

## Comparison of the Oral, Rectal, and Vaginal Immunization Routes for Induction of Antibodies in Rectal and Genital Tract Secretions of Women

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**To determine which mucosal immunization routes may be optimal for induction of antibodies in the rectum and female genital tract, groups of women were immunized a total of three times either orally, rectally, or vaginally with a cholera vaccine containing killed *Vibrio cholerae* cells and the recombinant cholera toxin B (CTB) subunit. Systemic and mucosal antibody responses were assessed at 2-week intervals by quantitation of CTB-specific antibodies in serum and in secretions collected directly from mucosal surfaces of the oral cavity, rectum, cervix, and vagina with absorbent wicks. The three immunization routes increased levels of specific immunoglobulin G (IgG) in serum and specific IgA in saliva to similar extents. Rectal immunization was superior to other routes for inducing high levels of specific IgA and IgG in rectal secretions but was least effective for generating antibodies in female genital tract secretions. Only vaginal immunization significantly increased both specific IgA and specific IgG in both the cervix and the vagina. In addition, local production of CTB-specific IgG in the genital tract could be demonstrated only in vaginally immunized women. Vaginal immunization did not generate antibodies in the rectum, however. Thus, generation of optimal immune responses to sexually transmitted organisms in both the rectal and the genital mucosae of women may require local immunization at both of these sites.**

Secretory immunoglobulin A (S-IgA) antibodies play an important role as a first line of defense against microorganisms that infect via mucosal surfaces (15, 17, 26). Thus, an important goal in halting the spread of sexually transmitted diseases is the development of vaccines that induce local production and secretion of pathogen-specific, neutralizing S-IgA antibodies in both the genital tract and the rectum. Even the most promising vaccine formulations may fail to establish protective immunity, however, if the route of vaccine administration is not optimal for induction of local immune responses in these mucosal tissues.

Systemic immunization routes do not generally induce secretion of specific S-IgA or protective immunity in mucosal tissues (24, 27, 29). Mucosal immune responses are initiated by uptake of antigens from mucosal surfaces into organized lymphoid tissues located in the mucosa or in nearby lymph nodes, where antigen-specific B cells are generated (31). Primed B cells subsequently enter the circulation and migrate to local and distant mucosal tissues and glands where terminal differentiation occurs (25). The predominantly dimeric IgA antibodies produced by these cells are transcytosed into secretions via the polymeric Ig receptor expressed on the basolateral surfaces of epithelial and glandular cells (18).

This cellular migration explains the well-documented fact that administration of antigen in one mucosal region may generate S-IgA antibodies at distant mucosal sites (8, 26–28). For example, oral immunization allows antigen uptake at inductive sites of the oral cavity and upper intestine but can elicit antibodies not only in salivary and intestinal secretions but also in

mammary gland and vaginal secretions (1, 8, 37). The convenience of delivering vaccines orally, along with the relative ineffectiveness of vaginal immunization for producing immune responses in the female genital tract in animals (28), has raised interest in the potential use of oral immunization for generating protective immunity to sexually transmitted organisms.

On the other hand, there is evidence that local exposure to antigen can result in much higher levels of specific IgA in the region of exposure than at distant sites (6, 12, 32, 34). This has led to testing of rectal and vaginal immunization strategies for vaccines against sexually transmitted diseases (3, 6, 10, 21–23, 39). Numerous M cells and lymphoid aggregates are present in the rectal mucosa (20), and rectal immunization of mice and macaques has been shown to generate specific antibodies in local rectal secretions (6, 10, 22, 40). Rectal immunization can also lead to antibody secretion at distant sites via the common mucosal immune system. In humans, for example, rectal administration of the Ty21a typhoid vaccine induced appearance of typhoid-specific B cells in the circulation and secretion of specific IgA in saliva (5). Rectal immunization of mice with cholera toxin has also been shown to generate specific IgA in the vagina, whereas vaginal immunization did not (6), perhaps because of the lack of organized mucosal lymphoid tissue and antigen-transporting M cells in the female genital tract. This raised the possibility that the rectal immunization route could be used to establish protective immune responses in the female genital tract, in addition to the rectum. However, recent studies with rhesus macaques and humans suggest that the vaginal immunization route can be used effectively for inducing local immune responses in the female genital tract (3, 39). These contradictory observations imply that there may be important differences among species in the induction and dissemination of antibody-producing cells in mucosal tissues. Systematic studies are needed to determine which immunization route(s) will

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be most effective for induction of protective immunity in the genital tract or rectum in humans.

In the present study, the recombinant cholera toxin B subunit (rCTB) was used as a model mucosal immunogen to assess the oral, rectal, and vaginal immunization routes for their abilities to induce antibodies in the rectum and genital tract in women. CTB can be safely administered to humans in the form of the whole cell/recombinant B subunit (WC/rBS) cholera vaccine and is known to be a potent mucosal immunogen, capable of stimulating local and distal immune responses following oral administration in humans (8, 9). A preliminary study has shown that vaginal administration of WC/rBS in women can induce a local antibody response to rCTB, establishing the immunogenicity of this protein in the human female genital tract mucosa (39). To obtain information about actual concentrations of specific and total antibodies on mucosal surfaces, we adapted a recently developed absorbent wick technique (6, 11) to collect local secretions from the rectum, in addition to the endocervix and vagina. We show here that this method allows accurate determination of antibody or Ig concentrations at discrete areas of the genital mucosa.

#### MATERIALS AND METHODS

**Subjects.** Fourteen healthy women aged 19 to 45 years (mean, 32) with regular menstrual periods and no history of cholera vaccination or infection were chosen to participate in the study. The women were routinely monitored throughout the study to confirm the absence of gynecological abnormalities or infections. Written informed consent was obtained from each woman, and the study was approved by The Miriam Hospital Clinical Research Review Board (protocol TMH 93-006).

**Vaccine and immunizations.** The WC/rBS cholera vaccine was obtained from the United States Army Medical Research and Materiel Command (Frederick, Md.). The components of this vaccine have been described previously (13). Briefly, one dose of WC/rBS contains 1 mg of rCTB,  $10^{11}$  inactivated cholera vibrios, and 0.4 mg of thimerosal in 3 ml of phosphate-buffered saline (PBS). One dose of vaccine was administered on three separate occasions to each of five, five, and four women either orally, rectally, or vaginally, respectively. Each woman was immunized via the same route at roughly 14-day intervals. Some deviation from this interval occurred, as vaginal immunization could not be performed during menstruation.

For oral immunizations, WC/rBS was ingested after mixing with 150 ml of H<sub>2</sub>O and Samarin (14), a sodium bicarbonate-containing effervescent powder (Cederroth's Nordic AB, Upplands Väsby, Sweden). For rectal or vaginal immunization, women were placed in supine position with feet in stirrups. Vaccine was directly instilled into the rectum, 5 cm from the anus, through a plastic transfer pipette (104-mm fine tip; Sarstedt, Newton, N.C.) which had been fitted to the needle attachment of a 5-ml syringe after both the bulb and the opposing 2-cm fine tip were cut off. For vaginal immunizations, one dose of vaccine was mixed with 0.5 g of Eldexomer (generously provided by Jan Holmgren, University of Göteborg, Göteborg, Sweden), an inert powder that forms a gel emulsion when mixed with water (39). A speculum was inserted into the vagina and then slightly retracted before the emulsion was applied with a wood spatula to the posterior fornix of the vagina and the underside of the cervix. The speculum was then removed, allowing the cervical os to fall and contact the vaccine preparation. Women were turned on their sides and remained immobile for 3 min after vaginal or rectal immunization. Mucosal inflammation or bleeding was not observed following vaginal or rectal administration of WC/rBS.

**Sample collection.** Preimmune serum and secretions were obtained from study participants immediately before the first immunization (T0). Samples of serum and secretions were collected again 2 weeks after the second immunization (T2), 2 weeks after the third immunization (T3), and 4 weeks after the third immunization (T4). Some deviation from this interval was necessary as genital tract secretions could not be collected from women during menstruation. Serum was obtained by simple venipuncture. Secretions were captured from mucosal surfaces with cylindrical cellulose acetate wicks (2 by 25 mm) (Polyfiltronics Group Inc., Rockland, Mass.). Prior to sample collection, each wick was placed in a 1.5-ml Eppendorf tube, and the combined weight of the wick and tube was recorded. Secretions were then absorbed onto wicks as described below. After absorption, each wick was quickly returned to the appropriate Eppendorf tube, postweighed, placed on ice, and frozen at  $-70^{\circ}\text{C}$ . The weight of the captured secretion was calculated as the difference between the weights before and after absorption.

Saliva was obtained by placing a wick in the oral cavity between the cheek and maxillary gingiva in an effort to maximize the parotid gland contribution to these secretions. Rectal, cervical, and vaginal secretions were collected from women in supine position with feet in stirrups. After insertion of a vaginal speculum, wicks

were grasped with forceps, carefully placed in the posterior vaginal fornix, and inserted into the cervical os. After 5 min, wicks were collected with the forceps, and the speculum was removed. To collect rectal secretions, 12 to 15 cm of cotton thread was first sewn through one end of a preweighed wick. The plunger was removed from a 1-ml tuberculin syringe, and with the syringe held needle end down, the threaded wick was lowered into the needle attachment. While the thread was held taut, the plunger was reinserted to a point just above the wick. The needle attachment was then cleanly sliced off with a razor. Lubricating jelly was applied to the anus, and the syringe was inserted to 5 cm. The barrel of the syringe was then pulled back 2.5 cm while the plunger was held in place, exposing the wick which contacted the rectal wall. This procedure was used since simply pushing the wick out of the syringe had a tendency to induce bleeding, presumably because the dry wick caused abrasion of the mucosal surface. After 5 min, the barrel was held in place while the plunger and thread were pulled out, drawing the wick back into the syringe. The entire apparatus was then removed, the thread was cut from the wick, and the wick in its Eppendorf tube was postweighed.

**Elution of proteins from wicks.** To extract protein from the wicks, 200  $\mu\text{l}$  of PBS containing 1% bovine serum albumin (BSA) and protease inhibitors at concentrations previously specified (6) was added to each Eppendorf tube. After 30 min on ice with brief vortexing, wicks were cut in half and each fragment was transferred with buffer to the upper compartment of a cellulose acetate microspin filter unit (pore size, 0.45  $\mu\text{m}$ ) (Whatman LabSales, Fairfield, N.J.). Secretions were simultaneously eluted from wicks and clarified by centrifuging at  $16,000 \times g$  for 10 min at  $4^{\circ}\text{C}$ . Fluids in the lower chambers of paired microspin filter units were recombined and maintained on ice for the enzyme-linked immunosorbent assays (ELISAs) described below. As the density of secretions is comparable to that of water (36), secretion weight (in milligrams) was assumed to be equivalent to volume (in microliters). The dilution factor associated with each eluted secretion was calculated as  $(200 \mu\text{l} + \text{secretion volume})/\text{secretion volume}$ . Nephur Test indicator strips (provided by Andreas Frey, University of Münster, Münster, Germany), capable of measuring hemoglobin (Hb) in a range corresponding to 10 to 250 lysed erythrocytes/ $\mu\text{l}$  were used to assess the extent of blood contamination in secretions as previously described (11). After adjustment for dilution factors and Hb in the 1% BSA-PBS diluent, mean levels of Hb in undiluted vaginal, cervical, and rectal secretions were found to be 0.0001, 0.02, and 0.12%, respectively, of those in whole blood. No Hb was detected in salivary secretions.

**Quantitation of total IgA and IgG concentrations.** The ELISA used to determine total IgA and IgG concentrations in serum and eluted secretions was performed as described elsewhere (16). Briefly, microtiter plates coated with antibodies specific for human IgA or IgG (Cappel, Durham, N.C.) were blocked and reacted with twofold serial dilutions of sera or eluted secretions and the appropriate standard. Moni-Trol control reagent (Baxter, Deerfield, Ill.) was used as standard for determination of total IgG in both sera and secretions and for serum IgA concentration. Purified colostrum IgA (Cappel) was used as standard for measurement of total IgA in secretions. Plates were incubated overnight at  $4^{\circ}\text{C}$  and then reacted with biotinylated goat F(ab')<sub>2</sub> specific for either human IgA or human IgG (Tago, Camarillo, Calif.) followed by avidin-labeled peroxidase. Color was developed with citrate-phosphate buffer containing 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) and H<sub>2</sub>O<sub>2</sub> (both from Sigma, St. Louis, Mo.). Absorbance values at 414 nm were recorded in a Spectramax 250 microplate spectrophotometer using the SoftMax 1.1 data analysis software (both from Molecular Devices, Sunnyvale, Calif.). Concentrations of unknowns were interpolated from four-parameter standard curves constructed by using this computer program.

**ELISA for quantitation of anti-CTB IgA and IgG antibodies.** Immulon 1 microtiter plates (Dynatech, Chantilly, Va.) were coated overnight at  $4^{\circ}\text{C}$  with 5  $\mu\text{g}$  of GM1 ganglioside (Sigma) per ml of PBS, washed twice in PBS, blocked with 0.1% BSA in PBS for 30 min at room temperature, and then reacted for 1 h at room temperature with 1  $\mu\text{g}$  of rCTB (List Biologicals, Campbell, Calif.) per ml of blocking buffer. Plates were washed three times with PBS containing 0.05% Tween 20 and reacted overnight at  $4^{\circ}\text{C}$  with twofold serial dilutions of sera or eluted secretions and either the IgA or the IgG anti-CTB standard (see below). Plates were washed and subsequently developed with biotinylated anti-human IgA or IgG (or IgM; Tago) as described above. Endpoint titers (ET) of anti-CTB antibodies having attached secretory component (SC) were also measured in some secretions by using rabbit polyclonal anti-human SC antibody conjugated to horseradish peroxidase (Dako, Carpinteria, Calif.) as the secondary reagent. In these experiments, the ET of antibodies was defined as the highest dilution of secretion producing an absorbance greater than or equal to the value corresponding to the mean absorbance plus 3 standard deviations (SD) for 12 blank wells which received diluent buffer rather than secretion. The ET was subsequently multiplied by the original dilution factor introduced into each secretion after being eluted from the wick into buffer.

As purified human anti-CTB IgA and IgG antibodies are not available, standards were generated in the laboratory by affinity column chromatography using sera from women previously immunized with WC/rBS. IgG and IgA were first isolated from pooled serum by using protein G-Sepharose (Pharmacia, Piscataway, N.J.) and anti-human IgA antibody immobilized on agarose (Sigma), respectively. CTB-specific antibodies in the IgA preparation were then purified by using rCTB coupled to CNBr-activated Sepharose as directed by the manu-

TABLE 1. Characteristics of secretions collected from mucosal surfaces by the wick method<sup>a</sup>

Sample source	Secretion vol (μl)	Dilution factor <sup>b</sup>	Total μg/ml				Intraindividual variation (CV) in IgA or IgG concn <sup>e</sup>	
			Diluted secretions <sup>c</sup>		Undiluted secretions and sera <sup>d</sup>		IgA	IgG
			IgA	IgG	IgA	IgG		
Rectum	15.40 ± 1.40	14.12 ± 1.25	219.81 ± 77.31	54.63 ± 12.95	3,104.48 ± 1,004.62	771.52 ± 119.56	0.81 ± 0.05	0.56 ± 0.04
Cervix	44.92 ± 13.35	5.59 ± 0.33	155.47 ± 20.64	228.05 ± 71.10	869.23 ± 242.62	1,275.05 ± 348.48	0.51 ± 0.07	0.60 ± 0.04
Vagina	44.76 ± 4.00	5.61 ± 0.55	56.11 ± 20.92	117.97 ± 47.34	314.91 ± 75.00	662.15 ± 164.46	0.56 ± 0.06	0.53 ± 0.04
Oral cavity	56.39 ± 1.33	4.56 ± 0.29	78.48 ± 7.80	9.98 ± 1.02	357.90 ± 52.78	45.51 ± 7.08	0.25 ± 0.04	0.39 ± 0.03
Blood					1,772.05 ± 90.56	11,664.22 ± 413.86	0.08 ± 0.02	0.08 ± 0.02

<sup>a</sup> Secretions and sera were collected from 14 women at four times during the study. Values are geometric means ± SEM for a total of 56 serum samples and 56 secretions collected from each mucosal site.

<sup>b</sup> After elution of the secretion from the wick into 200 μl of PBS containing 1% BSA and protease inhibitors.

<sup>c</sup> Measured by ELISA.

<sup>d</sup> The amount of IgA or IgG in each diluted secretion was multiplied by the corresponding dilution factor.

<sup>e</sup> The coefficient of variation (CV) is equivalent to SD/arithmetic mean and was calculated for each individual by using the total IgA or IgG concentrations found in the four serum samples or four undiluted secretions collected from each mucosal site during the study.

facturer (Pharmacia). The concentration of IgA eluted from this column was determined by ELISA and assumed to represent pure anti-CTB IgA antibody. Anti-CTB IgG antibodies were similarly isolated from the purified IgG. As amounts of specific IgA and IgG obtained in this manner were not sufficient to perform all assays, they were used to quantitate the concentrations of anti-CTB IgA and IgG antibodies in another pooled serum, which then served as standard in CTB ELISAs. Albeit very low, some level of reactivity to CTB was detected in all preimmune sera and in all preimmune secretions containing >30 μg of total IgA or IgG per ml. Therefore, similar reactivity to CTB was also assumed present, though below detectable levels, in preimmune genital tract secretions with <30 μg of total IgA or IgG per ml. The latter secretions were assigned antibody concentrations corresponding to the limit of detection of this assay (26 ng/ml for IgA; 32 ng/ml for IgG) to facilitate calculations and statistical analyses.

For each sample assayed, the concentration of anti-CTB IgA or IgG antibody measured was divided by the total IgA or IgG concentration, respectively, to determine the ratio or proportion of specific antibodies within each Ig isotype. Comparison of ratios obtained after repeated measurement of total IgG and specific IgG in 56 serum samples established that the average SD associated with ratios of specific IgG to total IgG determined by these ELISAs was 24.6% of the mean. Therefore, if the ratio of specific IgG to total IgG in a secretion was 75% (3 assay SD) above the mean ratio in the serum of the same individual, the secretion was judged to contain a significantly greater proportion of anti-CTB IgG antibodies.

**Statistics.** The Statview 4 computer program (Abacus Concepts, Berkeley, Calif.) was used for all calculations and statistical analyses. Results were logarithmically transformed to obtain geometric means, SD, and standard errors of the means (SEM). The paired *t* test was used to determine whether postimmunization proportions of antibodies were statistically greater than those in preimmune samples of the corresponding immunization group. Multiple (between-group) comparisons were performed by one-way analysis of variance (ANOVA) using Fisher's protected least-significant difference at the 5% level of significance. Fisher's *r*-to-*z* transformation of correlation coefficients was used to obtain *P* values in correlation analyses. Results of all statistical analyses were considered significant only if *P* values were less than 0.05.

## RESULTS

**Total IgA and IgG in mucosal secretions collected with wicks.** The amounts of fluid absorbed in 5 min by wicks placed in the endocervical canal, in the posterior fornix of the vagina, or in the oral cavity were very similar (Table 1). Smaller volumes of secretions were absorbed from the rectal mucosa in the same period. However, after secretions were eluted from wicks into buffer and analyzed for total IgA and IgG content, eluates from rectal wicks were found to contain the highest concentrations of IgA (Table 1). The level of total IgG in diluted rectal secretions was lower than the level of IgA but proved adequate for measurement of anti-CTB IgG antibodies. In contrast, CTB-specific IgG could not be detected in the majority of diluted salivary secretions, presumably because the total IgG was too low (Table 1) after further dilution of these samples 1:4 for measurement of antigen-specific IgG by ELISA.

The concentrations of IgA and IgG present on each mucosal

surface at the time of sample collection were calculated by correcting for the dilution made during elution of protein from each wick into buffer (Table 1). Vaccination did not appear to affect the concentrations of total IgA or IgG in these mucosal secretions. Very high concentrations of IgA (ranging up to 30 mg/ml) were found associated with the surface of the rectum (Table 1). The mean total IgA concentration in the rectum was fourfold greater than that of IgG at this site. It should be noted that very small amounts of feces were occasionally observed on rectal wicks and could have contributed to the weights of some rectal secretions. As this would lower the associated dilution factor, the mean concentration of IgA and IgG calculated on the rectal wall could have been slightly underestimated.

Variation in the total IgA and IgG concentrations at mucosal surfaces of each individual over the course of the study was also examined since rectal secretions collected at different times from several women displayed unexpectedly large fluctuations in total IgA concentration. The least intraindividual variation was observed for salivary IgA concentration (Table 1). Levels of total IgA and IgG in the cervix and vagina of each woman varied considerably more throughout the study. This was not surprising, as Ig levels in secretions of the female genital tract are known to change considerably over the course of the menstrual cycle (19). Curiously, IgA concentration in the rectum of each woman varied to a statistically greater extent than it did in the female genital tract ( $P < 0.005$ ). If this were due to changes in hydration of the mucosal surface or variations in sampling technique, one would expect similar variations in total IgG in these secretions. However, rectal IgG concentrations did not vary in parallel with IgA (Table 1). Indeed, concentrations of IgA were highly correlated with IgG in the cervix, vagina, saliva, and serum ( $P \leq 0.0001$  for all), but they did not correlate with IgG in the rectum ( $P > 0.6$ ). The reason for this difference is not clear. Nevertheless, it is important to note that rectal secretions collected at multiple times from one subject may contain very divergent total IgA concentrations. Accurately assessing postimmunization antibody responses in the rectal mucosae of individuals was not possible if the anti-CTB IgA antibody concentration measured for each rectal secretion was not adjusted relative to the total IgA concentration. If not adjusted, a large number of false-positive or false-negative antibody responses clearly would have been recorded (not shown), as previously described in detail by others measuring specific antibodies in the small intestine (4).

**Anti-CTB antibody responses in serum.** Vaccine-mediated protection against sexually transmitted human immunodeficiency



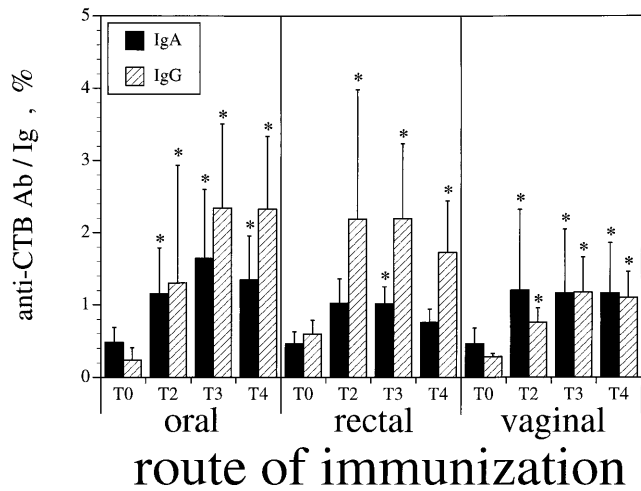


FIG. 1. CTB-specific IgA and IgG antibodies in serum after oral, rectal, or vaginal immunization of women with the WC/rBS cholera vaccine. Anti-CTB IgA and IgG antibody concentrations were measured by ELISA and divided by total IgA and IgG antibody concentrations, respectively, in each serum. The ratio obtained was subsequently multiplied by 100 to determine the percentage of specific antibodies within each isotype. Shown are the geometric mean percentages of specific IgA and IgG in serum at the four sampling times. Error bars represent SEM. Postimmunization proportions of antibodies found to be statistically greater than those in preimmune serum of the corresponding immunization group are indicated (asterisks).

ciency virus (HIV) will likely require induction of both mucosal and systemic immune responses. Therefore, mucosal immunization routes were examined for their abilities to produce a systemic antibody response to CTB. Concentrations of anti-CTB IgA and IgG antibodies in sera of mucosally immunized women were measured by ELISA and divided by the concentrations of total IgA and IgG, respectively, in the corresponding samples to determine the ratio or proportion of specific antibodies within these Ig isotypes. Mean proportions of specific IgA and IgG in serum for each immunization group before and after vaccination are shown in Fig. 1.

Two weeks after administration of the second vaccine dose, all immunization routes were observed to have increased proportions of anti-CTB IgG antibodies to levels significantly above those in preimmune serum. Further increases were detected 2 weeks after the third vaccination (T3) in sera of orally or vaginally immunized women. These antibody levels were maintained 2 weeks later (T4), at which time IgG antibodies in the rectal-immunization group had declined slightly. Serum anti-CTB IgA antibodies were also found to be significantly increased over preimmune levels at all times after oral or vaginal immunization but only at one time after rectal immunization (Fig. 1). Though oral or vaginal immunization produced a somewhat longer-lived serum IgA and IgG response than rectal immunization, no statistical differences could be demonstrated between groups when the percent anti-CTB IgG and IgA antibodies in serum were compared at each time point of sample collection.

No statistical differences were observed between or within immunization groups in regard to preimmune proportions of CTB-specific IgA and IgG in serum (and in secretions). We should mention that, although none of the women participating in this study had experienced *Vibrio cholerae* infection, they may have had prior exposure to the B subunit of *Escherichia coli* heat-labile enterotoxin (LTB), which is highly homologous to CTB. Infection by the latter pathogen could be largely

responsible for the presence of CTB-reactive antibodies in preimmune samples because anti-LTB antibodies often cross-react with CTB (30). Secondary exposure to rCTB does not, however, boost antibody responses to rLTB in mice (30). In this study, women with the highest proportions of preimmune antibodies in serum and secretions did not show evidence of a secondary immune response after immunization with WC/rBS: postimmune antibody responses to CTB did not develop earlier, nor were they of greater magnitude than those in women with the lowest preimmune levels of antibodies.

**Anti-CTB IgA antibody responses in saliva.** All immunization routes produced significant increases in specific salivary IgA at some time after immunization. Anti-CTB IgG antibodies were not detected in most salivary secretions, presumably due to the low levels of total IgG in these diluted samples. The greatest proportion of specific IgA in saliva was generally observed 2 weeks after the second or third vaccination, regardless of the immunization route (not shown). The mean peak proportion of specific IgA in saliva for each immunization group is shown in Fig. 2. The peak proportion of salivary anti-CTB IgA antibodies was highest for the oral group, but it did not differ statistically from those in other groups.

By using an anti-SC antibody as secondary reagent in the CTB ELISA, the ET of CTB-specific antibodies having attached SC were also measured in “peak” salivary secretions (Fig. 2) and found to be similar among immunization groups (data not shown). The mean ET (1:240; range, 1:50 to 1:2000) of SC-associated anti-CTB antibodies in postimmune saliva was statistically greater than the mean ET (1:60; range 1:20 to 1:160) in preimmune saliva ( $P < 0.0132$ ). CTB-specific IgM was detected in only one postimmune saliva sample (ET, 1:40). Therefore, titers of CTB-specific antibodies with attached SC most likely correspond to titers of specific S-IgA rather than specific S-IgM. Though it is not possible to determine how much of the specific IgA in these secretions may have attached SC, these data do suggest that oral, rectal, or vaginal immuni-

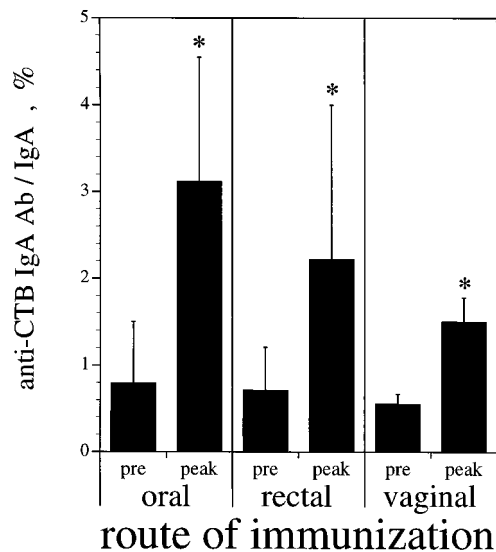


FIG. 2. Maximal anti-CTB IgA antibody responses in saliva after mucosal immunization. The greatest (peak) percentage of anti-CTB IgA antibodies within the total IgA in salivary secretions at times T2 to T4 after immunization is shown for each immunization group in comparison to the preimmune (pre) percentage. The data are geometric means with SEM. Proportions of specific antibodies significantly above those in preimmune of the same immunization group are indicated (asterisks).

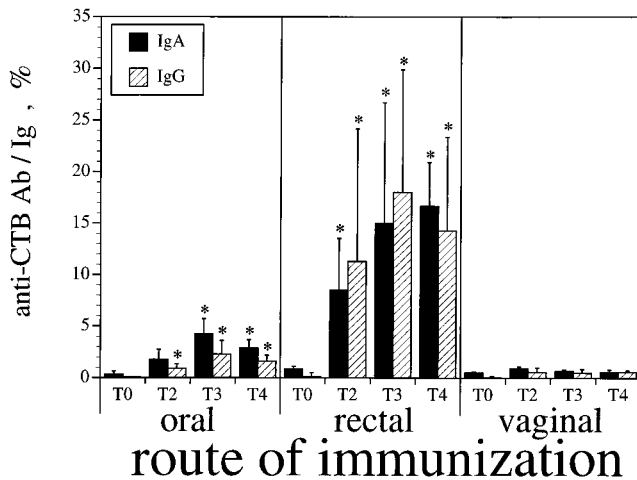


FIG. 3. CTB-specific IgA and IgG antibodies in rectal secretions. Shown are the geometric mean percentages of anti-CTB IgA and IgG antibodies constituting the total IgA and IgG in rectal secretions collected from women before ( $T_0$ ) and after ( $T_2$  to  $T_4$ ) immunization with WC/rBS. Error bars denote SEM. Proportions of antibodies significantly greater than the preimmunization proportion of the relevant immunization group are indicated (asterisks).

zation is capable of inducing specific secretory antibodies in the oral cavity.

**Anti-CTB antibody responses in the rectum.** The mean proportion of specific antibodies in rectal secretions was highest for women in the rectal-immunization group (Fig. 3). Oral immunization also significantly increased specific IgG and IgA in rectal secretions, but these responses were statistically lower than those evoked by rectal immunization at each postvaccination time point ( $P < 0.05$  for all). The vaginal immunization route was least effective for generating antibodies in the rectum; significant increases in anti-CTB IgA or IgG antibodies were not observed in rectal secretions at any time after vaginal vaccination.

A very large percentage of the total IgA and IgG in rectal secretions of women in the rectal group was directed against CTB after the third immunization (Fig. 3). At the final time point in this study less than 1% of the total IgA and 2% of the total IgG in sera of rectally immunized women reacted with CTB, whereas anti-CTB antibodies constituted 17 and 15% of the total IgA and IgG, respectively, in their rectal secretions ( $T_4$ ; Fig. 1 and 3). Therefore, it is likely that a large fraction of the anti-CTB antibodies measured in rectal secretions of rectally immunized women were of local origin and not derived from serum transudate or blood contamination of wicks.

To confirm that mucosally derived specific antibodies were present in these secretions, the remainder of rectal secretions collected at  $T_3$  and  $T_4$  from each of four rectally immunized women were combined, depleted of IgM by using agarose-conjugated anti-human IgM (Sigma), and analyzed by ELISA for the presence of SC on specific antibodies (data not shown). The mean ET of anti-CTB SC-bound antibodies found in these samples was 1:1,450 (range, 1:680 to 1:5,000), considerably higher than the 1:50 ET found in pooled preimmune rectal secretions of these four women. Since no anti-CTB IgM antibodies were detected in these samples (not shown), these titers likely represent specific S-IgA. These data indicate that rectal immunization did induce specific secretory antibodies in the intestinal tract.

**Anti-CTB antibody response in female genital tract secretions.** Cervical and vaginal secretions of women contained the

greatest mean levels of anti-CTB IgA and IgG antibodies after three vaccinations via the vaginal route (Fig. 4). Proportions of specific IgA and IgG in genital tract secretions varied so greatly among individuals in each group that no statistical differences could be demonstrated between immunization routes. As there are no data available regarding the effects of the menstrual cycle on specific antibody activity in the female genital tract in humans, it is not clear whether this variation may be related to the collection of secretions at different stages of the menstrual cycle or other factors.

Despite the inability to demonstrate statistical differences, the vaginal immunization route could be judged more effective than the rectal route for generating antibodies in the female genital tract; rectal immunization only transiently increased specific IgG in the vagina and failed completely to increase specific IgA in the genital tract (Fig. 4). Specific IgA and IgG were found in the cervix after oral immunization, but the cervical IgA antibody response was of short duration and this immunization route failed to increase specific antibodies of either isotype in the vagina. Only vaginal immunization was found to significantly increase proportions of both specific IgA and specific IgG in secretions collected from both the cervix and the vagina.

The large variation around the means shown for the vaginal immunization group in Fig. 4 stemmed from the fact that two women in this group exhibited widely divergent antibody responses in the female genital tract. One of these women was a low responder, with increases in anti-CTB antibodies not exceeding 0.25 and 1% of the total IgA and IgG, respectively, in the cervix or vagina (not shown). The other woman was the highest responder in this group: 40 to 70% of the total IgA and IgG in genital tract secretions reacted with CTB after the third

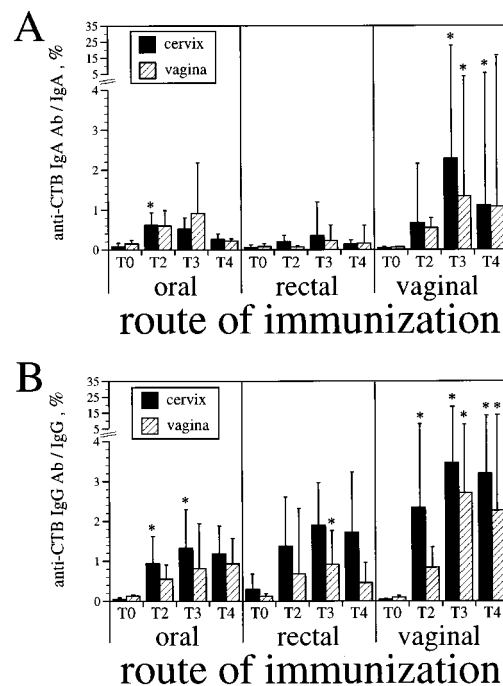


FIG. 4. Anti-CTB IgA and IgG antibodies in female genital tract secretions. Shown are the geometric mean percentages of anti-CTB IgA (A) and IgG (B) antibodies found within each isotype in cervical and vaginal secretions collected with wicks before ( $T_0$ ) and after ( $T_2$  to  $T_4$ ) immunization. Error bars represent SEM. Proportions of specific IgA and IgG significantly above those found in preimmune secretions are indicated (asterisks).

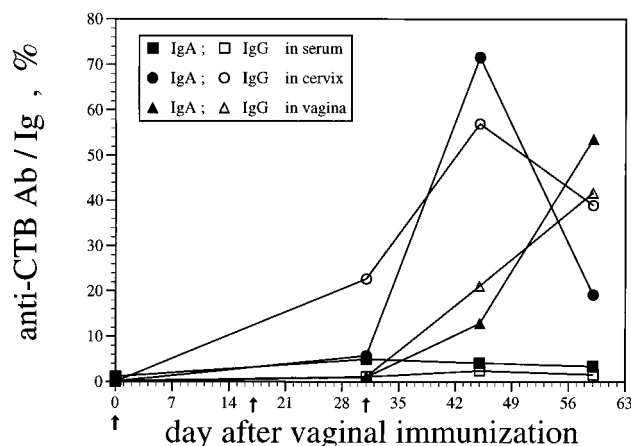


FIG. 5. Differing kinetics associated with development of specific antibody responses in the cervix and vagina. The percentages of specific IgA and IgG in secretions collected from the cervix and vagina of a woman vaginally immunized with the WC/rBS cholera vaccine are shown in addition to those in the subject's serum. Arrows indicate days on which immunization was performed.

vaginal immunization (Fig. 5). The ability to independently sample secretions in the cervix and vagina with absorbent wicks could be clearly demonstrated with secretions from the latter woman, as proportions of specific IgA or IgG found at these sites after immunization did not correlate (Fig. 5). The three immunizations of both this highest-responding woman and the lowest responder were performed on almost identical days during the luteal, follicular, and then luteal phase of the menstrual cycle. The remaining women, who were both vaginally immunized during the follicular, luteal, and then follicular phase of the cycle, demonstrated intermediate antibody responses in genital tract secretions: peak proportions of specific IgA and IgG ranged from 1 to 4.7% (not shown). Thus, in this small study population, no clear relationship could be established between the magnitude of the antibody response in the female genital tract and time during the menstrual cycle when vaginal immunizations were performed.

**Local IgG antibody production after local immunization.** It has been suggested that a large fraction of the IgG in female genital tract secretions may originate from serum, rather than local production (38). If IgG antibodies in genital tract or rectal secretions were solely derived from serum, the ratio of specific IgG to total IgG in these secretions should be less than or equal to those in serum. Indeed, in all five orally immunized women, the ratios of anti-CTB IgG antibody to total IgG in both genital tract and rectal secretions were consistently below or equal to that in serum collected at the same time (Table 2). The fact that the ratios of specific IgG to total IgG in rectal, cervical, and vaginal secretions of these women correlated with those in their sera ( $P < 0.05$  for all) further suggests that these antibodies may have been derived from serum.

In contrast, vaginal immunization induced specific IgG/total IgG ratios in vaginal and cervical secretions from two of four and three of four women, respectively, that were significantly greater than those in sera collected at the same time (Table 2). This implies that specific IgG in the genital tract in vaginally immunized women was derived at least in part from local production. However, none of these women showed evidence of local IgG antibody production in the rectum. Conversely, all five rectally immunized women developed proportions of anti-CTB IgG antibodies locally in the rectum that were significantly above those in their sera (Table 2), but none of these

women showed evidence of local IgG antibody production in the genital tract. Taken together, these data suggest that, while mucosal immunization can induce synthesis and secretion of specific IgG at or near the site of antigen administration, little or no local production of IgG antibodies occurs at distant mucosal sites.

**Local production of rectal IgA antibodies after oral immunization.** IgA antibodies in secretions of immunized women cannot be directly compared to those in sera because serum IgA is primarily monomeric whereas secreted IgA is polymeric (35). In this regard, it should be noted that, since anti-CTB IgA antibodies were quantitated relative to a serum standard in this study, the concentration of these antibodies (and hence the percentage within the total IgA) may have been underestimated two- to threefold in secretions (2). However, rectal secretions collected from four of five orally immunized women contained proportions of specific IgA which were 2.6–7.4-fold greater than those in serum (not shown). Anti-CTB SC-bound antibodies were also found in IgM-depleted T3 and T4 rectal secretions collected from two of three of these women (ET 1:280 and 1:456 versus 1:40 in pooled preimmune secretion). This suggests that, in contrast to IgG, some of the specific IgA in rectal secretions of these women was locally produced, even after immunization at a distant site.

The origin of anti-CTB IgA antibodies in genital tract secretions of orally immunized subjects is not as clear; the majority of secretions collected from the cervix and vagina for these women contained proportions of specific IgA below those in serum (not shown). We were not able to examine genital tract secretions of these (or other) women for the presence of specific antibodies with bound SC because sufficient quantities of these secretions were not available.

## DISCUSSION

In this study, the rCTB-containing WC/rBS cholera vaccine was used to demonstrate that rectal administration of antigen can induce a local immune response in the rectum in humans, as previously observed with mice and macaques (3, 6, 10, 40). Indeed, local immunization in the rectum in women induced very high levels of antibodies at this site: mean concentrations of anti-CTB IgA and IgG antibodies in secretions absorbed from the rectal surface for rectally immunized women were found to represent as much as 17% of the total IgA or IgG present. The majority of these specific antibodies were likely synthesized in the rectal mucosa and not derived from blood, as less than 2% of the IgA or IgG in sera of these women reacted with CTB. In addition, high titers of specific secretory

TABLE 2. Immunized women with secretions containing greater anti-CTB IgG antibody/IgG ratios than those in serum<sup>a</sup>

Route of immunization (n)	No. of women		
	Rectal secretions	Cervical secretions	Vaginal secretions
Oral (5)	0	0	0
Rectal (5)	5	0 <sup>b</sup>	0
Vaginal (4)	0 <sup>b</sup>	3	2

<sup>a</sup> The anti-CTB IgG antibody/IgG ratio in a secretion was considered significantly greater than that in serum if it was 3 assay SD above the mean ratio in serum collected simultaneously from the same woman (see Materials and Methods). Shown are numbers of women who had significantly greater ratios in secretions at all three postimmunization times of sample collection (T2 to T4).

<sup>b</sup> One individual had a significantly greater ratio of anti-CTB IgG antibody to IgG at one sampling time after immunization.



antibodies, most likely of the IgA type, were detected in these rectal secretions.

The possibility that a portion of the IgA (or IgG) measured in secretions collected from the surface of the rectum may have been synthesized in upper intestinal regions, such as the colon, cannot be excluded. Regardless of its exact origin within the gastrointestinal tract, the ability of a rectal vaccine to induce such high proportions of antigen-specific IgA at the rectal surface is encouraging for development of protective vaccines, especially in view of the high total IgA concentrations found at this site. However, generating large proportions of specific IgA (and IgG) in the rectum clearly required local administration of vaccine. No anti-CTB antibodies were detected in the rectum after vaginal immunization, and much lower mean proportions of these antibodies were measured after oral immunization.

Although the rectal route alone may be used for generating optimal immune responses in the rectum, rectal immunization was generally ineffective for eliciting CTB-specific antibodies in the genital tract in women. Therefore, it is likely that establishing protective immune responses in both the rectum and the female genital tract will require combining rectal immunization with another immunization route. Our data suggest that a combination of rectal and vaginal immunizations may be preferable to combined rectal and oral immunizations. The greatest proportions of specific IgA and IgG were found in the cervix and vagina in women immunized vaginally. Although oral immunization did increase anti-CTB IgA antibodies in the cervix, the response was transient and this immunization route did not significantly increase the level of specific IgA in the vagina. In addition, local immunization in the female genital tract stimulated a local IgG antibody response that was not evident in orally immunized women. Some care should be exercised in interpreting these results, as secretions were collected from women irrespective of the stage of the menstrual cycle. However, our data are in agreement with findings of others (39) who have reported that vaginal administration of WC/rBS was more effective than oral administration for generating local production and higher titers of specific IgA and IgG in the cervix and vagina in women.

The ability to induce high levels of specific antibodies in the rectum and genital tract in these women by using local immunization routes alone contrasts with results obtained after immunization of macaques with a CTB-conjugated simian immunodeficiency virus p27 preparation. Despite repeated oral, rectal, or vaginal administration of antigen, these immunization routes alone did not generate anti-p27 antibodies in rectal or vaginal fluid (23), and induction of specific antibodies in the rectum and genital tract was found to require a combination of local and oral immunizations (21, 22). However, others studying these immunization routes in macaques reported that rectal or vaginal immunization with HIV gp160 conjugated to CTB was capable of inducing HIV-specific IgA antibody responses in the rectum and vagina (3). These investigators concluded that an immunization strategy combining rectal immunization with vaginal immunization may be optimal for inducing immune responses in the rectal and female genital tract mucosae, and our results support this conclusion.

It is important to note that, although specific antibodies were not significantly increased in the rectum after vaginal immunization or in the female genital tract after rectal immunization of women in this study, both of these immunization routes were capable of inducing systemic antibodies and secretion of antibodies at other distant mucosal sites, as evidenced by the appearance of anti-CTB IgA antibodies in saliva. However, both rectal and vaginal immunizations produced higher

proportions of specific IgA locally in the rectum and genital tract, respectively, than in salivary secretions.

These data are consistent with previous studies which have demonstrated that greatest levels of antigen-specific IgA antibodies may be generated in regions that are closest to the site of antigen exposure (6, 12, 32, 34). Our data show that in humans this phenomenon includes production of specific IgG in the locally immunized mucosa, both in the rectum and the female genital tract. Although the female genital tract contains diffuse mucosa-associated lymphoid tissue typical of immune effector sites, it differs from the intestinal mucosa in that a larger fraction of the total Ig in associated secretions is of the IgG isotype (19). Early work with humans suggested that IgG in the female genital tract may be passively derived from serum (38). However, a significant fraction of the B cells in the mucosae of human cervix and vagina are IgG positive (19). The postulate that local synthesis of antibodies by these cells contributes IgG to genital tract secretions is supported by the finding that the distribution of the IgG subclasses in cervicovaginal fluid differs from that in serum (7). In addition, vaginal immunization of women with polio vaccine has been shown to induce IgG antibodies in cervicovaginal fluid in the absence of a systemic IgG response (33). There is also evidence that IgG antibodies in cervical secretions of women experiencing local bacterial infection originate locally (11). Our findings lend further support to the local origin of IgG antibodies in secretions of the female genital tract. Although the presence of specific S-IgA is generally considered to be the major factor in host defense at mucosal surfaces, it is likely that locally produced IgG antibodies do contribute to protection in the female genital tract.

The striking differences in local immune responses that we have observed after immunization via different routes in women emphasize the need to more clearly define the relationships between mucosal inductive sites and secretion of antibodies in humans. To that end, we are currently investigating the effects of the menstrual cycle on induction of immune responses in women in order to define the optimal timing of vaginal immunization. In addition, parallel studies with men are needed to define optimal immunization strategies for induction of immune responses in the male genital tract. Such studies will help to guide the development and use of novel mucosal vaccines for prevention of sexually transmitted diseases.

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