MATERIALS AND METHODS FOR DETECTION AND TREATMENT OF INSULIN-DEPENDENT DIABETES

The method and compositions of this invention provide an assay for diabetes. By providing a method for detecting autoantibodies to GAD_{65}, IA-2 and an antigen termed IA-29 herein, the method provides a chemical assay. In addition, these antigens may be employed in therapeutic regimens aimed at achieving amelioration of the clinical condition.
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DESCRIPTION

MATERIALS AND METHODS FOR DETECTION AND TREATMENT OF INSULIN-DEPENDENT DIABETES

This invention was made with government support under National Institutes of Health grant numbers R01 HD 19469, P01 DK39079 and GCRC M01 RR00082. The government has certain rights in the invention.

Background of the Invention

Field of the Invention

The method and compositions of this invention provide an effective and reliable substitute for the currently employed ICA assay for diabetes. By providing a method for detecting autoantibodies to IA-2β and other molecules such as the GAD65 and IA-2 auto-antigens, the method provides a chemical assay which has improved reliability. In addition, these antigens may be employed in therapeutic regimens for the amelioration of the clinical condition.

Background Literature

Diabetes is a term that refers to a collection of diseases resulting in disordered energy metabolism and varying degrees of blood glucose elevations or hyperglycemia. One of the best characterized forms of the disease is that resulting in immunologically mediated destruction of the insulin secreting pancreatic beta cells. This severe form of the disease is termed Insulin-dependent Diabetes (IDD) since it is associated with progressive insulin deficiency and coincident symptoms such as weight loss, glycosuria and polyuria, and increased thirst or polydipsia. Other terms for this form of diabetes are Type 1 Diabetes (cf. Type 2 Diabetes which
results from an inherent resistance to insulin action); Ketosis Prone Diabetes because there is abnormal generation of ketone bodies as a result of excessive breakdown of body fats due to the severe insulin deficiency; or Juvenile Diabetes, since virtually all diabetes that appears in childhood and adolescence is of this type (see Atkinson and Maclaren [1994] N. Engl. J. Med. 331:1428-1436).

Diabetes is a major public health problem, especially in Western countries. The incidence rates vary greatly worldwide, from as high as 40 per 100,000 persons in Finland to as low as 1-2 per 100,000 among Japanese, with the US in between. The peak incidence is during the pubertal years associated with increasing bodily demands for insulin associated with muscle growth. The prevalence rates in the US population under age 20 years is 0.25% and it approaches 0.4% over a lifetime, albeit an estimated 10-20% of patients with Non Insulin-dependent Diabetes (NIDD) or Type 2 or Maturity Onset Diabetes also have, in reality, slowly progressive IDD. Thus, it is estimated that there should be at least 1 million Americans affected by IDD.

Diabetes results in progressive damage to the blood vessels of the body, to a degree that depends upon the severity of hyperglycemia and its duration. The incident mortality rate for IDD has been calculated to be 7-fold higher than for age matched non diabetic controls. Whereas the decade long Diabetes Control and Complications Trial (DCCT) concluded in 1994 by the National Institutes of Health in the US showed that meticulous insulin replacement therapy would slow the appearance of damaged arteries, it was not able to prevent this since blood glucose levels were never kept within normal limits.
Ocular complications of diabetes are the leading cause of new blindness in persons of 20-74 years of age. The risk of lower extremity amputation is 15-fold higher in those with diabetes, while more than half of the approximately 125,000 persons undergoing lower limb amputation do so as a direct consequence of diabetes. Approximately 40% of persons undergoing renal transplantations have kidney failure because of their diabetes, and the proportion due to diabetes continues to rise each year. Women with diabetes produce newborn infants with a 7% newborn mortality rate, albeit this outcome can be greatly improved with tight glycemic control during the gestation period. Other complications of diabetes include increased heart disease and stroke, loss of nerve cells or neurones innervating the limbs and intestine, impotence and infertility, cataract formation in the lens of the eyes, increased periodontal disease, and predisposition to infectious diseases especially from bacteria and yeast. Of all patients with diabetes, those with IDD have a disproportionate share of these complications because of its severity and usual early age of onset. In the US, the direct health care costs attributable to diabetes in 1994 have been estimated to exceed $120 billion. Thus it is important that the pathogenesis of IDD be understood and strategies be developed to prevent it as a fully expressed clinical disease.

Patients with IDD are unusually prone to other diseases that have become recognized to have autoimmune origins. These diseases include thyroiditis or Hashimoto disease, Graves disease, Addison disease, atrophic gastritis and pernicious anemia, celiac disease and vitiligo (Maclaren, N.K. [1985] Diabetes Care 8(suppl.):34-38). Evidence that IDD itself has an autoimmune nature began with histological studies of
patients that succumbed at diagnosis which indicated that the islets were infiltrated with a chronic inflammatory (lymphocytic) infiltrate termed insulitis. This was supported in the early 1970s by reports of islet cell autoantibodies reactive to antigens within the cytoplasm (ICA) (Lendrum et al. [1975] Lancet 1:880-882) or confined to the islet cell surfaces (ICSA) (Maclaren et al. [1975] Lancet 1:977-1000) as detectable by indirect immunofluorescence. Later it was recognized that many patients also develop autoantibodies to insulin (IAA) before their diagnosis (Palmer et al. [1983] Science 222:1337-1339) as well as to insulin receptors (Maron et al. [1983] Nature 303:817-818). Autoantibodies were also reported to an islet cell protein composition of 64,000 M.Wt. in man (Baekkeskov et al. [1982] Nature 298:167-169), in the Biobreeding (BB) rat model (Baekkeskov et al.[1984] Science 224:1348-1350) and in the Non Obese Diabetic (NOD) mouse model (Atkinson and Maclaren [1988] Diabetes 37:1587-1590). 64 kDa antigen has subsequently been reported to be the lower molecular weight isoform of glutamic acid decarboxylase (GAD_65) (Baekkeskov et al. [1990] Nature 347:151-156) (Kauffman et al. [1992] J. Clin. Invest. 283-292). GAD is an enzyme that converts glutamate into the membrane stabilizing neurotransmitter called gamma amino butyric acid or GABA. In addition to autoantibodies to GAD, peripheral blood mononuclear cells were shown to be autoreactive in patients developing IDD (Atkinson and Maclaren et al. [1992] Lancet 339:458-459; and Harrison et al. [1993] Lancet 341:1365-1369). Indeed a leading possible cause for IDD is that immunity to enteroviral proteins (developed through infection by Coxsackie or closely related viruses) that have structural homologies to GAD, may in the genetically predisposed individual, trigger an autoimmune response to islet cells.

Since immunological markers predate the clinical onset of IDD often by many years, their possible value in disease prediction became increasingly realized (Maclaren, N.K. [1988] *Diabetes* 37:1591-1594), permitting in turn options for therapeutically induced delays in diabetes onset to be considered (Muir and Maclaren [1993] *J. Autoimmunity* 16:301-310). Indeed by 1994, multicenter trials attempting to prevent IDD through prophylactic parenteral insulin or oral insulin therapies had been initiated in the US (the DPT-1 trial), as well as in Europe using prophylactic nicotinamide (the ENDIT trial). Among relatives, the appearance of IAA was shown to predate onset of IDD (Atkinson and Maclaren [1985] *Diabetes* 35:894-898) while ICA proved to be valuable to the prediction of IDD in relatives (Riley, Maclaren et al. [1990] *N. Engl. J. Med.* 323:1167-1172) as well as in the general population (Schatz, Maclaren et al. [1994] *J. Clin. Invest.* 93:2403-2407), as modifiable on the basis of coincident IAA (Krischer, Maclaren et al. [1993] *J. Clin. Endo. Metab.* 77:743-749). While not ideal, the predictability of IDD based upon the ICA test provided the basis for the DPT-1 and ENDIT trials mentioned above. Furthermore, autoantibodies to the 64 kDa islet cell protein also proved to have utility in IDD prediction (Atkinson, Maclaren et al. [1990] *Lancet* 335:1357-1360), as eventually realized by the chemical assay for autoantibodies to GAD65 (Schott, Maclaren et al. [1994] *J. Autoimmunity* 7:865-872). These studies made it important to resolve the nature of all of the islet cell

Whereas ICA, as determined by indirect immunofluorescence of human cryocut pancreatic sections, was likely to represent multiple autoantigens (Genovese et al. [1992] Diabetologia 35:385-388), GAD soon proved to be one of these (Atkinson, Maclaren et al. [1993] J. Clin. Invest. 91:350-356). Insulin, however, was not a component of ICA unless the pancreatic sections were first chemically "fixed" before being used as tissue substrate.

Recently, a 3.6-kb cDNA with a 2,937-bp open reading frame was isolated from a human insulinoma subtraction library (ISL-153) as described by Lan et al. ([1994] DNA and Cell Biology 13:505-514, herein incorporated by reference). The predicted amino acid sequence and in-vitro-translated product (IA-2) of the cDNA revealed a 979-amino acid protein with a PI value of 7.09 and a molecular mass of 105,847 daltons. The protein sequence is consistent with a signal peptide, an extracellular domain, a transmembrane domain and an intracellular domain. The extracellular domain contains an unusual cysteine-rich region following the signal peptide. The intracellular cytoplasmic domain of IA-2 possesses highly conserved regions similar to the catalytic domains in members of the protein tyrosine phosphatase (PTP) family. Northern blot analyses showed that IA-2 mRNA was expressed in five of five freshly isolated human insulinomas, rat and mouse insulinoma cell lines, and in enriched normal mouse islets. It was also found in normal human brain, pituitary, pancreas, and brain tumor cell lines, but not in a variety of other normal or tumor tissues. Based on the sequence and expression data, it appears that IA-2 is a new member of the receptor-type PTP family that is expressed in islet and brain tissues. The involvement of
the molecule in beta cell autoimmunity or IDD was queried but was not disclosed or suggested in that work.

More recently, Payton et al. ([1995] J. Clin. Invest. 96:1506-1511) reported on the relationship of 37 kDa and 40 kDa tryptic fragments of islet antigens in IDD to the IA-2 molecule reported by Lan et al., supra. Payton et al. concluded that the 40 kDa fragment is a tryptic product of the IA-2 molecule, but that the 37 kDa molecule, while sharing some antigenic determinants with the 40 kDa molecule, was a fragment of an as yet unidentified molecule.

The invention provides, for the first time, the amino acid sequence and nucleotide sequence of both the mouse and the human counterparts of a new IDD associated autoantigen, referred to herein as IA-2β. This antigen, alone or in combination with other IDD associated antigens such as IA-2 and GAD65, is useful in the prediction (diagnosis), treatment (therapy), and prevention (prophylaxis) of diabetes.

**Brief Description of the Drawings**

**Figure 1.** Amino acid sequences of mouse IA-2β and IA-2. Protein sequences were lined up with a GCG BESTFIT program. Identical amino acid residues are shown by (||), highly similar amino acids by (:), and similar amino acids by (.). The putative transmembrane segment (TM) is shown in an open box and the PTP core sequence in a shaded box. Residue numbers represent mouse IA-2 (Lu, J., A.L. Notkins, M.S. Lan [1994] Biochem. Biophys. Res. Commun. 204:930-936). The amino acid terminal of the partial mouse IA-2β protein sequence was arbitrarily designated residue number one. Arrows indicate the start of the
intracellular sequences constructed in the pCRII vector for the in vitro translation study.

**Figures 2A-2B.** Northern analysis. Total RNA (20 μg) from normal mouse tissues and cell lines were separated on a 1% agarose/formaldehyde gel, transferred to a Nytran membrane and hybridized with $^{32}$P-labeled IA-2β cDNA probe (2A) or IA-2 cDNA probe (2B). Message sizes of IA-2, 3.8 kb (arrow), and IA-2β, 5.5 kb (arrow), were estimated by ribosomal RNAs. Ribosomal RNAs are shown at the bottom of the blot.

**Figure 3.** Sera from 50 patients with clinically documented IDD and 50 normal controls were tested for autoantibodies to the intracellular domain of murine IA-2β by radioimmunoprecipitation. The intensity of bands with a molecular weight of 41 kDa were scored on a 1+ to 4+ scale. Representative sera with different band-intensities are illustrated in the insert. Diabetic sera (D), control sera (C).

**Figures 4A-4B.** Competitive radioimmunoprecipitation. Figure 4A: Radiolabeled recombinant intracellular domain of IA-2 and IA-2β were immunoprecipitated by two different hyperimmune rabbit antisera or a mouse monoclonal antibody raised against the PTP domain of the IA-2 molecule. Figure 4B: In vitro radiolabeled intracellular domains of IA-2 and IA-2β were precipitated with sera from IDD patients. Sera were pre-incubated with PBS or unlabeled (*) in vitro translated recombinant IA-2 or IA-2β (10-fold excess as compared to radiolabeled antigen) for 2 hours, then the radiolabeled antigen was added. IA-2R and IA-2BR are the respective anti-sense in vitro translation products. "H" represents higher concentration (50-fold excess as compared to radiolabeled antigen) of in vitro
translated recombinant IA-2 or IA-2β for blocking.

**Figure 5.** Trypsin treatment converts IA-2 and IA-2β into 40 and 37 kDa fragments, respectively. In vitro translated full-length IA-2 (lanes 1 and 2), intracellular domain of IA-2 (lanes 3 and 4) and IA-2β (lanes 5 and 6) were trypsinized (50 µg/ml) before electrophoresis on a 10% SDS-PAGE gel.

**Figures 6A-6B.** Blocking of 37/40 kDa tryptic fragments by unlabeled (*) recombinant intracellular domains of IA-2β and/or IA-2. In vitro radiolabeled preparations of αTC-1 and βTC-1 lysates (1x10⁷ cpm) were immunoprecipitated with control or diabetic sera from two different patients (Figure 6A and Figure 6B). Precipitates were treated with trypsin (100 µg/ml) before loading onto a 10% SDS-PAGE gel. Blocking reactions were performed by incubating IDD sera for 2 hours with the unlabeled recombinant intracellular domain of IA-2 or IA-2β before adding the radiolabeled lysate from αTC-1 and βTC-1 cells.

**Brief Description of the Sequences**

**SEQ ID NO. 1** is the amino acid sequence of human IA-2β protein (shorter version).

**SEQ ID NO. 2** is the nucleotide sequence of full length human IA-2β cDNA (shorter version).

**SEQ ID NO. 3** is the amino acid sequence of human IA-2β protein (longer version).

**SEQ ID NO. 4** is the nucleotide sequence of full length human IA-2β cDNA (longer version).
SEQ ID NO. 5 is the amino acid sequence of mouse IA-2β protein.

SEQ ID NO. 6 is the nucleotide sequence of mouse IA-2β cDNA.

Detailed Description of the Invention

The invention described here relates to the detection of antibodies to insulinoma-associated antigen-2 (IA-2), and insulinoma-associated antigen-2β (IA-2β) alone or in combination with other antigens, as an accurate and specific early indicator of the onset of Insulin-dependent Diabetes (IDD).

IA-2 has been recently identified as a member of the transmembrane protein tyrosine phosphatase family. The complete amino acid sequence of IA-2 has been determined and the protein can be expressed as described herein in bacteria or eukaryotic cells.

Of sera from 55 diabetic patients, 45.4% reacted by ELISA with IA-2 expressed as intracellular and extracellular components in E. coli as compared to only 7.5% of normal controls. Analysis of newly-diagnosed (less than one year) IDD patients revealed that 50% reacted with IA-2 as did 38% of patients with longer term diabetes. Reactivity of IDD sera with glutamic acid decarboxylase (GAD65) as compared to reactivity with IA-2 showed that 60% of GAD65-negative IDD sera were positive for IA-2. Further experiments revealed that both rabbit antibody raised to IA-2, and human IDD sera positive for IA-2 antibody and affinity purified by passage through an IA-2 column, specifically stained cells in the pancreatic islets of Langerhans, but not surrounding acinar tissue.
We provide herein a novel cDNA, IA-2β, isolated from a mouse neonatal brain library and a human counterpart thereof cloned using the mouse cDNA. Our data indicates that IA-2β is a major autoantigen in IDD. We have developed a very sensitive radioimmune-precipitation assay to test for autoantibodies to the intracellular domain of IA-2β in sera of patients with IDD and individuals who are at high risk of developing IDD. Our data show that 46% of sera from IDD patients react with the intracellular domain of IA-2β, whereas none of the sera from normal controls react with IA-2β. The intracellular domain of mouse IA-2β yields considerable information. An even higher percentage of IDD sera scored positive when the entire sequence of IA-2β is used, particularly when the human protein is used as the ligand. IA-2β is also useful as a therapeutic reagent in preventing diabetes. Both the diagnostic and therapeutic values of IA-2β autoantigen will have a profound impact on the treatment of the diabetes.

One aspect of the subject invention is the discovery of IA-2β and that it is an autoantigen in IDD and is responsible for some of the staining of islets by islet-cell autoantibody positive sera, especially those negative for reactivity to the GAD₆₅ antigen and the IA-2 antigen. These findings indicate that testing for autoantibodies to GAD₆₅, IA-2, and IA-2β can be used to provide a reliable method for identifying IDD patients using chemical assays which are more reproducible than possible using the indirect immunofluorescence for ICA.

IA-2 is a 105,847 kDa transmembrane protein that belongs to the protein tyrosine phosphatase family. Immunoperoxidase staining with antibody raised against IA-2 confirms that this protein is expressed in human
pancreatic islet cells. The subject invention, the full-length cDNA clone of IA-2 can be expressed in a rabbit reticulocyte transcription/translation system and the recombinant radiolabelled IA-2 used as an antigen to detect autoantibodies by immunoprecipitation. IA-2β can be used in a similar fashion.

IA-2 and IA-2β can be expressed, isolated and used as antigens to produce immune tolerance and/or immunosuppression to ameliorate or prevent IDD. IA-2 or IA-2β may also be introduced into a patient with an adjuvant, such as alum, or as the diphtheria pertussis and tetanus (DPT) vaccine, or any other adjuvant accepted for introduction into people such as to create an immunization to the antigen. Furthermore, these antigens can be expressed in a recombinant viral vaccine or the DNA coding for IA-2 or IA-2β or both could be introduced into an individual for expression in muscle or other cells to achieve immune tolerance and thus prevent or ameliorate IDD. Further, the antigen or fragments thereof can be given intravenously to down regulate autoimmune islet responses and thereby to prevent or treat IDD.

One hundred coded sera were tested by this method, 50 from patients with newly diagnosed IDD and 50 from age-matched normal controls. Sixty-six percent of the sera from patients, but none of the sera from controls, reacted with IA-2. The same diabetic sera tested for autoantibodies to glutamic acid decarboxylase (GAD$_65$Ab) by depletion-ELISA (d-ELISA) and to islet cells by indirect immunofluorescence showed 52% and 68% positivity, respectively. Up to 86% of the IDD patients had autoantibodies to IA-2 and/or GAD$_65$. Patients diagnosed with IDD before age 20 were more likely to have autoantibodies to IA-2 than patients diagnosed after age
20. Over 90% (14 of 15) of sera that were ICA-positive, but GAD$_{65}$ Ab-negative, had autoantibodies to IA-2. Absorption experiments showed that the immunofluorescence reactivity of sera containing ICA was greatly reduced by prior incubation with recombinant IA-2 or GAD$_{65}$ when the respective antibody was present. Thus IA-2 is a major islet cell autoantigen in IDD pathogenesis, and is responsible, in part, for the reactivity of ICA with pancreatic islets. Tests for the detection of autoantibodies to recombinant IA-2 and GAD$_{65}$ have advantages over ICA as a predictor and identifier of patients with IDD.

Full-length IA-2 cDNA expressed in an eukaryotic expression system, can be used to create a radioimmunoassay for detecting autoantibodies to IA-2. Two thirds of our IDD patients had autoantibodies to IA-2, as compared to none of the controls. The radioimmunoassay is considerably more sensitive and specific than an ELISA test which employs the full-length of the intracellular domain of IA-2. Moreover, the radioimmunoassay used here is a liquid-phase assay and is therefore more likely to detect conformational epitopes than solid-phase ELISA. Fragments of the full-length protein can also be used.

We have isolated a novel cDNA, IA-2$\beta$ from a mouse neonatal brain library. The predicted protein sequence revealed an extracellular domain, a transmembrane region and an intracellular domain. Northern analysis showed that the message size of IA-2$\beta$ is approximately 5.5 kb. Its intracellular domain is 376 amino acids long and 74% identical to the intracellular domain of IA-2, a major autoantigen in IDD. A partial sequence of the extracellular domain of IA-2$\beta$ indicates that it differs substantially (only 26% identical) from that of IA-2.
Both molecules are expressed in islets and brain tissue, but based on analysis of islet cell tumor lines, IA-2β appears to be expressed predominantly in β cells, and IA-2 in α cells. The recombinant intracellular domain of IA-2β was tested for reactivity with sera from IDD patients by immunoprecipitation. Forty-six percent (23/50) of diabetic sera, but none of the sera from normal control (0/50) reacted with IA-2β. Competitive inhibition experiments showed that diabetic sera have autoantibodies that recognize both common and distinct determinants on IA-2 and IA-2β.

Many IDD sera are known to immunoprecipitate 37 kDa and 40 kDa tryptic fragments from insulinoma and islet cells. The identity of the precursor protein(s) from which these fragments are derived has remained elusive. The current disclosure shows that treatment of recombinant IA-2β and IA-2 with trypsin yields 37 kDa and 40 kDa fragments of these molecules, respectively, and that these fragments are immunoprecipitated with diabetic sera. Absorption of diabetic sera with unlabeled recombinant IA-2 or IA-2β, prior to incubation with radiolabeled tryptic fragments from insulinoma or glucagonoma cells, blocks the immunoprecipitation of both the radiolabeled 37 kDa and 40 kDa tryptic fragments. We conclude that IA-2β and IA-2 are the precursors of the 37 kDa and 40 kDa islet cell autoantigens, respectively, and that both IA2 and IA-2β are major autoantigens in IDD.

Accordingly, it is noted that IA-2 is a novel receptor-type PTP isolated from a human insulinoma subtraction library (Lan, M.S., J. Lu, Y. Goto, A.L. Notkins [1994] DNA and Cell Biology 13:505-514) and is now known to be a major autoantigen in IDD. We identified 21 PTPs in pancreatic islets, three of which were novel. In
the present disclosure, the entire sequence of the murine intracellular and transmembrane domain and a partial sequence of the extracellular domain of one of these novel PTPs, IA-2β, is revealed. The complete human sequences are disclosed herein. Our data indicates that IA-2β is closely related, but different from IA-2. The intracellular domain of IA-2β shows 74% identity to the intracellular domain of IA-2. The PTP core sequence differs by only one amino acid. A partial sequence (322 amino acids) of the extracellular domain of IA-2β, however, shows only 26% identity to the extracellular domain of IA-2.

As disclosed herein the tissue distribution of IA-2β and IA-2 shows similarities, but also some differences. IA-2β and IA-2 are expressed primarily in pancreatic islets and brain. Of particular interest is the fact that IA-2β is preferentially expressed in our β cell line (βTC-1), whereas IA-2 is preferentially expressed in our α cell line (αTC-1). Accordingly, this disclosure predicts that those proteins may be used differentially in disease diagnosis and treatment, based on whether or not α or β cells are the primary sites for the disease.

The intracellular domain of IA-2β, expressed in a reticulocyte transcription/translation system, was used as antigen to search for autoantibodies in the sera of diabetic patients. Our studies showed that close to 50% of sera from diabetic patients reacted with IA-2β. It is clear that many sera that react with IA-2β also react with IA-2 because of common antigenic determinants. However, screening indicates that there are unique epitopes on both IA-2 and IA-2β, and that certain diabetic sera preferentially recognize one or the other of these autoantigens. Examination of a large number of diabetic
sera reveals the clinical importance of these unique epitopes. The intracellular domain of mouse IA-2β has yielded considerable information. Even more data is obtained when the entire sequence of IA-2β is used, particularly when the human sequence disclosed herein is used. Human IA-2β was isolated, as disclosed herein, using mouse IA-2β cDNA as the probe.

Ongagna, J.C., C. Levy-Marchal [1995] Diabetologia 38:370-375. Since autoantibodies to the 37 kDa and 40 kDa tryptic fragments showed a strong positive correlation, it was suggested that these antibodies bound to epitopes common to both fragments (Christie, M.R., J.A. Hollands, T.J. Brown, B.K. Michelsen, T.L. Delovitch [1993] J. Clin. Invest. 92:240-248). However, the identity of the precursor protein(s) from which these tryptic fragments were derived remained unclear. Recently, Christie reported, based on blocking experiments with the intracellular domain of IA-2, that IA-2 appears to be the precursor of the 40 kDa, but not the 37 kDa fragment (Payton, M.A., C.J. Hawkes, M.R. Christie [1995] J. Clin. Invest. 96:1506-1511). Our data show that recombinant IA-2 and IA-2β yield tryptic fragments of 40 kDa and 37 kDa, respectively. Moreover, both unlabeled recombinant IA-2 and IA-2β block the binding of diabetic sera to the 40 kDa and 37 kDa tryptic fragments prepared from insulinoma cells. Therefore, we conclude that IA-2 is the precursor of the 40 kDa and IA-2β is the precursor of 37 kDa tryptic fragment, respectively.

The cloning and sequencing of IA-2 and IA-2β, as well as the identification of these molecules as major autoantigens, are critical steps in elucidating their role in the pathogenesis of IDD. The development of a panel of autoantibody assays using recombinant IA-2 and/or IA-2β together with recombinant GAD₆ permits screening large populations and assessing their relative predictive values in identifying individuals at high risk for IDD.

According to the disclosure provided herein, it is clear that one of ordinary skill could use either the murine or human sequences of IA-2 or IA-2β to clone the IA-2 or IA-2β sequences of other mammalian species. The
IA-2 or IA-2β from these other sources could then be used in the therapeutic and diagnostic procedures of the subject invention. In addition, as those of ordinary skill in the art will appreciate, any of a number of different nucleotide sequences can be used, based on the degeneracy of the genetic code, to produce the IA-2β protein. Accordingly, any nucleotide sequence which encodes full-length mammalian IA-2β comes within the scope of this invention and the claims appended hereto.

Also, as described herein, fragments of IA-2 and IA-2β are an aspect of the subject invention so long as such fragments retain the immunological activity so that such fragments are useful in therapeutic and diagnostic procedures as described herein. Such fragments can easily and routinely be produced by techniques well known in the art, for example, by time-controlled Bal31 exonuclease digestion of the full-length DNA, followed by expression of the resulting fragments and routine screening of the expression products for the desired activity. An antigenic fragment can be selected by applying the routine technique of epitope mapping to IA-2β to determine the regions of the proteins that contain epitopes reactive with serum antibodies or are capable of eliciting an immune response in an animal. Once the epitope is selected, an antigenic polypeptide containing the epitope can be synthesized directly, or produced recombinantly by cloning nucleic acids encoding the polypeptide in an expression system, according to the standard methods. Alternatively, an antigenic fragment of the antigen can be isolated from the whole antigen or a larger fragment by chemical or mechanical disruption. Fragments can also be randomly chosen from a known IA-2β sequence and synthesized. The purified fragments thus obtained can be tested to determine their antigenicity and specificity by
routine methods.

The term "nucleic acid" refers to a deoxyribonucleotide or ribonucleotide polymer in either single- or double-stranded form, and unless otherwise limited, would encompass known analogs of natural nucleotides that can function in a similar manner as naturally-occurring nucleotides.

The phrase "nucleic acid encoding" or "nucleic acid sequence encoding" refers to a nucleic acid which directs the expression of a specific protein or peptide. The nucleic acid sequences include both the DNA strand sequence that is transcribed into RNA and the RNA sequence that is translated into protein. The nucleic acid sequences include both full-length nucleic acid sequences as well as shorter sequences derived from the full-length sequences. It is understood that a particular nucleic acid sequence includes the degenerate codons of the native sequence or sequences which may be introduced to provide codon preference in a specific host cell. The nucleic acid includes both the sense and antisense strands as either individual strands or in the duplex. The terms "hybridize" or "hybridizing" refer to the binding of two single-stranded nucleic acids via complementary base pairing.

The phrase "hybridizing specifically to" refers to binding, duplexing, or hybridizing of a molecule only to a particular nucleotide sequence under stringent conditions when that sequence is present in a preparation of total cellular DNA or RNA.

The term "stringent conditions" refers to conditions under which a probe will hybridize to its target sub-
sequence, but to no other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (Tm) for the specific sequence at a defined ionic strength and pH. The Tm is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a complementary probe. Typically, stringent conditions will be those in which the salt concentration is at least about 0.1 to 1.0 N Na ion concentration at pH 7.0 to 7.5 and the temperature is at least about 60°C for long sequences (e.g., greater than about 50 nucleotides) and at least about 42°C for shorter sequences (e.g., about 10 to 50 nucleotides).

The terms "isolated" or "substantially pure" when referring to nucleic acid sequences encoding IA-2 or IA-2β proteins or fragments thereof refers to isolated nucleic acids which do not encode proteins or peptides other than IA-2 or IA-2β proteins or peptides.

The terms "isolated" or "substantially purified" when referring to IA-2 or IA-2β proteins, means a chemical composition which is essentially free of other cellular components. It is preferably in a homogenous state although it can be in either a dry or aqueous solution. Purity and homogeneity are typically determined using analytical chemistry techniques such as polyacrylamide gel electrophoresis or high performance liquid chromatography. A protein which is the predominant species present in a preparation is substantially purified. Generally, a substantially purified or isolated protein will comprise more than 80% of all macromolecular species present in the preparation. Preferably, the protein is purified to
represent greater than 90% of all macromolecular species present. More preferably, the protein is purified to
greater than 95%, and most preferably the protein is
purified to essential homogeneity, wherein other
macromolecular species are not detected by conventional
techniques.

The phrase "specifically binds to an antibody" or
"specifically immunoreactive with," when referring to a
protein or peptide, refers to a binding reaction which is
determinative of the presence of the protein in a
heterogeneous population of proteins and other biologics.
Thus, under designated immunoassay conditions, the
specified antibodies bound to a particular protein do not
bind in a significant amount to other proteins present in
the sample. Specific binding to an antibody under such
conditions may require an antibody that is selected for
its specificity for a particular protein. A variety of
immunoassay formats may be used to select antibodies
specifically immunoreactive with a particular protein.
For example, solid-phase ELISA immunoassays are routinely
used to select antibodies specifically immunoreactive with
a protein. See Harlow and Lane (1988) Antibodies, A
Laboratory Manual, Cold Spring Harbor Publications, New
York, for a description of immunoassay formats and
conditions that could be used to determine specific
immunoreactivity. The subject invention further concerns
antibodies raised against the purified IA-2 or IA-2β
molecules or their fragments.

The term "biological sample" as used herein refers to
any sample obtained from a living organism or from an
organism that has died. Examples of biological samples
include body fluids, tissue specimens, and tissue cultures
lines taken from patients.
The term "recombinant DNA" or "recombinantly-produced DNA" refers to DNA which has been isolated from its native or endogenous source and modified either chemically or enzymatically to delete naturally-occurring flanking nucleotides and/or to provide flanking nucleotides that do not naturally occur. Flanking nucleotides are those nucleotides which are either upstream or downstream from the described sequence or sub-sequence of nucleotides.

The term "recombinant protein" or "recombinantly-produced protein" refers to a peptide or protein produced using non-native cells that do not have an endogenous copy of DNA able to express the protein. The cells produce the protein because they have been genetically altered by the introduction of the appropriate nucleic acid sequence. The recombinant protein is not found naturally in association with proteins and other subcellular components normally associated with the cells producing the protein.

The following terms are used to describe the sequence relationships between two or more nucleic acids or polynucleotides: "reference sequence," "comparison window," "sequence identity," and "percentage of sequence identity."

A "reference sequence" is a defined sequence used as a basis for a sequence comparison; a reference sequence may be a subset of a larger sequence, for example, as a segment of a full-length cDNA or gene sequence given in a sequence listing, or may comprise a complete cDNA or gene sequence.

"Percentage of sequence identity" is determined by comparing two optimally aligned sequences or sub-sequences over a comparison window or span, wherein the portion of
the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical subunit (e.g., nucleic acid base or amino acid residue) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison, and multiplying the result by 100 to yield the percentage of sequence identity. Percentage sequence identity when calculated using the programs GAP or BESTFIT is calculated using default gap weights. In a preferred embodiment, IA-2β sequences of the subject invention will have at least about 80% sequence identity with SEQ ID NO. 3 or SEQ ID NO. 4.

When percentage of sequence identity is used in reference to proteins or peptides, it is recognized that residue positions which are not identical may differ by conservative amino acid substitutions where amino acid residues are substituted for other amino acid residues with similar chemical properties (e.g., charge or hydrophobicity) and therefore do not change the functional properties of the molecule. Where sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of this substitution. Means for making this adjustment are well known to those of ordinary skill in the art. Typically, this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity.

Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given
a score of 0, a conservative substitution is given a score between 0 and 1. The scoring of conservative substitutions is calculated according to the algorithm of Meyers and Milleer (1988) *Computer Applic. Biol. Sci.* 4:11-17 as implemented in the program PC/GENE (Intelligenetics, Mountain View, CA, USA). The following six groups each contain amino acids that are conservative substitutions for one another:

1. Alanine (A), Serine (S), Threonine (T);
2. Aspartic acid (D), Glutamic acid (E);
3. Asparagine (N), Glutamine (Q);
4. Arginine (R), Lysine (K);
5. Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and
6. Phenylalanine (F), Tyrosine (Y), Tryptophan (W).

A "comparison window," as used herein, refers to a segment of at least about 20 contiguous positions, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned.

Methods of alignment of sequences for comparison are well known in the art. Optimal alignment of sequences for comparison may be conducted by the local homology algorithm of Smith and Waterman (1981) *Adv. Appl. Math.* 2:482, which is incorporated herein by reference; by the homology alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48:443, which is incorporated herein by reference; by the search for similarity method of Pearson and Lipman (1988) *Proc. Natl. Acad. Sci. USA* 85:2444, which is incorporated herein by reference; by computerized implementations of the algorithms (including, but not limited to, CLUSTAL in the PC/GENE program by
Intelligenetics, GAP, BESTFIT, PASTA, and TFASTA in Wisconsin Genetics Software Package, Genetics Computer Group (GCG), 575 Science Drive, Madison, WI, USA) or by inspection. In particular, methods for aligning sequences using the CLUSTAL program are well described by Higgins and Sharp (1988) Gene 73:237-244 and (1989) CABIOS 5:151-153, both of which are incorporated herein by reference.

Expression of IA-2 or IA-2β proteins. Once DNA encoding IA-2 or IA-2β proteins is isolated and cloned, one can express the IA-2β proteins in a variety of recombinantly engineered cells. It is expected that those of ordinary skill in the art are knowledgeable in the numerous expression systems available for expression of DNA encoding IA-2 or IA-2β proteins.

In brief summary, the expression of natural or synthetic nucleic acids encoding proteins will typically be achieved by operably linking the DNA or cDNA to a promoter (which is either constitutive or inducible), followed by incorporation into an expression vector. The vectors can be suitable for replication and integration in either prokaryotes or eukaryotes. Typical expression vectors contain transcription and translation terminators, initiation sequences, and promoters useful for regulation of the expression of polynucleotide sequences encoding IA-2 or IA-2β proteins. To obtain high level expression of a cloned gene such as those polynucleotide sequences encoding IA-2 or IA-2β proteins, it is desirable to construct expression plasmids which contain at the minimum a strong promoter to direct transcription, a ribosome binding site for translational initiation, and a transcription/translation terminator. The expression vectors may also comprise generic expression cassettes containing at least one independent terminator sequence,

Expression in prokaryotes. A variety of prokaryotic expression systems may be used to express IA-2 or IA-2β proteins. Examples include E. coli, Bacillus, Streptomyces, and the like. For example, IA-2 or IA-2β proteins may be expressed in E. coli.


Proteins produced by prokaryotic cells may not
necessarily fold properly. During purification from *E. coli*, the expressed protein may first be denatured and then renatured. This can be accomplished by solubilizing the bacterially produced protein in a chaotropic agent such as guanidine HCl and reducing all the cysteine residues with a reducing agent such as β-mercaptoethanol. The protein is then renatured, either by slow dialysis or by gel filtration. See U.S. Patent No. 4,511,503.

Detection of the expressed protein is achieved by methods known in the art such as radioimmunoassay or Western blotting techniques or immunoprecipitation. Purification for *E. coli* can be achieved following procedures described in U.S. Patent No. 4,511,503.

**Expression in eukaryotes.** A variety of eukaryotic expression systems such as yeast, insect cell lines, bird, fish, and mammalian cells, are known to those of ordinary skill in the art. As explained briefly below, IA-2 or IA-2β proteins may be expressed in these eukaryotic systems.


Suitable vectors usually have expression control sequences, such as promoters, including 3-phosphoglycerate kinase or other glycolytic enzymes, and an origin of replication, termination sequences, and the like, as desired. For instance, suitable vectors are described in the literature (Botstein *et al.* [1979] Gene 8:17-24; Broach *et al.* [1979] Gene 8:121-133).
Two procedures are used in transforming yeast cells. In one case, yeast cells are first converted into protoplasts using zymolase, lyticase, or glucosylase, followed by addition of DNA and polyethylene glycol (PEG). The PEG-treated protoplasts are then regenerated in a 3% agar medium under selective conditions. Details of this procedure are given in the papers by Beggs, J.D. (1978) Nature 275:104-109 and Hinnen et al. (1987) Proc. Natl. Acad. Sci. USA 75:1929-1933. The second procedure does not involve removal of the cell wall. Instead, the cells are treated with lithium chloride or acetate and PEG and put on selective plates (Ito et al. [1983] J. Bact. 153:163-168).

IA-2 or IA-2β proteins, once expressed, can be isolated from yeast by lysing the cells and applying standard protein isolation techniques to the lysates. The monitoring of the purification process can be accomplished by using Western blot techniques or radioimmunoassay or other standard immunoassay techniques.

The sequences encoding IA-2 or IA-2β proteins can also be ligated to various expression vectors for use in transforming cell cultures of, for example, mammalian, insect, bird, or fish origin. Illustrative of cell cultures useful for the production of the polypeptides are mammalian cells. Mammalian cell systems often will be in the form of monolayers of cells though mammalian cell suspensions may also be used. A number of suitable host cell lines capable of expressing intact proteins have been developed in the art, and include the HEK293, BHK21, and CHO cell lines, and various human cells such as COS cell lines, HeLa cells, myeloma cell lines, Jurkat cells, etc. Expression vectors for these cells can include expression control sequences, such as an origin of replication, a
promoter (e.g., the CMV promoter, a HSC tk promoter or pgk [phosphoglycerate kinase] promoter), an enhancer (Quenn et al. [1986] Immunol. Rev. 89:49), and necessary processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites (e.g., an SV40 large T ag ply A addition site), and transcriptional terminator sequences. Other animal cells useful for the production of proteins are available, for example, from the American Type Culture Collection Catalogue of Cell Lines and Hybridomas (7th Edition, 1992).

Appropriate vectors for expressing proteins in insect cells are usually derived from the SF9 baculovirus. Suitable insect cell lines include mosquito larvae, silkworm, armyworm, moth, and Drosophila cell lines such as a Schneider cell line (see Schneider, J. [1987] Embryol. Exp. Morphol. 27:353-365).

As indicated above, the vector, e.g., a plasmid, which is used to transform the host cell, preferably contains DNA sequences to initiate transcription and sequences to control the translation of the protein. These sequences are referred to as expression control sequences.

As with yeast, when higher animal host cells are employed, polyadenylation or transcription terminator sequences from known mammalian genes need to be incorporated into the vector. An example of a terminator sequence is the polyadenylation sequence from the bovine growth hormone gene. Sequences for accurate splicing of the transcript may also be included. An example of a splicing sequence is the VP1 intron from SV40 (Sprague et al. [1983] J. Virol. 45:773-781).
Additionally, gene sequences to control replication
in the host cell may be incorporated into the vector such
as those found in bovine papilloma virus-type vectors.
213-238.

The host cells are rendered competent or rendered
competent for transformation by various means. There are
several well-known methods of introducing DNA into animal
cells. These include: calcium phosphate precipitation,
fusion of the recipient cells with bacterial protoplasts
containing the DNA, treatment of the recipient cells with
liposomes containing the DNA, DEAE dextran,
electroporation and micro-injection of the DNA directly
into the cells.

The transformed cells are cultured by means well
known to one of ordinary skill in the art. Kuchler, R.J.
(1977) Biochemical Methods in Cell Culture and Virology,
Hutchinson and Ross, Inc. The expressed polypeptides are
isolated from cells grown as suspensions or monolayers.
The latter are recovered by well-known mechanical,
chemical, or enzymatic means.

Following are examples which illustrate procedures,
for practicing the invention. These examples should not
be construed as limiting. All percentages are by weight
and all solvent mixture proportions are by volume unless
otherwise noted.

Example 1 – Evidence That Autoantibodies to IA-2 Occur in
Patients With IDD

The intracellular (a.a. 603-979) and extracellular
(a.a. 129-472) domains of IA-2 were expressed as fusion
proteins with glutathione transferase (GST) from separate subclones in a pGEX bacterial expression vector. The cDNAs were verified by direct DNA sequencing, and the fusion proteins were induced by IPTG and purified by glutathione-agarose (Sigma). The fusion proteins were further processed by cleavage with human thrombin (Sigma) and the GST fusion partner removed by passage through a glutathione-agarose affinity column.

New Zealand rabbits were immunized against the extra and intra-cellular fragments of IA-2 to provide hyperimmune sera. An ELISA for IA-2 autoantibodies was developed as follows. Polyvinyl microtiter plates (Becton-Dickerson, Oxnard, CA) were coated with 0.4 μg/100 μl/well of the purified IA-2 fragments or GST expression proteins. The sera from 55 patients with IDD and 53 normal control persons were tested. Some 34% of the patients had IDD diagnosed within a year. One hundred μg of the human sera was diluted 1:50 (IC domain) or 1:100 (EC domain) in BLOTTO-Tween (10 mM Tris-HCL, pH 8.0; 150 mM NaCl; 5% Carnation nonfat dry milk; 0.05% Tween 20; 0.05% NaN₃) and reacted with the antigens. The autoantibodies were in turn detected using a 1:2000 dilution of alkaline phosphatase labelled goat anti-human IgG (Southern Biotechnology Assoc. Birmingham AL) in BLOTTO-Tween. Polyacrylamide gel electrophoresis of the recombinant IA-2 fragments revealed bands of the predicted molecular weights, as confirmed by reactivity with the specific polyvalent rabbit antibodies, which also stained pancreatic islets. Of the patients, 21 reacted to the intracellular domain and 10 to the extracellular domain of IA-2, compared to 3 and 2 control sera respectively while 6 patients but only 1 control serum reacted to both fragments. Thus 25 patients (45.5%) had autoantibodies to the IA antigen overall, compared to only 4 controls.
(7.5%). Of the newly diagnosed patient group, 17 of 34 (50%) had IA-2 autoantibodies, while fewer (38.1%) had antibodies persisting beyond 1 year after diagnosis. Autoantibodies were also determined to GAD$_{65}$ produced by a baculoviral insect cell expression system, using a depletion ELISA assay (d-ELISA). Some 20 of the patients (37.7%) were found to be negative while all of the controls were negative. Strikingly, 21 of 29 IA-2 autoantibody negative patients (72%) were found to be GAD$_{65}$ autoantibody positive. Therefore, determination of both antibodies would correctly identify considerably more of the patients than would be possible by either antibody alone.

Table I.

Reactivity of Sera From Diabetic Patients to IA-2 in Relationship to the Presence or Absence of Autoantibodies of GAD$_{65}$

<table>
<thead>
<tr>
<th>Diabetic Patients (n=53)</th>
<th>IA-2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td>GAD$_{65}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>20</td>
<td>37.7</td>
</tr>
<tr>
<td>Positive</td>
<td>33</td>
<td>62.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive*</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>60</td>
</tr>
<tr>
<td>Negative</td>
<td>8</td>
<td>40</td>
</tr>
<tr>
<td>Positive</td>
<td>21</td>
<td>64</td>
</tr>
</tbody>
</table>

(*) - Samples with reactivity to the intracellular and/or extracellular domains of IA-2.

Example 2 - Evidence That IA-2 is a Major Autoantigen in IDD

Since IA-2 is an integral membrane protein expressed in its native state in restricted cell types in humans, the full length cDNA was next expressed in an eukaryotic rabbit reticulocyte transcription/translation system, and the recombinant protein radiolabelled with S$^{35}$ methionine
was used to detect IA-2 autoantibodies by immunoprecipitation. The full length cDNA without its leader sequence was cloned into a pCRll cloning vector (Invitrogen, San Diego CA) with a perfect Kozak translational start sequence (GCCGCCACCATGG). One microgram of plasmid DNA was added to TNT coupled rabbit reticulocyte lysate system (Promega, Madison WI) in the presence of [35S] methionine (Amersham, Arlington Heights, IL) at 30°C for 2 hours. The translated reticulocyte lysate (at approximately 50,000-75,000 cpm) and 5 µl of each test serum was mixed in 100 µl of immunoprecipitation buffer (20 mM Tris, pH 7.4, 150 mM NaCl and 1% Triton X-100). The reaction mixture was incubated overnight and 50 µl of 50% (v/v) protein A-agarose (Life Technologies, Gaithersburg, MD) was added to the solution at 4°C for one hour. After washing four times with immunoprecipitate buffer, the immunoprecipitation mixture was boiled in sample buffer and applied to an 8% SDS-PAGE gel. The gels were fixed with acetic acid/methanol (12.5%/12.5%) and then exposed to X-ray sensitive film overnight. The intensity of the IA-2 bands (approximately 106 kDa) was scored independently from 1-4+ by two independent investigators. One hundred coded sera comprising 50 from newly diagnosed patients and an equal number of matched controls were studied. Using this method, 66% of the patient sera but none of the controls were positive for autoantibodies to IA-2. Autoantibodies to GAD65 were also performed by a D-ELISA method, and 52% were positive. In all, 86% of the patient sera but none of the controls were positive for autoantibodies to GAD65 and/or IA-2, with 34% being positive to both antigens. There was an age-associated bias to the results. Of the patients diagnosed before age 20 years, 68% had IA-2 autoantibodies and 60% GAD65 autoantibodies. However, of the patients diagnosed after age 20 years, only 46% were positive for IA-2
autoantibodies while 86% were positive for GAD₆₅ autoantibodies. These results were greatly improved from the earlier study using ELISA assays and IA-2 fragments. There are several possible explanations. It is probable that the disease associated autoantibodies react to the antigen through conformational epitopes. Thus reactivity may be greatly enhanced using the whole protein rather than its fragments. The method also involves antibody reactivities with the IA-2 protein in its native undenatured state, conditions which enhance reactions to conformational epitopes. Such is also the case for autoantibody reactivities to GAD₆₅.

Example 3 — Evidence That IA-2 and GAD₆₅ Are Antigens That Are Components of the ICA Reaction

There was an excellent correlation between occurrence of autoantibodies either to IA-2 or GAD₆₅ and ICA, in that only one of 15 ICA positive sera that did not react to GAD₆₅ was not positive for IA-2 autoantibodies. This suggested that GAD₆₅ and IA-2 are component antigens of the ICA response. This conclusion is proven by an experiment in which 6 sera were selected because they were positive for ICA, and only GAD₆₅ (n=2) or only IA-2 (n=4) and subjected to absorption studies. Recombinant baculoviral expressed human GAD₆₅ was used to absorb out the corresponding autoantibody before the sera were applied to the ICA reaction. We found that this procedure reduced the ICA reactivity only of the sera which were found to be positive for GAD₆₅ autoantibodies, as expected from Atkinson et al. ([1993] J. Clin. Invest. 91:350-356). Conversely, we also absorbed out the 6 sera after passage through an IA-2 affinity column to remove autoantibodies to IA-2, before applying them to pancreatic sections for the ICA reaction, and found only those with autoantibodies to IA-2 to be reduced.
Table II. Absorption of ICA-Containing Sera with rIA-2 and rGAD$_{65}$

<table>
<thead>
<tr>
<th>Patient Serum</th>
<th>Reactivity of Sera With</th>
<th>Absorption of ICA-Containing Sera With</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Islet Cells</td>
<td>rIA-2</td>
</tr>
<tr>
<td>1</td>
<td>Pos</td>
<td>Pos</td>
</tr>
<tr>
<td>2</td>
<td>Pos</td>
<td>Pos</td>
</tr>
<tr>
<td>3</td>
<td>Pos</td>
<td>Pos</td>
</tr>
<tr>
<td>4</td>
<td>Pos</td>
<td>Pos</td>
</tr>
<tr>
<td>5</td>
<td>Pos</td>
<td>Neg</td>
</tr>
<tr>
<td>6</td>
<td>Pos</td>
<td>Neg</td>
</tr>
</tbody>
</table>

Reactivity of ICA-containing sera with islet cells as measured by intensity of immunofluorescence: greatly reduced (↓); reduced (↓); slightly reduced (↓); no change (_).

These experiments document that GAD$_{65}$ and IA-2 are both antigens involved in the ICA response; however, some sera showed that double absorptions with both antigens did not ablate any of the ICA responses, suggesting that autoantibodies to additional autoantigen(s) must be often present in ICA positive sera. A candidate antigen is IA-2β. Preliminary data in fact suggest that more than 45% of sera from patients with IDD are autoantibody positive, compared to none of normal controls.

Example 4 – Evidence That Autoantibodies to IA-2 Are Useful to Predict IDD

The human leukocyte antigens HLA-DR/DQ have been analyzed by a novel PCR based molecular typing system in more than 50 subjects with IDD. The high risk DRB1*03/DQB1*0201 and DRB1*04/DQB1*0302 haplotypes were over-represented in those positive for either
autoantibodies to GAD$_{65}$ and/or IA-2. Thus, risk for IDD among patients and their relatives indicated a strong association between the IDD associated high risk HLA and appearance of these autoantibodies suggesting that the autoantibodies themselves would be expected to be associated with a high risk for IDD also.

In studies with 2500 normal school children and a similar number of first degree relatives of patients with IDD, we found that testing autoantibodies to IA-2, GAD$_{65}$, IAA and ICA, the combined predictive power of the IA-2 and GAD$_{65}$ test was greater than 90% in both groups for subsequent development of IDD.

Example 5 — Therapeutic Regimens

From the current disclosure and experimental results it is evident that IA-2 and IA-2β are major antigens in the pathogenesis of IDD, and are thus valuable in the diagnosis and therapy of the disease. Autoantibodies to these proteins are useful in disease prediction both in non-diabetic relatives of patients affected by IDD, as well as in the general population. Such autoantibodies may react mainly to determinants on the intracellular domain of IA-2 or IA-2β. Such antibodies will be detectable by radioimmunoassay using recombinant IA-2 or IA-2β, depletion or D-ELISA and/or by ELISA or immunoprecipitation as outlined here. Based upon experience of the inventors with GAD$_{65}$ and insulin autoantibody analyses, baculoviral based eukaryotic expression systems are likely to be preferred to fold the protein appropriately, and glycosylate the protein if this enhances the antibody binding ability. However, such expression systems as COS cells, yeast cells, and bacterial cells such as E. coli could be used for this purpose as those skilled in the art are readily able to
appreciate.

Fluid-based immunoassays using the antigens and antibodies of this invention provide the greatest sensitivity to the method since autoantibodies of relevance mostly react to their respective autoantigen through conformational rather than through linear epitopes. The RIA and d-ELISA methods are most useful in fulfilling these properties. Accordingly, autoantibodies to human GAD_{65}, IA-2, and IA-2β antigens can replace the ICA method for prediction of IDD, with chemically based assays giving greater precision, reproducibility, and specificity without compromising sensitivity or positive predictive power.

Cellular responses, such as by proliferation or by cytokine elaboration after in vitro exposure to IA-2 or IA-2β, are also useful in disease prediction.

The IA-2 or IA-2β molecule or peptide derivatives of IA-2 or IA-2β are used in antigen based therapies, including giving the antigens intravenously to induce anergy; deliberately immunizing against the antigen such as to induce an antibody response mediated by T helper-2 type lymphocytes to induce immunosuppressive effects on the pathogenic T helper-1 lymphocyte subset; or orally fed antigen such as to induce anergy and suppressive effects. Intravenous GAD_{65} antigens have been given in early life in NOD mice and shown to induce reductions in the degree of the inflammatory infiltrates or insulitis lesions and prevent the onset of hyperglycemia (Kauffman et al. [1994] Nature 366:69-72). Subcutaneous immunizations by insulin and insulin B chains in incomplete Freund's adjuvant will prevent diabetes in NOD mice for prolonged periods without reductions in the insulitis lesions. The infiltrating
cells however change their phenotype from ones that make large amounts of interferon gamma to ones that do not. Transfer of splenic lymphocytes from mice protected from diabetes in this way also convey protection for periods of up to a month. The intervention thus induces an active immunosuppressive effect and an insulitis lesion that is protective rather than destructive associated with a switch from Th1 to Th2 responses. Further, the effect must be beyond that merely involving autoimmunity to insulin, since beta cell destruction is arrested. The release of protective cytokines into the milieu of the islet must then also inhibit adjacent autoimmunity responses to other self-antigens through a bystander effect (Muir, Maclaren et al. [1995] J. Clin. Invest. 95:628-634; Ramiya, Muir, Maclaren [1995] Clin. Immunotherapy 3:177-183.) Repeated feedings of defined autoantigens may also be used to inhibit ongoing autoimmune diseases. In the case of the NOD mice, this has occurred through orally administered insulin (Weiner et al. [1991] Proc. Natl. Acad. Sci. USA 88:10252-10256) as well as through the use of oral feedings of insulin and GAD (Muir, Maclaren et al. [1994] Diabetes/Metabolism Reviews 9:279-287). Accordingly, therapeutic methods employing IA-2 or IA-2β, alone or in combination with other antigens, improves the therapeutic efficacy of such treatments by providing an additional component of the ICA reaction.

Example 6 — Collection of Biological Fluid for Detection of Autoantibodies

A volume of greater than 500 microliters of whole blood is collected from the individual to be tested for IA-2 or IA-2β autoantibodies. The blood is drawn into a glass vacutainer tube directly, or into a syringe followed by transfer into a glass vacutainer tube. In order to
obtain sera (blood devoid of clotting factors), the common vacutainer tubes used are termed a red top tube (devoid of sodium heparin), or a serum separator (STS) tube. If a common red top tube is used, the tube is allowed to clot (a period of greater than 10 minutes), and the clot removed. At this period of time, either sample tube may be centrifuged for 5 minutes at 1000 rpm at room temperature. The serum within the sample is removed and placed into a plastic storage vial and sealed tightly. The sample can be frozen at -20° until IA-2 or IA-2β autoantibody analysis.

**Example 7 — Methods of Detecting IA-2 or IA-2β Antibodies**

In addition to the use of immunoprecipitation techniques, the subject invention can be practiced utilizing any other procedures which facilitate detecting the presence of antibodies to IA-2 or IA-2β. For example, other immunological methods which can be used include enzyme linked immunosorbent assay (ELISA) and radioimmunoassay (RIA). The principles and experimental methods of these procedures are well known to those skilled in the art. The assays can be carried out rapidly and efficiently by the use of natural or recombinant proteins which bind with the antibodies to IA-2 or IA-2β. Both whole cell and cell lysate procedures are familiar to those working in this field and can be readily employed to detect the IA-2 or IA-2β antibodies.

The amino acid sequences of IA-2 and IA-2β can be analyzed to ascertain immunologically reactive epitopes. These epitopes are amino acid sequences which will react immunologically with the antibodies to IA-2 or IA-2β. These sequences can then be produced recombinantly. For recombinant production, the DNA coding for the epitopes is inserted into a vector which is then used to transform an
appropriate host cell to express the desired amino acid sequence. Although bacteria, insects, yeasts, and mammalian cells could all serve as appropriate hosts, if protein folding is an important factor in the reactivity of the epitope, then a eukaryotic cell would be a preferred host.

Purified protein or lysate of the cells producing the protein could be used for the assays.

Also, an alternative to using IA-2 or IA-2β antigens would be to use antibodies generated to IA-2 or IA-2β, otherwise known as an anti-antibody. This antibody would immunoprecipitate with IA-2 or IA-2β, and the detection could be carried out as described above.

Example 8 – Treatment of IDD

The specific event or agent which triggers the onset of diabetes has not been identified. A virus carrying an antigen similar to the IA-2 or IA-2β protein may provoke both a normal immune response to the virus and also an abnormal, autoimmune response to IA-2 or IA-2β through its molecular mimicry with the viral proteins. The genetic susceptibility is thus expressed by an exaggerated or prolonged immune response to the environmental agent which initiates the disease process. It is also possible that the IA-2 or IA-2β protein may have a delayed expression in the development of islet cells in ontogeny, rendering it antigenic because tolerance to it would not have been developed in the early stages of life.

A novel therapy of the subject invention involves the injection into the bloodstream of a toxin bound to a purified form of the IA-2 or IA-2β antigen. The antigen-toxin complex would quickly reach the lymph nodes where it
is taken up by immune cells that normally produce the antibodies to IA-2 or IA-2β. Also, the antigen-toxin complex would be bound by the T-lymphocytes that recognize the antigens on β-cells. Thus, the specific immune cells involved in β-cell destruction are poisoned and inactivated, leaving non-destructive immune cells unharmed. The hybrid protein could comprise, for example, a diphtheria toxin joined together with the IA-2 or IA-2β antigen. The construction of such a hybrid toxin could proceed, for example, according to the disclosure of United States Patent Number 4,675,382 (Murphy) relating to hybrid proteins.

In a preferred method of the subject invention, prevention or treatment involves the administration of autoantigens to the susceptible individual. IDD has an autoimmune etiopathogenesis, as discussed above. Various mechanisms have been proposed that would account for the beneficial value of administering autoantigens as a preventive treatment. In addition, it is also well known in the art that the administration of autoantigens can be used to induce immunological non-responsiveness, that is, specific tolerance of the antigen. See U.S. Patent No. 5,114, 844; Nagler-Anderson et al. (1986) Proc. Natl. Acad. Sci USA 83:7443-7446; Miller et al. (1984) Clin. Immunol. Immunopathol. 31:231-240; Silverman et al. (1983) J. Immunol. 131:2651-2661; Michael (1989) Immune Invest. 18:1049-1054. The administration of the IA-2 or IA-2β antigens according to the subject invention can be done using procedures, formulations, and administration routes well known in the art. As one skilled in the art having the benefit of this disclosure would appreciate, the administration of the IA-2 or IA-2β protein or peptide can be by, for example, parenteral, oral, intranasal, or by modification of the patient's genome to express an
antigenic epitope.

**Example 9 - Use of IA-2 Antibodies in Conjunction with Pancreas Transplantation**

One approach for treatment of a patient with IDD is to transplant normal islets as replacements for the damaged or destroyed β-cells. Segmental and whole pancreas transplantations have been performed successfully in a number of patients with diabetes. However, permanent immunosuppressive therapy is required to maintain the grafts and prevent rejection. Segmental or whole pancreas transplants under continuous immunosuppressive therapy have produced normal levels of blood glucose in some patients with diabetes. Pancreatic transplants are done late in the course of diabetes and will probably not reverse complications such as nephropathy and indeed may worsen retinopathy.

Importantly, successful pancreatic grafts between identical twins have been maintained without immunosuppressors; however, autoimmune islet cell destruction has occurred with recurrence of diabetes. Thus, even when the graft is not rejected, there is obligatory need for immunotherapies to prevent disease recurrence. The destruction (rejection) of transplanted islets may be due, at least in part, to the representation of autoantigens responsible for the autoimmune destruction. There is no specific immunotherapy to prevent the autoimmune destruction (rejection of transplanted islets/pancreas) at present. In order to prevent the autoimmune destruction of either transplanted islet cells or pancreas, a specific immunotherapy using a hybrid toxin or tolerance strategy, as detailed above, can be used to prevent islet cell destruction. The combined use of the immunotherapies
could make islet cell/pancreas transplantation a therapeutic tool for the treatment of IDD.

Example 10 – Kits for Assay of IA-2 or IA-2β Autoantibodies and IDD

A reagent kit can be provided which facilitates convenient analysis of serum samples using the novel procedures described here. Kits can be prepared which utilize recombinant or synthetically produced intact IA-2 or IA-2β protein(s) or immunoreactive peptides to serve as an antigen for the detection of antibodies to IA-2 or IA-2β. Alternatively, antibodies specifically developed to detect antibodies to IA-2 or IA-2β may also be useful. The principles and methods for ELISA and RIA technologies to detect antibodies are well-established.

As an example, for the ELISA assay, one such kit could comprise the following components:

1. IA-2 protein, peptide, or antibodies to IA-2 antibodies;
2. Enzyme (e.g., peroxidase);
3. Conjugated animal anti-human immunoglobulin; and
4. Positive and negative controls.

The above kit could be modified to include 96 well plastic plates, colorimetric reagents, ELISA readers, blocking reagents, and wash buffers. Inclusion of GAD₆₅ antigen would also be highly preferred.

Also by way of example, for the RIA, one such kit could comprise the following components:

1. Radiolabeled IA-2 protein(s), peptide, or antibodies to IA-2 antibodies;
2. Wash buffers;
3. Polyethylene glycol (PEG);
4. Goat or sheep antihuman precipitating (second) antibodies; and
5. Positive and negative controls.

Either of the above kits may be modified to include any appropriate laboratory supplies or to exclude non-essential compounds such as the buffers, PEG, or controls. Presence of IA-2 autoantibodies as detected by using this kit is indicative of IDD or susceptibility to IDD, especially if in addition, GAD_{65} reactive autoantibodies are detected. To preserve the conformational epitopes in their reactivities to their respective autoantibodies, the results of a ligand phase antibody reaction can be determined by measurement of the remaining antigens by an ELISA. Analogous kits can be prepared using IA-2β.

**Example 11 – Cloning and Sequencing of Mouse IA-2β cDNA Clones**

Twenty-one different members of the protein tyrosine phosphatase (PTP) family were identified from short nucleotide sequences isolated from a polymerase chain reaction (PCR) amplified cDNA library which was constructed with cDNAs reverse transcribed from pancreatic beta cells and a pair of degenerate primers derived from known PTP nucleotide sequences. Three of these 21 PTPs were previously unknown. One of them is referred to herein as IA-2β. A 300 bp fragment of IA-2β isolated from the PCR-based PTP library was used as a probe to screen a mouse neonatal brain lambda ZAPII cDNA library (Stratagene, La Jolla, CA). Two clones extending approximately 2 kb upstream from the polyA tail were isolated by screening 400,000 plaques. The insert of the longer clone was used to re-screen the same library. Seven additional cDNA clones were obtained that contained
sequences overlapping with original clones. The nine different cDNA clones were isolated and their nucleotide sequences were determined by double strand sequencing by the chain termination method using sequenase version 2.0 sequencing kit (U.S. Biochemical Corporation, Cleveland, OH). The insert sizes ranged from 0.6 kb to 2.5 kb and the total overlapped sequence was approximately 3.5 kb. Comparison of the nucleotide sequence of the different clones revealed that the 3'-untranslated region of some of the clones lacked a stretch of 306 bp, perhaps the result of alternative splicing. A compressed GA-rich stretch at the 3' end of the untranslated region further hampered resolution of the exact sequence. Nonetheless, all nine cDNA clones possessed the same open reading frame that translated into a protein of 723 amino acids with an intracellular, transmembrane and extracellular domain (Figure 1). Sequence analysis using the GenBank database showed that IA-2β is a member of the transmembrane PTP family. Its intracellular segment contains a single PTP domain which shows 74% identity to IA-2. The PTP core sequence of IA-2β (VHCSDGAGRS/TG) differs from that of IA-2 by only one amino acid. In contrast, the partial extracellular sequence of IA-2β shows only 26% identity with the extracellular domain of IA-2.

**Example 12 — Northern Analysis**

Total RNAs were isolated from normal mouse tissues and tumor cell lines, αTC-1 and βTC-1, by the acid guanidinium thiocyanate/phenol/chloroform extraction method (Chomczynski, P., N. Scchi [1987] Anal. Biochem. 162:156-159). RNA samples (20 μg each) were electrophoresed in a 1% agarose/5.4% formaldehyde gel, transferred to nitrocellulose membrane (Schleicher & Schuell, Keene, WH) via capillary blotting. Hybridization was performed as described previously (Lan, M.S., J. Lu,
Y. Goto, A.L. Notkins [1994] DNA and Cell Biology 13:505-514. IA-2β cDNA (a.a. 3 to a.a. 470) void of PTP domain or full-length IA-2 cDNA were used as the probe for Northern analysis. Ribosomal RNAs were used to verify the quality of RNAs.

Since the intracellular domain of IA-2β shows high similarity to the intracellular domain of IA-2, Northern analysis was performed using as the probe a ^32P-labeled IA-2β sequence void of the PTP domain. Figure 2A shows that a 5.5 kb mRNA band was prominent in the insulinoma cell line, βTC-1 and brain, less prominent in the glucagonoma cell line, αTC-1, pancreas and stomach, and barely detectable in colon. The other tissues tested including thymus, ovary, muscle, skin, heart, kidney, spleen, and liver were negative. For comparison, Northern analysis was performed on αTC-1 and βTC-1 cells with a ^32P-labeled IA-2 sequence as the probe. Figure 2B shows that a 3.8 kb mRNA band was very prominent in αTC-1 cells, but less prominent in βTC-1 cells. A weaker signal of larger message was detected in αTC-1 cells that may be the result of alternative splicing. Because of its sequence similarity with IA-2 and strong expression in βTC-1 cells, we refer to this new cDNA as IA-2β.

Example 13 – Radioimmunoprecipitation of In Vitro-Translated Intracellular Domain of IA-2β Autoantigen With IDD Sera and Competitive Immunoprecipitation

The intracellular domain of mouse IA-2β cDNA (a.a. 345 to a.a. 732) was PCR amplified with a perfect Kozak translational start sequence (GCCGCCACCATGG) that was engineered at the 5’-end of the sequence (Kozak, M. [1987] Nucl. Acids Res. 15:8125-8148) and subcloned into a pCRII cloning vector (Invitrogen, San Diego, CA). Polymerase chain reaction (PCR) was performed with 10 ng of IA-2β
cDNA as template. The PCR conditions were as follow: 1 minute at 94°C, 1 minute at 55°C, 1.5 minutes at 72°C for 35 cycles. A similar strategy, with a perfect Kozak sequence, was employed to clone the intracellular domain of mA-I-2 (a.a. 598 to a.a. 979). The in vitro transcription/translation product (41 kDa) was prepared with 1 µg of plasmid DNA in a TNT coupled rabbit reticulocyte lysate system (Promega, Madison, WI) in the presence of [35S] methionine (Amersham, Arlington Heights, IL) at 30°C for 2 hours. Radiolabeled protein was determined by 10% trichloroacetic acid precipitation. Immunoprecipitation was performed as described below. Translated reticulocyte lysate (approximately 50,000-75,000 cpm) and 5 µl of tested serum were mixed in 100 µl of immunoprecipitation buffer (20 mM Tris, pH 7.4, 150 mM NaCl, and 1% Triton X-100). The reaction mixture was incubated overnight at 4°C and 50 µl of 50% (v/v) Protein A-Agarose (Life Technologies, Gaithersburg, MD) was added to the solution at 4°C for one hour. The immunoprecipitation mixture was washed four times with immunoprecipitation buffer, boiled in sample buffer and applied to 10% SDS-PAGE gel. The intensity of the IA-2β band (approximately 41 kDa) was scored from 1+ to 4+ by two independent investigators.

**Human subjects.** Fifty newly onset IDD patients who had been diagnosed within a week of their blood sampling and 50 age-matched controls with no history of autoimmune disease, were studied. Blood samples were collected under informed consent as approved by the University of Florida Institutional Review Board.

**Preparation of rabbit hyperimmune serum and mouse monoclonal antibody.** Rabbit polyclonal antisera were prepared against the intracellular PTP domain of the IA-2
molecule by immunizing two male New Zealand White rabbit
with multiple subcutaneous injections at the back of 125
µg of bacterial expressed GST-IA-2 fusion protein
emulsified in incomplete Freund's adjuvant. Injections
were performed every two weeks. Serum was collected and
tested for immunoreactivity with bacterial expressed
fusion protein. Mouse monoclonal antibody, IA-2/161, was
prepared against the FTP domain of the IA-2 molecule by
conventional hybridoma technology.

Radiolabeled IA-2β then was immunoprecipitated with
serum from IDD patients and separated on a 10% SDS-PAGE
gel. The insert in Figure 3 shows seven representative
sera from IDD patients that recognized the in vitro
translated product. The intensity of the bands ranged
from 1+ to 4+.

Fifty coded sera from diabetic patients and 50 coded
sera from controls then were tested by
radioimmunoprecipitation for autoantibodies to the IA-2β
intracellular domain. As seen in Figure 3, 46% of the
sera from IDD patients, but none of the sera from
controls, reacted with IA-2β.

Example 14 - Immunoprecipitation and Blocking of 37/40
Tryptic Fragments From IA-2β/IA-2 Autoantigens

A mouse insulinoma cell line, βTC-1, and a mouse
glucagonoma cell line, αTC-1, were maintained at low
glucose DMEM medium supplemented with 10% fetal calf
serum. Before labeling, a 70% confluent culture was
incubated in methionine-free medium supplemented with 10%
dialysed fetal calf serum for 1 hour to deplete the
intracellular methionine pool. The culture was
subsequently added 35S-methionine (100 µCi/ml) for 5 hours.
The labeled cells were harvested and prepared for membrane
fraction as described (Christie, M.R., et al., 1990, supra). Radiolabeled cell lysates (1 x 10^6 cpm) were precipitated with diabetic serum for overnight and co-precipitated by Protein A-Agarose beads. After being washed three times with precipitation buffer, the beads were washed with water once and incubated with trypsin (50 or 100 µg/ml) on ice for 20 minutes. The precipitate was washed once again with water, boiled in 1X SDS sample buffer and separated on a 10% SDS-PAGE gel. Blocking reaction was performed by pre-incubation of the serum with rabbit reticulocyte lysate containing unlabeled intracellular domain of IA-2 (a.a. 598 to 979) or IA-2β (a.a. 354 to 723), translated into 42 kDa and 41 kDa products respectively, for 2 hours. Radiolabeled cell lysate was added, precipitated and trypsinized as described above. Labeled reticulocyte lysates of full-length IA-2, intracellular domain of IA-2 and IA-2β were also directly treated with trypsin (50 µg/ml) on ice for 20 minutes before loading on a 10% SDS-PAGE gel.

As seen in Figure 4A, hyperimmune rabbit sera and mouse monoclonal antibody to the intracellular domain of IA-2 reacted not only with IA-2, but also with IA-2β, precipitating a 42 kDa and 41 kDa protein, respectively. The difference in the intensity of the reactivity of the rabbit hyperimmune sera with IA-2 and IA-2β may reflect a difference in the epitopes recognized on these two molecules. Sera from diabetic patients (#77 and #85) recognized and precipitated radiolabeled IA-2 and IA-2β and both unlabeled IA-2 and IA-2β blocked this precipitation (Figure 4B). Some diabetic sera (e.g., #91a) that recognized both IA-2 and IA-2β could be blocked by 10-fold excess of unlabeled IA-2β (as compared to radiolabeled antigen), but not by the same concentration of IA-2. Higher concentrations of unlabeled IA-2 (50-fold
excess) were required for blocking the same serum (e.g., #91b), arguing that the autoantibodies in this serum have a higher affinity for and/or recognize predominantly the IA-2β epitopes. Still other diabetic sera (e.g., #79) recognized only IA-2, and the reaction could be blocked by IA-2, but not by IA-2β. Taken together, these studies show that autoantibodies to IA-2 and IA-2β have a high degree of cross-reactivity, but that both common and distinct epitopes are present on these molecules.

Trypsin treatment converts IA-2β and IA2 into 37 and 40 kDa fragments. The in vitro translated products of full-length IA-2 (106 kDa) and intracellular IA-2 (42 kDa) were subjected to trypsin treatment. As seen in Figure 5, the predominant tryptic fragment migrated as a 40 kDa band. In contrast, the tryptic fragment of intracellular domain of IA-2β (41 kDa) migrated as a 37 kDa band. Both tryptic fragments could be precipitated by diabetic sera.

Blocking of 37/40 kDa tryptic fragments from islet cells by intracellular domain of IA-2β and/or IA-2. To further show that the 37 and 40 kDa tryptic fragments were derived from IA-2β and IA-2, tryptic fragments from αTC-1 and βTC-1 cell lines were precipitated with diabetic sera. As seen in Figures 6A-6B, diabetic sera precipitated only a 40 kDa band from αTC-1 cells and a 37/40 kDa doublet from βTC-1 cells. These findings are consistent with the relative abundance of IA-2 mRNA in αTC-1 and IA-2β mRNA in βTC-1 cells (Figures 2A-2B). Blocking experiments (Figure 6A) showed that both unlabeled recombinant IA-2 and IA-2β were capable of preventing the precipitation of radiolabeled IA-2 and IA-2β by diabetic sera from patient A. Similarly, unlabeled recombinant IA-2 completely blocked the precipitation of radiolabeled IA-2 by diabetic serum from patient B, but unlabeled recombinant IA-2β was
somewhat less effective (e.g., αTC-1 cells) (Figure 6B). This may be due to the different epitopes recognized on IA-2 and IA-2β molecules by sera from different diabetic patients.

**Example 15 – Cloning of the Human IA-2β**

The mouse cDNA (4 kb) was used as a probe to screen a human brain cDNA library. Several clones were identified and sequenced. Among the clones identified, there were different sized versions. Close to the N-terminus of the longer version there are an additional 51 nucleotides as compared to other clones (the shorter version). The additional 51 nucleotides gives rise to an additional 17 amino acids in the longer version.

**Example 16 – Use of IA-2β in Diagnosis, Therapy and Prophylaxis of Diabetes**

All of the techniques and methods for using IA-2 disclosed herein, including the detection methods, prophylactic and therapeutic methods and diagnostic kits are directly applicable for use with IA-2β.

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims.
SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: The United States of America, as represented by the Secretary, Department of Health and Human Services, c/o National Institutes of Health Suite 325, 6011 Executive Boulevard Rockville, MD20852
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(ii) TITLE OF INVENTION: Materials and Methods for Detection and Treatment of Insulin-dependent Diabetes

(iii) NUMBER OF SEQUENCES: 6

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(F) ZIP: 30303

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: U.S. Serial Nos. 08/514,213 and 08/548,159
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(viii) ATTORNEY/AGENT INFORMATION:
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(C) REFERENCE/DOCKET NUMBER: 14014.0200/P

(ix) TELECOMMUNICATION INFORMATION:
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(B) TELEFAX: 404-688-9880

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 969 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(C) INDIVIDUAL ISOLATE: Human IL-2 Beta Protein (shorter version)
Met Gly Pro Pro Leu Pro Leu Leu Leu Leu Leu Leu Leu Leu Pro
1

Pro Arg Val Leu Pro Ala Ala Pro Ser Ser Val Pro Arg Gly Arg Gln
20

Leu Pro Gly Arg Leu Asp Gly Val Phe Gly Arg Cys Gln Lys Val Pro
35

Ala Met Asp Phe Tyr Arg Tyr Glu Val Ser Pro Val Ala Leu Gln Arg
50

Leu Val Ala Leu Gln Lys Leu Ser Gly Thr Gly Phe Thr Trp Gln
65

Asp Asp Tyr Thr Gln Tyr Val Met Asp Gln Glu Leu Ala Asp Leu Pro
85

Lys Thr Tyr Leu Arg Arg Pro Glu Ala Ser Ser Pro Ala Arg Pro Ser
100

Lys His Ser Val Gly Ser Glu Arg Arg Tyr Ser Arg Glu Gly Gly Ala
115

Ala Leu Ala Asn Ala Leu Arg Arg His Leu Pro Phe Leu Glu Ala Leu
130

Ser Gln Ala Pro Ala Ser Asp Val Leu Ala Arg Thr His Thr Ala Gln
145

Asp Arg Pro Pro Ala Glu Gly Asp Asp Arg Phe Ser Glu Ser Ile Leu
165

Thr Tyr Val Ala His Thr Ser Ala Leu Thr Tyr Pro Gly Pro Arg
180

Thr Gln Leu His Glu Asp Leu Leu Pro Arg Thr Leu Gly Gln Leu Gln
195

Pro Asp Glu Leu Ser Pro Lys Val Asp Ser Gly Val Asp Arg His His
210

Leu Met Ala Ala Leu Ser Ala Tyr Ala Ala Gln Arg Pro Pro Ala Pro
225

Pro Gly Glu Gly Ser Leu Glu Pro Gln Tyr Leu Arg Ala Pro Ser
245

Arg Met Pro Arg Pro Leu Leu Ala Pro Ala Ala Pro Gln Lys Trp Pro
260

Ser Pro Leu Gly Asp Ser Glu Asp Pro Ser Thr Gly Asp Gly Ala
275

Arg Ile His Thr Leu Leu Lys Asp Leu Gln Arg Gln Pro Ala Glu Val
290

Arg Gly Leu Ser Gly Leu Glu Leu Asp Gly Met Ala Glu Leu Met Ala
305

Gly Leu Met Gln Gly Val Asp His Gly Val Ala Arg Gly Ser Pro Gly
Arg Ala Ala Leu Gly Glu Ser Gly Glu Gln Ala Asp Gly Pro Lys Ala
Thr Leu Arg Gly Asp Ser Phe Pro Asp Asp Gly Val Gln Asp Asp Asp
Asp Arg Leu Tyr Gln Glu Val His Arg Leu Ser Ala Thr Leu Gly Gly
Leu Leu Gln Asp His Gly Ser Arg Leu Leu Pro Gly Ala Leu Pro Phe
Ala Arg Pro Leu Asp Met Glu Arg Lys Ser Glu His Pro Glu Ser
Ser Leu Ser Ser Glu Glu Glu Thr Ala Gly Val Gln Asn Val Lys Ser
Gln Thr Tyr Ser Lys Asp Leu Leu Gly Gln Gln Pro His Ser Glu Pro
Gly Ala Ala Ala Phe Gly Glu Leu Gln Asn Gln Met Pro Gly Pro Ser
Lys Glu Glu Gln Ser Leu Pro Ala Gly Ala Gln Glu Ala Leu Ser Asp
Gly Leu Gln Leu Glu Val Gln Pro Ser Glu Glu Ala Arg Gly Tyr
Ile Val Thr Asp Arg Glu Val Leu Gly Pro Ala Val Thr Phe Lys Val
Ser Ala Asn Val Gln Asn Val Thr Thr Glu Asp Val Glu Lys Ala Thr
Val Asp Asn Lys Asp Leu Glu Glu Thr Ser Gly Leu Lys Ile Leu
Gln Thr Gly Val Gly Ser Lys Ser Lys Leu Lys Phe Leu Pro Pro Gln
Ala Glu Gln Glu Asp Ser Thr Lys Phe Ile Ala Leu Thr Leu Val Ser
Leu Ala Cys Ile Leu Gly Val Leu Leu Ala Ser Gly Leu Ile Tyr Cys
Leu Arg His Ser Ser Gln His Arg Leu Lys Glu Leu Ser Gly Leu
Gly Gly Asp Pro Gly Ala Asp Ala Thr Ala Ala Tyr Gln Glu Leu Cys
Arg Glu Arg Met Ala Thr Arg Pro Pro Asp Arg Pro Glu Gly Pro His
Thr Ser Arg Ile Ser Ser Val Ser Ser Gln Phe Ser Asp Gly Pro Ile
Pro Ser Pro Ser Ala Arg Ser Ser Ala Ser Ser Trp Ser Glu Glu Pro
Val Gln Ser Asn Met Asp Ile Ser Thr Gly His Met Ile Leu Ser Tyr
660 665 670
Met Glu Asp His Leu Lys Asn Lys Asn Arg Leu Glu Lys Glu Trp Glu
680 690 695 700
Ala Leu Cys Ala Tyr Gln Ala Glu Pro Asn Ser Ser Phe Val Ala Gln
710 715 720
Arg Glu Glu Val Pro Lys Asn Arg Ser Leu Ala Val Leu Thr Tyr
725 730 735
Asp His Ser Arg Val Leu Leu Lys Ala Glu Asn Ser His Ser His Ser
740 745 750
Asp Tyr Ile Asn Ala Ser Pro Ile Met Asp His Asp Pro Arg Asn Pro
755 760 765
Ala Tyr Ile Ala Thr Gln Gly Pro Leu Pro Ala Thr Val Ala Asp Phe
770 775 780
Trp Gln Met Val Trp Glu Ser Gly Cys Val Val Ile Val Met Leu Thr
785 790 795 800
Pro Leu Ala Glu Asn Gly Val Arg Gln Cys Tyr His Tyr Trp Pro Asp
805 810 815
Glu Gly Ser Asn Leu Tyr His Ile Tyr Glu Val Asn Leu Val Ser Glu
820 825 830
His Ile Trp Cys Glu Asp Phe Leu Val Arg Ser Phe Tyr Leu Lys Asn
835 840 845
Leu Gln Thr Asn Glu Thr Arg Thr Val Thr Gln Phe His Phe Leu Ser
850 855 860
Trp Tyr Asp Arg Gly Val Pro Ser Ser Ser Ser Arg Ser Leu Leu Asp Phe
865 870 875 880
Arg Arg Lys Val Asn Lys Cys Tyr Arg Gly Arg Ser Cys Pro Ile Ile
885 890 895
Val His Cys Ser Asp Gly Ala Gly Arg Ser Gly Thr Tyr Val Leu Ile
900 905 910
Asp Met Val Leu Asn Lys Met Ala Lys Gly Ala Lys Glu Ile Asp Ile
915 920 925
Ala Ala Thr Leu Glu His Leu Arg Asp Gln Arg Pro Gly Met Val Gln
930 935 940
Thr Lys Glu Gln Phe Glu Phe Ala Leu Thr Ala Val Ala Glu Glu Val
945 950 955 960
Asn Ala Ile Leu Lys Ala Leu Pro Gln
965

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
Molecule Type: cDNA

Original Source:
Individual Isolate: Human IL-2 Beta cDNA (shorter version)

Sequence Description: Seq ID No:2:

CAGGCAGCCG GATGGG GCC CGCTCCCG TGCTACTGCT GTGCTGCTCGG 60
CCAGCGCTCC TGCTCGCCG CTCCTTCGCC GTCCCCCGC CGCCGGCAGCT CCGGGGGCGT 120
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GTGCTCGCGG TGGGCACTGCA CGCCCTGCGG GTGGGTTGCG AGAAGCTTTC CGGGCACGCT 240
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   (B) TYPE: amino acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
   (C) INDIVIDUAL ISOLATE: Human IL-2 Beta Protein (longer
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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2994 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(C) INDIVIDUAL ISOLATE: Human IL-2 Beta cDNA (longer version)

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(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 723 amino acids
   (B) TYPE: amino acid
   (C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(vi) ORIGINAL SOURCE:
(C) INDIVIDUAL ISOLATE: Mouse IA-2 Beta Protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

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(2) INFORMATION FOR SEQ ID NO:6:

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(A) LENGTH: 2351 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:
(C) INDIVIDUAL ISOLATE: Mouse IA-2 Beta cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

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What is claimed is:

1. A method for detecting or predicting susceptibility to insulin-dependent diabetes in a patient or distinguishing between type 1 and type 2 diabetes, which comprises detecting the presence of antibodies in the serum of said patient which bind to IA-2β, or fragments thereof, wherein the presence of said antibodies is indicative of the presence of insulin-dependent diabetes or susceptibility to insulin-dependent diabetes in said patient.

2. The method of claim 1 which further comprises detecting the presence of antibodies in the serum of said patient which bind to IA-2 or GAD$_{65}$ or both.

3. The method of claim 1 which is a radioimmunoassay, an ELISA assay, a depletion ELISA (d-ELISA), or an immunoprecipitation method.

4. The method of claim 1 in which recombinant IA-2β is used.

5. The method of claim 4 in which the recombinant IA-2β is produced in a baculovirus expression system.

6. A method to treat a patient suffering from insulin-dependent diabetes or a patient at risk of developing insulin-dependent diabetes which comprises administering an effective amount of IA-2β, or fragments thereof, to said patient.

7. The method according to claim 6 in which the IA-2β is administered intravenously, orally, or subcutaneously with an adjuvant or expressed as a recombinant protein in a recombinant viral vaccine, alone or in combination with IA-2, GAD$_{65}$ or insulin, administered by the same or a different
route.

8. The method of claim 6 which comprises immunizing the patient with an amount of IA-2β or immunogenic fragments thereof to induce an antibody response mediated by TH₂ lymphocytes to induce immunosuppressive effects on pathogenic TH₁ lymphocytes.

9. A composition comprising an isolated IA-2β molecule, or fragment thereof, for use in the immune detection, treatment or prophylaxis of diabetes.

10. The composition of claim 9 which further comprises an isolated GAD₆₅ molecule, an isolated IA-2 molecule or both.

11. A kit for detection of autoantibodies associated with diabetes which comprises IA-2β protein, antigenic fragments thereof or antibodies to IA-2β.

12. The kit of claim 11 which further comprises labeled animal anti-human immunoglobulin.

13. The kit of claim 12 which further comprises GAD₆₅ protein, antigenic fragments thereof, or antibodies to GAD₆₅, or IA-2 protein, antigenic fragments thereof, or antibodies to IA-2, or both.

14. The kit of claim 13 which further comprises a microtiter plate.

15. The kit of claim 11 which further comprises colorimetric agents, ELISA blocking reagents, positive and negative controls, or wash buffers.

16. The kit of claim 11 in which the IA-2β protein,
peptide or anti-IA-2β antibody is radiolabeled or conjugated to a marker molecule.

17. A method for detecting or predicting IDD in a patient which comprises detecting cellular responses, selected from lymphocyte proliferation and cytokine elaboration, after in vitro exposure of cells of said patient to IA-2β, or a fragment thereof.

18. Isolated, full-length mammalian IA-2β protein, or a fragment thereof.

19. The isolated, full-length mammalian IA-2β protein of claim 18 comprising the sequence of SEQ ID NO. 1, SEQ ID No. 3, or SEQ ID NO. 5 or a sequence substantially similar to said sequence which is not IA-2.

20. An isolated nucleic acid sequence encoding full-length mammalian IA-2β or a fragment thereof.

21. The isolated nucleic acid sequence of claim 20 comprising the sequence of SEQ ID NO. 2, SEQ ID NO. 4, or SEQ ID NO. 6 or a sequence substantially similar to said sequence which is not IA-2.

22. An isolated nucleic acid encoding an IA-2β protein, said nucleic acid capable of hybridizing specifically to a second nucleic acid consisting of the nucleic acid sequence of SEQ ID NO. 4 in the presence of a human genomic library under stringent conditions.

23. The nucleic acid sequence of claim 22, wherein said nucleic acid sequence has at least about 80% sequence identity with the nucleic acid of SEQ ID NO. 4.
24. An isolated nucleic acid encoding an IA-2β protein, wherein said isolated nucleic acid sequence encodes a protein which is recognized by antibodies to IA-2β that do not recognize the IA-2β antigens.

25. An isolated IA-2β protein, wherein said protein has at least about 80% sequence identity with the amino acid sequence of SEQ ID NO. 3.

26. A DNA construct encoding an IA-2β protein or a polypeptide fragment thereof, which comprises the following operably-linked elements:
   (a) a transcriptional promoter;
   (b) a DNA sequence encoding an IA-2β protein or a polypeptide fragment thereof; and
   (c) a transcriptional terminator.

27. An expression system for producing the IA-2β protein or polypeptide fragment thereof wherein the DNA construct of claim 26 is incorporated into a suitable host.

28. A method for detecting or predicting susceptibility to insulin dependent diabetes in a patient, which does not depend on immunohistological examination such as in the ICA method, which comprises detecting the presence of antibodies in the serum of said patient which bind to IA-2 alone or to IA-2 and GAD$_{65}$, or to IA-2 and GAD$_{65}$ or IA-2-β, wherein the presence of said antibodies is indicative of the presence of diabetes or susceptibility to diabetes in said patient.

29. The method of claim 28 which is a radioimmunoassay, an ELISA assay, a depletion ELISA, or an immunoprecipitation method.
30. The method of claim 29 in which recombinant IA-2 is used.

31. The method of claim 30 in which the recombinant IA-2 is produced in a baculovirus expression system.

32. A method to treat a patient suffering from insulin dependent diabetes or a patient at risk of developing insulin dependent diabetes which comprises administering an anergy promoting amount of IA-2 to said patient, to produce immunosuppressive effects, and to elicit protective peripheral blood lymphocyte cytokine responses to IA-2 or components thereof.

33. The method according to claim 32 in which the IA-2 is administered intravenously orally, or subcutaneously with an adjuvants or expressed as a recombinant protein in a recombinant viral vaccine, alone or in combination with GAD65 or insulin, administered by the same or a different route.

34. The method of claim 32 which comprises immunizing the patient with an amount of IA-2 or immunogenic fragments thereof to induce an antibody response mediated by TH-2 lymphocytes to induce immunosuppressive effects on pathogenic TH-1 lymphocytes.

35. A composition comprising both an isolated GAD65 and an isolated IA-2 antigen for use in the immune detection, treatment or prophylaxis of insulin dependent diabetes.

36. A kit for detection of autoantibodies associated with diabetes which comprises IA-2 protein, antigenic peptides thereof or antibodies to IA-2.

37. The kit of claim 36 which further comprises labeled
animal anti-human immunoglobulin.

38. The kit of claim 36 which further comprises GAD_{65} protein, antigenic peptides thereof, or antibodies to GAD_{65}.

39. The kit of claim 36 which further comprises a microtiter plate.

40. The kit of claim 36 which further comprises colorimetric agents, ELISA blocking reagents, positive and negative controls, or wash buffers.

41. The kit of claim 36 in which the IA-2 protein, peptide or anti-IA-2 antibody is radiolabeled or conjugated to a marker molecule.

42. A method for detecting or predicting IDD in a patient which comprises detecting cellular responses, selected from lymphocyte proliferation and cytokine elaboration, after \textit{in vitro} exposure of cells of said patient to IA-2.
Fig. 1
FIG. 3
### FIG. 4A

<table>
<thead>
<tr>
<th>Rabbit Antiserum</th>
<th>Mouse MAb</th>
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<tbody>
<tr>
<td>IA-2</td>
<td>IA-2β</td>
</tr>
<tr>
<td>IA-2</td>
<td>IA-2β</td>
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<td>IA-2</td>
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<td>IA-2</td>
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![Images of protein bands with 42 kDa and 41 kDa markers]

### FIG. 4B

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<tr>
<th>IDDM Sera</th>
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![Images of protein bands with 42 kDa and 41 kDa markers]

SUBSTITUTE SHEET (RULE 26)
FIG. 5
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/12 C12N15/55 G01N33/53 A61K38/17 C07K14/47
C07K14/705 C12N9/16 C12N15/86 A61K38/43 A61K39/395

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N G01N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>DNA AND CELL BIOLOGY, vol. 13, no. 5, May 1994, pages 505-514, XP000612611</td>
<td>1-18, 20, 24, 26-42</td>
</tr>
<tr>
<td></td>
<td>MICHAEL S. LAN ET AL.: *Molecular cloning and identification of a receptor-type</td>
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<tr>
<td></td>
<td>protein tyrosine phosphatase, IA-2, from human insulinoma* cited in the</td>
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<tr>
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<td>see abstract</td>
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</table>

X Further documents are listed in the continuation of box C.

* Special categories of cited documents:
  *A* document defining the general state of the art which is not considered to be of particular relevance
  *E* earlier document but published on or after the international filing date
  *L* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
  *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
  *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
  *S* document member of the same patent family

Date of actual completion of the international search
7 January 1997

Date of mailing of the international search report
30.01.97

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Fax (+31-70) 340-3016

Authorized officer
Montero Lopez, B
<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
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<tr>
<td>A</td>
<td>BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 204, no. 2, 28 October 1994, ORLANDO, FL US, pages 930-936, XP000561251 JIA LU ET AL.: &quot;Isolation, sequence and expression of a novel mouse brain cDNA, mIa-2, and its relatedness to members of the protein tyrosine phosphatase family&quot; cited in the application see abstract see page 932, paragraph 2 - page 935, last paragraph ---</td>
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<tr>
<td>P,X</td>
<td>PROC. NATL. ACAD. SCI. U. S. A. (1996), 93(6), 2307-11 CODEN: PNASAA; ISSN: 0027-8424, 1996, XP000612013 LU, JIA ET AL: &quot;Identification of a second transmembrane protein tyrosine phosphatase, IA-2 beta., as an autoantigen in insulin-dependent diabetes mellitus: precursor of the 37-kDa tryptic fragment&quot; see abstract see page 2307, left-hand column, paragraph 1 - right-hand column, paragraph 2 see page 2308, right-hand column, paragraph 2 - page 2309, left-hand column, paragraph 1; figure 1 see page 2309, right-hand column, paragraph 2 - page 2310, left-hand column, paragraph 1 see page 2310, right-hand column, paragraph 3 - page 2311, right-hand column, paragraph 3 ---</td>
</tr>
<tr>
<td>P,X</td>
<td>THE JOURNAL OF CLINICAL INVESTIGATION, vol. 96, no. 3, September 1995, pages 1506-1511, XP000612574 MARK A. PAYTON ET AL.: &quot;Relationship of the 37,000- and 40,000-Mr tryptic fragments of islet antigens in insulin-dependent diabetes to the protein tyrosine phosphatase-like molecule IA-2 (ICA512)&quot; cited in the application see abstract see page 1506, right-hand column, paragraph 2 see page 1507, right-hand column, paragraph 3 - page 1511, left-hand column, paragraph 2 ---</td>
</tr>
</tbody>
</table>
**INTERNATIONAL SEARCH REPORT**

**Box I  Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)**

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. **X** Claims Nos.: 6-8, 32-34
   because they relate to subject matter not required to be searched by this Authority, namely:
   
   **Remark:** Although claims 6-8 and 32-34 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compounds.

2. ☐ Claims Nos.: because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II  Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.
The method and compositions of this invention provide an assay for diabetes. By providing a method for detecting autoantibodies to GAD65, IA-2 and an antigen termed IA-2beta herein, the method provides a reliable chemical assay. In addition, these antigens may be employed in therapeutic regimens aimed at achieving amelioration of the clinical condition.