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Induction of Antigen-Specific Antibodies in Vaginal Secretions by Using a Nontoxic Mutant of Heat-Labile Enterotoxin as a Mucosal Adjuvant

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Immunization of the female reproductive tract is important for protection against sexually transmitted diseases and other pathogens of the reproductive tract. However, intravaginal immunization with soluble antigens generally does not induce high levels of secretory immunoglobulin A (IgA). We recently developed safe mucosal adjuvants by genetically detoxifying *Escherichia coli* heat-labile enterotoxin, a molecule with a strong mucosal adjuvant activity, and here we describe the use of the nontoxic mutant LTK63 to induce a response in the mouse vagina against ovalbumin (Ova). We compared intravaginal and intranasal routes of immunization for induction of systemic and vaginal responses against LTK63 and Ova. We found that LTK63 is a potent mucosal immunogen when given by either the intravaginal or intranasal route. It induces a strong systemic antibody response and IgG and long-lasting IgA in the vagina. The appearance of vaginal IgA is delayed in the intranasally immunized mice, but the levels of vaginal anti-LTK63 IgA after repeated immunizations are higher in the intranasally immunized mice than in the intravaginally immunized mice. LTK63 also acts as a mucosal adjuvant, inducing a serum response against Ova, when given by both the intravaginal and intranasal routes. However, vaginal IgA against Ova is stimulated more efficiently when LTK63 and antigen are given intranasally. In conclusion, our results demonstrate that LTK63 can be used as a mucosal adjuvant to induce antigen-specific antibodies in vaginal secretions and show that the intranasal route of immunization is the most effective for this purpose.

Because the majority of pathogenic microorganisms initiate infection by interacting with the host mucosae, induction of mucosal immune responses can be very important in controlling infection and preventing disease. However, most of the vaccines used so far are parenteral vaccines, in spite of the fact that parenteral immunization generally induces high serum immunoglobulin G (IgG) and little or no secretory IgA responses. One of the limitations in the development of mucosal vaccines has been the lack of safe and effective mucosal adjuvants. In fact, while replicating microorganisms can be good mucosal immunogens (19), mucosal immunization with nonviable microorganisms or soluble antigens generally fails to elicit local responses (19, 23). Furthermore, induction of a local response in certain mucosal sites, such as the female reproductive tract, is particularly difficult to achieve (15, 24). Induction of a local response in the vagina could, however, be very important for protection against viral and bacterial pathogens such as human immunodeficiency virus, papilloma virus, herpes simplex virus type 2, and *Chlamydia trachomatis*. Indeed, intravaginal (IVAG) immunization with live microorganisms has been shown in some cases to produce a protective local response (8), but the response induced with soluble antigens has always been quite poor and protection has never been demonstrated in such systems. In some studies, combined oral-vaginal or parenteral-vaginal immunizations have been used to induce a local response in the vagina to soluble antigens (6, 12, 20). Conventional adjuvants such as alum hydroxide have been shown to increase the local secretory response if large amounts of soluble antigen were used, but the IgA titers in vaginal fluids were only slightly higher than those obtained by parenteral immunization (21, 22). On the other hand, IVAG immunization with cholera toxin (CT), which is a potent mucosal immunogen, failed to induce a local response (4).

Cholera toxin and *Escherichia coli* heat-labile enterotoxin (LT) are known to be very powerful mucosal adjuvants, as well as strong mucosal immunogens (5, 9, 18). Nevertheless, a drawback to the use of these molecules as mucosal adjuvants is their toxicity. In the attempt to overcome this problem, we have recently constructed, by site-directed mutagenesis, a series of nontoxic mutants of LT (16) and have shown that intranasal (IN) immunization with one of these mutants (LTK7) is very effective in inducing mucosal and systemic immunity to coadministered antigens (3). Thus, nontoxic LT molecules represent a new generation of powerful and safe mucosal adjuvants that could be used at high doses to induce mucosal immune responses even in poorly responsive mucosal sites such as the female reproductive tract.

We describe here the use of a new nontoxic mutant of LT (LTK63) as an adjuvant to induce a local immune response in the mouse vagina against ovalbumin (Ova). Two routes of immunization were used (IVAG and IN), and the local and systemic responses to both LTK63 and Ova were compared for the two routes. Our results show that LTK63 is highly immunogenic when delivered IVAG, inducing a systemic response as well as a long-lasting local response in vaginal secretions. Anti-LTK63 antibodies were also found in serum samples and nose, lung, and intestinal lavages of animals immunized IN, whereas anti-LTK63 IgA and IgG antibody-secreting cells (ASC) were found in the nasal mucosa and spleen tissues of mice immu-

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nized by both routes. Furthermore, LTK63 acts as a mucosal adjuvant, since codelivery of LTK63 and Ova IN induces anti-Ova IgA in the vagina.

MATERIALS AND METHODS

Mice. Female BALB/c mice aged 6 to 8 weeks were obtained from Charles River (Calco, Como, Italy).

Antigens. LTK63 is a mutant of *E. coli* LT containing the single mutation Ser-63→Lys. This mutant was obtained and purified at our institution (3, 16); Ova was obtained from Sigma (St. Louis, Mo.).

Immunization schedule. Groups of five mice were immunized five times (days 0, 14, 21, 28, and 35) with either (i) 20 μg of LTK63 and 50 μg of Ova IVAG or (ii) 1 μg of LTK63 and 20 μg of Ova IN. A sixth dose was given 60 days after the last one to mice from which nasal, lung, and gut washes were collected. Two control groups received Ova alone either IN (20 μg) or IVAG (50 μg). For the IN immunization, the mice were partially anesthetized with an anesthetic delivered intraperitoneally. The volume of immunization was 15 μl per nostril, delivered with a Gilson pipette. The mice immunized IVAG each received a volume of 20 μl and were kept immobilized in a cylinder for 2 to 3 h before being placed in single cages. This procedure was used in order to prevent nasal or oral contact with an immunized vagina. Serum samples and vaginal fluids were collected 24 h before each immunization and every week after the last immunization.

Collection of mucosal fluids. Nasal, lung, and gut washes were collected 14 days after the sixth immunization. Nasal, lung, and gut lavages were performed on the sacrificed animal by repeated flushing and aspiration of 1, 1.5, and 1 ml, respectively, of phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) and 1 mM phenylmethylsulfonyl fluoride (Fluka, Buchs, Switzerland) as a protease inhibitor. Vaginal washes were performed on the live animal by repeated flushing and aspiration of a total of 0.3 ml of the same buffer used for other lavages. All fluids were kept frozen at −20°C.

ELISA for LT and Ova. LTK63-specific antibodies were measured in a GM1 capture enzyme-linked immunosorbent assay (ELISA). Each well on 96-well plates (Greiner GmbH, Kremsmunster, Austria) was first coated with 0.2 ml (0.75 μg/ml) of GM1 ganglioside (Sigma) overnight at 4°C. The wells were then washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween 20, and LTK63 (0.1 ml per well) (0.5 μg/ml) was added for 2 h at 37°C. For estimation of Ova-specific antibodies, each well (on 96-well plates) was directly coated with 0.1 ml of Ova (45 μg/ml). Both LTK63 and Ova wells were washed and blocked for 1 h at 37°C with 0.2-ml portions of PBS containing 1% BSA. Serum samples from individual mice were serially diluted starting with a dilution of 1:50 in PBS, and vaginal, nasal, lung, and gut washes were serially diluted starting with a dilution of 1:10 in PBS. Diluted samples were added to washed plates (0.1 ml per well) and incubated for 2 h at 37°C. The wells were then washed, and those containing serum samples were incubated with 0.1 ml of rabbit anti-mouse Ig–horseradish peroxidase (HRP) conjugate (Dako, Glostrup, Denmark) diluted 1:2,000 in PBS containing 0.1% BSA and 0.025% Tween 20 for 2 h at 37°C. Wells containing mucosal washes were incubated with 0.1-ml portions of α- or γ-chain-specific biotin-conjugated goat anti-mouse serum diluted 1:1,000 (Sigma) for 2 h at 37°C. HRP-conjugated streptavidin (0.1 ml per well) (Dako) was added to washed plates for 2 h at 37°C. Antigen-bound antibodies were visualized by adding *o*-phenylenediamine substrate (Sigma) to washed plates, and A_{450} was read. Titers were determined arbitrarily as the reciprocal of the sample dilution corresponding to an optical density at 450 nm of 0.3. Sera or fluids with absorbance values lower than 0.3 above the background value were considered negative.

ELISAs for total IgA and IgG in body fluids. Total IgA and IgG antibodies were measured in ELISAs as described above, the only difference being that the wells on these plates were each coated overnight at 4°C with 0.1 ml of unconjugated α- or γ-chain-specific goat anti-mouse Igs (Sigma) at a concentration of 2.5 μg/ml in carbonate buffer. The wells were washed and blocked as described above before the addition of serial dilution of lavages (from 1:10 to 1:6,250) and incubated for 2 h at 37°C. Each well was then washed and incubated with 0.1 ml of α- or γ-chain-specific biotin-conjugated goat anti-mouse serum (Sigma) for 2 h at 37°C. HRP-conjugated streptavidin (0.1 ml per well) (Dako) was added to washed wells for 2 h at 37°C, and the assay was completed as described above. Titers were determined as described above.

Calculation of ratios of specific antibodies/total antibodies. Titers of LTK63- or Ova-specific antibodies and titers of total IgA or IgG present in lavages were determined in parallel ELISAs. The ratios of specific antibody titers/total antibody titers were then calculated as follows: [titer of LTK63- or Ova-specific IgA (or IgG)]/[titer of total IgA (or IgG)] × 100.

ELISPOT. To determine the number of anti-LTK63 ASC, spleen and nasal mucosa tissues were disrupted by a glass mortar. After removal of tissue debris, cell suspensions were spun down and resuspended in ACK lysing solution (0.15 M ammonium chloride, 0.01 M potassium hydrogen carbonate, and 0.1 mM disodium EDTA [pH 7.4]) for 5 min at room temperature to lyse erythrocytes. Cells were then washed three times, resuspended in medium, and counted. A modified protocol of the originally described ELISPOT (1, 17) was then used. Briefly, cell suspensions were added to the wells on 48-well plates that had been precoated with GM1 ganglioside (0.5 ml per well at 1.5 μg/ml, overnight at 4°C) and then LTK63 (0.25 ml per well at 1 μg/ml for 2 h at 37°C) and blocked with BSA, by a procedure similar to that described above for ELISA plates. Each cell suspension represents a pool of lymphocytes from four to five animals and is used either undiluted or diluted 1:4 up to five times. The tissue culture medium used was Dulbecco modified Eagle medium (Gibco Laboratories, Grand Island, N.Y.) supplemented with 1% glutamine, 0.5% 2-mercaptoethanol, antibiotics, and 10% fetal calf serum. Plates were incubated overnight at 37°C in a CO_2 atmosphere, and then washed and unconjugated α- or γ-chain-specific goat anti-mouse Igs (Sigma) were added to wells for 2 h at 37°C. After further washings, rabbit anti-goat Igs conjugated with alkaline phosphatase were added for 2 h at 37°C. The substrate (5-bromo-4-chloro-3-indolylphosphate disodium salt [Sigma]) was added to washed plates and incubated at 4°C until blue spots became visible. Spots in each well were counted, and the number of ASC was expressed per 10^6 cells seeded.

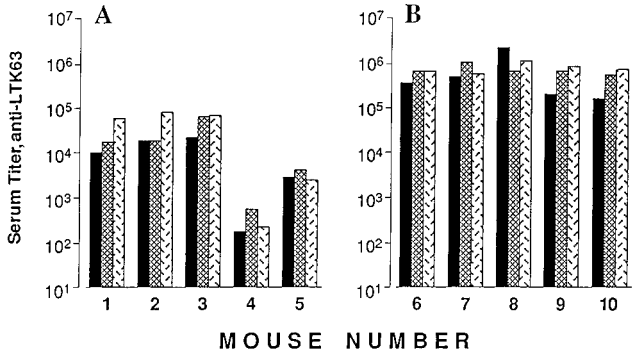

FIG. 1. Titers of anti-LTK63 total Igs in the sera of individual mice after two (■), three (▨), or four (▩) immunizations with LTK63. The animals were immunized IVAG (A) and IN (B). The data presented are from one representative experiment of four performed.

RESULTS

Systemic response to LTK63 following IVAG immunization. IN immunization with LTK63 has been shown to be very effective for the induction of specific local and systemic immune responses (2a). Thus, in this study the immunogenicity of LTK63 following IVAG immunization is analyzed in parallel with the response obtained with the IN route of immunization. We first analyzed the systemic response to LTK63. Figure 1 shows the individual anti-LTK63 serum Ig titers for mice that received four immunizations with LTK63 and Ova, either IVAG (Fig. 1A) or IN (Fig. 1B). Both groups of mice developed high anti-LTK63 titers that reached a plateau after the second immunization. Although the five animals immunized IN have serum anti-LTK63 titers 10-fold higher than those of IVAG immunized mice, these results show that LTK63 is able to induce a high serum response when delivered IVAG.

To investigate whether the serum anti-LTK63 response induced by IVAG and IN immunizations were qualitatively similar, we pooled serum samples from mice in each group and tested them for their capability to neutralize the effect of wild-type LT on Y-1 cells in vitro (2). We found that sera from IVAG and IN immunized mice had similar neutralizing titers (1:1,000), even though IVAG immunized mice had lower anti-LTK63 ELISA titers.

Anti-LTK63 IgA and IgG in vaginal washes. To assess whether LTK63 induces a local response in the vagina, we followed the appearance of anti-LTK63 IgA antibodies in vaginal washes after two and three immunizations. We also investigated whether anti-LTK63 IgA appeared in the vaginas of IN immunized animals. We found that four of the five IVAG immunized animals developed anti-LTK63 IgA in their vaginal washes after two immunizations (Fig. 2a). After the third immunization, IgA titers increased, and mouse 1 developed spe-

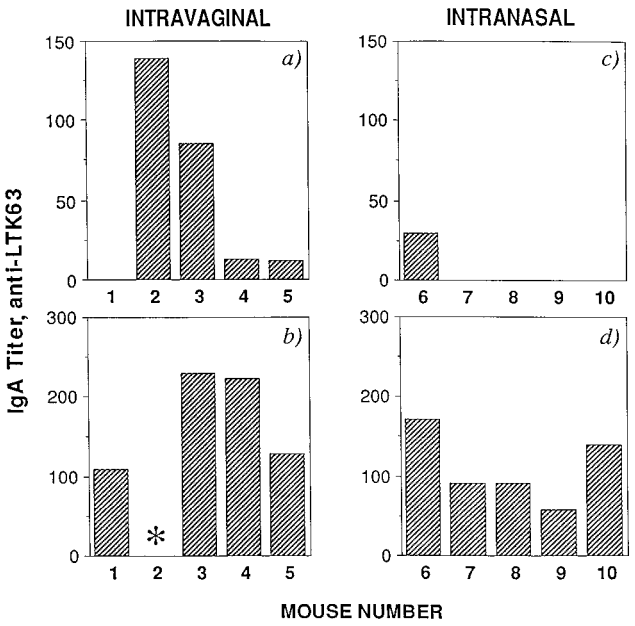

FIG. 2. Kinetics of appearance of anti-LTK63 IgA in vaginal secretions of mice immunized IVAG two (a) or three (b) times and mice immunized IN two (c) or three (d) times. Vaginal washes were performed 6 days after immunization. The asterisk indicates that the vaginal wash was not tested.

cific IgA (Fig. 2b). On the other hand, of the five mice immunized IN, only one had specific anti-LTK63 IgA in the vagina after the second immunization (Fig. 2c). However, all five IN immunized mice developed anti-LTK63 IgA in their vaginas after the third immunization (Fig. 2d). Remarkably, the IgA titers of these mice were comparable to those of the IVAG immunized mice (Fig. 2b). These results demonstrate that LTK63 is able to induce a local response in the vagina and also show that IN immunization induces antigen-specific IgA in the vagina, although the appearance of vaginal responses by this route is delayed. Indeed, two IVAG or three IN immunizations are required to induce anti-LTK63 IgA in vaginal secretions.

We next investigated the induction of LTK63-specific IgG in the vagina, since this antibody isotype is generally present in the vagina together with IgA (11). Table 1 reports the ratios of specific antibodies/total antibodies for both IgG and IgA in animals that received five immunizations. We found that both IVAG and IN immunized mice have anti-LTK63 IgG in vaginal washes and that the levels of specific IgG are similar in the two groups of mice. On the other hand, the level of specific IgA is higher, after five immunizations, in the group that received LTK63 IN. This could be due to the induction of a high number of ASC in the nasal mucosa that migrate to the vaginal wall. Furthermore, anti-LTK63 IgG and IgA induced by both routes of immunization decreased but still persisted in vaginal secretions up to 2 months following the last immunization (Table 1).

TABLE 1. Anti-LTK63 IgA and IgG in vaginal secretions

Immunization route	Ratio of specific antibodies/total antibodies (100)[a]			
	IgG		IgA	
	6 days	60 days	6 days	60 days
IVAG	38.9 ± 1.2	7.9 ± 1.8	42.6 ± 1.4	11.4 ± 1.9
IN	42.6 ± 1.4	3.8 ± 1.0	87.1 ± 1.1	54.9 ± 1.47

[a] Geometric mean ± standard deviation of ratio of LTK63-specific IgG (or IgA)/total IgG (or IgA) antibodies measured in mice 6 or 60 days after the fifth immunization.

TABLE 2. Levels of LTK63-specific IgA in body fluids of mice immunized IVAG and IN[a]

Immunization route and mouse no.	Ratio of LTK63-specific IgA/total IgA (100) (total IgA titer)			
	Vagina	Gut	Nose	Lung
IVAG				
1	80 (86)	10 (713)	0 (10)	0 (10)
2	79 (382)	7.1 (703)	0 (10)	0 (10)
3	13 (581)	4.4 (1271)	0 (26)	0 (23)
IN				
6	85.8 (368)	5 (788)	90 (44)	88 (202)
7	90 (30)	9.9 (522)	95 (20)	90 (419)
8	95 (422)	11.4 (719)	92 (66)	90 (106)
9	98 (89)	5.8 (357)	95 (17)	85 (40)
10	86.8 (61)	9.1 (360)	90 (23)	92 (119)

[a] Mice received a booster dose 2 months after the fifth immunization, and body fluids were collected 2 weeks later.

Altogether these results indicate that while IVAG and IN immunization routes induce similar levels of anti-LTK63 IgG in the vagina, repeated immunizations with LTK63 by the IN route are more effective in inducing a long-lasting anti-LTK63 IgA response in vaginal secretions.

Anti-LTK63 IgA in other mucosal sites. The observation that IN immunization with LTK63 induces a mucosal response in the vagina indicates that upon immunization, there is recirculation of antibodies or ASC from one compartment of the body to another, confirming the existence of a common mucosal system (10). Thus, we investigated first whether IVAG immunization could induce an anti-LTK63 mucosal response in the nose and second whether there was recirculation of specific IgA in other mucosal sites following both IN and IVAG immunization. The mice received a booster dose 2 months after the fifth immunization and were sacrificed 2 weeks later to collect the body fluids. The results in Table 2 show that following IVAG immunization, high levels of anti-LTK63 IgA are found mainly in vaginal washes and to a lesser extent in the gut. Nose and lung lavages are completely devoid of anti-LTK63 IgA, although they have measurable total IgA titers. On the other hand, IN immunization induces very high levels of specific IgA in the vagina, nose, and lung and lower levels in the gut. These results suggest that recirculation of antibodies or ASC in the mucosal system is somehow regulated.

Quantitation of cells secreting anti-LTK63 IgA and IgG in systemic and mucosal tissues. In parallel with the data shown in Table 2, the anti-LTK63 IgA and IgG ASC were measured by ELISPOT in the noses and spleens of the same mice (Fig. 3). In accordance with the results shown in Table 2, IN immunization induced very large numbers of IgA ASC in the nose and smaller but significant numbers in the spleen. A large number of IgG ASC was also present in the nose. Indeed, anti-LTK63 IgG antibodies were detected in nasal and lung washes of these mice (data not shown). In spite of the fact that anti-LTK63 antibodies were not detected in the noses of IVAG immunized mice (Table 2), some IgA and IgG ASC were found in the nasal tissues of these animals. Furthermore, IVAG immunization elicited similar numbers of IgA and IgG

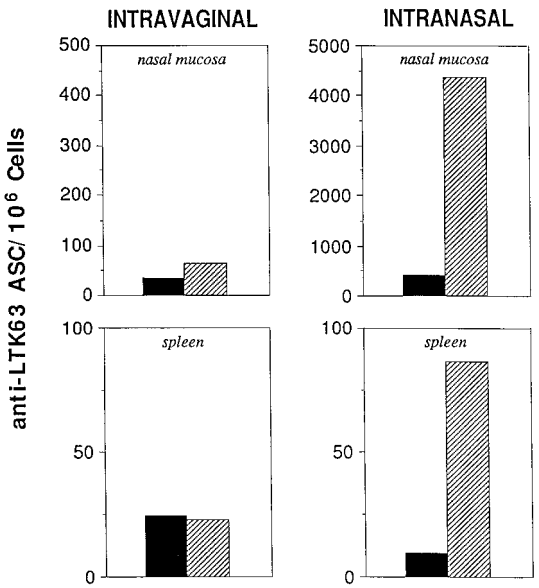

FIG. 3. Number of anti-LTK63 ASC per 10^6 cells in the noses and spleens of IVAG and IN immunized animals. Cells secreting IgG (■) and cells secreting IgA (▨) are shown.

ASC in the spleen. However, the number of IgA ASC was threefold higher in the spleens of IN immunized mice.

Adjuvant effect of LTK63 on systemic response to Ova. Once we assessed that IVAG delivered LTK63 is immunogenic in terms of both systemic and local responses, we investigated whether this molecule acted as a mucosal adjuvant for induction of a mucosal and systemic response to an antigen coadministered IVAG. We then analyzed the response to Ova in animals immunized with LTK63 and Ova or with Ova alone and compared the responses of the IN and IVAG immunized animals. The results in Fig. 4 show that both groups of mice developed a systemic response to Ova, although there were some differences between the two groups. The IN immunized animals (Fig. 4B) all developed an anti-Ova serum response after two immunizations and exhibited antibody titers that were generally higher than those of IVAG immunized animals. The IVAG immunized animals (Fig. 4A) were more heterogeneous in their responses. Generally, they required three to five immunizations to develop a serum response to Ova, depending on the individual mouse considered, although one mouse of this group developed anti-Ova antibodies after the second IVAG immunization. On the other hand, one mouse failed to respond to Ova even after five immunizations. The variability in antibody response within the group of IVAG immunized mice could reflect hormone influences on the immune response (15, 24), since the menstrual cycles of the animals were not synchronized. None of the IVAG immunized animals responded to Ova alone even after five immunizations (data not shown), whereas two of four IN immunized mice developed low anti-Ova titers (100 and 200) after the second immunization. These titers, however, did not increase during the course of the immunizations (data not shown). In conclusion, these results show that repeated IVAG delivery of Ova together with LTK63 as an adjuvant induces a systemic response to the antigen. Thus, LTK63 acts as a mucosal adjuvant for IVAG immunization.

TABLE 3. Anti-Ova IgA levels in vaginal secretions[a]

Immunization route	Mouse no.	Ratio of anti-Ova IgA/total IgA (100)
IVAG	1	0
	2	0
	3	5
	4	0
	5	0
IN	6	3
	7	22
	8	15
	9	0
	10	0

[a] Vaginal washes were performed 6 days after the fifth immunization. Mice immunized with Ova alone IVAG or IN did not have any anti-OVA IgA response in their vaginal secretions.

Anti-Ova IgA in vaginal secretions. To investigate whether LTK63 exerts an adjuvant effect locally at the site of immunization, we measured the anti-Ova IgA in vaginal washes following IVAG immunization and compared the response with that of IN immunized animals. Table 3 shows that only one of the IVAG immunized animals has 5% of Ova-specific IgA, whereas three of five IN immunized animals have 3 to 22%

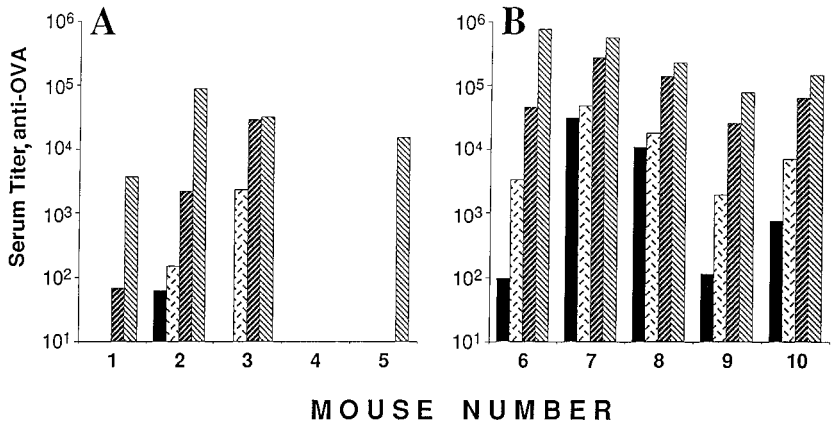

FIG. 4. Titers of anti-Ova total Igs in the sera of mice after two (■), three (▩), four (▨), or five (▧) immunizations with LTK63 plus Ova in IVAG (A) and IN (B) immunized animals. Mice immunized with Ova alone IVAG did not respond to the antigen. Two of four mice immunized IN with Ova alone developed a low anti-Ova response, which did not increase during the course of the immunizations (titers ranged between 100 and 200).

Ova-specific IgA in vaginal washes. Mice immunized IN or IVAG with Ova alone did not have any anti-Ova IgA in their vaginal secretions (data not shown). Thus, it can be concluded that IN immunization with the adjuvant LTK63 is more effective than IVAG immunization for induction of Ova-specific IgA in vaginal secretions. This suggests that the common mucosal system can be exploited for the induction of a local IgA response in the vagina by the IN route.

DISCUSSION

We have demonstrated that it is possible to induce a systemic response and a mucosal response in the vagina immunizing at mucosal sites with a nontoxic mutant of LT. Previous attempts to immunize mice IVAG with wild-type cholera toxin, another potent mucosal immunogen, stimulated a serum response but failed to induce local IgA (4). In this study, we used LTK63 not only as an immunogen but also as a mucosal adjuvant by assaying its capability to stimulate a response in the vagina to a coadministered antigen by both the IVAG and IN routes of immunization.

The availability of the nontoxic LTK63 mutant allowed us to use high concentrations, and we found that the optimal concentration for IVAG immunization was 20 μg. However, comparing the efficacy of the IVAG and IN routes, we found that IN delivery of a much lower dose (as little as 1 μg) of LTK63 (and lower doses of Ova) induces a higher response to LTK63, as well as to the coadministered antigen in vaginal secretions. This confirms that the female reproductive tract is a mucosal site where it is difficult to induce a local response and offers the prospective to exploit the common mucosal system for induction of an antigen-specific response in the vagina (25). Indeed, the advantages of the IN administration route are (i) lower adjuvant and antigen dosages, (ii) easier administration, (iii) induction of systemic and local responses higher than those achieved through IVAG immunization, (iv) long-lasting memory, and (vi) independence from sexual hormone influences. Indeed, it has been shown that the mucosal response in the vagina is very much dependent on the menstrual cycle (15, 24).

The relation between inductive and effector sites in the vaginal tissue is not yet clear. There is neither the presence of a histologically identified organized lymphoid tissue nor the presence of specialized M cells in the epithelium of the female reproductive tract (4). It has been speculated that under hormone influences, the epithelial cells of the vaginal wall, in some periods of the cycle, can sample antigens from the lumen and deliver them to the few macrophages, dendritic cells, and lymphocytes present in the lamina propria (13, 14). However, even the number of these lymphoid cells in the lamina propria may change during the menstrual cycle (14). Interestingly, in our case, whichever cells took part in the presentation and recognition of LTK63 in vaginal and nose tissues, both routes of immunization induced serum antibodies with neutralizing activity against wild-type LT.

Another issue that is not clear yet is whether vaginal IgG is produced by local B cells or whether they transude from the serum (11). Our results indicate that there is local production of IgG by B cells stimulated locally (in the IVAG immunized mice) or by cells that were primed in the nose and that migrated to the vagina. Three lines of evidence support these conclusions. (i) We found similar titers of anti-LTK63 IgG in the vaginal washes of animals immunized either IVAG or IN. If those IgG had transuded from the serum, then the IN immunized animals should have had higher titers of vaginal IgG, since they have higher titers of anti-LTK63 antibodies in the serum. (ii) The IVAG immunized animals have IgG ASC in the nose and spleen, indicating that these cells have been stimulated during local immunization. (iii) We did not find antigen-specific IgG in vaginal washes from mice immunized subcutaneously, in spite of a high titer of specific antibodies in the serum (not shown).

In conclusion, our results demonstrate that three to five IN immunizations with low concentrations of a soluble antigen plus LTK63 as an adjuvant represent an effective way to induce specific antibodies in the vagina. Whether the level of the antibodies elicited is enough to confer protection against pathogens has to be proven. Certainly, the ratios of specific/total antibodies we found in vaginal washes are quite high compared with those induced by IN immunization with a live vector, in which 8% of specific IgA against the outer surface protein A of *Borrelia burgdorferi* were found (7).

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