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Optimizing Oral Vaccines: Induction of Systemic and Mucosal B-Cell and Antibody Responses to Tetanus Toxoid by Use of Cholera Toxin as an Adjuvant

RAYMOND J. JACKSON,[1,2,3,4]* KOHTARO FUJIHASHI,[1,2,4] JIANGCHUN XU-AMANO,[1,2,3] HIROSHI KIYONO,[1,2,4] CHARLES O. ELSON,[1,2,5] AND JERRY R. McGHEE[1,2,3,4]

Immunobiology Vaccine Center,[1] Mucosal Immunization Research Group,[2] and Departments of Microbiology,[3] Oral Biology,[4] and Medicine,[5] University of Alabama at Birmingham Medical Center, Birmingham, Alabama 35294

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Cholera toxin (CT) is an effective mucosal antigen and acts as an adjuvant when given orally with various antigens; however, few studies have compared the levels of antibody responses to CT and coadministered protein in systemic and mucosal tissues. In this study, we used tetanus toxoid (TT) for assessment of immune responses. Time course and dose-response studies established that 250 μg of TT given orally with 10 μg of CT three times at weekly intervals induced high serum and gastrointestinal tract anti-TT and anti-CT antibody responses. Oral immunization with TT alone induced no detectable mucosal immunoglobulin A (IgA) antibodies in fecal extracts and only weak serum IgG anti-TT responses. The coadministration of CT and TT induced peak serum IgG anti-TT responses following two oral doses that remained constant after the third oral immunization, while optimal mucosal IgA responses were seen after the third oral immunization. The serum anti-TT response obtained with CT and TT proved protective against TT challenge (100 minimum lethal doses), whereas mice orally given CT or TT alone died. Antigen-specific B-cell responses were assessed with an isotype-specific Elispot assay of isolated lymphoid cells from the spleen, Peyer's patches, and the small intestinal lamina propria. Interestingly, approximately fourfold-higher numbers of IgA anti-CT than of anti-TT antibody-producing (spot-forming) cells occurred in lymphocytes from the lamina propria of mice orally immunized with both TT and CT. The adjuvant CT did not induce polyclonal B-cell responses in mice given CT by the oral route, since no significant differences in total numbers of B cells producing IgA, IgG, or IgM were found compared with the numbers in mice given TT alone. The results clearly indicate that serum and mucosal antibody responses develop with different kinetics and that protective TT-specific antibody responses are generated in the systemic compartment when TT is administered with CT via the oral route.

Most bacterial and viral infections are acquired through mucous membranes, most notably in the respiratory, gastrointestinal (GI), and urogenital tracts. It is now established that protection of these mucosal surfaces is mediated in large part by local production of secretory immunoglobulin A (S-IgA) antibodies (27). Furthermore, parenterally administered vaccines are generally ineffective for induction of S-IgA responses (27). Thus, the mucosal immune system is separate from the systemic compartment and consists of distinct lymphoid sites where antigens are encountered and processed and initial B- and T-cell triggering occurs (inductive sites) and separate areas where immune cells actually function (effector sites) (24, 25, 27). In this regard, the gut-associated lymphoreticular tissues, e.g., the Peyer's patches (PP), are well characterized inductive sites for IgA memory B cells and for precursors of IgA plasma cells (3, 13), while the lamina propria regions of the GI tract are major effector regions rich in IgA plasma cells (24, 25, 27). Although oral administration of protein vaccine might be expected to induce IgA responses in the GI tract and in other distant mucosal effector sites, in reality this mode of delivery generally results in either poor or short-lived antibody responses. Furthermore, oral administration of large doses of protein often induces unresponsiveness to peripheral immunization (oral tolerance) (32).

* Corresponding author.

A major exception to poor immune responses to orally administered proteins and vaccines is cholera toxin (CT), which can induce both mucosal S-IgA and serum IgG antibodies following oral administration of only microgram quantities to mice (10, 24, 29). High numbers of anti-CT IgA-producing plasma cells lodge in the lamina propria of the GI tract following oral immunization (24); however, the origins of anti-CT IgG antibody-producing cells and serum IgG antibodies are less clear. Studies by Lycke and colleagues have shown that oral immunization with CT results in memory B-cell responses in both mice and humans (16, 18–20). The high-affinity binding of CT, specifically the B subunits of CT to GM1 gangliosides on intestinal epithelial cells, appears to contribute to its strong immunogenicity (22). Recent studies suggest that microaggregates of CT are found in the domed epithelium of murine PP, which are enriched in M cells that function in translocation of antigens into the gut-associated lymphoreticular tissues (28).

Another unique function of CT is an ability to serve as an adjuvant for enhancement of mucosal S-IgA and serum IgG responses to coadministered proteins (6). Initial studies showed that when CT was orally administered to mice with an otherwise tolerance-inducing dose of keyhole limpet hemocyanin, good mucosal IgA and serum IgG anti-keyhole limpet hemocyanin antibody responses occurred (8). These results have now been extended to show that CT can enhance antibody responses to other protein antigens and to bacterial and viral vaccines (2, 5, 8, 14, 26, 31). Despite

numerous studies with CT in the mucosal immune system, the precise mechanism for CT adjuvancy is still unknown. The adjuvant effect occurs only when CT is administered simultaneously with an antigen and by the same route, clearly suggesting that CT has direct effects on mucosal tissues and lymphoid cells (17, 22, 24). Nevertheless, CT (and CT subunit B [CT-B]) inhibit mitogen-induced T-cell responses (11, 34) and interleukin-2 production (15). This inhibition of T-cell responses by CT is difficult to reconcile with its known adjuvancy. Recent studies have shown that CT and CT-B induce increased switching of surface IgM-positive B cells to those which secrete IgG and IgA in lipopolysaccharide-triggered B-cell cultures (15, 23). Switches to IgG and IgA would explain the presence of these antibody isotypes in serum and in mucosal secretions, respectively, following oral immunization with CT and other antigens (23).

We have used CT as an adjuvant to optimize antigen-specific B-cell responses to orally administered tetanus toxoid (TT). The kinetics of GI tract IgA and serum IgG anti-TT antibody responses were assessed following oral immunization with TT and CT as an adjuvant. By use of isotype-specific endpoint antibody analysis and antigen-specific single-B-cell analysis, we were able to compare CT and TT responses quantitatively in the mucosal and systemic immune compartments of mice given TT and CT. In addition, we investigated whether oral immunization with TT and CT provided protective immunity by challenging mice with tetanus toxin. The results of these studies are discussed from the standpoint that oral delivery of TT with CT results in significant peripheral as well as mucosal immune responses which differ in antigen dose requirements, antibody isotypes, and kinetics of B-cell and antibody responses.

MATERIALS AND METHODS

Mice. C57BL/6 mice which were barrier maintained and antibody negative for a panel of mouse virus and bacterial pathogens were obtained from the Frederick Cancer Research Facility (National Cancer Institute, Frederick, Md.). These mice were maintained in horizontal laminar flow cabinets and provided sterile food and water ad libitum. All mice used in this study were between 8 and 12 weeks of age.

Immunogens. CT and CT-B were purchased from Sigma Chemical Co., St. Louis, Mo. TT containing 400 Lf units/ml was kindly provided by Subramonia Pillai, Lederle-Praxis Biologicals, Rochester, N.Y.

Tetanus toxin challenge. In this study, we obtained tetanus toxin (lot T-1) from Jean Halpern and William Habig, Division of Bacterial Products, Food and Drug Administration, Bethesda, Md. The toxin was diluted in gelatin-saline, and appropriate doses were injected subcutaneously in normal and immunized mice. The mice were monitored daily for paralysis and death.

Immunization of mice. Groups of five mice each of either sex were immunized by an oral intubation procedure. The mice were deprived of food for 2 h prior to oral immunization, and 30 min before immunization the mice were gavaged with 0.5 ml of a solution consisting of 8 parts Hanks' balanced salt solution and 2 parts 7.5% sodium bicarbonate in order to neutralize stomach acidity. Antigens were administered in 0.25 ml of phosphate-buffered saline (PBS). Groups of mice were immunized on days 0, 7, and 14. Four experimental groups were used in the study. Group one consisted of mice receiving 10 μg of TT and 10 μg of CT; group two was given 100 μg of TT and 10 μg of CT; group three received 250 μg of TT and 10 μg of CT; and group four were orally immunized with 250 μg of TT only. Fecal extracts prepared by the method of deVos and Dick (7) and serum samples were obtained from mice and monitored for IgM, IgG, and IgA anti-TT and anti-CT antibody responses throughout the experiment.

ELISA. Antibody titers in serum and fecal extracts were determined by an enzyme-linked immunosorbent assay (ELISA). Briefly, the assay was conducted in Falcon Microtest III assay plates (Becton Dickinson, Oxnard, Calif.). Plates were coated with an optimal concentration of TT (100 μl of 5-μg/ml TT, equivalent to 0.8 Lf units/ml) or 100 μl of CT-B (5 μg/ml) in PBS. Plates were incubated at 4°C overnight in a humid atmosphere and washed three times with PBS. Blocking was done with 200 μl of 1% bovine serum albumin (BSA) in PBS for 1 h at 37°C. The plates were washed three times with PBS. Serial dilutions of serum or fecal extracts in 1% BSA–PBS were prepared, 100 μl was added in duplicate, and the plates were incubated for 2 h at 37°C. Normal serum and fecal extracts at similar dilutions were included on the same plate as controls. Wells incubated with 1% BSA–PBS (i.e., no antibody) served as substrate controls. Following incubation at 37°C for 2 h, the plates were washed four times with PBS containing 0.05% Tween 20. The secondary antibody (stock reagents were 0.5 mg/ml; Southern Biotechnology Associates, Birmingham, Ala.) consisted of 100 μl of a 1:1,000 dilution of biotinylated goat anti-mouse IgM, IgG, or IgA in PBS–0.05% Tween 20. Plates were incubated at 37°C for 2 h or overnight at 4°C and then washed four times with PBS–0.05% Tween 20. The detection enzyme consisted of a 1:1,000 dilution of horseradish peroxidase-conjugated streptavidin (2.5 mg/ml; Zymed Labs, South San Francisco, Calif.) in PBS–0.05% Tween 20. The plates were incubated at room temperature for 1 h, washed four times with PBS, and developed at room temperature with 100 μl of 1.1 mM ABTS [(2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)] in 0.1 M citrate–phosphate buffer (pH 4.2) containing 0.01% H_2O_2. Endpoint titers were expressed as the reciprocal $\log_2$ of the last dilution which gave an optical density at 414 nm (OD_{414}) of ≥0.1 OD unit above the OD_{414} of negative controls after a 15-min incubation. Incubations were terminated by addition of 50 μl of 10% sodium dodecyl sulfate in 0.1 M citrate–phosphate buffer.

Preparation of single-cell suspensions. Single-cell suspensions were obtained from spleens by gently teasing the tissue through a sterile stainless steel screen as described previously (1). PP lymphoid cells were isolated by excising the PP from the small intestine wall. Cells were dissociated in Joklik's modified medium (GIBCO, Grand Island, N.Y.) containing 1.5 mg of the neutral protease enzyme Dispase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) per ml as described previously (12). Lamina propria lymphocytes (LPLs) were isolated after removal of PP and mesenteric lymph nodes from the small intestine by a modification of a previously described method (30). Briefly, the small intestine was cut longitudinally, washed thoroughly in incomplete RPMI 1640 medium, and cut into segments of approximately 1 to 2 cm. To remove intraepithelial lymphocytes, the tissue was placed in RPMI 1640 containing 2% fetal calf serum (FCS), stirred for 30 min at 37°C, transferred to a 50-ml centrifuge tube, and shaken vigorously for 15 s. The supernatant was discarded, fresh medium was added, and the shaking procedure was repeated two additional times. Two additional 30-min incubations followed by vigorous agitation were required to deplete the intraepithelial lymphocytes. The tissue was minced and then suspended in

Joklik's modified minimal essential medium containing 1.5 mg of Dispase per ml. The medium was adjusted to pH 7.4, and the tissues were digested for 30 min at 37°C with stirring. Cells were harvested by centrifugation and resuspended in RPMI 1640 containing 2% FCS. The digestion was repeated two additional times. After a wash in RPMI 1640–2% FCS, lymphocytes were obtained by the use of a discontinuous Percoll gradient (Pharmacia Fine Chemicals, Uppsala, Sweden). Nine parts Percoll were mixed with one part 10× Hanks' balanced salt solution and used as 100% Percoll. Gradients were prepared in 15-ml centrifuge tubes by first placing 2 ml of a 75% Percoll solution diluted in RPMI 1640–2% FCS at the bottom of the tube and then adding the cells suspended in 4 ml of 40% Percoll. Gradients were centrifuged at 25°C for 20 min at 600 × *g*. The interface between the 40 and 75% Percoll layers, containing lymphocytes, was removed, and the cells were washed and resuspended in RPMI 1640 containing 10% FCS.

Elispot assay. An Elispot assay (4) was used to detect total Ig-secreting cells and antigen-specific spot-forming cells (SFCs). Ninety-six-well nitrocellulose plates (Millititer HA; Millipore Corp., Bedford, Mass.) were used for the assay. In order to detect SFCs, the plates were coated with goat anti-mouse Ig (Southern Biotechnology Associates) at 2 μg/ml (100 μl per well) for total IgM, IgG, and IgA SFCs, CT-B at 5 μg/ml (100 μl per well) for anti-CT-specific SFCs, or TT at 5 μg/ml (100 μl per well) for anti-TT-specific SFCs. After incubation overnight at room temperature, the plates were washed four times with PBS. Plates were then blocked with 1% BSA–PBS for 1 h at 37°C. The blocking solution was discarded, and 100 μl of cells in RPMI 1640–10% FCS at various dilutions was added; each dilution was tested in duplicate. Cell suspensions were incubated for 4 h at 37°C in 5% CO_2. Plates were washed three times with PBS and then three times with PBS–0.05% Tween 20. The detection antibody consisted of 100 μl of a 1:1,000 dilution (stock concentration, 0.4 mg/ml) of goat anti-mouse IgM, IgG, or IgA conjugated to horseradish peroxidase (Southern Biotechnology Associates) in PBS–0.05% Tween 20 containing 1% FCS. Plates were incubated overnight at 4°C in a humid atmosphere. Following incubation, plates were washed four times with PBS and developed by the addition of 200 μl of 1.6 mM 3-amino-9-ethylcarbazole dissolved in 0.1 M sodium acetate buffer (pH 5.0) containing 0.015% H_2O_2 per well. Plates were incubated at room temperature for 15 to 20 min and washed with water, and SFCs were quantitated with the aid of a stereomicroscope.

RESULTS

Titration of CT for adjuvancy effects. In an initial experiment, we titrated various amounts of CT with a constant amount of TT to determine the quantity of CT to use as adjuvant (Table 1). Ten micrograms of CT not only induced the highest CT-specific serum and fecal titers, but proved to be the most effective adjuvant dose for the generation of TT-specific antibody in both serum and fecal extracts. Higher doses of CT were not tested because of its known toxicity. Since 10 μg of CT caused no discernible toxic effects and had good adjuvancy for TT, this dose was used throughout the study.

Dose-response and time course for GI tract antibody responses to TT and CT. In these studies, oral vaccines consisting of different amounts of TT (10, 100, and 250 μg) in the presence of 10 μg of CT were used to determine optimal TT concentrations for induction of serum and mucosal antibody responses. No detectable fecal anti-TT-specific antibody responses were observed in mice receiving TT alone (250 μg per dose per mouse) or the group receiving 10 μg of TT plus 10 μg of CT (Fig. 1). Mice given 100 or 250 μg of TT with CT by the oral route exhibited IgA titers of 1:32 and 1:128, respectively. It should be noted that oral administration of CT induced brisk fecal anti-CT antibody responses (Fig. 1B). Mucosal responses, as reflected by antibody titers in fecal extracts, were primarily of the IgA isotype. The endpoint titer of fecal IgA anti-CT antibodies in each of the groups receiving 10 μg of CT corresponded to an endpoint titer of 1:512. The dose of TT did not influence the response to CT, and as expected, mice given TT only did not produce antibodies to CT.

The time course of anti-TT-specific antibody production in mice given 250 μg of TT together with 10 μg of CT is depicted in Fig. 2A. Fecal IgA antibody responses were initially detected at day 14 and rose by day 21 to a titer of 1:128. However, no fecal IgM or IgG anti-TT antibodies were detected at any time point tested. The anti-CT antibody responses in fecal extracts of mice immunized with 250 μg of TT and 10 μg of CT is illustrated in Fig. 2B. Peak IgA

TABLE 1. Dependence of TT-specific antibody responses on oral CT adjuvant dose[a]

CT dose (μg)	Titer			
	Serum IgG		Fecal IgA	
	CT-B	TT	CT-B	TT
0.1	10	9	6	0
1.0	14	13	8	4
10.0	19	15	10	6

[a] Groups of five mice were orally immunized with various amounts of CT and 160 μg of TT on days 0, 7, and 14. The pooled samples were assayed on day 21. Titers are expressed as the reciprocal $\log_2$ of the endpoint dilution (see text).

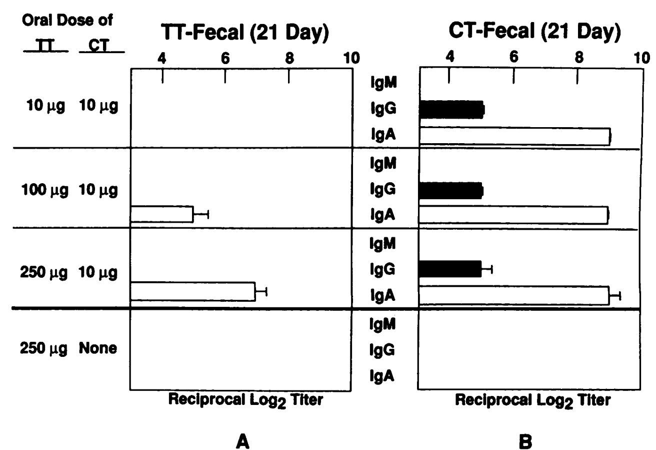

FIG. 1. Optimal antigen dose for the induction of TT-specific and CT-specific mucosal responses. (A) TT-specific antibody responses at 21 days in fecal extracts from mice immunized with TT alone and with various doses of TT plus 10 μg of CT as an adjuvant. IgA was the only isotype detected. (B) Isotype responses to CT-specific antibody. The dose of TT did not affect the CT-specific response. In this and subsequent figures, results are representative of two or three experiments and error bars denote the standard error of the mean.

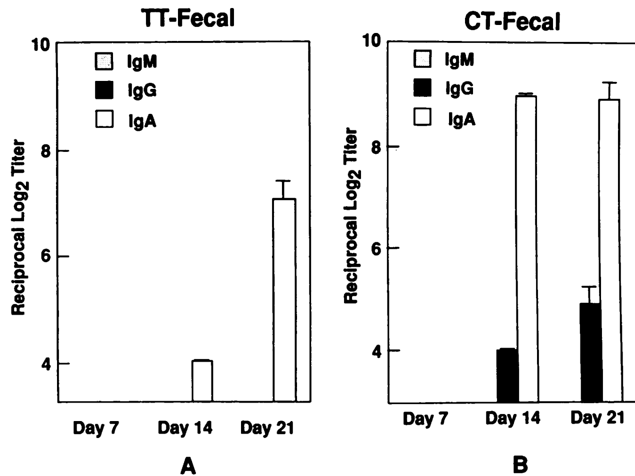

FIG. 2. Time course of mucosal antibody responses. (A) TT antibody response in mice immunized orally with 250 μg of TT plus 10 μg of CT. Optimal IgA responses occurred on day 21. (B) CT-specific response. Both IgG and IgA isotypes were detected. Peak IgA titers were observed on day 14 and remained constant at day 21.

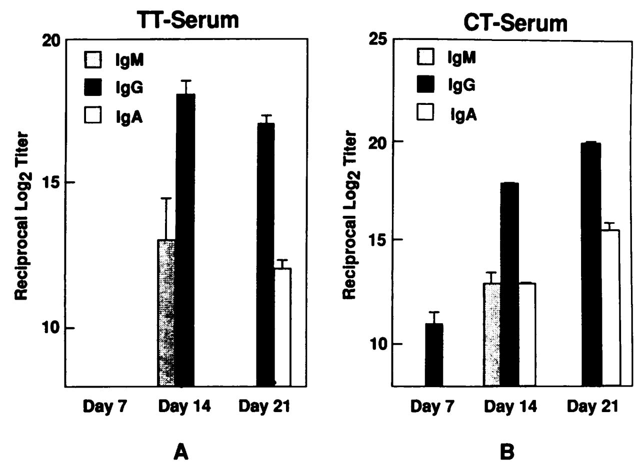

FIG. 4. Time course of serum antibody responses. (A) Anti-TT antibody isotypes detected after a dose of 250 μg of TT plus 10 μg of CT. Serum IgG levels peaked by day 14, while IgA antibody responses were observed by day 21. A transient IgM response was seen at day 14. (B) IgG anti-CT antibodies were detected by day 7 and peaked by day 21. A transient IgM response was observed at day 14, and a significant serum IgA level was attained by day 21.

responses were found by day 14 and remained constant at day 21. Furthermore, IgG anti-CT-specific antibody responses were also found in fecal samples from immunized mice (Fig. 2B). In contrast, no fecal IgG antibodies to TT were found.

Dose-response and kinetics of serum antibody responses. Serum anti-TT-specific antibody levels in mice that received oral vaccine containing TT in the presence or absence of CT are depicted in Fig. 3A. In the absence of CT, 250 μg of TT induced a weak (1:2,000) antibody response at day 21, whereas in the presence of CT, a dose of TT as low as 10 μg elicited elevated serum IgG antibody levels (1:32,000). While serum IgG levels peaked with 100 μg of TT in the presence of CT, serum IgA levels were higher in mice given the 250-μg TT dose in the presence of CT. Good serum anti-CT antibody responses were induced when 10 μg of CT was

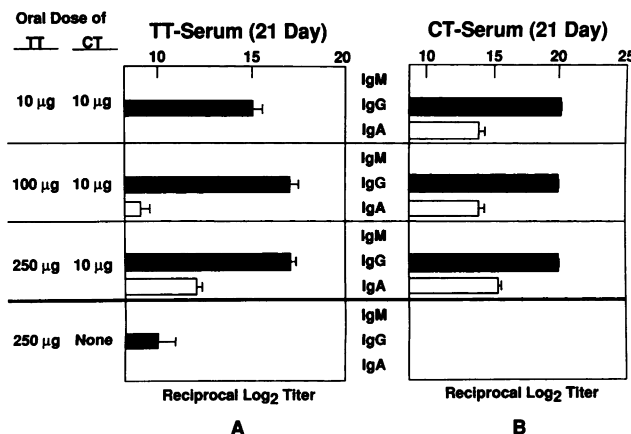

FIG. 3. Optimal antigen dose for the induction of TT-specific and CT-specific serum antibody responses. (A) TT-specific antibody production of IgG and IgA isotypes at various doses of TT with and without CT. An IgG response to 250 μg of TT given without CT was not seen prior to day 21. (B) Anti-CT response was not influenced by the TT dose.

administered with various concentrations of TT. The highest serum responses to CT were of the IgG isotype; however, significant serum IgA responses were also noted (Fig. 3B). The dose of TT did not influence the response to CT.

We next measured the isotypes and titers of serum anti-TT antibodies in mice orally immunized with the vaccine containing 250 μg of TT and 10 μg of CT throughout the 21-day period (Fig. 4A). No antibodies were detectable 1 week following the first immunization; however after two consecutive oral immunizations, both IgM and IgG antibodies to TT were detected (Fig. 4A). By day 21, IgM antibodies were no longer found, and IgG anti-TT antibody levels remained relatively constant (titer of 1:130,000). Three consecutive oral doses were required to induce serum IgA anti-TT antibody responses. Thus, IgA anti-TT-specific titers of 1:4,000 were seen at day 21. CT-specific antibody levels and their isotypes are depicted in Fig. 4B. Serum IgG anti-CT antibodies were present at day 7 and increased to a titer of >1:1,000,000 by day 21. A transient IgM response was noted at day 14. In contrast to TT-specific IgA responses in serum (Fig. 4A), IgA anti-CT antibodies were already evident by day 14 and reached titers of 1:32,000 by day 21 (Fig. 4B).

Polyclonal and antigen-specific B-cell responses. Having established the optimal dose of TT and the number of immunizations required for induction of peak mucosal IgA responses with CT as an adjuvant, we subsequently examined both total (polyclonal) and antigen-specific B-cell responses in mice that received 250 μg of TT only or 250 μg of TT plus 10 μg of CT by the Elispot assay. Both total Ig-secreting cells and antigen-specific antibody-producing SFCs were evaluated in the spleen, as an example of systemic lymphoid tissue, and in PP and the lamina propria from the GI tract, as mucosal IgA inductive and effector sites, respectively.

In order to determine whether administration of CT influenced the distribution of B cells capable of secreting antibody, total numbers of IgA, IgG, and IgM SFCs in each of the tissues were compared. Clearly, CT did not influence the frequency of cells secreting total IgA, IgG, or IgM in the intestinal lamina propria (Fig. 5). As expected, the dominant

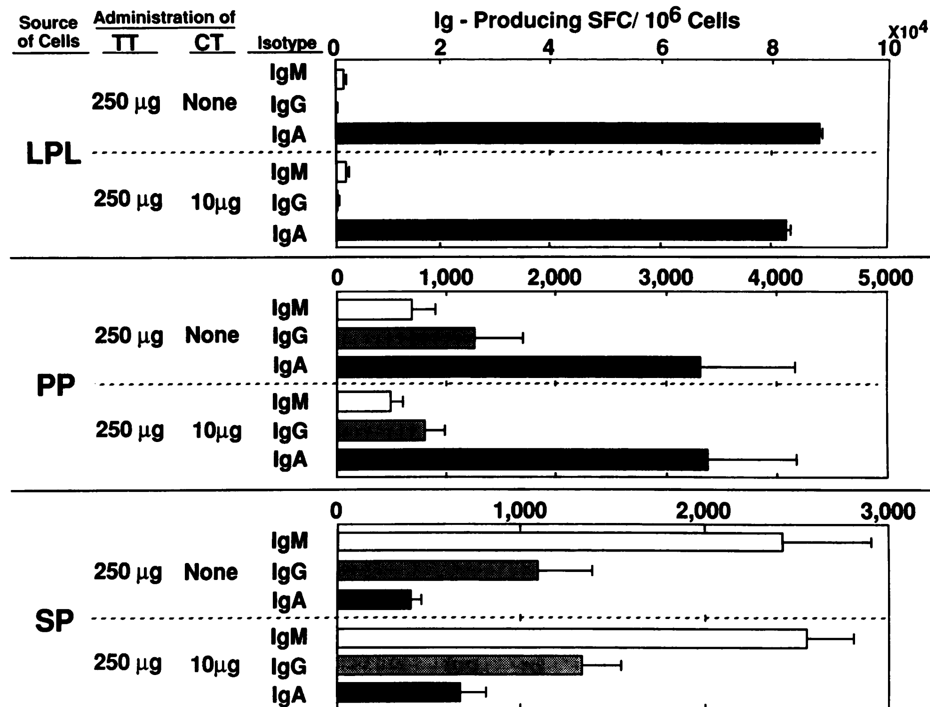

FIG. 5. Isotype profile comparisons of total SFCs. The numbers and isotype SFC profiles of mice receiving 250 μg of TT alone or 250 μg of TT plus 10 μg of CT were compared in LPLs, PP, and spleen cells (SP).

Ig-producing cells were of the IgA isotype in both groups of mice that received vaccine containing TT only or TT and CT. In the case of PP, both groups of mice contained high numbers of IgA-producing cells, followed by IgG and IgM (Fig. 5). Spleen cells from both groups had elevated IgM SFCs together with IgG and low-IgA SFCs. These findings suggested that neither TT nor CT affected the total number of IgM-, IgG-, or IgA-producing cells in either mucosa-associated or systemic tissues of orally immunized mice.

The levels of antigen-specific B-cell responses in mice orally immunized with 250 μg of TT and 10 μg of CT were also assessed. The LPLs isolated from mice orally immunized with TT and CT contained a large population of cells secreting anti-CT-specific IgA in addition to TT-specific IgA SFCs (Table 2). An average of 4,700 SFCs per 10^6 CT-specific antibody-producing cells was found in this mucosal effector site. PP exhibited approximately 10-fold fewer SFCs than LPLs, consistent with this tissue's role as an IgA inductive site. When isolated cells secreting TT-specific antibodies were quantitated, the IgA isotype predominated, and LPLs contained approximately 1,200 SFCs per 10^6 total cells. In contrast to mice that received 250 μg of TT and 10 μg of CT, those orally immunized with 250 μg of TT alone exhibited few TT-specific IgA-secreting cells (Table 2). In these studies, no TT-specific IgA-producing cells were seen in the spleen. However, although the numbers were low, TT-specific IgA SFCs were noted in IgA inductive tissues (PP).

We next assessed the kinetics of splenic B-cell responses in mice orally immunized with 250 μg of TT and 10 μg of CT. Mice given a single oral dose showed low IgM anti-TT and anti-CT SFC responses 7 days later; however, significant numbers of IgA anti-CT SFCs were seen (day 7, Fig. 6). Peak IgG anti-CT and anti-TT SFC responses together with lower numbers of IgM and IgA SFCs occurred in the spleens of mice given two oral doses (day 14, Fig. 6). Splenic SFC

TABLE 2. Oral vaccination of mice with TT and CT induces antigen-specific antibody-producing cells in mucosa-associated tissues[a]

Antigen-specific response	Isotype	Mean no. of antigen-specific SPCs/10^6 cells ± SEM in tissues from mice immunized with:					
		TT + CT			TT alone		
		LPL	PP	Spleen	LPL	PP	Spleen
TT	IgA	1,166 ± 162	80 ± 5	7 ± 0.3	90 ± 45	125 ± 55	3 ± 1
	IgG	25 ± 14	1 ± 1	11 ± 1	0	17 ± 17	1 ± 1
	IgM	0	16 ± 3	5 ± 4	1 ± 1	22 ± 17	26 ± 24
CT	IgA	4,733 ± 588	426 ± 54	67 ± 17	0	0	0
	IgG	133 ± 35	138 ± 20	47 ± 21	0	0	0
	IgM	3 ± 3	15 ± 5	10 ± 5	0	0	0

[a] Groups of five mice were orally immunized with 250 μg of TT plus 10 μg of CT or 250 μg of TT alone on days 0, 7, and 14. Mice were killed on day 21. Values are the means for three experiments (TT plus CT) or two experiments (TT alone).

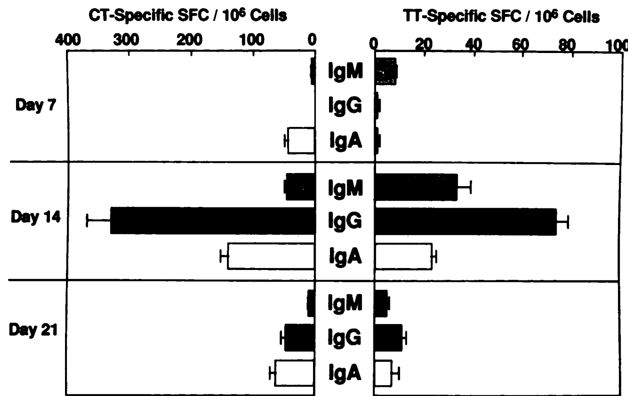

FIG. 6. Spleen SFC isotype profile at 7, 14, and 21 days. CT-specific (left) and TT-specific (right) SFCs were assessed on the days shown. Numbers of SFCs for each isotype peaked on day 14.

responses to both CT and TT declined markedly 1 week after the third oral dose and were in sharp contrast to IgA anti-CT and anti-TT SFC responses in the lamina propria of these mice (Table 2).

Oral immunization induces protective antibodies to TT. It was important to determine the quality of anti-TT antibody responses induced by oral immunization. We therefore determined the minimum lethal dose of tetanus toxin in small groups of naive mice. Subsequently, different groups of mice were orally immunized with TT alone, CT alone, or a combination of TT and CT. As additional controls, mice were systemically immunized as described in Table 3, footnote *a*. All groups of mice were challenged with 100 minimum lethal doses of tetanus toxin on day 21. While mice systemically immunized with TT plus CT survived, as expected, oral immunization with TT and CT also provided complete protection, clearly demonstrating the effectiveness of this route of immunization (Table 3). In current experiments, we are performing in vitro neutralization assays to determine whether the fecal IgA anti-TT antibodies are also protective.

DISCUSSION

In the present study, we used a well-characterized protein-based vaccine, TT, in order to study the kinetics and likely origin of B-cell and antibody responses of the major isotypes in systemic and mucosal compartments of mice after oral administration of TT with the mucosal adjuvant CT. Despite numerous past studies in which CT and proteins were used for oral immunization, the origin of antigen-specific B cells which contribute to antibodies in serum or in external secretions is ambiguous. It is, however, well established that oral immunization of mice with protein antigens and vaccines together with CT effectively induces serum antiprotein and anti-CT antibodies, most notably of the IgG and IgA isotypes. However, the actual locale where B cells, whose progeny give rise to the serum antibody response, are induced remains unclear. Even more surprisingly, it is not yet formally established that oral immunization with protein together with CT initially triggers precursor IgA B cells in PP for ultimate IgA plasma cell responses to both protein and CT in mucosal effector sites such as the lamina propria of the GI tract. These are important issues in mucosal vaccination, since ideally the goal will be to optimize priming of both systemic and mucosal immune compartments following oral immunization.

It was important to first establish optimal doses of TT for induction of both serum and mucosal antibody responses. Our studies as well as those of others (9, 17, 32) have shown that 10 μg of CT is an effective oral dose for induction of both serum and mucosal anti-CT antibody responses, and furthermore, this dose also effectively promotes antibody responses to the coadministered protein (Table 1). Dose-response studies with increasing amounts of TT indicated that 250 μg of TT plus 10 μg of CT was required for induction of maximum IgA antibodies in fecal extracts (Fig. 1). However, a surprising finding was that lower doses of oral TT, even as low as 10 μg, coadministered orally with CT induced elevated serum anti-TT antibodies which were solely of the IgG isotype (Fig. 3). This result suggested that one could selectively induce serum antibodies by use of small antigen doses, and we are currently testing this assumption with other protein-based vaccines. This finding could open new vistas for vaccines, in which oral immunization can be used to induce systemic responses as well. The challenge study also demonstrated that orally induced TT-specific antibody responses in the systemic compartment provided protection against tetanus toxin challenge (Table 3). This would indicate that the specific B-cell epitopes of TT associated with the induction of protective antibodies survive in the GI tract environment. Current studies are comparing fecal S-IgA and serum IgG anti-TT antibodies for effective neutralization of tetanus toxin. For practical purposes, if we can establish oral immunization regimens for the generation of protective antigen-specific responses in serum, this could substitute for currently existing protocols in which vaccines are administered by the systemic route.

We noted that increased doses of oral TT plus CT induced some serum IgA anti-TT antibody responses, and this correlated with the presence of IgA antibodies in fecal extracts. Two possibilities could be suggested for the source of TT-specific IgA antibody in serum. First, oral immunization with TT and CT may induce IgA responses in the spleen and peripheral lymph nodes, since CT has been shown to induce switching of IgM B cells to IgA-producing cells in lipopolysaccharide-triggered splenic B-cell cultures (23). We favor the alternative explanation, that the IgA anti-TT antibodies in serum may be derived from mucosal sites, e.g., the lamina propria of the GI tract, and this point is discussed in more detail below. In any case, it will be important to study the contribution of orally induced antigen-specific serum IgA

TABLE 3. Oral immunization with TT and CT induces protective immunity to systemic challenge with tetanus toxin[a]

Immunization route	Antigen and dose (μg)	Serum IgG anti-TT titer (reciprocal log_2)	No. of mice dead at 96 h/4 per group
Oral	CT (10)	0	4
	TT (250)	11	4
	TT (250) + CT (10)	17	0
Systemic	CT (0.2)	0	4
	TT (10)	12	3
	TT (10) + CT (0.2)	19	0

[a] The minimum lethal dose (MLD) of toxin was established as 1 μg per mouse in naive mice (data not shown). Oral immunizations with TT and CT were performed on days 0, 7, and 14 at the doses indicated. Systemic immunizations were given intraperitoneally on day 0, and mice were boosted on day 14. All groups were challenged on day 21 by subcutaneous injection of 100 MLDs of toxin in 0.5 ml of PBS–0.2% gelatin.

responses in addition to the IgG isotype in protection against challenge.

It appears that different kinetics of immune response occurred in serum and external secretions. For example, the time course study suggested that fecal IgA anti-CT responses were first present 1 week following the second oral immunization. Serum IgA anti-CT antibodies were also noted at day 14 and reached higher titers after the third oral immunization with CT (Fig. 4). Interestingly, IgG anti-CT antibody responses were noted in serum after only one oral dose (day 7) and rose to high levels after the second and third immunizations. The presence of increasing levels of IgG anti-CT antibodies in fecal extracts on days 14 and 21 corresponds to the marked increases in serum IgG titers and suggests that these fecal IgG responses may be serum derived.

More definitive proof for the origin of anti-TT and anti-CT antibodies in serum and in the GI tract were provided by single-cell Elispot analysis. For example, only IgA SFCs to TT or CT were found in LPLs isolated from the GI tract of mice given oral TT and CT (Table 2). Since only one time point was sampled, it could be argued that plasma cells producing IgG anti-CT or anti-TT in the lamina propria followed different kinetics; however, our antibody analyses indicated that IgG antibodies in fecal extracts actually increased with time and reached their highest concentrations at day 21. Thus, the highest levels of IgG anti-TT and anti-CT were present at the time chosen for B-cell analysis. Thus, it is likely that the IgA antibodies present in the GI tract were derived from local production by plasma cells in the lamina propria, whereas the IgG antibodies were serum derived. It is tempting to speculate that CT induces increased transudation of serum antibodies after oral delivery, especially after booster immunizations.

The presence of high but transient numbers of IgG anti-TT and anti-CT SFCs in the spleens of mice orally immunized with these antigens provides additional support that the serum IgG antibodies are derived from peripheral lymphoid tissue, e.g., the spleen. This does not prove that orally administered TT plus CT reaches peripheral lymphoid tissue and elicits an immune response. However, recent studies have shown that orally administered CT enhances the uptake of bystander antigens across the epithelial cell barrier (21), and it is logical to assume that antigen reaches peripheral lymphoid tissue in sufficient amounts for induction of antibody responses (21). However, our results, unlike those of earlier studies, suggest that this is an efficient process, since small amounts of TT (10 μg) induced significant serum IgG anti-TT antibody responses. Furthermore, higher oral doses of TT given with CT resulted in elevated serum antibody responses.

The finding that 250 μg of TT given orally without CT induced small but significant serum IgG anti-TT responses also merits some discussion. We purposely chose not to use higher doses of TT because we found that oral administration of 500 μg or 1 mg on more than one occasion induced systemic unresponsiveness (data not shown). Generally, soluble proteins (other than CT) are not strongly immunogenic when given by the oral route. However, it is a misconception to indicate that lower doses of proteins given orally induce oral tolerance. In almost all instances, milligram amounts of soluble proteins are required for the induction of oral tolerance (32). We thus chose a weakly immunogenic rather than a tolerance-inducing dose of TT for the present study.

The above studies suggest that systemic immune responses are more effectively induced than accompanying mucosal IgA responses. In this regard, low antigen doses induced antibodies in serum more quickly than in GI tract secretions. The induction of maximum fecal IgA responses required higher doses of TT and was detected only after three consecutive immunizations. Additional studies with other protein vaccines given orally with CT will be required to confirm these results; however, it is clear that some commonly accepted ideas about mucosal responses may require reevaluation. For example, it is often assumed that CT, when given orally with other proteins, mainly primes the immune system for enhanced mucosal IgA responses. It has been suggested that oral CT induces long-lived memory B cells for IgA responses, and compelling data to support this have been presented (16, 18–20). However, it is equally likely that oral CT, together with proteins such as TT, induces significant memory in the systemic immune compartment as well. In fact, this priming for serum-derived antibody responses may be as effective as has been shown for CT (16, 18–20) and more recently for keyhole limpet hemocyanin in mice (33).

We should emphasize that our studies with CT as an oral adjuvant were done in order to optimize antibody responses to the coadministered vaccine. It is realized that CT is toxic in humans and thus would not be a suitable adjuvant. However, it remains the optimal method for induction of mucosal S-IgA and serum IgG responses to orally administered protein antigens, which normally induce poor responses when given alone.

In summary, our present study has provided compelling evidence that oral immunization with a vaccine containing TT and the mucosal adjuvant CT induced antigen-specific responses in both mucosal and systemic tissues. Furthermore, TT-specific responses generated in the latter compartment by oral immunization provided protective immunity. Thus, one can envision the extension of oral antigen delivery for the induction of systemic responses in addition to classical mucosal antibody responses. Immunization of subjects with protein vaccines such as TT and an optimal dose of mucosal adjuvant may provide double protection in both systemic and mucosal sites.

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