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Intramuscular injection of plasmid DNA encoding intracellular or secreted glutamic acid decarboxylase causes decreased insulitis in the non-obese diabetic mouse

Research Article

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Abbreviations: **NOD**, non-obese diabetic; **IDDM**, insulin-dependent diabetes mellitus; **GAD**, glutamic acid decarboxylase; **GABA**, gamma-aminobutyric acid; **aa**, amino acids.

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Summary

Our goal is to determine whether gene vaccination can be used for the treatment of insulin-dependent diabetes mellitus (IDDM), an autoimmune disease. In this work, weanling non-obese diabetic (NOD) mice, an animal model system for the study of IDDM, received intramuscular injections of "naked" plasmid DNA encoding either intracellular or secreted human glutamic acid decarboxylase (GAD), one of the major autoantigens recognized during the onset of IDDM. Seven weeks later, each pancreas was scored for insulitis, an inflammation indicative of the disease. Mice treated with either type of *gad* gene-carrying plasmid showed a significant decrease in the severity of insulitis when compared to controls. These results suggest that vaccination using autoantigen-encoding genes may provide a means of treating IDDM.

I. Introduction

Insulin dependent diabetes mellitus (IDDM), or type I diabetes, is a disease with high morbidity and mortality that affects 1 in 300 persons in North America, with a prevalence ever increasing in small children (for a review see Mandrup-Poulsen, 1998). Although also called juvenile diabetes because it often affects young people, a similar disease has been diagnosed in patients 50 years of age and older (Molbak et al., 1994). IDDM is thought to be caused by both genetic and environmental factors, and is associated with the autoimmune destruction of insulin-producing beta cells found in the pancreatic islets of Langerhans. Loss of these insulin-secreting cells results in the inability to metabolize glucose, leading to hyperglycemia and ketoacidosis, which in turn cause a variety of debilitating and life-threatening ailments such as blindness, kidney disease, heart attack, stroke, and neuropathy. Although injection of the hormone insulin can prolong life of IDDM patients, it does not provide a cure for the disease, likely due to lack of proper regulation of insulin levels within the body. A cure for IDDM could be achieved if the destruction of beta cells were averted.

IDDM has been characterized as an autoimmune disease based on the observations that patients suffering from this illness have high levels of islet cell autoantibodies in their sera (Bottazo et al., 1974), and chronic mononuclear cell infiltration of their pancreatic islets (Gepts and Lecompte, 1981). Presence of autoantibodies can be detected years

before the onset of symptoms, and is considered to be diagnostic for IDDM (Maclaren, 1988), although it does not always imply occurrence of the disease. In humans, the nature of these antibodies varies with age: autoantibodies against insulin and tyrosine phosphatase IA-2 are associated with early childhood, glutamic acid decarboxylase (GAD) and islet cell cytoplasmic protein autoantibodies with late childhood and adolescence, while late onset can be associated with other typical immune markers (Mandrup-Poulsen, 1998). Inflammatory infiltration of the islets (insulitis) and beta cell destruction are due mostly to T lymphocytes, both $CD4^+$ helper and $CD8^+$ cytotoxic (Itoh et al., 1993; Peakman et al., 1994), and result in loss of islet cell mass. When this cell mass drops below 10% of normal, hyperglycemia and ketosis develop.

A large part of what is known about IDDM comes from studies of animal model systems, in particular the non-obese diabetic (NOD) mouse. The NOD mouse possesses most of the characteristics of human IDDM, such as genetic predisposition due to MHC II linkage, development of insulitis with infiltration of T lymphocytes selectively toxic to insulin-producing beta cells, and humoral reactivity to beta cells (for a review see Bowman et al., 1994). However, unlike humans, NOD mice have a strong gender bias in the appearance of the disease: 91% of females NOD/Lt mice manifest diabetes at 250 days of age, while only 21% of males show a similar symptom at that age (Baxter et al., 1991).

Studies of NOD mice (Kaufman et al., 1993; Tisch et al., 1993) and patients (Baekkeskov et al., 1990) indicate that the GAD protein is a major autoantigen recognized during the onset of IDDM. GAD is an enzyme found mostly in neurons (Erlander et al., 1991) and pancreatic islet cells (Christgau et al., 1991), where it catalyzes the synthesis of gamma-aminobutyric acid (GABA). GABA is an inhibitory neurotransmitter in the central nervous system, and may be a paracrine signaling molecule in the pancreas. Two forms of GAD are encoded by different genes in mammals, a 65 kDa (previously called 64) and a 67 kDa (previously called 65) molecular weight form. GAD65 is a membrane-anchored intracellular protein, while GAD67 is found soluble in the cytosol (Christgau et al., 1991; Christgau et al., 1992). Both GAD65 and GAD67 are recognized by the immune system of IDDM patients (Baekkeskov et al., 1990; Honeyman et al., 1993). In addition, the first T cell response against beta cell antigens in 4-week old NOD mice is against GAD65 (Kaufman et al., 1993;Tisch et al., 1993), and both $CD8^+$ cytotoxic (Panina-Bordignon et al., 1995) and $CD4^+$ T helper 1 (Th1) (Tabata et al., 1998) lymphocytes specific for GAD65 can be found in patients suffering from IDDM. Together with the finding that adoptive transfer of GAD-reactive T cells can cause diabetes in NOD/SCID mice (Zekzer et al., 1998), these observations strongly indicate that GAD65 plays an important role as an autoantigen during onset of IDDM.

The NOD mouse serves not only as a model to study IDDM, it is also an excellent system for the development of new methods for preventive transfer of this form of diabetes. Such therapies include immunosuppression, immunostimulation, tolerance induction, manipulation of hormonal/dietary milieu, and anti-inflammatory agents (Bowman et al., 1994). In this work, we have investigated whether gene vaccination could be used to prevent insulitis in the NOD mouse. Specifically, we have used intramuscular injection of "naked" plasmid DNA encoding human GAD65 and SGAD55, an engineered secreted form of this protein. We report that injection of DNA encoding these proteins resulted in significant decreases in insulitis, suggesting the possibility that this form of gene therapy might be useful to prevent clinical manifestation of IDDM.

II. Results

A. Construction of a secreted form of human GAD65

Extracellular antigens can be used for tolerization or for suppression of MHC class II restricted Th1 inflammatory response, probably through a MHC class II restricted $CD4^+$ Th2 lymphocyte response, as Th1 and Th2 responses appear to be mutually exclusive (Mosmann and Sad, 1996). Therefore, two genes encoding GAD proteins that had the potential of being secreted by mammalian cells were constructed.

The first construct consisted of the leader peptide from human interleukin-2 (IL-2) protein fused to full-length human GAD65, generating a fusion protein encoded by the *sgad65* gene. This leader sequence was previously shown to cause secretion by mammalian cells of normally intracellular proteins (Okano et al., 1990; Liu et al., 1997). However, because GAD65 is a membrane-anchored protein, the protein region responsible for the anchoring could have interfered with secretion. The sequence corresponding to approximately the first 100 amino acids (aa) of human GAD65 contains a Golgi-targeting sequence (Solimena et al., 1994), as well as cysteine residues that are palmitoylated and responsible for membrane anchoring (Christgau et al., 1992). In addition, this sequence is not recognized by autoantibodies from IDDM patients (Richter et al., 1993). The first 88 aa of the human GAD65 protein were therefore deleted, and the remainder of the protein was fused to the IL-2 leader sequence, generating a fusion protein (SGAD55) encoded by the *sgad55* gene (**Fig. 1B**).

Simian COS-7 cells were transiently transfected with the two gene constructs coding for these proteins, and immunoblot analysis of intracellular GAD protein was performed using a monoclonal human GAD65 antibody.

Results confirmed the synthesis of SGAD65 (**Fig. 2A**, lane 3), and of the lower molecular weight SGAD55 (**Fig. 2A**, lane 4). To determine whether SGAD65 and SGAD55 were secreted by mammalian cells, proteins from COS-7 cells transiently transfected with the different gene constructs were labeled *in vivo* with ^{35}S-methionine. Culture media from these cells were then used for immunoprecipitation using the same antibody used for immunoblot analysis, and immunoprecipitates were fractionated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Results showed that SGAD55 protein was immunoprecipitated from cell culture media (**Fig. 2B**, lane 3). In contrast, no secreted SGAD65 protein could be detected (**Fig. 2B**, lane 4). The gene construct *sgad55* was therefore selected for further use in animal experiments.

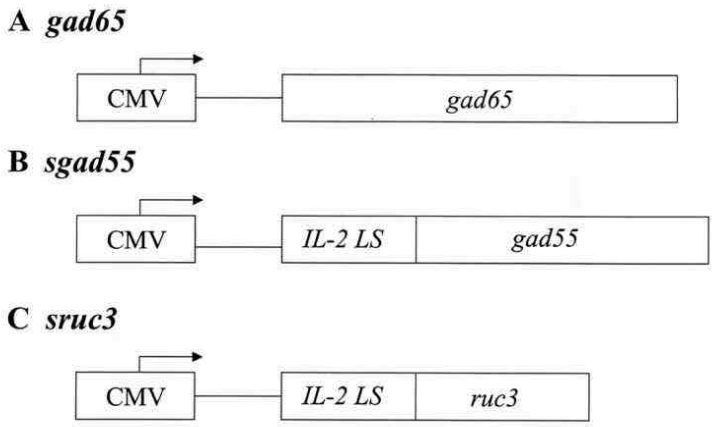

Figure 1. Gene constructs used for intramuscular DNA injection. Three genes were placed under transcriptional control of the cytomegalovirus promoter (CMV) into an expression plasmid, *gad65*, encoding a wild type intracellular human GAD65 protein (**A**), *sgad55*, encoding a fusion of the IL-2 leader sequence (IL2-LS) to a truncated human GAD65 protein (**B**), and *sruc3*, encoding a secreted *Renilla* luciferase (**C**).

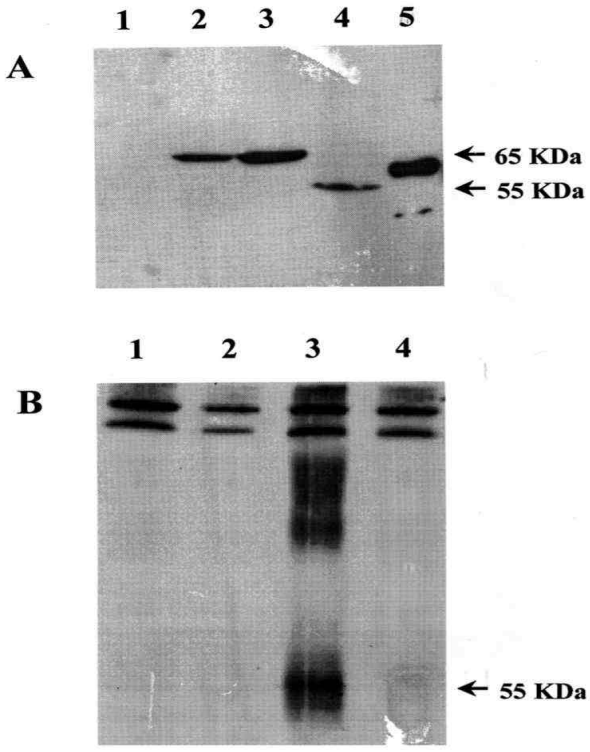

Figure 2. Detection of GAD proteins from lysates (**A**) and culture media (**B**) of mammalian cells grown in vitro. **A**. Immunoblot analysis of simian COS-7 cells transiently transfected with different gad genes. Cells were transfected with plasmid vector only (lane 1), plasmid carrying gene *gad65* (lane 2), *sgad65* (lane 3), or *sgad55* (lane 4). Lane 5 contains a truncated version of *GAD*65 isolated from *Escherichia coli* as control. Total cells lysates were fractionated using SDS-PAGE, transferred onto a membrane, and reacted with a mouse monoclonal antibody raised against wild-type human GAD65, and subsequently to a secondary antibody bound to alkaline phosphatase for chemiluminescent detection. **B**. Immunoprecipitation of ^{35}S-methionine-labeled proteins from culture media. Culture media from COS-7 cells transiently transfected with plasmid vector only (lane 1), plasmid carrying gene *gad65* (lane 2), *sgad55* (lane 3), or *sgad65* (lane 4) were used for immunoprecipitation using the same antibody used in A. Immunoprecipitates were then fractionated using SDS-PAGE and exposed to X-ray film.

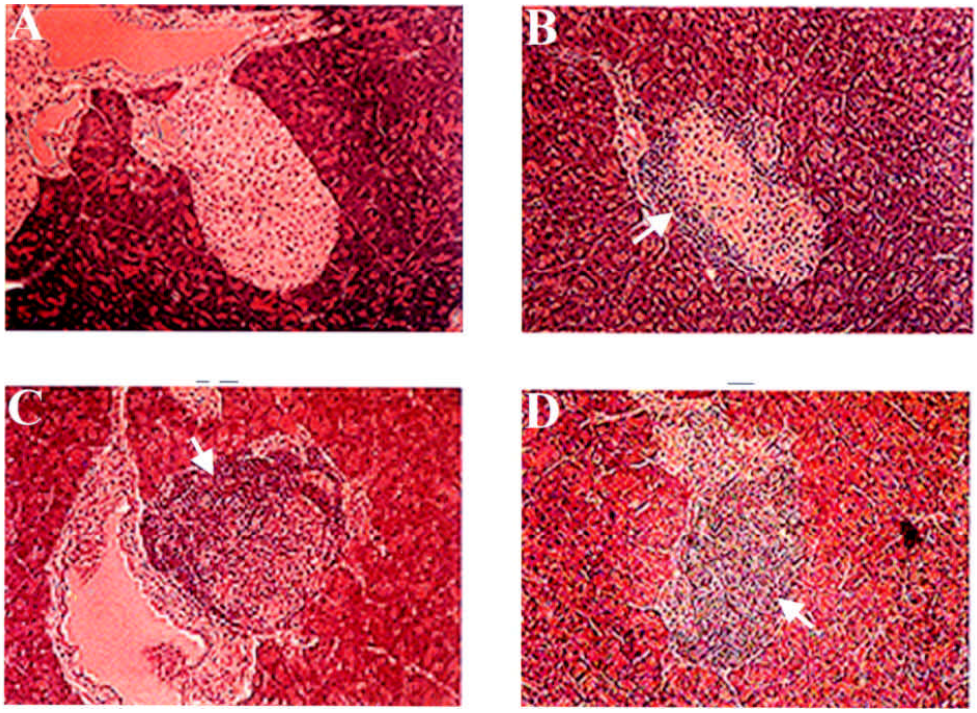

Figure 3. Histopathological examination of pancreatic islets. Islets with score 0 (**A**), and 3 (**B**) from a GAD65-treated mouse are shown for comparison with islets with score 5 (**C**) and 6 (**D**) from a control animal. Arrows point to T cell infiltration.

B. Effects of intramuscular injection of GAD-encoding genes on insulitis and cytokine profile

Each of four groups of three-week old female NOD mice received injections of one of four plasmid DNAs, and injections were repeated after three days. Mice were injected with either plasmid vector only, plasmid vector carrying the *sruc3* gene encoding a stable mutant (JL and AE, unpublished data) of a secreted soft coral luciferase (Liu et al., 1997), the human *gad65* gene, or the *sgad55* gene encoding secreted GAD protein. The *sruc3* gene was used as control for possible non-specific effects of synthesis of a plasmid-encoded antigen on insulitis. Another group of NOD mice was used as a non-injected control (N.B. this group was kept in a different animal room at Loma Linda University, and at a different time).

Mice were killed when 10 weeks old for histopathological analysis, insulitis scoring, and immune assay. None of the mice had developed diabetes, as determined by urine and blood glucose analysis (data not shown). **Figure 3** shows representative islets illustrating the levels of insulitis that were observed. **Figure 4A** shows that a significant reduction in the severity of insulitis was detected in mice receiving injections of plasmids carrying the *gad65* and *sgad55* genes, when compared to the three control groups. In addition, mice injected with these *gad* genes also had a higher percentage of insulitis-free islets (**Fig. 4B**). Cytokine profiles of GAD65-stimulated spleen lymphocytes (splenocytes) tended to support the histological findings. While the Th1-type cytokines (IFNγ and IL-2) were not different between groups (**Fig. 5A** and **B**), IL-4 production (Th2-type) was higher in the gene-vaccinated groups (**Fig. 5C**, 3 and 4) than in the controls (**Fig. 5C**, 1 and 2), when challenged in vitro with recombinant human GAD65 protein.

III. Discussion

Gene vaccination consists of the introduction and expression of a gene into an organism, with the purpose of generating an immune response against its encoded product. The simplest way of achieving this purpose is to use the method of intramuscular or subcutaneous "naked" DNA injection, originally presented as a means of expressing plasmid-encoded genes after direct injection of DNA into mouse muscle (Wolff et al., 1990). This method has since been used to generate immune responses to a wide variety of antigens, such as human immunodefficiency virus 1 glycoproteins, and malarial circumsporozoite protein (for a review see Tighe et al., 1998). Although the majority of studies have focused on infectious diseases, "naked" DNA

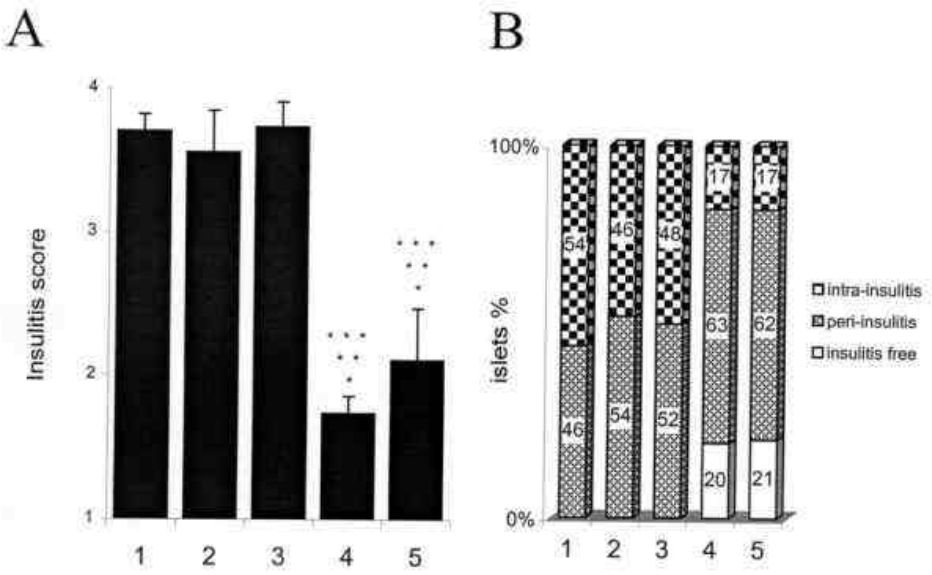

Figure 4. Insulitis scores of 10-week old female NOD mice. Severity of insulitis is presented based on a 0-6 scale (**A**), and as percentage of islets showing intra-, peri-, or no insulitis (**B**). Insulitis was scored with untreated mice (group 1, n= 5), mice receiving injections of plasmid vector only (group 2, n=5), or vector carrying gene *sruc3* (group 3, n= 6), gene *gad65* (group 4, n=7), or gene *sgad55* (group 5, n=7). Data are presented as the mean score ± SEM. When individually compared to control groups 1 (*), 2 (* *), or 3 (* * *), group 4 or 5 showed statistically significant differences (P value <0.05). No statistically significant differences were found among groups 1, 2, 3, or groups 4 and 5.

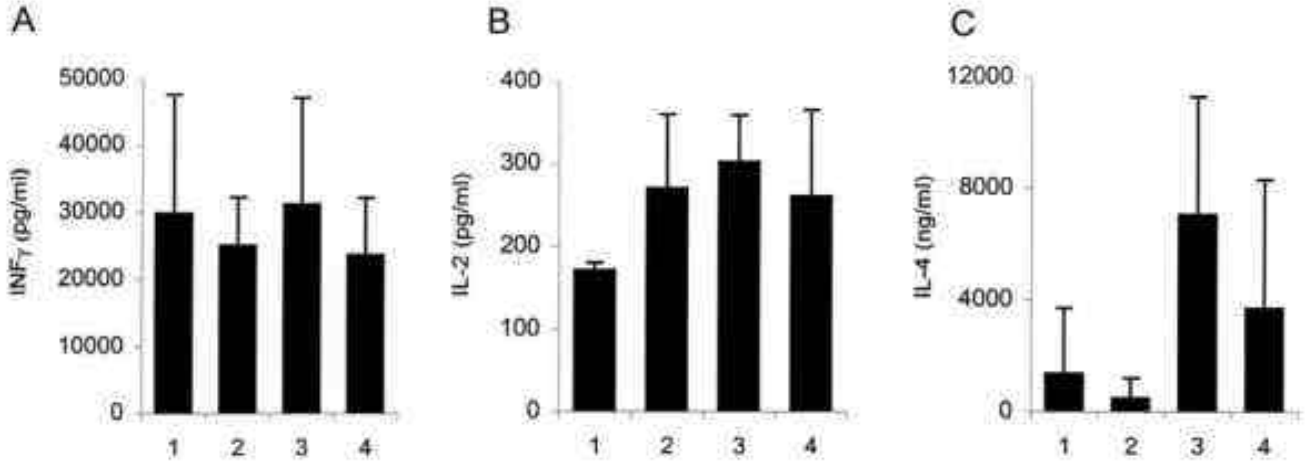

Figure 5. Cytokine profile of GAD65 protein-stimulated splenocytes. Splenocytes from mice receiving injections with plasmid vector only (1), or plasmid carrying the *sruc3* (2), *gad65* (3), or *sgad55* (4) gene were stimulated with 1.5 μg/mL of isolated recombinant human GAD65 protein. After 72 hrs, culture supernates were assayed for IFNγ (**A**), IL-2 (**B**), and IL-4 (**C**).

gene vaccination can also be applied to studies of alloimmunity (Geissler et al., 1994) and treatment of cancer (Condon et al., 1996). Recently, gene vaccination was used to suppress the symptoms of autoimmune encephalomyelitis in rats through synthesis of an autoantigenic peptide (Lobell et al., 1998).

Glutamic acid decarboxylase (GAD) is thought to be a major autoantigen contributing to the onset of insulin-

dependent diabetes mellitus (IDDM), and injection of GAD protein can delay the onset of the disease in NOD mice (Kaufman et al., 1993; Tisch et al., 1993; Elliot et al., 1994; Petersen et al., 1994; Sai et al., 1996). In addition, similar results are obtained with oral feeding of the protein (Ma et al., 1997; Ramiya et al., 1997). In this work, we investigated whether expression of genes encoding two forms of human GAD could cause reduction of insulitis in the NOD mouse, an inflammation of pancreatic islets which is characteristic of IDDM in this model.

Two *gad* genes were used for expression in muscle tissue, a *gad65* cDNA encoding human GAD65 protein, and *sgad55*, a gene construct based on *gad65*, encoding the interleukin-2 (IL-2) leader sequence fused to a GAD65 protein lacking its first 88 amino acids (SGAD55) (**Fig.1**). The N-terminal region of GAD65 was removed in the SGAD55 fusion protein because we suspected that it could interfere with its secretion, since this region contains a Golgi-targeting and membrane-anchoring sequence. This was confirmed by the finding that a fusion of the IL-2 leader sequence to full-length GAD65 could not be detected in the culture media of mammalian cells expressing its encoding gene **(Figure 2B**, lane 4). In contrast, SGAD55 protein was detected in culture media of cells expressing the *sgad55* gene (**Fig. 2B**, lane 3).

Intracellular and secreted forms of GAD65 were used in this study because of the known differences in the type of immune response that intra- and extra-cellular antigens can generate. Intracellular antigens are presented by MHC class I molecules and generate a $CD8^+$ cytotoxic T lymphocyte response, while extracellular antigens are presented by MHC class II molecules on the surface of antigen presenting cells, generating a $CD4^+$ helper T lymphocyte response (Tighe et al., 1998). Although secreted proteins are synthesized within a cell, they appear to be less likely to be presented by MHC class I molecules than cytosolic proteins (Yewdell et al., 1998).

Synthesis of intracellular GAD65 by muscle cells was not expected to effect insulitis. Considering the small number of muscle cells able to express injected genes, the levels of intracellular GAD65 protein found in injected and non-injected mice NOD should not have differed greatly enough to generate an immune response influencing T cell infiltration of islets. This supposition was corroborated by the finding that in NOD mice transgenic for murine *gad65*, only those mice showing the highest levels of transgene expression could exacerbate insulitis and diabetes (Geng et al., 1998). In contrast, secretion of SGAD55 could have caused either reduced insulitis (through an anti-inflammatory Th2 response) or increased insulitis (through an inflammatory Th1 response), depending on the levels of extracellular antigens attained (Hosken et al., 1995).

Our results show that injections of *gad65*-carrying plasmids caused a reduction of insulitis similar, if not greater, to that resulting from injections of *sgad55*-carrying plasmid (**Fig. 4**). A variety of non-exclusive mechanisms are thought to lead to Th1 and Th2 immune responses after plasmid DNA injection, such as release of antigens from intact cells expressing the plasmid-carried gene, or from lysed cells after a cytotoxic T lymphocyte response, and direct transfection of antigen presenting cells (Davis et al., 1993; Xiang et al., 1994; Condon et al., 1996; Gregoriadis et al., 1997). Our results suggest that one (or both) of the latter two putative mechanisms was likely to be responsible for the similar reduction of insulitis after intramuscular injection of *gad65* or *sgad55* gene, since neither the intra- nor extra-cellular nature of the plasmid-encoded GAD antigens appeared to affect the extent of insulitis differently.

To determine the nature of the immune response generated in plasmid-treated NOD mice, cytokine secretion by splenocyte was measured after challenge with recombinant human GAD65 protein. Splenocytes of all plasmid-injected mice secreted similar levels of Th1-specific IFNγ (**Fig. 5A**) and IL-2 (**Fig. 5B**). However, cells from mice receiving injections of *gad65* or *sgad55* genes maintained higher levels of Th2-specific IL-4 than the controls when challenged with GAD65 autoantigen (**Fig. 5C**). No differences in splenic memory cell numbers (immunophenotyping) or blood levels of cytokines could be demonstrated between groups (data not shown). These results suggest that the reduced levels of insulitis observed after injection of *gad65* or *sgad55* genes could have been the result of a Th2-mediated response. This would be in accordance with the observation that suppression of insulitis is associated with elevated synthesis of IL-4 and IL-10 (for a review see Rabinovitch, 1998), and that suppression of the diabetogenic response in NOD mice after injection of GAD65 protein is mediated by the induction of GAD65-specific regulatory Th2 cells (Tisch et al., 1998). Failure to detect systemic changes in circulating cytokine levels (IFNγ 75-1365 pg/mL) and splenic phenotypes (13-15% CD62Lneg CD44pos T helper cells) was expected in light of the well-established organ-specificity of this disease. Further work on the cellular infiltrate should reveal more relevant information.

Immunomodulatory gene therapy has been previously considered as a possible approach for the prevention of IDDM. In one study, islet-specific Th1 cells transduced with engineered retroviruses carrying a gene encoding the anti-inflammatory cytokine IL-10 were able to cause reduced insulitis and delayed onset of diabetes when injected into NOD mice (Moritani et al., 1996). In contrast, intramuscular injection of plasmid DNA encoding IL-10 did not cause reduced insulitis, but did result in delay of diabetes onset (Nitta et al., 1998). In another study, intramuscular injection of DNA encoding TGF-β1 caused both reduced insulitis and delayed onset of diabetes

(Piccirillo et al, 1998). Our data suggest that intramuscular injection of DNA coding for an IDDM autoantigen could also be used for this purpose. Plasmid injection offers potentially both therapeutic and economical advantages. Injection of plasmid DNA could permit the development of plasmid "cocktails" encoding combinations of different autoantigens and immunomodulating cytokines. When compared to injection of isolated proteins, the availability, quality, and cost of these therapeutic proteins would not be a concern, since their synthesis would occur within the host. Clearly, injection of plasmid DNA is a promising approach for suppressing symptoms of IDDM or other autoimmune diseases in the future.

IV. Materials and Methods

A. Gene and plasmid construction

The *sgad65* gene encodes a fusion of the leader peptide from human IL-2 to full-length human GAD65 protein. This gene was constructed by ligating an 89 base pair (bp) DNA fragment encoding the first 23 amino acids of IL-2 (isolated previously by PCR from human cell line A293 as described by Liu et al., 1997) in frame with a 1.8 Kilobase pair (Kb) NcoI-XhoI DNA fragment carrying a human GAD65 cDNA. The *sgad55* gene encodes a fusion of the leader peptide from human IL-2 to a truncated version of human GAD65 with 88 aa deleted at its N-terminus. Two oligonucleotides were used to amplify the 89 bp DNA fragment encoding the IL-2 leader peptide from gene *sgad65*, IL-01 (TTT TCT AGA ATG TAC AGG ATG CAA CTC CTG) and IL-03 (TTT ACG CGT AAG TAG GTG CAC TGT TTG TGA). IL-03 introduced an MluI site which was used to clone the PCR product in frame with the MluI-XhoI 1.5 Kb DNA fragment encoding GAD55, the truncated version of human GAD65. The identity of PCR products and gene fusion junctions were confirmed using automated DNA sequencing.

For cell culture work, the *gad65*, *sgad65*, and *sgad55* genes were cloned under transcriptional control of the cytomegalovirus (CMV) promoter into plasmid vector pLNCX (Miller and Rosman, 1989). For muscle injection, all genes were cloned under transcriptional control of the CMV promoter in plasmid pND-2, a vector known to provide high gene expression in muscle tissues (Gary Rhodes and Robert Malone, unpublished data).

B. Mammalian cell culture and transfection

Simian COS-7 cells were grown in 60 mm tissue culture dishes containing 3 mL DMEM medium with 10% fetal bovine serum (FBS). Media were changed 3 hrs prior to transfection when cells were 70% confluent. Cell transfection was performed using the ProFection calcium phosphate system (Promega, Madison, WI) using 40 μg of plasmid DNA per plate. Cells were incubated with the DNA-calcium phosphate complex for 6 hours, washed twice with phosphate-buffered saline (PBS), and 3 mL DMEM medium +10% FBS was added into each plate. Culture plates were then incubated for 48 hrs before harvesting cells and media for analysis.

C. Immunoblot analysis

Simian COS-7 cells were washed twice with cold PBS 48 hrs after transfection, and harvested in 100 μl hot 2x gel-loading buffer (100mM Tris.HCl, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.01% bromophenol blue) using a rubber policeman. Cells were lysed by sonication, boiled for 3 min, and lysates were centrifuged at 1000x g for 10 min to pellet cell debris. Twenty microliters from each sample was loaded on a 12% SDS-polyacrylamide gel for fractionation. Proteins were then transferred onto a nylon membrane by electroblotting, and a GAD65 monoclonal antibody was used to detected GAD protein using a method previously described (Liu et al., 1997).

D. Protein radiolabeling and immunoprecipitation

To detect secreted GAD proteins in cell culture media, ^{35}S-methionine (specific activity>1000 Ci/mmol, from DuPont NEN, Boston, MA) was used to label total cell protein from COS-7 cells. Media were removed after incubation with the DNA-calcium phosphate complex, and cells were rinsed twice with 1 x PBS and once with medium without methionine and serum. Cells were then incubated in 3 mL DMEM medium without methionine + 1% dialyzed FBS for 20 min to deplete intracellular pools of endogenous methionine. One hundred microcuries of ^{35}S-methionine was then added directly into media for protein labeling. Cells were incubated for 24 hrs before being harvested. Media were collected and concentrated using a Centricon spin column (15 kDa molecular weight cut-off, from Amicon) to a final volume of 500 μL. ^{35}S-labeled GAD protein was immunoprecipitated from media using the Protein A Immunoprecipitation Kit (Boehringer Mannheim, Indianapolis, IN) and a monoclonal antibody raised against human GAD65. Immunoprecipitates were fractionated in a 12% SDS-polyacrylamide gel, and protein bands were detected by exposure to X-ray film.

E. Isolation of plasmid DNA for muscle injection

Plasmid DNA was amplified in *Escherichia coli* strain DH5-α, using the alkaline-lysis method, and isolated by standard double-round cesium chloride purification (Maniatis et al., 1989). The quality and quantity of DNA was determined by U.V. spectrophotometry (A_{260}/A_{280} ratio greater than 1.8) and by agarose gel electrophoresis. Plasmid DNA was dissolved under sterile conditions in double distilled water at a final concentration of 2 μg/μL, and stored at -20°C.

F. Intramuscular DNA injection

Three-week old female NOD mice were purchased from Taconic Laboratories (Germantown, NY) and kept at Loma Linda University animal facilities. Mice were injected with DNA (200 μg/100 μL/leg) into each quadriceps muscle with a 27-gauge needle under general anaesthesia (Ketamine, 66 mg/Kg body weight, from Phoenix Scientific, St Joseph, MO; Oxylazine, 7.5 mg/Kg body weight, from LLOYD Laboratories,

Shenandoa, IO; and Acepromazine Maleate, 1.5 mg/Kg body weight, from Fermenta Animal Health Co., MO), and injections were repeated three days later. Urine glucose levels were monitored weekly with Clinistix Reagent Strips for Urinalysis (Bayer Corporation, Elkhart, IN). Mice were killed for insulitis scoring at the age of 10 weeks, and blood glucose levels were checked with ACC.-CHEK Advantage (Boehringer Mannheim Corporation, Indianapolis, IN).

G. Histopathological analysis of insulitis

Pancreatic tissues were fixed with 10% buffered Formalin, stained with hematoxylin, and counterstained with eosin, and an average of fifteen islets/mouse were scored. A 7-level semi-quantitative scoring scale (Zhang et al., 1991) was used for insulitis scoring: 0, normal islet tissue without any detectable T cell infiltration; 1, focal peri-islet T cell infiltration with less than one-third of the peri-islet area; 2, more extensive peri-islet T cell infiltration with less than two-thirds of the peri-islet area; 3, peri-islet T cell infiltration with more than two-thirds of the peri-islet area; 4, intra-islet T cell infiltration with less than one-third of the islet area; 5, intra-islet T cell infiltration with less than two-thirds of the islet area; 6, severe intra-islet T cell infiltration with more than two-thirds of the islet area. Scoring of 1-3 indicated peri-insulitis, and scoring of 4-6 indicated intra-insulitis. Scoring was conducted using the double-blind method by two different scorers.

H. In vitro challenge of splenocytes

Lymphocytes were flushed from splenic pulp and washed in complete media (RPMI, 10% FBS, 2% L-Glutamine, and 4 x 10^{-5} M 2-mercaptoethanol). In a 24-well plate, $1x10^6$ cells in 1 mL complete media (unstimulated control) or 1 mL GAD65 (1.5 μg/mL) were cultured (37°C, 5% CO_2) for 72 hrs. Cell culture supernatants and blood plasma (from terminal bleeds) were assayed by standard sandwich ELISA (Endogen, Woburn, MA) for IFNγ, IL-2 and IL-4.

I. Immunophenotyping of splenocytes

Since antigen-specific memory cells of the Th1-type T lymphocytes express CD44 and lose expression of CD62 ligand (CD62L) (Mocci and Coffman, 1997; Bradley et al., 1992), splenocytes were stained with three fluorochrome-conjugated monoclonal antibodies (Becton Dickinson, Immunocytochemistry Systems, San Jose, CA) to CD4, CD44 and CD62L. After red cells lysis, the phenotypes were analyzed by 3-color flow cytometry. Phenotyping controls included untreated (autofluorescence) and isotype antibody-treated cells (nonspecific staining). CD4 T cells (phycoerythrin) were back-gated and these were analyzed for the expression of CD44 (PerCP) and CD62L (FITC).

J. Statistical analysis

Comparison between groups was done using a ONE-WAY ANOVA and Duncans post-hoc test for multiple comparisons.

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