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Intranasal Vaccination with Proinsulin DNA Induces Regulatory CD4+ T Cells That Prevent Experimental Autoimmune Diabetes

Alison L. Every,* David R. Kramer,† Stuart I. Mannering,* Andrew M. Lew,* and Leonard C. Harrison2*

Insulin, an autoantigen in type 1 diabetes, when administered mucosally to diabetes-prone NOD mice induces regulatory T cells (Treg) that protect against diabetes. Compared with protein, Ag encoded as DNA has potential advantages as a therapeutic agent. We found that intranasal vaccination of NOD mice with plasmid DNA encoding mouse proinsulin II-induced CD4+ Treg that suppressed diabetes development, both after adoptive cotransfer with “diabetogenic” spleen cells and after transfer into NOD mice given cyclophosphamide to accelerate diabetes onset. In contrast to prototypic CD4+CD25+ Treg, CD4+ Treg induced by proinsulin DNA were both CD25+ and CD25− and not defined by markers such as glucocorticoid-induced TNFR-related protein (GITR), CD103, or Foxp3. Intriguingly, despite induction of Treg and reduced islet inflammation, diabetes incidence in proinsulin DNA-treated mice was unchanged. However, diabetes was prevented when DNA vaccination was performed under the cover of CD40 ligand blockade, known to prevent priming of CTL by mucosal Ag. Thus, intranasal vaccination with proinsulin DNA has therapeutic potential to prevent diabetes, as demonstrated by induction of protective Treg, but further modifications are required to improve its efficacy, which could be compromised by concomitant induction of pathogenic immunity. The Journal of Immunology, 2006, 176: 4608–4615.

Studies in the NOD mouse model of type 1 diabetes demonstrate that autoreactive T cells are responsible for the destruction of insulin-secreting pancreatic β cells (1, 2). In common with humans at risk for type 1 diabetes, NOD mice exhibit spontaneous immunity to insulin (3). NOD mice, however, are completely protected from diabetes by transgenic expression of proinsulin in APCs (4) or by reconstitution with hemopoietic stem cells that encode proinsulin expression in APC progeny (5). Thus, although proinsulin is an autoantigen, its expression can be manipulated to prevent β cell autoimmunity. Approaches to autoantigen-specific therapy for humans have centered on exploiting mucosa-mediated immunoregulation or “tolerance” (6, 7). The administration of soluble protein Ag to mucosal surfaces, classically by the oral route, suppresses subsequent priming to the same Ag. Mucosal tolerance has been attributed to deletion and/or anergy of Ag-specific T cells and, particularly after low-dose Ag, to induction of regulatory T cells (Treg)3 (6, 7). In the NOD mouse, repeated low-dose oral insulin (8, 9), intranasal insulin B chain peptide (10), intranasal proinsulin B-C chain peptide (11), or inhaled insulin aerosol (12) induce tolerance which is transferable by Treg into nontolerized recipients. Unique markers for Treg have not been identified. However, CD25 (IL-2Rα) distinguishes a population of naive CD4+ Treg in the thymus (13), and CD4+CD25+ Treg were reported to be induced peripherally by oral OVA (14, 15). The glucocorticoid-induced TNFR-related protein (GITR), a member of the TNFR superfamily, is constitutively expressed on thymic CD4+CD25+ Treg (16, 17), but also acts as a costimulatory molecule on activated T cells (18, 19). αβ integrin (CD103), expressed predominantly by intraepithelial lymphocytes on mucosal epithelia, has also been reported to be a marker of a subset of CD4+ Treg (20). In mice, the forkhead/winged helix transcription factor gene, Foxp3, is the most specific marker of naturally occurring thymic CD4+CD25+ Treg (21–23), but its expression can be induced in peripheral CD4+CD25− Treg upon exposure to TGF-β (24, 25).

As a candidate therapeutic, autoantigen could also be delivered as DNA. Compared with Ag delivered as protein, Ag encoded as DNA has several potential advantages. These include ease of handling, stability, purity (less risk of contaminants), production of native protein (nature does the work) with no requirement for protein purification, and sustained delivery (less frequent dosing). Intramuscular vaccination with plasmids that express “naked” DNA for autoantigens has been shown to induce protective immunity in experimental models of autoimmune diseases, including type 1 diabetes (26–28) and experimental autoimmune encephalomyelitis (EAE) (29, 30). For the prevention of autoimmune disease, a potential drawback of mucosally delivered DNA is that, like viral nucleic acid, it may elicit strong CD8+ CTL responses to encoded MHC class I-restricted epitopes. CTL induction is not, however, unique to DNA and also occurs in response to protein delivered mucosally. For example, in C57BL/6 mice, we found that oral, aerosol, or nasal OVA protein induced both tolerance and a pathogenic CTL response (31). Importantly, however, blockade of CD40 ligand (CD40L)-mediated costimulation prevented priming of CTL but spared tolerance (32). In this study, we report for the
first time the effect of mucosal vaccination with a DNA-encoded autoantigen, namely, intranasal plasmid DNA encoding mouse proinsulin II, an autoantigen in type 1 diabetes.

Materials and Methods

**DNA**

The mouse proinsulin II gene or an OVA minigene was subcloned into a mammalian expression plasmid containing a CMV immediate-early promoter and a polyadenylation signal sequence from bovine growth hormone (33). The OVA minigene was derived by replacing the 378-bp SacI fragment of OVA cDNA with 1.6-kb SacI fragment of the OVA gene. The proinsulin II and OVA constructs each contained one GAGCT CgG motif per molecule; this sequence is thought to be the optimal ligand for the mouse TLR9 (34). Gene expression was confirmed by transfection into Chinese hamster ovary cells. The proinsulin II gene insert was previously shown to be expressed in vivo (4). Plasmids prepared from *Escherichia coli* were purified by polyethyleneglycol precipitation and Triton X-114 phase partition to remove endotoxin, as described previously (35). This reduced the endotoxin concentration, measured by chemiluminescence in the Limulus lysate assay (BioWhittaker), from 10^7 to 1 IU/ml plasmid DNA diluted to 2 mg/ml in PBS.

**Mice and treatment**

NOD/LtJ Jax mice were bred and maintained in the Walter and Eliza Hall Institute of Medical Research. Nonanesthetized female mice were given 50 μg of plasmid DNA intranasally in 25 μl of PBS routinely at 3 and 5 wk of age. In other experiments, mice were given a range of intranasal DNA doses (25, 50, and 100 μg of plasmid) weekly from 3 to 10 wk of age.

In the CD40L blocking experiments, mice were injected i.p. with 300 μg of anti-CD40L mAb (clone MR-1) or hamster IgG control (clone 6C8, specific for human Bcl-2) just before intranasal DNA treatment at 4, 6, and 8 wk old. Abs were purified on protein G-Sepharose from supernatants of hybridomas grown in our mAb facility.

For in vivo cytotoxicity assays, female NOD mice were given 100 μg of proinsulin or OVA DNA intradermally at 6 and 8 wk of age. C57BL/6 mice used as positive controls were injected with 100 μg of OVA DNA intradermally at the base of the tail or were given 50 μg of OVA DNA intranasally at 3 and 5 wk old.

**Detection of diabetes**

Blood glucose was measured fortnightly using the Advantage monitor (Boehringer Mannheim) on a drop of blood obtained via a fine glass capillary tube from the retro-orbital venous plexus. Mice were considered to be diabetic if their blood glucose was >11 mM, confirmed the following day, except in the cyclophosphamide experiment when blood glucose was measured only on days 10, 17, and 24.

**Adaptive transfer of diabetes**

Male NOD mice at 8–10 wk old were irradiated (8 Gy) from a cobalt source. Four hours later, they were injected via the tail vein with 2 × 10^6 pooled spleen cells from recently diabetic female NOD mice along with cells from 7- to 10-wk-old mice that had been treated with intranasal DNA. The recently diabetic mice had been hyperglycemic for >1 wk. Cells from test mice were either 5 × 10^6 nylon wool nonadherent T cell-enriched spleen cells, 4 × 10^6 splenic CD4^+ T cells or 1 × 10^6 splenic CD8^+ T cells that had been positively selected by magnetic sorting (MACS MicroBeads; Miltenyi Biotech), or 2.5 × 10^6 splenic CD4^+ T cells depleted of CD4^+ cells by magnetic sorting. The yield of CD4^+ and CD8^+ T cells from magnetic sorting was 70–80% and 12–15%, and their purity by flow cytometry was >95 and ≥85%, respectively. Depletion of CD4^+ cells was >98%. Puriﬁcation and depletion of CD25^+ T cells was performed by magnetic sorting with anti-PE microbeads bound to CD25-PE Ab (PC61; BD Pharmingen) on the autoMACS (Miltenyi Biotech). Briefly, 4 × 10^6 CD25^+ purified cells or 10^6 CD25^+ depleted cells were cotransferred with 10^6 diabetic antigen-sensitized spleen cells. CD25-depleted cells were ≥90% pure and CD25-depleted cells were examined ≥95% CD25^− cells.

To investigate the roles of CD103 and GITR, spleen cells from intranasal proinsulin DNA-treated mice were incubated with 10 μg of anti-GITR mAb (DTA-1, hybridoma provided by Prof. Shimon Sakaguchi, Kyoto University, Kyoto, Japan), anti-CD103 mAb (M290; BD Pharmingen), or isotype control (GL117, prepared in-house) for 5 min on ice, and then treated with anti-asialoGM1 to suppress the adoptive transfer of diabetes. In subsequent experiments, GITR-expressing cells from intranasal proinsulin DNA-treated mice were depleted by magnetic separation before adoptive cotransfer.

**Cyclophosphamide-induced diabetes**

Female NOD mice (8 wk old) were injected with cyclophosphamide (300 mg/kg body weight) to accelerate the onset of diabetes (36). Two days later, they were injected via the tail vein with either 5 × 10^6 nylon wool nonadherent T cell-enriched spleen cells, 4 × 10^6 splenic CD4^+ T cells, or 2.5 × 10^6 CD4^+ depleted spleen T cells.

**Detection of Foxp3 RNA**

CD4^+ CD25^+ and CD4^+ CD25^- T cells from DNA-treated mice were purified by magnetic cell sorting on an autoMACS following further purification on a FACSARIA (BD Biosciences). Total cellular RNA was prepared with the RNeasy mini kit (Qiagen). RNA was boiled for 5 min, reverse transcribed to cDNA, and PCR performed with the following primers (Sigma Genosys): Foxp3, 5'-cagctgcgtcactacgccc-3', 5'-cattctcgagtaggtgagc-3' actin, 5'-gtggcgcctgcacca-3', 5'-cattctgtagacgattgc-3'. Forward and reverse primers were designed so that they bridged intron-exon boundaries to prevent amplification of genomic DNA. The cycling conditions were: 30 s at 94°C, 30 s at 60°C, 30 s at 72°C for 25 cycles (actin) or 35 cycles (Foxp3).

**Cytokine assays**

Lymph node or spleen cells suspended in mouse tonicity-RPMI 1640 medium with 10% FCS at 2 × 10^6 cells/ml were stimulated with 25 ng/ml PMA (Sigma-Aldrich) and 1 μg/ml ionomycin (Sigma-Aldrich) for 3 h at 37°C in 10% CO2. After a 20-min stimulation, 0.6 μl/ml monensin (GolgiStop; BD Pharmingen) was added and included in subsequent incubations until fixation. Cells were stained with FITC-labeled anti-CD4. After fixation in Cytofix/Cytoperm (BD Pharmingen), cells were washed twice and permeabilized in Perm/Wash buffer (BD Pharmingen), stained with PE-labeled anti-CD25 mAb (BD Pharmingen) and immediately analyzed on a FACScan (BD Biosciences) using CellQuest software (BD Biosciences).

**Assessment of pancreatic islet inflammation (“insulitis”)**

Mice (n = 6 from each treatment group) were killed by CO2 asphyxiation at 14 wk old, at the late preclinical stage of disease. The pancreas was fixed in Bouin’s solution overnight, embedded in paraffin, and serial 5-μm sections were stained with H&E. The insulitis score (mean ± SD) was determined by microscopically grading the degree of cellular infiltration in 10–15 islets/mouse (4).

**In vivo cytotoxicity assay**

An assay for detecting Ag-specific CTL in vivo (37) was used in an attempt to identify CTL induced by proinsulin DNA. Following RBC lysis, spleen cells from naïve donors were divided into two populations and labeled with a low (0.5 μM) or a high (5 μM) concentration of CFSE dye. The CFSEhigh population was pulsed with either 10^-4 M proinsulin peptide B25-C34 (FYTPMSREEV) (11) or B15-23 (LYLVCGGERG) (38) known to be K^b- restricted CTL epitopes in the NOD mouse or with 10^-6 M of OVA257-264 (SHINEKLV), a K^b-restricted CTL epitope in the C57BL/6 mouse. In studies of NOD mice treated with intradernal DNA, spleen cells from transgenic mice that express proinsulin on a MHC class II promoter (4) were labeled CFSEhigh and tested as targets. Both the CFSElow and CFSEhigh populations were injected i.v. into naïve NOD mice and into mice treated 2 wk before, at 6 and 8 wk of age, with intradernal proinsulin DNA or OVA DNA. In separate experiments, cells were injected i.v. into naive C57BL/6 mice and into C57BL/6 mice treated 3 wk before, at 6 and 8 wk of age, with intradernal or intranasal OVA DNA. Twenty-four hours after injection of target cells, flow cytometric analysis of the differentially labeled populations in the spleen allowed calculation of the ratio of unlysed cells, i.e., ratio (r) = (percent CFSEhigh/pctent CFSElow), with high r values being indicative of specific lysis.

**Statistics**

In some experiments, Fisher’s exact test (two tailed) was used to compare diabetes incidence between treatment groups. Differences between Kaplan-Meier survival curves were analyzed by the log rank test. The Mann-Whitney U test was used to compare group mean insulitis scores and mean number of IFN-γ-producing cells. Statistical analyses were performed using GraphPad Prism version 3.0c for Macintosh (GraphPad).

**Results**

**Intranasal proinsulin DNA induces anti-diabetogenic CD4^+ Treg**

Spleen cells were harvested by guest on July 27, 2017

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of age. After passage through nylon wool, enriched T cells were either 1) cotransferred i.v. with spleen cells from recently diabetic mice into irradiated, young male mice or 2) transferred i.v. into young female mice that had been treated with cyclophosphamide to accelerate the onset of diabetes. In both experimental models, a significant reduction in diabetes incidence was observed in recipients that received cells from proinsulin DNA-treated compared with OVA DNA-treated donors (Fig. 1; Tables I and II). In three experiments in which splenic T cells from DNA-treated mice were cotransferred with diabeticogenic spleen cells, the combined incidence of diabetes 4 wk after transfer was 14% in recipients of cells from proinsulin DNA-treated donors compared with 64% in recipients of cells from OVA DNA-treated donors (p = 0.003; Table I). In parallel experiments, the peak incidence of diabetes observed 17 days after cyclophosphamide treatment was 12.5% in recipients of cells from proinsulin DNA-treated donors compared with 64% in recipients of cells from OVA DNA-treated donors (p = 0.02; Table II).

To identify the phenotype of T cells that mediate protection, splenic T cell populations were fractionated before transfer. CD4+ or CD8+ cells, positively selected by magnetic sorting, or splenic T cells depleted of CD4+ cells, were cotransferred with 2 × 10^7 diabeticogenic spleen cells in numbers approximately equivalent to those in total splenic T cells. Diabetes incidence 4 wk after transfer was 36% in recipients of CD4+ cells from proinsulin DNA-treated donors compared with 71% in recipients of CD4+ cells from OVA DNA-treated donors (p = 0.02; Table I). Splenic T cells from proinsulin DNA-treated donors had no suppressive effect when depleted of CD4+ cells (diabetes incidence 84%; data not shown). Diabetes incidence in recipients of cotransferred CD8+ cells from proinsulin DNA-treated mice was consistently higher than from OVA DNA-treated mice (e.g., 94 vs 83%, Table I). The findings were similar when CD4+ cells or CD4+-depleted spleen cells were transferred to cyclophosphamide-treated mice. Thus, diabetes incidence in recipients of CD4+ cells from proinsulin DNA-treated donors was 17% compared to 56% in mice that received CD4+ cells from OVA DNA-treated donors (p = 0.04). This suppressive effect was abrogated when CD4+ cells were depleted (Table II).

**CD25 does not distinguish CD4+ Treg induced by intranasal proinsulin DNA**

Prototypic CD4+ Treg can be enriched based on their constitutive expression of CD25 (13, 39). After intranasal proinsulin DNA, spleen cells, either unfractonated or separated into CD25+ and CD25− cells, were cotransferred with diabeticogenic spleen cells into irradiated NOD male recipients (Fig. 2). By 56 days post-transfer, no mouse that received whole spleen cells from proinsulin DNA-treated donors had developed diabetes, compared to 50% of recipients of whole spleen cells from OVA DNA-treated mice (p = 0.01) or 75% of recipients of diabeticogenic spleen cells alone (p = 0.002). Diabetes incidence was suppressed by cotransfer of either CD25+ (14%, p = 0.05) or CD25−-depleted (18%, p = 0.05) cells from proinsulin DNA-treated mice, compared with diabeticogenic spleen cells alone. There was no quantifiable increase in the frequency of CD4+CD25+ Treg.

<table>
<thead>
<tr>
<th>Intranasal Treatment of Donor</th>
<th>Donor Spleen Cells Cotransferred (with 2 × 10^7 diabetic spleen cells)</th>
<th>Diabetes Incidence 4 wk after Cotransfer (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proinsulin DNA</td>
<td>5 × 10^6 T cells</td>
<td>4/28 (14)</td>
</tr>
<tr>
<td>OVA DNA</td>
<td>5 × 10^6 T cells</td>
<td>18/28 (64)</td>
</tr>
<tr>
<td>Proinsulin DNA</td>
<td>4 × 10^6 CD4+ T cells</td>
<td>10/28 (36)</td>
</tr>
<tr>
<td>OVA DNA</td>
<td>4 × 10^6 CD4+ T cells</td>
<td>20/28 (71)</td>
</tr>
<tr>
<td>Proinsulin DNA</td>
<td>1 × 10^6 CD8+ T cells</td>
<td>17/18 (94)</td>
</tr>
<tr>
<td>OVA DNA</td>
<td>1 × 10^6 CD8+ T cells</td>
<td>15/18 (83)</td>
</tr>
</tbody>
</table>

*p = 0.003.

**CD25 does not distinguish CD4+ Treg induced by intranasal proinsulin DNA**

Prototypic CD4+ Treg can be enriched based on their constitutive expression of CD25 (13, 39). After intranasal proinsulin DNA, spleen cells, either unfractonated or separated into CD25+ and CD25− cells, were cotransferred with diabeticogenic spleen cells into irradiated NOD male recipients (Fig. 2). By 56 days post-transfer, no mouse that received whole spleen cells from proinsulin DNA-treated donors had developed diabetes, compared to 50% of recipients of whole spleen cells from OVA DNA-treated mice (p = 0.01) or 75% of recipients of diabeticogenic spleen cells alone (p = 0.002). Diabetes incidence was suppressed by cotransfer of either CD25+ (14%, p = 0.05) or CD25−-depleted (18%, p = 0.05) cells from proinsulin DNA-treated mice, compared with diabeticogenic spleen cells alone. There was no quantifiable increase in the frequency of CD4+CD25+ Treg.

<table>
<thead>
<tr>
<th>Intranasal Treatment of Donor</th>
<th>Donor Spleen Cells Injected (2 days after cyclophosphamide)</th>
<th>Peak Diabetes Incidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proinsulin DNA</td>
<td>5 × 10^6 T cells</td>
<td>2/16 (12.5)</td>
</tr>
<tr>
<td>OVA DNA</td>
<td>5 × 10^6 T cells</td>
<td>9/16 (56)</td>
</tr>
<tr>
<td>Proinsulin DNA</td>
<td>4 × 10^6 CD4+ T cells</td>
<td>3/18 (17)</td>
</tr>
<tr>
<td>OVA DNA</td>
<td>4 × 10^6 CD4+ T cells</td>
<td>10/18 (56)</td>
</tr>
<tr>
<td>Proinsulin DNA</td>
<td>2.5 × 10^6 CD4+ T cells</td>
<td>5/6 (83)</td>
</tr>
<tr>
<td>OVA DNA</td>
<td>2.5 × 10^6 CD4+ T cells</td>
<td>4/6 (67)</td>
</tr>
</tbody>
</table>

*a Seventeen days after cyclophosphamide was administered i.p.

*b p = 0.02.

c p = 0.04 compared to OVA DNA control; Fisher’s exact test.
Nevertheless, as shown, CD25 expression in CD4+ T cells is suppressed by cotransfer of either CD25+ or CD25- spleen cells from intranasal proinsulin DNA-treated mice. Cells were sorted magnetically into CD25+ and CD25- populations and transferred i.v. with 10^7 spleen cells from recently diabetic mice into young, irradiated male NOD mice as detailed in Materials and Methods. Mice received 10^7 diabetogenic spleen cells alone or with 10^7 unfractionated spleen cells from intranasal proinsulin or OVA DNA-treated mice, or with 4 x 10^6 CD25+ or 10^7 CD25- spleen cells from intranasal proinsulin DNA-treated mice.

Treatment of spleen cells from intranasal proinsulin DNA-treated mice with anti-GITR, but not anti-CD103, mAb impairs their ability to inhibit adoptive transfer of diabetes

GITR is constitutively expressed by CD4+CD25+ Treg (16, 17). When spleen cells from intranasal proinsulin DNA-treated mice were incubated with anti-GITR mAb, their ability to inhibit adoptive transfer of diabetes was impaired (p = 0.05, Fig. 3a). However, incubation of cells with mAb to CD103, a candidate Treg marker expressed by both CD4+ and CD4- Treg (20), had no effect on their ability to suppress adoptive transfer of diabetes (Fig. 3a).

Spleen cells from intranasal proinsulin DNA-treated mice, depleted of GITRhigh cells, maintain their ability to inhibit adoptive transfer of diabetes

GITR acts as a costimulatory molecule (18, 19). Therefore, rather than inhibiting Treg anti-GITR mAb could act agonistically on effector cells to enhance their diabetogenicity. When GITRhigh cells were depleted from spleen cells of intranasal proinsulin DNA-treated mice before cotransfer with diabetogenic T cells, the ability of the cells to inhibit adoptive transfer of diabetes was unchanged (Fig. 3b). This indicates that anti-GITR mAb was acting not to inhibit Treg but to promote effector cells.

Foxp3 is expressed in CD25+ but not CD25-CD4+ T cells

One week after the second intranasal treatment with proinsulin or OVA DNA, spleen cells were fractionated into CD25+ and CD25-CD4+ cells, and Foxp3 mRNA was measured semiquantitatively by RT-PCR with normalization to actin expression. Foxp3 transcription was detected in CD25+CD4+ T cells from both intranasal proinsulin and OVA DNA-treated mice but not in CD25-CD4+ T cells from either treatment group (Fig. 4). Nevertheless, as shown, CD25- cells also prevented adoptive transfer of diabetes.

Treatment with intranasal proinsulin DNA is associated with increased expression of IFN-γ by pancreatic lymph node CD4+ T cells

Nasal administration of protein Ag induces CD4+ Treg that express the anti-inflammatory cytokine IL-10 (40). In addition, some CD4+ Treg express not only IL-10 but also the prototypic proinflammatory cytokine IFN-γ (41). Expression of IL-10 and IFN-γ by pancreatic and inguinal lymph node CD4+ T cells following intranasal proinsulin or OVA DNA was assessed by intracellular labeling, after stimulation of lymph node cells by PMA and ionomycin (Fig. 5a). No difference was found between treatment groups for IL-10 expression, but in proinsulin DNA-treated mice IFN-γ expression by CD4+ T cells was significantly higher in the pancreatic than inguinal lymph node (p = 0.03, Fig. 5b).

Intranasal proinsulin DNA treatment is associated with a decrease in islet pathology but not diabetes incidence

The development of clinical diabetes in NOD mice is preceded by mononuclear cell infiltration of pancreatic islets (insulitis), which progresses from a peri-islet accumulation of cells at the vascular pole or boundary of the islet to massive infiltration into the islet associated with β cell destruction. When islets of 14-wk-old NOD mice that had received intranasal DNA at 3 and 5 wk of age were examined, the degree of insulitis (mean ± SD insulitis score) was significantly less in proinsulin DNA- than in OVA DNA-treated mice (0.89 ± 0.08 vs 1.6 ± 0.12, p < 0.01; Mann-Whitney U test). Nevertheless, in five experiments using three different batches of plasmid, the mean incidence of diabetes was identical at 14 wk (10%, 10%), 22 wk (38%, 40%), and 78 wk (58%, 58%) in proinsulin DNA- and OVA DNA-treated mice. Neither more frequent treatment (weekly from 3 to 10 wk old) nor varying plasmid doses (25, 50, and 100 μg) altered diabetes incidence between treatment groups (data not shown).
Intranasal proinsulin DNA prevents diabetes when administered under cover of transient CD40L blockade

The observation that intranasal proinsulin DNA induced antidiabetogenic CD4+ Treg and decreased islet infiltration, yet did not reduce the incidence of diabetes, suggested that proinsulin DNA also had a counter effect to promote diabetes. Previously, we found that nasal, aerosol, or oral OVA induced pathogenic CTL as well as tolerance (31), but that the induction of CTL was prevented by blockade of CD40-CD40L costimulation (32). Therefore, we administered a single 300-μg i.p. dose of anti-CD40L mAb (clone MR-1) just before intranasal DNA at 4, 6, and 8 wk old. The combination of intranasal proinsulin DNA and anti-CD40L Ab prevented diabetes (p = 0.007, compared with intranasal proinsulin DNA or OVA DNA and control Ab) (Fig. 6). Anti-CD40L Ab (with OVA DNA) had a partial but nonsignificant effect to reduce diabetes incidence. In a replicate experiment, the same effect of intranasal proinsulin DNA in combination with anti-CD40L Ab was seen.

Proinsulin epitope-specific CTL are undetectable after proinsulin DNA

We attempted to identify proinsulin-specific CTL induced by proinsulin DNA by an in vivo cytotoxicity assay. The assay was validated by demonstrating CTL that could lyse targets pulsed with the Kd-restricted OVA257–264 epitope after intradermal immunization of C57BL/6 mice with OVA DNA. However, specific lysis of target cells was not detected after intranasal OVA DNA treatment (Fig. 7). In view of this, NOD females were immunized intradermally with proinsulin DNA to maximize the possibility of detecting CTL. Specific lysis of target cells pulsed with the Kd-restricted proinsulin CTL epitopes B15–23 or B25–34 was not detected after intradermal immunization with proinsulin DNA. Specific lysis was also not detected after intradermal proinsulin DNA when spleen cells from transgenic mice expressing proinsulin on a MHC class II promoter (4) were used as targets.

Discussion

This is the first report of Treg induction in response to mucosal administration of a DNA-encoded Ag. Intranasal proinsulin DNA induced CD4+ T cells that blocked diabetogenic T cells in two experimental models: adoptive transfer of diabetes into irradiated recipients and acceleration of spontaneous diabetes by cyclophosphamide. Inhibition of adoptive transfer of diabetes was used previously to identify Treg induced by oral (9) or nasorespiratory (10–12) delivery of (pro)insulin peptides or protein. The effect of intranasal proinsulin DNA-induced T cells on cyclophosphamide-accelerated diabetes was examined because this is a model of accelerated spontaneous diabetes that may be more stringent than the adoptive transfer model. The mechanism by which cyclophosphamide accelerates diabetes onset in NOD mice is not clear, but may involve ablation of endogenous regulatory cells (42). Inhibition of cyclophosphamide-accelerated diabetes by splenic CD4+ T cells specifically from mice given intranasal proinsulin DNA confirms the regulatory function of these cells. In studies of EAE, CpG motifs in the plasmid construct were reported to be necessary for protection by i.m. DNA encoding myelin oligodendrocyte glycoprotein peptide (29, 30). CpG DNA binds TLR9 and the optimal CpG ligand for murine TLR9 is GACGTT (34). However, because the proinsulin and OVA plasmid DNA constructs each contain one GACGTT CpG motif, the differential effect of intranasal proinsulin DNA on experimental diabetes is unlikely to be accounted for by CpG motifs. Furthermore, results with OVA DNA were no different than those with the intranasal PBS carrier solution used for intranasal delivery.

Recently, Treg defined as CD4+CD25+ have been the subject of intense interest (39). Although originally identified as a subset of
thymocytes (13), CD4⁺CD25⁺T_{reg} have also been found in the periphery (43). The CD4⁺T_{reg} induced by intranasal proinsulin DNA were not confined to either the CD25⁺ or the CD25⁻ subset; both populations suppressed the adoptive transfer of diabetes. Others have reported that mucosal OVA induces CD4⁺CD25⁺T_{reg} (14, 15), but our findings show that CD4⁺T_{reg} induced by intranasal proinsulin DNA are not uniquely defined by CD25 expression. Similarly, Unger et al. (44) demonstrated that tolerance to intranasal OVA protein was transferable by CD25⁻ cells. Moreover, homeostatic expansion of CD4⁺CD25⁺T_{reg} in lymphopenic hosts resulted in decreased CD25 expression but correlated with enhanced suppressor function (45). This is not surprising because CD25 is also a marker of activated T cells (46) and its use to enhanced suppressor function (45). This is not surprising because CD25 is also a marker of activated T cells (46) and its use to denote T_{reg} in the periphery of adult mice is problematic (47).

GITR, a member of the TNFR superfamily constitutively expressed on natural CD4⁺CD25⁺T_{reg} (16, 17), was an alternative marker to CD25 to distinguish T_{reg} induced by intranasal proinsulin DNA. Ligation of GITR with GITR ligand (19, 48) or signaling through GITR by anti-GITR Ab (16, 17) was reported to abrogate suppression mediated by CD4⁺CD25⁺T_{reg}. GITR⁺CD4⁺T cells were shown to prevent inflammatory bowel disease in scid mice regardless of CD25 expression (49). Thus, we investigated whether CD4⁺T_{reg} induced by intranasal proinsulin DNA could be distinguished by GITR expression. Spleen cells from intranasal proinsulin DNA-treated mice mixed with anti-GITR mAb lost their protective effect in adoptive cotransfer. However, it was unclear whether the agonistic anti-GITR mAb was on T_{reg} or pathogenic effector T cells. Kohm et al. (50) reported that anti-GITR mAb directly enhanced CD4⁺T cell effector function through a costimulatory-type action that exacerbated EAE. Depletion of GITR⁺spleen cells failed to abolish the protective effect of intranasal proinsulin DNA. Thus, CD4⁺T_{reg} induced by intranasal proinsulin DNA were not distinguished by GITR expression, and the effect of GITR mAb was therefore most likely on effector T cells to enhance their diabetogenicity. The forkhead/winged helix transcription factor gene, Foxp3 is expressed by natural CD25⁺CD4⁺T_{reg} but has also been shown to be expressed by peripheral CD4⁺CD25⁻T_{reg} treated with TGF-β (24, 25). Foxp3 expression was confined to the CD25⁺ subset of splenic T cells in both intranasal proinsulin DNA- and OVA DNA-treated mice, yet CD25⁻ cells were also regulatory. Thus, Foxp3 was also not a marker that distinguished T_{reg} induced by intranasal proinsulin DNA.

Further studies are required to illuminate the mode of action of CD4 T_{reg} induced by intranasal proinsulin DNA. The increase in IFN-γ-expressing CD4⁺T cells in the pancreatic compared with the inguinal lymph node of proinsulin DNA-treated mice is intriguing but of uncertain significance. Nasal Ag has been reported to induce CD4⁺T_{reg} that mediate protection from autoimmune disease via the anti-inflammatory cytokine IL-10 (40), and these T_{reg} may also express IFN-γ (41). Furthermore, CD4⁺T_{reg} that cosecrete IL-10 and IFN-γ have been detected in NOD mice after i.p. administration of peptides from glutamic acid decarboxylase, another autoantigen in type 1 diabetes (51). However, we did not detect an increase in IL-10-expressing CD4⁺T cells following intranasal proinsulin DNA. Ideally, proinsulin-specific T cells induced by intranasal proinsulin DNA should be analyzed, but the
sensitive and reproducible detection of these cells in NOD mice has been problematic (52).

At 14 wk of age, when the islet lesion is normally well advanced but most mice are not yet diabetic, the degree of insulitis was modestly but significantly reduced in mice that had received proinsulin DNA. This suggests that treatment with proinsulin DNA, even though 9 wk earlier, retarded islet pathology. Intriguingly, however, the incidence of spontaneous diabetes was similar in proinsulin DNA- and OVA DNA-treated mice. Increasing the frequency of treatments or the dose did not alter diabetes incidence between treatment groups. We considered that the lack of a clinical effect of Treg might be due to coinduction of diabetogenic T cells by proinsulin DNA. Previously, in C57BL/6 mice bearing transgenic T cell receptors specific for OVA and expressing OVA as a transgene in β cells, we found that nasal, aerosol, or oral OVA not only induced tolerance but also induced CD8⁺ T cells that destroyed β cells and caused diabetes (31). A clue to this possibility in the present studies is the observation that disease incidence was consistently higher after transfer of CD8⁺ T cells or CD4⁻ depleted T cells from proinsulin DNA-treated mice. In the transgenic OVA mice, blockade of costimulation through CD40L prevented induction of CTL and diabetes by oral OVA (32). In the present study, CD40L blockade at the time of intranasal proinsulin DNA treatment prevented diabetes, consistent with the hypothesis that proinsulin DNA induces CTL. CD40L blockade alone was reported to prevent diabetes in NOD mice if administered before 9 wk of age (53). We observed that CD40L blockade (with intranasal OVA DNA) had a partial but not significant effect on reducing diabetes incidence. The magnitude of this effect was similar to that in separate experiments in which anti-CD40L Ab was given alone (in PBS) (N. R. Martinez and L. C. Harrison, unpublished data). Although CD40L blockade was permissive for a protective effect of intranasal proinsulin DNA, we were unable to demonstrate that proinsulin DNA induced CTL. There may be several reasons for this. First, we would expect the frequency of autoantigen-specific CTL to be very low. Second, if priming of CTL to islet Ags occurs early in the NOD mouse, because expression of MHC class I on β cells is required for initiation of insulitis (2), a background of proinsulin-specific CTL could make it difficult to detect an increase in CTL activity measured by comparing ratios of specific lysis in primed and unprimed mice. Third, it is conceivable that CTL induced by intranasal proinsulin DNA could be located in sites (e.g., mucosa or pancreatic lymph nodes) other than the spleen. Finally, detection of cytotoxicity requires that target cells express appropriate MHC class I-restricted peptide epitopes. Although evidence suggests that proinsulin contains CTL epitopes in the insulin B chain (aa 15–23) (38) and across the B-connection (C chain junction (aa 25–34)) (11), these may not be the epitopes presented after intranasal or intradermal proinsulin DNA. However, with no assumptions about the CTL epitope and on the premise that transgenic proinsulin is processed in the MHC class I pathway, we could not detect lysis of target spleen cells from mice expressing proinsulin on a MHC class II promoter.

The finding that intranasal proinsulin DNA induces CD4⁺ Treg that prevent experimental diabetes provides a rationale for vaccination strategies to prevent autoimmune disease based on the advantages of DNA-encoded autoantigen. The evidence presented here, although indirect, suggests that the therapeutic potential of this vaccination strategy may be compromised by coinduction of pathogenic immunity. Thus, as with protein, the efficacy of mucosal vaccination with DNA may require the careful selection of tolerogenic epitopes as demonstrated for the proinsulin B-C peptide (11) or simultaneous blockade of costimulation (32).
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