Original Article

Inhibition of Autoimmune Diabetes by Oral Administration of Anti-CD3 Monoclonal Antibody

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Anti-CD3 monoclonal antibody (mAb) has been shown to induce tolerance and to be an effective treatment for diabetes both in animal models and in human trials. We have shown that anti-CD3 mAb given orally is biologically active in the gut and suppresses experimental autoimmune encephalitis by the induction of a regulatory T-cell that expresses latency-associated peptide (LAP) on its surface. In the present study, we investigated the effect of oral anti-CD3 mAb on the prevention of autoimmune diabetes in AKR mice in which the low-dose streptozocin (STZ) model induces autoimmunity to the β-cells of the islets. We found that oral anti-CD3 mAb given at doses of 50 and 250 μg/feeding suppressed the incidence of diabetes in this model with the best effects seen at the 50 μg/dose. Associated with suppression, we observed decreased cell proliferation in the spleen and conversion of T-helper (Th)1 responses into Th2/Th3 responses in the periphery, including the pancreatic lymph nodes. Oral anti-CD3 mAb increased the expression of LAP on $CD4^+$ T-cells, and these cells could adoptively transfer protection. Protection by oral anti-CD3 was transforming growth factor-β dependent. Our results demonstrate that oral anti-CD3 is effective in the model of STZ-induced diabetes and may be a useful form of therapy for type 1 diabetes in humans.
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Immunological tolerance is mediated by a number of mechanisms, and it is generally believed that autoimmune processes such as those that occur in type 1 diabetes are in some way related to defects in immunological tolerance (1). One approach for the treatment of autoimmunity has been the parenteral administration of anti-CD3 monoclonal antibody (mAb), which is efficacious in animal models of autoimmunity including autoimmune diabetes (2–7) and experimental allergic encephalomyelitis (EAE) (8,9) and in human trials of type 1 diabetes (10–12). Intravenous (IV) anti-CD3 mAb is an approved therapy for transplant rejection in humans (13). We have been interested in immune therapy of autoimmune diseases by mucosal administration of autoantigens designed to induce regulatory T-cells (14,15). We have recently found that oral anti-CD3 mAb is biologically active in the gut and induces a $CD4^+CD25^-$latency-associated peptide $(LAP)^+$ regulatory T-cell that suppresses EAE in a transforming growth factor (TGF)-β–dependent fashion (16). LAP is the NH_2-terminal domain of the TGF-β precursor peptide; it remains noncovalently associated with TGF-β peptide after cleavage and forms the latent TGF-β complex. We previously identified $CD4^+CD25^-LAP^+$ T-cells that suppress colitis by a TGF-β–dependent mechanism (17).

Given this finding, we investigated the effect of oral anti-CD3 mAb in AKR mice on the prevention of autoimmune diabetes using the low-dose streptozocin (STZ) model, which induces autoimmunity to β-cells (18). Diabetes in the STZ model can be prevented by administration of anti–T-cell monoclonal antibodies, and diabetes can be adoptively transferred with splenocytes from diabetic animals (19). This model is useful for the testing of novel immunotherapeutic interventions because hyperglycemia and insulitis can be easily induced in a relatively short period of time in a high percentage of animals. Furthermore, treatment can be given before irreversible tissue damage and before T-cells have become sensitized to islet antigens. It was previously shown by Herold et al. (2) that IV anti-CD3 mAb is effective in the STZ model. Given our results in the EAE model, we investigated the effect of oral anti-CD3 mAb in the STZ model of diabetes.

RESEARCH DESIGN AND METHODS

AKR/J male mice (5–6 weeks old) were purchased from The Jackson Laboratory and housed in a pathogen-free animal facility at the Harvard Institutes of Medicine according to the animal protocol guidelines of the Committee on Animals of Harvard Medical School. For recovery of anti-CD3 mAb after feeding, SJL mice were used (The Jackson Laboratory).

Induction of diabetes by STZ and treatment protocols. Mice were injected intraperitoneally for 5 consecutive days with 40 mg/kg body weight of STZ (Sigma Aldrich, St. Louis, MO) in 0.01 mol/l citrate buffer (pH 4.5) to induce diabetes. Day 1 was defined as the first STZ injection. An intraperitoneal glucose tolerance test (IPGTT) was performed to diagnose diabetes using a glucose meter (Hypoguard, Minneapolis, MN). Blood glucose levels were estimated from the tail vein 30 min after an intraperitoneal injection of glucose (1.33 g/kg body weight). Mice with a glucose level of >250 mg/dl for ≥2 consecutive weeks were considered to be diabetic.

Anti-CD3 mAb, whole antibody (Ab), and (145-2C11) $F(ab')_2$ were purchased from Bio Express (West Lebanon, NH). Hamster IgG whole Ab and $F(ab')_2$ were purchased from Jackson ImmunoResearch Laboratories (Charlestown, MA) and were used as control Ig. Mice were fed with anti-CD3 mAb, control IgG, or PBS followed by STZ injection (40 mg/kg) daily for 5 consecutive days (days 1–5). In combination with anti-CD3 mAb feeding in some experiments, mice were injected with 500 μg neutralizing anti–TGF-β

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Ab, antibody; APC, antigen-presenting cell; EAE, experimental allergic encephalomyelitis; IL, interleukin; IFN, interferon; IPGTT, intraperitoneal glucose tolerance test; IV, intravenous; LAP, latency-associated peptide; mAb, monoclonal antibody; MAPC, immunomagnetic cell sorting; MLN, mesenteric lymph node; PE, phycoerythrin; PLN, pancreatic lymph node; STZ, streptozocin; TGF, transforming growth factor; Th, T-helper.

Ab (Bio Express) or mouse IgG (Jackson ImmunoResearch Laboratories) as control Ig on days 0, 2, 4, 6, and 14.

Histological examination of pancreatic islets. For histological examination of pancreatic islets, the pancreas was removed on day 28 and fixed in 10% buffered formalin. Specimens embedded in paraffin wax were cut by microtome, and sections were stained with hematoxylin and eosin. The slides were analyzed for presence of insulitis by an individual without knowledge of the identity of samples.

Proliferation and cytokine assays. Single-cell suspensions were prepared from spleen and mesenteric lymph node (MLN). To measure proliferation, cells were plated in 96-well round-bottom plates (2.5×10^5 cells/well) in cRPMI containing: RPMI 1640 medium with 10% FBS (BioWhittaker, Walkersville, MD), 100 units/ml penicillin (BioWhittaker), 100 μg/ml streptomycin (BioWhittaker), and 10 mmol/l HEPES (BioWhittaker) and stimulated with 1 μg/ml soluble anti-CD3 mAb (BD Pharmingen, San Diego, CA). After 48 h in culture at 37°C with 7% CO_2, 1 μCi [^{3}H]thymidine was added to each well, and cells were harvested 12 h later. Proliferation was detected using a 1450 Microbeta liquid scintillation counter (PerkinElmer, Boston, MA). For cytokine production, cells were cultured at 2.5×10^5 cells/well in X-VIVO 20 medium (BioWhittaker) and stimulated with 1 μg/ml anti-CD3 mAb. The culture supernatants were collected at 48 h for interleukin (IL)-2, IL-4, IL-10, and interferon (IFN)-γ and at 72 h for TGF-β. Enzyme-linked immunosorbent assays for IL-2, IL-4, IL-10, and IFN-γ were performed as reported previously (20). TGF-β was assayed using a mouse/rat/porcine TGF-β1 immunoassay kit according to the manufacturer's protocol (R&D Systems, Minneapolis, MN).

Flow cytometry analysis. Flow cytometry analysis was performed on single-cell suspensions isolated from MLN, spleen, and pancreatic lymph node (PLN). Cells were stained in PBS with 2% BSA and fixed with 1% paraformaldehyde. Flow cytometry analysis was performed by a FACScan using Cellquest software. The antibodies used were anti-CD16/CD32 mAb (2.4G2) as the Fc blocker, fluorescein isothiocyanate-conjugated anti-CD4 mAb (L3T4), phycoerythrin (PE)-conjugated anti-CD3 mAb (145 2C11), PE-conjugated anti-CD25 mAb (7D4), antigen-presenting cell (APC)-labeled streptavidin, PE-conjugated anti-CD62L Ab (MEL14), PE-conjugated anti-CD45RB Ab (16A) (BD Pharmingen), and biotinylated anti-human LAP Ab (BAF246; XO02) (R&D Systems).

Cell purification and culture. $CD4^+$ T-cells from spleen cells were purified using a mouse T-cell CD4 enrichment column kit (R&D Systems) according to the manufacturer's instructions. LAP^+CD4^+ cells were purified by immunomagnetic cell sorting (MACS) (Miltenyi Biotec, Auburn, CA). All procedures were performed in accordance with the manufacturer's instructions. Purified $CD4^+$ T-cells were stained with biotinylated anti-human LAP Ab and streptavidin-magnetic microbeads in the presence of Fc block. Then LAP^+CD4^+ cells were positively separated and LAP^-CD4^+ cells negatively separated by the MACS column. In some experiments, $CD25^+$ cells were excluded from LAP^-CD4^+ cells or $CD4^+$ T-cells followed by staining with biotinylated anti-human LAP Ab and streptavidin-magnetic microbeads. Cells stained in a combination of PE-conjugated anti-CD25 Ab and anti-PE magnetic microbeads were separated into $CD25^+$ cells and $CD25^-$ cells by the MACS column. To prepare APCs depleted of T-cells, spleen cells from naive mice were stained with a combination of PE-conjugated anti-CD3 mAb and anti-PE magnetic microbeads, and stained cells were negatively isolated by the MACS column. Purified cells were used for cytokine assays or suppressive function assays. For cytokine assays, 2.5×10^5 LAP^+CD4^+ cells, $LAP^-CD25^+CD4^+$ cells, or $LAP^-CD25^-CD4^+$ cells were cultured with 10 μg/ml plate-bound anti-CD3 mAb in X-VIVO 20 (BioWhittaker) in 96-well round-bottom plates. The culture supernatants were collected at 48 h for IL-2, IL-4, IL-10, and IFN-γ and at 72 h for TGF-β. For measuring suppressive function in a coculture system, titrated purified cells were cultured with an equal ratio of responder cells ($LAP^-CD25^-CD4^+$ cells) and APCs and stimulated with 1 μg/ml soluble anti-CD3 mAb in cRPMI. Cocultured cells were maintained for 60 h, and 1 μCi [^{3}H]thymidine was added at 12 h before harvest. In some experiments, 50 μg/ml neutralizing Ab, anti–IL-10 Ab (JES052A5), or anti–TGF-β Ab (1D11), purchased from R&D systems, was added to the culture system.

Adoptive transfer. One day after the last feeding, $CD4^+$ T-cells or LAP^-CD4^+ cells were purified from spleen and MLN of mice fed with 50 μg anti-CD3 mAb or control IgG for 5 consecutive days. The purified cells (2.5×10^6) were injected intravenously to naive AKR/J mice on day 0. STZ (40 mg/kg) was administrated to the recipient AKR/J mice from days 1 to 5, and an IPGTT was performed on days 7, 14, 21, and 28 using a glucose meter.

Recovery of anti-CD3 mAb from the intestines of mice fed anti-CD3 mAb. Six hours after feeding, the upper part of the small intestine from three mice fed 50 μg anti-CD3 mAb or control isotype Ab was digested using 1 ml PBS containing 10 mg collagenase D (Roche) for 30 min at 37°C. The tissue was homogenized and centrifuged for 30 min at 70,000 rpm. After centrifugation, the soluble fraction was added to a protein-G column, and the bound IgG was eluted in the presence of proteinase inhibitors (Roche).

The biological activity and the concentration of the eluted IgG were ascertained by the ability to induce T-cell proliferation of naive splenocytes compared with known concentrations of anti-CD3 mAb. The culture conditions for proliferation with anti-CD3 mAb were as follows. Single cell suspensions were prepared from spleens of naive SJL mice. Cells were plated in 96-well round-bottom plates (2.5 × 105 cells/well) in X-VIVO 20 and stimulated with either soluble known concentrations of anti-CD3 mAb (0.0025, 0.005, or 0.01 μg/ml) or with 0.5- to 5-μl eluates. After 72 h in culture 1 μCi [^{3}H]thymidine was added to each well, and cells were harvested 12 h later. Each experiment was repeated twice. The statistic used was an unpaired Student's t test.

Statistical analysis. Statistical significance was calculated using ANOVA with Bonferroni post hoc analysis, the unpaired Student's t test assuming unequal variance, or a χ^2 test for in vitro assays. For in vivo assays the Mantel-Cox log-rank test or a χ^2 test was used.

RESULTS

Oral anti-CD3 mAb treatment prevents the development of diabetes induced by STZ. Mice were orally administered 5, 50, or 250 μg anti-CD3 mAb and injected with STZ to induce diabetes on days 1–5. Thereafter, blood glucose levels were monitored once a week, and mice with a glucose level >250 mg/dl by IPGTT for 2 or more consecutive weeks were classified as diabetic. As shown in Fig. 1, diabetes progression was not suppressed in mice fed with 5 μg anti-CD3 mAb compared with the PBS group or control IgG group. On the other hand, mice treated with 50 or 250 μg anti-CD3 mAb had significantly reduced development of diabetes compared with mice treated with either PBS or control IgG. On day 14, diabetes was diagnosed in 67% (8 of 12) of mice treated with PBS and 83% (10 of 12) of mice treated with control IgG. However, development of diabetes in mice treated with anti-CD3 mAb was only 33% (4 of 12). Mice treated with 50 μg anti-CD3 mAb were followed for up to 42 days. At this time, there was no statistically significant difference between the treated and control groups although there were fewer diabetic animals in the treated group (8 of 12) than in the control group (10 of 12). Significant suppression of diabetes was also observed in animals fed 250 μg anti-CD3 mAb. Correspondingly, as shown in Fig. 1*D*, the blood glucose levels on day 14 were also significantly reduced in mice treated with 50 μg anti-CD3 mAb compared with mice treated with either PBS or control IgG. Moreover, as shown in Table 1, the mice treated with 50 μg anti-CD3 mAb had a significantly lower incidence of insulitis in the pancreas than that of mice treated with either control IgG or PBS on day 28.

Oral anti-CD3 mAb treatment suppresses IL-2 and IFN-γ cell proliferation and enhances secretion of TGF-β. It has been reported that IV anti-CD3 mAb can treat diabetes in NOD mice and that the effect is related to the immunoregulatory function of TGF-β (7). Therefore, we investigated whether the regulatory function in the periphery was enhanced in the STZ model after 50 μg oral anti-CD3 mAb treatment and STZ injection. On day 6, spleens were removed from each group, and cells were cultured with soluble anti-CD3 mAb to measure cell proliferation and cytokine production. As shown in Fig. 2, spleen cells from mice treated with either PBS or control IgG proliferated to anti-CD3 mAb in a dose-dependent manner. On the other hand, proliferation of spleen cells from mice treated with oral anti-CD3 mAb was significantly suppressed compared with either the PBS group or the control IgG group ($P < 0.009$). For cytokines, in comparison to both the PBS group and the control IgG group, IL-2 ($P < 0.04$) and IFN-γ ($P < 0.008$) secretions

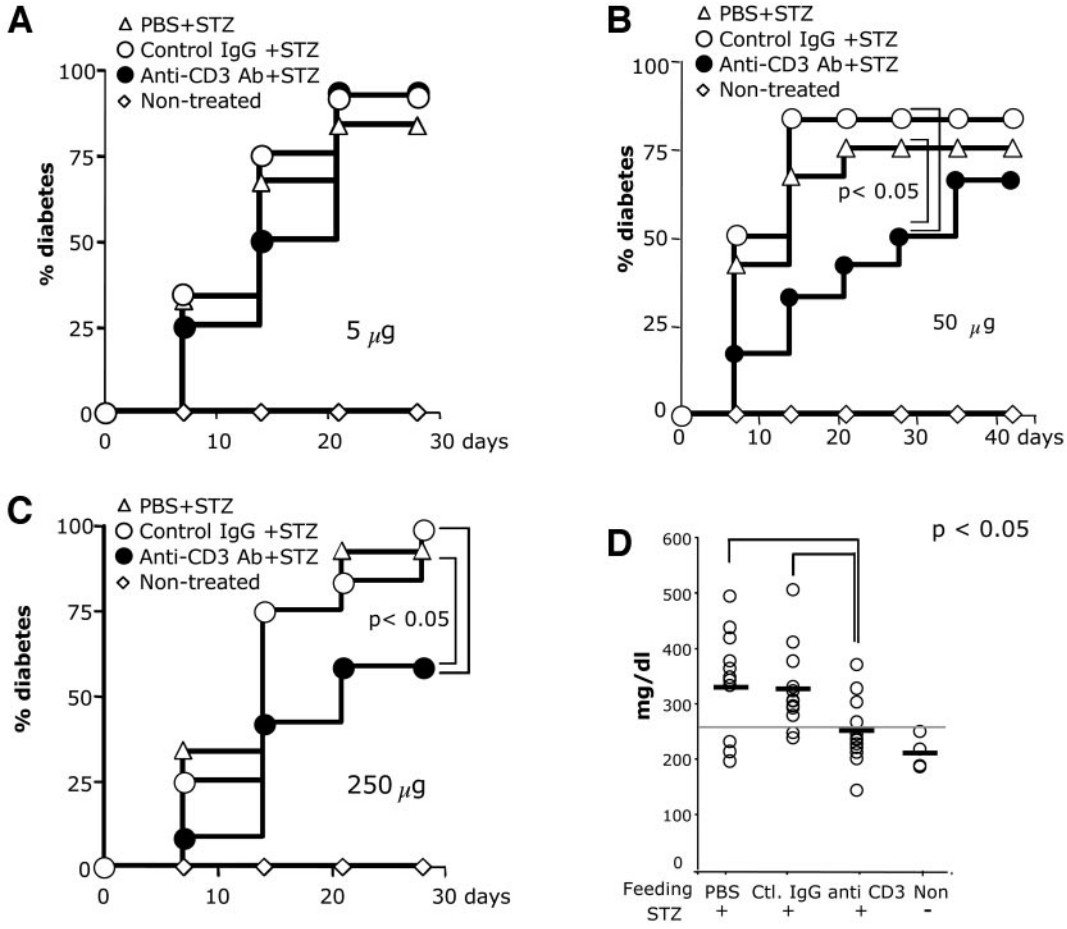

FIG. 1. Oral administration of anti-CD3 mAb suppresses incidence of STZ-induced diabetes in AKR mice. AKR mice were fed with 5 μg (*A*), 50 μg (*B*), or 250 μg (*C*) of anti-CD3 mAb and injected with STZ from days 1 to 5 and monitored once a week. Diabetes was diagnosed in mice with blood glucose level readings >250 mg/dl by IPTTG. △, PBS + STZ (n = 12); ○, control IgG + STZ (n = 12); ●, anti-CD3 mAb + STZ (n = 12); ◇, nontreated (n = 4). *D*: Blood glucose levels measured at day 14 by IPGTT in mice treated with 50 μg anti-CD3 mAb and injected with STZ. Both incidence of diabetes and the blood glucose levels on day 14 were significantly suppressed in mice fed with 50 or 250 μg anti-CD3 mAb compared with mice fed with either control IgG or PBS (P < 0.05).

were significantly decreased and TGF-β secretion (P < 0.01) was significantly increased in the spleens of mice fed with anti-CD3 mAb.

Recovery of biologically active anti-CD3 mAb from the intestines of mice fed anti-CD3 mAb. We previously found that oral anti-CD3 mAb accumulates in the gut villi of mice after feeding as measured by immunohistochemistry (16). To determine whether we could recover biologically active anti-CD3 mAb after feeding, mice were fed 50 μg anti-CD3 mAb or isotype control antibody, and 6 h after feeding, anti-CD3 mAb or isotype control antibody was extracted from the upper intestine using a protein-G column. The biologic activity of the eluted anti-CD3 mAb was tested by measuring its ability to stimulate T-cell proliferation and compared with known concentrations of anti-CD3 mAb. As shown in Fig. 3, biologically active anti-CD3 mAb could be eluted from oral anti-CD3–fed animals as measured by proliferation and was equivalent to ~0.01 μg/ml of anti-CD3 mAb.

Increase of LAP$^+$ on CD4$^+$ cells after oral administration of anti-CD3 mAb. To address whether any cell populations with regulatory function were induced after oral anti-CD3 mAb, MLN, spleen, and PLN cells from each group were prepared on day 6. Cells were stained with a combination of anti-CD4 mAb and antibody to CD25, LAP, CD45RB, and CD62L. The frequency of CD25$^+$CD4$^+$ cells did not change in MLN, spleen, or PLN in any groups (Table 2), whereas the frequency of LAP$^+$CD4$^+$ cells in

TABLE 1
Incidence of insulitis in pancreas after 50 μg anti-CD3 Ab

Feeding	IP	Islets	Normal islets	Peri-insulitis	Invasive insulitis
PBS	STZ	72	50 (69)	15 (21)	7 (10)
Control IgG	STZ	88	62 (71)	15 (17)	11 (13)
Anti-CD3 Ab	STZ	135	125 (93)**	8 (6)**	2 (2)*
None	None	66	66 (100)	0 (0)	0 (0)

Data are n or n (%). Sections of pancreas from 6–8 mice/group (nontreated group; n = 4) on day 28 were scored for the presence of insulitis. Pooled insulitis score data were statistically analyzed by χ^2 test. *P < 0.01 compared with the PBS group or the control IgG group. IP, intraperitoneal injection.

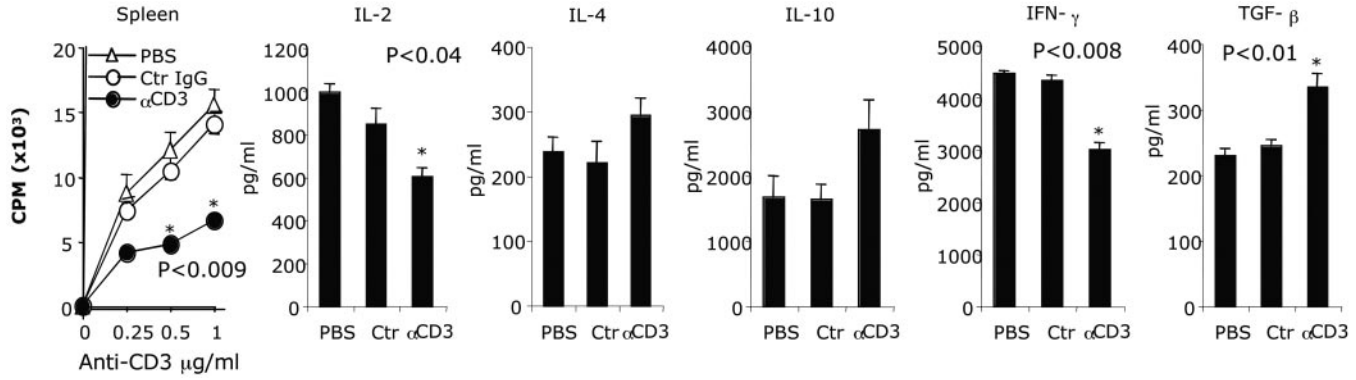

FIG. 2. Cell proliferation and cytokine production after oral anti-CD3 mAb. AKR mice were fed with 50 μg anti-CD3 mAb, injected with STZ from days 1 to 5, and killed on day 6. Spleen cells (2.5×10^5 cells/well) were cultured with 0.25–1 μg/ml anti-CD3 mAb in cRPMI medium for 60 h. [^{3}H]Thymidine was added in the last 12 h of culture. To measure cytokine production, cells were cultured with 1 μg/ml anti-CD3 mAb in X-VIVO 20 medium. Supernatants were collected at 48 h for IL-2, IL-4, IL-10, and IFN-γ at 72 h for TGF-β. Means and SE are shown. *For proliferation, $P < 0.009$ compared with either the control IgG (Ctr) or PBS group; for IL-2, $P < 0.04$; for IFN-γ, $P < 0.008$; and for TGF-β, $P < 0.01$. Similar results were obtained in three independent experiments.

spleen and PLN significantly increased in mice treated with oral anti-CD3 mAb compared with either the PBS group or the control IgG group. The frequency returned to control levels by day 21. No significant changes in frequency of either $CD45RB^{low}CD4^+$ or $CD62L^+CD4^+$ were observed in MLN, spleen, and PLN after oral anti-CD3 mAb (not shown), and, as in our studies of oral anti-CD3 mAb in the EAE model, we did not find that CD3 expression was decreased and/or downregulated after oral anti-CD3 mAb treatment (16).

LAP^+CD4^+ cells from mice treated with oral anti-CD3 mAb have enhanced regulatory function in vitro. To investigate whether the in vitro suppressive function of LAP^+CD4^+ cells was enhanced after oral anti-CD3 mAb, purified LAP^+CD4^+ cells and $LAP^-CD25^+CD4^+$ cells from spleen on day 6 were cultured with $LAP^-CD25^-CD4^+$ cells as responder cells, APCs, and soluble 1 μg/ml anti-CD3 mAb. As shown in Fig. 4*A* and *B*, LAP^+CD4^+ cells, $LAP^-CD25^+CD4^+$ cells, and $LAP^+CD25^-CD4^+$ cells were anergic to anti-CD3 mAb stimulation in vitro, and they suppressed responder cell proliferation in a dose-dependent fashion. The suppressive function of LAP^+CD4^+ cells was augmented more than two to threefold after oral anti-CD3 mAb treatment compared with that of control IgG-treated mice. However, the suppressive function of $LAP^-CD25^+CD4^+$ cells was not affected by anti-CD3 mAb treatment. To rule out a possible effect of $CD25^+$ cells on LAP^+CD4^+ cell suppressive function, $CD25^+$ cells were excluded from LAP^+CD4^+ cells. As shown in Fig. 4*B*, $LAP^+CD25^-CD4^+$ cells also suppressed responder cells with increased suppressive function after oral anti-CD3 mAb treatment. We next examined in vitro kinetics of suppressive function of LAP^+CD4^+ cells after anti-CD3 mAb feeding. LAP^+CD4^+ cells from mice fed with anti-CD3 mAb had enhanced suppressive function compared with LAP^+CD4^+ cells from mice fed with IgG on days 6 and 14, which was no longer observed on day 21 (Fig. 4*C*).

To determine whether the in vitro suppressive function of $LAP^+CD25^-CD4^+$ cells after oral anti-CD3 mAb is mediated through IL-10 or TGF-β neutralizing, anti–IL-10 Ab or anti–TGF-β Ab was added in vitro. As shown in Fig. 4*D*, neutralizing anti–IL-10 Ab had no effect, whereas neutralizing anti–TGF-β Ab partially reversed the suppressive function by $LAP^+CD25^-CD4^+$ cells.

Neutralization of TGF-β in vivo abrogates the effect of oral anti-CD3 mAb on diabetes. To determine whether TGF-β is involved in preventing diabetes progression after oral anti-CD3 mAb in vivo, 500 μg neutralizing anti–TGF-β Ab was given on days 0, 2, 4, 6, and 14 and blood glucose levels measured on day 21. As shown in

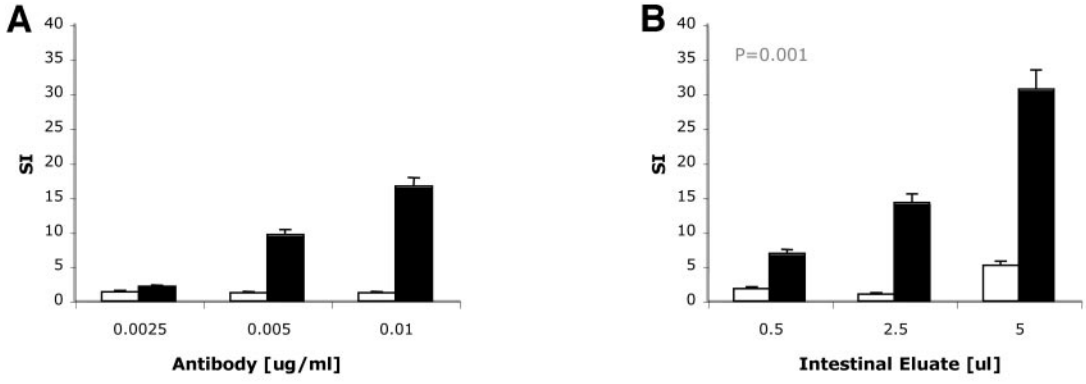

FIG. 3. Activity of eluted anti-CD3 mAb IgG from the intestines of mice fed with anti-CD3 mAb as measured by T-cell proliferation. Three mice were fed with 50 μg anti-CD3 mAb or isotype control antibody, and 6 h after feeding the upper intestine was processed to elute anti-CD3 mAb. The eluted material contained 105 μg/ml protein. Splenocytes from naive mice were cultured with anti-CD3 mAb or intestinal eluates, and proliferation induced by eluates was measured and compared with cells stimulated with known concentrations of anti-CD3 mAb as described in RESEARCH DESIGN AND METHODS. Eluates from anti-CD3 mAb–fed mice had a higher proliferation as compared with control fed mice, $P = 0.001$. *A*: □, RPMI; ■, anti-CD3. *B*: □, isotype control fed; ■, anti-CD3 fed.

TABLE 2
Percentages of $CD4^+$ cell populations in MLN, spleen, and PLN

		MLN		Spleen		PLN	
Feeding	IP	$CD25^+CD4^+$	LAP^+CD4^+	$CD25^+CD4^+$	LAP^+CD4^+	$CD25^+CD4^+$	LAP^+CD4^+
PBS	STZ	7.9 ± 1.1	2.2 ± 0.7	8.4 ± 0.7	3.0 ± 0.4	13.8 ± 1.9	3.7 ± 0.7
Ctl IgG	STZ	7.4 ± 1.1	2.4 ± 0.7	8.0 ± 0.6	3.2 ± 0.6	12.6 ± 2.3	3.9 ± 0.6
aCD3 Ab	STZ	7.6 ± 1.4	2.5 ± 0.6	8.5 ± 0.9	4.3 ± 0.5*	13.3 ± 2.2	4.5 ± 0.7†

Data are means ± SD from three experiments (n = 10–14). *$P < 0.01$; †$P < 0.05$, compared with either the PBS group or the control (Ctl) IgG group. IP, intraperitoneal injection.

Table 3, when mice treated with oral anti-CD3 mAb were injected with neutralizing anti–TGF-β Ab, the preventive effect on diabetes progression was abrogated.

Adoptive transfer of diabetes prevention after oral anti-CD3 mAb is dependent on LAP^+ T-cells. We investigated whether the suppressive function of oral anti-CD3 mAb could be adoptively transferred to naive recipients. To investigate this, $CD4^+$ T-cells or $CD4^+$ cells depleted of LAP^+ cells from spleen and MLN of mice fed with anti-CD3 mAb or control IgG were transferred to naive AKR/J mice on day 0. STZ was injected to the recipient AKR/J mice on days 1–5, and blood glucose was monitored once weekly. As shown in Fig. 5, development of diabetes was significantly suppressed in the mice transferred with $CD4^+$ T-cells from mice fed anti-CD3 mAb compared with the mice transferred with $CD4^+$ T-cells from mice fed control IgG. No suppression was observed when $CD4^+LAP^-$ cells were transferred. Of note, deple-

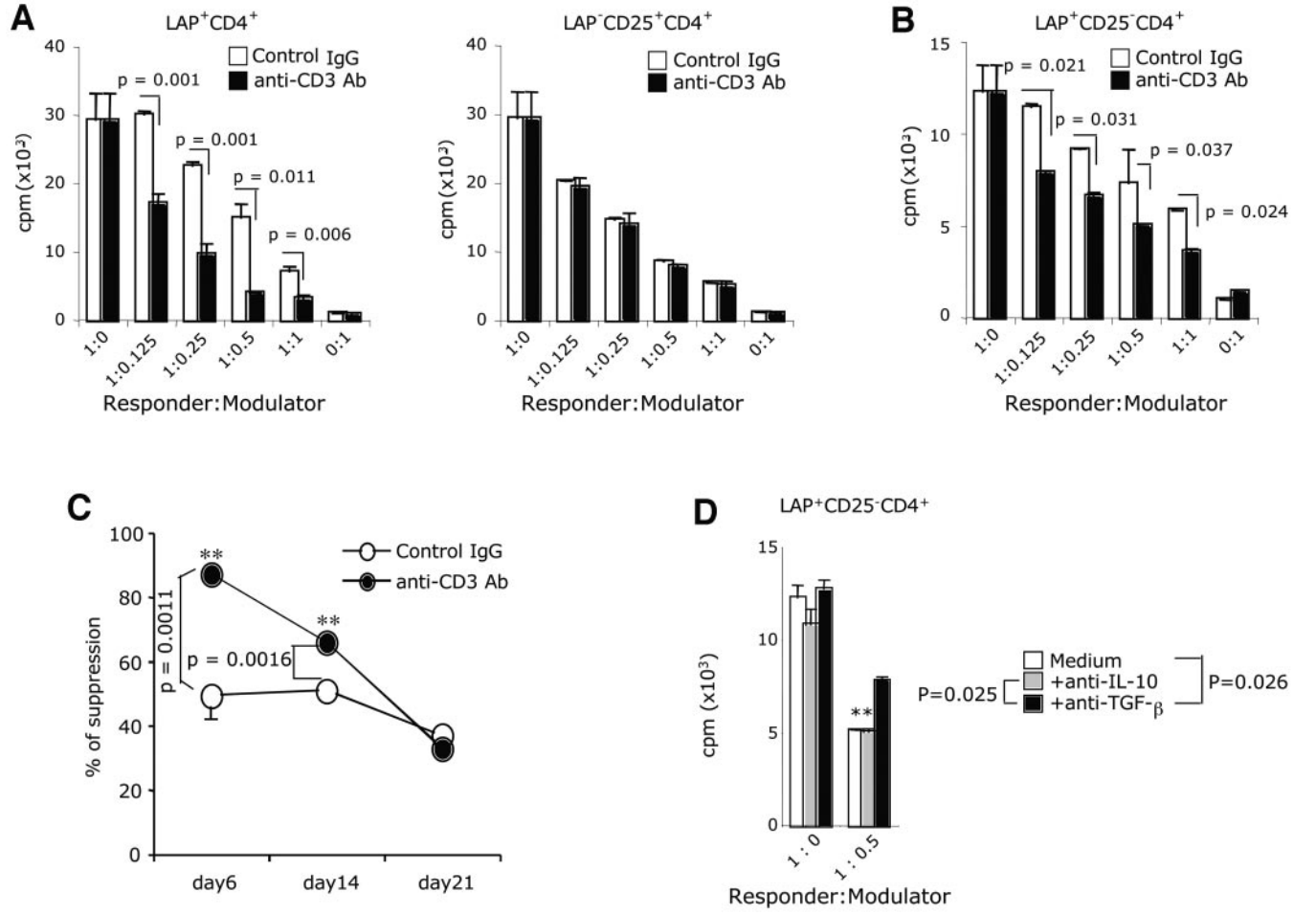

FIG. 4. Enhancement of LAP^+CD4^+ in vitro suppression after oral anti-CD3 mAb. Mice were fed with 50 μg anti-CD3 mAb and injected with STZ from days 1 to 5 and killed on day 6. LAP^+CD4^+, $LAP^-CD25^+CD4^+$, or $LAP^+CD25^-CD4^+$ cells as modulator were purified from spleens as described in RESEARCH DESIGN AND METHODS. $LAP^-CD25^-CD4^+$ cells used as responder cells were purified from spleens of mice fed with control IgG. *A*: Titrated modulator cells from the control IgG or anti-CD3 mAb group were cocultured with responder cells (2.0×10^5 cell/well) in the presence of APCs and 1 μg/ml anti-CD3 mAb. *B*: Titrated modulator $LAP^+CD25^-CD4^+$ cells from the control IgG or anti-CD3 mAb group were cocultured with responder (1.0×10^5 cell/well) in the presence of APCs and 1 μg/ml anti-CD3 mAb. *C*: On day 6, 14, or 21, modulator LAP^+CD4^+ cells (1.0×10^5 cell/well) from the control IgG or anti-CD3 mAb group were cocultured with responder cells (2.0×10^5 cell/well) in the presence of APCs and 1 μg/ml anti-CD3 mAb. Modulator LAP^+CD4^+ cells from mice fed anti-CD3 mAb were more suppressive ($P = 0.0011$ for day 6 and $P = 0.0016$ for day 14) than those from control IgG–fed mice. *D*: Modulator $LAP^+CD25^-CD4^+$ cells (0.5×10^5 cell/well) from spleens of mice fed with anti-CD3 mAb were cocultured with responder cells (1.0×10^5 cell/well) in the presence of APCs and 1 μg/ml anti-CD3 mAb. To neutralize IL-10 or TGF-β, either 50 μg/ml anti–IL-10 Ab or 50 μg/ml anti–TGF-β Ab was added in culture. Anti–TGF-β significantly increased proliferation compared with medium ($P = 0.026$) or anti–IL-10 ($P = 0.025$). These results are representative of two or three independent experiments.

TABLE 3
Neutralization of TGF-β reverses the protective effect of oral anti-CD3 Ab in STZ-induced diabetes

Treatment		Incidence of diabetes	Blood glucose (mg/dl)
Feeding	IP		
PBS		11/12	381 ± 87
Control IgG	Control IgG	10/12	394 ± 113
Control IgG	Anti–TGF-β Ab	10/12	355 ± 73
Anti-CD3 Ab	Control IgG	6/12*	275 ± 73*
Anti-CD3 Ab	Anti–TGF-β Ab	11/12	358 ± 81

Data are *n* or means ± SD from three experiments. Mice were fed with 50 μg anti-CD3 Ab and injected with STZ on days 1–5. Neutralizing anti–TGF-β Ab was injected intraperitoneally (IP) at days 0, 2, 4, 6, and 14. Blood glucose was measured at day 21. *$P <$ 0.05, compared with other groups.

tion of LAP^+ cells from control Ig–fed animals also enhanced the incidence of diabetes. These results confirm that LAP^+CD4^+ cells play a crucial role in prevention of autoimmune diseases followed by restoration of peripheral immune tolerance and that the regulatory function of LAP^+CD4^+ cells was reinforced by anti-CD3 mAb feeding.

DISCUSSION

In the present study we demonstrated the effectiveness of oral anti-CD3 mAb in the STZ model of diabetes. Oral anti-CD3 mAb induces an LAP^+ regulatory T-cell that can adoptively transfer protection and functions in a TGF-β–dependent fashion. We do not know at this time whether LAP^+ cells are a subset of T-helper (Th)3 cells, although our working hypothesis is that they are precursors of Th3 cells (21) and either differentiate into Th3 cells upon secretion of TGF-β or induce regulatory T-cells by the secretion of TGF-β. Once Th3 cells are stimulated, they may lose LAP on their surface.

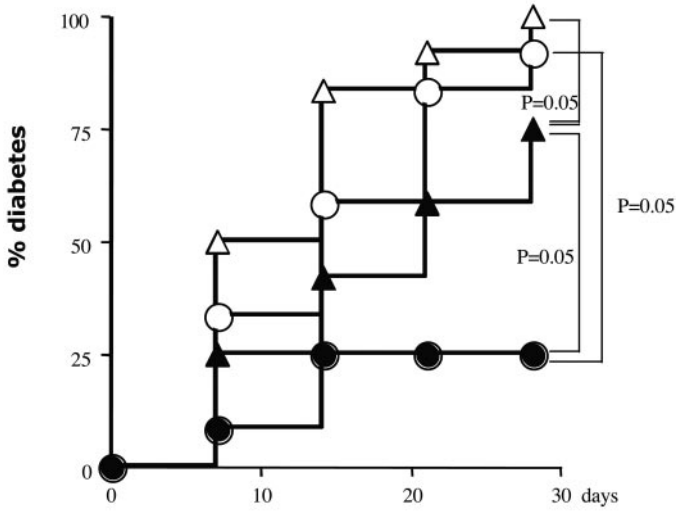

FIG. 5. Adoptive transfer of $CD4^+$ cells, but not LAP^-CD4^+ cells, from mice fed with anti-CD3 mAb suppresses development of diabetes in recipient mice. $CD4^+$ T-cell or LAP^-CD4^+ cells were purified 1 day after the last feeding for 5 consecutive days from spleens and MLN of mice fed with 50 μg control IgG or anti-CD3 mAb. Purified cells (2.5×10^6) were injected intravenously to naive AKR/J mice on day 0. Recipient mice were injected with 40 mg STZ from days 1 to 5, and the blood glucose level was monitored by IPGTT once weekly. ●, $CD4^+$ T-cells from anti-CD3 mAb–fed mice ($n = 12$); ▲, $CD4^+$ T-cells from control IgG Ab–fed mice ($n = 12$); ○, $CD4^+LAP^-$ T-cells from anti-CD3 mAb–fed mice ($n = 12$); △, $CD4^+LAP^-$ T-cells from control IgG–fed mice ($n = 12$). Mice transferred with $CD4^+$ cells from anti-CD3–fed mice developed significantly less diabetes ($P = 0.05$) than those that were given $CD4^+$ cells from control IgG–fed mice or LAP^- cells from either CD3- or control IgG–fed mice.

Our results are analogous to what we observed with administration of oral anti-CD3 mAb in the mouse model of EAE (16). One of the features of the induction of regulatory T-cells by oral anti-CD3 mAb is a dose response in which lower doses are more effective. This was seen in the EAE model in which 5 μg was the most effective dose, no effect was observed at 50 μg, and worsening of EAE occurred with 500 μg. This dose response correlated with the induction of regulatory cells in the MLN. Thus, it appears that the dose of anti-CD3 mAb is very important for the induction of regulatory T-cells and that LAP^+ cells will not be induced if the signal delivered to the T-cell receptor by anti–CD-3 is either too weak or too strong. We observed a similar dose-response pattern in the STZ model although the most effective dose was 50 μg, and we also observed positive effects at 250 μg. We did not test doses higher than 250 μg. The shifted dose response may be related to different strains being used and the use of complete Freund's adjuvant for the induction of EAE versus the use of STZ to induce diabetes. Of note, at 42 days after treatment of animals with 50 μg oral anti-CD3 mAb, there was no statistically significant difference between the treated and control groups although there were fewer diabetic animals in the treated group (8 of 12) than in the control group (10 of 12). For a more prolonged effect, repeat or chronic dosing may be required.

Unlike the EAE model in which oral anti-CD3 mAb enhanced recovery in animals treated at the peak of disease, we did not observe a protective effect of oral anti-CD3 mAb once animals were diabetic. The lack of effect may be related to the fact that the STZ diabetes model is an induced model with rapid onset. The NOD model of diabetes serves as one of the primary models for human disease, and we are currently testing the effect of oral anti-CD3 mAb in the NOD model given both as prevention and therapeutically at the onset of diabetes. In preliminary experiments we have found that oral anti-CD3 mAb is effective in preventing diabetes when given to neonatal NOD mice (R.M., M. Abraham, unpublished observations). Although the cause of type 1 diabetes is not known, there is the suggestion that the gut may play a role as the site at which newborns are sensitized to islet antigens related to cross-reactivity with cow milk and/or defective mechanisms of mucosal tolerance (22–24). Oral anti-CD3 mAb may thus serve to boost the natural induction of regulatory T-cells at the gut mucosa.

There is a difference in the effect of anti-CD3 mAb depending on the route it is given (IV versus oral) and when it is administered. In the EAE model, IV anti-CD3 mAb is effective when it is given at the height of the disease but not before disease induction (9). This phenomenon is felt to be related to the mechanism of action of IV anti-CD3 mAb. It is believed that IV anti-CD3 mAb kills Th1 cells that have been generated and are present at the height of disease. Oral anti-CD3 mAb, on the other hand, is effective before disease onset in the EAE model as it directly induces regulatory T-cells, which prevent the induction of effector cells. In the EAE model, it is also effective at the height of disease, presumably by boosting a naturally occurring regulatory T-cell response that occurs before the recovery phase (20). The effect of oral anti-CD3 mAb appears to be different in the STZ-induced diabetes model. We postulate that unlike EAE, regulatory

T-cells have not been generated in the STZ-induced diabetes model and that the induction of regulatory cells would not be effective in later stages of STZ-induced diabetes unless Th1 responses are also downregulated. Consistent with this hypothesis, in the NOD model of diabetes, IV anti-CD3 mAb appears to eliminate the Th1 milieu, allowing the emergence of regulatory T-cells at a late time point. Unlike IV anti-CD3 mAb, oral anti-CD3 mAb directly induces regulatory T-cells; thus, in diabetes it is effective prophylactically and may also be effective after IV anti-CD3 mAb to boost a naturally occurring T-cell response that arises after IV anti-CD3 mAb (25).

After oral anti-CD3 mAb, LAP^+ regulatory T-cells are first induced in the MLN after which they migrate to sites of inflammation (16). Thus, we observed increased numbers of LAP^+ T-cells in the popliteal lymph nodes in EAE animals after immunization with protedlipid protein/CFA. In the present study we observed an increase of LAP^+ T-cells in the PLNs of mice with STZ-induced diabetes. It thus appears that one of the properties of LAP^+ regulatory T-cells is their ability to migrate to sites of inflammation where they exert their regulatory effects. In our studies of anti-CD3 mAb in the EAE model (16), we found that anti-CD3 mAb appears in the villi of fed mice within 1 hour after feeding. We now present data showing that biologically active anti-CD3 mAb can be recovered from the intestine as measured by the ability of recovered antibody to stimulate naive T-cells in vitro.

The immunological effects of oral anti-CD3 mAb are antigen nonspecific because anti-CD3 mAb binds to CD3 on the surface of T-cells independent of the antigen specificity of the T-cell receptor. Thus, it would be expected to be effective in other models of autoimmunity besides EAE. The present study is the first demonstration that oral anti-CD3 mAb is effective in an autoimmune model different from EAE and suggests that oral anti-CD3 mAb may be applicable for the treatment of human type 1 diabetes. For clinical application, it may be more effective when given to subjects at risk rather than to those with new-onset diabetes or after treatment with IV anti-CD3 mAb in subjects with new-onset diabetes.

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