Helper T Cell Subsets for Immunoglobulin A Responses: Oral Immunization with Tetanus Toxoid and Cholera Toxin as Adjuvant Selectively Induces Th2 Cells in Mucosa Associated Tissues

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Summary

Antigen-specific B cell responses to mucosally delivered proteins are dependent upon CD4-positive T helper (Th) cells, and the frequency of Th1 and Th2 cell responses after oral immunization may determine the level and isotype of mucosal antibody responses. We have used a protein-based vaccine, tetanus toxoid (TT), together with the mucosal adjuvant cholera toxin (CT), for oral immunization of mice to study the nature of antigen-specific Th cell subsets induced in Peyer's patches (PP) of the gastrointestinal (GI) tract and in the spleen (SP) during peak antibody responses. Mice orally immunized with TT and CT responded with antigen-specific secretory immunoglobulin A (S-IgA) antibodies in the GI tract, and with both IgG and IgA antibody responses in serum. PP and SP $CD4^+$ T cells from mice orally immunized with TT plus CT were cultured with antigen-coated latex microspheres for induction of proliferative responses and for enumeration of cytokine producing $CD4^+$ T cells. Interestingly, both PP and SP $CD4^+$ T cell cultures showed increased numbers of IL-4- and IL-5 (Th2-type)-producing, spot-forming cells (SFCs) after 21 d of immunization, while essentially no interferon-γ (IFN-γ) or IL-2 (Th1-type) SFCs were noted. Cytokine-specific Northern blots and RT-PCR also revealed that significant IL-4 and IL-5 mRNA levels, but not IFN-γ or IL-2 mRNA, were present in $CD4^+$ T cells isolated from antigen-stimulated cultures. However, systemic immunization with TT and CT induced antigen-specific IgG and IgM but not IgA antibodies in serum. Further, both IL-2- and IFN-γ-producing Th1-type cells as well as IL-4- and IL-5-secreting Th2-type cells were generated in SP. Our results show that oral immunization with TT and the mucosal adjuvant CT selectively induced antigen-specific Th2-type responses which may represent the major helper cell phenotype involved in mucosal IgA responses in the GI tract.

Studies of cytokine production by murine Th cell clones have provided direct evidence for the existence of at least two different Th subsets that may have a common precursor (Th0) (reviewed in references 1–4). Th1 cells selectively produce IL-2, IFN-γ, and TNF-β, whereas Th2 cells are unique in the production of IL-4, IL-5, IL-6, and IL-10 (1–4). These distinct cytokine profiles contribute to pronounced differences in Th cell function. The Th1 cell subset is preferentially involved in cell-mediated immunity, while Th2 cells are most effective in the regulation and support of B cell responses (1–4). In the case of IgA responses, IL-5 and IL-6 have been shown to induce committed surface IgA^+ B cells to secrete IgA (5–10).

It has also been established that certain antigens and microbial infections elicit responses predominantly associated with one of the two Th cell subsets (11–14). Downregulation of Th1 cytokine production accompanied by the induction of Th2 cell responses is a characteristic of the immune response to the parasitic helminth, *Schistosoma mansoni* (11). Furthermore, it was shown that the murine nematode *Trichuris muris* induced predominantly IFN-γ-secreting Th1-type cells in susceptible mouse strains, while IL-4- and IL-5-producing

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Th2-type cells were noted in resistant strains (13). Different routes of immunization may also induce different subsets of Th cells which may in turn influence the isotype of B cell responses. Oral immunization of mice with SRBC resulted in predominantly IL-5-producing, Th2-type cells in both Peyer's patches (PP)[1] and spleen (SP), while higher frequencies of IFN-γ-producing Th1-type cells occurred in SP of mice given antigen by the systemic route (15). Furthermore, it has been shown that higher numbers of IL-5-producing, Th2-type cells, when compared with IFN-γ-producing T cells, were seen in mucosal effector tissues, i.e., the lamina propria (LP) of the gastrointestinal (GI) tract and in the salivary glands (16, 17).

Cholera toxin (CT), a protein exotoxin produced by *Vibrio cholerae*, is a multi-subunit molecule composed of two structurally and functionally distinct components. The B subunit consists of five identical polypeptides that bind with high affinity to GM1 ganglioside cell surface receptors and facilitate the entry of the A subunit into the cell (18, 19). CT has been widely used because of its strong immunogenicity in IgA responses, and also because of its capacity to act as a mucosal adjuvant for other antigens or vaccines (20–26). However, the precise mechanism for CT adjuvancy in the mucosal immune system is not yet understood.

This work has focused on the Th cell subsets associated with optimal IgA responses in mucosal inductive sites (e.g., PP) in the GI tract and in the SP of mice orally immunized with a protein vaccine in the presence of the mucosal adjuvant CT. For these studies, we chose tetanus toxoid (TT) as a typical protein vaccine and assessed immunity in both T and B cell compartments to both TT and CT. Unlike past in vitro studies which suggest that CT inhibits T cell responses, this study has shown that CT facilitates the induction of Th2 cell responses to both itself and to coadministered antigen (TT) in PP and SP when given orally, but enhances both Th1 and Th2 responses when administered by the systemic route.

Materials and Methods

Mice. C57BL/6 mice that had been barrier maintained and were pathogen–antibody negative were obtained from the Frederick Cancer Research Facility (National Cancer Institute, Frederick, MD) at 5–6 wk of age, maintained in horizontal laminar flow cabinets, and provided with sterile food and water ad lib. All mice used in this study were between 8 and 12 wk of age.

Immunization of Mice. Mice were deprived of food for 2 h and given 0.5 ml of a solution containing eight parts HBSS and two parts 7.5% sodium bicarbonate by gastric intubation to neutralize stomach acidity. After 30 min, mice were orally immunized by intubation of 0.25 ml PBS containing a mixture of TT (250 μg/mouse) kindly provided by PRAXIS Biologics, Inc. (West Henrietta, NY) and CT (10 μg/mouse; Sigma Chemical Co., St. Louis, MO). The mice were boosted 1 and 2 wk later. Dose–response and time course studies showed that this schedule induced optimal mucosal and serum antibody responses to TT (Jackson, R. J., K. Fujihashi, J. Xu-Amano, H. Kiyono, C. D. Elson, and J. R. McGhee, manuscript in preparation). Mice were killed 1 wk after each oral immunization. For systemic immunization, mice were intraperitoneally immunized with TT (10 μg/mouse) and CT (1 μg/mouse) or TT (10 μg/mouse) only and boosted in the same manner 2 wk later. Mice were killed 1 wk after each immunization. Other groups of mice were immunized by the intraperitoneal route with TT (100 μg/mouse) in the presence of CFA and boosted intravenously with TT (10 μg/mouse) and CT (1 μg/mouse) 4 mo later. Mice were killed 1 wk after the booster immunization.

Detection of TT- and CT-specific Antibody Production by ELISA. Antibody responses were determined by ELISA. Briefly, TT (0.08 Lf units/well) or CT-B at 5 μg/ml in PBS was coated to Falcon (Microtest III) 96-well assay plates (Becton Dickinson, Oxnard, CA) overnight at 4°C. Plates were blocked with 1% BSA-PBS for 1 h at 37°C. Fecal extract samples were obtained by vortexing fecal pellets in PBS as described by others (27). The serum and fecal extracts were serially diluted in 1% BSA-PBS and incubated for 2 h at 37°C with coating antigens. Detection antibodies consisting of a 1:1,000 dilution of biotinylated goat anti-mouse-μ, -γ, or -α (Southern Biotechnology Associates, Birmingham, AL) in PBS with 0.5% Tween-20 (PBS-Tween) were then added to the plate and incubated overnight at 4°C. A 1:1,000 dilution of detection enzyme, horseradish peroxidase–streptavidin (2.5 mg/ml; Zymed Laboratories, Inc., South San Francisco, CA) was added to the plate and incubated at room temperature for 1 h. The reaction was developed by a substrate solution consisting of 1.1 mM ABTS (2,2′ azino-bis [3-ethylbenz-thiazoline-6-sulfonic acid], diammonium salt; Sigma Chemical Co.) in 0.05 M citrate-phosphate buffer, pH 4.2, containing 0.01% H_2O_2 for 15 min. The developing reaction was terminated by the addition of 50 μl per well of 10% SDS solution in 0.05 M citrate-phosphate buffer, pH 4.2. Optical density was recorded in a plate reader at 414 nm (model EL 312; Bio-Tek Instruments, Winooski, VT). End point titers were expressed as the last dilution of sample which gave an OD value of ≥0.1 after subtraction of nonimmunized control serum or normal fecal extract values (28).

Preparation of Cell Suspensions from SP, PP, and Intestinal LP. SP were aseptically removed and single cell suspensions were prepared as described previously (8, 16, 17). PP were carefully excised from the intestinal wall and dissociated using the neutral protease enzyme Dispase® (Boehringer Mannheim Corp., Indianapolis, IN) in Joklik-modified medium (GIBCO BRL; Gaithersburg, MD) to obtain single cell preparations (8, 16, 17).

Purification of $CD4^+$ T Cells. Single cell suspensions prepared from SP were incubated in complete medium consisting of RPMI 1640 (Whittaker Bioproducts, Walkersville, MD) supplemented with nonessential amino acids, sodium pyruvate, Hepes, penicillin, streptomycin, gentamycin (incomplete medium), and 10% FCS for 4 h at 37°C, 5% CO_2 in air to remove adherent cells. The $CD3^+$ T cells were obtained by negative panning using goat anti-mouse Ig $F(ab')_2$ in order to avoid T cell stimulation as previously described (15). For enrichment of PP T cells, enzymatically isolated PP cells were incubated in complete medium for at least 6 h at 37°C, 5% CO_2 in air to allow full reexpression of surface molecules and to remove adherent cells before further T cell purification by panning with mAb anti-L3T4 as described elsewhere (15). This procedure resulted in cell fractions containing >99% $CD4^+$, $CD8^-$ T cells.

Antigen-coated Latex Microspheres for Induction of $CD4^+$ T Cell Responses. The proteins TT and CT-B were coated to latex microspheres by a modification of a previously described method

[1] *Abbreviations used in this paper:* CT, cholera toxin; E/C, experimental/control; GI, gastrointestinal; LP, lamina propria; LPL, lamina propria lymphocytes; MLN, mesenteric lymph nodes; PP, Peyer's patches; RT, reverse transcriptase; SFC, spot forming cells; SP, spleen; TT, tetanus toxoid.

(29). Briefly, 1 ml of Polybead®–hydroxylate latex microspheres (1.0 μm; Polysciences Inc., Warrington, PA) was washed and resuspended in 400 μl of 0.1 M bicarbonate buffer (pH 8.8) and TT or CT-B (80 μg/400 μl) in 0.5 M Tris HCl buffer (pH 8.0) was slowly added with continuous mixing. This suspension was further diluted with 400 μl of bicarbonate buffer and then incubated for 24 h at 25°C with continuous rocking. The protein-coated microspheres were washed twice with sterile PBS, resuspended in RPMI 1640 with 500 μg/ml gentamycin, and incubated for 48 h at 37°C to effect sterility. This procedure routinely yielded 0.5 μg of TT or CT-B/10^8 beads and represented ~30% of the original sample as determined by measuring the protein concentration remaining in the buffer phase. $CD4^+$ T cells (10^5/well) were cultured in flat-bottom, 96-well microculture plates (Corning Inc., Corning, NY) in the presence of IL-2 (10 U/ml), T cell–depleted, irradiated (3,000 rad) splenic feeder cells (10^4–10^5 cells/well) and with different doses of TT- or CT-B-coated latex microspheres (particle/cell ratios of 50:1, 10:1, and 2:1) or soluble proteins (0.1–10 μg/ml). As controls, wells contained cells only or cells incubated with the unrelated antigens KLH- or diphtheria toxoid–coated latex microspheres. Cultures were maintained at 37°C and 5% CO_2 in air for various periods (1, 3, and 6 d) and 0.5 μCi of [^{3}H]thymidine (Amersham Corp., Arlington Heights, IL) was added to all wells 6 h before harvest. Cell proliferation was determined by standard liquid scintillation counting. The stimulation index E/C (experimental/control) was determined in each experiment.

For analysis of antigen-specific Th1 and Th2 cell responses, SP or PP $CD4^+$ T cells were cultured at a density of 10^6 cells/ml together with either TT- or CT-B-coated latex microspheres. In addition, T cell–depleted, irradiated (3,000 rad) splenic feeder cells and IL-2 (10 U/ml) were added to individual cultures. Antigen-stimulated T cells were harvested at various intervals (1, 3, and 6 d) for the enumeration of cytokine (e.g., IFN-γ, IL-2, IL-4, or IL-5) producing cells by use of cytokine-specific ELISPOT assay, and for RT-PCR and Northern blot analysis of mRNA expression (see below).

Enumeration of IFN-γ, IL-2-, IL-4-, and IL-5-producing Cells. The ELISPOT assay was adapted for cytokine analysis in antigen-specific T cell cultures. For the IFN-γ-specific ELISPOT assay, mAb anti-IFN-γ R4-6A2 (30) and XMG 1.2 (31) were used as coating and detection antibodies, respectively, as described previously (16, 32). For measurement of IL-2 specific spot forming cells (SFC) mAb anti-mouse IL-2 (CR No. 40014; Collaborative Research, Inc., Bedford, MA) was used as the coating antibody, while rabbit IgG anti-mouse IL-2 (Collaborative Research, Inc.) was used for detection as recently described (33). A similar assay was developed for assessment of IL-4–specific SFC with rat mAb anti-mouse IL-4 (BVD4-1D11; PharMingen, San Diego, CA) used for coating and a biotinylated mAb anti-mouse IL-4 (BVD6-24G2; PharMingen) (2 μg/ml) followed by peroxidase-conjugated goat anti-biotin (5 μg/ml; Vector Labs, Inc., Burlingame, CA) used in detection (33). For the elucidation of IL-5–specific SFC, two monoclonal anti-mouse IL-5 mAb (TRFK-5 and TRFK-4; 34) were used for coating and detection, respectively, as described in our previous studies (16, 32).

Cytokine-specific Northern Blot Analysis. For Northern blot analysis of cytokine-specific mRNA present in $CD4^+$ T cell cultures, a method described by others (35, 36) was modified and used here (15). Total RNA (20 μg) isolated by the guanidinium thiocyanate method (37) from $CD4^+$ T cell culture sample was electrophoretically fractionated in 1.2% agarose-formaldehyde gels and then stained with ethidium bromide to ensure the quality of RNA loaded and to localize the ribosomal RNA. The separated RNA was then transferred to nitrocellulose transfer membranes (Micron Separations Inc., Westboro, MA) and UV cross-linked. Blots were prehybridized at 42°C in 5× SSC containing 0.1% SDS, 50 mM NaH_2PO_4, 50% formamide, 10 mM $Na_4P_2O_8$, 5× Denhardt's solution, and 250 μg/ml sonicated salmon sperm DNA for 6 h. Hybridizations were carried out at 42°C in the same solution with the addition of ^{32}P-labeled cDNA for mouse IFN-γ, IL-2, IL-4, or IL-5 (Clontech Laboratories, Palo Alto, CA) and reacted for 48 h. Blots were washed sequentially (twice in each step) using 2× SSC and 0.1% SDS for 20 min at 42°C followed by more stringent washes with 0.2× SSC and 0.1% SDS for 20 min at 42°C. Blots were exposed to x-ray film (Eastman Kodak Co., Rochester, NY) for 96 h and then developed. The total RNA preparations were also hybridized with an actin-specific cDNA probe as an internal control for each blot.

Cytokine-specific PCR Analysis. RT-PCR analysis was also used to detect IFN-γ, IL-2, IL-4, and IL-5 mRNA expression. Briefly, SP or PP $CD4^+$ T cells were washed twice with PBS and total cytoplasmic RNA was extracted from the cells using vanadyl ribonucleoside complexes as an RNase inhibitor (38). Reverse transcription was carried out using oligo $(dT)_{16}$ primer and the specific cDNA fragment was amplified by using RT-PCR (39). The IL-2, IFN-γ, and IL-4 primers used for amplification during the PCR were obtained from Clontech Laboratories. The sequences used for IL-5 primers were: sense: 5′ ATGACTGTGCCTCTGTGCCTGGAGC 3′, antisense: 5′ CTGTTTTTCCTGGAGTAAACTGGGG 3′ (40). To verify that equal amounts of RNA were added in each RT-PCR reaction, primers for β-actin mRNA were used to amplify the cDNA reverse transcribed from the total RNA. To detect the amplified products, a total of 10 μl of the final reaction mix was run on a 2% agarose gel at 100 V for 2 h. The gels were then stained with ethidium bromide (0.5 μg/ml), visualized under UV light, and photographed. To ensure the specificity of the PCR products, the gels containing amplified IL-2, IL-4, IL-5, and IFN-γ PCR product were Southern blotted to nitrocellulose membranes (Micron Separations Inc.) and hybridized with the appropriate cytokine-specific cDNA probes. Single bands that corresponded with the size of the PCR products were detected for all cytokines tested.

Results

Anti-TT and -CT-specific Antibody Responses. We first determined the oral dose and number of immunizations required for maximum anti-TT and -CT antibody responses in serum and in GI tract secretions. We have shown that mice orally immunized with 250 μg of TT and 10 μg of CT three times at weekly intervals gave maximum IgA anti-TT and -CT responses on day 21 in fecal samples (1:128 for TT and 1:512 for CT). Further, serum IgG anti-TT and -CT antibody responses were also highest with this regimen (1:131,000 and 1:1,048,000, respectively). As a comparison, mice systemically immunized with TT and CT contained serum TT-specific IgM (1:4,000) and IgG (1:32,000) antibodies 7 d after the first immunization and markedly increased IgG (but not IgA) responses (fourfold) 1 wk after boosting. Again, CT was necessary for induction of these antibody responses, since mice systemically immunized with TT alone showed 65-fold lower TT-specific IgG responses in serum 1 wk after boosting. Thus, we chose to assess Th cell subsets in PP and SP cell cultures during peak antibody responses.

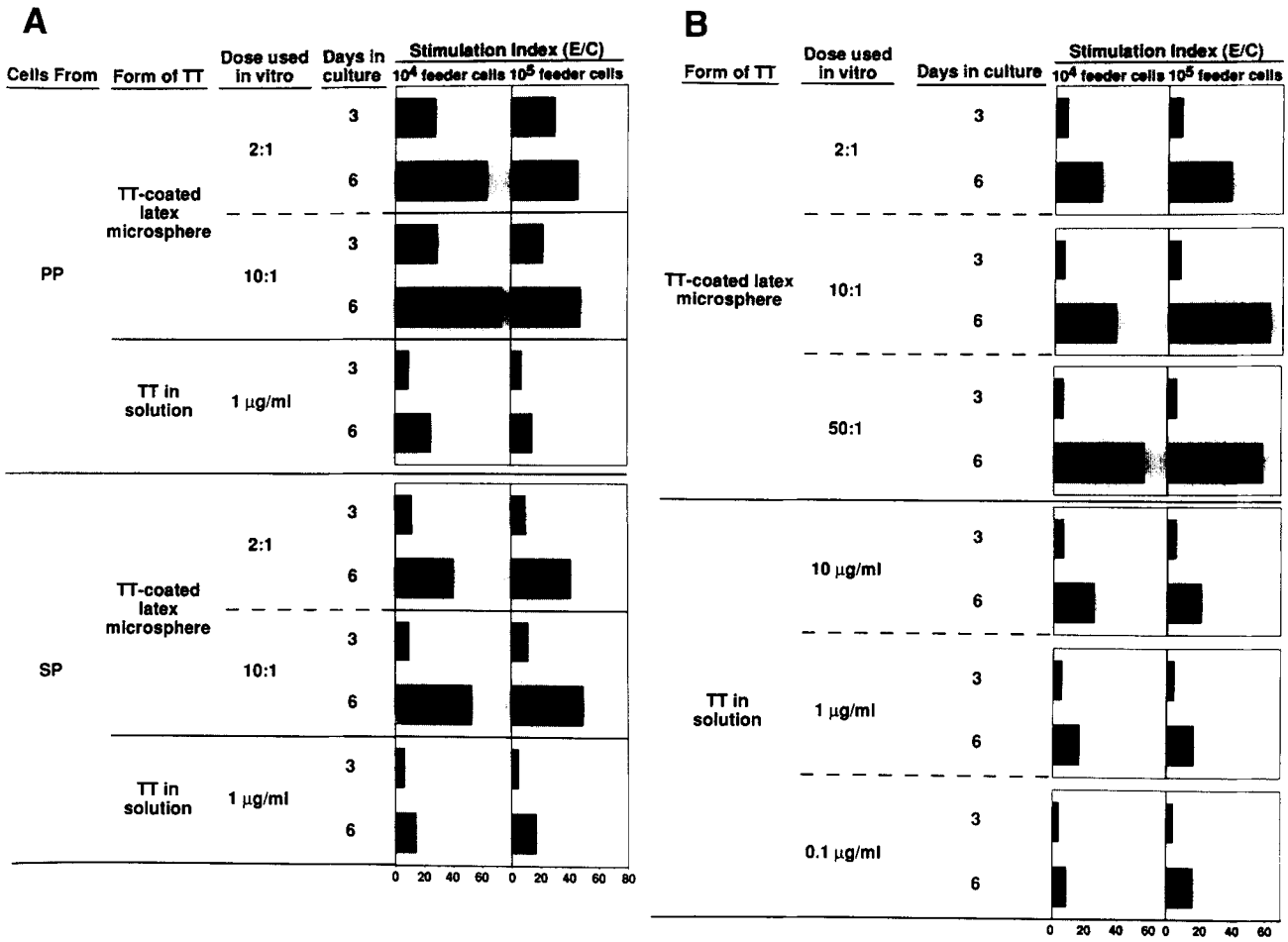

Figure 1. Antigen-specific CD4+ T cell proliferative responses in PP and SP of orally immunized mice (*A*) and in SP of systemically immunized mice (*B*). Mice were immunized by gastric intubation with TT (250 μg/mouse) plus CT (10 μg/mouse) and reimmunized 1 and 2 wk later. As control, mice were immunized by the intraperitoneal route with 0.5 ml of TT (100 μg/mouse) in CFA and boosted intravenously with TT (10 μg/mouse) and CT (1 μg/mouse) 4 mo later. 1 wk after the last immunization, mice were killed and CD4+ T cells were isolated from PP and SP and cultured at a density of 10^5 cells/well in vitro with TT-coated latex microspheres at different bead to cell ratios (2:1 to 50:1) or with soluble TT (0.1–10 μg/ml) in the presence of IL-2 and different numbers of feeder cells (10^4 or 10^5 cells/well). 6 h before cell harvest, 0.5 μCi of [^{3}H]thymidine was added to each well. The proliferative responses of CD4+ T cells after 3 and 6 d in culture were measured by assessing the amount of [^{3}H]thymidine incorporation. The results are expressed as stimulation index E/C (nonstimulated cells from naive mice). Control cultures ranged from 140 to 300 cpm.

Characterization of TT-specific T Cell Proliferative Responses. To examine antigen-specific T cell responses in vitro, CD4+ T cells were isolated from PP and SP of mice that had received three oral immunizations with TT plus CT at weekly intervals and from SP of mice initially immunized with TT in CFA, and boosted intravenously with TT and CT. CD4+ T cells were cultured in wells containing feeder cells (either 1×10^4/well or 1×10^5/well) in the presence of different doses of TT-coated latex microspheres or TT in solution (Fig. 1). After 3 and 6 d of incubation, T cell proliferative responses were measured by [^{3}H]thymidine incorporation (Fig. 1). PP and SP CD4+ T cells from mice orally immunized with TT plus CT responded vigorously to TT (Fig. 1 *A*). In this regard, stimulation indices of up to 74-fold were noted when PP CD4+ T cell cultures were stimulated with TT-coated latex microspheres. High E/C indices were always induced by TT coupled to latex microspheres when compared with TT in solution. TT-coated latex microspheres also gave significant stimulation indices for SP CD4+ T cells from the mice intravenously immunized with TT plus CT (Fig. 1 *B*). The addition of unrelated antigens (KLH- or diphtheria toxoid–coated latex microspheres) to cultures containing CD4+ T cells from TT- plus CT-immunized mice resulted in minimum stimulation (less than twofold). A 10:1 ratio of TT-coated latex microspheres to CD4+ T cells gave optimal T cell stimulation indices in 6-d cultures (Fig. 1). Furthermore, CT-B-coated latex microspheres at a bead to cell ratio of 10:1 also gave significant stimulation of CD4+ T cells from mice orally or systemically immunized with TT plus CT (data not shown). Thus, antigen-specific CD4+ Th cell proliferative responses were induced by stimulating the Th cells with TT-coated latex microspheres, and the optimal in vitro conditions for TT-specific CD4+ T cell responses were shown to be a 10:1 ratio of microspheres to T cells.

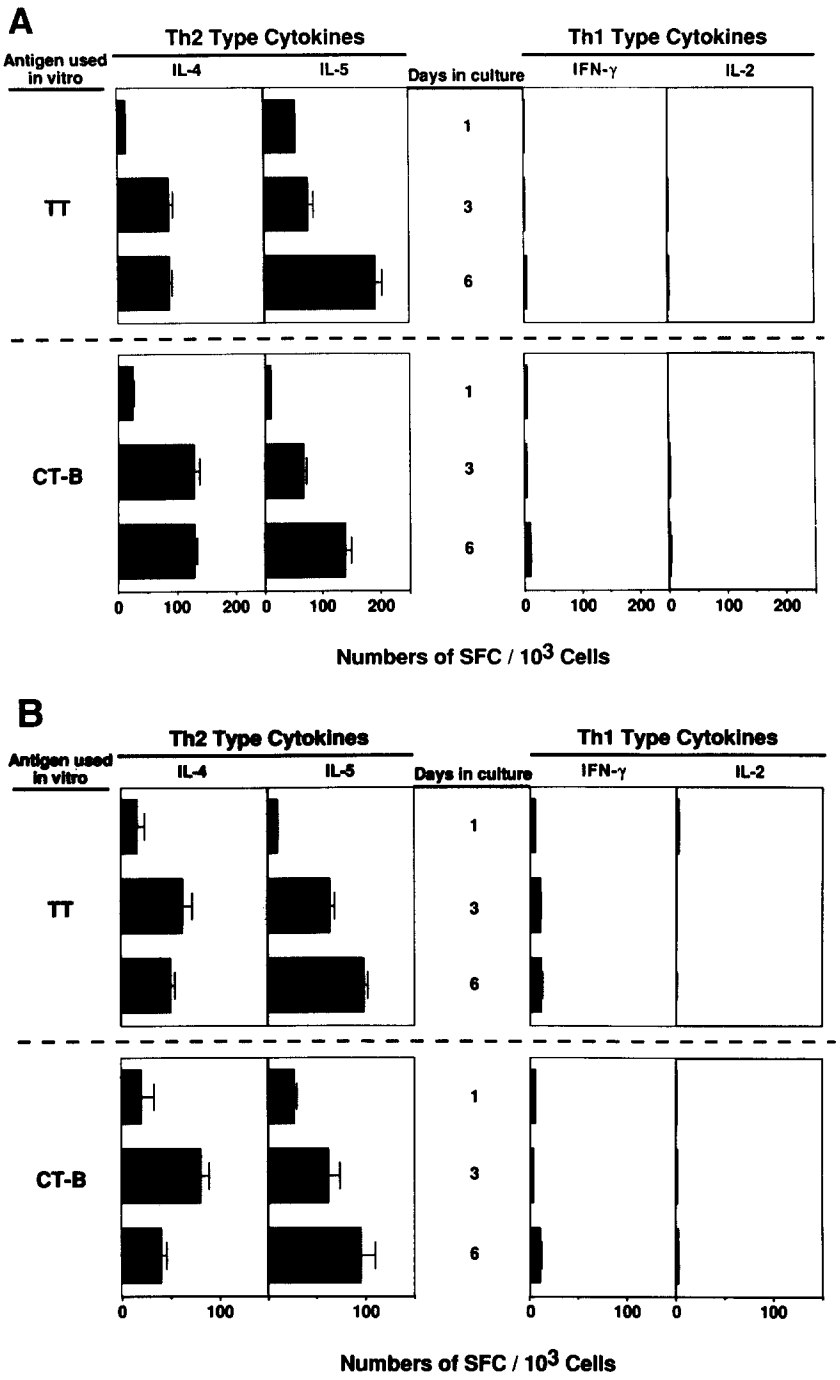

Figure 2. Oral immunization of mice induces TT- and CT-B-specific, IL-4- and IL-5-producing Th2 cells in PP (*A*) and SP (*B*). Mice were orally immunized as described in the legend to Fig. 1. 1 wk after the third oral immunization, mice were killed and PP and SP $CD4^+$ T cells were isolated and stimulated in vitro with TT- or CT-B-coated latex microspheres under optimal conditions (see Results). Control cultures were not pulsed with Ag-coated microspheres. At various culture intervals (days 1, 3, and 6), $CD4^+$ T cells were assessed for the numbers of IFN-γ, IL-2, IL-4, and IL-5 SFC by cytokine-specific ELISPOT assays. Antigen-specific cytokine-producing cells are calculated as the numbers of SFC in antigen-stimulated cultures after subtraction of the number of SFC in control cultures. The results are presented as the mean antigen-specific cytokine SFC ± 1 SE.

Oral Immunization with TT in the Presence of CT Adjuvant Preferentially Induces IL-4- and IL-5-producing Th2-Type Cells in PP and SP. We next determined the nature of Th1 and Th2 cell responses induced by oral immunization of mice with TT plus CT. The $CD4^+$ T cells were purified as described above and then cultured with either TT- or CT-B-coated latex microspheres. At various intervals (1, 3, and 6 d), the numbers of IL-2-, IFN-γ-, IL-4-, and IL-5-producing cells were determined by respective cytokine-specific ELISPOT assays (Fig. 2). The frequency of antigen-specific IL-4- and IL-5-producing cells increased with time in culture, while IL-2 and IFN-γ SFC were the same as background (Fig. 2). The predominance of Th2-type responses was maintained throughout the culture period and the numbers of IL-4 and IL-5 SFCs were generally higher in PP than in SP.

Further, when mice were orally immunized with TT only, low numbers of IL-2, IFN-γ, IL-4-, and IL-5-producing $CD4^+$ T cells were seen (Fig. 3). After in vitro antigen

stimulation, significant numbers of IL-4- and IL-5-producing Th2-type cells were seen when compared with IFN-γ- or IL-2-producing Th1-type cells. However, much higher frequencies of Th2 cytokine-producing cells were found in PP $CD4^+$ T cell cultures from mice orally immunized with TT plus CT (Fig. 3). This finding indicates that CT is important for induction of higher TT-specific Th2-type responses in orally immunized mice. Taken together, these results show that oral immunization preferentially induces antigen-specific Th2-type cells in PP and SP.

Analysis of Cytokine-specific mRNA. To determine whether the induction of Th2 cell responses by oral immunization with TT plus CT resulted from increased mRNA production, we assessed cytokine-specific mRNA expression by using Northern blot analysis with IL-2-, IFN-γ-, IL-4-, and IL-5-specific cDNA probes (Fig. 4). The results of the mRNA analyses correlated well with the ELISPOT assay. High levels of a 1.5-kb IL-5 message were detected in both PP and SP $CD4^+$ T cell cultures from mice orally immunized with TT and CT and stimulated in vitro with TT-coated latex microspheres, with higher levels noted in the PP T cell cultures. Significant amounts of 2.0-kb IL-4 message were also found in PP and SP $CD4^+$ T cell cultures. On the other hand, no signals for IL-2 or IFN-γ were seen in PP or SP $CD4^+$ T cell cultures while β-actin mRNA levels remained the same in all cultures (data not shown). These findings further support the cytokine-specific ELISPOT results and indicate that de novo synthesis of Th2-specific cytokine mRNA occurs in antigen-stimulated T cell cultures.

Since our results suggested that oral administration of the protein TT together with CT led to increased expression of cytokine-specific mRNA and synthesis of Th2-type cytokines, it was important that more sensitive methods be used to confirm this result. Accordingly, we have used cytokine-specific RT-PCR analyses to determine the cytokine mRNA expression in PP and SP $CD4^+$ T cell cultures. A 243-bp band that corresponded to IL-5-specific RT-PCR product was consistently seen in RNA preparations obtained from both PP and SP $CD4^+$ T cells of orally immunized mice (Fig. 5), and in vitro stimulation with TT-coated microspheres resulted in marked increases in IL-5-specific mRNA. The IL-4 mRNA expression was weak in PP $CD4^+$ T cells at day 0; however, after in vitro stimulation, increases in IL-4 mRNA were noted in PP $CD4^+$ T cells (Fig. 5). Interestingly, IL-4-specific mRNA was low but detectable in SP $CD4^+$ T cells stimulated in vitro, and this is probably due to the low numbers of IL-4-producing T cells present in SP when compared with PP. In other studies, we could detect IL-4 mRNA in SP $CD4^+$ T cell cultures stimulated with TT by using higher numbers of amplifying cycles in RT-PCR. This sensitive PCR amplification method did not allow detection of either IL-2 or IFN-γ-specific mRNA in SP or PP $CD4^+$ T cells from mice orally immunized with TT in the presence of CT (day 0) (data not shown). After in vitro antigen stimulation, IFN-γ- or IL-2-specific mRNA expression remained undetected in PP or SP $CD4^+$ T cells (days 3 and 6; data not shown). These results clearly show that oral immunization preferentially induces antigen-specific Th2-type responses in mucosal inductive sites (e.g., PP) and probably accounts for the IgA responses associated with effector tissues (e.g., LP).

Kinetics of T Cell Responses after Oral Immunization. To study the antigen entry and T cell priming process in PP after oral immunization with TT and CT, we next performed the T cell proliferation assay for PP and SP $CD4^+$ T cells isolated from mice orally immunized with TT and CT at days 0, 7, and 14 and determined the frequency of Th1- and Th2-type cytokine-producing cells. PP and SP $CD4^+$ T cells from mice 1 and 2 wk after initial oral immunization responded weakly to antigen stimulation; however, 1 wk after the third booster immunization (day 21), maximum proliferative responses were observed in day 6 PP and SP $CD4^+$ T cell cultures (Fig. 6 *A*). Further, cytokine-specific ELISPOT assays

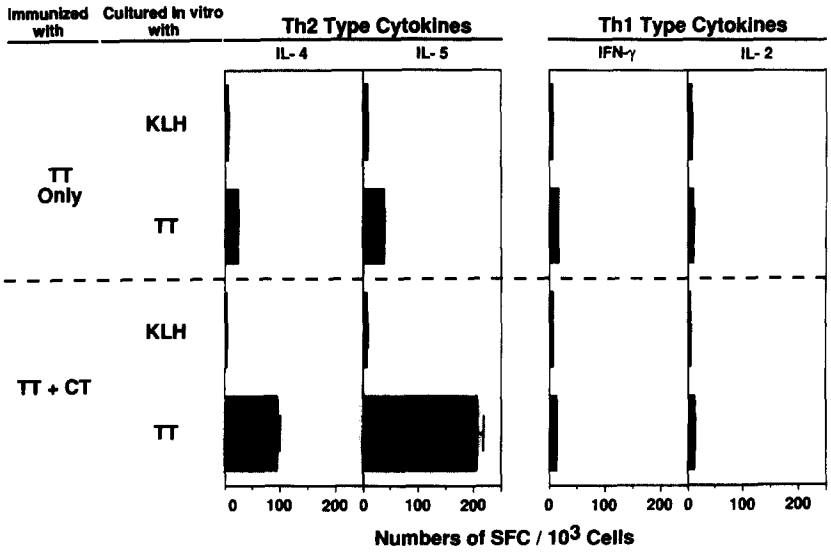

Figure 3. Oral immunization of mice with TT and CT selectively upregulates Th2 responses to TT. Mice were immunized by gastric intubation with TT (250 μg/mouse) plus CT (10 μg/mouse) or with TT (250 μg/mouse) only at days 0, 7, and 14. 1 wk after the last oral immunization, mice were killed and PP $CD4^+$ T cells were isolated and cultured in vitro with TT-coated latex microspheres under optimal conditions (see Results). Control cultures were not pulsed with TT-coated or received KLH-coated latex microspheres. After 6 d of culture, $CD4^+$ T cells were assessed for the numbers of IFN-γ, IL-2, IL-4, and IL-5 SFC by cytokine-specific ELISPOT assays. The results are presented as the mean antigen-specific cytokine SFC ± 1 SE.

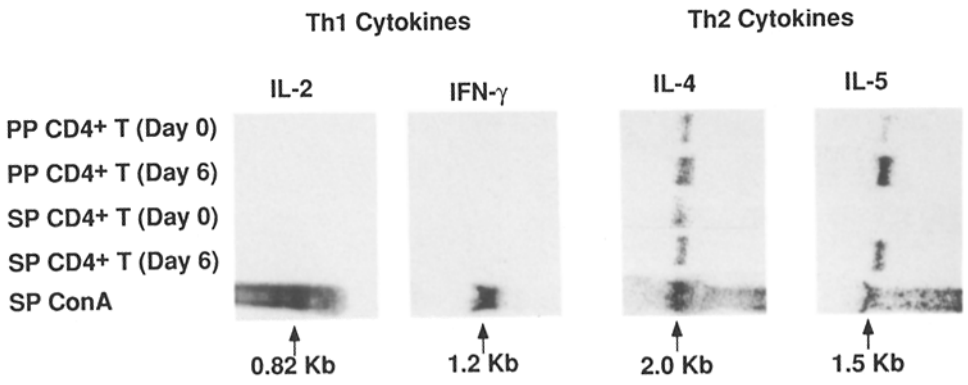

Figure 4. Determination of cytokine-specific mRNA expression by Northern blot analysis in mice orally immunized with TT plus CT. Mice were orally immunized with TT and CT as described in the legend to Fig. 3. Mice were killed 1 wk after the last oral immunization and PP and SP $CD4^+$ T cells were isolated and stimulated in vitro with TT-coated latex microspheres in the presence of IL-2 and SP feeder cells. Total RNA was isolated from both PP and SP $CD4^+$ T cells at the time of sacrifice (*day 0*) and at the end of in vitro stimulated cultures (*day 6*). The RNA was then subjected to Northern blot analysis by hybridization with mouse IFN-γ, IL-2, IL-4, and IL-5 cDNA probes to determine the amount of specific cytokine mRNA expression. Positive controls were RNA from SP cells stimulated with Con A for 20 h.

showed that PP and SP $CD4^+$ T cells isolated from mice 7 and 14 d after oral immunization did not contain significant numbers of IL-4- or IL-5-producing Th2-type cells, nor did they possess high levels of IL-2 or IFN-γ-secreting Th1-type cells (Fig. 6 *B*). However, by day 21 (i.e., after three oral doses) we could detect a high frequency of IL-4- and IL-5-producing Th2-type cells in PP and SP $CD4^+$ T cell cultures with higher numbers noticed in PP $CD4^+$ T cell cultures. These results clearly indicated that three oral immunizations with TT and CT were required to induce maximum antigen-specific Th cell responses, namely, Th2 cell responses in PP. This correlated with the antigen-specific antibody responses where we see maximum TT-specific IgA responses in the intestine at day 21 of immunization.

Systemic Immunization with TT and CT Induces Both IL-4-, IL-5-producing Th2-Type Cells and IL-2-, IFN-γ-secreting Th1-type Cells in SP. To determine the possible role of CT in directing Th cell responses into a particular Th cell pathway, we have determined the type of Th cell responses in SP of mice intraperitoneally immunized with TT alone or with TT plus CT. SP $CD4^+$ T cell cultures established from mice 1 wk after primary or booster immunization with TT only or with TT plus CT contained both IL-4- and IL-5-producing Th2-type cells and IL-2- and IFN-γ secreting Th1-type cells.

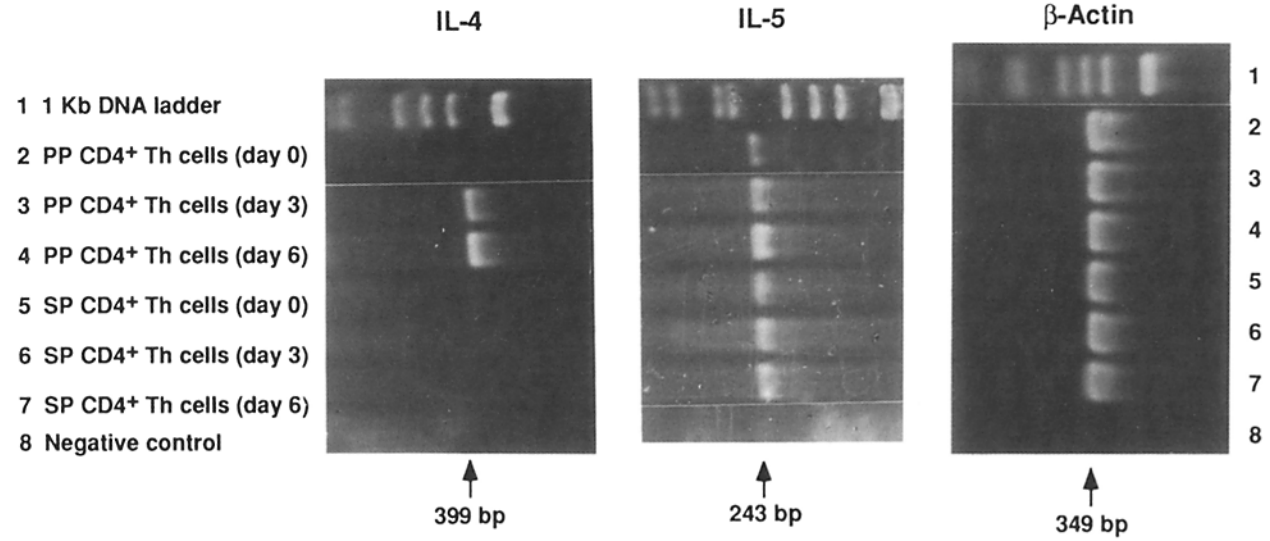

Figure 5. Levels of IL-4 and IL-5 mRNA expression in PP and SP $CD4^+$ T cells from mice orally immunized with TT plus CT. Mice were given TT and CT orally as indicated in the legend to Fig. 1. Mice were killed and PP and SP $CD4^+$ T cells were isolated and stimulated in vitro with TT-coated latex microspheres as described in Materials and Methods. At various culture intervals (days 0, 3, and 6) total RNA was extracted and the levels of IL-4 and IL-5 mRNA expression were determined by RT-PCR using cytokine-specific primers.

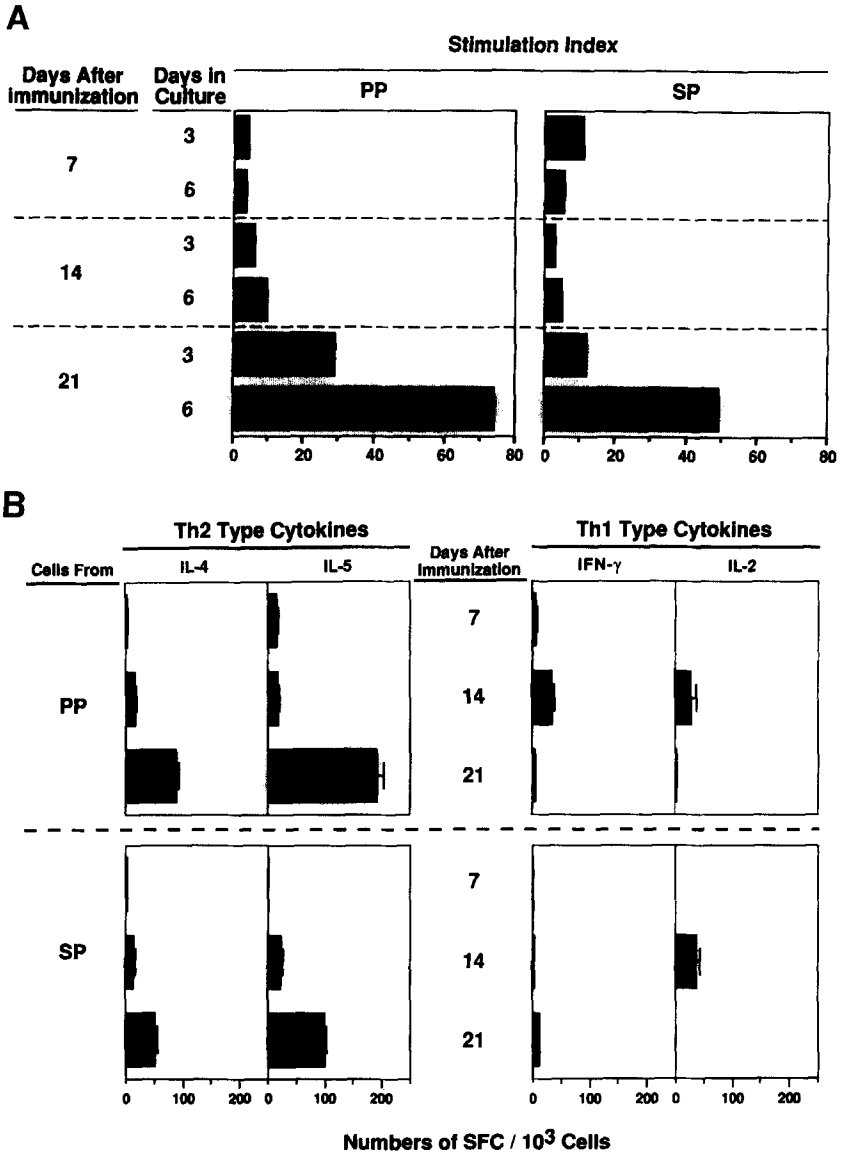

Figure 6. Kinetics of Th cell responses after oral immunization. Mice were orally immunized as described in Fig. 1, and 7, 14, and 21 d after immunization mice were killed. The PP and SP $CD4^+$ T cells were isolated and stimulated in vitro with TT-coated latex microspheres for the determination of Th cell proliferative responses at 3 and 6 d of cultures (*A*) and the numbers of Th1- and Th2-type cytokine-producing cells at 6 d of culture (*B*) as described in the legends to Figs. 2 and 3. Results are expressed as stimulation index E/C (*A*) and the mean antigen-specific cytokine SFC ± 1 SE (*B*).

Higher frequencies of both Th1- and Th2-type cells were seen in mice immunized with TT plus CT. This finding showed that CT also acted as an adjuvant at the Th cell level for antigens coadministered systemically with CT (Fig. 7). This result also indicated that it is not the CT that directs the Th cell responses, but rather, the route of immunization. While both Th1- and Th2-type cells were induced in SP by systemic administration of TT plus CT, the oral route of immunization predominantly induced Th2 cells in mucosa-associated tissues.

Discussion

A major goal of our research has been to characterize the nature of Th cells and derived cytokines for induction of B cells to become IgA-producing plasma cells in mucosal effector sites. Therefore, in this study we used the well-characterized protein vaccine, TT, and CT as an adjuvant in the mouse model, since it has been shown that oral administration of microgram amounts of CT resulted in substantial serum and mucosal antibody responses to CT itself and to coadministered proteins (20–26). However, it has remained unclear whether immune responses were first induced in PP, followed by an exodus of immune B and T cells to mucosal effector sites, or whether CT enhanced immune responses directly in the LP of the GI tract. This study would strongly argue that oral immunization with TT and CT induces both T and B cell responses in the PP. In vitro culture of PP $CD4^+$ T cells with either TT- or CT-B-coated microspheres resulted in significant T cell proliferative responses, suggesting that direct antigen uptake and immunization occurred in the PP itself. On the other hand, stimulation of $CD4^+$ Th cells from PP of nonimmunized mice with TT-coated microspheres induced minimal proliferative responses (data not shown).

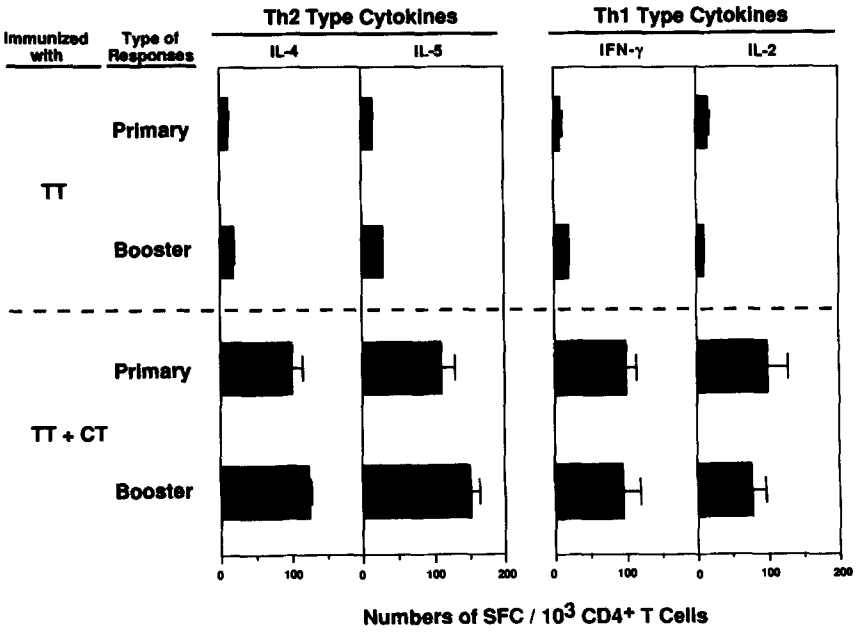

Figure 7. Systemic immunization with TT and CT induces both IL-4- and IL-5-producing Th2-type cells and IL-2- and IFN-γ-secreting Th1-type cells in SP. Mice were immunized by the intraperitoneal route at days 0 and 14 and were killed at either day 7 (primary) or day 21 (booster). The SP $CD4^+$ T cells were isolated and stimulated with TT-coated latex microspheres for 6 d, and the numbers of IL-4-, IL-5-, IL-2-, and IFN-γ-producing cells were determined by using cytokine-specific ELISPOT assays. The results are presented as the mean antigen-specific cytokine SFC ± 1 SE.

We adapted a protein-coated microsphere method to induce $CD4^+$ T cell responses in vitro. This method was significant for these analyses since soluble antigens are sometimes poor stimulants for T cells and are suboptimally processed by APCs, especially in mucosa-associated tissues (41). Our results are the first to show that TT-coated latex microspheres induced significant proliferation of $CD4^+$ T cells from mice orally immunized with TT and CT, and allowed detection of individual cytokine-producing T cells. The results obtained in this study have shown that oral immunization with TT and CT preferentially induced IL-4- and IL-5-producing, TT-specific Th2-type cells in PP $CD4^+$ T cell cultures. This result contrasted with our previous study with SRBC, where it was shown that although predominant Th2 cell responses were induced, significant Th1 cell responses were seen (17). In the present study, we showed that specific IgA antibody responses from mice orally immunized with TT plus CT correlate with a preferential Th2 cell response in PP and SP.

Other groups have studied cytokine production in vitro using lymphoid cells isolated from SP, mesenteric lymph nodes (MLN), PP, and LP of mice orally immunized with KLH and CT (42). For example, high levels of IL-4, IL-5, and IL-6 with lower levels of IL-2 and IFN-γ were seen in lamina propria lymphocyte (LPL) cultures, while PP gave lower Th2-type cytokines when compared with LPL cultures. The IL-2 levels were similar in PP and LPL, while low levels of IFN-γ were present in PP (42). In our studies, by using the cytokine-specific ELISPOT assay, we were able to show that the numbers of antigen-specific IL-4- and IL-5-producing cells could be enhanced by antigen restimulation in vitro; however, essentially no antigen-specific IFN-γ- or IL-2-producing Th1-type cells were noted. Our experiments also differed from the other study (42) which used whole LPL populations that contained other cell subsets in addition to $CD4^+$ T cells. Thus, we used purified $CD4^+$ T cells to ensure that the antigen effects noted were due to direct stimulation of T cells and not to stimulation of other cell types that could influence the cytokine pattern observed. Further, since no mRNA analyses were carried out in the earlier study (42), it remained to be established that the effect of oral immunization and in vitro antigen stimulation induced antigen-specific Th cells to produce cytokines de novo. Good correlations were noted in our studies between the numbers of cytokine-specific cells and the levels of cytokine mRNA expression and clearly indicated that antigen-specific Th2 cell responses were induced by oral immunization with TT and CT. Therefore, the increase in Th2-type cytokine-producing cells was probably a direct consequence of increased Th2 cytokine mRNA expression.

An interesting result was found when SFC and mRNA levels for IL-4 and IL-5 were determined in $CD4^+$ T cell cultures from PP and SP. The numbers of IL-4- and IL-5-producing cells were generally lower in SP than in PP $CD4^+$ T cell cultures. Further, we could detect only weak IL-4 mRNA expression by PCR in SP $CD4^+$ T cell cultures from mice orally immunized with TT and CT; however, under the same conditions IL-4 mRNA was higher in PP $CD4^+$ T cell cultures. On the other hand, we could detect IL-5 mRNA in both PP and SP $CD4^+$ T cell cultures. These results suggest that orally primed TT-specific Th2-type cells in PP probably migrate to SP before residence in the LP of the GI tract. Further, under the influence of a particular microenvironment, IL-4 expression is low for Th2 cells in SP for reasons that are not yet understood. It is tempting to speculate that decreased IL-4 production is a necessary characteristic for T cells that temporarily reside in the spleen be-

fore migration to effector sites. This would avoid the nonspecific activation of mature splenic B cells and prevent their terminal differentiation in this site.

Although CT has been shown to be a potent oral adjuvant, the precise mechanism for adjuvancy in the mucosal immune system is not yet understood. The results from in vitro studies with isolated T and B cells and APC suggest that CT can affect all of the cell types involved in the induction of antigen-specific immune responses (43, 44). In this regard, CT has been shown to promote isotype-switching of LPS-stimulated surface IgM-bearing B cells to expression of IgG or IgA (44). CT can act synergistically with IL-4 to promote switches to the IgG1 subclass and subsequent differentiation to IgG1 synthesis in LPS-stimulated B cell cultures (45). CT can inhibit some T cell functions, including IL-2 production and IL-2-dependent proliferation (46, 47). Further, CT stimulates IL-1 production and antigen presentation by macrophages (48). Other studies (49) showed that rCT-B enhanced the expression of MHC class II molecules and minor lymphocyte-stimulating determinants on B cells. However, the exact mechanisms for CT adjuvant activity at the level of antigen-specific Th cells, including Th1 and Th2 cell responses to orally administered protein-based vaccines, have not been elucidated.

The studies of others (50) have shown that CT inhibited T cell receptor-mediated IL-2 production and proliferation in Th1 cells while it failed to block IL-4 production and proliferation in Th2 cells. Since CT led to elevated levels of intracellular cAMP, it was concluded that CT discriminates between Th1 and Th2 cells in TCR-mediated activation (50). Our results of preferential induction of Th2-type cells by oral immunization with TT and CT as adjuvant could be used as evidence for this hypothesis. However, other factors may also determine the nature of Th cell responses. Recent reports indicate that CT may influence cellular growth via cAMP-independent mechanisms (51–54). It should be emphasized that the results that support the hypothesis that CT blocks IL-2R expression on Th1 cells were obtained from the study of only two long-term T cell clones, one for Th1- and the other for Th2-type responses (50). In this study, we have compared the type of Th cell responses induced by systemic primary and booster immunization with TT only or with TT plus CT. The results showed that systemic immunization with TT only or with TT plus CT induced both Th1- and Th2-type cells in SP of primary or booster immunized mice. On the other hand, predominant Th2 cell responses were always seen in mice orally immunized with TT only (Fig. 3). When CT was coadministered with TT, this enhanced Th2 cell responses and represents one mechanism for CT adjuvant activity at the level of Th cells. This would suggest that the route of immunization and the innate features of PP, e.g., specialized antigen uptake and unique APCs, are important for Th2 cell responses and that CT is less important in modulating specific types of Th cell responses.

In conclusion, oral immunization with TT and CT as adjuvant induces antigen-specific Th2 cells in mucosa-associated tissues and this has significant implications in the development of oral vaccines. Studies for the evaluation of mucosal vaccines should include analysis of IgA antibody responses as well as Th cell responses since the T cell–derived cytokines significantly influence the isotype and level of B cell responses. Our results provide the first compelling evidence that high titers of IgA antibodies directly correlate with the presence of antigen-specific Th2-type cells. Since antigen-specific Th1 cells were either absent or below the level of detection by the methods used here, this would for the first time argue that Th1-type cell responses are not required for mucosal S-IgA immune responses. This may indicate that IgA anti-protein/vaccine responses are totally Th2 cell dependent; that is, to obtain optimal mucosal IgA responses, Th2 cell responses are required. To further test this idea, we are currently determining the type of Th cell responses in mice treated with either anti-IFN-γ, anti-IL-4, or anti-IL-10 followed by challenging with TT together with CT to determine whether anti-cytokine treatment can alter Th1 and Th2 cell responses and thus affect antigen-specific IgA antibody responses to both TT and CT in the GI tract.

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