Induction of Autoimmune Diabetes Through Insulin (but Not GAD65) DNA Vaccination in Nonobese Diabetic and in RIP-B7.1 Mice

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Insulin has been used to modify T-cell autoimmunity in experimental models of type 1 diabetes. In a large clinical trial, the effect of insulin to prevent type 1 diabetes is currently investigated. We here show that insulin can adversely trigger autoimmune diabetes in two mouse models of type 1 diabetes, using intramuscular DNA vaccination for antigen administration. In female nonobese diabetic (NOD) mice, diabetes development was enhanced after preproinsulin (ppIns) DNA treatment, and natural diabetes resistance in male NOD mice was diminished by ppIns DNA vaccination. In contrast, GAD65 DNA conferred partial diabetes protection, and empty DNA plasmid was without effect. In RIP-B7.1 C57BL/6 mice (expressing the T-cell costimulatory molecule B7.1 in pancreatic β-cells), autoimmune diabetes occurred in 70% of animals after ppIns vaccination, whereas diabetes did not develop spontaneously in RIP-B7.1 mice or after GAD65 or control DNA treatment. Diabetes was characterized by diffuse $CD4^+CD8^+$ T-cell infiltration of pancreatic islets and severe insulin deficiency, and ppIns, proinsulin, and insulin DNA were equally effective for disease induction. Our work provides a new model of experimental autoimmune diabetes suitable to study mechanisms and outcomes of insulin-specific T-cell reactivity. In antigen-based prevention of type 1 diabetes, diabetes acceleration should be considered as a potential adverse result. *Diabetes* 51:3237–3244, 2002

In experimental models of type 1 diabetes, prophylactic treatment with β-cell antigens has been used specifically to modify T-cell autoimmunity and diabetes development. For example, insulin (1–4), insulin β chain (5), GAD67 (6), GAD65 (7,8), heath shock protein 65 (9), and ICA69 (10) have been shown to reduce the development of diabetes in nonobese diabetic (NOD) mice, involving different routes of antigen administration. A variety of immunological mechanisms, including T-cell tolerance and immune deviation, have been suggested to explain disease protection observed in these studies. On the basis of these observations and after pilot studies in prediabetic patients, a large clinical trial has been designed to evaluate the efficacy of prophylactic insulin treatment to prevent overt type 1 diabetes in islet cell antibody-positive high-risk individuals (11).

Evidence from diabetes-prone BB rats (12) and rat insulin promoter (RIP) ovalbumin (Ova)-transgenic (13) mice has shown that treatment with β-cell antigens may adversely promote diabetes development. Similarly, T-cell–mediated experimental autoimmune encephalitis (14) or autoimmune ovarian disease (15) can be induced by injection of myelin basic protein and ZP3 glycoprotein, respectively, both autoantigens expressed in affected target tissues.

For antigen administration, recombinant proteins or synthetic peptides have been used in most experimental studies. DNA vaccination is a more recent and simple strategy of antigen administration to induce T-cell immunity (16). In DNA vaccination, transient in vivo protein expression is induced in the host through injection of a DNA plasmid vector containing cDNA of a desired gene, thus circumventing difficulties with recombinant protein production and purification. We have recently shown that genetic vaccination using GAD65 cDNA is suitable to induce diabetes protection in young NOD mice (17).

To assess further the therapeutic potential of antigen-specific intervention in type 1 diabetes, we extended our DNA vaccination studies to other β-cell proteins. Instead of disease protection, we observed diabetes induction in both female and male NOD mice after insulin DNA vaccination. Similarly, insulin (but not GAD65) DNA vaccination specifically induced $CD4^+CD8^+$ insulitis, β-cell destruction, and autoimmune diabetes in RIP-B7.1 transgenic mice, even in the absence of diabetes-risk major histocompatibility cell (MHC) alleles. Disease acceleration must therefore be considered as a potential outcome in antigen-specific diabetes prevention. In this process, β-cell expression of T-cell costimulatory molecules such as B7.1 may be critical. Our novel experimental autoimmune diabetes (EAD) model should allow to address this and other aspects of insulin-induced diabetic autoimmunity.

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aa, amino acids; APC, antigen-presenting cell; CMV, cytomegalovirus; CTL, cytotoxic T-cell; EAD, experimental autoimmune diabetes; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IFN-γ, interferon-γ; LCMV, lymphocytic choriomeningitis virus; MHC, major histocompatibility cell; ppIns, preproinsulin; Ova, ovalbumin; RIP, rat insulin promoter.

RESEARCH DESIGN AND METHODS

Animal studies. Breeding stocks of nonobese diabetic mice (NOD/Bom) were purchased from M&B Bomholtgard (Ry, Denmark). Diabetes incidences at 32 weeks of age were stable during the entire study period of 2 years, with average incidences of 65–75% in female and 20–30% in male mice at 32 weeks of age. The generation of RIP-B7.1 mice expressing B7.1 (CD80) in pancreatic β-cells under control of the RIP backcrossed (>15 generations) to the C57BL/6 (H-2b) background has been described (18,19). C57BL/6 wild-type mice (H-2b) were obtained from the Animal Research Center, University of Ulm. Mice were fed a standard rodent diet (Altromin 1314; Altromin, Lage, Germany) and water ad libitum.

For vaccination studies, 4- to 6-week-old mice were given an intramuscular injection of 50 μg of DNA plasmid (dissolved in 50 μl of PBS) into each anterior tibialis muscle after shaving and skin desinfection. Injections were repeated once after 1 week (boost), and no adjuvants, other additives, or pretreatment were used. Animals were screened for diabetes development by weekly measurement of glucosuria (Tes-Tape; Lilly, Indianapolis, IN), and diabetes was confirmed (Glucometer Elite; Bayer, Pittsburgh, PA) by blood glucose levels ≥13.8 mmol/l (≥250 mg/dl). Blood glucose was in addition measured weekly in RIP-B7.1 mice for 8 weeks after vaccination. Animal studies were conducted after institutional board approval according to the German Federal Animal Protection Law.

Construction of DNA vaccination vectors. Complementary DNA of β-cell antigens was inserted into plasmid vectors containing the cytomegalovirus (CMV) promoter to achieve eukaryotic gene expression. Mouse preproinsulin (ppIns) II cDNA (Genbank X04727, 110 amino acids [aa]) was generated by RT-PCR from murine islet cell RNA (20), followed by ligation into the *Eco*RI and *Xba*I sites of pCI (Promega). Similarly, murine proinsulin II cDNA (86 aa, lacking signal peptide) was generated by RT-PCR, with start codon and Kozak consensus incorporated in the sense primer. For the construction of murine insulin II (51 aa, lacking signal peptide and C-peptide), cDNAs encoding B chain (aa position B1-B30) and A chain (aa position A1-A21) were independently RT-PCR amplified, ligated between position B30 and A1, and inserted into the pCI vector as above. Islet cell antigen 69 cDNA (clone is10, 483 aa) (21) was subcloned into *Xba*I and *Sma*I sites of pCI (Promega). The construction of the GAD65 cDNA vector (rat GAD65, Genbank M72422, 586 aa) has been described (17). Except for murine proinsulin and insulin cDNA, complete coding sequences and natural translation initiation and termination sites were used in all constructs, and plasmids contained the Amp^r gene characterized by CpG-rich immunostimulatory sequences. All vector sequences were confirmed by bidirectional DNA sequencing.

Plasmids were transformed into supercompetent XL1-blue MRF' *Escherichia coli* (Stratagene, La Jolla, CA), grown in selection medium, and isolated using the Plasmid Mega Prep Kit (Qiagen, Hilden, Germany). Endotoxin levels in plasmid preparations (final DNA concentration, 1 μg/μl) were determined by Dr. J. Endl (Roche Diagnostics, Penzberg, Germany), using the Limulus lysate assay (ppIns 0.06 EU/μl, pGAD65 0.12 EU/μl, pCI control vector 0.06 EU/μl, medium 0.003 EU/μl; BioWhittaker, Walkersville, SC).

Vector-induced gene expression. Protein synthesis of target autoantigens was studied after transient transfection in HEK 293 cells in vitro. Semiconfluent cells were transfected with 0.5–10 μg of plasmid vector DNA by standard $CaCl_2$ precipitation, grown for 48 h in Dulbecco's modified Eagle's medium/10% FCS medium (Gibco, Karlsruhe, Germany) and harvested. Culture supernatants and cell lysates were analyzed as indicated. GAD65 and ICA69 expression was detected by Western blotting using GAD6 (gift of Dr. W. Richter, Heidelberg, Germany) and polyclonal anti-ICA69 (20) as primary antibodies after 10% SDS-PAGE and semi-dry blotting onto Hybond C membranes (Amersham, Freiburg, Germany). Expression of murine ppIns, proinsulin, and insulin was studied using a radioimmunoassay as described below, with an average intra-assay coefficient of variation of 4.5%.

PpIns mRNA in mouse muscle tissue was analyzed by quantitative real-time RT-PCR (TaqMan, Perkin Elmer), with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as endogenous reference gene. Ratios of ppIns/GAPDH mRNA levels were calculated after triplicate quantifications, and values are expressed as means ± SD. RNA was DNase-treated (Qiagen) before reverse transcription. Mice were DNA-vaccinated with 100 μg of plasmid as a single injection, and groups of two to three mice were subsequently studied at a given time as indicated.

Histology. Pancreatic cryosections (5 μm thick) from B7.1 mice were treated with the Avidin/Biotin Blocking Kit (Vector Labs) and incubated with primary antibodies to insulin (Biotrend/Linco Research, St. Charles, MO), CD4 (Phar Mingen, San Diego, CA), or CD8 (PharMingen) at 4°C overnight. Slides for CD4/CD8 staining were incubated with biotin-conjugated goat anti-rat IgG secondary antibody (Jackson Immuno Research, Westgrove, PA), followed by incubation with horseradish peroxidase–conjugated avidin (Vectastain Elite ABC; Vector Labs). Sections for insulin staining were incubated with a peroxidase-conjugated rabbit anti-guinea pig IgG secondary antibody (A5545; Sigma, St. Louis, MO). Finally, slides were developed with diaminobenzidine (DAB Substrate Kit; Vector Labs) and counterstained with hematoxylin. Specificity of immunostaining was confirmed by using irrelevant primary antibody (goat anti-human IgA) as control. Insulitis scores in NOD mice were determined in pancreatic sections as described (22).

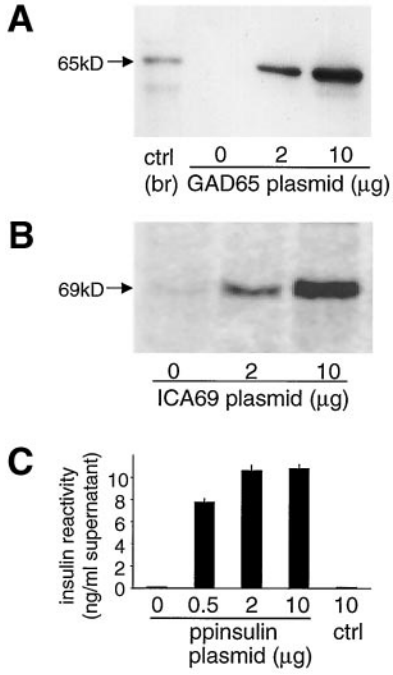

FIG. 1. DNA vector-induced gene expression of β-cell antigens. Dose-dependent protein expression of GAD65 (*A*), ICA69 (*B*), or ppIns (*C*) after transient transfection with DNA plasmids in HEK293 cells, as detected by Western blot (*A* and *B*) or radioimmunoassay (*C*). Ctrl, control. Plasmid encoding murine interleukin-4 cDNA was used as control in *C*. See RESULTS for details.

Pancreatic insulin contents. Insulin was isolated from snap-frozen and cryoconserved (−80°C) pancreatic tissue by standard 80% ethanol/0.1 mol/l hydrochloric acid extraction. For measuring extractable insulin, a radioimmunoassay for rat insulin (with 100, 69, and 100% cross-reactivity to murine insulin, human proinsulin, and human insulin, respectively) was used (Linco Research). Insulin contents are expressed as nanograms per milligram of wet-weight tissue.

Statistical analysis. Statistical analyses were made with SAS software, version 8.2 (SAS Inc., Cary, NC). For comparison of diabetes incidences, Fisher's exact test was used. Means were compared using the Wilcoxon or Kruskal-Wallis test, assuming nonparametric statistics. Statistical significance was defined as $P < 0.05$.

RESULTS

Vector-induced gene expression of β-cell autoantigens. To confirm vector-mediated gene expression of β-cell proteins under control of the eucaryotic CMV promoter, we performed in vitro transfection studies in HEK293 cells. Cell lysates of cells transfected with vaccination plasmid (2–10 μg) coding for GAD65 (Fig. 1*A*) or ICA69 (Fig. 1*B*) were studied by immunoblotting, showing a dose-dependent protein expression. In addition, after transfection with plasmids (0.5–10 μg) containing cDNA for ppIns (Fig. 1*C*), proinsulin, or insulin (data not shown), dose-dependent protein expression of these antigens was observed by radioimmunoassay. In addition to DNA sequence verification, these data confirmed the structural and functional integrity of DNA vector systems used for in vivo antigen expression in subsequent vaccination studies.

In addition, in vivo gene expression was studied in mice

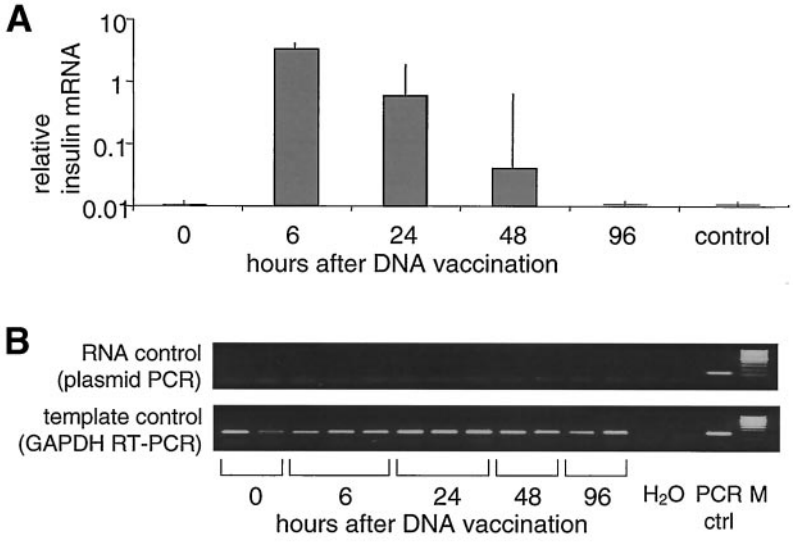

FIG. 2. In vivo insulin gene expression is detectable up to 48 h after intramuscular DNA vaccination (*A*) as shown by real-time RT-PCR. Muscle cDNA used as template was intact in all individual mice (*B*, bottom) and was not contaminated by plasmid DNA (*B*, top). See RESULTS for details. H_2O, negative water control; PCR ctrl, positive PCR amplification control; M, DNA size marker.

(n = 12) after DNA vaccination. At the site of DNA injection (muscle), local ppIns gene transcription was detectable up to 48 h after vaccination by specific quantitative real-time RT-PCR (Fig. 2), reaching its maximum 6 h after injection.

No diabetes protection after insulin DNA vaccination in female NOD mice. It was shown previously that DNA vaccination with GAD65 can prevent diabetes development in female NOD mice (17). As GAD is not an exclusive autoantigen in type 1 diabetes, we now extended these studies to other target β-cell antigens. DNA vaccination was performed in 4- to 6-week-old female NOD mice, and animals were monitored for diabetes onset until 32 weeks of age.

Diabetes development in mice that were vaccinated with murine ppIns (containing insulin B chain, A chain, signal, and connecting peptide) was 88.0% (Fig. 3), i.e., disease development was not reduced in comparison with control DNA-treated or untreated NOD mice. Instead, there was a trend toward higher cumulative diabetes incidences in ppIns-vaccinated mice compared with control groups (P = 0.09). In female NOD mice that were DNA-vaccinated with GAD65 (Fig. 3), however, diabetes development was significantly reduced (P = 0.0013), confirming previous observations (17).

For studying the effects of multiple-antigen treatment, a group of female animals were vaccinated with a mixture of ppIns, islet cell antigen ICA69, and GAD65 DNA (Fig. 3). The cumulative diabetes incidence in these mice at 32 weeks was similar (P = 1.0) to untreated or control DNA-vaccinated animals and lower than in mice that were treated with ppIns alone (P = 0.09). Thus, the protective effects of GAD65 vaccination is abolished by the coadministration of ppIns and ICA69 DNA vaccine.

The degree of pancreatic islet cell inflammation, expressed as the mean insulitis score at 32 weeks of age (22), was similar (P = 0.41, Kruskal-Wallis) in nondiabetic NOD mice after GAD treatment (score 2.39 ± 0.41), ppIns treatment (score 1.92 ± 0.57), and control DNA treatment (score 2.08 ± 0.38).

Notably, diabetes incidences were not different in mice that were treated with control DNA plasmid compared with unvaccinated NOD mice (Fig. 3). Thus, major antigen-independent effects of the DNA plasmid vector backbone carrying the immunostimulatory CpG motif or of contaminating agents in the plasmid DNA preparation, which may affect diabetes development (23), can be ruled out in our studies.

Loss of natural diabetes resistance in male NOD mice after insulin DNA vaccination. In contrast to female NOD mice, male NOD mice are characterized by a considerable natural (spontaneous) diabetes resistance, despite the presence of autoimmune islet cell infiltration (insulitis) (24). We therefore chose male NOD mice as model system with low spontaneous diabetes probability to test for disease acceleration after insulin DNA vaccination, as hypothesized from our observations in female animals.

Male NOD mice were DNA-vaccinated at 5–6 weeks of

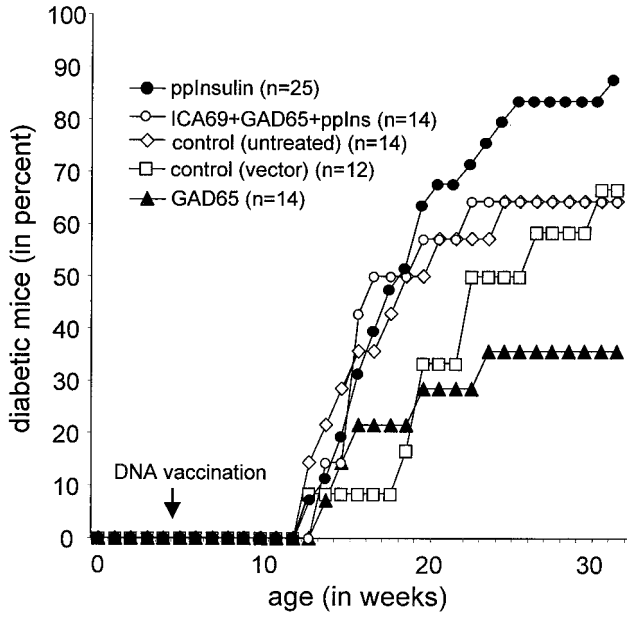

FIG. 3. Diabetes development in female NOD mice after DNA vaccination. Cumulative diabetes incidences (in percentage) after treatment with different DNA plasmids (insert) in 4- to 6-week-old animals are shown. The umber of treated animals is in parentheses.

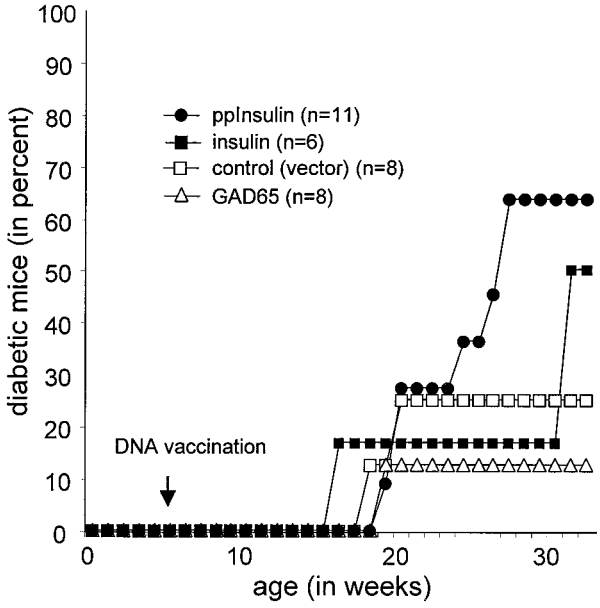

FIG. 4. Loss of natural diabetes resistance in male NOD mice after (prepro)insulin DNA vaccination. Cumulative diabetes incidences (in percentage) after treatment with different DNA plasmids (insert) in 4- to 6-week-old animals are shown. The number of treated animals is in parentheses.

age, using the same protocol as in female mice (Fig. 4). Diabetes incidences after DNA vaccination with ppIns were increased to 63.6% at 32 weeks of age, compared with 12.5% in GAD65-vaccinated mice ($P = 0.03$). Similarly, in mice that were vaccinated with insulin DNA, a trend toward higher diabetes incidences was observed compared with control or GAD65-treated mice ($P = 0.17$). Taken together, despite different natural diabetes rates in male and female animals, (prepro)insulin DNA vaccination does not protect but rather promotes diabetes development in both male and female NOD mice.

Diabetes induction by insulin DNA vaccination in RIP-B7.1 transgenic mice. Costimulatory signals by antigen-presenting cells (APCs) through B7.1 (CD80) enhance T-cell activation and effector function after cognate antigen–T-cell receptor engagement. With the use of lymphocytic choriomeningitis virus (LCMV) as a neo-autoantigen expressed in β-cells, it has been shown that transgenic β-cell expression of B7.1 increases diabetes susceptibility in this classic autoimmunity model (25).

We translated these findings from LCMV to insulin as a "natural" diabetes autoantigen. RIP-B7.1 transgenic mice expressing B7.1 in pancreatic β-cells under control of the RIP were used for DNA vaccination studies (Table 1). Importantly, these mice have a C57BL/6 (H-2b) MHC background, thus lacking genetic diabetes risk alleles required for diabetes development in NOD mice. RIP-B7.1 mice ($n = 8$) did not develop diabetes spontaneously, even after an extended follow-up of 48 weeks. Similarly, RIP-B7.1 mice that were vaccinated with DNA control plasmid ($n = 16$) did not develop hyperglycemia or other signs of disease (Table 1). However, DNA vaccination with ppIns was followed by the development of diabetes (Table 1) in 14 of 20 RIP-B7.1 mice (incidence 70%, $P < 0.001$). Diabetes was diagnosed in these animals after a median of 39 days (range 22–134 days). Hyperglycemia was characterized by severe insulin deficiency and heavy lymphocytic infiltration of pancreatic islets by $CD4^+$ and $CD8^+$ T-cells, as described below. In contrast, diabetes did not occur in any of 15 mice that were DNA-vaccinated with GAD65 (incidence 0%; Table 1), despite effective vector-induced gene expression. Similarly, nontransgenic (wt/wt) C57BL/6 mice that were vaccinated with ppIns did not develop diabetes (Table 1). When ppIns DNA vaccination doses for were reduced from 100 to 10 μg, diabetes development was abolished (7 of 13 vs. 0 of 14; $P < 0.002$). Taken together, diabetes induction in C57BL/6 mice is linked to the β-cell expression of the B7.1 costimulatory molecule. In addition, the disposition to develop diabetes after DNA vaccination in RIP-B7.1 mice seems limited to particular β-cell autoantigens.

To map diabetes induction to functional domains of the insulin gene product, we constructed deletion-mutant vaccination vectors lacking insulin signal peptide (proinsulin) or signal and connecting peptide sequences (insulin). After vaccination with proinsulin and insulin (Table 1), diabetes occurred in 4 of 6 and 4 of 10 treated RIP-B7.1 mice, respectively, with no significant difference compared with ppIns-treated animals ($P = 0.27$). Thus, neither insulin signal peptide nor connecting peptide sequence seems to be essential for diabetes induction in RIP-B7.1 mice. This finding confirms previous findings in male NOD mice (Fig. 4), in which increased diabetes incidences were observed after both insulin and ppIns treatment.

Diabetes after DNA vaccination occurred equally in male and female RIP-B7.1 mice. A total of 15 of 23 (65.2%) female mice and 7 of 13 (53.8%) male mice developed diabetes after ppIns, proinsulin, or insulin DNA vaccination ($P = 0.72$), with identical disease onset after vaccination in both sexes (median 50 days vs. 39 days). Similarly,

TABLE 1
Diabetes development in RIP-B7.1 transgenic mice after insulin DNA vaccination

Mouse strain	DNA vaccination	Diabetes development	Diabetes incidence*	Diabetes onset (median)
C57BL/6	ppIns	No	0/6	—
RIP-B7.1	None	No	0/8†	—
RIP-B7.1	Vector (control)	No	0/16	—
RIP-B7.1	ppIns	Yes	14/20 (70%)	39 days
RIP-B7.1	Proinsulin	Yes	4/6 (66%)	57.5 days
RIP-B7.1	Insulin	Yes	4/10 (40%)	42 days
RIP-B7.1	GAD65	No	0/15	—

*Diabetes incidences were not different in ppIns-, proinsulin-, and insulin-treated animals ($P = 0.27$, Fisher's exact). †Extended follow-up of 48 weeks.

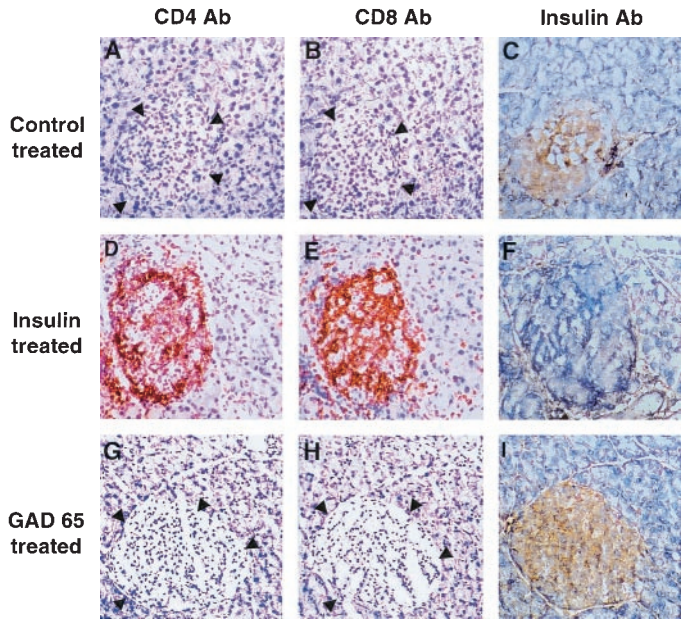

FIG. 5. $CD4^+CD8^+$ islet infiltration and β-cell destruction after insulin DNA vaccination. Serial pancreatic sections from RIP-B7.1 mice after DNA vaccination with insulin (*D–F*), GAD65 (*G–I*), or control DNA (*A–C*), immunostained for CD4 (left), CD8 (middle), or insulin (right). Localization of islets in *A*, *B*, *G*, and *H* indicated by arrowheads. Magnification ×200, counterstaining with hematoxylin.

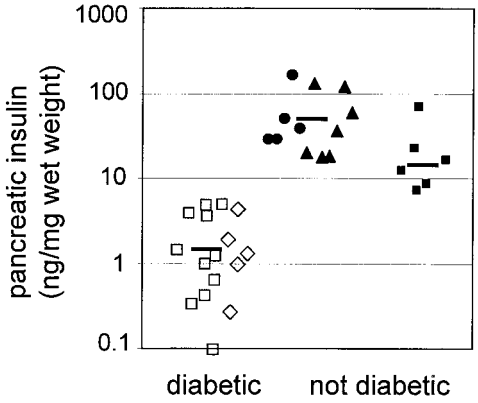

FIG. 6. Severe pancreatic insulin deficiency in RIP-B7.1 mice DNA-vaccinated with insulin (□) or ppIns (◇). In contrast, normal pancreatic insulin after treatment with GAD65 (●) or empty control plasmid (▲) is shown. For comparison, untreated C57BL/6 mice (■) are shown. Data from individual mice are shown, with means indicated by horizontal bars. Note the logarithmic scale.

diabetes rates in mice that were vaccinated at 2–3 weeks of age were not different from those of animals treated at weeks 4–6 ($P > 0.05$). In contrast to NOD mice, EAD after insulin DNA vaccination in RIP-B7.1 mice is characterized by an equal disease susceptibility in male and female individuals without sex bias, thus resembling human type 1 diabetes.

Characteristics of EAD in RIP- B7.1 transgenic mice. To analyze mechanisms of diabetes development in RIP-B7.1 mice after DNA vaccination, we first studied islet morphology in serial pancreatic sections after hematoxylin and eosin staining. Pancreatic islets in mice that were vaccinated with insulin or ppIns DNA showed a dense, homogeneously distributed mononuclear cell infiltration in the islets of Langerhans, affecting all islets at similar cell density. In contrast, sections from mice after GAD65 or control DNA vaccination did not exhibit cellular infiltration in the islets (data not shown). For further characterizing islet infiltration in these animals, immunostaining for CD4 and CD8 was used. In mice that were treated with insulin DNA, a dense accumulation of $CD4^+$ (Fig. 5*D*) and $CD8^+$ cells (Fig. 5*E*) was observed, whereas such cells were absent in the islets of GAD65-treated (Fig. 4*G* and *H*) or control DNA-treated (Fig. 4*A* and *B*) mice. Some $CD8^+$ cells were detectable in surrounding exocrine pancreatic tissue, although at low density (Fig. 4*E*). In mice that were vaccinated with ppIns (data not shown), identical immunostaining was observed as in insulin-treated mice. Taken together, insulin and ppIns DNA-vaccinated animals are characterized by a prominent $CD4^+CD8^+$ insulitis.

Immunohistology further displayed a marked reduction of insulin-positive islet cells in insulin DNA-vaccinated animals (Fig. 5*F*), whereas the majority of islet cells in sections from control (Fig. 5*C*) or GAD65-treated (Fig. 5*I*) mice showed insulin immune reactivity. To quantify further the degree of insulin deficiency, we analyzed pancreatic insulin contents after extraction from freshly collected tissue in these animals. Extractable insulin as measured by radioimmunoassay (Fig. 6) was strongly decreased ($P < 0.001$) in insulin or ppIns DNA-vaccinated diabetic mice (mean 2.0 ± 1.8 ng/mg) compared with GAD65 DNA- and control DNA-treated mice (mean 61.4 ± 54.0 ng/mg). Pancreatic insulin levels were similar (Fig. 6) in healthy RIP-B7.1 mice and nontransgenic C57BL/6 mice (mean 23.3 ± 24.0 ng/mg). In summary, diabetes after insulin DNA vaccination results from severe insulin deficiency.

Insulin DNA vaccination does not cause metabolic effects. To address potential hypoglycemic effects of vector-induced in vivo insulin gene expression, venous glucose levels were followed up in healthy mice after DNA vaccination for 120 h (Table 2). Animals were studied under prolonged fasting conditions to unmask subtle hypoglycemic changes potentially compensated by oral carbohydrate intake. Glucose values after ppIns or insulin DNA vaccination were not different from those of control DNA-vaccinated male NOD mice ($P > 0.15$, Wilcoxon), with glucose levels equally declining in all groups during fasting as expected (Table 2). All mice were asymptomatic and showed normal behavior. These findings indicate that insulin and ppIns DNA at concentrations used in this study do not decrease blood glucose levels in vaccinated mice. PpIns and proinsulin have been shown to be metabolically inactive, and conversion to insulin by PC2/PC3 endopeptidase-mediated cleavage is restricted to pancreatic β-cells. Similarly, "linear" insulin generated in vivo after cDNA vaccination has a different predicted conformation than native insulin, precluding biological activity. Taken together, observed changes in diabetes incidences in NOD and RIP-B7.1 mice after DNA vaccination are unlikely to result from metabolic effects of the DNA vaccine.

DISCUSSION

The major finding of this work is that insulin (and its precursor molecules ppIns and proinsulin) can induce autoimmune diabetes in two mouse models of type 1 diabetes, using intramuscular DNA vaccination for antigen

TABLE 2
Fasting blood glucose (mmol/l) in DNA-vaccinated NOD mice, showing lack of metabolic (hypoglycemic) effects of insulin and ppIns DNA

Hours after DNA vaccination*	Duration of fasting (h)	Blood glucose (mmol/l) ppIns DNA (n = 5 mice)†	Insulin DNA (n = 5 mice)†	Control DNA (n = 4 mice)†
0	6	3.3 ± 0.2	3.3 ± 0.4	3.0 ± 0.2
12	18	2.5 ± 0.1	2.5 ± 0.1	2.5 ± 0.1
24	30	2.2 ± 0.1	2.1 ± 0.1	2.2 ± 0.2
48	54	2.0 ± 0.2	2.1 ± 0.1	2.0 ± 0.1
120	6	2.9 ± 0.3	2.7 ± 0.2	3.4 ± 0.8

*Blood glucose at hour 0, 12, 24, and 48 was measured during a fasting period starting 6 h before DNA vaccination. Mice were allowed to feed from hour 48 until hour 114. †Glucose values of ppIns, insulin, and control DNA-treated mice were not significantly different at any time ($P > 0.15$, Wilcoxon).

administration. Experimental evidence supporting this observation is solid and reproducible, obtained from a total of 112 NOD mice and 81 RIP-B7.1 mice from independent litters, using independent DNA preparations for antigen treatment. The extent of diabetes induction was considerable, as RIP-B7.1 mice are naturally diabetes-free, whereas 61.1% of antigen-treated animals became diabetic (Table 1). Similarly, diabetes could be induced in the majority of male NOD mice, characterized by a high natural diabetes resistance. Even in female NOD mice with a high spontaneous diabetes incidence, an increased diabetes incidence was observed after ppIns treatment.

Diabetes occurred specifically after DNA vaccination with (prepro)insulin, whereas GAD65 treatment conferred partial diabetes protection, confirming previous findings in NOD mice (17). From these observations, diabetes induction upon DNA vaccination with β-cell antigens is not a general feature in RIP-B7.1 and NOD mice but seems limited to particular autoantigens. High gene expression levels and nearly exclusive tissue specificity may in this context set apart insulin from other β-cell proteins. The potential effect of autoantigens other than insulin and GAD65 in diabetes intervention will require additional study.

Prevention of NOD mouse diabetes after DNA vaccination with hsp60 has been described (23), but treatment with control plasmid (not containing antigen cDNA) was equally effective in that report. In contrast, in our studies, treatment with empty DNA vector neither promoted nor prevented diabetes development, so major antigen-independent treatment effects of DNA vaccination (e.g., mediated by plasmid CpG motifs or contaminants) may be excluded in our work.

In a recent study (26), DNA vaccination with insulin β chain conferred diabetes protection in young NOD mice, whereas DNA constructs encoding the entire (prepro)insulin protein were not studied in that work. Insulin β chain contains major diabetes-associated T-cell epitopes, and it is conceivable that treatment outcomes reflect the differential choice of antigenic determinants expressed by the DNA vaccine. In addition, plasmid design and vaccination modalities may affect the clinical outcome of DNA vaccination in NOD mice (26,27).

To map diabetes induction to functional domains of the insulin molecule, we constructed and tested in vivo deletion-mutant vectors for DNA vaccination. Insulin and its precursor molecules proinsulin and ppIns all were effective to induce autoimmune diabetes in our experimental system. Therefore, insulin α (A1–21) and β (B1–30) chain domains seem sufficient to confer diabetogenic effects of the DNA vaccine. Ongoing vaccination studies using insulin minigene constructs will help to define T-cell epitopes that mediate in vivo diabetogenicity in this novel EAD model.

Diabetes development in RIP-B7.1 mice after insulin treatment was characterized by a diffuse infiltration of pancreatic islets with $CD4^+$ and $CD8^+$ T-cells, whereas insulitis was absent after GAD65 or control DNA vaccination. In RIP-LCMV/B7.1 transgenic mice, we previously observed insulitis and diabetes development upon DNA vaccination with LCMV glycoprotein, accompanied by the induction of $CD8^+$ cytotoxic T-cell (CTL) responses against the immunodominant LCMV epitope gp33 (K. Pechhold et al., unpublished observations). T-cell proliferative responses to proinsulin were not significantly different in NOD mice that were vaccinated with ppIns or control plasmid (data not shown), reminiscent of T-cell proliferative responses in GAD65-vaccinated animals (17). A recent workshop report (28) concluded that instead of proliferation, more sensitive assays (including ELISPOT or tetramer technology) may be preferred to study autoreactive T-cells. Thus, the exact contribution of individual T-cell subsets to diabetes induction in NOD and B7.1 mice after (prepro)insulin DNA vaccination remains to be clarified.

In RIP-B7.1 mice, diabetes developed on a wild-type C57BL/6 (H-2b) genetic background, not carrying diabetes-associated MHC alleles or other established disease risk loci, thus resembling most cases of sporadic human type 1 diabetes. As nontransgenic C57BL/6 mice remained healthy after DNA vaccination with insulin, diabetes susceptibility is directly linked to the expression of B7.1 in pancreatic β-cells. In mice and humans, professional APCs upregulate B7.1 upon various activation signals, including viral and bacterial products, CD40:CD40L interaction, or interferon-γ (IFN-γ) and other cytokines. We showed previously that functional B7.1 expression can be induced on nonprofessional APCs after treatment with IFN-γ (29). It is conceivable that ongoing β-cell inflammation (e.g., through enterovirus infection) thus may increase diabetes susceptibility independent of MHC risk alleles. Indeed, enteroviral infection has been implicated frequently (e.g., 30) as a relevant environmental risk factor for human type 1 diabetes. In this scenario, T-cell sensitization to insulin

after its release from β-cell damage or through exogenous insulin administration may adversely promote insulitis and autoimmune diabetes, as shown in our work.

In a variety of animal studies (1–5), local or systemic application of insulin or insulin fragments using different treatment protocols has been shown to be effective to reduce diabetes development in NOD mice, with antigen therapy commonly initiated at early stages of autoimmunity to achieve disease protection. Protection was associated with T-cell regulation or tolerance induction in some of these studies. However, oral administration of an Ova to mice expressing Ova as a transgene in pancreatic β-cells led to the induction of cytotoxic T-cells and autoimmune diabetes (13). Similarly, insulin treatment in conjunction with bacterial adjuvant resulted in diabetes acceleration in diabetes-prone BB rats (12). This effect was attributed to increased enteral expression in the gut of IFN-γ, a potent enhancer of Th1 and CTL immunity and T-cell costimulation (12). In other T-cell–mediated autoimmune disorders, experimental autoimmune encephalitis (14) and autoimmune ovarian disease (15) have been induced by myelin basic protein and ZP3 glycoprotein, respectively, both autoantigens expressed in affected tissues. We now demonstrate for the first time the induction of EAD by insulin in naturally diabetes-resistant mice, as well as disease promotion in the classic NOD diabetes model. Taken together, these observations suggest that treatment of autoimmune disorders with autoantigen may confer either disease protection or acceleration, depending on factors (e.g., T-cell costimulation, cytokine environment) other than the antigenic determinant itself.

Our work may have significant clinical implications. Insulin is being used in a large clinical trial (11) to prevent autoimmune diabetes in high-risk individuals with detectable islet cell antibodies and impaired insulin secretion, marking progressive diabetic autoimmunity. In advanced insulitis preceding overt diabetes in NOD mice, secretion of IFN-γ (a potent inducer of B7.1 costimulation) prevails (31). We have shown that co-vaccination with B7.1 cDNA abrogates GAD65-mediated diabetes protection in NOD mice (17). Antigen treatment in the presence of B7.1 upregulation, e.g., during advanced diabetic autoimmunity, may result in a substantially different outcome compared with treatment in unprimed individuals. When therapeutically given at time of diabetes manifestation, insulin has indeed been shown to be ineffective to arrest β-cell destruction in patients with type 1 diabetes (32). In a clinical setting (33), antigen treatment seems particularly problematic during episodes of systemic or viral infections or other states of increased IFN-γ secretion.

In conclusion, clinical deterioration of autoimmune disease may be a potential adverse outcome after antigen-specific immune therapy. Additional study of its dual role in disease prevention and acceleration should be considered before insulin (and other autoantigens) may be fully accepted as immunologically safe for the prevention of human type 1 diabetes.

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