Kinetics of Local and Systemic Immune Responses after Vaginal Immunization with Recombinant Cholera Toxin B Subunit in Humans

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Vaginal vaccination seems to be the best strategy for inducing specific immunoglobulin A (IgA) and IgG antibody responses in the female genital tract. The relative efficiencies of one, two, and three vaginal doses of recombinant cholera toxin B subunit (CTB) in generating mucosal and systemic immune responses in healthy women were evaluated, and the kinetics of the immune responses were monitored for responding volunteers for up to 12 months after the last vaccination. A single dose of CTB failed to generate CTB-specific IgA antibody responses in cervical secretions. Two vaccinations induced significant increases in IgA antitoxin titers in seven of nine volunteers, and four volunteers also developed IgG antitoxin responses. The magnitudes of the responses were 20-fold for IgA antitoxin and 7.1-fold for IgG antitoxin. A third vaccination did not significantly increase the antitoxin responses, although the frequency of IgG responses was slightly higher than that after the second vaccination. In serum, CTB-specific antibodies were observed already after a single vaccination. However, two vaccinations were required to induce marked IgA as well as IgG antitoxin titer increases in the majority of volunteers. The postvaccination levels of antitoxin antibodies in serum were comparable after two and three vaccinations. At 12 months after vaccination, significantly elevated IgA and IgG antitoxin levels in cervical secretions could still be detected in approximately half of the volunteers who had initially responded to the vaccine. Antitoxin titer increases in serum were found in most of the vaccinees at follow-up.

Sexually transmitted diseases (STDs) are a major global health problem causing morbidity and mortality worldwide. The onset of the AIDS epidemic has focused interest on the development of vaccines to control the spread of STDs. To provide specific protection against microorganisms which invade mucosal surfaces, it is important to define mucosal vaccination strategies which could induce pathogen-specific, neutralizing antibodies in secretions of the genital tract (16, 24). Human vaginal washes and cervical fluids contain both immunoglobulin A (IgA) and IgG antibodies (9, 18, 19). Most of the IgA in the female genital tract originates from local production (18, 20, 21), whereas the origin of IgG has not been conclusively determined. However, it is likely that a certain portion of the IgG originates in plasma (1, 9).

It is generally believed that direct application of an antigen at the target mucosa is the most efficient way of inducing a protective mucosal immune response (4-6, 8, 22). We and others have shown that vaginal administration is superior to both oral vaccination and rectal vaccination in generating strong IgA and IgG antibody responses in cervical and vaginal secretions of humans (15, 27). According to recent studies, the nasal route can also be considered for the induction of antibodies in the female genital tract (2, 12, 17). However, information concerning the kinetics of immune responses in the human genital tract after mucosal vaccination is scarce.

The aim of the present study was to examine the kinetics of the local and systemic immune responses in healthy fertile women given three vaginal vaccinations with an inactivated cholera vaccine containing recombinant cholera toxin B subunit (CTB). CTB is one of the best-characterized mucosal antigens with regard to both safety and immunogenicity in humans (2, 7, 10, 27). CTB-specific IgA and IgG antibodies in cervical secretions along with antitoxin antibodies in serum were determined, and the immune responses after one, two, and three vaginal doses of CTB were compared. The kinetics of the immune responses were also monitored for responding volunteers for up to 12 months after the last vaccination.

METHODS

Study design. Twelve healthy Swedish women 31 to 44 years old (mean, 37 years) volunteered and gave informed consent to participate in the study, which was approved by the Human Research Ethical Committee of the medical faculty of Göteborg University, Göteborg, Sweden.

None of the volunteers had previously been vaccinated against cholera or had traveled to areas where cholera or enterotoxigenic Escherichia coli is endemic during the 5 years preceding the study. All women were regularly menstruating, and none used oral contraceptives.

Each volunteer was vaginally immunized with three doses of a licensed, inactivated B-subunit–whole-cell (B-WC) cholera vaccine (Dukoral; SBL Vaccin AB, Stockholm, Sweden) administered at 2-week intervals. The vaccine contained 1 mg of recombinant CTB and 1011 inactivated cholera vibrios per dose (7). Each dose (3 ml) of the vaccine was mixed with 650 mg of a biologically inert polysaccharide (Eldexomer, batch 020; Perstorp Pharma, Perstorp, Sweden). The freshly made vaccine-gel mixture was deposited in the upper fornix of the vagina, and the women remained in a horizontal position for 10 min after each vaccination (12, 27). The first immunization was initiated on day 10 of the menstrual cycle (i.e., 10 days after the last menstrual bleeding had started). The second
immunization was given on day 24 of the menstrual cycle, and the third immunization was given on day 10 of the following menstrual cycle.

Nine volunteers participated in the first part of the study, in which the immune responses after one, two, and three vaginal immunizations with CTB were evaluated.

Cervical secretions and serum were collected immediately before immunization (day 0, i.e., day 10 of the menstrual cycle), 14 days after the first immunization (day 14, i.e., day 14 of the menstrual cycle), 14 days after the second immunization (day 28, i.e., day 24 of the following menstrual cycle), and 8 to 10 days after the third immunization (days 36 to 38, i.e., days 18 to 20 of the following menstrual cycle).

In the second part of the study, the kinetics of the immune responses in seven of the nine volunteers who had participated in the first part of the study were monitored. The remaining volunteers were withdrawn from the study; one woman was a nonresponder, and the other woman did not give her consent to continue. In addition, three women were recruited and vaginally immunized three times with CTB according to the above-mentioned schedule. Thus, 10 volunteers who responded with significant IgA antitoxin titer increases in cervical secretions after vaginal vaccination participated in the second part of the study.

In this part of the study, cervical and serum specimens were obtained 1, 2, 3, 6, and 12 months after the third vaccination. Samples of cervical secretions were obtained with a syringe (Aspiglaire; Biotechnologies International, Aigle, France), and the volume was recorded. Before determination of the antibodies, the cervical samples were diluted 1:10 with phosphate-buffered saline and treated with bromelain (Sigma Chemical Company, St. Louis, Mo.) to solubilize the mucus (27). Bromelain-treated specimens were stored at −70°C until analyzed. Serum specimens were stored in aliquots at −20°C until analyzed.

**FIG. 1.** Geometric mean ± standard error of the ratios of IgA (A) and IgG (B) antitoxin titers to total immunoglobulin concentrations in cervical secretions from nine healthy women before and after one, two, and three vaginal immunizations with recombinant CTB. The values have been multiplied by 100. The numbers of responding volunteers are indicated above the bars. Immunizations are indicated by arrows.

Determination of total immunoglobulin and specific antibodies. The total IgA and IgG antibody contents in cervical specimens were determined by a modified enzyme-linked immunosorbent assay (ELISA) (2, 26). Specimens with low IgA and/or IgG concentrations (≤40 μg ml⁻¹) were excluded from further analyses, since our previous studies showed that antibody titers in such specimens were unreliable.

IgA and IgG antibodies to cholera toxin in cervical secretions and serum were determined by the GMI ELISA method as previously described (25, 27). The CTB-specific IgA and IgG antibody activities (in units per microgram) in cervical secretions were determined by dividing the ELISA immunoglobulin titer (in units per milliliter) by the total immunoglobulin concentration (in micrograms per milliliter) in the specimens to compensate for variations in immunoglobulin contents in specimens collected on different days. Responders were defined as having a greater than twofold increase in specific antibody activities between pre- and postvaccination specimens (11). In serum, a twofold or greater increase in endpoint titers between pre- and postvaccination specimens was used to signify seroconversion at a P value of ≤0.05 (10, 11).

**RESULTS**

Antitoxin responses in cervical secretions and serum. The local immune responses in cervical secretions as well as the antibody responses in serum were studied after one, two, and three vaginal immunizations with CTB in nine volunteers.

A single vaccination failed to induce any CTB-specific IgA antibody responses in cervical secretions (Fig. 1A), and an
increase in the ratios of IgG antitoxin titers to total IgG concentrations was found only in one of nine individuals (Fig. 1B). Two doses of CTB induced significant increases in IgA antitoxin titers in seven of nine volunteers, and four volunteers developed IgG antitoxin responses. A 20-fold increase in CTB-specific IgA titers was induced in the entire group of vaccinees, and the IgG responses were increased 7.1-fold; the magnitudes of the titer increases in the responders were 50-fold for IgA antitoxin and 47-fold for IgG antitoxin. The postvaccination levels of IgA as well as IgG antitoxin were significantly higher after two vaccinations than after a single dose of vaccine ($P = 0.0078$ and $P = 0.0336$, respectively) (Fig. 1). A third dose of CTB did not significantly increase the antitoxin responses in cervical secretions, although the frequency of IgG responses was slightly higher than that after the second vaccination. No differences in the postvaccination levels of IgA and IgG antitoxins were found after three versus two vaccinations (Fig. 1).

At variance with the kinetics of antibody titers in cervical secretions, significant increases in IgA antitoxin titers in serum appeared already after a single vaccination in five of nine volunteers, and three volunteers developed IgG antitoxin responses. A second dose of CTB further increased the number of volunteers responding with CTB-specific IgA and IgG titers (Table 1). The geometric mean fold increases in IgA and IgG antitoxin titers for the entire group of vaccinees were also higher after the second vaccination than after the first vaccination ($P = 0.049$ and $P = 0.012$, respectively). The frequencies and magnitudes of the antitoxin responses in serum were comparable after two and three vaccine doses (Table 1).

**Kinetics of antitoxin responses.** The kinetics of the local and systemic antitoxin responses were monitored for up to 12 months in 10 volunteers who had responded with significant IgA antitoxin titers in cervical secretions after three vaginal doses of CTB. The frequencies of CTB-specific IgA and IgG titer increases in cervical secretions and serum from prevaccination to postvaccination at different time points are shown in Table 2. Of the 10 initially responding volunteers, 9 had significantly elevated IgA antitoxin levels in cervical secretions 3 months after vaccination, and in 5 volunteers, the antitoxin levels were still elevated at 12 months. Maximal IgA antitoxin titer increases were achieved 8 to 10 days after the third vaccination, the geometric mean level being 29-fold higher than that before vaccination (Fig. 2A). From 1 month onward, the antitoxin levels declined but still showed a 2.3-fold mean increase at 12 months of follow-up. Significant increases in cervical IgG antitoxin levels were observed in seven of the eight initially responding volunteers 3 months after the third vaccination, and in four volunteers, the antitoxin levels remained elevated at 12 months (Table 2). The IgG antitoxin responses peaked 8 to 10 days after the third vaccination, the geometric mean level being 14-fold higher than that before vaccination (Fig. 2B). Thereafter, the IgG antitoxin levels declined but still showed a 2.9-fold mean increase 12 months after vaccination.

The IgA as well as IgG antitoxin responses in serum lasted longer than the corresponding responses in cervical secretions. Significantly elevated antitoxin levels were found in most of the volunteers 12 months after vaccination (Table 2), but the magnitudes of the responses had decreased from 9.1-fold to 3.8-fold for IgA antitoxin and from 6.1-fold to 2.8-fold for IgG antitoxin.

**TABLE 1. Antitoxin antibody responses in serum from healthy women after one, two, and three vaginal administrations of recombinant CTB**

| Day(s) postimmunization | IgA | IgG |
|-------------------------|-----|-----|
|                         | No. of responders/total no. tested | Fold increase | No. of responders/total no. tested | Fold increase |
| 14                      | 5/9 | 3.1 | 6.4 |
| 28                      | 7/9 | 8.4 | 14.4 |
| 36–38                   | 8/9 | 9.0 | 11.5 |
| 28                      | 7/9 | 5.6 | 8.7 |
| 36–38                   | 8/9 | 7.4 | 9.4 |

* Recombinant CTB was administered vaginally on days 0, 14, and 28 (i.e., day 10 and day 24 of the menstrual cycle and day 10 of the following menstrual cycle).

b Responders were defined as showing a twofold or greater increase in CTB-specific titers between pre- and postimmunization specimens.

c Fold increase is the geometric mean titer increase in relation to the preimmunization titer.

**TABLE 2. Kinetics of frequencies of IgA and IgG antitoxin responses in cervical secretions and serum from healthy women after three vaginal doses of recombinant CTB**

| Time interval after the third immunization | Cervical secretions | Serum |
|-------------------------------------------|---------------------|-------|
|                                           | IgA/total no. tested | IgG   | IgA/total no. tested | IgG   |
| 8–10 days                                 | 10/10               | 8/10  | 8/10               | 8/10  |
| 1 mo                                      | 10/10               | 9/10  | 9/10               | 8/10  |
| 2 mo                                      | 9/9                 | 7/9   | 9/10               | 8/10  |
| 3 mo                                      | 9/10                | 7/10  | 9/10               | 8/10  |
| 6 mo                                      | 8/9                 | 6/9   | 8/10               | 8/10  |
| 12 mo                                     | 5/9                 | 4/10  | 8/10               | 8/10  |

* Responders were defined as showing a greater than twofold increase in the ratio of CTB-specific immunoglobulin antibody titers to total immunoglobulin concentrations in cervical secretions and a twofold or greater increase in CTB-specific titers in serum between pre- and postimmunization specimens.

b One cervical specimen was excluded because of low concentrations of IgA and IgG, i.e., $<40$ µg/ml.

c Cervical specimens were not obtained from one volunteer.

d One cervical specimen was excluded because of low concentrations of total IgA, i.e., $<40$ µg/ml.
DISCUSSION

Antibody responses in genital tract secretions after various modes of mucosal administration of recombinant CTB have been thoroughly evaluated in humans (2, 12, 15, 17, 23, 27). To our knowledge, this is the first study to examine the kinetics and long-term duration of local mucosal antibody responses in the female genital tract after vaginal vaccination. We found that two vaginal doses of CTB were far more efficient than a single dose in generating CTB-specific IgA and IgG antibody responses in cervical secretions, and a third dose did not further increase the responses. Twelve months after vaccination, increased antitoxin levels in cervical secretions could still be found in half of the volunteers who had initially responded to the vaccine.

The endocervix appears to be the predominant immunological organ in the female genital tract (18), although no organized inductive sites containing membranous epithelial cells have been found in the genital mucosa. Immunohistochemical examinations of tissue sections or dispersed cells have indicated that the human endocervix contains larger numbers of immunoglobulin-secreting cells than do the ectocervix, fallopian tubes, and vagina (3, 18). Almost all IgA-producing cells contain joining chains and the secretory component (SC), a marker of synthesis of polymeric IgA. Further, the single-layer epithelium of the endocervix as well as the fallopian tubes, uterus, and ectocervix express SC, which is necessary for the transportation of locally produced polymeric IgA into genital secretions. In contrast, the multilayer epithelium of the vagina does not stain for SC (18; J. Mestecky, R. P. Edwards, P. A. Crowley-Nowick, A. M. Pitts, and W. H. Kutteh, unpublished data). For the determination of local immune responses in the female genital tract after various routes of mucosal vaccination, cervical secretions and/or vaginal fluids have been used. Vaginal vaccination has been shown to be the route of choice for stimulating both IgA and IgG antibody responses in cervical secretions (12, 16, 27), while nasal vaccination seems to be superior in inducing IgA responses in vaginal fluids (12). These findings indicate that there is compartmentalization within the genital tract and that the induction of specific antibodies in cervical secretions is regulated in a manner different from that in vaginal secretions (12).

The present study shows that two vaginal vaccinations were required for the induction of strong IgA and IgG antitoxin responses in the human genital tract. None of the vaccinees responded with IgA antitoxin titer increases in cervical secretions after a single vaccination, and only one of nine vaccines developed a significant IgG antitoxin response. These results agree with the notion that one nasal dose of CTB was less efficient than two doses in stimulating IgA and IgG antibody responses in nasal and vaginal secretions (23). Two oral doses of B-WC cholera vaccine have also been reported to be optimal for the induction of an effective local IgA immune response in the intestine (26). Our results also showed that two vaginal doses of CTB were as effective as three doses in generating local IgA as well as IgG antibody responses in cervical secretions.
secretions. In contrast, Kozlowski et al. (15) reported higher levels of CTB-specific antibodies in cervical and vaginal secretions after three compared to two vaginal vaccinations. However, the number of vaccinees was small, and there was a large distribution of CTB-specific antibody titers around the mean value in their study. According to our data, immunity at the systemic level does not directly reflect the local antibody responses in the genital tract. CTB-specific IgA and IgG antibody responses in serum were found already after a single vaginal vaccination, and the magnitudes of the responses in serum after two and three vaginal vaccinations were lower than those in cervical secretions.

Several studies have evaluated the kinetics of local immune responses to CTB in various secretions after various routes of mucosal vaccination. In volunteers given two oral doses of B-WC cholera vaccine, peak levels of IgA antitoxin antibodies in intestinal, nasal, and vaginal fluids appeared 2 weeks after the second vaccination (13, 23). After nasal administration of CTB, maximal increases in specific IgA antibodies in nasal and vaginal secretions were found 1 week after the second vaccination, whereas IgG antibodies showed a delayed onset, with peak responses appearing after 6 weeks (23). Our analyses of the kinetics of antibody responses in cervical secretions after vaginal administration of B-WC cholera vaccine indicated that IgA as well as IgG antitoxin antibodies showed peak responses 8 to 10 days after the third vaccination. Kozlowski et al. (15) also reported similar findings of higher levels of antitoxin antibodies in cervical secretions 2 weeks compared to 4 weeks after vaginal vaccination.

Immune responses at mucosal surfaces are generally regarded to be short-lived. However, analyses of fecal antibody responses in Swedish volunteers have shown that increases in IgA antitoxin and antibacterial antibodies can be demonstrated in 50 and 43% of initially responding subjects 6 months after oral immunization with B-WC cholera vaccine (14). Nasal administration of CTB has also been shown to result in persisting IgA and IgG antibody responses in nasal as well as vaginal fluids for at least 6 months after vaccination (2). Prior to this study, the duration of local IgA and IgG antibody responses in cervical secretions after vaginal vaccination had been monitored for only 2 months (17). The relative ease in collecting cervical specimens made it possible for us to monitor local mucosal immune responses in the genital tract for a longer period. After 3 months, significant IgA antitoxin responses could be demonstrated in eight of nine initially responding volunteers, and in five vaccinees, the antitoxin levels were still elevated at 12 months of follow-up. The IgG antibody titer increases in cervical secretions displayed a similar pattern, with persisting responses being found in half of the initially responding volunteers 12 months after vaccination. The effective induction and long-term persistence of locally produced antibodies in cervical secretions suggest that CTB and probably also other antigens linked to CTB may be used for effective mucosal immunization of the genital tract against specific pathogens. These findings may be of relevance for the development of vaccines against human immunodeficiency virus infection as well as other STDs.

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