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Induction of Specific Immunoglobulin A in the Small Intestine, Colon-Rectum, and Vagina Measured by a New Method for Collection of Secretions from Local Mucosal Surfaces

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In order to study patterns of local antibody responses following mucosal immunization of mice via different routes, a method for collection of secretions directly from mucosal surfaces was developed. Mice were immunized on days 0, 10, 17, and 24 by administration of cholera toxin into the oral cavity, stomach, colon-rectum, or vagina. At sacrifice on day 32, absorbent wicks were placed in the oral cavity and, via an applicator tube, into the vagina and distal colon-rectum and along the entire small intestine after flushing of luminal contents. Protein was quantitatively extracted from wicks, and specific anti-cholera toxin immunoglobulin A (IgA) and IgG were measured by enzyme-linked immunosorbent assay. Concentrations of specific IgA in secretions at various mucosal sites were dramatically influenced by the route of immunization. Oral immunization effectively induced IgA in saliva, and the intragastric route was optimal for induction of IgA in the small intestine. High levels of specific IgA appeared on the colonic-rectal mucosal surface only after rectal delivery of antigen. Oral, gastric, and rectal immunizations also produced distant responses in the vagina. Following vaginal immunization, however, neither local nor distant IgA responses were detected. These results suggest that vaccines intended for protection of colonic-rectal and vaginal mucosal surfaces might best be administered by the rectal route.

Uptake and sampling of antigens in the mucosal immune system occur locally, at specific sites on mucosal surfaces that are identified by the presence of organized lymphoid follicles and a specialized follicle-associated epithelium containing M cells (6, 21, 34, 38). In the digestive tract, these "inductive sites" are clustered in tonsils, intestinal Peyer's patches, and the appendix and are also distributed as isolated follicles throughout the small and large intestines (42, 43). In humans, isolated lymphoid follicles associated with specialized epithelia are numerous in the lower colon and rectum (26, 41). In mice, aggregated lymphoid follicles have also been demonstrated in the colon and rectum (43). In both mice and humans, the secretory immune response to foreign antigens and microorganisms may be detected both at the site of initial sampling and in distant mucosal and glandular tissues (6, 31, 34). This is apparently due to dissemination via the bloodstream of effector and memory cells which migrate into mucosal and glandular connective tissues throughout the body, where they differentiate into plasma cells that produce immunoglobulin A (IgA), the effector molecules of mucosal secretions (reviewed in references 6, 21, and 34).

Although the existence of a common mucosal immune system is well documented (7, 10, 11, 15, 17, 29, 50), there is evidence that local exposure to an antigen can result in much higher levels of specific secretory IgA in the region of exposure than at distant sites (36, 39). Even within the intestine, administration of cholera toxin (CT) into proximal or distal segments evoked the highest levels of specific antitoxin secretory IgA in the segment of antigen exposure (47), and antibodies to poliovirus were most abundant in the region of the colon which had been selectively immunized (39). This regional nature of the mucosal immune response may be functionally important, since it would concentrate production of secretory IgA at the site of potential microbial colonization or invasion (31).

Induction of specific IgA in rectal and genital secretions may be particularly important in protection against sexually transmitted pathogens such as the human immunodeficiency virus (16, 32). Alternate immunization routes for induction of IgA in the female reproductive tracts of rodents, monkeys, and humans have been tested, but the optimal route is not established (23, 24, 27). Intravaginal immunization with live microorganisms or virus-infected cells has produced local antibody responses in humans (40) and mice (30), but in most studies, the response to local genital immunization was poor (23, 45, 51). Combined oral and vaginal immunizations have induced significant local mucosal responses in the genital system (9, 27, 28), but the role of the genital mucosa in induction of these responses was not clear. The route of mucosal immunization that is most effective for induction of local immunity in the colon and rectum has received much less attention. The future design of mucosal immunization strategies will require more detailed information about the relationship between inductive and effector sites in these mucosal tissues.

A major obstacle to obtaining such information from the digestive tract has been the technical difficulty of collecting secretions from mucosal surfaces and distinguishing locally secreted IgA from that produced at distant sites. In humans,

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intestinal secretory immune responses have been assessed by sampling of feces (18, 19) or measurement of specific IgA in saliva or small intestinal fluids (17), and mucosal immune responses have been monitored by quantitation of IgA-producing cells in blood (10, 11, 15). In experimental animals, intestinal secretory IgA is usually collected by flushing of luminal contents at sacrifice (33) or secretagogue-stimulated intestinal lavage in vivo (14). These methods provide an overview of IgA in the luminal contents of the entire gastrointestinal tract but do not provide information about concentrations of specific IgA on local epithelial surfaces that are vulnerable to region-specific pathogens.

We therefore sought to develop a method for direct retrieval of mucous secretions and fluid associated with epithelial surfaces of the gastrointestinal system and vagina. This required identification of a suitable absorbent filter material that could be applied directly to these mucosal surfaces without epithelial damage and from which IgA could be quantitatively eluted. Absorbent discs made of synthetic fibers were previously used for collection of buccal and nasal secretions in humans (22). We used an analogous material to design absorbent wicks and applicators for collection of local secretions from intestinal and vaginal mucosae. Using this approach and CT as a test antigen, we have shown that the levels of specific IgA associated with local mucosal surfaces of the gastrointestinal and female genital tracts are highly dependent on the route of mucosal immunization and that high secretory IgA levels on the rectal mucosal surface can be most readily achieved by rectal immunization.

MATERIALS AND METHODS

Animals. Female BALB/c mice used in immunization studies and in the development of the wick procedure were 5 to 8 weeks of age and were obtained from Charles River Laboratories, Wilmington, Mass.

Immunizations. CT type Inaba 569B (Calbiochem Corp., La Jolla, Calif.) was dissolved in sterile water (1 mg/ml), divided into aliquots, immediately frozen in liquid nitrogen, and stored at −80°C until used for immunizations or immunologic assays. Four groups of mice, originally consisting of five mice each, were immunized with CT on day 0 (20 μg), day 10 (10 μg), day 17 (10 μg), and day 24 (10 μg). CT was delivered to each of the groups by either the oral, gastric, rectal, or vaginal route. For oral immunization, 20 μl (initial immunization) and 10 μl (boost) of antigen solution were delivered with a micropipette as the mice sucked the fluid from the tip. For gastric immunization, the antigen was diluted to a volume of 200 μl in 0.1 M sodium bicarbonate, (pH 8.1) and delivered by a blunt steel feeding tube (Popper & Sons, New Hyde Park, N.Y.). For colonic-rectal immunization, the antigen was diluted with phosphate-buffered saline (PBS) (pH 7.2), and volumes of 40 to 100 μl were delivered by a feeding tube inserted via the anus until the tip was approximately 3 cm from the anal opening. An undetermined fraction of the antigen solution in the first three doses was lost by leakage; for the fourth (last) dose, 10 μl containing 10 μg of the antigen was therefore given without further dilution. For vaginal immunization, 20 μl (initial immunization) and 10 μl (boost) were delivered into the vagina by a micropipette.

A high initial dose of CT was used to increase the likelihood of inducing immune responses by the rectal and vaginal routes. Oral delivery of 20 μg of CT is known to be potentially toxic in mice; indeed, two of five animals immunized orally died within a few days, and the other three showed signs of illness after the first dose. Subsequent administration of 10 μg to these three mice, however, had no visible effect. CT delivery by other routes, including gastric administration in bicarbonate buffer, had no apparent effect; thus, the toxicity of oral CT may have been partially due to toxin action on the respiratory epithelium. Mice receiving rectal or vaginal immunizations were briefly anesthetized by ether inhalation before the first dose, but subsequent doses were given without anesthesia and produced no evidence of damage or discomfort. Two mice died from anesthetic overdose, however, during sampling of blood. Thus, 16 mice in four immunization groups (three oral, five gastric, four rectal, and four vaginal) underwent the complete analysis that is reported here.

Collection of secretions. In a series of pilot experiments, filter materials of 10 types from three commercial sources were tested by application of preweighed filter disks to the surfaces of mouse intestinal mucosa that had been cleared of luminal contents. The filters varied widely in their abilities to efficiently absorb fluid and mucus and to release adsorbed proteins during subsequent elution with a small volume of PBS. The only filter material that fulfilled the criteria consisted of a mixture of synthetic fibers and cellulose produced by Polyfiltronics Group, Inc., Rockland, Mass. Cylindrical wicks composed of this material were custom made by Polyfiltronics and were used to collect samples of secretions. The dimensions of the wicks were 3 by 10, 2 by 25, and 2 by 5 mm for salivary, intestinal, and vaginal insertion, respectively. Glass tubes, 9 to 10 cm long and with an outer diameter of 3.8 mm (custom made by the Biomedical Engineering Department, Children's Hospital, Boston, Mass.), were used as applicators for insertion of the wicks into segments of the gut or into the vagina. Wicks to be used for collection of secretions were placed in separate 1.5-ml microcentrifuge tubes, and the weight of each tube containing a dry wick was recorded. The weights of the captured secretions were calculated as the differences between the weights before and after collection.

For collection of secretions, mice were anesthetized with tribromoethanol (Chemical Dynamics Corp., South Plainfield, N.J.) which was dissolved in tertiary amyl alcohol (2:1, wt/wt) and diluted 1:80 in PBS, pH 7.2. Each animal received 0.5 ml injected intraperitoneally. Salivation was induced by a single intraperitoneal injection of 0.1 mg of pilocarpine-HCl (Sigma Chemical Co., St. Louis, Mo.) in 100 μl of PBS. Saliva was collected by inserting the tip of a wick into the mouth for 1 to 2 min until it was saturated. The net weights of captured saliva were 47 to 65 mg (mean, 57 mg). Ten microliters of PBS was instilled into the vagina; this was followed by introduction of a glass applicator and insertion of a wick up to the cervix. The wick, which presumably absorbed secretions from both the exocervix and vagina, was removed after 2 to 4 min, and the net weights of fluid collected varied from 6 to 18 mg (mean, 11 mg).

Blood was then collected by cardiac puncture, and the animals were sacrificed by cervical dislocation. The abdomen was opened; the small intestine was clamped at both ends, removed, and measured; and the serosal surface was rinsed in cold PBS with protease inhibitors. The following concentrations of protease inhibitors were added to all intestinal wash and extraction fluids: 0.2 mM 4-(2-aminoethyl)-benzenesulfonylfluoride (Calbiochem), 1 μg of aprotinin (Sigma) per ml, 10 μM leupeptin (Sigma), and 3.25 μM bestatin (Boehringer Mannheim, Indianapolis, Ind.). The

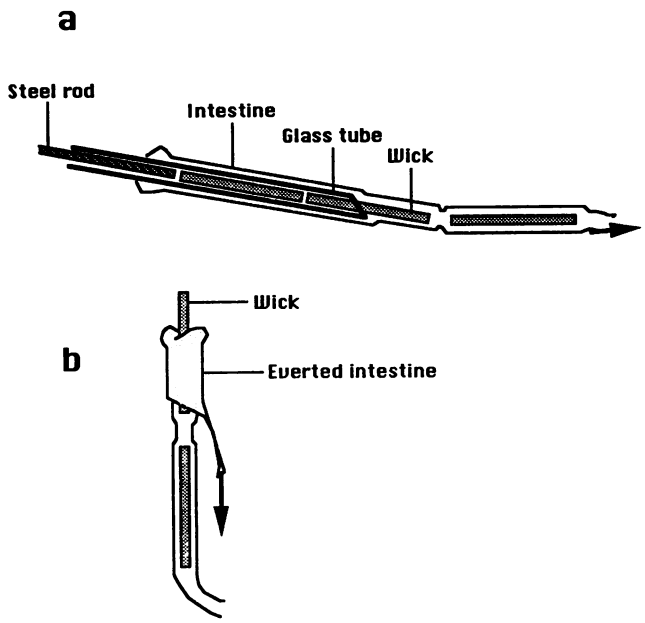


FIG. 1. Schematic drawings illustrating the wick method for collection of mucosal surface secretions from the excised small intestine of a mouse. (a) Wicks are introduced into the lumen of the intestine via a glass applicator tube. A series of wicks are held in place by a steel rod as the applicator is withdrawn, and the intestine slides off the applicator (in the direction of the arrow). (b) After absorption of secretions, wicks are sequentially removed by eversion of the intestine. The arrow indicates the direction of removal of the intestinal wall from the wick.

lumen of the small intestine was filled with 2.5 ml of PBS, the distal 4-cm segment was severed to allow for release and collection of intestinal contents and wash fluid, and the intestine was placed on an ice-cold glass plate. The entire small intestine was gently slipped onto a glass applicator tube inserted into the proximal end, and a chain of wicks was placed in the tube. The wicks were sequentially inserted into the intestine as follows: each wick was gently pushed to the opening of the tube with a steel rod at the same time as a short segment of intestine was slid off of the tube, thus capturing the wick (Fig. 1a). Care was taken during this process to ensure that the wicks themselves did not move along mucosal surfaces. The wicks were sequentially removed after 2 to 4 min by severing the gut distal to each wick and everting the gut along the wick (Fig. 1b). The mean net weights of the secretions captured varied from 11 to 22 mg per wick, except for the most distal segment (mean, 52 mg) which retained significant amounts of wash fluid. The most proximal one or two wicks representing duodenum and upper jejunum were heavily bile stained.

For collection of secretions from the mucosal surfaces of the rectum and descending colon, a glass applicator tube was introduced through the anus into the descending colon, displacing any fecal pellets proximally. As the applicator was withdrawn, a wick was inserted onto a relatively clean mucosal surface to a distance of approximately 0.5 cm from the anal opening and was left in place for 3 to 4 min. The wick was then removed by eversion of the colon and rectum, taking care to avoid contamination by peritoneal fluid or feces. The net weights of colonic-rectal samples varied from 2 to 11 mg (mean, 6 mg).

To assess the effect of pilocarpine on the volumes and IgA contents of collected secretions, a separate experiment was done in which secretions were collected as outlined above from four nonimmunized mice, two of which were injected with pilocarpine. In mice without pilocarpine, the salivary secretions captured on oral wicks amounted to 1 mg or less (compared with about 60 mg after pilocarpine). However, the weights of secretions captured from other sites and the concentrations of total IgA and IgG measured by enzyme-linked immunosorbent assay (ELISA) were unaffected by pilocarpine.

Extraction of protein from wicks. Wicks containing captured secretions were stored in microcentrifuge tubes at −20°C. Proteins were extracted by addition of 300 μl of PBS with 5% nonfat dry milk (that was immunoglobulin-free by ELISA) and protease inhibitors, vortexing twice for 15 s each, and centrifugation at 16,000 × *g* for 2 min at 4°C to drive fluid from the wick. The extracts were assayed immediately or stored at −20°C. To ascertain the efficiency of recovery of IgA from wicks, two pilot experiments were conducted. First, either buffer containing a known amount of standard mouse monoclonal IgA (Sigma) or intestinal wash with a known concentration of total IgA was added to wicks that were subsequently extracted as described above. Measurements of total IgA in these extracts by ELISA showed greater than 90% recovery. Second, known amounts of monoclonal anti-CT IgA, diluted over a wide concentration range (0.03 to 7.23 μg/ml), were added to dry wicks, or to wicks containing but not saturated with intestinal secretions, after insertion and removal from a nonimmunized mouse. Specific IgA measured in eluates by ELISA consistently showed over 85% (mean, 96%) recovery from dry wicks and over 93% recovery from wicks with secretions.

Collection and extraction of feces. Before immunizations and just before collection of secretions, three to six pieces of freshly voided feces were collected into 1.5-ml microcentrifuge tubes, frozen at −20°C, and subsequently vacuum dried in a Speed Vac Concentrator (model SVC 100H) with refrigerated condensation trap (model RT 100) (Savant Instruments, Inc., Farmingdale, N.Y.). After net dry weights were recorded, extracts of feces were made by using a modification of methods previously described (20). Briefly, PBS containing 5% nonfat dry milk and protease inhibitors was added to samples at a ratio of 20 μl/mg of dry feces. Solid matter was suspended by extensive vortexing and separated by centrifugation at 16,000 × *g* for 10 min. The clear supernatants were used for ELISAs as described below.

Quantitation of antibodies and immunoglobulins. IgA and IgG antibodies to CT and concentrations of monoclonal IgA and IgG antibodies were determined by ELISA using Nunc-Immuno Plates (MaxiSorp F96; A/S Nunc, Roskilde, Denmark). Monoclonal IgA and IgG antibodies directed against the B subunit of CT served as standards for the ELISA. These were produced by hybridomas generated by combined oral and intravenous immunization of mice followed by isolation and fusion of Peyer's patch cells with P3X63/Ag8U.1 mouse myeloma cells (3). Hybridoma cells were cultured with Hybri-max serum-free medium (Sigma); culture supernatants were pooled and concentrated by Amicon filtration (Amicon, Beverly, Mass.).

Plates for specific anti-CT antibody assays were coated by overnight incubation at 4°C with CT (Calbiochem) at 1.7 μg/ml in carbonate-bicarbonate buffer (pH 9.6). Nonspecific protein-binding sites were blocked with PBS (pH 7.2) containing 5% nonfat dry milk. Twofold serial dilutions (in PBS

with 5% nonfat dry milk) of both test samples and standard solutions were applied to ELISA plates (100 μl per well) and incubated at room temperature for 1.5 h. After being washed with PBS containing 0.1% Tween, plates were incubated at room temperature with affinity-purified, peroxidase-conjugated goat polyclonal antibodies specific for either the alpha chain of mouse IgA or the Fc fragment of mouse IgG (both from Sigma) diluted 1:1,000 or 1:5,000, respectively. Plates were developed with *o*-phenylene-diamine (Sigma) in citrate-phosphate buffer, pH 5.0. Optical densities were read at 492 nm with an Easy Reader 400 AT (SLT Labinstruments, Groedig/Salzburg, Austria) equipped with a Citizen 120-D printer (Citizen America Corp., Santa Monica, Calif.). Standard curves were generated by using known amounts of monoclonal anti-CT IgA or IgG. Anti-CT antibody concentrations in unknown samples were determined and then corrected for the weights of the original samples and for dilutions made during extraction from wicks and preparation for ELISA.

Concentrations of total IgA and IgG in concentrated hybridoma culture supernatants containing anti-CT IgA and IgG antibodies were determined by ELISA as described above, except that the plates were initially coated with a 1:500 dilution of affinity-purified goat polyclonal antibodies directed either against the alpha chain of mouse IgA or against the Fc portion of mouse IgG (both from Cappel Research Products, Durham, N.C.). Purified monoclonal IgA (MOPC-315) and polyclonal mouse IgG (both from Sigma) were used as reference standards. After incubation with standard and unknown samples, bound immunoglobulins were detected by using horseradish peroxidase-conjugated goat antibodies to mouse IgA or IgG (Sigma). The limit of detection for both of these standards was 1 ng/ml.

Statistics. Analysis of variance was calculated by using the StatView 512+ program for Macintosh computers. Differences among groups of animals at a significance level of 95% were calculated by a two-tailed analysis using Fisher's protected least-significant-difference test. Simple linear regression and calculation of Pearson's correlation coefficient were done with the same program.

Light microscopy and immunocytochemistry. For histologic analysis of the areas of contact between absorbent wicks and intestinal mucosa, wicks were inserted into the small intestine and colon-rectum of nonimmunized mice treated or not treated with pilocarpine as described above. Segments of small intestine and colon were excised with wicks in place and were immediately immersed in a fixative solution consisting of 2% freshly depolymerized formaldehyde–2.5% glutaraldehyde–4 mM $CaCl_2$–2 mM $MgCl_2$ in 0.1 M sodium cacodylate, pH 7.2. The lumens of the intestine and colon were also injected with fixative. After 4 h of fixation at room temperature, the intestine and colon with wicks were cut in 2.5-mm slices, returned to the fixative solution for 4 to 6 h, and stored overnight in 0.1 M sodium cacodylate buffer containing 4% sucrose.

For cryosectioning, samples were infused with 10% bovine gelatin in PBS at 37°C for 15 min and placed in fresh fixative solution for 1 h at 4°C. Samples were then placed in PBS containing 2.1 M sucrose at room temperature for several hours, mounted on machine screws (2 by 10 mm) (Metric Tool and Screw Company, Wakefield, Mass.) with Cryoform embedding medium (International Equipment Company, Needham, Mass.), frozen by immersion in Freon-22 (E.I. DuPont de Nemours Co., Inc., Wilmington, Del.) cooled by liquid nitrogen, and stored in liquid nitrogen. Frozen sections of approximately 1-μm thickness were cut at −80°C on a Reichert Ultracut E ultramicrotome fitted with an FC4D cryosectioning attachment.

For plastic embedding, samples were rinsed in 0.1 M sodium cacodylate buffer (pH 7.2) at 4°C, postfixed in 1% osmium tetroxide in the same buffer for 1 h at 4°C, and stained en bloc in a solution of 0.5% uranyl acetate in 0.1 M acetate buffer at pH 6. Tissue blocks were dehydrated in graded ethanol solutions and propylene oxide and embedded in Epon-Araldite. Plastic sections 1 μm in thickness were cut on a Reichert Ultracut E ultramicrotome and stained with 1% toluidine blue in sodium borate buffer. Specimens were photographed with T-Max 400 film (Eastman Kodak, Rochester, N.Y.), using a Zeiss Axiophot photomicroscope.

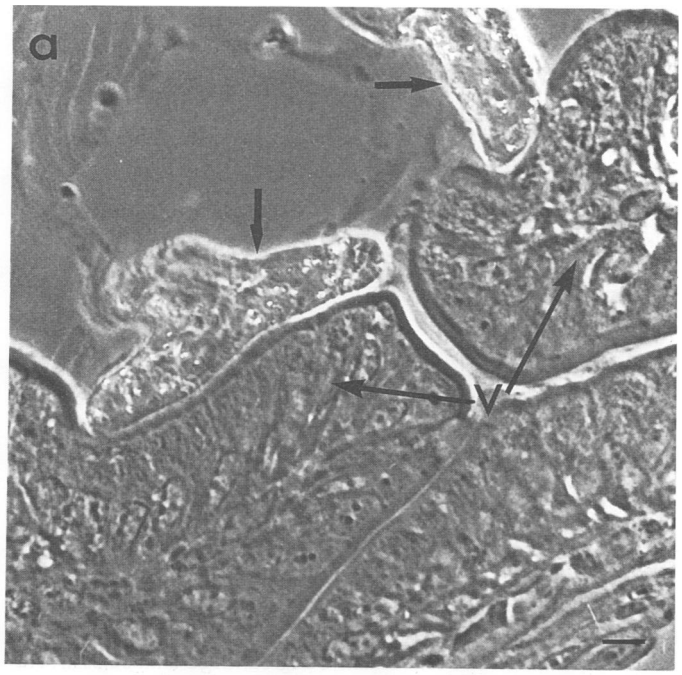
RESULTS

Wicks make close contact with epithelial surfaces. The wicks used for collection of secretions from mucosal surfaces of the small intestine, colon-rectum, and vagina were put into place and removed in a way designed to avoid abrasion and mucosal damage. Frozen sections of small intestine fixed with a wick in situ allowed direct visualization of wick fibers (Fig. 2a). The fibers came into close contact with the villus epithelium, and in some areas the unbroken epithelium conformed to the shape of the fibers. High-resolution light microscopy of semithin plastic sections confirmed that in most mucosal areas of both the small intestine and colon, the epithelial barrier was continuous and intact (Fig. 2b).

Local mucosa-associated secretions are efficiently sampled with use of wicks. The range of specific anti-CT antibody concentrations in mucus and fluid samples captured by the wicks varied with the source and dilution of the secretions (Fig. 3). In general, the highest levels were found in mucous secretions collected in undiluted form, including those from mucosal surfaces of the duodenum (up to 132 μg/ml), ileum (up to 152 μg/ml), and distal colon-rectum (up to 384 μg/ml). Lower levels were measured in vaginal fluids which had been diluted by preinstillation of 10 μl of buffer solution and in saliva which was secreted in diluted form following pilocarpine injections.

IgG antibodies to CT were also demonstrated in secretions and fecal extracts from some animals (data not shown). In most mice, these IgG concentrations were very low, averaging less than 1% of the corresponding serum IgG concentrations. Two mice showed relatively high levels of IgG antibodies in secretions (i.e., up to 7% of serum levels in colonic-rectal mucus and in vaginal fluid), but in these animals IgG antibodies were elevated in secretions obtained from all mucosal sites. This indicates that enhanced release of IgG was not due to local mucosal damage caused by the wicks but to some unknown factor specific to these individuals.

Patterns of local concentration of specific IgA reflect routes of immunization. Within each mucosal location sampled, the concentrations of anti-CT IgA antibodies varied greatly, depending on the site of prior immunization (Fig. 3). Except after vaginal immunization (which consistently failed to evoke specific IgA), the highest anti-CT IgA concentrations were generally seen at collection sites in the regions likely to have been directly exposed to CT. Thus, oral administration of the antigen resulted in significantly ($P < 0.05$) higher salivary antibody levels than did gastric, rectal, or vaginal administration. Likewise, gastric delivery of CT resulted in significantly ($P < 0.05$) higher antibody levels in small intestinal secretions than did oral or vaginal delivery. Colon-



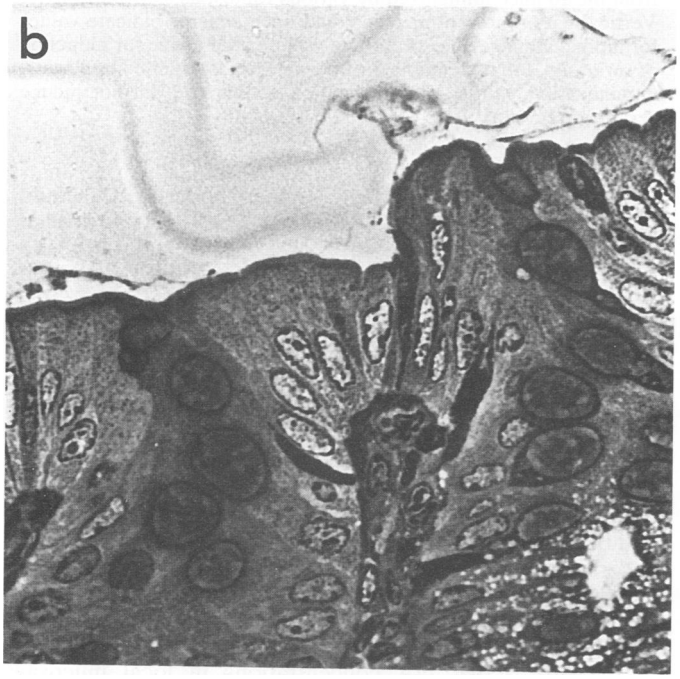


FIG. 2. Light micrographs of intestinal tissue fixed and processed with wicks in place, showing the relationship of wick fibers to epithelial surfaces. Bar, 10 μm. (a) One-micron cryosection of small intestine viewed by phase-contrast microscopy. Portions of two wick fibers (short arrows) are in contact with two villi (V) whose surfaces seem to conform to the shape of the fibers. The fibers come into close contact with enterocyte brush borders, but the epithelial barrier is intact. (b) One-micron plastic section of colon stained with toluidine blue, viewed by bright-field microscopy. The wick fibers are not visible, having been dissolved during plastic embedment. Although the surface epithelium shows indentations due to wick fibers, the epithelium is intact.

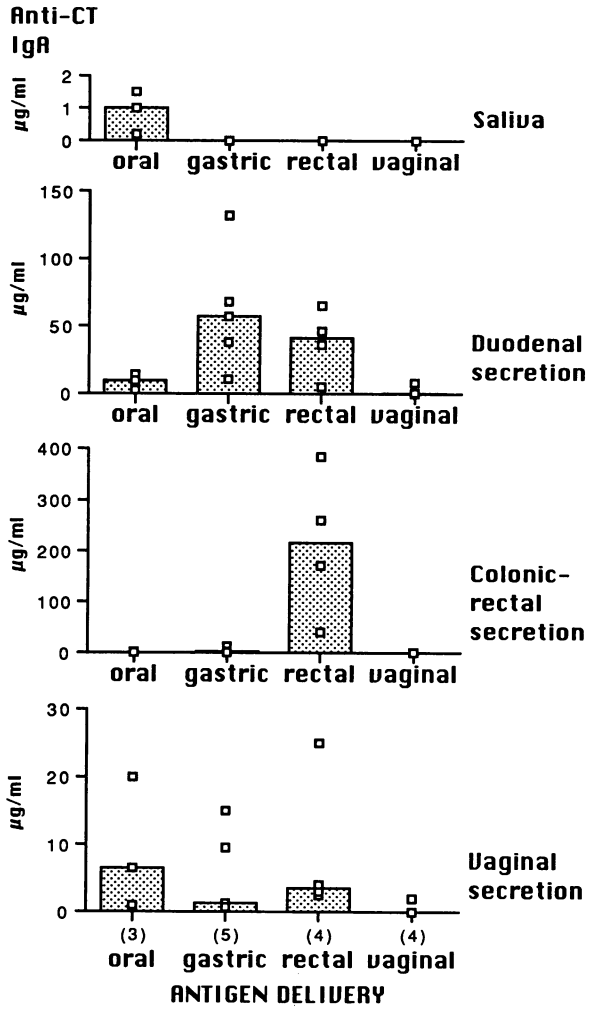


FIG. 3. Concentrations of specific anti-CT IgA antibodies in saliva and mucosal surface secretions collected by wicks from the small and large intestines and vagina of 16 mice immunized by either the oral, gastric, rectal, or vaginal route. The number of mice in each immunization group is indicated in parentheses. Samples of saliva, secretions in the distal colon-rectum, and cervical-vaginal fluid were each obtained from single wicks. Samples from the duodenum represent secretions eluted from the most proximal wick inserted in the small intestine. Vertical bars represent medians, and open squares indicate values for individual mice.

ic-rectal delivery produced significantly ($P < 0.05$) higher antibody levels in colonic-rectal secretions than did immunizations at other sites. Indeed, a strong IgA antibody response in colonic and rectal secretions was restricted to mice that received the antigen by the rectal route.

Immunizations via oral, gastric, and rectal (but not vaginal) routes induce IgA secretion at distant sites. After colonic-rectal and gastric delivery of CT, high levels of anti-CT IgA were found in the samples obtained from the most proximal segments of small intestine (Fig. 4). Secretions from these mucosal surfaces were heavily bile stained, as were the luminal contents. After immunization by the oral, gastric, and colonic-rectal routes, vaginal secretions consistently contained specific IgA antibodies (Fig. 3). Administration of CT via the vagina, however, failed to elicit IgA antibodies either locally or at distant sites (Fig. 3).

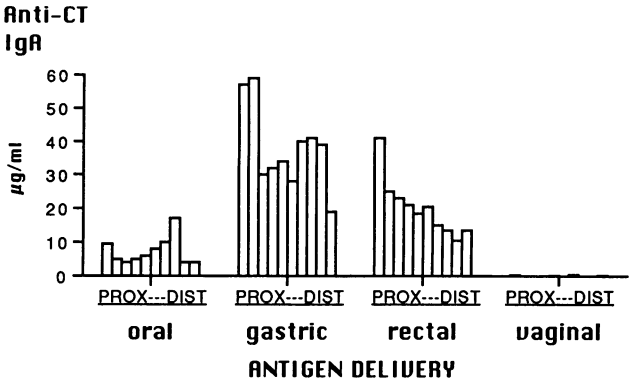


FIG. 4. Concentrations of anti-CT IgA antibodies in secretions recovered by wicks from 10 successive segments of the small intestines of the four groups of mice shown in Fig. 3, immunized by either the oral, gastric, rectal, or vaginal route. Within each group, the most proximal segment (duodenum) is on the left and the most distal segment (lower ileum) is on the right. Each bar represents the median for one segment. High specific IgA levels in proximal segments appeared after gastric and rectal immunization. A second peak appeared distally after oral or gastric (but not rectal) immunization.

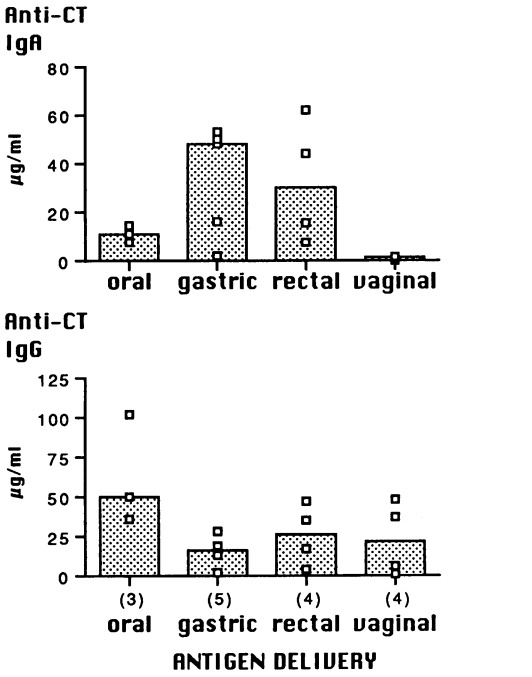


FIG. 5. Concentrations of anti-CT IgA and IgG antibodies in sera from the 16 mice immunized by various routes, as shown in Fig. 3. Vertical bars represent medians, and open squares indicate values for individual mice. The oral route was most effective for induction of serum IgG. A systemic IgG response was also elicited by vaginal immunization, although immunization at this site did not induce serum or secretory IgA.

Different routes of mucosal immunization induce distinct patterns of serum antibodies. All immunized animals developed serum IgA and IgG antibodies to CT. Concentrations of anti-CT IgA in serum generally reflected the levels of specific IgA in secretions. For example, high levels were found in serum after gastric and rectal delivery of the antigen, while concentrations were low after oral delivery and very low (1 ± 0.4 µg/ml) after vaginal delivery (Fig. 5). Specific IgA and IgG levels could not be directly compared with each other because the affinities of the monoclonal standards used were not determined. Nevertheless, the data show that patterns of serum anti-CT IgG concentrations were distinctly different from those of IgG in secretions. The highest levels of anti-CT IgG antibodies were found in serum from orally immunized animals, and vaginal immunization also induced a substantial serum IgG response.

Fecal extracts can be used to monitor the intestinal IgA response. IgA antibodies to CT could be measured in extracts of feces from all animals that received the antigen by the oral, gastric, or colonic-rectal (but not vaginal) route (Fig. 6a). Fecal samples showed somewhat higher antibody levels after rectal than after gastric delivery of the antigen, most likely because of addition of IgA-rich colonic and rectal mucus to the surfaces of the fecal pellets. Oral immunization resulted in low antibody levels in feces. Thus, levels of fecal IgA showed a rough correlation with levels of IgA collected from intestinal mucosal surfaces.

Washes of the small intestinal lumen, representing the entire luminal content diluted by the PBS solution, had approximately 10-fold-lower levels of IgA antibodies than did the secretions collected by wicks from mucosal surfaces after the luminal wash (Fig. 3 and 6a). Because of variable recovery of wash fluid, it was not possible to calculate the exact antibody concentrations in luminal contents. Nevertheless, the concentrations of CT-specific IgA antibodies in extracts of feces were positively correlated with concentrations in small intestinal washes after oral and gastric (and, to a lesser degree, rectal) immunization (Fig. 6b). Antibodies in feces therefore may be used to estimate the total secretion of IgA antibodies in the intestine. IgA antibodies to CT could be measured in feces after administration of only one dose of the antigen by the oral, gastric, or colonic-rectal route, and subsequent gastric and rectal doses produced high and persistent fecal IgA antibody levels. Repeated testing for fecal IgA antibodies thus provides a sensitive and convenient way to follow intestinal antibody responses over time.

DISCUSSION

The wick method for collection of mucosa-associated secretions allowed direct measurement of specific antibodies on local mucosal surfaces. After luminal contents and feces had been cleared, surface mucus and liquid secretions were absorbed by wicks from which antibodies could later be extracted. We were thus able to distinguish antibodies associated with epithelial surfaces from those moving with the luminal contents through the length of the intestine and to measure actual IgA concentrations in local mucosa-associated secretions.

High specific IgA levels were recovered in wicks from the small intestine, where the epithelial surface area is large. In this region, IgA concentrations in undiluted secretions adherent to the mucosal surface were correlated with IgA levels in gut washes but were an order of magnitude higher. In future studies, the wick method should prove useful for determining the local IgA levels correlated with protection of intestinal epithelia against infectious agents. IgA antibodies do not specifically bind to mucin glycoproteins (8, 37), and like other immunoglobulins, they can probably diffuse through mucus gels (48). Thus, the IgA recovered by wicks

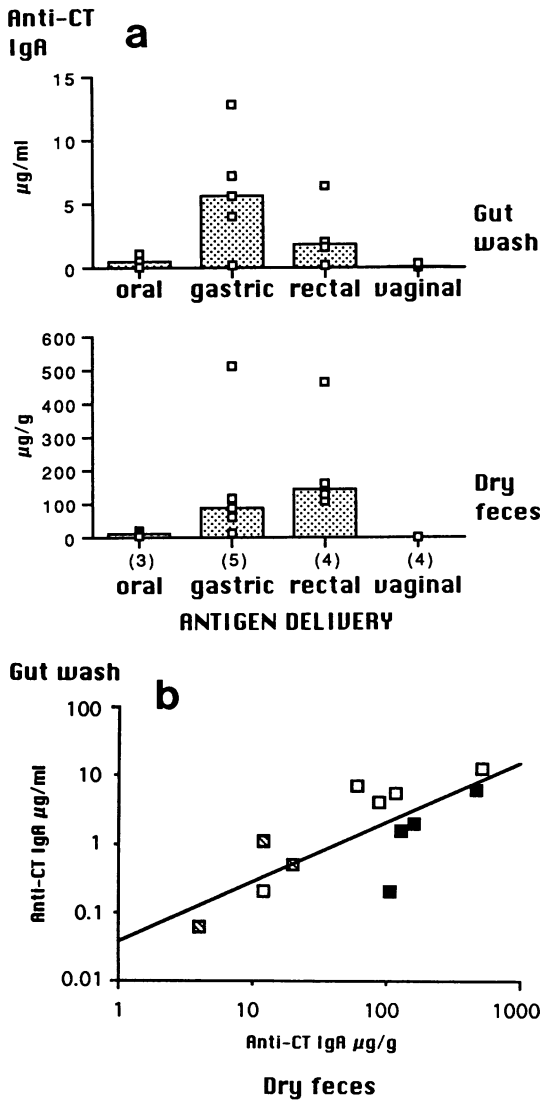


FIG. 6. Use of small intestinal washes and fecal samples for assessment of the IgA secretory immune response in the intestine. (a) Concentrations of anti-CT IgA antibodies recovered in luminal washes of excised small intestine and in extracts of dry feces from the 16 mice immunized by various routes, as shown in Fig. 3. Vertical bars represent medians, and open squares indicate values for individual mice. (b) Correlation between concentrations of anti-toxin IgA antibodies in small intestinal washes and extracts of dry feces from 12 individual mice after oral ▨, gastric □, or rectal ■ immunization with CT. A simple regression line is shown; Pearson's correlation coefficient was 0.77, and $P = 0.0034$.

from the small intestine may have included IgA secreted by hepatocytes into bile (1, 12, 20) as well as that secreted locally. The highest specific IgA concentrations were present in undiluted samples of surface mucus recovered from the distal colon and rectum, regions in which there is abundant mucus and IgA secretion as well as resorption of excess fluid by the epithelium (37). The fact that there were clear differences between the patterns of specific IgA concentrations measured in gut washes and those measured in local colonic-rectal mucus by the wick method demonstrates the unique usefulness of this method for assessing the mucosal response to vaccines intended for protection of colonic or rectal mucosal surfaces.

The salient finding of this study was that concentrations of specific IgA antibodies on mucosal surfaces in different regions of the gastrointestinal tract and in the vagina varied greatly, depending on the route of immunization. Our results confirm and extend previous studies in which differential responses have been produced in the gastrointestinal versus respiratory tracts by peroral or tracheal immunizations (36) and in small intestine versus colon by immunization in isolated intestinal segments (47). Since the collection of surface mucus directly from the airways was not feasible by the wick method and our primary concerns were intestinal and genital immune responses, we focused this study on analysis of mucosal surface secretions along the entire length of the intestine, as well as the oral cavity and vagina. Each immunization route produced a distinctive distribution of mucosal surface IgA.

Administration of antigen directly into the oral cavity was the only method that induced detectable levels of specific IgA in saliva. This suggests that the key inductive sites for this response were located locally, perhaps at lymphoid follicles in the oropharyngeal mucosa (5, 13). The relatively weak IgA response in the small intestine after oral delivery may reflect sampling of toxin in proximal Peyer's patches or may reflect cell migration from oropharyngeal inductive sites or regional lymph nodes to liver (1) and proximal gut mucosa (4). The oral route was clearly effective in induction of IgA in distant secretions, as observed by others in mice (9) and monkeys (27, 28), since it resulted in specific IgA in vaginal fluid, presumably from IgA lymphoid cells, generated either locally or in regional lymph nodes, that had migrated into the uterine cervix (23, 44). Intragastric immunization also induced specific IgA in the vagina but failed to induce IgA in saliva or the colon-rectum. Although others have observed parallels between the IgA-producing cells in the distal gastrointestinal and female genital tracts (23, 24, 35), our results suggest that the cell proliferation, migration, and homing phenomena that underlie the integration of mucosal immune responses are organ selective even in these regions and that a single oral vaccine may not protect both rectal and genital mucosa.

Recovery of specific IgA from cervical-vaginal fluids confirmed previous studies with mice, monkeys, and humans which had demonstrated that the female reproductive tract is an effector organ for IgA secretion after immunization at distant oral and intestinal inductive sites (23, 45). Under the conditions of this study, the female tract was not an inductive site, however, since we were unable to detect secretory immune responses to CT at any location after immunization by the vaginal-cervical route. This contrasts with previous studies with mice (30) and humans (40, 49) in which intravaginal and intrauterine immunizations with live microorganisms elicited antibodies in local secretions. On the other hand, local immune responses to intravaginal protein immunogens have generally been poor (23, 45, 51). The lack of a secretory IgA response after vaginal immunization is consistent with the apparent absence of histologically identified organized mucosal lymphoid tissue or specialized follicle-associated epithelial cells in the mouse female reproductive tract (46). The stratified vaginal epithelium contains antigen-presenting dendritic cells, a motile cell type associated with systemic immune responses (25). The systemic IgG response that we observed may have been due to sampling by these cells or to movement of antigen via the uterus and fallopian tubes into the peritoneal cavity.

Immunization via the rectal route was the only method that induced high levels of specific IgA in the mucous secretions coating the rectum and distal colon. The wick method demonstrated that the concentration of specific anti-toxin IgA attained on the rectal mucosa after rectal immunization was over 200 μg/ml, higher than at any other site tested. Our results also confirm that the colonic-rectal mucosa is a site of induction of distant mucosal immune responses, as previously demonstrated by others using live attenuated bacterial and viral vectors (17, 27, 28). The colonic and rectal mucosae of mice (43) as well as of humans (26, 41) are known to be rich in organized mucosal lymphoid tissue and associated antigen-transporting M cells, assemblies thought to be key in initiating IgA immune responses (21, 34, 42). M cells in both small and large intestines, including the rectum, also provide a portal of entry for infectious agents, however (38), and these may include the human immunodeficiency virus (2). It now is timely to exploit this accessible inductive site to obtain secretory IgA protection against pathogens that infect mucosal surfaces of the colon, rectum, and vagina.

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