Induction and Distribution of Intestinal Immune Responses after Administration of Recombinant Cholera Toxin B Subunit in the Ileal Pouches of Colectomized Patients

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The induction and dissemination of mucosal immune responses to recombinant cholera toxin B subunit (rCTB) administered into the ileal pouches of patients, who had been colectomized because of ulcerative colitis, was analyzed. Biopsies from the duodenum and ileal pouch were collected, along with peripheral blood and ileostomy fluids. Two immunizations induced strong CTB-specific immunoglobulin A (IgA) antibody-secreting cell (ASC) responses in the duodenum in five of five patients, whereas weaker and less-frequent ASC responses were noted in the ileal pouch. Intestine-derived CTB-specific IgA ASCs were found in peripheral blood in three of the five patients. The vaccination also induced significant IgA antitoxin titer rises in ileostomy fluid in all of the patients. Increased production of gamma interferon in cell cultures from the ileal pouch was found in four of five patients after the vaccination. These results clearly indicate that rCTB administered into the distal ileum is capable of inducing B-cell responses in the “entire” small intestine and that homing of immunocompetent cells occurs preferentially to the duodenum.

Induction of mucosal immune responses has been studied mainly after oral administration of antigens (11–16, 22). Mucosal immune responses are initiated by uptake of antigens from mucosal surfaces into organized lymphoid tissues located in the mucosa or in nearby lymph nodes, where antigen-specific B cells are generated. B-cell immunoblasts recruited at mucosal inductive sites subsequently enter the circulation and migrate to local and distant mucosal tissues and glands, where terminal differentiation occurs. This cellular migration is an important feature of the mucosa-associated lymphoid tissue, since administration of antigens in one mucosal region may generate secretory immunoglobulin A (IgA) antibodies at distant mucosal sites (19, 20). However, a number of studies have shown that local exposure to antigen results in much higher levels of specific IgA antibodies in the region of exposure than at distant sites (6–8).

In the present study, recombinant cholera toxin B subunit (rCTB) was used as a model immunogen to assess the induction and dissemination of mucosal immune responses after the administration of rCTB into the ileal pouch of patients who had had colectomies due to ulcerative colitis. Cholera toxin B subunit (CTB) is a well defined and potent mucosal immunogen which can be safely administered to humans in the form of the inactivated B-subunit-whole-cell (B-WC) cholera vaccine (11, 12). Several studies have shown that rCTB gives rise to strong IgA immune responses at various mucosal sites, especially within the intestine (3, 12, 15, 17, 22). Recently, we have also demonstrated that two oral doses of rCTB induced signifi-

cant CTB-specific IgA antibody responses in ileostomy fluid of patients colectomized due to ulcerative colitis (14).

The aim of the present study was to examine whether CTB-specific immune responses could be induced by antigen exposure in the distal ileum and to determine to what extent such responses could disseminate to the proximal small intestine. This was studied by collecting biopsies from the ileal pouch and duodenum along with peripheral blood and ileostomy fluid specimens from colectomized patients before and after the administration of rCTB. The T-cell responses after vaccination were also studied by assessing the cytokine production in ileostomy fluid and cell supernatants from intestinal biopsies.

Study design. Five adult patients (two women and three men), aged 43 to 52 years, who had undergone colectomies due to ulcerative colitis, were recruited from the regular follow-up program for patients with inflammatory bowel disease at the Department of Surgery of the Sahlgrenska University Hospital in Göteborg. Continence surgery had been performed 5 to 12 years earlier by construction of a pelvic pouch with an ileoanal anastomosis. The maximal extent of the small bowel resection was limited to 10 cm of the distal ileum. All patients were in general good health and had had no episodes of acute pouchitis or signs of extraintestinal manifestations of ulcerative colitis for the 3 years immediately preceding the study. None of the subjects had previously been vaccinated against cholera. All subjects agreed to participate in the study, which was undertaken with due approval from the Human Research Ethical Committee of the Medical Faculty, Göteborg University.

Each subject received two doses of an inactivated B-WC cholera vaccine 2 weeks apart; the first dose was given at least 3 days after preimmune sampling of the specimens. The vaccine, containing 1.0 mg of rCTB and 10¹¹ heat- and formalin-killed O1 vibrios per dose, was produced by SBL Vaccin, Stockholm, Sweden (9). Each dose of vaccine (3 ml) was sus-

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pended in 20 ml of phosphate-buffered saline (PBS) and deposited into the ileal pouch, which had been emptied immediately before the immunization. No coadministration of bicarbonate buffer was needed, since the pH of the ileal pouch secretion was found to be neutral. The participants remained resting for 30 min, alternating between the supine and side positions, after vaccine administration.

Specimen collection. Mucosal biopsies (duodenum and ileal pouch), ileostomy fluids, and blood specimens were collected before the first immunization (day 0) and 7 days after the second vaccine dose. In addition, ileostomy fluids were collected 21 days after the second immunization. The duodenal biopsies were obtained under local anesthesia by using a gastrointestinal fibroscope and a pair of biopsy forceps (FB 24 K; Olympus, Stockholm, Sweden). On each occasion, 12 pinch biopsies, 1 to 2 mm in diameter, were collected approximately 4 cm distal to the pyloric sphincter. Five biopsies, 3 to 4 mm in diameter, were also collected from the ileal pouch by means of a pediatric rectoscope and rectal biopsy forceps (4 by 2.6 mm; Instrumenta AB, Partille, Sweden). The extent of inflammation in the duodenal and ileal pouch mucosa was assessed by light microscopy examination of formalin-fixed biopsy specimens.

Ileostomy fluids were collected within 3 h after the last emptying of the reservoir. A 50-ml portion of fluid was immediately chilled on ice, centrifuged, and treated with enzyme inhibitors as previously described (14). The ileostomy fluid was frozen at -70°C until used. For determination of circulating vaccine-specific antibody-secreting cell (ASC) responses, 20 ml of heparinized venous blood was collected from the patients before and 7 days after the last vaccination. Serum specimens were obtained on the same occasions.

Detection of total and specific immunoglobulin-secreting cells. Intestinal mononuclear cells (MNCs) were isolated from duodenal and ileal pouch biopsies using an enzymatic dispersion technique as previously described (21). A pool of 12 duodenal biopsies yielded a mean of 1.4×10^6 viable MNCs (range, 0.6 to 2.0×10^6), and a pool of 5 biopsies from the ileal pouch 1.1×10^6 MNCs (range, 0.4 to 2.5×10^6). MNCs from heparinized venous blood were isolated by standard gradient centrifugation on Ficoll-Paque (Pharmacia Biotech AB, Uppsala, Sweden). Intestinal and peripheral blood cell suspensions were assayed for numbers of IgA-secreting cells and CTB-specific IgA ASCs by the enzyme-linked immunospot (ELISPOT) technique described elsewhere (5, 27). For the analyses of IgA-secreting cells, we added 50 to 500 intestinal MNCs per well or 10^4 to 10^5 peripheral blood MNCs per well. For the CTB-specific IgA ASC analyses, the corresponding numbers were 10^4 to 10^5 intestinal MNCs per well or 10^5 to 10^6 peripheral blood MNCs per well. Spots were enumerated under low magnification ($\times 40$), and the number of ASCs was calculated as the mean for four wells. IgA-secreting cells in the intestine were expressed per 10^5 MNCs. CTB-specific IgA ASCs were expressed per 10^4 IgA-secreting cells in the intestine and per 10^6 MNCs in peripheral blood to allow comparison with results in other studies (12, 17, 22). Vaccinees who as a mean had ≥ 2 CTB-specific IgA ASCs per 10^4 IgA-secreting cells in their intestinal biopsies after vaccination were considered as responders on the condition that they had < 1 CTB-specific IgA ASC per 10^4 IgA-secreting cells in each of the four

wells prior to immunization. The corresponding figure for a response in peripheral blood was as a mean a postvaccination value of ≥ 2 CTB-specific IgA ASCs per 10^6 MNCs. In no instances were CTB-specific IgA ASCs detected when we examined the highest available number of MNCs (10^6 cells) before immunization.

Antibody determinations. Levels of total IgA in ileostomy fluids were determined by a modified microplate enzyme-linked immunosorbent assay (ELISA) method as previously described (3, 30). The IgA antibody responses to cholera toxin in ileostomy fluid were studied by the GM1 ELISA method (14, 29). The specific IgA antitoxin activity in ileostomy fluid was determined by dividing the IgA ELISA antitoxin titer by the total IgA concentration (given in micrograms per milliliter) of the sample to adjust for variations in the IgA content in specimens collected from different persons and on different days. Based on previous calculations, a > 2 -fold increase in the mean IgA antitoxin titer/total IgA between pre- and postimmunization specimens was regarded as a significant response (1, 12). Serum antibody responses of IgA and IgG classes to cholera toxin were determined by the GM1 ELISA method (10, 11).

In vitro stimulation of MNCs. MNCs from intestinal biopsies were obtained according to methods described above. Circulating T cells were separated from blood MNC suspensions by rosetting with 2-aminoethyliso-thiuroniumbromide (Sigma Chemical Co., St. Louis, Mo.)-treated sheep red blood cells (26). The non-T-cell fraction was then used as accessory antigen-presenting cells. According to previous experiments, these cells consisted of approximately one-third B cells ($\text{CD}19^+$) and two-thirds monocytes ($\text{CD}14^+$) and contained $< 10\%$ of T cells (25). Isolated intestinal MNCs (2×10^5 cells per well) were cultured in duplicate together with accessory cells (2×10^4 cells per well) in round-bottom 96-well plates (Nunc, Roskilde, Denmark) in Iscove medium (Biochrom KG, Berlin, Germany) supplemented with 5% of human AB^+ serum, 3 μg of L-glutamine (Biochrom KG) ml^{-1} , and 100 μg of gentamicin (Schering-Plough AB, Stockholm, Sweden) ml^{-1} . Thereafter, the T-cell cultures were incubated without further additives or stimulated by CTB at final concentrations of 5 and/or 10 μg ml^{-1} . Ten micrograms of phytohemagglutinin (Murex Diagnostics, Ltd., Temple Hill, United Kingdom) ml^{-1} was used as a positive control to assess polyclonal T-cell activation. The culture medium was collected after 48 h, pooled, and stored at -70°C until further analyzed for cytokine content.

Cytokine detection. The concentrations of interleukin-4 (IL-4), IL-10 and gamma interferon ($\text{IFN-}\gamma$) in ileostomy fluids and cell supernatants from intestinal biopsies and peripheral blood were determined by different ELISAs. Ninety-six-well plates (Nunc) were coated with mouse anti-human monoclonal antibodies specific for the respective cytokine in 0.05 M carbonate buffer (pH 9.6) at 4°C overnight. After blocking of the remaining binding sites of the plates with 1% bovine serum albumin (BSA) in PBS at room temperature for 1 h, cell culture supernatants or ileostomy fluid specimens diluted in PBS-Tween (0.05%) containing 0.1% BSA were added to the individual wells and incubated at 4°C overnight. The cytokine concentrations were determined by stepwise addition of biotinylated antibodies reacting with the respective cytokine, peroxidase-labeled extravidin (Sigma Chemical Co.), and *o*-phen-

FIG. 1. Frequencies of CTB-specific IgA ASCs in duodenal and ileal pouch biopsies (A) and peripheral blood (B) before and 7 days after two administrations of rCTB into the ileal pouch of colectomized patients. The data are expressed as numbers of vaccine-specific IgA ASCs per 10^4 IgA-secreting cells (A) or per 10^6 MNCs (B). The dotted lines indicate the level for a significant response. The numbers correspond to the individual patients. For patient 1, CTB-specific IgA ASCs in the ileal pouch after vaccination could not be analyzed due to insufficient numbers of MNCs.

ylenediamine substrate (Sigma Chemical Co.). The coating and detecting antibodies to IL-4 and IL-10 were purchased from Pharmingen (San Diego, Calif.), and the IFN- γ -specific reagents were from Chromogenix (Mölnådal, Sweden). Standard curves were constructed using recombinant human cytokines obtained from Genzyme (Cambridge, Mass.).

B-cell responses in the intestine. CTB is a potent immunogen which has been used in numerous studies to assess the induction of immune responses at various mucosal sites (3, 12, 15, 17, 22, 23). Large numbers of CTB-specific IgA ASCs in the duodenum of healthy volunteers have been demonstrated after oral immunization with the B-WC cholera vaccine (17, 22). Rectal and intratonsillar immunization of healthy humans with CTB have also induced substantial IgA ASC responses to CTB at the site of antigen exposure (9a, 23).

In the present study, suspensions of MNCs obtained from enzymatically dispersed duodenal and ileal pouch biopsies were assayed for CTB-specific IgA ASCs after administration of rCTB in the ileal pouch of colectomized patients. Prior to immunization, <1 CTB-specific IgA ASC per 10^4 IgA-secreting cells were found in the duodenum and the ileal pouch of all patients. Two administrations of rCTB into the ileal pouch induced weak CTB-specific IgA ASC responses in the distal ileum in two of four patients (one postvaccination specimen had to be excluded from the analysis due to insufficient numbers of ileal pouch MNCs) (Fig. 1A). Interestingly, all of the five vaccinees responded with substantial increases in CTB-specific IgA ASCs per 10^4 IgA-secreting cells in the duodenum, and the magnitude of the response had a mean value of 95-fold (Fig. 1A). Thus, considerably stronger CTB-specific IgA ASC responses were observed in the duodenum than at the site of

CTB exposure, i.e., in the ileal pouch of colectomized patients. The response in the duodenum was most likely not due to a gastrointestinal reflux of CTB, since radiologic evaluations in colectomized patients with continent ileostomy reservoirs have shown a maximum reflux of 20 cm after volume infusions of 100 to 300 ml into the reservoirs (2). As the total volume administered into the empty ileal pouch was no more than 23 ml in the present study, retrograde migration of antigen seems highly unlikely. Furthermore, we have recently shown that installation of CTB into the small intestine approximately 30 cm beyond the pyloric sphincter does not result in any detectable reflux as determined by coadministration of C^{13} -labeled polyethylene glycol, as well as antigen analysis of gastric fluid for CTB (M. Quiding-Järbrink, H. Lönnroth, I. Ahlstedt, J. Holmgren, and A.-M. Svennerholm, unpublished results). Our findings indicate the possibility of inducing B-cell responses within the gastrointestinal tract of humans without the presence of antigen at the effector site. The high frequencies of CTB-specific IgA ASCs found in the duodenum of all the colectomized patients clearly show that immunocompetent cells initially activated in the distal ileum have the capacity to disseminate to a more proximal part of the small intestine. The observed homing of ASCs to the duodenum might be due to an upregulated expression of mucosa-specific adhesion molecules in the duodenal mucosa, resulting in an increased migration of plasma cell precursors into the duodenal mucosa.

Studies of the compartmentalization of local immune responses indicate that even though responses can be recorded at distant sites from that of the antigen administration, the highest responses are generally seen at the site of antigen administration (6–8). In the present study, however, considerably

TABLE 1. Antitoxin IgA antibody responses in ileostomy fluid samples from colectomized patients before immunization and after two doses of rCTB into the ileal pouch

Days postimmunization ^a	Total IgA content ($\mu\text{g ml}^{-1}$) ^b	Antitoxin IgA titer/total IgA ($\text{U } \mu\text{g}^{-1}$) ^c	Frequency ^d	Magnitude ^e
0	1,780 (1,150–2,690)	0.11 (0.015–0.82)		
21	1,620 (1,050–2,510)	1.05 (0.15–7.2)	4/5	9.5 (15.8)
35	1,500 (1,050–2,090)	0.55 (0.043–6.9)	5/5	4.9 (4.9)

^a Recombinant CTB was administered into the ileal pouch 2 weeks apart.

^b Geometric mean total IgA concentration (± 1 standard deviation) for the whole group.

^c Geometric mean antitoxin IgA antibody titer/total IgA concentration (± 1 standard deviation) for all subjects. The values have been multiplied by 10.

^d The number of responders/total number of patients is shown. Responders were defined as having a >2-fold increase in specific IgA antibody titer/total IgA concentration between pre- and postimmunization specimens.

^e Geometric mean increase in antitoxin IgA antibody titer/total IgA concentration for the whole group (responding subjects).

stronger responses were seen in the duodenum than at the site of CTB exposure. The weak IgA ASC response found in the ileal pouch after vaccination might be explained by differences in the distribution of IgA-secreting cells within the small intestine. However, similar frequencies of IgA-secreting cells were found in the intestinal biopsies; the geometric mean number of IgA-secreting cells in duodenum was 19,000 per 10^5 MNCs (range, 9,000 to 36,000), and in the ileal pouch 17,000 per 10^5 MNCs (range, 7,000 to 35,000). The frequencies of IgA secreting cells in the duodenum and ileal pouches were similar in biopsies obtained before and after vaccination. Another explanation for the decreased responsiveness to CTB at the inductive site could be that there are differences in the expression of endothelial adhesion molecules and/or chemokines within the small intestine. The mucosal addressin cell adhesion molecule-1 (MadCAM-1), which is the only characterized mucosa-specific addressin involved in lymphocyte trafficking to mucosal lymphoid tissues, is localized to the intestinal mucosa and gut-associated lymphoid tissue, and expression appears to be increased at inflammatory foci associated with ulcerative colitis and Crohn's disease (4). In spite of the weak response in the ileal pouch, administration of rCTB in this location clearly resulted in a strong efferent B-cell response. Thus, significant (>2-fold) increases in CTB-specific IgA antibodies/total IgA were observed in the ileostomy fluid in all of the five vaccinees. The geometric mean fold increase of the antitoxin response was 9.5-fold on day 7 and 4.9-fold on day 21 after the second vaccination (Table 1). However, the magnitude of the IgA antitoxin responses was lower than that observed in colectomized patients given CTB by the oral route (14).

Inflammation per se has been suggested to facilitate the induction of immune responses (18). Recently, high frequencies of vaccine-specific IgA ASCs were found locally in the inflamed gastric mucosa of *Helicobacter pylori*-infected patients after oral immunization with the B-WC cholera vaccine, whereas none of noninfected controls responded to the vaccination in antrum (17). The inflammation in the gastric mucosa of *H. pylori*-infected patients is characterized by a massive infiltration of lymphocytes and neutrophils (31). Although a mild chronic lymphocytic infiltration was observed in the lamina propria of the ileal pouches, no evidence for an increased B-cell response following administration of rCTB into the pouches was noted. One explanation for the variable responsiveness to mucosal administration of antigen might be that the recruitment of inflammatory cells in *H. pylori*-infected gastric mucosa and ileal pouches differs.

B-cell responses in peripheral blood. Monitoring of different homing receptors on circulating ASCs induced by various routes of immunization has clearly shown that ASCs of the IgA isotype assayed 7 days after antigen administration by the oral or rectal routes largely represent cells of gut origin (13, 24). After administration of rCTB into the distal part of the small intestine, circulating CTB-specific IgA ASCs were detected in peripheral blood in three of the five colectomized patients, but the magnitude of the response was modest (Fig. 1B). The frequency and magnitude of the ASC responses were lower than those observed in healthy volunteers given CTB by the oral (12) or rectal (9a) route. According to the concept of a common mucosal immune system, the destination of these circulating B cells initially activated in the gut is the lamina propria and other mucosal tissues distant from the initial site of antigen exposure (19, 20).

Also in serum, significant increases in IgA antitoxin titer were found in three of five vaccinees, and four of them developed IgG antitoxin responses. The magnitudes of the increases in serum antitoxin titer among the responders were 14-fold for IgA and 3.3-fold for IgG. The seroconversion rate to CTB was similar to what has been noted after oral vaccination (14).

Cytokine responses in the intestine. The human intestinal mucosa seems to be a rich source of cells producing IFN- γ . High frequencies of cells spontaneously secreting IFN- γ have been found in duodenal biopsies from healthy volunteers (22). In patients with ulcerative colitis, mucosal biopsies from uninfamed ileoanal pouches have been shown to contain slightly increased numbers of IFN- γ -producing cells (28). In the present study, all of the patients had detectable amounts of IFN- γ in the supernatants of unstimulated cells isolated from the ileal pouch (Fig. 2) and duodenal (not shown) biopsies before immunization. The IFN- γ production from ileal pouch and duodenal cells was similar, i.e., the mean IFN- γ concentration ± 1 standard deviation was $156 \pm 89 \text{ ng ml}^{-1}$ for ileal pouch cells (Fig. 2) and $137 \pm 59 \text{ ng ml}^{-1}$ for duodenal cells. Secretion of IL-4 and IL-10, on the other hand, was found more often and in higher levels in unstimulated cell cultures from the duodenum (246 ± 196 and $277 \pm 199 \text{ ng ml}^{-1}$, respectively) than from the ileal pouch (20 ± 14 and $34 \pm 27 \text{ ng ml}^{-1}$, respectively).

Administration of rCTB into the ileal pouch resulted in increased production of IFN- γ in unstimulated cultures of ileal pouch cells for three of the five patients (Fig. 2). These findings are consistent with a previous report showing increased numbers of IFN- γ -producing cells in the duodenal mucosa of

FIG. 2. IFN- γ production in cell supernatants of ileal pouch MNCs from colectomized patients before and 7 days after administration of rCTB into the ileal pouch. Results from unstimulated cultures are shown before and after immunization, and in three patients, from whom sufficient cell numbers were available, the data are also presented after stimulation with 5 μ g of CTB ml $^{-1}$ for ileal pouch MNCs collected after vaccination. The numbers correspond to the individual patients.

healthy volunteers after oral immunization with the B-WC cholera vaccine (22). Stimulation with CTB of ileal pouch cells collected after immunization induced a further increase in IFN- γ secretion in the three patients from whom sufficient numbers of cells were available for analyses (Fig. 2). We believe that this further increase in IFN- γ production is a result of an antigen-specific stimulation. Histopathological examinations of the biopsies revealed no difference in the inflammatory score before and after administration of rCTB. In contrast to our findings in the ileal pouch, duodenal cells from one vaccinee only (subject 4) showed an increase in IFN- γ production after immunization, and no further increase was observed after stimulation with CTB. The production of IL-4 and IL-10 was only increased in the supernatant of unstimulated duodenal cells from one patient (subject 5) after vaccination; in cultures of ileal pouch cells increased amounts of IL-10 were also found in the same vaccinee. Prevacination ileostomy fluids rarely contained detectable amounts of the cytokines studied, and administration of rCTB into the ileal pouch only induced increased levels of IFN- γ , IL-4, and IL-10 in one of the patients (subject 3).

In conclusion, the present study clearly shows that it is possible to induce B-cell responses within the small intestine without the actual presence of antigen at the effector site. Administration of rCTB into the ileal pouches of colectomized patients resulted in considerably stronger CTB-specific IgA ASC responses in the duodenum than at the site of antigen exposure, suggesting that the homing of immunocompetent B cells occurs preferentially to the duodenum. The observation that the cytokine response was limited to the induction site suggests a lower extent of distribution for the T-cell response than the B-cell response.

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