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Immune Response of the Female Rat Genital Tract after Oral and Local Immunization with Keyhole Limpet Hemocyanin Conjugated to the Cholera Toxin B Subunit

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The immune response of the female rat genital tract was evaluated with Lewis rats given primary and secondary immunizations with keyhole limpet hemocyanin (KLH) alone or coupled to the cholera toxin (CT) B subunit (CTB) by the oral or intravaginal-uterine route or a combination of routes. CT (2 to 5 μg) was administered as an adjuvant with the KLH-CTB conjugate. While a significant mucosal immunoglobulin A (IgA) response was induced by KLH, there were no significant differences among the immunized groups in the levels of IgA antibodies in salivary gland, gut, vaginal, and uterine secretions, with the exception that rats immunized only orally with the KLH-CTB conjugate lacked a detectable vaginal response. Levels of IgA antibodies to CT, however, were significantly increased in genital tract secretions of rats immunized locally versus orally with the KLH-CTB conjugate. Antibody activity of the IgG isotype against both KLH and CT was significantly elevated in genital tract secretions of rats immunized with KLH-CTB by the oral or intravaginal-uterine route and given genital tract boosters, in comparison with the results for the other groups. IgM antibody titers were generally negligible in the different secretions. An enzyme-linked spot-forming assay revealed IgA and IgG antibody-secreting cells in salivary gland and uterine tissues. A highly significant correlation between the numbers of antibody-secreting cells and antibody titers existed for uterine IgG but not IgA responses to KLH among the different groups of rats. In conclusion, a vigorous local immune response was induced after immunization of the female rat reproductive tract alone or in combination with peroral challenge with the KLH-CTB conjugate.

The induction of a strong immune response in the female reproductive tract has proven difficult, although natural immunity has been widely observed. Genital tract secretions from different female mammals, including humans, have been found to possess antibodies against various microorganisms as well as sperm in some infertile women (11). These antibodies, primarily of the immunoglobulin A (IgA) and IgG isotypes, originate from the circulation and also from plasma cells located in the genital tract mucosa (3, 16). The secretory activity of the latter appears to be under the influence of the gonadal hormones (25). IgA is found mostly in the secretory form in mucosal secretions from the genital tract as well as from other tissues and organs that constitute the common mucosal immune system (16). Major components of this system are the respiratory and gastrointestinal tracts and lacrimal, mammary, and salivary glands. Mucosal organs contain lymphoid tissue that is morphologically and functionally different from that of the spleen and lymph nodes. The lymphoid tissue in the intestines constitutes the largest mucosal immune system organ (3). This tissue is the major source of precursors for IgA-secreting plasma cells. The premise of the common mucosal immune system is that exposure to an antigen at one inductive site results in IgA-expressing B cells being dispersed to different mucosal effector sites, at which they mature into plasma cells and secrete specific antibodies of the IgA isotype. Adoptive transfer studies indicate that IgA-producing mesenteric lymph node cells preferentially repopulate mucosal surfaces, including the female genital tract, whereas IgG-producing cells migrate primarily to the peripheral lymph nodes (15).

IgA secreted by mucosal tissues is polymeric (pIgA), possesses J chains, and originates primarily from plasma cells located in the lamina propria (3, 16). There may also be some contribution, at least in rodents, of pIgA to the secretions from the circulation; circulatory pIgA is apparently transported across the epithelium by the same secretory component mechanism as that for pIgA produced locally by plasma cells (22). Intravenous injection of anti-influenza virus antibodies in mice revealed a specific transfer of pIgA into nasal secretions but a lack of transfer of monomeric IgA.

The induction of significant mucosal immune responses in the female reproductive tract by use of either systemic or local immunizations has proven difficult and requires the use of strong adjuvants, high doses of antigen, or numerous injections (19, 20, 26). A combination of systemic and local injection sites has proven effective in inducing uterine immunoglobulins in some studies (28).

Intragastric immunization with different types of antigens has resulted in specific antibodies appearing in genital tract secretions. Oral administration of sperm (1), bacteria (17), or viruses (2) in mice resulted in the appearance of IgA as well as IgG antibodies in vaginal washes.

The induction of mucosal immunity against most nonviable or soluble antigens has been difficult; however, the administration of antigen with cholera toxin (CT) or conjugated with the B subunit of CT (CTB) has been shown effective in inducing immune responses (4, 5, 23). CT not

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only is a strong oral immunogen but also has potent adjuvant activity (14).

The purpose of this study was to evaluate the magnitude of the immune response in the secretions and tissues of the genital tract and other mucosal organs and the blood as measured by levels of antibodies and antibody spot-forming cells (SFC) of different isotypes after immunization by the oral or local route with keyhole limpet hemocyanin (KLH) or a KLH-CTB conjugate.

MATERIALS AND METHODS

Animals. Female Lewis rats approximately 8 weeks old were obtained from Harlan Sprague-Dawley (Indianapolis, Ind.) for use in these studies. Immediately before the experiments were started, the estrous cycles of the rats were determined by use of vaginal smears and microscopic evaluation of vaginal cells.

Materials. KLH and bovine GM1 ganglioside were obtained from Calbiochem Corp. (San Diego, Calif.). CT and CTB were purchased from List Biological Laboratories, Inc. (Campbell, Calif.). 2,2-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), 5-bromo-4-chloro-3-indolylphosphate (BCIP), EDTA, nitroblue tetrazolium, *N*-succinimidyl 3-(2-pyridyldithio)-propionate (SPDP), phenylmethylsulfonyl fluoride (PMSF), hydrogen peroxide, avidin-horseradish peroxidase conjugate, and soybean trypsin inhibitor were from Sigma Chemical Co. (St. Louis, Mo.). Mouse anti-rat IgA antibodies were from Zymed Laboratories, Inc. (San Francisco, Calif.), and goat anti-rat IgG and IgM antibodies were from Southern Biotechnology Associates, Inc. (Birmingham, Ala.). Each antibody was heavy chain specific.

Antigen preparation. The CTB-KLH conjugate was prepared from equimolar amounts of CTB (2 mg) and KLH (12 mg) derivatized with SPDP at molar ratios of 10:1 and 3:1, respectively (23). The conjugate eluted mostly in the voided volume from a Superose 6 column (Pharmacia LKB Biotechnology, Piscataway, N.J.). An enzyme-linked immunosorbent assay (ELISA) (see below) done with plates coated with GM1 ganglioside and developed with rat antibody to KLH and then with a labeled antibody to rat immunoglobulin indicated that the conjugate retained the ability of CTB to bind to GM1 ganglioside and the antigenic property of KLH.

Alum-precipitated KLH (AL-KLH) was prepared by mixing equal volumes of 1% potassium alum with a 1% KLH suspension in phosphate-buffered saline (PBS) and adjusting the pH to 7.0. The final concentration used for immunization was 1 mg/ml.

Immunization. Five groups of rats were immunized as follows: group i, KLH given orally for priming and boosting (O-O), $n = 4$; group ii, KLH-CTB given orally for priming and boosting (O-O), $n = 5$; group iii, KLH-CTB given orally for priming and by the intravaginal-uterine route for boosting (O-VU), $n = 6$; group iv, KLH-CTB given by the intravaginal-uterine route for priming and boosting (VU-VU), $n = 6$; and group v, AL-KLH given by the intravaginal-uterine route for priming and boosting (VU-VU), $n = 5$.

Oral immunizations with 250 μg of KLH alone or conjugated to CTB in 250 μl of 0.2 M $NaHCO_3$ were given by gastric intubation with a 20-gauge feeding needle. The primary immunization consisted of two doses at an 8- to 10-day interval, and the secondary (booster) immunization was given 28 to 36 days later. Intravaginal-uterine immunizations with 100 μg of KLH as AL-KLH or conjugated to CTB in 100 μl of PBS were given at the proestrus or estrus stage of the cycle by use of a 1.5-in. (ca. 3.8-cm) blunt 20-gauge needle. The needle was carefully passed through the cervix into the uterine lumen, 50 μl was dispensed into the uterus, and the remaining 50 μl was placed in the vagina. When the needle could not easily be inserted into the uterus, the entire 100 μl was deposited in the vagina. A cotton plug was then placed in the vagina to retain the antigen solution. Three primary intravaginal-uterine immunizations were given at 4- to 5-day intervals and were followed 30 to 35 days later by two booster immunizations 4 to 5 days apart. CT was included as an adjuvant with the KLH-CTB conjugate at doses of 5 μg per oral immunization and 2 μg per intravaginal-uterine immunization. Each of the rats given intravaginal-uterine immunizations received at least one secondary uterine immunization and, when appropriate, one or more primary uterine immunizations.

Sample collection. Control blood samples were obtained from the rats by retro-orbital bleeding before immunizations were begun. For 3 days before the rats were killed, vaginal washes were obtained by repetitive washing (three or four times) of the vagina with 100 μl of PBS. A small portion of each sample was examined microscopically to determine the stage of the estrous cycle. After collection, the samples were centrifuged (1,000 × *g*, 10 min), the supernatants were removed, and sodium azide was added to each sample to a final concentration of 0.02%. The processed samples from each rat were pooled and stored frozen (−20°C) until assayed.

At 7 to 9 days after the last secondary immunization, the rats were anesthetized with Ketalar (10 mg of ketamine plus 1 mg of xylazine per ml) at 0.1 ml/100 g of body weight. They were then injected subcutaneously with pilocarpine HCl (1 mg/100 g of body weight) to stimulate the flow of saliva. Approximately 1 ml of saliva was collected per rat. Blood obtained by cardiac puncture was treated with heparin, diluted 1:3 with PBS, and processed to obtain the peripheral blood mononuclear cells by centrifugation over Histopaque-1077 (Sigma). The plasma was saved for antibody analysis. The anesthetized rats were killed by cervical vertebra dislocation, and the salivary glands, small intestine, and reproductive tract were excised.

After being freed of fat, connective tissue, and lymph nodes, the salivary glands were minced and digested five times with 0.15% Dispase (9).

The small intestine was trimmed of connective tissue and washed through with approximately 4 ml of buffered salt solution to collect the contents for antibody assays (7). The gut washes were thoroughly mixed, and tubes containing them were placed on ice until they were centrifuged at 4°C and 1,000 × *g* for 20 min. To each sample was added approximately 0.1 mg of soybean trypsin inhibitor, 5% 1 M EDTA, and 1% 0.1 M PMSF. The samples were recentrifuged at 2,000 × *g* for 20 min, the supernatants were removed, and additional PMSF, sodium azide, and fetal calf serum (FCS) were added to each sample to yield final concentrations of 1 mM, 0.02%, and 5%, respectively.

The lateral blood vessels of the uterus were perfused with PBS before the luminal contents of the uterine horns were obtained by inserting a blunt syringe needle through the cervical canal into each horn, clamping the cervix with a hemostat, and then washing the lumen with several injections and withdrawals of 400 μl of PBS. After centrifugation at 1,000 × *g* for 10 min, the samples were stored frozen until assayed. The uterus then was trimmed of extraneous tissue, blotted, and cut open longitudinally, and the cervix and uterine horns were cross-sectioned into approximately 5-mm slices. After being washed in cold PBS, the tissue fragments

were digested with 2 mg of collagenase and 5 μg of DNase per ml in RPMI 1640 culture medium buffered with 15 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (18). Three digestions of 30 min each were performed at 37°C with a magnetic stirring bar. The digested cell suspensions were allowed to settle in 15-ml conical centrifuge tubes for 15 to 20 min on ice, at which time the supernatants were centrifuged and the cells were washed with cold PBS by centrifugation (10 min, 200 × *g*) and stored on ice. Samples from individual rats were pooled, filtered through nylon filters, given a final wash, and resuspended in 10% FCS in RPMI 1640, and the lymphocytes were counted in a hemacytometer chamber.

Lymphocytes from the different sources were adjusted to a concentration of 10×10^6 cells per ml in RPMI 1640 with 10% FCS. When cell numbers from a particular tissue were inadequate, samples from two rats in the same group were pooled.

ELISPOT assay. The enzyme-linked spot-forming (ELISPOT) assay was done with 96-well plates with a nitrocellulose base (Millititer HA; Millipore Corp.) and coated with KLH (20 μg/ml), GM1 (2.5 μg/ml) and then CT (1 μg/ml), or affinity-purified goat $F(ab')_2$ anti-rat IgA, IgG, or IgM (5 μg/ml) (4, 13, 23). After blocking was done with 5% FCS in RPMI 1640, cell suspensions from blood, salivary glands, and uterus were added, and the plates were incubated for 4 h at 37°C in a humidified atmosphere of 5% CO_2. The plates were washed with PBS and then with PBS–0.4% Tween 20 (PBS-T), and then biotinylated anti-rat immunoglobulin class-specific antibodies were added to the wells for overnight incubation at 4°C. The labeled antibody dilutions were determined in preliminary trials. After the plates were washed, streptavidin-alkaline phosphatase (0.5 μg/ml) was added, and the mixtures were incubated for 60 min at room temperature. The spots representing antibody-secreting cells were developed with nitroblue tetrazolium-BCIP substrate in 0.1 M bicarbonate buffer (pH 9.8) containing 1 mM $MgCl_2$. After the spots were developed to optimal intensity (approximately 20 min), the reaction was stopped by a tap water wash. The plates were dried, and the spots were counted under a dissecting microscope.

ELISA. Samples obtained on the day the rats were killed as well as some preimmune serum and vaginal wash samples were assayed for antibody activity of the IgA, IgG, and IgM isotypes against KLH and CT by a solid-phase ELISA (4, 8, 10). Sample dilutions were incubated in 96-well microELISA plates Immulon 4; Dynatech) coated with either KLH or CT. KLH was used at a concentration of 20 μg/ml of PBS containing 0.02% NaN_3. CT-coated plates were prepared by coating the wells overnight with 100 μl of GM1 at 5 μg/ml in 0.1 M carbonate-bicarbonate buffer, washing the wells with PBS-T, and then adding 100 μl of CT at 1 μg/ml in PBS-T to the wells for 4 h at room temperature. The wells were blocked with 5% FCS in PBS–0.02% NaN_3 for 60 min at 37°C before being washed with PBS-T, and duplicate dilutions of primary antibody samples were added. After incubation, the wells were washed with PBS-T, and biotinylated anti-immunoglobulin antibodies were added at predetermined optimal dilutions. Avidin-horseradish peroxidase conjugate was added to the wells and incubated for 60 min, the wells were washed with PBS, and ABTS-hydrogen peroxide substrate was added. The plates were read with a V_{max} microtiter plate reader (Molecular Devices Co., Palo Alto, Calif.) at 414 nm.

In each plate, a positive standard sample was run to provide valid comparisons between plates for each immunoglobulin isotype and antigen. Initially, serum, gut wash, and saliva samples from the experimental groups expected to have the highest antibody titers were screened to provide a standard for each immunoglobulin isotype against the two antigens. Each reference sample was assigned a value of 1,000 ELISA units of anti-KLH and anti-CT antibodies per ml for each isotype, and these samples were used to generate standard curves. The absorbances of the different samples were determined and converted into ELISA units per milliliter by use of a computer program based on the four-parameter logistic plot. The ELISA and ELISPOT results were transformed to logarithm base 10 for graphing and statistical analysis. Samples with antibody titers below detectable levels were arbitrarily assigned a value of 1 $\log_{10}$ unit for the purpose of analysis. In the figures, the data are presented as means ± standard errors of the mean $\log_{10}$ titers, with differences determined by signed rank test. Analysis of variance, Student *t* test, Wilcoxon signed rank test, and correlation analyses were performed by use of the University of Michigan Interactive Data Analysis computer program.

RESULTS

The majority of the rats (21 of 26) were in the proestrus or estrus stage of the estrous cycle when killed. The remaining five rats (one each in the KLH-CTB groups and two in the AL-KLH group) had vaginal smears suggesting mixed diestrus-proestrus stages.

The ELISA results revealed that IgA anti-KLH antibodies were not detected at dilutions of ≥1:9 in any preimmune sera and were detected in the sera of only four rats after immunization: two in the KLH group (O-O) and two in the KLH-CTB groups (O-O and VU-VU). KLH immunization induced measurable levels of IgA antibodies in all of the secretions evaluated. The mucosal IgA responses were not significantly affected by the different immunization regimens, except for the lack of detectable antibody levels in the vaginal washes (diluted ≥1:10) of rats receiving KLH-CTB (O-O) and reduced antibody levels in the saliva of rats receiving AL-KLH (VU-VU) (Fig. 1). The uterine responses were not significantly different among the groups. Preimmune sera and vaginal washes at dilutions of 1:10 lacked detectable IgA anti-KLH antibodies.

The IgG response against KLH, however, was significantly elevated in the sera and secretions of rats immunized by the intravaginal-uterine route with KLH-CTB (Fig. 2). Overall, the IgG antibody levels were lowest in the saliva and gut washes and highest in the uterine washes. Compared with preimmune sera, which showed negative titers at dilutions of ≥1:90, sera from all the immunized groups exhibited high titers of IgG anti-KLH antibodies, except for sera from rats immunized by the oral route with KLH, which exhibited low titers.

Only low levels of IgM anti-KLH antibodies were present in the sera, gut washes, and uterine washes of the different groups of rats (Fig. 3). None of the saliva samples and only one of the vaginal wash samples possessed detectable IgM antibody levels. The only positive uterine wash samples were from rats immunized locally in the genital tract with KLH-CTB.

All of the secretions from the rats not receiving CTB and CT assayed negative for anti-CT antibodies of the three isotypes. Assay of the serum samples from the different groups of rats and preimmune serum samples revealed no significant differences in the levels of IgA antibodies against

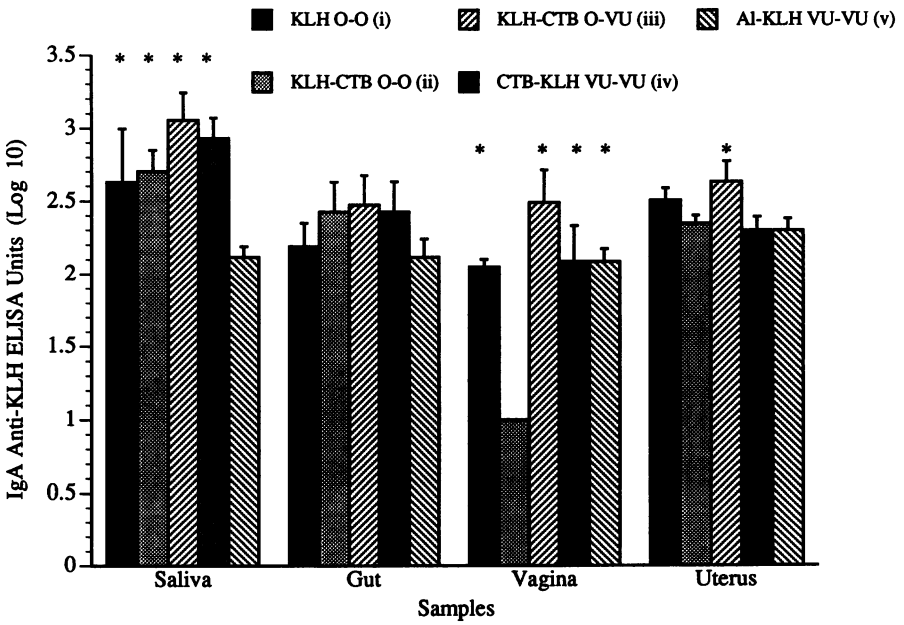

FIG. 1. IgA antibody responses against KLH in secretions from rats immunized with KLH by the oral and/or intravaginal-uterine routes. All preimmune serum and vaginal samples and most immune serum samples were negative at the dilutions assayed. Bar graphs without error bars represent experimental groups with antibody titers below the limits of detection. Group differences (asterisks) for antibody titers in the various secretions were as follows: saliva, groups i to iv > group v ($P < 0.05$); vagina, groups i and iii to v > group ii ($P < 0.01$) and group iii > groups i and v ($P < 0.05$); uterus, group iii > groups ii, iv, and v ($P < 0.05$).

CT (Fig. 4). The salivary gland and intestinal responses were similar among the rats immunized by the different schedules with KLH-CTB, whereas the vaginal and uterine secretions from rats given O-O immunizations possessed low IgA anti-CT antibody activity compared with that found in rats given O-VU or VU-VU immunizations.

IgG antibodies against CT were found in high levels in serum samples from rats immunized with KLH-CTB (Fig. 5). However, antibodies were undetectable in dilutions (≥1:90) of all preimmune sera and sera from rats immunized only with KLH. Secretions from the salivary glands, gut, and vagina possessed low levels of antibody activity, with the trend for the titers to be higher in rats given O-VU VU-VU immunizations. This in IgG anti-CT antibodies increase was significant in the uterine washes from these two groups with intermediate antibody titers.

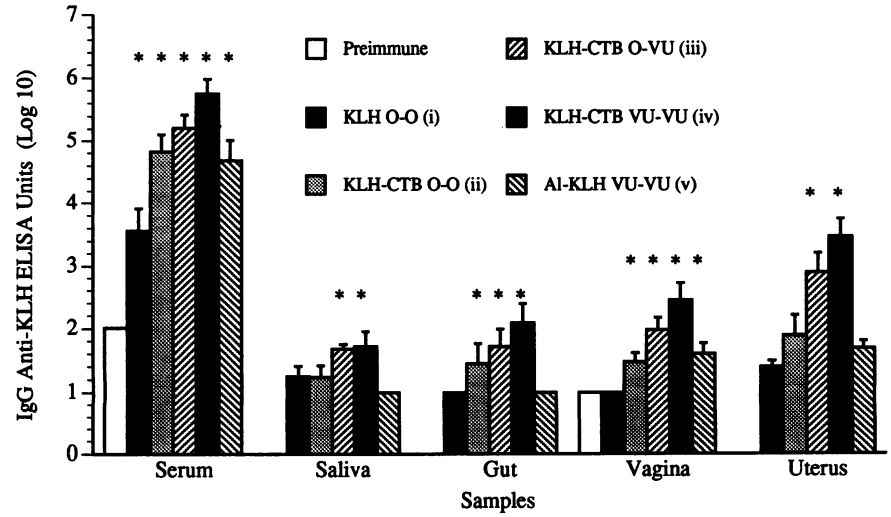

FIG. 2. IgG antibody responses against KLH. Bar graphs without error bars represent experimental groups with antibody titers below the limits of detection. Group differences (asterisks) for antibody titers in serum and the various secretions were as follows: serum, group iv > groups ii, iii, and v > group i > preimmune sera ($P < 0.05$); saliva, groups iii and iv > groups i, ii, and v ($P < 0.05$); gut, groups ii to iv > groups i and v ($P < 0.01$); vagina, groups ii to v > group i and preimmune sera ($P < 0.01$) and group iv > groups ii, iii, and v ($P < 0.05$); uterus, groups iii and iv > groups i, ii, and v ($P < 0.01$).

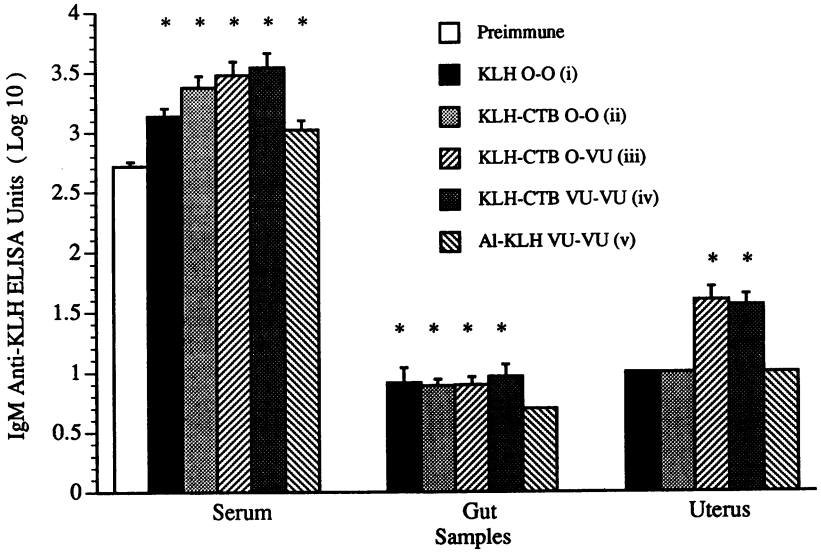

FIG. 3. IgM antibody responses against KLH. Antibody titers were not detected in salivary gland or vaginal secretions. Bar graphs without error bars represent experimental groups with antibody titers below the limits of detection. Group differences (asterisks) for antibody titers in serum and the various secretions were as follows: serum, groups ii to iv > groups i and v > preimmune sera ($P < 0.01$); uterus, groups iii and iv > groups i, ii, and v ($P < 0.01$).

Levels of circulating antibodies of the IgM class against CT were significantly increased in samples from rats immunized with KLH-CTB in comparison with samples from rats immunized with KLH only or preimmune serum samples (Fig. 6). The salivary gland response was marginal but significant compared with that in non-CTB-immunized rats. As with the anti-KLH IgM antibody response, the IgM antibody response to CT in the uterus was detectable only in rats given the CTB-KLH conjugate locally in the genital tract. IgM antibodies were undetectable in the gut and vaginal washes at the dilutions examined (1:5 and 1:10, respectively).

Multiple correlation coefficients among the variables revealed that vaginal levels of IgA antibodies against KLH and CT were highly associated ($P < 0.01$) with concentrations of IgA antibodies in uterine fluids, whereas the vaginal levels of

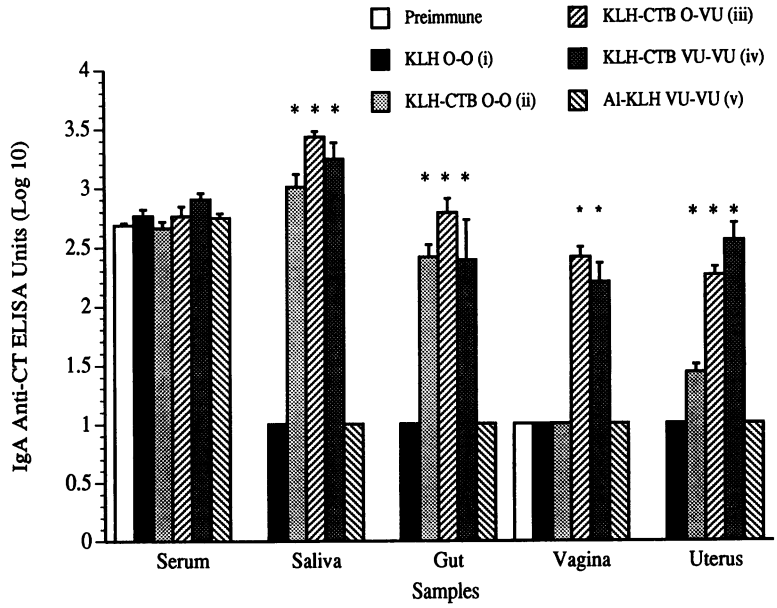

FIG. 4. IgA antibody responses against CT in serum and secretions of rats from groups i to v. Non-CTB-immunized rats served as controls for anti-CT responses. Bar graphs without error bars represent experimental groups with antibody titers below the limits of detection. Group differences (asterisks) for antibody titers in the various secretions were as follows: saliva and gut, groups ii to iv > groups i and v ($P < 0.01$) and group iii > group ii ($P < 0.05$); vagina, groups iii and iv > groups i, ii, and v and preimmune sera ($P < 0.01$); uterus, group iv > group iii > group ii > groups i and v ($P < 0.01$).

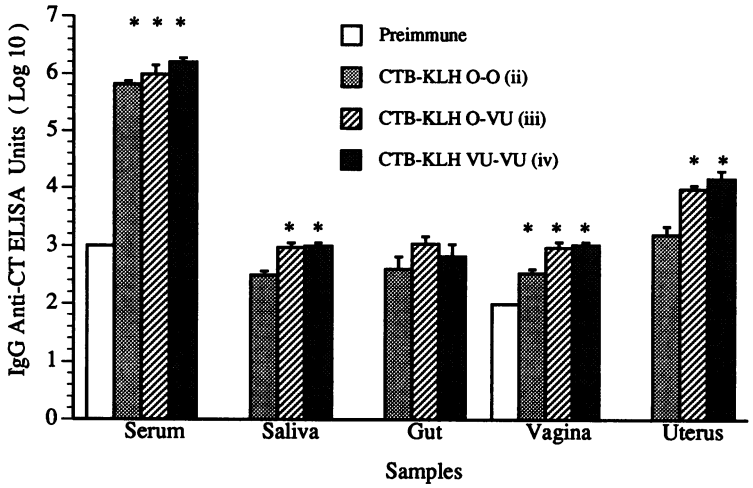

FIG. 5. IgG antibody responses against CT (groups ii to iv). Bar graphs without error bars represent experimental groups with antibody titers below the limits of detection. Group differences (asterisks) for antibody titers in serum and the various secretions were as follows: serum, groups ii to iv > preimmune sera ($P < 0.01$); saliva, groups iii and iv > group ii ($P < 0.01$); vagina, groups iii and iv > group ii > preimmune sera ($P < 0.01$); uterus, groups iii and iv > group ii ($P < 0.01$).

IgG antibodies were more closely associated with titers found in the serum of rats in groups ii to iv (Table 1). Levels of both uterine IgA and IgG antibodies were more closely associated with titers found in the saliva than in the serum. There was a lack of significant correlation of antibody titers in the genital tract and those in the gut washes.

The results of the ELISPOT assays are shown in Fig. 7 and 8. The assay for CT-specific SFC was not performed for cells from rats not immunized with CTB or for anti-CT IgG- and IgM-producing cells from rats immunized orally with the conjugate because of insufficient cell numbers. There were few SFC specific for either KLH or CT in the peripheral blood 7 to 9 days after the last immunization. The number ranged from 1 to 30 specific SFC per 10^6 peripheral blood mononuclear cells, a number 4 to 12% of the number recovered from uterine tissue. Overall, the numbers of SFC of different isotypes in the different tissues showed a wide variation, except for the IgG SFC activity against KLH and CT of the uterine lymphocytes. Of the lymphocytes recovered from the different sources, those from the uterus possessed the largest number of total IgG SFC and overall the largest number of cells forming spots against KLH and CT. In general, cells from the tissues showed more SFC activity against CT than against KLH for the three immunoglobulin isotypes, with the exception of the anti-KLH IgG SFC from the uterus, especially in rats immunized via the

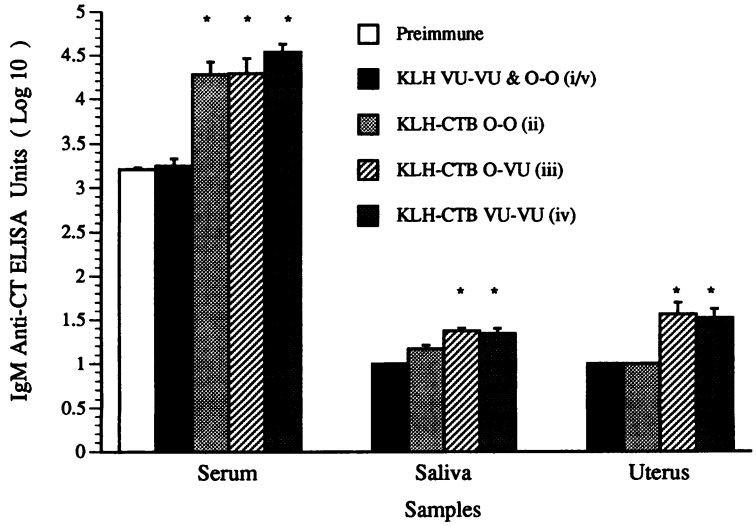

FIG. 6. IgM antibody responses against CT (groups ii to iv). Samples from non-CTB-immunized rats served as controls. No titers were detected in gut or vaginal secretions. Bar graphs without error bars represent experimental groups with antibody titers below the limits of detection. Group differences (asterisks) for antibody titers in serum and the various secretions were as follows: serum, groups ii to iv > group i or v and preimmune sera ($P < 0.01$); saliva, groups iii and iv > group ii > group i or v ($P < 0.05$); uterus, groups iii and iv > group i or v and ii ($P < 0.01$).

TABLE 1. Significant simple and multiple correlation coefficients ($P < 0.05$) among ELISA antibody titers for serum and salivary, vaginal, and uterine secretions

Antibody response	Correlation type	Independent variable (serum or secretion)	Correlation coefficient for the following dependent variable (secretion):			
			Groups ii to iv[a]		Groups i and v[b]	
			Vaginal	Uterine	Vaginal	Uterine
IgA anti-KLH	Simple	Salivary	0.48	0.76	NS[c]	NS
		Uterine	0.66		−0.86	
	Multiple	Salivary	NS	0.65	NS	NS
		Uterine	0.66		−0.86	
		R^{2d}	56	44	76	NS
IgG anti-KLH	Simple	Serum	0.92	0.82	NS	0.71
		Salivary	0.79	0.85	NS	NS
		Uterine	0.80		NS	
	Multiple	Serum	0.92	NS	NS	0.71
		Salivary	NS	0.85	NS	NS
		Uterine	NS		NS	
		R^2	85	72	NS	50
IgA anti-CT	Simple	Salivary	NS	0.50		
		Uterine	0.78			
	Multiple	Salivary	NS	0.50		
		Uterine	0.78			
		R^2	61	25		
IgG anti-CT	Simple	Serum	0.74	0.55		
		Salivary	0.84	0.82		
		Uterine	0.77			
	Multiple	Serum	0.60	NS		
		Salivary	0.84	0.82		
		Uterine	NS			
		R^2	81	67		

[a] Groups ii to iv were immunized with KLH-CTB conjugate plus CT.
[b] Groups i and v were immunized with KLH without CTB but with CT.
[c] NS, not significant.
[d] R^2 is the percent variation in the dependent variable because of significant associations with independent variables.

genital tract. For the three CTB-immunized groups, there was a correlation coefficient of 0.91 ($P < 0.10$) between the averaged values of the ELISA and ELISPOT assays for anti-KLH IgG in the uterine samples.

DISCUSSION

The capability to stimulate a strong immunologic response in the female genital tract has clinical applications in the prevention of sexually transmitted diseases as well as fertility control. Our study suggested that conjugating an antigen to CTB and including a small amount of CT per immunization resulted in an increased immune response in the female reproductive tract. The elevated response, which was primarily of the IgG class of antibodies, tended to be highest in rats receiving both the primary and the secondary immunizations in the genital tract, although a combination of oral and local immunizations also was successful. That the increased levels of IgG antibodies may have originated not only from serum but also from antibody-secreting cells in the mucosa is suggested by two observations: first, the numbers of KLH- and CT-specific IgG SFC were increased in the uterine tissue of the responding rats, and second, titers of IgG antibodies in the uterine tissue were more closely associated with salivary gland IgG titers than with blood IgG titers. In addition, the low levels of specific SFC in the peripheral circulation relative to the levels detected in the uterine tissue reduced the possibility that there was gross contamination with blood components.

The titers of IgA antibodies against KLH suggest that all of the immunization schemes were successful in inducing similar mucosal immune responses in the uterus but not in the vagina. Vaginal antibody titers in rats immunized only orally with the KLH-CTB conjugate were below assay limits for IgA antibodies against both CT and KLH. In addition, the levels of uterine anti-CT IgA antibodies were significantly lower in rats given O-O immunizations. This observation agrees with those of other studies suggesting that secondary responses are achievable at mucosal sites other than the site primed with an antigen but that the maximal response to a secondary challenge usually occurs at the originally primed mucosal surface (24, 28). Studies with mice have shown that the induction of IgA responses in the genital tract occurs after oral immunization, intranasal challenge, or repetitive vaginal immunization with large antigen loads (19, 26, 29). These studies with mice have also suggested that the induction of IgG antibodies requires the use of an adjuvant with either local or systemic immunizations. Our results

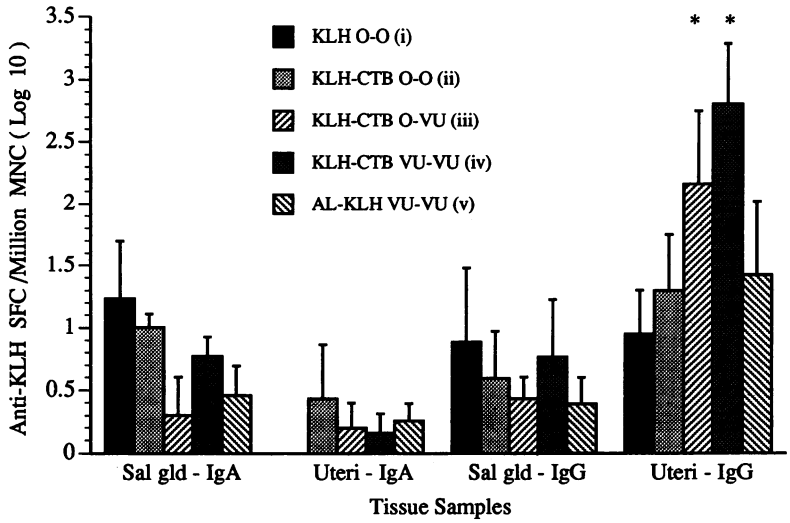

FIG. 7. ELISPOT data for anti-KLH IgA and IgG SFC per 10^6 mononuclear cells (MNC) from salivary gland (Sal gld) and uterine (Uteri) samples. Group differences (asterisks) in the numbers of SFC were as follows: uterus (IgG), groups iii and iv > group i ($P < 0.05$) and group iv > group ii ($P < 0.05$).

support an earlier report (19) suggesting that immunoglobulins in vaginal fluids of mice have different origins depending on isotype, in that IgA arises largely from uterine or cervical secretions and that IgG arises from serum.

Antibodies of the IgM class, which were present at low titers, also tended to reflect the enhanced immune response occurring in the uteri of rats immunized locally in the genital tract with the KLH-CTB conjugate. None of the other groups had detectable titers of uterine IgM antibodies.

It is of interest to note that the antibody titers in serum and the different secretions against both KLH and CT antigens were consistently highest in the two groups immunized via the intravaginal-uterine route with KLH-CTB. There exist at least two explanations that are not mutually exclusive: (i) the inclusion of the genital tract in the common mucosal immune system, so that immunization at one site results in immunoglobulin-secreting cells migrating to other mucosal sites; and (ii) ingestion by rats immunized in the genital tract of antigen draining from the external genitalia during the cleaning and grooming process. It is likely that both of these events occurred. However, it seems improbable that the rats given VU-VU immunizations consumed sufficient antigen to exhibit equal or more pronounced immune responses in the gut and salivary glands than the rats given O-O immunizations.

For mice, oral administration of KLH has been reported to induce oral tolerance (6). Although we did not assay for

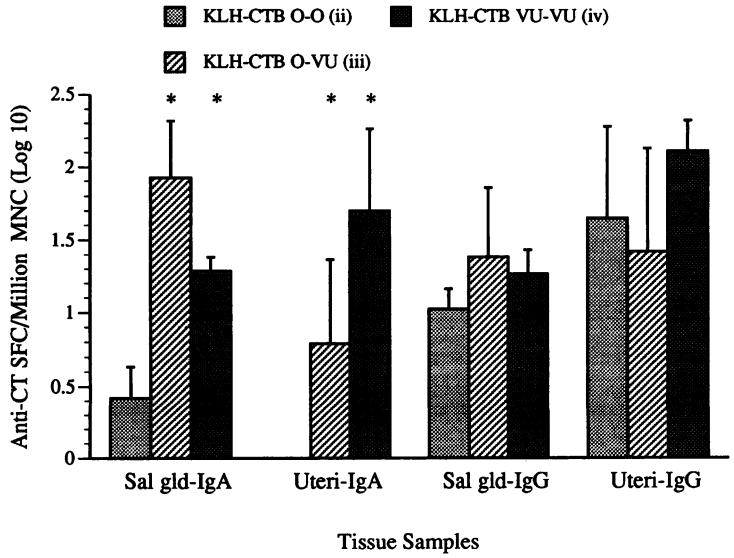

FIG. 8. ELISPOT data for anti-CT IgA and IgG SFC per 10^6 mononuclear cells (MNC) from salivary gland (Sal gld) and uterine (Uteri) samples. Group differences (asterisks) in the numbers of SFC were as follows: salivary gland (IgA), group iii > group iv > group ii ($P < 0.05$); uterus (IgA), groups iii and iv > group ii ($P < 0.05$).

oral tolerance induction, we did find that rats given only KLH orally responded with IgA antibodies in the secretions assayed. However, IgG antibody levels were significantly lower in all secretions except saliva in these rats (group i) than in rats of the other groups.

In the present study, coupling KLH with CTB did not have a marked effect on IgA antibody titers against KLH, as has been noted with some other antigens. This result may be due in part to the immunogenicity and dose of KLH compared with those reported in other studies. Those studies noted increased mucosal levels of IgA and serum IgA and IgG titers in response to antigens conjugated to CTB and given orally (4, 5, 23). The CTB-CT combination has a synergistic effect in the induction of a mucosal immune response of the intestine against KLH in orally immunized mice (27). The enhanced adjuvant effect has been ascribed to the binding of CTB to GM1 on the epithelial cell surface, resulting in increased antigen presentation and also possibly inhibiting suppressor cell activity (5). The enhanced mucosal immune response is thought to be due to increased uptake of the coupled antigen and an adjuvant effect of CT.

Although the IgA response against KLH in the genital tract was not significantly affected by the conjugate, the local and systemic levels of IgG were dramatically increased. The rat uterus was previously reported to be inefficient as an antigen delivery site for primary immunizations; however, intrauterine deposition of alum-precipitated dinitrophenyl-bovine gamma globulin primed the rats sufficiently to produce strong systemic secondary responses to soluble antigen placed in the uterus (12), whereas secondary immunizations at other sites in rats primed in the uterus induced lower antibody titers. Similarly, immunization of uterine horns of rats with sheep erythrocytes was more effective in inducing IgA and IgG antibodies in uterine secretions than Peyer's patch immunization alone or followed by an intrauterine challenge (28). Our results suggested that the maximal genital tract immune response occurred after local primary and secondary immunizations. These results agree with reports for other mucosal tissues (21, 24).

In conclusion, the use of CTB-antigen conjugates administered either locally only in the genital tract or in the genital tract after oral immunization appears to result in mucosal immune responses in the female genital tract consisting of high levels of IgA and IgG antibodies. The origin of the IgG antibodies is presently unclear. Other studies have reported that CTB-antigen conjugates result in prolonged immune responses and elevated secondary responses to later challenges with the antigen (23, 24).

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