjuvanticity of CTB was not clearly established in this study because AgI/II by itself was not administered in other monkeys. The fact that N_{imm} in our study subjects did not produce rectal CTB-specific IgA Ab responses comparable to those induced by R_{imm} argues against a strong adjuvant effect. In addition, the presence of rCTB in the vaccine did not seem to enhance the frequency or magnitude of LPS-specific

Ab responses in the genital tract and rectum, where 100% of V-FP_{imm} and R_{imm} women demonstrated much greater LPS Ab responses than those seen after N_{imm}. Determining with certainty whether rCTB has adjuvant activity in the human nasal cavity will require comparative studies designed specifically to address this issue by nasally administering peptide or protein-based vaccines with or without rCTB.

Although N_{imm} with low-dose cholera vaccine induced rectal CTB-specific Ab, it did not produce the high magnitude CTB- and LPS-specific rectal Ab responses generated by R_{imm}. Others have shown that N_{imm} with 1 mg rCTB induced 3- to 5-fold greater CTB-specific IgG and IgA in serum and vaginal secretions than N_{imm} with 0.1 mg rCTB (27). If N_{imm} with 1 mg rCTB also generated 3- to 5-fold more CTB-specific IgA in rectal secretions, this would closely approximate the amounts induced by local R_{imm} with this dose. Unfortunately, we could not compare N_{imm} to R_{imm} using 1 mg rCTB, because this dose produces adverse local reactions in the nasal cavity of humans (27). Thus, the possibility remains that a high dose of a safe, nontoxic vaccine given via the nasal route could be as effective as the same dose given rectally.

An unexpected finding was that N_{imm} failed to induce CTB-specific IgA in saliva. High proportions of influenza-specific IgA Ab have been generated in saliva after N_{imm} of humans with live attenuated flu vaccine (28). N_{imm} may be more effective for induction of salivary Ab in previously primed individuals or by administering replication-competent vaccines. A lack of durable salivary IgA Ab responses to CTB was also observed previously in women who received biweekly R_{imm} or V_{imm} with cholera vaccine (8). In that study, the majority of R_{imm} or V_{imm} women demonstrated peak levels of CTB-specific IgA in saliva collected 2 wk after the second or third vaccination. However, these Ab had dropped to insignificant levels 1 mo after the third immunization (8), which is consistent with the lack of CTB-specific IgA in saliva collected in the current study at monthly intervals.

This is the first human study that examines the outcome of V_{imm} in relation to the phase of the menstrual cycle during which vaccine is administered. If CTB Ab responses alone had been assessed, we might have erroneously concluded that V_{imm} during the midfollicular and midluteal phases were equally effective for induction of local genital tract Ab responses. However, CTB is an atypical Ag in several respects. It is a very strong immunogen when administered mucosally, in part because its binding and uptake at mucosal surfaces is very efficient. CTB binds with extremely high affinity to GM-1 ganglioside, a ubiquitous component of plasma membranes of epithelial cells, APC, and T lymphocytes (44). Since most other Ag are not as immunogenic as CTB, it was important to compare the responses to another Ag after V_{imm} at different stages of the menstrual cycle. Clear differences in the outcome of V-FP_{imm} vs V-LP_{imm} (and N_{imm}) with cholera vaccine were revealed by analysis of mucosal anti-LPS Ab. The finding that only V-FP_{imm} could consistently induce LPS-specific (IgA2-restricted) IgA Ab in cervical secretions suggests that the environment in the female genital tract may be more favorable for uptake of Ag or induction of immune responses during the midfollicular phase.

It is not clear whether the lack of LPS-specific IgA2 Ab responses in V-LP_{imm} women was due to reduced uptake of free LPS and/or LPS-expressing cholera vibrios, or to reduced immune induction during the luteal phase of the menstrual cycle. There is very little information available regarding the mechanisms of Ag uptake and induction of immune responses in the human reproductive tract at any time of the menstrual cycle. Based in part on the low numbers of B and T cells and the lack of characteristic mucosal inductive site follicle-associated epithelium, M cells, and organized lymphoid follicles (45, 46) in the murine reproductive tract, it has been proposed that induction of immune responses to vaginally administered Ag may be initiated by migratory Ag-presenting DC in draining lymph nodes, some of which are shared with the rectum (20–22). The induction of rectal CTB-specific IgA after V-FP_{imm}, but not V-LP_{imm}, might reflect activation in these lymph nodes of more B cells destined to home to the rectal mucosa. However, in women and macaques, greater numbers of lymphocytes are present in the reproductive tract, and small lymphoid aggregates consisting of a B cell core surrounded by either $CD8^+$ or $CD4^+$ T cells and macrophages have been detected in the vagina, cervix, and endometrium during both the midfollicular and -luteal menstrual cycle phases (22–24). Although their function remains unclear, the presence of these aggregates suggests that induction of immune responses may occur locally, to some extent, in the reproductive tract of humans and nonhuman primates.

In the reproductive tract of women, the endocervix has been shown to contain most concentrated numbers of DC, macrophages, and $CD4^+$ T cells (20), which may favor Ag presentation and immune induction at this site in particular. If this is true, the lack of cervical LPS Ab responses in V-LP_{imm} women might reflect reduced uptake of cholera vibrios in the endocervix due to blockade of the cervical canal by the mucus plug that forms in the cervical os after ovulation (33). The differential ability to induce LPS-specific Ab in V-FP_{imm} and V-LP_{imm} women could also be related to distributional changes in Th1- vs Th2-type cytokine-secreting cells during the menstrual cycle. It has been proposed that immune responses in women shift toward the Th2-type during the luteal phase, since more Th2 cytokine-secreting cells have been detected in the circulation at this time of the menstrual cycle (47). In addition, Th1 cells have been found to predominate in the human endometrium during the follicular phase, but decrease during the luteal phase when the percentage of Th2 cells increase 3-fold (48). Because LPS has been found to drive Th1-type immune responses in animals (49), it is possible that reduced numbers of genital tract Th1 T cells during the luteal phase may be related to poor induction of LPS-specific Ab responses in V-LP_{imm} women. However, it is presently unclear how Th1 and Th2 cells may influence induction of reproductive tract immune responses in humans. There is a need to obtain a better understanding of immune induction mechanisms in both the upper and lower human reproductive tract.

We previously showed that O_{imm} with cholera vaccine was less effective than V_{imm} or R_{imm} for induction of specific IgA in the female genital tract and rectum, respectively (7). These findings have been supported by others using different vaccines in humans and nonhuman primates (29, 30, 37, 50). The results of the present study extend our understanding of mucosal sites for administration of nonreplicating vaccines in humans by showing that either N_{imm} or R_{imm} can be effective for induction of mucosal IgA Ab responses in the rectum. For induction of S-IgA and IgG Ab in the female genital tract, either N_{imm} or V_{imm} routes should be considered. However, if V_{imm} is selected for this purpose, it may be necessary to administer vaccine during the follicular phase of the menstrual cycle. This complicating factor could be avoided by using the N_{imm} route, which offers the additional advantage of potentially inducing high levels of specific IgG Ab in the circulation, and requiring lower Ag doses. Therefore, the N_{imm} route in particular deserves further testing with vaccines and potential adjuvants for prevention of STDs.

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