Oral Administration of Cholera Toxin B–Insulin Conjugates Protects NOD Mice From Autoimmune Diabetes by Inducing CD4$^+$ Regulatory T-cells

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Restoration of peripheral tolerance to target autoantigens during autoimmune diseases has met with several limitations because of the limited efficacy of this approach in an already immune host. To optimize the induction of tolerance, we have shown that feeding insulin conjugated to cholera toxin B-subunit (CTB), a potent mucosal adjuvant, reduced by 5,000 the amounts of antigen necessary for delaying diabetes onset in NOD mice. To analyze these protective mechanisms, we have performed cotransfer experiments using splenocytes from young females fed once with 10 µg of CTB-insulin, mixed with diabetogenic T-cells, and intravenously injected into irradiated syngeneic male recipients. We demonstrated that the delayed onset of diabetes relied on CD4$^+$ T-cells. We studied the cytokine production from plate-bound anti-CD3–stimulated cells. Higher interleukin (IL)-4 amounts were observed in both splenocytes and pancreatic lymph node (PLN) cell cultures from CTB-insulin–fed mice as soon as 4 h after the feeding. An increase in the levels of transforming growth factor-β was seen after 24 h only in the mesenteric lymph nodes (MLN). In both of these organs, a reduction of γ-interferon (IFN-γ) production occurred after CTB-insulin treatment, at 24 h in the PLN and at 7 days in the MLN. Reverse transcription–polymerase chain reaction analysis indicated an increase in the level of IL-4 and a reduction in IFN-γ transcripts in the PLN of mice treated orally with CTB-insulin and of the recipients of regulatory T-cells. Using different strains of congenic NOD mice at the *Thy1* locus, we showed that protection was associated with the accumulation of T-cells from CTB-insulin–fed mice in the lymph nodes from draining sites containing functional islets, i.e., the PLN in normal mice and the renal lymph nodes after a syngeneic islet graft under the kidney capsule of streptozotocin-treated mice. Taken together, our results clearly indicate that oral administration of CTB-insulin conjugates in NOD mice produced a shift from a T-helper type 1 to a type 2 profile with the induction of antigen-specific regulatory CD4$^+$ T-cells in the vicinity of the mucosal barrier and close to the inflamed islets. *Diabetes* 48:2150–2156, 1999

Type 1 (insulin-dependent) diabetes is a T-cell–mediated autoimmune disease localized to the endocrine pancreas that occurs spontaneously in genetically predisposed individuals (1). As in other organ-specific autoimmune diseases, lack of tolerance to self-antigens leads to the expansion of autoreactive T-cells. In type 1 diabetes, this autoreactivity results in mononuclear cell infiltration of the pancreatic islets and specific β-cell destruction. The importance of cellular immunity in the etiology of the disease has been demonstrated in both BB (Bio-Breeding) rats and NOD (nonobese diabetic) mice, two experimental models of spontaneous diabetes with immunopathological features resembling those of the human disease (1,2). A major role has been ascribed to autoreactive T-cells, and especially CD4$^+$ T-helper (Th) type 1, in both rodents (3,4) and humans (5) in promoting disease and β-cell destruction. Splenic T-cells from diabetic animals are able to adoptively transfer the disease to naive syngeneic recipients, and anti–T-cell strategies prevent the onset of the disease or delay β-cell loss in both humans and rodents (1). CD4$^+$ T-cells fall into two major classes: Th1-cells, which secrete inflammatory cytokines such as γ-interferon (IFN-γ) and interleukin (IL)-2, and Th2-cells, which produce IL-4, IL-10, and transforming growth factor-β (6). In this regard, β-cell destruction has been attributed to excessive Th1-cell function (4), with a defect in Th2-cell function (5).

Oral tolerance is an attractive strategy for treatment of autoimmune diseases (7). For instance, oral administration of insulin has been shown to protect NOD mice from both spontaneous (8) and accelerated diabetes (9). The mechanism by which the immune system selects the tolerance pathway is largely unknown. Depending on the dose of antigen fed, three effector primary mechanisms involved in this form of tolerance have been described (7): active suppression, clonal anergy, and clonal deletion involving either CD4$^+$ (8–11) or CD8$^+$ (12) T-cells.

Mucosal administration of an antigen linked to cholera toxin B-subunit (CTB), the nontoxic receptor binding moiety of cholera toxin (CT), strongly enhances peripheral T-cell tolerance to the conjugated antigen even when given after systemic sensitization (13,14). We have previously demonstrated that feeding minute amounts of CTB-conjugated insulin

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APC, antigen-presenting cells; CT, cholera toxin; CTB, cholera toxin B-subunit; CTB-INS, CTB-conjugated insulin; CTB-OVA, CTB-conjugated ovalbumin; FITC, fluorescein isothiocyanate; IFN-γ, γ-interferon; IL, interleukin; mAb, monoclonal antibody; MLN, mesenteric lymph node; PCR, polymerase chain reaction; PLN, pancreatic lymph node; RT, reverse transcription; STZ, streptozotocin; Th, T-helper.

(CTB-INS) conjugates enhanced the tolerogenic properties of insulin and significantly delayed diabetes onset in adult female NOD mice (15). Both the dose of antigen and the administration rate necessary for suppression of systemic T-cell reactivity could be dramatically reduced. The aims of the present study were to elucidate the cellular mechanisms governing tolerance after oral administration of CTB-INS in NOD mice. We report that feeding prediabetic NOD mice with microgram amounts of insulin conjugated to CTB induces active suppression mediated by regulatory $CD4^+$ T-cells. The adjuvant capacity of CTB was associated with an upregulation of Th2 secretory T-cells in the mesenteric lymph nodes (MLN) and in the draining lymph nodes of the inflamed islets.

RESEARCH DESIGN AND METHODS

Mice. NOD Thy-1.2 mice were bred under standard conditions in our animal facility. Spontaneous diabetes starts as early as 12 weeks of age in female mice, and the incidence of diabetes in our colony reaches 70% in females and 15–20% in males by 30 weeks of age. Diabetes was characterized by polydipsia, weight loss, glycosuria as assessed by urine chemstrips (Bayer, Puteaux, France), and persistent hyperglycemia (>200 mg/dl), which was determined with blood glucose chemstrips (Lifescan, Mountain View, CA). Diabetic NOD females served as donors of autoreactive T-cells during cotransfer experiments. C3H and Balb/c mice were obtained from Charles River (St Aubin les Elbeuf, France).

Antigens. CTB was purified by a combination of hexametaphosphate precipitation and Sephadex G-75 gel filtration from the culture filtrate of a mutant strain of Vibrio choleras deleted of the CT genes and transfected with a recombinant overexpression plasmid encoding CTB (16). Recombinant human insulin (Novo-Nordisk, Baegsvaerd, Denmark) was conjugated to CTB, using N-succinimidyl (3-[2-pyridyl]dithio)propionate (SPDP; Pharmacia, Orsay, France) as bifunctional coupling reagent (13). The resulting CTB-INS conjugates were purified by gel filtration and characterized for their receptor binding and serological reactivity by a solid-phase enzyme-linked immunosorbent assay using immobilized GM1 ganglioside as the capture system and enzyme-linked antibodies to CTB and insulin as the detection systems (13). The purified conjugates were found to contain, on average, 20% (wt/wt) of insulin. The same coupling and purification protocols were used to obtain the CTB-ovalbumin (CTB-OVA) conjugate. In all protocols, the doses of CTB-INS or CTB-OVA given represent the amount of insulin or ovalbumin in such conjugates.

Oral tolerization protocols. The 8-week-old female mice were fed once with either 10 µg of recombinant human insulin conjugated to CTB, 10 µg of CTB-OVA, or 10 µg of unconjugated CTB, given in a volume of 400 µl 0.35 mol/l $NaHCO_3$ in phosphate-buffered saline by gastric intubation with an 18-gauge stainless steel feeding needle.

Cell preparation. After feeding, spleens, pancreatic, and mesenteric lymph nodes were aseptically removed, and single-cell suspensions were prepared by mechanical dispersion, according to standard procedures, in Hanks' balanced salt solution. T-cell–enriched populations were obtained by elimination of B-cells and macrophages by panning on plastic dishes coated with rabbit anti-mouse IgG (H + L) antibody (Biosys, Compiègne, France). The resulting cell populations comprised >90% Thy-1.2^+ cells by flow cytofluorometry using a fluorescein isothiocyanate (FITC)-labeled hamster anti-mouse CD3 antibody (clone CT-CD3; Caltag, San Francisco, CA) and anti-Thy-1.2 monoclonal antibody (mAb)-FITC (clone 30-H12; Pharmingen, San Diego, CA) on a Facscan flow cytometer (Becton Dickinson, Mountain View, CA). For $CD4^+$ or $CD8^+$ T-cell depletion, lymphocyte suspensions were first incubated for 30 min on ice with rat anti-mouse CD4 or CD8 mAbs (clones GK-1.5 and 53–67, respectively) under constant agitation and for 30 min with magnetic particle beads coated with a sheep anti-rat IgG covalently linked antibody (Dynabeads M-450; Dynal, Oslo, Norway) with a bead-to-cell ratio of 20:1. Nonadherent cells were collected after the removal of free and cell-bound beads using a magnetic bead concentrator (MPC 6; Dynal). The resulting T-cell subpopulations were found to consist of >90% $CD4^+$ or >80% $CD8^+$ T-cells, as demonstrated by flow cytofluorometry using an FITC-labeled anti-mouse CD4 (clone CT-CD4; Caltag) or FITC-labeled anti-mouse CD8 mAbs (clone 53–6.7; Pharmingen).

Adoptive transfers. At 1 week after feeding, five million splenic T-cells from NOD Thy-1.2 mice fed with either CTB-INS, CTB-OVA, or CTB alone were mixed with 5×10^6 T-cells from diabetic NOD females. The resulting cell mixture was injected intravenously into 8- to 10-week-old irradiated (750 rads) NOD males (17). To evaluate the relative radiosensitivity of the transferred cells contributing to the protective effects, 10×10^6 splenocytes from CTB-INS–fed mice were irradiated at 900, 1,500, or 3,000 rads (X-rays) before being coinjected with 10×10^6 diabetogenic splenocytes into irradiated NOD male recipients. The incidence of diabetes in the recipients was compared with those from mice injected with 10×10^6 diabetogenic splenocytes and 10×10^6 nonirradiated splenocytes from CTB-INS– or CTB-OVA–fed mice.

Islet isolation and transplantation. Pancreases from 15-week-old nondiabetic Thy-1.2 NOD males were excised, and islets were isolated by collagenase digestion. Briefly, pancreases were distended by injection of a collagenase solution (1.5 mg/ml collagenase P; Sigma, St. Louis, MO), and enzymatic digestion was then performed at 37°C for 20 min in a shaking water bath. Islets were harvested at the interfaces of 11–20% and 20–23% of a discontinuous density ficoll gradient, washed in Ca/Mg-free phosphate-buffered saline, and isolated by a double hand-picking procedure. After an overnight incubation at 37°C, 350–400 islets were transplanted under the left kidney capsule of Thy-1.2 streptozotocin (STZ)-induced diabetic NOD males, as described previously (18). Normalization of glycemic values occurred the following day. To evaluate the role of functional islet tissue in targeting the homing of regulatory T-cells, irradiated experimental mice harboring islet grafts were adoptively reconstituted with 5×10^6 splenic T-cells from Thy-1.1 mice fed either with CTB-INS or CTB together with 5×10^6 splenic T-cells from diabetic Thy-1.2 mice. The percentages of Thy-1.1 T-cells in spleen, pancreatic, and renal lymph nodes were analyzed by flow cytometry.

Cytokine secretion and cytokine mRNA production. Spleen and lymph nodes were removed 4 h, 24 h, and 1 week after feeding. Purified T-cells (5×10^5) were cultured in flat-bottomed 96-well microtiter plates, and the concentration of a given cytokine in cell-free supernatants was determined after stimulation with plate-bound anti-CD3 mAbs (10 µg/ml, clone 145–2C11; Pharmingen). This approach was used because direct restimulation of splenocytes or mesenterial node cells with heterologous insulin failed to yield any detectable response. Supernatants were collected after 48 h (IL-4, IL-2, IFN-γ) or 72 h (TGF-β) of culture and analyzed for cytokine content using specific enzyme-linked immunosorbent assay kits (Genzyme, Cergy, France) following manufacturer's instructions. Two independent experiments were conducted, using 8 mice per group. The optical density of samples was determined at 405 nm using an automated microplate reader (Labsystems Multiskan MCC/340; Cergy, Pontoise, France).

Evaluation of cytokine mRNA content was performed as described previously (19). Briefly, RNAs from lymph nodes and spleens were extracted by acid-guanidinium-thiocyanate. The first-strand cDNA was synthesized by thermostable reverse transcriptase (*Thermus thermophilus* DNA polymerase; Promega, Charbonnières, France) with specific primer and amplified by 37 cycles of polymerase chain reaction (PCR) (Taq polymerase; Life Technologies, Cergy, France). The 216- to 227-bp PCR products were analyzed by agarose gel electrophoresis (2%) in the presence of ethidium bromide (0.5 mg/ml). A multispecific plasmidic RNA (a gift from D. Shire, Sanofi Labège, France) corresponding to an amplicon length of 322 bp was used as control for the efficiency of cDNA synthesis.

Statistical analysis. Cytokine levels were compared using Wilcoxon's rank test. The efficacy of diabetes transfer was compared between animal groups using 2×2 contingency tables and $\times 2$ analysis.

RESULTS

Oral CTB-INS administration generates regulatory $CD4^+$ T-cells. Since both $CD4^+$ and $CD8^+$ T-cells have been identified as mediators of oral tolerance, we analyzed the respective contribution of these cell subsets in a model of CTB-driven oral tolerization. Splenocytes from animals orally tolerized with CTB-INS were tested for their ability to interact with diabetogenic T-cells after adoptive cotransfers into syngeneic irradiated male recipients. Systemic effectiveness was evaluated by the prevalence of clinical diabetes. As shown in Fig. 1, recipients receiving the mixture of $CD4^+$ T-cells from CTB-INS–fed mice and diabetogenic T-cells exhibited a 90% reduction in diabetes prevalence 45 days after cell transfer, in contrast with recipients receiving $CD8^+$ T-cells from CTB-INS–fed donors coinjected with the same diabetogenic cells (1 of 10 vs. 5 of 5; $\chi^2 = 11$, $P < 0.01$). Furthermore, clinical onset of diabetes in the latter group injected with $CD8^+$ T-cells appeared to be accelerated when compared with control mice transferred with cells from CTB-fed mice, although the shapes of the incidence curves were comparable (Fig. 1). Thus, we conclude that protection from adoptively transferred diabetes after feeding NOD mice with CTB-INS involves the generation of a subpopulation of regulatory $CD4^+$ T-cells.

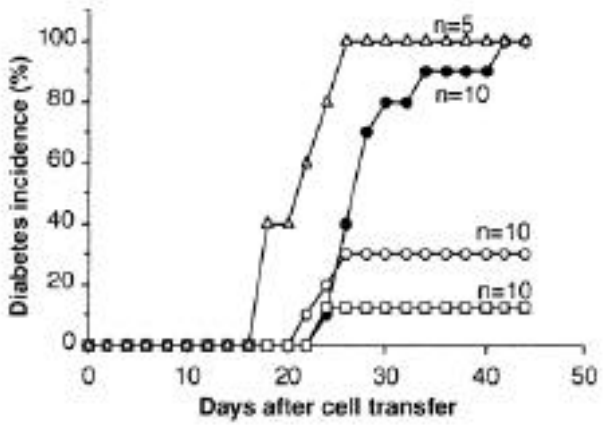

FIG. 1. Eight-week-old irradiated males were transferred with 5 × 10^6 splenic T-cells from diabetic mice together with 5 × 10^6 splenic T-cells (○), $CD4^+$ T-cells (□), or $CD8^+$ T-cells (△) from mice fed once with 10 µg of CTB-INS 7 days before cell transfer, in comparison with splenic T-cells from mice fed once with 10 µg of CTB alone (●).

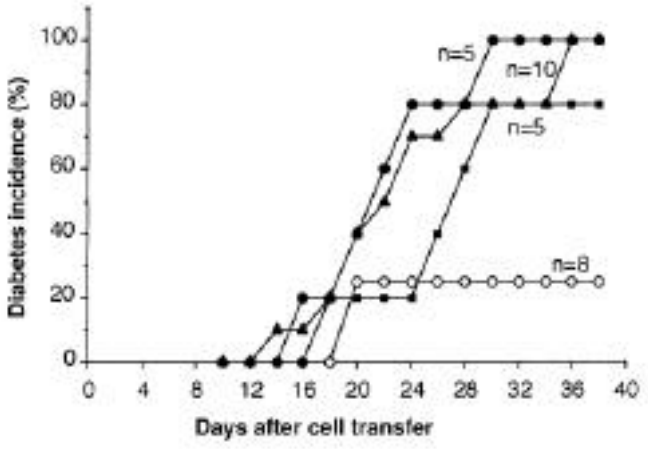

FIG. 2. Eight-week-old irradiated males were transferred with 10 × 10^6 splenocytes from diabetic mice together with 10 × 10^6 splenocytes from mice fed once with 10 µg of CTB-INS either nonirradiated (○) or irradiated with 900 rad (●), 1,500 rad (■), or 3,000 rad (▲).

To rule out the possibility that donor antigen-presenting cells (APC) could anergize diabetogenic T-cells in the host, and hence contribute to the protective effect observed, splenocytes from CTB-INS–tolerized mice were exposed to three different irradiation doses immediately before their cotransfer with diabetogenic T-cells. These doses were chosen to separate conditions where only lymphocytes or both lymphocytes and APC were deleted (20). Representative results of such experiments are depicted in Fig. 2, which summarizes two independent cotransfer experiments and demonstrates that all three irradiation doses abolish the capacity of splenocytes from CTB-INS–fed donors to protect recipients from developing diabetes. Therefore, these experiments suggest that oral administration of CTB-INS relies on the expansion of radiosensitive regulatory T-cells that are capable of suppressing disease.

Oral CTB-INS induced Th2 T-cells and inhibited Th1 T-cells. In an attempt to determine the Th1 and/or Th2 phenotype of the T-cells present in the lymphoid organs after feeding CTB-INS or CTB, the amounts of IFN-γ, as well as IL-4 and TGF-β, from culture supernatants were evaluated after stimulation by plate-bound anti-CD3 antibodies. Results are shown in Table 1.

When compared with CTB, a single oral administration of 10 µg of CTB-INS was followed after 24 h by an increase in TGF-β production in the MLN (1.4 ± 0.4 vs. 0.86 ± 0.3 ng/ml, $P = 0.02$), compared with CTB-fed mice. Amounts of IFN-γ production were comparable after 4 h (6 ± 2.3 vs. 5.6 ± 1.9 ng/ml, respectively) and were also similar to unfed control mice (4.5 ± 1.4 ng/ml). Levels of IFN-γ in the MLN increased significantly 7 days after feeding CTB compared with CTB-INS (8.2 ± 3.2 vs. 4.9 ± 0.8 ng/ml, $P < 0.05$).

In the spleen, CTB-INS–fed mice produced elevated amounts of IL-4 at 4 and 24 h after feeding (487 ± 76 vs. 297 ± 108 pg/ml, $P = 0.03$, and 480 ± 53 vs. 276 ± 108 pg/ml, $P = 0.01$, respectively) and initially lower TGF-β amounts at 4 h (1.7 ± 0.2 vs. 2.3 ± 0.2 ng/ml, $P = 0.03$).

Oral administration of CTB-INS was followed in the PLN by a marked increase in IL-4 production after 4 h (41 ± 20

TABLE 1
Cytokine production from anti-CD3–stimulated T-cells 4 h, 24 h, and 7 days after one single oral administration of 10 µg of CTB-INS or CTB

	PLN			MLN			Spleen		
	CTB-INS	CTB	*P* value	CTB-INS	CTB	*P* value	CTB-INS	CTB	*P* value
IL-4 (pg/ml)									
4 h	41 ± 20	8 ± 7	<0.05	34 ± 22	53 ± 6	NS	487 ± 76	297 ± 108	0.03
24 h	13 ± 4.3	14 ± 5.5	NS	12.5 ± 7	33.6 ± 27	NS	480 ± 53	276 ± 108	0.01
7 days	0.9 ± 1	0.65 ± 0.8	NS	55 ± 14	45.6 ± 17	NS	397 ± 125	373 ± 130	NS
TGF-β (ng/ml)									
4 h	0.9 ± 0.05	1.2 ± 0.3	NS	0.95 ± 0.05	1.2 ± 0.4	NS	1.7 ± 0.2	2.3 ± 0.2	0.03
24 h	0.9 ± 0.1	0.9 ± 0.1	NS	1.4 ± 0.4	0.86 ± 0.3	0.02	1.8 ± 0.2	1.75 ± 0.2	NS
7 days	0.85 ± 0.3	1.0 ± 0.12	NS	1.2 ± 0.2	1.0 ± 0.1	NS	1.8 ± 0.1	1.52 ± 0.2	NS
IFN-γ (ng/ml)									
4 h	3.3 ± 1.2	0.95 ± 0.7	NS	6 ± 2.3	5.6 ± 1.9	NS	16 ± 8.6	15.3 ± 2	NS
24 h	0.6 ± 0.1	1.4 ± 0.4	0.03	3.12 ± 1	4.4 ± 2.8	NS	19.1 ± 4	17.2 ± 2	NS
7 days	3.4 ± 3.5	3.7 ± 3.4	NS	4.9 ± 0.8	8.2 ± 3.2	<0.05	18.6 ± 5	15.8 ± 3.5	NS

Data are means ± SD and are taken from two independent experiments using eight mice per group.

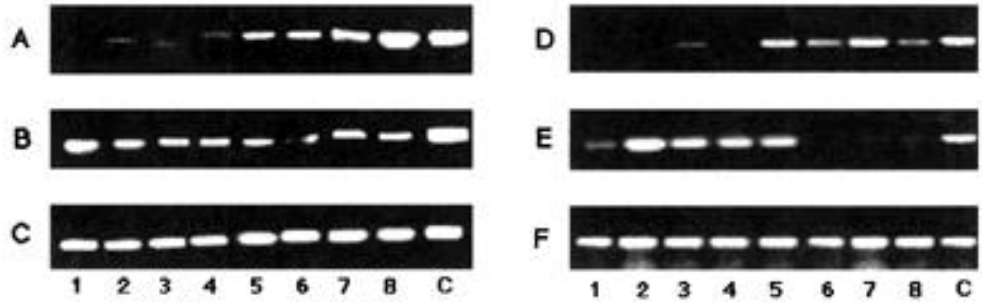

FIG. 3. RT-PCR analysis of IL-4 (*A*, *D*), IFN-γ (*B*, *E*), or β-2 microglobulin (*C*, *F*) specific mRNAs from MLN (*left*) or PLN (*right*) 24 h after a single oral administration of 10 μg of CTB (1–4) or CTB-INS (5–8). Columns 1–8 correspond to individual mice. The amplicon length of the plasmidic control (C) is 322 bp.

vs. 8 ± 7 pg/ml, $P < 0.05$), whereas TGF-β levels remained comparable both 4 h (0.9 ± 0.05 vs. 1.2 ± 0.3 ng/ml), 24 h (0.9 ± 0.1 vs. 0.9 ± 0.1 ng/ml), and 7 days (0.85 ± 0.3 vs. 1.0 ± 0.12 ng/ml) after the feeding. Interestingly, IFN-γ production was significantly reduced (0.6 ± 0.12 vs. 1.4 ± 0.4 ng/ml, $P = 0.03$) 24 h after feeding CTB-INS compared with feeding CTB alone.

To determine whether Th2 type responses could be due to de novo synthesis, reverse transcription (RT)-PCR analysis was used to detect IFN-γ–, TGF-β–, and IL-4–specific mRNAs. As shown in Fig. 3, the 216-bp band corresponding to IL-4 mRNA was detected in the MLN and the PLN from 4 of 4 mice fed with CTB-INS and 3 of 4 mice fed with CTB. Under similar conditions of mRNA amplification (as shown in Fig. 3, *lane C*), IL-4 mRNA expression was increased after CTB-INS administration in comparison to CTB. When IFN-γ mRNAs were studied, no significant differences could be noticed between mice in the MLN, but the corresponding band in the PLN was absent in 1 of 4 and reduced in 2 of 4 CTB-INS–fed mice. When RT-PCR analysis was conducted in cotransferred mice (Fig. 4), IL-4 mRNAs were not detected in the MLN, but were only found in the PLN of 3 of 3 mice receiving cells from CTB-INS–fed mice. TGF-β mRNAs were detected in the PLN of 2 of 3 mice receiving cells from CTB-INS–fed mice in contrast to 0 of 3 in the CTB-fed group. IFN-γ mRNAs were detected at similar levels in both MLN and PLN of experimental mice.

Taken together, both cytokine production studies and mRNA studies indicate that oral administration of CTB-INS elicits a Th2 type T-cell response with preferential expression of TGF-β and IL-4.

Homing studies. As shown in Table 2, 7 days after an adoptive transfer, recipients of 5×10^6 splenic T-cells originated from CTB-INS–fed mice showed a significant increase in the percentages of donor Thy-1.1$^+$ T-cells in the PLN when compared with control mice (52 ± 14 vs. 21 ± 7%, $P < 0.04$) demonstrating that this accumulation occurred even in absence of diabetogenic T-cells and inflammatory environment. To determine whether this specific recruitment was linked to anatomic proximity or to antigen-specific interactions, we performed adoptive cotransfer experiments in STZ-induced diabetic mice with syngeneic islet graft under the kidney capsule. As shown in Table 3, the absence of functional β-cells in the pancreas was associated with the loss of specific accumulation of Thy-1.1$^+$ T-cells in the PLN of mice receiving T-cells from CTB-INS–fed mice. On the other hand, a striking increase in the percentages of Thy-1.1 T-cells was observed in the lymph node draining the kidney with the syngeneic islet graft compared with the ungrafted kidney (Table 3). Therefore, we can conclude that the specific homing of regulatory T-cells to the PLN was linked to antigen-specific interactions in the draining lymph nodes of an organ containing functional islets and not by the presence of proinflammatory T-cells.

DISCUSSION

The findings detailed herein extend our previous studies on the protective effects of oral administration of CTB-INS conjugates against autoimmune diabetes (15). We indicate here that suppression of disease is mediated by regulatory CD4$^+$ Th2 type T-cells. The ability of CD4$^+$ T-cells to suppress the adoptive transfer of diabetes can be explained in terms of gen-

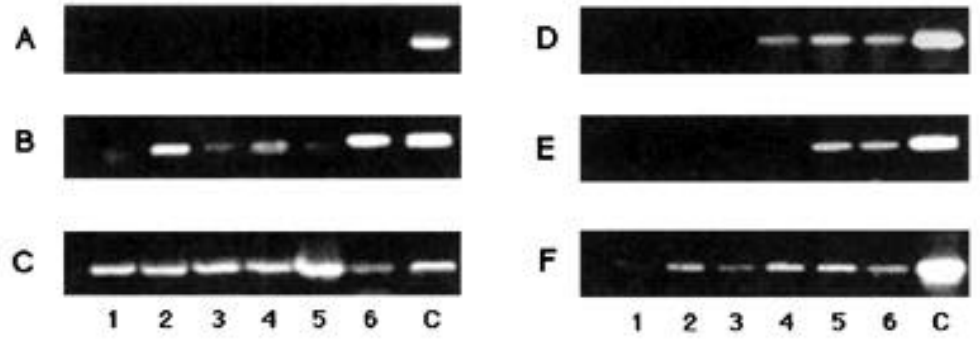

FIG. 4. RT-PCR analysis of IL-4 (*A*, *D*), TGF-β (*B*, *E*), and IFN-γ (*C*, *F*) specific mRNAs from MLN (*left*) or PLN (*right*) 7 days after the coinjection of 5×10^6 splenic T-cells from diabetic mice together with 5×10^6 splenic T-cells from mice fed with 10 μg of CTB (1–3) or 10 μg CTB-INS (4–6). Control mRNAs for IL-4 and IFN-γ were obtained from specific transfected cell lines (C).

TABLE 2
Homing studies after adoptive T-cell transfer

	+ Diabetogenic T-cells			– Diabetogenic T-cells		
	CTB-INS	CTB	*P* value	CTB-INS	CTB	*P* value
Spleen	3 ± 2	2 ± 0	NS	7 ± 1	10 ± 3	NS
PLN	38 ± 4	19 ± 8	0.04	52 ± 14	21 ± 7	<0.04
MLN	15 ± 2	12 ± 8	NS	20 ± 3	16 ± 6	NS

Data are mean ± SD percentages of T-cells determined in three to five animals per experimental group. Flow cytometric analysis was performed to determine the percentages of donor Thy-1.1$^+$ T-cells in the spleen and lymph nodes of Thy-1.2$^+$ NOD males, 7 days after the transfer of 5×10^6 Thy-1.1$^+$ T-cells from mice fed CTB-INS or CTB mixed or not with 5×10^6 Thy-1.2$^+$ T-cells from syngeneic diabetic females.

eral bystander suppression mediated by Th2 anti-inflammatory cytokines.

T-cell regulation of immune responses is a major tenet in mucosal immunology. Oral administration of an autoantigen has been used successfully to treat autoimmune diseases in animal models with the generation of active T-cell suppression. In the NOD mouse model, we have previously demonstrated that both insulin (9) or CTB-INS (15) administration induced regulatory T-cells that were able to counteract the migration of autoreactive T-cells and the final destruction of the β-cells. During a follow-up of 90 days after cell cotransfer, we observed that oral CTB-INS only delayed β-cell destruction and did not induce permanent protection with a reduction in diabetes incidence that remained significant up to 60 days (data not shown). Because of the strong mucosal immunostimulatory properties of CT and CTB, it was not known, however, whether feeding a protein in its native form or conjugated to CTB induced the same regulatory mechanisms. We showed that the suppressor cell population was inhibited by low irradiation doses, which is consistent with the strong involvement of T-cells (20). In contrast with what has been described with insulin by Polanski et al. (21), cells transferring the disease could not be tolerized by antigen fed to the irradiated recipients (data not shown), which reinforces the concept of an active cell-mediated suppression mechanism requiring an immune competent host. T-cell subset analysis revealed that oral CTB-INS was associated with CD4$^+$ T-cells with suppressive capacities. This result is interesting, since the gut immune system is composed of a majority of CD8$^+$ T-cells located to the intraepithelial compartment, whereas CD4$^+$ T-cells predominate in the lamina propria of the gut mucosa, as well as in the Peyer's patches and lymphoid follicles (22). The induction of CD4$^+$ regulatory T-cells may correspond, therefore, to a highly compartmentalized phenomenon. Although CD8$^+$ T-cells exert important downregulatory function in normal gut mucosa during oral tolerance in myelin basic protein–fed animals (12,23), most studies have shown that feeding antigens induced systemic hyporesponsiveness requiring CD4$^+$ T-cells (10,11), including ours using oral insulin in NOD mice (9). The normal appearance of oral tolerance to keyhole limpet hemocyanin in CD8$^{-/-}$ mice (24) is consistent with the importance of CD4$^+$ T-cells to maintain systemic hyporesponsiveness. In the NOD mouse model, several studies have identified CD4$^+$ T-cells with suppressive capacities. In NOD male mice, CD4$^+$ regulatory T-cells have been previously reported (25), which could explain the lower diabetes incidence rate in comparison to female mice. More recently, an insulin-reactive CD4$^+$ T-cell clone has been isolated from the pancreatic lymph nodes of diabetic mice after an adoptive transfer (26). This clone was able to block both spontaneous diabetes and adoptive transfer of diabetes. However, because of the distinct phenotypic characteristics and receptor usage of the different protective T-cell clones that have been yet identified, it is likely that multiple protective cellular mechanisms may be operative in the NOD mouse. The selective homing of regulatory T-cells to the PLN favors an important regulatory function of this lymphoid structure during the pathogenesis of autoimmune diabetes. The increased percentage of T-cells from CTB-INS–fed animals in the PLN after adoptive cell transfer suggests that protective mechanisms might also occur before islet cell invasion. Surprisingly, this specific T-cell homing was not dependent on the simultaneous addition of diabetogenic T-cells. Dendritic cells have been shown to take up antigens in tissues and to travel via the afferent lymphatics to the draining lymph nodes, where they stimulate antigen-specific immune responses (27). Our group has previously demonstrated a significant accumulation and activation of commit-

TABLE 3
Homing studies after adoptive T-cell transfer

	Feeding regimen of Thy-1.1 donor mice			
	CTB-INS		CTB	
Recipient Thy-1.2 mice	Thy-1.1	Thy-1.2	Thy-1.1	Thy-1.2
Spleen	0.4 ± 0.2	77.9 ± 0.5	1.2 ± 0.5	78.5 ± 0.6
PLN	4.4 ± 1.1	82.1 ± 9.9	6.8 ± 0.9	58.7 ± 5.3
MLN	5.2 ± 4	76.9 ± 5	3.56 ± 1.7	79.8 ± 5.2
Left renal lymph node	25.3 ± 11*	69.6 ± 13	3.65 ± 4.3*	44.3 ± 23
Right renal lymph node	4.7 ± 0.5	38.2 ± 3	4.8 ± 3.0	32.4 ± 9

Data are mean ± SD percentages of T-cells determined in three to five animals per experimental group. *$P < 0.05$, CTB-INS vs. CTB. Flow cytometric analysis was performed to determine the percentages of donor Thy-1.1$^+$ T-cells in the spleen and lymph nodes of Thy-1.2$^+$ NOD males, 7 days after the cotransfer of 5×10^6 Thy-1.2$^+$ T-cells from syngeneic diabetic females and 5×10^6 Thy-1.1$^+$ T-cells from mice fed CTB-INS or CTB. STZ-induced diabetes was cured by syngeneic islet grafts under the left kidney capsule, 10 days before irradiation and adoptive transfer.

ted T-cells in the PLN 2 days after adoptive cell transfer (28) that can be reduced by anti L-selectin antibodies (29). The presence of sufficient amount of autoantigens may be a critical issue to trigger either bystander suppression or antigen-specific downregulation. Our capacity to target the specific migration of regulatory T-cells from the PLN to the draining lymph nodes of the kidney containing functional islet grafts in an STZ-induced diabetic NOD mice clearly favors this hypothesis. Altogether, we can postulate that local expression of islet antigens dictates the homing of regulatory T-cells, which may inhibit autoreactive T-cells through local IL-4 secretion.

Demonstration that NOD mice have defective IL-2 and IL-4 production as well as lower suppressive capacities suggests that the NOD mouse model is associated with abnormalities of immune regulation. Administration of IL-4 can prevent diabetes in NOD mice (30). Defective T-suppressor activity is a common feature of several autoimmune disorders in mice (31). It can be hypothesized that resistance to autoimmune diseases is closely linked to the maintenance in the periphery of T-cell tolerance to autoantigens through regulatory T-cell activity and a Th1/Th2 balance. This lack of suppression in the NOD mouse has been attributed to deficiency of $NK1^+$-like thymocytes (32). Although CTB may influence mucosal immune responses by itself, it may act as a carrier enhancer for insulin to generate suppressive effects. Although CT and CTB have been shown in vitro to promote antibody responses (33) and IgA isotype switching by splenic B-cells (34) , we have not been able to detect any difference in the levels of IgG or IgA antibodies to insulin in the sera of both CTB-INS– and CTB–fed animals (data not shown). This suggests that oral CTB-INS may influence systemic rather than local immune responses. CTB-pretreated APC have been shown to enhance IL-4 production from anti-CD3 stimulated T-cells (35), and CTB can modulate antigen processing and presentation (35,36). It can be hypothesized, although more definite proof will be required, that the lymphocyte repertoire in the NOD mouse contains insulin-specific $CD4^+$ suppressor cells and that CTB-INS may act on APC to provide a costimulatory signal to these preformed insulin-specific $CD4^+$ T-cells secreting IL-4.

Because only splenocytes from CTB-INS–fed mice are able to counteract the activation and migration of autoreactive T-cell to the pancreas, it is likely that only Th2 cytokines produced at the site of tissue injury can exert a suppressive effect. Insulitis appears to be necessary, but not sufficient, for progression to diabetes (37,38). It is likely that interactions between pathogenic and regulatory cells occur within the insular lesion of the pancreas. We and others have attributed to the PLN important regulatory functions in autoimmune diabetes. We previously demonstrated that PLN were early infiltrated by autoreactive T-cells and were early sites for lymphocyte activation (28). This was confirmed by recent data obtained in BDC 2.5 T-cell receptor transgenic mice, which express a receptor recognizing an islet β-cell antigen (39). We have previously shown, using adoptive transfers with congenic mice at the Thy1 locus, that PLN were selectively infiltrated by regulatory cells originated from CTB-INS–fed mice (15). Although in vitro–induced cytokine production may not reflect the situation at sites of tissue injury, the demonstration of increased levels of IL-4 production in the PLN as well as reduced levels of IFN-γ production shortly after a single oral administration of CTB-INS reinforces the concept of immune deviation from a pathogenic Th1 response to a protective Th2 response in tolerized animals.

In conclusion, this study provides evidence that oral administration of minute amounts of CTB-INS conjugates in the NOD mouse model induced regulatory $CD4^+$ T-cells. Selective homing of these regulatory cells to the pancreas and its draining lymph nodes has been demonstrated. These results obtained with minute amounts of antigen can be a highly effective approach to prevent human type 1 diabetes.

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