PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C07K 3/00, G01N 33/567
A61K 39/00, C07H 19/00
A61M 37/00

A1

(11) International Publication Number: **WO 92/06105**

(43) International Publication Date: 16 April 1992 (16.04.92)

(21) International Application Number: PCT/US91/07052

(22) International Filing Date: 25 September 1991 (25.09.91)

(30) Priority data:
591,608 2 October 1990 (02.10.90) US

(71) Applicant: TRIGEN INCORPORATED [US/US]; 2211 Michigan Avenue, Santa Monica, CA 90404 (US).

(72) Inventors: LIN, Hun-chi ; 11452 Clarkson Road, Los Angeles, CA 90064 (US). THAI, Toha ; 851 S. Mansfield Avenue, No. 104, Los Angeles, CA 90036 (US). LEI, Shau-Ping ; 11452 Clarkson Road, Los Angeles, CA 90064 (US).

(74) Agents: OLDENKAMP, David, J. et al.; Poms, Smith, Lande & Rose, 2121 Avenue of the Stars, Suite 1400, Los Angeles, CA 90067 (US).

(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), NO, SE (European patent).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: ANTIBODIES AND METHODS FOR DIAGNOSIS AND TREATMENT OF DIABETES

(57) Abstract

The present invention provides a continuous cell line producing a DM monoclonal antibody reactive with beta cell autoantigen on human pancreatic beta cells having a molecular weight of approximately 64.000 daltons and associated with the onset of insulin dependent diabetes mellitus (IDDM). The DM antibody is used in diagnostic assay methods and in methods for the treatment and prevention of IDDM. The invention further provides the beta cell autoantigen of approximately 64,000 daltons molecular weight associated with the onset of IDDM, reactive with the DM monoclonal antibody. The beta cell autoantigen is used to diagnose the early onset of IDDM. Also disclosed are immunotherapeutic methods employing anti-id monoclonal antibodies and the beta cell autoantigen. These methods are employed for inhibiting the binding of islet cell autoantibodies to pancreatic islet cells, and for removing islet cell autoantibodies and lymphocytes reactive with beta cell autoantigen from the peripheral circulation of a subject.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MN	Mongolia
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GN	Guinea	NL	Netherlands
BJ	Benin	GR	Greece	NO	Norway
BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland			SN	Senegal
CI	Côte d'Ivoire	KR	Republic of Korea	SU+	Soviet Union
CM	Cameroon	LI	Liechtenstein	TD	Chad
CS	Czechoslovakia	LK	Sri Lanka	TG	Togo
DE*	Germany	LU	Luxembourg	US	United States of America
DK	Denmark	MC	Monaco		

+ Any designation of "SU" has effect in the Russian Federation. It is not yet known whether any such designation has effect in other States of the former Soviet Union.

ANTIBODIES AND METHODS FOR DIAGNOSIS AND TREATMENT OF DIABETES

FIELD OF THE INVENTION

The present invention relates to antibodies useful in the diagnosis and treatment of insulin dependent diabetes mellitus (IDDM), and more particularly to a hybridoma cell line producing a novel monoclonal antibody, reactive with beta cell autoantigen on pancreatic islet cells. The invention also relates to isolation of the beta cell autoantigen reactive with the monoclonal antibody, and to methods of diagnosing and treating IDDM patients using the antibodies and autoantigen produced in accordance with the present invention.

BACKGROUND OF THE INVENTION

New onset, type I diabetes, also known as Insulin Dependent Diabetes Mellitus or IDDM is an autoimmune disease characterized by immune destruction of the beta cells in the pancreas. The beta cells produce insulin, and are clustered together in the pancreas in structures called the Islets of Langerhans. Islet tissue is composed of approximately 80 percent beta cells. The affected individual's immune system produces autoantibodies against the antigens associated with pancreatic islet beta cells ("beta cell autoantigens"). The islet cell autoantibodies attack and progressively destroy all the pancreatic islet beta cells, resulting in the loss of the ability to produce insulin. Also in the affected individual, the antigens of the beta cells trigger B- and T-lymphocytes and macrophages to "autoreact" with the body's own pancreatic tissue. B-lymphocytes triggered by the specific beta cell autoantigen begin to produce islet cell autoantibodies

that react with pancreatic beta cells, leading to a cytotoxic killing reaction. T-lymphocytes stimulated by the specific beta cell autoantigen carry out cell-mediated immune killing reactions that wipe out the pancreatic beta cells. The result is that the patient requires insulin treatment for life because insulin is required for the normal metabolism of blood sugar.

In the United States, from 30,000 to 60,000 new cases of IDDM occur annually. The disease presently affects 600,000 Americans. Thus, for this disease there is a critical need for an early diagnostic test to determine the presence of beta cell autoantibodies before beta cell destruction takes on clinical significance, and for treatment strategies.

IDDM is currently diagnosed by the abrupt and sometimes fatal clinical symptoms of ketoacidosis. This represents an inability in a thinner, younger patient to adjust blood glucose levels without receiving a therapeutic dose of insulin. Other than ketoacidosis, there are a variety of methods currently used by physicians to make a positive diagnosis, such as the glucose tolerance test and measurement of endogenous insulin level. However, these tests are able to detect IDDM only after 90% of the beta cells are already destroyed by the islet cell autoantibodies.

IDDM is currently treated through multiple, daily subcutaneous injections of insulin. Recently, immunological methods and tissue culture techniques have been developed which permit transplantation of pancreatic islets into animals to treat diabetes (Charles et al., Cellular Immunol. 79:403-406 (1983)). Transplanted islets, however, may be rejected through the action of immune leukocytes, such as lymphocytes and macrophages, or, by antibody and complement (Charles et al., Diabetes Res. (1984)). Mammals typically respond by producing autoantibodies against surface autoantigens

of the islet beta cells, precipitating progressive destruction of the beta cells. In order to kill a cell, autoantibodies, like antibodies, require the action of complement to lyse the cell. The antibody is first bound to a cell through a cell surface marker. The complement then binds to the antibody and triggers the complement's cell lysing activity. There are presently no generally accepted preventive or therapeutic approaches for preventing autoimmune destruction of islet cells in IDDM.

The possibility of identifying a single beta cell autoantigen, e.g. a glycolipid or protein specifically associated with type I diabetes has proven difficult due to immunoheterogeneity of the disease. Of the glycolipids and proteins reactive with human or animal islet cell autoantibodies (ICA) found in sera, no pancreatic beta cell autoantigen has been identified with a known specific function related to diabetes. Furthermore, because beta cells are difficult to isolate, they are not abundantly available, making it very difficult to obtain a significant quantity of the autoantigen for characterization.

Identification of the beta cell specific autoantigen is of prime importance in understanding the pathogenesis of IDDM, in development of diagnostic techniques for early detection of IDDM, and for developing therapies for IDDM. Using monoclonal antibodies, researchers have attempted to characterize islet beta-cell specific autoantigens that play a role in the pathogenesis of type I diabetes. (Eisenbarth, Diabetes 36:355-364 (1987); Alejandro et al., J. Clin. Invest. 74:25-38 (1984), and Brogen et al., Diabetologia 29:330-333 (1986)). Recently, a proteinaceous islet cell specific autoantigen having a molecular weight of 64,000 daltons has been identified in man, rat and mouse using islet cell autoantibodies. (Atkinson et al.,

Lancet 335:1357-60 (1990), Baekkeskov et al, Nature, 298:167-169 (1982); Baekkeskov et al., Science 224:1348-50 (1984); Colman et al., Diabetes, 36:1432-40 (1987); and Atkinson et al., Diabetes, 37:1587-90 (1988)). However, its significance in the pathogenesis of IDDM is not presently understood. In addition, the autoantigen has not yet been purified and characterized, nor has the gene encoding the autoantigen been cloned, sequenced, and expressed. It is believed (ibid) that the beta cell autoantigen having a molecular weight approximately 64 kd causes the production in humans of islet cell autoantibodies, hereinafter referred to as anti-64 kd autoantibody, which progressively destroy islet cells, resulting in the disease. Anti-64 kd autoantibodies are a component of a polyclonal human set of islet cell autoantibodies.

Polyclonal human islet cell autoantibody is one reagent capable of characterizing the complexity of islet proteins. The use of polyclonal human islet cell autoantibody has resulted in identifying only one major autoantigen of 64 kd (Baekkekov et al., Nature 298:167-169 (1982); and Colman et al., Diabetes 36:1432-1440 (1987)). The problems remain of identifying whether the 64 kd autoantigen is the major autoantigen associated with the earliest pathogenic stages of IDDM, purifying and characterizing the 64 kd autoantigen.

Autoimmune destruction of the beta cells caused by the immune system is carried out, in part, by circulating autoantibodies, which attack beta cell autoantigen. Because the major autoantigen associated with IDDM has not been positively isolated, identified and characterized, immunoassays for detecting beta cell autoantibodies that react with the autoantigen are not yet available for making an early diagnosis of IDDM.

The circulating autoantibodies, like all antibodies, carry a variable region for specific binding

to an antigen. The variable region of an antibody contains antigenic determinant sites, known as idiotypes. Antibodies directed to the variable region of an antibody, so-called "anti-idiotypic" or "anti-id" antibodies, may be produced when an animal is injected with specific idiotypic antibodies. Anti-id antibodies can bind to that site on the idiotypic antibodies which stimulated their production, thereby blocking the antibodies from binding to their natural target. Idiotypic interactions have been proposed for therapy of a wide variety of disease conditions ranging from cancer to autoimmune diseases. (Geha, Advances in Immunology, 39:255-297 (1986)). It would be desirable to provide anti-id antibodies reactive with autoantibodies against beta cell autoantigen for use in an immunotherapeutic plasma treatment system, such as plasmapheresis, to specifically and quickly remove the autoantibodies from patient blood, without affecting other immunoglobulins of the patient.

It would also be advantageous to be able to destroy T-and B-lymphocytes in the peripheral circulation that are involved in beta cell destruction in the pathogenesis of IDDM. The specific binding of T-and B-lymphocytes involved in beta cell destruction provides a basis for specific destruction of these lymphocytes. These T- and B- lymphocytes comprise the subset of lymphocytes that present on their surface and produce anti-64 kd autoantibodies. It would thus be desirable to be able to introduce into a human subject a sufficient amount of anti-id antibody reactive with the anti-64 kd autoantibodies. Alternatively, anti-id antibodies conjugated with a toxin, or beta cell autoantigen conjugated with a toxin, or combinations thereof may be used to bind and destroy specifically these T and B lymphocytes.

Thus, there is a recognized set of needs for the present invention to produce anti-beta cell autoantigen antibodies, substantially purified beta cell autoantigen, and anti-id antibodies in the diagnosis, prevention, and treatment of IDDM.

SUMMARY

It was unexpectedly found that cell lines not derived from pancreatic tissue in culture produced substantial quantities of beta cell autoantigen. This finding permitted the cloning and expression of beta cell autoantigen and its production in substantially purified form. In conjunction with this advance, the use of monoclonal antibody procedures provides a means of satisfying the needs recognized previously.

In particular, the present invention satisfies these needs by providing (a) a continuous cell line producing monoclonal antibodies reactive with beta cell autoantigen on human pancreatic beta cells and associated with the onset of insulin dependent diabetes mellitus (IDDM); (b) monoclonal antibodies reactive with beta cell autoantigen produced by the cell line; (c) substantially purified beta cell autoantigen associated with the onset of IDDM; (d) vaccines for the treatment and prevention of IDDM; (e) a continuous cell line producing anti-id monoclonal antibody reactive with islet cell autoantibodies reactive with beta cell autoantigen; (f) an anti-id monoclonal antibody produced by the cell line reactive with islet cell autoantibodies; (g) an immunotherapeutic method for inhibiting the binding of islet cell autoantibodies to pancreatic islet cells, (h) an immunotherapeutic method for removing islet cell autoantibodies from the peripheral circulation of a subject; and (i) an immunotherapeutic method for removing T and B lymphocytes autoreactive with beta cell autoantigen and

anti-id antibodies from the peripheral circulation of a subject.

The monoclonal antibody embodied by the present invention is produced by hybridoma cell line ATCC No. HB 10502. Alternatively, the monoclonal antibody can be produced by recombinant DNA methods. The specific monoclonal antibody embodied by this invention is classified as IgM, and recognizes and binds to an immunodominant epitope on a plasma membrane bound autoantigen of human pancreatic beta cells.

The invention also provides substantially purified beta cell autoantigen. The monoclonal antibody of the invention can be used to isolate beta cell autoantigen. The autoantigen may also be obtained from body tissues such as pancreatic islet cells or from body fluids such as blood serum. The beta cell autoantigen of the invention has a molecular weight of approximately 64,000 daltons as determined by polyacrylamide gel electrophoresis and has an immunodominant epitope associated with the presence of IDDM. The autoantigen may also be isolated from cell lines, including insulinoma D115 or HEp2.

The invention further comprises a cDNA library which contains cDNA sequences coding for the beta cell autoantigen. The antigen can be recognized and bound by anti-64 kd monoclonal antibodies (hereinafter referred to as "DM monoclonal antibodies"). This cDNA can be cloned. The autoantigen can be produced by an expression system transcribing and translating this cloned cDNA. This autoantigen produced by recombinant DNA techniques can be used to generate monoclonal antibody by immunizing susceptible animals, isolating antibody-producing spleen cells, and using techniques such as those of Kohler and Milstein to produce monoclonal antibodies.

The beta cell autoantigen or recombinant autoantigen derived from a cDNA library of the present invention may be used in an in vitro diagnostic method for diagnosing IDDM by detecting in human body fluids anti-64 kd autoantibodies. The method involves examining the body fluids by reacting a sample of purified labeled autoantigen with the sample of human body fluid suspected of containing anti-64 kd autoantibodies. This method can be based on immunoassay techniques involving antigen capture or immunoprecipitation methods.

The monoclonal antibody of the invention may be used to prepare a vaccine composition for preventing pathogenesis of IDDM. This method prevents destruction of pancreatic beta cells by introducing a vaccine comprising the monoclonal antibody into a human.

Another therapeutic application of the present invention for treating IDDM involves removing circulating anti-64 kd autoantibodies. The step of removing circulating anti-64 kd autoantibodies employs the use of immobilized ligands specific for binding circulating islet cell autoantibodies reactive with beta cell autoantigen. These ligands can be mounted in an apparatus. One ligand can be the beta cell autoantigen prepared either by substantial purification from cell lines or by recombinant means. Another ligand can be the anti-id antibody that specifically recognizes and binds anti-64 kd autoantibodies. These anti-id antibodies are derived from the use of the DM monoclonal antibody of the present invention produced by the hybridoma cell line of the present invention which produces monoclonal antibody reactive with beta cell autoantigen.

The present invention further contemplates a method for immunotherapy of IDDM that involves destroying lymphocytes that produce autoantibodies that attack beta

cells, resulting in IDDM. This method involves injection of toxins conjugated to either the anti-id antibodies or beta cell autoantigen of the invention for destruction of lymphocytes involved in the immune destruction of beta cells. This method can be used alone but preferably is used in combination with the removal of circulating anti-64 kd autoantibodies.

BRIEF DESCRIPTION OF DRAWINGS

Fig. 1 is a restriction map for plasmid pUH14 indicating a DNA insert comprising about 1400 base pairs coding for the beta cell autoantigen specifically reactive with the DM monoclonal antibody and the anti-64 kd autoantibody.

DETAILED DESCRIPTION

In order that the invention herein described may be more fully understood, the following detailed description is set forth.

The present invention concerns hybridoma cell lines and novel monoclonal antibodies produced by the hybridoma cell lines. One of the novel monoclonal antibodies, designated DM monoclonal antibody, is specifically reactive with an autoantigen bound to the plasma membrane of pancreatic beta cells.

Another embodiment is an anti-id antibody which is specifically reactive with autoantibodies produced in a subject reactive with beta cell autoantigen. The DM monoclonal antibody of the invention and the autoantibodies compete for binding with the same beta cell autoantigen. The DM monoclonal antibody and the anti-64 kd autoantibodies have the same or a similar idiotope which recognizes the beta cell autoantigen. Thus, anti-id antibodies can be developed which are specific for the antigenically determinant idiotope of the DM monoclonal antibody and will also be specific for

the anti-64 kd autoantibody. Accordingly, the anti-id antibodies of the present invention specifically react with the DM monoclonal antibody and the autoantibodies reactive with beta cell autoantigen.

5 The DM monoclonal antibody of the invention can be prepared by hybridoma fusion techniques or by techniques that utilize EBV-immortalization technologies.

Hybridoma fusion techniques were first introduced by Kohler and Milstein (see Kohler and Milstein, Nature,
10 256:495-97 (1975); Brown et al., J. Immunol., 127 (2):539-45 (1981); Brown et al., J. Biol. Chem., 255:4980-83 (1980); Yeh et al., Proc. Nat'l. Acad. Sci. (USA), 76 (6):927-31 (1976); and Yeh et al., Int. J. Cancer, 29:269-75 (1982)).

15 These techniques involve the use of an animal which is primed to produce a particular antibody. The animal can be primed by injection of an immunogen (e.g., purified antigen or cells or cellular extracts carrying the antigen) so as to elicit a desired immune response,
20 i.e. production of antibodies from the primed animal. A primed animal is also one which is expressing a disease. Lymphocytes derived from the lymph nodes, spleens or peripheral blood of primed, diseased animals can be used to search for a particular antibody. For example, when
25 either lymphocytes from prediabetic BB rats or lymphocytes from patients with Type I diabetes are fused with myeloma cells, hybridomas can be selected which produce antibodies that preferentially bind to beta cells. The lymphocyte chromosomes encoding desired
30 immunoglobulins are immortalized by fusing the lymphocytes with myeloma cells, generally in the presence of a fusing agent such as polyethylene glycol (PEG). Any of a number of myeloma cell lines may be used as a fusion partner according to standard
35 techniques; for example, the P3-NS1/1-Ag4-1, P3-x63-Ag8.653, Sp2/0-Ag14, or HL1-653 myeloma lines. These

myeloma lines are available from the American Type Culture Collection (ATCC), Rockville, Maryland.

The resulting cells, which include the desired hybridomas, are then grown in a selective medium such as HAT medium, in which unfused parental myeloma or lymphocyte cells eventually die. Only the hybridoma cells survive and can be grown under limiting dilution conditions to obtain isolated clones. The supernatants of the hybridomas are screened for the presence of the desired specificity, e.g. by immunoassay techniques using the antigen that has been used for immunization. Positive clones can then be subcloned under limiting dilution conditions, and the monoclonal antibody produced can be isolated.

Novel antibodies of mouse or human origin can be also made to the beta cell autoantigen having the appropriate biological functions. For example, human monoclonal antibodies may be made by using the antigen, e.g. the beta cell autoantigen of the present invention, to sensitize human lymphocytes in vitro followed by EBV transformation or hybridization of the antigen-sensitized lymphocytes with mouse or human lymphocytes, as described by Borrebaeck et al. (Proc. Nat'l. Acad. Sci. (USA), 85:3995-99 (1988)).

Various conventional methods can be used for isolation and purification of the monoclonal antibodies so as to obtain them free from other proteins and contaminants. Commonly used methods for purifying monoclonal antibodies include ammonium sulfate precipitation, ion exchange chromatography, and affinity chromatography (see Zola et al., in Monoclonal Hybridoma Antibodies: Techniques and Applications, Hurell (ed.) pp. 51-52 (CRC Press, 1982)). Hybridomas produced according to these methods can be propagated in vitro or in vivo (in ascites fluid) using techniques known in the

art (see generally Fink et al., Prog. Clin. Pathol., 9:121-33 (1984), Fig. 6-1 at p. 123).

Generally, the individual cell line may be propagated in vitro, for example, in laboratory culture vessels, and the culture medium containing high concentrations of a single specific monoclonal antibody can be harvested by decantation, filtration, or centrifugation.

For certain therapeutic applications, chimeric (mouse-human) or human monoclonal antibodies may be preferable to murine antibodies because patients treated with mouse antibodies generate human anti-mouse antibodies. (Shawler et al., J. Immunol., 135:1530-35 (1985)). Chimeric mouse-human monoclonal antibodies reactive with the beta autoantigen bound to the plasma membrane of pancreatic beta cells can be produced, for example, by techniques recently developed for the production of chimeric antibodies (Oi et al., Biotechnologies, 4(3):214-221 (1986); Liu et al., Proc. Nat'l. Acad. Sci. (USA), 84:3439-43 (1987); Reichman et al., Nature 332:323-327 (1988); and Fell et al., "Homologous recombination in hybridoma cells: Heavy chain chimeric antibody produced by gene targeting", Proc. Natl. Acad. Sci. USA 86:8507-8511 (1989)). Accordingly, genes coding for the constant regions of the murine DM monoclonal antibody molecule are substituted with human genes coding for the constant regions of an antibody with appropriate biological activity (such as the ability to activate human complement and mediate antibody dependent complement-mediated cytotoxicity).

Other recombinant antibodies include fusion proteins where the antibody is combined with a second protein having a desired biological function (Neuberger et al., "Recombinant Antibodies Possessing Novel Effector Functions", Nature 312:604-608 (1984)).

Fragments of the monoclonal antibodies containing the active binding region, such as Fab, $F(ab)'_2$ and Fv fragments may be produced from the monoclonal antibody using techniques well-established in the art (Rousseaux et al., Methods Enzymol. 121:663-669, Academic Press (1986)).

A typical method for producing the monoclonal antibody by recombinant means involves a recombinant gene expression system situated in *E. coli* bacteria, yeast, or in a mammalian cell system. The gene expression system is constructed with a DNA sequence taken from hybridoma cell line ATCC No. HB 10502 and encodes at least a portion of the immunoglobulin molecule. It should be understood that the scope of the present invention encompasses the DM monoclonal antibody produced by recombinant means. The recombinant means can be a recombinant gene expression system situated in *E. coli* bacteria. The gene expression system can be constructed to express a DNA sequence containing the DM monoclonal antibody. Such a DNA sequence, which can be derived at least in part from hybridoma cell line ATCC No. HB 10502, encodes at least a portion of the DM immunoglobulin molecule.

According to a preferred embodiment, the antibody of this invention, designated DM monoclonal antibody, was produced via hybridoma techniques as described by Kohler and Milstein, with minor modifications as described in Example 1, *infra*. Spleen cells from a spontaneously diabetic mouse were used as a source of cells producing an antibody against the autoantigen located on the mouse beta cells. The DM hybridoma, producing the DM monoclonal antibody, has been deposited with the ATCC, Rockville, Maryland. The deposit was received by the ATCC July 11, 1990, and has been there identified as follows:

DM Monoclonal Antibody Accession No.: HB 10502

The DM monoclonal antibody is of the IgM class. As described below in Example 2, the antibody displays a very strong reactivity with pancreatic beta cells and cell lines which express a specific plasma membrane bound antigen similar to the 64 kd autoantigen located on pancreatic beta cells. The DM monoclonal antibody shows no detectable binding to human stomach or kidney cells, nor to rat stomach, heart, liver and kidney cells.

It should be understood that the present invention encompasses the DM monoclonal antibody described above and any fragments thereof containing the active binding region of the antibody, such as Fab, F(ab)'2 and Fv fragments. Such fragments can be produced from the DM monoclonal antibody using techniques well established in the art (see e.g. Rousseaux et al., in Methods Enzymol., 121:663-69, Academic Press (1986)).

As contemplated by the present invention, the DM monoclonal antibody recognizes and binds to an immunodominant epitope of the approximately 64 kd beta cell autoantigen. The immunodominant epitope is the antigenic determinant site on an immunogen to which most of the immune response is directed. In autoimmune diseases, and in particular IDDM, the immunodominant epitope is the antigenic determinant site to which the major reactivity of autoantibodies are targeted. (Saus et al. J. of Biol. Chem. 263: 13374-13380 (1988)). The antigenic determinant is that portion of an antigen that makes contact with a particular antibody or T cell receptor. In a typical protein antigen, it is likely that any residue accessible from the surface is part of one or another antigenic determinant. A protein therefore has sequential and/or conformational determinants. The molecular weight of the beta cell

autoantigen is approximately 64,000 daltons, as determined by polyacrylamide gel electrophoresis. This autoantigenic protein component of beta cell plasma membrane is the primary target for autoantibodies, namely anti-64 kd autoantibodies, responsible for immune destruction of islet beta cells. This autoantigen is considered to be primarily involved in the beta cell autoimmunity underlying IDDM based on the concurrence of the anti-64 kd autoantibody with the onset of IDDM in man, mouse and rat, by the early appearance of anti-64 kd autoantibody in the natural history of islet cell inflammation in these species, and by the restriction of this protein autoantigen to beta cells and perhaps to cell membranes (Atkinson et al., Lancet, 335:1357-60, (1990)).

The specificity of the DM monoclonal antibody for the immunodominant epitope of the beta cell autoantigen is indicated by the observation, as illustrated in Example 2, infra, that the DM monoclonal antibody and the autoantibody reactive with the beta cell autoantigen exclude each other from binding to the beta cell autoantigen. Accordingly, the DM monoclonal antibody inhibits the binding of human islet cell autoantibody positive serum to human pancreatic beta cells.

It should be understood that the specificity of the DM monoclonal antibody encompassed by the present invention includes recognizing and binding functional equivalents of the immunodominant epitope of the beta cell autoantigen.

The specificity of the DM monoclonal antibody for the 64,000 dalton beta cell autoantigen is further indicated, as shown in Example 3 infra, by the DM monoclonal antibody's capability of precipitating antibody-dependent complement-mediated cytotoxicity of rat insulinoma cells. The DM monoclonal antibody

specifically mediates cytotoxicity of these insulinoma cells.

The present invention further embodies the conjugation of the DM monoclonal antibody or purified beta cell autoantigen to a label capable of producing a detectable signal for use in various types of quantitative immunoassays. These immunoassays include monoclonal competitive immunoassays, monoclonal "sandwich" direct immunoassays and indirect immunoassays to carry out the method of the invention involving detection of beta cell autoantigen. The three most common quantitative immunoassays are radioimmunoassay (both soluble phase and solid phase), quantitative fluorescence immunoassays, and quantitative enzyme-linked assays. Any of these may be used to practice the invention. These assays involve the formation of an antigen-antibody complex that includes a label and the detection of such complexes via the label. As used herein, the term "label" is intended to include moieties that may be detected directly such as fluorochromes and radiolabels, as well as moieties like enzymes that must be reacted or derivatized to be detected.

The particular label that is used will depend on the type of quantitative immunoassay used. The assay should be such as to provide sensitivities sufficient for detection by labeled DM monoclonal antibody of beta cell autoantigen or detection of anti-64 kd autoantibodies by labeled beta cell autoantigen. Examples of labels that may be used are radiolabels such as ^{32}P, ^{125}I, ^{3}H, ^{14}C, fluorescent labels such as fluorescein and its derivatives, rhodamine and its derivatives, dansyl, and umbelliferone, chemiluminescers such as luciferin and 2,3-dihydrophthalazinediones, and enzymes, such as horseradish peroxidase, alkaline phosphatase, lysozyme, and glucose-6-phosphate dehydrogenase. The antibody, or, as described below,

purified beta cell autoantigen, may be tagged with such labels by known methods. For instance, coupling agents, such as aldehydes, carbodiimides, dimaleimide, imidates, succinimides, bisdiazotized benzidine, and the like may be used to tag the antibodies or antigens with fluorescent, chemi-luminescent, or enzyme labels.

The label detection means used in the immunoassay will depend upon the particular label involved. For instance, enzyme labels may be read with colorimeters that detect the magnitude of color change caused by the reaction between the enzyme and the substrate or spectrophotometers (e.g. microtiter plate readers), radiolabels may be read with scintillation counters or gamma counters, and fluorescent labels with fluorescent microscopes (Fluorimeters).

An indirect immunoassay, such as enzyme linked immunosorbent assay (ELISA) is a common method for detecting reactivity between antigen and antibody. In the ELISA, an antigen or antibody is typically bound (immobilized) to a solid phase, such as a tube, bead or well surface. For instance, an antigen may be immobilized to a solid phase. Then, a patient's serum sample containing a first antibody reactive with the bound antigen is added, and a second enzyme-labeled antibody against the retained antibody is added. Immobilized antigen-antibody complexes are then detected via reaction of the enzyme with an appropriate substrate. For example, the enzyme horseradish peroxidase may be used along with its substrate orthophenylene diamine as a color indicator.

Further, in accordance with the embodiments of the DM monoclonal antibody provided, anti-id antibodies can be developed which exhibit selectivity toward antigenically distinct features of immunoglobulins, in particular, the DM monoclonal antibody of the present invention.

Anti-id antibodies are prepared according to Rivas et al., Proc. Nat. Acad. Sci. USA, 85:6092-6096 (1988) and Miller et al. New Engl. J. Med., 321: 851-857 (1989). Small and active fragments of the DM monoclonal antibody for immunizing mice are prepared according to the method of Mathew and Reichardt in J. Immunol. Methods, 50:239-253 (1982). Briefly, one part of 500 mM Tris, pH 8.0, 10mM $CaCl_2$ is added to nine parts of purified DM monoclonal antibody in PBS. The final concentration of monoclonal antibody is adjusted to 0.3 mg/ml with 50 mM Tris, pH 8.0, 150 mM NaCl, 1 mM $CaCl_2$. The sample is digested with 0.005 mg/ml trypsin (Sigma) for two hours at 30°C. 10 mg/ml of soybean trypsin inhibitor is added to a final concentration of 0.1 mg/ml and incubated for 5 minutes at 37°C. Iodoacetamide is added to a final concentration of 60 mM and incubated at room temperature for ten minutes. The samples are then dialyzed against four changes of 1 liter of 10 mM phosphate buffer, pH 7.4 at 4°C over 48 hours. Active small fragments are further purified by ion exchange chromatography. One hundred micrograms of purified $F(ab')_2$ are used to immunize a rabbit or mouse. Sera are collected after boosting twice. Anti-id antibodies are purified from the sera by immunoaffinity column containing purified DM monoclonal antibody ligand. Purified DM monoclonal antibodies are coupled to a CNBr activated Sepharose 4B gel (Pharmacia) according to the manufacturer's recommendation. The anti-id antibodies are precipitated with ammonium sulfate at 50 percent of saturating concentration. The precipitant is dissolved in PBS and dialyzed extensively against PBS. The preparation is then incubated with mouse DM monoclonal antibodies affinity column overnight at 4°C. Anti-id antibodies from the sera specifically bound to the column is eluted with elution buffer purchased from Bio-Rad and dialyzed extensively against PBS.

Immunoaffinity purified anti-id antibodies are then coupled to the CNBr-activated Sepharose 4B gel or Affigel HZ (Bio-Rad Laboratories, Richmond, CA).

The anti-id antibodies produced are characterized using standard techniques (Miller et al., New Engl J. Med. 321:851-857 (1989); Rivas et al., Proc. Natl. Acad. Sci. USA, 85:6092-96 (1988)). Patients' sera is obtained from subjects previously proved to be positive for IDDM by indirect fluorescent antibody (IFA). The sera is diluted with PBS containing 5% BSA and incubated with rabbit anti-idiotypic antibodies using an affinity column and a non-specific column. Unbound material is collected after overnight incubation at 4°C. The antibody titers of both unbound samples are determined by incubating serially diluted sera on an EIA plate coated with beta autoantigen or DM monoclonal antibodies. The percentage of removal is determined by the ratio of dilution between the rabbit anti-id column and the non-specific column at OD_{405}=0.6.

Development of a hybridoma cell line that produces anti-id antibodies is accomplished using conventional techniques described above with modifications according to Miller et al., New England J. Med, 321:851-857(1989). The major difference involved in selecting for a hybridoma producing anti-id antibodies is that the screening procedures intails excluding selections for variable regions other than the active site.

The invention further concerns the beta cell autoantigen, recombinant methods for producing the beta cell autoantigen, diagnostic methods for the early detection of IDDM using the beta cell autoantigen and DM monoclonal antibody, and immunotherapeutic methods for preventing destruction of pancreatic beta cells in a subject using the beta cell autoantigen, the anti-id antibodies, or combinations of the autoantigen and anti-id antibodies.

The beta cell autoantigen is recognized and bound by the DM monoclonal antibody of the present invention. The monoclonal antibody of the present invention provides an advantage because its specificity of binding to the beta cell autoantigen makes possible the specific isolation of the autoantigen from very small amounts of tissue or fluids. The beta cell autoantigen is further recognized by anti-64 kd autoantibodies, a component of human islet cell positive serum. An epitope on the beta cell autoantigen is the primary target of anti-64 kd autoantibodies that mediate pancreatic islet cell destruction in the pathogenesis of IDDM. The same or a different epitope on the beta cell autoantigen is the primary target of the DM monoclonal antibody. The invention also provides a method for purifying the beta cell autoantigen from cells. In particular, the method employs a cellular source for the beta cell autoantigen, as described in Example 5, _infra_. The cellular source is preferably any tissue or cell line rich in the approximately 64,000 dalton beta cell autoantigen. These sources can be chosen from among pancreatic islet cells or various cell lines characterized by a high concentration of the beta cell autoantigen. Unexpectedly, a most preferred source of the autoantigen is human epidermoid carcinoma (Hep2) cells. (Moore, A.E. et al., _Cancer Res_. 15: 598 (1955)). Other preferred sources, which are also unexpected, are neuronal cell lines, such as human glioblastomas U87MG (ATCC No. HTB14) (Ponten, J. et al., _Acta Path. Microbiol. Scand._ 74: 465:486 (1968)) and U138MG (ATCC No. HTB16) (ibid), human neuroblastomas SKNMC (ATCC No. HTB10) (Spengler et al., _In Vitro_ 8:410 (1973)) and SKNSH (ATCC No. HTB11) (ibid). Rat insulinoma D115 cells (derived from CRI-D11, Carrington, C.A. et al., _J. Endocrinol_. 109: 193-200 (1986)) are another preferred source.

The invention embodies a further method for the production of recombinant beta cell autoantigen, as described in Example 7, _infra_. In particular, the method involves construction of a cDNA library encoding the beta cell autoantigen. The source of nucleic acid for the cDNA library can be a cell line of non-pancreatic origin. It is preferably a cell line which has a high constitutive rate of producing the approximately 64,000 dalton beta cell autoantigen, such as HEp2 cells. Other preferred sources include the neuronal cell lines described above, and rat insulinoma cells.

The techniques for sequencing, cloning and expressing DNA sequences encoding the beta cell autoantigen e.g synthesis of oligonucleotides, probing a cDNA library, transforming cells, constructing vectors, extracting messenger RNA, preparing cDNA libraries, and the like are well-established in the art, and most practitioners are familiar with the standard resource materials for specific conditions and procedures. However, the following paragraphs are provided for convenience and notation of modifications where necessary, and may serve as a guideline.

Sequencing

Isolated DNA encoding the beta cell autoantigen of the invention is analyzed by restriction and/or sequenced by the dideoxy method of Sanger et al., _Proc. Nat. Acad. Sci. USA_ 74:5463 (1977) as further described by Messing et al., _Nucleic Acids Res_. 9:309 (1981) or by the method of Maxam et al., _Methods in Enzymol._ 65:499 (1980).

Cloning

The DNA sequences encoding the beta cell autoantigen are obtained from a cDNA library prepared

from mRNA isolated from cells expressing the beta cell autoantigen according to procedures described in Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, pp. 198-198 (1982), with particular reference to
5 Young et al., Nature, 316:450-452 (1988).

The cDNA insert from the successful clone, excised with a restriction enzyme such as EcoRI, is then used as a probe of the original cDNA library to obtain the additional clones containing inserts encoding other
10 regions of the autoantigen which, together with this probe, span the nucleotides containing the complete coding sequence of the beta cell autoantigen.

Probing cDNA

15 A cDNA library is screened using the methods described in Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, pp. 198-198 (1982), with particular reference to Young et al., Nature, 316:450-452 (1988).

20 cDNA Library Production

Double-stranded cDNA is synthesized and prepared for insertion into a lambda vector using the technique of Okayama and Berg, Molecular and Cellular Biology, 2:263 (1982). First strand cDNA is synthesized by the
25 RNA-dependent DNA polymerase (reverse transcriptase) from a source such as Avian Myeloblastosis Virus by priming with oligo (dT) 12-18 or random primer on 5 μg mRNA. Second strand DNA is synthesized by using *E. coli* DNA polymerase I and the nicked RNA as primers. Any
30 nicks in the double-stranded cDNA were repaired by *E. coli* ligase. These molecules were further blunt-ended by adding T4 DNA polymerase. Linkers containing EcoRI and NotI sites were added. The blunt-ended, double stranded cDNA is then fractionated by agarose gel
35

electrophoresis. cDNA roughly greater than 500 base pairs is recovered for further library construction.

Vector Construction

5 Construction of a suitable lambda vector containing the desired coding and control sequences employs standard ligation and restriction techniques which are well understood in the art. (Young et al., Nature 316:450-452 (1988)).

10 Site specific DNA cleavage is performed by treating with the suitable restriction enzyme, such as EcoRI, (or enzymes) under conditions which are generally understood in the art, and the particulars of which are specified by the manufacturer of these commercially available 15 restriction enzymes. See, e.g., New England Biolabs, Product Catalog. In general, about 1 μg of phage DNA sequence is cleaved by one unit of enzyme in about 20 μl of buffer solution; in the examples herein, typically, an excess of restriction enzyme is used to ensure 20 complete digestion of the DNA substrate. Incubation times of about one hour to two hours at about 37°C are workable, although variations can be tolerated. After each incubation, protein is removed by extraction with phenol/chloroform, and may be followed by ether 25 extraction and the nucleic acid recovered from aqueous fractions by precipitation with ethanol.

In vector construction employing "vector fragments", the vector fragment is commonly treated with bacterial alkaline phosphatase (BAP) or calf intestinal 30 alkaline phosphatase (CIP) in order to remove the 5' phosphate and prevent religation of the vector. Digestions are conducted at pH 8 in approximately 150 mM Tris, in the presence of Na+ and Mg^{+2} using about 1 unit of BAP or CIP per μg of vector at 60°C or 37°C, 35 respectively, for about one hour. In order to recover the nucleic acid fragments, the preparation is extracted

with phenol/chloroform and ethanol precipitated. Alternatively, religation can be prevented in vectors which have been double digested by additional restriction enzyme digestion of the unwanted fragments.

5 Ligations are performed in 15-50 μl volumes under the following standard conditions and temperatures: 20 mM Tris-Cl, pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 33μg/ml BSA, 10 mM-50 mM NaCl, and either 40 μM ATP, 0.01-0.02 (Weiss) units T4 DNA ligase at 0°C. (for "sticky end" 10 ligation) or 1 mM ATP, 0.3-0.6 (Weiss) units T4 DNA ligase at 14°C (for "blunt end" ligation). Intermolecular "sticky end" ligations are usually performed at 33-100 μg/ml total DNA concentrations (5-100 nM total end concentration). Intermolecular blunt 15 end ligations (usually employing a 10-30 fold molar excess of linkers) are performed at 1 μM total ends concentration.

Verification of Construction

20 Correct ligations for lambda vector construction are confirmed according to the procedures of Young et al., Nature, 316:450-452 (1988).

Expression

25 The nucleotide sequences encoding the beta cell autoantigen may be expressed in a variety of systems as set forth below. The cDNA may be excised by suitable restriction enzymes and ligated into procaryotic or eucaryotic expression vectors for such expression.

30

Hosts and Control Sequences

Both procaryotic and eucaryotic systems may be used to express the DNA sequences encoding the beta cell autoantigen; procaryotic hosts are the most convenient 35 for cloning procedures. Procaryotes most frequently are represented by various strains of *E. coli*; however,

other microbial strains may also be used. Commonly used procaryotic control sequences which are defined herein to include promoters for transcription initiation, optionally with an operator, along with ribosome binding site sequences, include such commonly used promoters as the beta-lactamase (penicillinase) and lactose (lac) promoter systems (Chang et al., Nature 198: 1056 (1977)) and the tryptophan (trp) promoter system (Goeddel et al., Nucleic Acids Res. 8:4057 (1980)) and the lambda derived P_L promoter and N-gene ribosome binding site (Shimatake et al., Nature 292:128 (1981)).

In addition to bacteria, eucaryotic microbes, such as yeast, may also be used as hosts. Laboratory strains of *Saccharomyces cerevisiae*, Baker's yeast, are most used although a number of other strains are commonly available. Vectors employing, for example, the 2μ origin of replication of Broach, Meth. Enz. 101:307 (1983), or other yeast compatible origins of replications (see, for example, Stinchcomb et al., Nature 282:39 (1979)); Tschempe et al., Gene 10:157 (1980); and Clarke et al., Meth. Enz. 101:300 (1983)) may be used. Control sequences for yeast vectors include promoters for the synthesis of glycolytic enzymes (Hess et al., J. Adv. Enzyme Reg. 7:149 (1968); Holland et al., Biochemistry 17:4900 (1978)). Additional promoters known in the art include the promoter for 3-phosphoglycerate kinase (Hitzeman et al., J. Biol. Chem. 255:2073 (1980)), and those for other glycolytic enzymes. Other promoters, which have the additional advantage of transcription controlled by growth conditions are the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, and enzymes responsible for maltose and galactose utilization. It is also believed terminator sequences are desirable at the 3' end of the coding sequences.

Such terminators are found in the 3' untranslated region following the coding sequences in yeast-derived genes.

It is also, of course, possible to express genes encoding polypeptides in eucaryotic host cell cultures derived from multicellular organisms. (See, for example, Tissue Cultures, Academic Press, Cruz and Patterson, Eds. (1973)). These systems have the additional advantage of the ability to splice out introns and thus can be used directly to express genomic fragments. Useful host cell lines include VERO and HeLa cells, and Chinese hamster ovary (CHO) cells. Expression vectors for such cells ordinarily include promoters and control sequences compatible with mammalian cells such as, for example, the commonly used early and late promoters from Simian Virus 40 (SV 40) (Fiers, et al., Nature 273:113 (1973)), or other viral promoters such as those derived from polyoma, Adenovirus 2, bovine papilloma virus, or avian sarcoma viruses. The controllable promoter, hMTII (Karin, et al., Nature 299:797-802 (1982)) may also be used. General aspects of mammalian cell host system transformations have been described by Axel (U.S. Patent No. 4,399,216 issued Aug. 16, 1983). It now appears, that "enhancer" regions are important in optimizing expression; these are, generally, sequences found upstream or downstream of the promoter region in non-coding DNA regions. Origins of replication may be obtained, if needed, from viral sources. However, integration into the chromosome is a common mechanism for DNA replication in eucaryotes.

Transformations

Depending on the host cell used, transformation is done using standard techniques appropriate to such cells. The treatment employing calcium chloride, as described by Cohen, Proc. Natl. Acad. Sci. USA (1972) 69:2110 (1972) or the $CaCl_2$ method described in Maniatis,

et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, p. 254, (1982), may be used for procaryotes or other cells which contain substantial cell wall barriers. For mammalian cells without such cell walls, the calcium phosphate precipitation method of Graham and van der Eb, Virology 52:546 (1978), optionally as modified by Wigler et al., Cell 16:777-785 (1979), may be used. Transformations into yeast may be carried out according to the method of Van Solingen et al., J. Bact. 130:946 (1977), or of Hsiao et al., Proc. Natl. Acad. Sci. USA 76:3829 (1979).

Other representative transfection methods include DEAE-dextran mediated transfection techniques, lysozyme fusion or erythrocyte fusion, scraping, direct uptake, osmotic or sucrose shock, direct microinjection, indirect microinjection such as via erythrocyte-mediated techniques, and/or by subjecting host cells to electric currents. The above list of transfection techniques is not considered to be exhaustive, as other procedures for introducing genetic information into cells will no doubt be developed.

Protein Recovery

The beta cell autoantigen may be produced either as a mature protein or a fusion protein, or may be produced along with a signal sequence in cells capable of processing this sequence for secretion. It is advantageous to obtain the protein in its native conformation by obtaining it as it is secreted from the endoplasmic reticulum membrane to the membrane compartment. This minimizes the difficulties in purification; thus, it is preferred to express the beta cell autoantigen gene which includes the codons for native signal sequence in cells capable of appropriate processing. It has been shown that cultured mammalian cells are able to cleave and process heterologous

mammalian proteins containing signal sequences, and to secrete them into the membrane compartment. (McCormack et al., Mol. Cell. Biol. 4:166 (1984)). When secreted into the medium, the protein is recovered using standard protein purification techniques. However, while the procedures are more laborious, it is within the means known in the art to purify this protein from sonicates or lysates of cells in which it is produced intracellularly in fused or mature form.

The beta cell autoantigen so produced by recombinant means has a determinant site containing the immunodominant epitope associated with IDDM. Accordingly, the beta cell autoantigen is specifically recognized and bound by (a) the DM monoclonal antibody of the present invention, and (b) anti-64 kd autoantibodies produced in a subject in response to the beta cell autoantigen.

The beta cell autoantigen so produced by recombinant means can be used to produce monoclonal antibodies to the autoantigen by immunizing suitable animals, such as rats or mice, and then applying the monoclonal antibody techniques discussed above. Human monoclonal antibodies can also be made to the recombinant autoantigen by sensitization of human lymphocytes in vitro followed by EBV transformation or hybridization of the antigen-sensitized lymphocytes with mouse or human lymphocytes.

In accordance with the present invention, a diagnostic method is also provided for detecting autoantibodies reactive with the beta cell autoantigen in a sample from a human. The method can be useful in the diagnosis of IDDM, and in particular, the diagnosis of the early onset of IDDM. The method involves reacting the beta cell autoantigen of the present invention, labeled, for example with a fluorescent label, with a sample of tissue or fluid from a suspected

IDDM patient. Preferably, the sample is fluid, such as serum. The beta autoantigen, fragments or equivalents thereof, can be derived from purification of a cell line rich in the beta autoantigen, such as HEp2 cells. Alternatively, the beta autoantigen can be derived from recombinant DNA techniques, preferably a cDNA library. The beta autoantigen derived from cDNA methods can be a fusion protein comprised of a carrier protein and the DM antigen or DM antigen fragment. The carrier protein can be beta-galactosidase or arabinose isomerase. Standard techniques, such as direct or indirect immunofluorescence, immunoprecipitation, or enzyme linked immunoabsorbent assay (ELISA) are used to detect reaction between the beta cell autoantigen and autoantibodies reactive with the beta cell autoantigen. These techniques also apply to staining tissue sections to localize antigens or antibodies on the surfaces of or inside cells.

Also included within the scope of the invention is a method for diagnosing IDDM by detecting endogenous beta cell autoantigen. This method is a serologic diagnostic technique for detecting endogenous beta cell autoantigen that has been secreted or "shed" into the serum or other biological fluids of patients thought to be suffering from IDDM. The method involves reacting the purified, labeled DM monoclonal antibody of the invention with a sample of human tissue or fluid. The tissue sample is preferably pancreatic islet tissue. The fluid sample is preferably serum. Standard immunological techniques are used to then detect the interaction of the DM monoclonal antibody with endogenous beta cell autoantigen, fragments or equivalents of endogenous beta cell autoantigen. The method employs the DM antibody conjugated to a label capable of producing a detectable signal. The label can

be selected from the group consisting of a radionuclide, an enzyme, a fluorescer and a chromophore.

Further in accord with the embodiments of the invention is a vaccine and methods for treating IDDM patients with the vaccine. The vaccine contains an effective amount of the DM monoclonal antibody in association with a pharmaceutically acceptable carrier vehicle. The vaccine is effective because the DM monoclonal antibody contains the same idiotypic determinant site as the beta cell autoantigen autoantibody. Accordingly, a method of the present invention comprises introducing into individuals the vaccine comprising the monoclonal antibody, stimulating the production of anti-id antibodies directed toward the autoantibodies. The anti-id antibodies prevent or retard destruction of pancreatic islet cells by anti-64 kd autoantibodies. This method comprises introducing the vaccine preferably into subjects who are either already producing islet cell autoantibody or who are likely to begin producing islet cell autoantibody. Individuals who are likely to begin producing islet cell autoantibodies are those who are first degree relatives of probands with IDDM.

Preparation of Vaccines

Vaccines may be prepared from one or more immunogenic polypeptides encoded within cDNA sequences isolated by utilizing the cDNA sequences encoding the DM monoclonal antibody or from the genome of hybridoma ATCC No. HB 10502 to which they correspond. The preparation of vaccines which contain an immunogenic polypeptide(s) as active ingredients, is known to one skilled in the art. (See, for example, Voller and Friedman, Eds. New Trends and Developments in Vaccines, University Park Press, Baltimore, MD (1978)).

Administration to an IDDM patient of a vaccine which comprises immunogenic polypeptide(s) associated with the DM monoclonal antibody stimulates in vivo the production of anti-id antibodies against anti 64 kd autoantibodies. Specific reactivity between the anti-id antibodies and the autoantibodies eliminates autoantibodies from the pool of anti-64 kd autoantibodies reactive with beta cell autoantigen. The result is that the anti-id antibodies prevent or slow down the rate of pancreatic beta cell destruction by anti-64 kd autoantibodies.

Typically, such vaccines are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection, may also be prepared. The preparation may also be emulsified, or the protein encapsulated in liposomes. The active immunogenic ingredients are often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the vaccine may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, and/or adjuvants which enhance the effectiveness of the vaccine. Examples of adjuvants which may be effective include but are not limited to: aluminum hydroxide, N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-nor-muramyl-L-alanyl-D-isoglutamine (CGP 11637, referred to as nor-MDP), N-acetylmuramyl-L-alanyl-D-sialoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-ethylamine (CGP 19835A, referred to as MTP-PE), and RIBI, which contains three components extracted from bacteria, monophosphoryl lipid A, trehalose dimycolate and cell wall skeleton (MPL+TDM+CWS) in a 2% squalene/Tween 80 emulsion. The

effectiveness of an adjuvant may be determined by measuring the amount of antibodies directed against an immunogenic polypeptide containing DM monoclonal antibody antigenic sequence resulting from administration of this polypeptide in vaccines which are also comprised of the various adjuvants.

The vaccines are conventionally administered parenterally, by injection, for example, either subcutaneously or intramuscularly. Additional formulations which are suitable for other modes of administration include suppositories and, in some cases, oral formulations. For suppositories, traditional binders and carriers may include, for example, polyalkylene glycols, e.g., polyethylene glycol, or triglycerides; such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5% to 10%, preferably 1%-2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 10-95% of active ingredient, preferably 25%-70%.

The autoantigen may be formulated into the vaccine as neutral or salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with free amino groups of the peptide) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids such as acetic, oxalic, tartaric, maleic, and the like. Salts formed with the free carboxyl groups may also be derived from inorganic bases such as, for example, sodium potassium, ammonium, calcium, or ferric hydroxides, and such

organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

Dosage and Administration of Vaccines

5 The vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be prophylactically and/or therapeutically effective. the quantity to be administered, which is generally in the range of 5 10 micrograms to 250 micrograms of antigen per dose, depends on the subject to be treated, capacity of the subject's immune system to synthesize antibodies, and the degree of protection desired. Precise amounts of active ingredient required to be administered may depend 15 on the judgment of the practitioner and may be peculiar to each subject.

The vaccine may be given in a single dose schedule, or preferably in a multiple dose schedule. A multiple dose schedule is one in which a primary course of 20 vaccination may be with 1-10 separate doses, followed by other doses given at subsequent time intervals required to maintain and or reenforce the immune response, for example, at 1-4 months for a second dose, and if needed, a subsequent dose(s) after several months. The dosage 25 regimen will also, at least in part, be determined by the need of the individual and be dependent upon the judgment of the practitioner.

In addition, the vaccine containing the immunogenic DM monoclonal antibody antigen(s) may be administered in 30 conjunction with other immunoregulatory agents, for example, immunoglobulins.

Therapeutic procedures

The antibodies of the invention are also used in 35 therapeutic procedures for treating IDDM. Falling within the scope of the embodiments of the present

invention is a method of immunotherapy to treat IDDM that involves two steps. A first step employs an immunoaffinity column designed to remove autoantibodies specifically reactive with beta cell autoantigen from a patient's plasma. The immunoaffinity columns loaded with the anti-id antibodies and/or autoantigen of the present invention can remove a clinically significant percentage of autoantibodies specifically reactive with beta cell autoantigen from the plasma of an IDDM patient. Plasmapheresis, which is a method for the treatment of autoimmune diseases, can be used for carrying out this procedure. In plasmapheresis, plasma is removed from an individual and passed through columns containing ligands for specifically removing from the plasma the autoantibodies responsible for attacking and destroying specific tissue (Jones et al. Plasma Therapy Transfusion & Technology, 7:333-349 (1986). This method typically involves extracorporeal immunoadsorption for the reduction of plasma factors known to be associated with enhancement of immune response in IDDM. An immunoadsorptive column can have protein ligands covalently bound to an inert silica matrix. The patient's whole blood is separated using either an apheresis machine or centrifuge. A specified volume of the plasma is perfused through the column and then returned to the patient. In particular, the method of the present invention accordingly employs a plasmapheresis column loaded with anti-id antibodies and/or autoantigen. The column is tested with one patient's plasma at a predefined dilution. An individual preparation of anti-idiotypic antibody is initially immobilized on CNBr-activated Sepharose 4B gel. Plasma from an individual is run through the column using conventional plasmapheresis protocols.

This first step involves treating the peripheral circulation of a patient for a sufficiently long time to

remove substantially all of the circulating anti-64 kd autoantibodies (Jones et al., Plasma Therapy Transfusion & Technology, 7:333-349 (1986)). The peripheral circulation is that portion of the blood circulating from the heart through the arteries, capillaries, and veins back again to the heart. A typical plasmapheresis apparatus is made by Prosorba(TM), (Imre Corp. Seattle, WA). The ligand is preferably the anti-id antibodies that specifically recognize and bind autoantibody specifically reactive with beta cell autoantigen. Beta-cell autoantigen may also be used as the ligand. Using an immunoassay, such as ELISA, the peripheral circulation can be assayed after various treatment periods for the presence of autoantibodies by reacting a sample of plasma with anti-id antibody.

The second step of destroying lymphocytes involves introducing to a human a sufficient amount of anti-id antibody reactive with the autoantibody. Specifically, the invention uses anti-id antibodies to recognize and bind to lymphocytes that present anti-beta cell autoantigen autoantibody on their surface. Autoantibodies presented on the surface of these lymphocytes combine with the anti-id antibodies to form an antigen-antibody complex. This complex reacts with complement and results in destruction, that is, lysis of the cell. Geha, Advances in Immunology, 39: 255-297 (1986). Thus, the anti-id antibody suppresses the generation of the immune response to beta cell autoantigen.

Alternatively, anti-id antibodies or beta cell autoantigen are linked with toxins and carry the toxins to the surfaces of B-lymphocytes that are producing autoantibody. At the surfaces of these cells, the anti-id antibodies or autoantigen react and bind specifically with autoantibodies. The toxin molecule is then absorbed into the B-cell, killing it. Examples of

toxins include, but are not limited to, abrin and gelanin (Thorpe, P.E. et al., Eur. J. Biochem., 116:447-454 (1981)), and diphtheria toxin A chains and ricin A chains (Oeltmann, T.N. and Forbes, J.T., Arch. Biochem. Biophys. 209: 362-370 (1981)). Anti-id antibodies also cause destruction of a specific population of T-cells which had been previously presented beta cell autoantigen by a macrophage. These T-cells have receptors on their surfaces that specifically react with anti-id antibody of the present invention. Accordingly, anti-id antibody, by itself or conjugated with a toxin, will cause toxicity to these T-cells. Because autoantigen specifically reacts with autoantibody, autoantigen linked with a toxin will destroy B-lymphocytes and does so by specifically reacting with and binding to autoantibody located at the surface of these B-lymphocytes (Kernan et al., JAMA, 259:3154-3157 (1988), Blythman et al., Nature, 290:145-146 (1981)).

In order that the invention described herein may be more fully understood, the following examples are set forth. It should be understood that these examples are for illustrative purposes only and are not to be construed as limiting the scope of this invention in any manner.

EXAMPLE 1

Preparation of Hybridoma Cell Line ATCC No. HB 10502 Producing DM Monoclonal Antibody

Cloning of Hybridoma Cell Line ATCC No. HB 10502

A male, spontaneously diabetic, non-obese mouse (NOD) obtained from Clea Japan, Inc., Tokyo, Japan, was sacrificed at 12 weeks of age. The spleen was removed and the cells obtained therefrom obtained were fused with myeloma line HL1-653 (Ventrex Laboratories, Inc.,

Portland, Maine) according to the procedure described in the Ventrex product brochure, following the method of Taggart, R. et al. Science 219:1228 (1983). The myeloma cells were washed twice with complete medium without fetal calf serum (FCS). The spleen cells were washed twice with serum free medium. 10^8 spleen cells were combined with 10^7 myeloma cells in a 50 cc conical tube. This mixture was washed again with serum free medium then spun down. The supernatant was discarded and the pellet loosened. The tube containing the cell mixture was immersed in a 37° water bath. 1 ml of 50% PEG is added with gentle shaking over a minute. Over the next 7 minutes, with gentle shaking of the tube, 12 ml of RPMI medium without serum was added. The cell mixture was then centrifuged at 1000 rpm for 5 minutes. The pellet was gently resuspended in enough AAT medium (Adenine, 7.5×10^{-5} M, Aminopterin, 8×10^{-7} M, Thymidine, 1.6×10^{-5} M) to yield about 3×10^6 spleen cells per ml. About 150 microliters of cell mixture was plated per well. Fused cells were plated on 96-well culture plates. Selection of hybridoma clones was accomplished by testing the binding of supernatants of the clones to frozen human pancreas sections using the immunofluorescence assay procedure described in Example 2 below. Positive clones were further subcloned by limited dilution. Hybridoma ATCC No. HB 10502 was subcloned from the limited dilution of one of these positive clones. Binding of antibodies produced by hybridoma ATCC No. HB 10502 to frozen pancreas sections was confirmed according to the procedures in Example 2, infra. (Bonifacio et al., J. Immunol. Methods, 106:83-88 (1988)).

Adaptation of Hybridoma Cell Line ATCC No. HB 10502 to Serum Free Medium

Hybridoma cell line ATCC No. HB 10502 was adapted to serum free medium to simplify subsequent purification of the monoclonal antibody free of contaminating proteins, specifically other contaminating serum-originating immunoglobulins. Hybridoma line ATCC No. HB 10502 was originally grown in HL1 medium (Ventrex, Portland, Maine) plus 1% calf bovine serum. HL1 medium is optimized for the growth of hybridoma lines derived from myeloma lines HL1-653. The adaptation procedure was initiated by changing the growth medium to WRC935 medium (Amicon, Danvers, MA) plus 1% calf bovine serum. After one to two weeks of adaptation, the serum concentration was gradually reduced to 0.5%, then to 0.25%, and finally to medium free of serum supplement.

The production of the DM monoclonal antibody by the hybridoma of the present invention was doubled as estimated by an ELISA method using the goat anti-mouse IgM (mu chain specific)-alkaline phosphatase conjugate as secondary antibody. (Engvall and Perlmann, Immunochemistry 8:871-879 (1971); Van Weeman and Schuurs, FEBS Lett. 15:232-236 (1971)).

Purification of the DM Monoclonal Antibody From Cultured Medium of Hybridoma Cell Line ATCC No. HB 10502

The serum-free cultured medium was collected by centrifugation and concentrated 60 to 100 fold. The concentrate was treated with DNase I and RNase A at concentrations of 1 microgram/ml respectively at 4°C for 2 hours. Six milliliters of clarified concentrate was loaded on a Sephacryl S-300 column (1.5 cm x 120 cm) (Pharmacia, Piscataway, NJ) and eluted with PBS buffer. The fractions containing the antibody were identified by SDS-PAGE and pooled.

The purity of the DM monoclonal antibody was examined on SDS-PAGE. Purity was greater than approximately 95% and the preparation was free of bovine IgM.

5

EXAMPLE 2

Characterization of the DM Monoclonal Antibody

I. Binding

10 Immunofluorescence Assay

Tissues were obtained from different organs. These organs and the cell lines tested are listed in Table 1 ("Characterization of the specificity of DM Monoclonal Antibody to different tissues and cell lines by IFA 15 Method"). These tissues were islets from human, rat and dog; human pancreas and kidney, rat stomach, heart, liver and kidney. The following cell lines were obtained: myeloma cells HL1-653 (Taggart, R.T. et al., Science 219:1228-1230 (1983)) from mouse; rat insulinoma 20 cells D11D5 (Carrington, C.A. et al., J. Endocrin. 109:190-200 (1986)); and human epidermoid carcinoma cells - HEp2 (Moore, A.E. et al. Cancer Res. 15:598 (1955)).

25 Tissues

The tissues were prepared according to Bonifacio et al., J. Immunol. Methods, 106: 83-88 (1988). Tissue sections were cut at 4 microns thickness by cryostat and stored in a sealed plastic bag at -80°C until used.

30 The 4-micron thick tissue sections were thawed at room temperature and incubated with purified DM monoclonal antibody serially diluted in phosphate buffered saline (PBS), produced as described above in Example 1. The incubations were carried out in a 35 humidified chamber for two hours (or overnight) at room temperature.

The sections were washed once with PBS - 0.05% Tween 20 and three times with PBS alone with gentle shaking. After brief air drying, the sections were incubated with goat anti-mouse (IgG and IgM)-FITC conjugate (at 1:80 dilution) for one hour. After repeating the washing procedure as described above, the sections were coated with mounting buffer, covered with a cover slide and examined under a fluorescent microscope for binding of the DM monoclonal antibody to the various tissues.

The DM monoclonal antibody stained the frozen human pancreas sections but not sections of other organs, such as human kidney and stomach, and rat stomach, heart, liver and kidney. As shown in Table 1, these results demonstrate the specificity of the DM monoclonal antibody for pancreatic tissue, and for islet beta cells which are present in the pancreas. DM monoclonal antibody staining of the pancreas sections also was observed in the epidermal cell layers of the pancreas. For that reason, an epidermoid cell line was tested for reactivity with DM monoclonal antibody. Neuronal cell lines (Table 2, below) were also tested for reactivity with DM monoclonal antibody as neuronal cells and epidermal cells have a common embryonic origin.

<u>Cell lines</u>

Purified islet cells less than three days after harvesting were maintained in RPMI-1640 medium supplemented with 10% bovine serum. The cell lines (e.g. rat insulinoma and mouse myeloma) were grown to confluency in RPMI medium supplemented with 10% calf serum. Cells were detached from the culture flask using trypsin-EDTA (Irvine Scientific, CA) and reseeded on non-treated culture flasks. After 24 hours of cultivation, the cells were harvested by gentle suction and washed one time with PBS.

DM Monoclonal antibody was serially diluted with PBS and incubated with cell suspensions at 10^5 cells/ml for two hours at room temperature. After several washes with PBS, the cells were resuspended in PBS containing goat anti-mouse (IgG and IgM)-FITC conjugate at 1:80 dilution, and incubated at room temperature for another hour. After the cells were washed, they were examined under the microscope.

Table 1 shows that the DM monoclonal antibody was able to bind to the surface of live human, rat and dog islet cells. Binding to insulinoma cells was also observed.

The DM monoclonal antibody was not able to bind to the mouse myeloma line HL1-653, from which hybridoma cell line ATCC No.HB 10502 was derived. As shown in Table 1, the DM monoclonal antibody was able to bind to the cytoplasmic area of fixed human epidermoid carcinoma (HEp2) cells. These binding studies were conducted using the Fluorescent ANA Test, Immunoconcepts, Sacramento, CA.

TABLE 1: CHARACTERIZATION OF THE SPECIFICITY OF DM MONOCLONAL ANTIBODY TO DIFFERENT TISSUES AND CELL LINES BY IFA METHOD.

TISSUE OR CELL LINE	IFA	REMARKS
Human Islet	+	Live Cell, Surface Staining
Rat Islet	+	Live Cell, Surface Staining
Dog Islet	+	Live Cell, Surface Staining
Human Pancreas	+	Frozen Section
Human Kidney	-	Frozen Section
Rat Stomach	-	Frozen Section
Rat Heart	-	Frozen Section
Rat Liver	-	Frozen Section
Rat Kidney	-	Frozen Section
Rat Insulinoma D11D5	+	Live, Cell, Surface Staining
Mouse Myeloma HL1-653	-	Live Cell
Human Epidermoid Carcinoma HEp-2	+	Fixed Cell, Cytoplasm Staining

Identification of Beta Cell Autoantigen-Rich Cells

Different cell lines listed in Table 2 were grown in RPMI-1640 medium supplemented with 10% calf bovine serum. One day before harvesting, the cells were treated with trypsin-EDTA to detach them from the petri dish or culture flask walls. After being washed with RPMI medium the cells were resuspended in the same medium and grown in the non-treated petri dishes in which attachment of the cells to the plastic walls was reduced. Twenty million cells collected from the overnight culture were resuspended in 1 ml of lysis buffer. This buffer contained 20 mM HEPES, pH 7.4, 5 mM benzamidine, 10 mM N-ethylmaleimide, 0.1 mM Na vanadate,

5 mM Na pyrophosphate, 2.5 mM EDTA, 2.5 mM NaF, 0.01% trypsin inhibitor, and 2% Triton X-114. The cells were disrupted by continuously rocking for 4 hours at 4°C. The extracted proteins were obtained after removing the cell debris by centrifugation at 100,000 x g for 10 minutes at 4° C. 75 micrograms of extracted protein from each cell line was separated on 7.5% or 11.25% SDS-PAGE and electroblotted to the nitrocellulose paper. The nitrocellulose paper was then incubated with 10 micrograms/ml of purified DM monoclonal antibody in PBS buffer with 1% nonfat milk at room temperature overnight. After 4 brief washings with PBS, the nitrocellulose paper was incubated with goat-anti-mouse IgM horseradish peroxidase conjugate (at 1:500 dilution) for 2 hours and then developed. Table 2 shows that binding of the DM monoclonal antibody to HEp2 cells occurred with greater intensity than to other cell lines tested, including rat insulinoma. (See Example 5 for use of HEp2 cells as source of the beta cell autoantigen.)

Six cell lines were tested for their reactivities with the DM monoclonal antibody. Table 2 shows that all of the cell lines contain an approximately 64,000 dalton immunoreactive protein band, each with different relative intensities. HEp2 and D11D5 have an additional approximately 52,000 dalton protein that can react with this antibody. The ratio between the approximately 64,000 dalton and approximately 52,000 dalton proteins varied from batch to batch of preparation. Lengthier incubation of HEp2 extracts on nitrocellulose paper with the DM monoclonal antibody revealed two, faint, larger molecular weight bands. The larger weight bands had molecular weights of approximately 75,000 daltons and 85,000 daltons. Occasionally, a 45,000 dalton reactive band shows up and may be due to the low stringent washing conditions, as described.

TABLE 2: IMMUNOBLOT RESULTS OF DIFFERENT CELL LINES USING DM MONOCLONAL ANTIBODY

CELL LINE	INTENSITY	MW (kd) OF ANTIGEN
HEp2	++++	64>>52
D11D5	+++	64<52 or 64>52
U87	++	64
U138	++	64
SKNMC	++	64
SKNSH	+	64

HEp-2: Human Epidermoid Carcinoma
D11D5: Rat Insulinoma
U87: Human Glioblastoma
U138: Human Glioblastoma
SKNMC: Human Neuroblastoma
SKNSH: Human Neuroblastoma

II. Competition between the DM monoclonal antibody and islet cell autoantibody for the specific beta cell autoantigen

Two experiments were designed to test the ability of the DM monoclonal antibody and human islet cell autoantibodies to recognize the same immunodominant epitope, namely, the beta cell autoantigen.

The first experiment involved the ability of either the DM monoclonal antibody or autoantibodies to block the other from binding to the beta cell autoantigen *in situ*. This blocking experiment had two parts. In the first part, the ability of DM monoclonal antibody derived from mouse cells to block binding of human islet cell autoantibody to beta cell autoantigen was tested. In the second part, the ability of the human

autoantibody to block the binding of the DM monoclonal antibody to beta cell autoantigen was tested.

The second experiment involved the ability of a mixture of DM monoclonal antibody and autoantibodies to simultaneously exclude each other from binding to the beta cell autoantigen. This simultaneous exclusion experiment had two parts. In the first part, autoantibody concentration was held constant and mixed with various concentrations of DM monoclonal antibody. In the second part, DM monoclonal antibody concentration was held constant and mixed with various concentrations of autoantibodies.

First experiment: Blocking

Human pancreas sections, 4 microns in thickness, were first incubated for two hours at room temperature in a humidified chamber with different concentrations of purified DM monoclonal antibody. The sections were washed two times with PBS-0.05% Tween 20, then washed two times with PBS, and then incubated another two hours with different concentrations of pooled human ICA positive sera obtained from Specialty Laboratories, Inc., Santa Monica, CA.

The washing procedure was repeated. The sections were then incubated with goat anti-human (IgG and IgM)-FITC conjugate at 1:80 dilution for one hour. After the second washing step, the sections were mounted with mounting buffer and examined for immunofluorescent patterns that reflected the ability of the mouse DM monoclonal antibody to block human islet cell autoantibody. The results, as shown in Table 3, are expressed as relative intensities of fluorescence, the fluorescence being proportional to binding of islet cell autoantibodies to beta cells.

TABLE 3: BLOCKING EFFECT OF DM MONOCLONAL ANTIBODY ON THE BINDING OF HUMAN ICA POSITIVE SERUMTO HUMAN PANCREAS SECTION

MAb (μg/mL) DILUTION	HUMAN ICA POSITIVE SERUM		
	1:8 DILUTION	1:16 DILUTION	1:32
0	4+	3.5+	3.5+
21	3.5+	1.75+	1+
64	1.75+	±	±
191	1.25+	1+	±

Table 3 indicates that DM monoclonal antibody was able to partially inhibit human islet cell antibody (ICA) positive serum from binding to human pancreas sections. Inhibition of ICA binding by DM monoclonal antibody was more effective at lower ICA concentrations. These results demonstrate that occupation of a binding site on beta cells by the DM monoclonal antibody precludes binding of human ICA positive serum to the same or nearby site. Since ICA positive serum reflects the production of islet cell autoantibodies in response to an immunodominant epitope, and since such autoantibodies bind to this immunodominant epitope, it follows that either (a) the DM monoclonal antibody recognizes and binds to an epitope that is the immunodominant epitope of human ICA positive serum or (b) an epitopic site on the autoantigen which, when occupied by the DM monoclonal antibody, precludes binding of the human ICA positive serum to the autoantigen.

To examine whether human ICA positive sera can block mouse DM monoclonal antibody from binding to islet beta cells in pancreas sections, a similar procedure was carried out as described above. However, the cells were

first incubated with human ICA positive sera, and then incubated with mouse DM monoclonal antibody. The cells were then treated with goat anti-mouse (IgG and IgM)-FITC conjugate. Further blocking studies revealed that 5 human ICA positive sera were able to block mouse DM monoclonal antibodies from binding to islet beta cells.

Second experiment: Simultaneous exclusion

Different concentrations of DM monoclonal antibody 10 were mixed with 40 Juvenile Diabetes Foundation (JDF) units of pooled human ICA positive sera and incubated with human pancreas sections overnight at room temperature in a humidified chamber. The sections were then washed and incubated with anti-human (IgG and IgM)- 15 FITC conjugate for one hour as described above in this Example. The pancreas sections were then examined under fluorescence microscopy.

In a separate experiment, different concentrations of human ICA positive sera were mixed with 50 20 micrograms/ml of DM monoclonal antibody and incubated with human pancreas sections overnight at room temperature in a humidified chamber. The sections were then washed and incubated with anti-human (IgG and IgM)-FITC conjugate for one hour, and then examined under 25 fluorescence microscopy. The results are shown in Table 4.

TABLE 4: COMPETITION BETWEEN DM MONOCLONAL ANTIBODY ANDHUMAN ICA POSITIVE SERUM TO THE FROZEN HUMAN PANCREAS SECTION

ANTI-MOUSE (IgG & IgM) POSITIVE SERUM CONJUGATE (JDF UNITS) (1:80)	HUMAN ICA MAb (µg/mL)	GOAT ANTI-HUMAN FITC CONJUGATE (1:80)	GOAT (IgG & IgM) FITC
40	6.25	1+	
40	3.12	1+	
40	1.56	2+	
40	0.78	3+	
40	0.39	3+	
40	0	4+	
40	0		0
40	50		1+
20	50		1+
10	50		2+
5	50		2+
2.5	50		2-3+
1.25	50		2-3+
0	50		3+
0	50	0	

As shown in Table 4, more than 87% of the human ICA positive serum (40 JDF units) was prevented from binding to the frozen human islets when 3.12 micrograms/ml or more of the DM monoclonal antibody of the present
5 invention was coincubated with the human serum. The human positive ICA serum was able to inhibit up to 75%

DM monoclonal antibody from binding to the frozen human pancreas. This inhibition was concentration dependent. These results further suggest that DM monoclonal antibody and the human ICA positive serum can recognize and bind to the same or nearby epitope and exclude each other from binding. This finding is important since it indicates that the DM monoclonal antibody can be used to purify the beta cell autoantigen. The isolated beta cell autoantigen is critically important for development of a diagnostic reagent for the early detection of the early onset of IDDM. The autoantigen is of further importance for the development of therapeutic agents for the removal of islet cell autoantibodies and for the destruction of IDDM-causing lymphocytes.

EXAMPLE 3

Antibody-Dependent Complement-Mediated Cytotoxicity

Rat insulinoma D11D5 cells (Carrington, C.A. et al., J. Endocrin. 109:193-200 (1986)) were used to determine the antibody-dependent complement-mediated cytotoxicity of DM monoclonal antibody. One day before the assay, D11D5 cells were trypsinized and reseeded in non-treated petri dishes. The cells were harvested, resuspended in R10 medium (10% calf serum in RPMI medium), counted and adjusted to a final cell concentration of 10^6 cells/ml. 60 microcuries of ^{51}Cr (specific activity 421 mC/mg) per 10^6 cells were added and incubated at 37°C for one hour. The cells were centrifuged at 1500 rpm for 5 minutes and resuspended in the R10 medium and incubated for another one hour. After washing with 20 ml of PBS twice, the cells were resuspended at a concentration of 1.8×10^6 cells/ml. 0.1 ml of cell suspension (1.8×10^5 cells) was delivered to each tube and 0.1 ml of the DM monoclonal antibody solution-TBS buffer was added to make final

concentrations of 0, 0.5, 1, 2, 10 micrograms/ml. The mixture was then incubated at 37°C. 0.2 ml of rabbit complement (Lot 10, One Lambda Company, Los Angeles, CA) was added to each tube and incubated for another one hour at 37°C. The released ^{51}Cr was collected by centrifugation at 4000 rpm for five minutes. The residual ^{51}Cr in the cells was released by lysing the cells with 0.1% SDS and counted. The specific cytotoxicity was calculated by the formula as described in Table 5).

TABLE 5: ANTIBODY-DEPENDENT COMPLEMENT-MEDIATED CYTOTOXICITY OF DM MONOCLONAL ANTIBODY TO INSULINOMA D11D5

DM-MaB (μg/mL)	RABBIT COMPLEMENT (Dilution)	CI[a] (%)
10	1:2	7.3
2	1:2	10.7
1	1:2	18
0.5	1:2	13

[a] CI: Cytotoxicity Index =

$$100\% \times \left[\frac{(\%\ \text{release of (Ab + C)} - \%\ \text{release of C alone})}{(100\% - \%\ \text{release of C alone})}\right]$$

where C=Complement

The data compiled in Table 5 signifies that DM monoclonal antibody has antibody-dependent complement-mediated cytotoxicity to rat insulinoma D11D5 cells. The best killing effect of DM monoclonal antibody was achieved at a concentration of 1 microgram/ml. The results of Table 5 are consistent with the hypothesis that the humoral immune response to the approximately 64,000 dalton beta cell autoantigen may be in part responsible for the destruction of islet cells. Humoral

immunity is mediated by antibodies that are in the body fluids. The humoral immune response to the beta cell autoantigen is also discussed in Example 5.

5

EXAMPLE 4

In Vivo Effect of the DM Monoclonal Antibody

Twenty three Balb/C mice weighing from 20 g to 27 g were divided into two groups. The experimental group
10 (n=12) was injected intraperitoneally with 100 micrograms purified DM monoclonal antibody in 0.2 ml PBS five times a week. The control group (n=11) was injected intraperitoneally with 0.2 ml PBS five times a week. Starting from the 11th day, the monoclonal
15 antibody was increased to 150 micrograms per mouse and injected daily. The fasting blood glucose level was measured at day 38 by removing food 14 hours prior to collecting blood samples from the tail vein. The glucose concentration was determined by a commercial kit
20 (Sigma Chemical Co., St. Louis, MO).

Table 6 shows the in vivo effects of the DM monoclonal antibody on blood glucose level of Balb/C mice during the glucose tolerance test.

25

TABLE 6: EFFECT OF DM MONOCLONAL ANTIBODY ON FASTING BLOOD GLUCOSE LEVEL OF BalB/C MICE.

(mg/dL)	FASTING BLOOD GLUCOSE LEVEL	
	Day 38	Day 44
Control Group		
n10	7	11
Mean	66.5	80
SD	±6.4	±11
Mean + 2SD	79.3	102
Experimental Group (1+2)		
n	8	10
Mean	83.7	98.3
SD	±14.8	±20.8
% of Control Group	126	123
Experimental Group 1 (≤Mean + 2SD)		
n	4	4
Mean	65.1	72.2
SD	±4.7	±16.2
% of Control Group	97.9	90.2
Experimental Group 2 (> Mean + 2SD)		
n	4	6
Mean	102.4	115.7
SD	±9.0	±5.9
% of Control Group	154	145

Comparing experimental group 2 vs. the control group, Table 6 shows that 50% of the experimental mice had elevated fasting blood glucose levels (102.4 micrograms/dl +/- 9.0 micrograms/dl vs. 65.5 micrograms/dl +/- 5 micrograms/dl) after 38 days of injection. The elevation of blood glucose levels suggests that the DM monoclonal antibody caused an abnormality of beta cell function presumably by interacting with the beta cell autoantigen.

In addition, subacute lymphocyte infiltration in the pancreas tissue was observed in all experimental animals examined by Hematoxylin and Eosin staining. Although all of the islets appeared normal, some of them were surrounded by lymphocytes and many of them were detached from surrounding acini cells. These observations suggest that the reactivity of the DM monoclonal antibody with the beta cell autoantigen *in vivo* attracted lymphocytes to the islets.

It was further observed that several mice developed acute paralysis after one to two months of injecting the DM monoclonal antibody. Baekkeskov et al. (*Nature*, 347:151-156) have demonstrated that autoantibodies associated with the development of IDDM specifically react with a 64 kd antigen associated with a subpopulation of central nervous system neurons. As shown in Example 2, above, and Example 5, below, of the present application, neuronal tissues, e.g. human neuroblastoma and human glioblastoma react specifically with DM monoclonal and are a rich source of the beta cell autoantigen. Reactivity of the DM monoclonal antibody with the 64 kd autoantigen of neuronal tissue in the experimental rats *in vivo* would be consistent with the observed paralysis. Such reactivity may have precipitated autoimmune destruction of neuronal tissue, leading to paralysis.

EXAMPLE 5

Use of HEp2 cells as Source of Beta Cell Autoantigen

After the unexpected finding in Example 2 of relatively strong reactivity between DM monoclonal antibody and human epidermoid carcinoma cells and neuronal cells, the cell lines listed in Table 2 were evaluated to determine the richest source of beta cell autoantigen for subsequent isolation.

Six different cell lines as listed in Table 2 were grown in RPMI-1640 medium supplemented with 10% calf bovine serum. One day before harvesting, the cells were treated with trypsin-EDTA to detach them from the petri dish or culture flask wells.

After being washed with RPMI medium, the cells were resuspended in the same medium and grown in the non-treated petri dishes where attachment of the cells to the plastic wells was reduced.

Twenty million cells collected from the overnight cultures were frozen and thawed once and resuspended in 1 ml of lysis buffer containing 20 mM HEPES, pH 7.4, 5 mM benzamidine, 10 mM N-ethylmaleimide, 0.1 mM Na vanadate, 5 mM Na pyrophosphate, 2.5 mM EDTA, 2.5 mM NaF, 0.01% trypsin inhibitor, and 2% Triton X-114, following the modifications of Baekkeskov et al., Diabetes, 38:1133-1141 (1989) and Bordier, J. Biol. Chem. 256:1604-1607 (1981). The cells were disrupted by gentle, continuous tilt-rocking for four hours at 4°C. The preparation was centrifuged at 100,000 x g for thirty minutes at 4°C. The supernatant containing the extracted proteins was separated from the cell debris pellet.

75 micrograms of extracted protein from each cell line was separated on 7.5% or 11.25% SDS-PAGE and electroblotted to the nitrocellulose paper according to the methods of Burnette, Anal. Biochem., 112:195-203

(1981) and Towbin et al, Proc. Natl. Acad. Sci. USA, 76:4350-4354 (1979). The nitrocellulose paper was then incubated with 5 micrograms/ml of purified DM monoclonal antibody in TBS buffer (10 mM Tris, pH 7.5, 150 mM NaCl) with 1% nonfat milk at room temperature overnight. After four brief washings with TBS, the nitrocellulose paper was incubated with goat anti-mouse IgM-peroxidase conjugate (at 1:500 dilution) for two to four hours and then developed.

Six cell lines were tested for their reactivities with the DM monoclonal antibody. Prior to the unexpected findings of Example 2, it would not have been anticipated that the HEp2 cells, glioblastoma, and neuroblastoma cells would contain significant amounts of the 64 kd autoantigen. Table 2 shows that all of the cell lines contain an approximately 64,000 dalton immunoreactive protein band, each with different relative intensities. HEp2 and D11D5 have an additional approximately 52,000 dalton protein that can react with this antibody. The ratio between the approximately 64,000 dalton and approximately 52,000 dalton proteins varied from batch to batch of preparation. Lengthier incubation of HEp2 extracts on nitrocellulose paper with the DM monoclonal antibody revealed two, faint, larger molecular weight bands. The larger weight bands had molecular weights of approximately 75,000 daltons and 85,000 daltons. Occasionally, a 45,000 dalton reactive band shows up and may be due to the low stringent washing conditions, as described.

The HEp2 cell line surprisingly had the highest specific immunoreactivity with the DM monoclonal antibody, even higher than that of insulinoma cells. This cell line is preferably used for the preparation of the autoantigen, and for the preparation of a cDNA library encoding the autoantigen.

EXAMPLE 6

Purification of 64 kd Autoantigen from HEp2 Cells

HEp2 cells were grown as described in Example 5. After being trypsinized and regrown on non-treated petri dished for 8 to 16 hours at 37°C, the cells were collected by repeatedly pipetting using a 10 ml pipet and transferred to a 50 ml centrifuge tube. The cells were pelleted by centrifugation at 1000 x g for 5 minutes. The cell pellets were resuspended in one-fifth original volume of PBS. 1 ml of the resuspension was transferred to a 1.5 ml Eppendorf tube. The cells were repelletted at 1000 x g for two minutes at room temperature. After the supernatant was removed, the pellets were frozen at - 80°C.

To the frozen pellet was added, at a ratio of 1 ml per 20 to 40 million frozen cells, a solution of 20 mM phosphate buffer, pH 7, 10 mM N-ethylmaleimide, 0.01% trypsin inhibitor, and 2.5 mM EDTA. The pellets were thawed and resuspended by repeatedly pipetting at room temperature. The suspended cells were then rocked for 30 minutes at 4°C. The aqueous soluble fraction was collected by centrifugation at 1500 x g for 2 minutes at room temperature. The pellets (Pellet 1) were washed once with half volume of the same buffer and pooled. The combined supernatant was further centrifuged at 100,000 x g for 2 hours at 4°C. After removing the supernatant (supernatant 1 or SS1), the pellet (Pellet 2) was resuspended in 1 ml of 10 mM phosphate buffer, pH 7, 2% Triton X-114 (Buffer T) and rocked at 4°C for 2 to 16 hours. The extract was collected by centrifugation at 100,000 x g for 30 minutes at 4°C. The Triton X-114 detergent condensation was then performed as described in Example 5. The aqueous phase of the extract (supernatant 2 or SS2) was stored at 4°C or frozen at -80°C until further purification.

Immediately before the next purification step by FPLC - Fast Protein and Polynucleotide Liquid Chromatography (Pharmacia, Piscataway, NJ), the SS2 was centrifuged at 100,000 x g for 10 minutes to remove the aggregates. A MonoQ (Pharmacia) column was employed to further purify the 64 kd autoantigen. The clarified SS2 was directly loaded onto the MonoQ column pre-equilibrated with 10 mM phosphate buffer, ph &, 0.01% Triton X-114. The gradient extended from 0 to 0.5 M NaCl in the same buffer. The majority of 64 kd autoantigen was detected between 0.15 and 0.2 M NaCl. The peak fractions were pooled for further analysis. It was estimated that the 64 kd autoantigen had been enriched by at least 400-fold from the total cell extract. The purity of aqueous, soluble 64 kd autoantigen was estimated at 10% to 30 %.

Pellet 1, prepared as previously described, was used to further purify the aqueous-insoluble form of 64 kd autoantigen. 1 ml of 10 mM phosphate buffer, pH 7, with 2% Triton X-114 was added per amount of lysate equivalent to that derived from 20 to 40 million cells, i.e., equivalent to Pellet 1, and rocked for 2 hours at 4°C. The lysate was clarified at 100,000 x g for 30 minutes at 4°C. The detergent condensation procedure was performed to remove excess detergent as described previously.

The aqueous phase of the extract (pellet-supernatant 1 or PS1) was stored at 4°C or frozen at -80°C until further purification. The next purification step also employed the MonoQ column chromatography in a manner as described above. It was estimated that the 64 kd autoantigen was enriched more than 100-fold after this step, and was deemed suitable for serving as a reagent for diagnosing anti-64 kd autoantibodies in the sera of IDDM patients. (See Example 7).

The partially purified autoantigen was also suitable for undergoing protein sequencing or peptide mapping.

5

EXAMPLE 7

Diagnostic Method to Detect Early Onset of IDDM Using Beta Cell Autoantigen Partially Purified from HEp2 Cells

Beta cell autoantigen extracted from HEp2 cells and
10 partially purified as described in Example 6 was used to develop a diagnostic reagent for specific reactivity with islet cell autoantibody. In particular, an enzyme-linked immunoassay (EIA) method was developed to detect anti-64 kd autoantibodies in patients' sera.
15 Approximately 10 micrograms/ml of the partially purified autoantigen was coated on an Immunolon II plate (Dynatech Laboratories, Inc., Chantilly, VA) overnight at 4°C. Aqueous phase prepared from Buffer T (see Example 6) after detergent condensation was used as a
20 non-antigen background control. After coating, the wells were blocked with TBS-2% (Tris Buffer: 10 mM Tris, pH 7.4, 150 mM NaCl), BSA and 0.05% azide for 30 minutes with shaking at 500 rpm. 100 microliters of human sera at 1:100 dilution in the same buffer were added to each
25 well and incubated for 30 minutes at room temperature with shaking. After washing twice with TBS-0.5% Tween 20 and twice with TBS alone, 100 microliters of goat anti-human Ig-alkaline phosphatase conjugate (1:1000 dilution) in TBS-2% BSA and 5% azide buffer were added
30 to each well and incubated for another 30 minutes. The washing step was repeated and the antibody binding was determined using phosphatase substrate (1 mg/ml buffer, Sigma).

The results are summarized in Table 7.

35

TABLE 7: ENZYME IMMUNOASSAY RESULTS USING PARTIALLY PURIFIED 64 KD AUTOANTIGEN TO DIAGNOSE TYPE 1 DIABETES

	EIA	
	Positive	Negative
Normal sera (n=36)	30%	70%
ICA positive sera (n=37)	65%	35%

An average of 65% of the ICA positive patient sera tested have had an elevated level of autoantibody to the partially purified 64 kd autoantigen preparation. About 30% of the sera from healthy volunteers had an elevated level of antibody recognizing the same antigen preparation. Both patient and control sera with elevated antibody activities were further tested by Western immunoblot against the partially purified 64 kd autoantigen. It was apparent that none of the sera from healthy, normal volunteers and only about 50% of the IDDM patient sera could immunoreact with a protein band at approximately 64 kd in size. However, the intensity of the signal was considerably weaker than the immunoreactivity of the DM monoclonal antibody.

The results suggested that the partially purified 64 kd autoantigen may be useful for the diagnosis of IDDM. Further purification is likely to reduce the level of false positive results. The autoantigen would be suitable for use as an immunodiagnostic for IDDM in methods employing, for example, immunoprecipitation or immunoblot.

EXAMPLE 8

Construction of cDNA Library From HEp2 Cells

mRNA Isolation

5 A general procedure for obtaining total cellular RNA encoding beta cell autoantigen was followed according to the method of Chirgwin et al., Biochemistry 18:5294 (1979). While selection of polyadenylated mRNA from total cellular RNA may be accomplished with a
10 procedure described in Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, pp. 197-198, (1982), a Fast Track mRNA Kit (Invitrogen, San Diego, CA) was employed to isolate mRNA directly from HEp2 cells without having to isolate total cellular RNA as an intermediate.
15 The cells were grown to confluency in RPMI medium supplemented with 10% calf serum. Cells were detached from the culture flask using trypsin-EDTA (Irvine Scientific, Irvine, CA) and reseeded on culture flask. After 24 hours of cultivation, the cells were harvested
20 by gentle suction and washed one time with PBS. The harvested cells were frozen under liquid nitrogen until used. 10^8 cells were used to isolate the mRNA according to the manufacturer's protocol (Invitrogen, San Diego, CA). Approximately 20 micrograms of purified mRNA was
25 obtained.

cDNA Synthesis and lambda gt11 Construction

The procedures for efficient conversion of mRNA to an RNA-cDNA hybrid and then into double-stranded cDNA
30 were based on those first described by Okayama and Berg, Molecular and Cellular Biology 2: 263 (1982). The procedure involves using reverse transcriptase, E. coli DNA polymerase I, RNase, and E. coli DNA ligase. Reverse transcriptase was used to synthesize a cDNA from
35 an RNA template in the presence of oligo dT primer and random primer. The E. coli RNase then cleaved RNA while

in a hybrid RNA-DNA form. The enzyme E. coli DNA polymerase I then used the fragments of nicked RNA as primers to synthesize the second strand of cDNA. Any nicks in the double-stranded cDNA were repaired by E. coli ligase. A modification of this protocol was used involving the addition of T4 DNA polymerase to the reaction after the E. coli DNA polymerase I reaction was completed.

A Librarian™ cDNA Library Construction system (Invitrogen, San Diego, CA) was used to construct a bacteriophage lambda gt11 cDNA library. (Young et al., Nature, 316:450-452 (1988)). Approximately 5 micrograms of purified mRNA from HEp2 cells were used to prepare the lambda gt11 library according to the manufacturer's procedure. It is estimated that approximately 1.24 x 10^6 primary phage clones were obtained from one preparation and among them 9 x 10^5 primary clones (72%) contained inserts as determined by a lack of beta-galactosidase activity. The primary library was divided into four parts and amplified separately. The insert frequency dropped from 72% to 62% after amplification.

Library Screening

The lambda gt11 cDNA library prepared from the mRNA isolated from the HEp2 cell lines was screened using the DM monoclonal antibody as a probe. E. coli Y1090 (Young et al., Nature, 316:450-452 (1988) cells were infected with approximately 15,000 plaque forming units (pfu) of phage and plated per 82 mm plate. After five hours of incubation at 37°C, nitrocellulose paper presoaked with IPTG (isopropyl beta-D thiogalactopyranoside) was overlaid on the plate and incubated for another five hours or overnight at 37°C. The plates were cooled to 4°C before lifting the nitrocellulose papers. The nitrocellulose papers were incubated with PBS containing 1% skim milk and 5 micrograms/ml DM monoclonal antibody

for four to five hours, or overnight, at room temperature with shaking. After washing with PBS four times, the papers were incubated with goat anti-mouse IgM-horse radish peroxidase conjugate (1:500 dilution) in the same buffer for two hours. After washing with PBS four times, the papers were developed with substrate solution. Approximately 30 positive clones were identified after initial screening of 600,000 plaques. Eighteen positive clones were obtained after subsequent secondary screening.

Characterization of the Positive Clones: Determination of the Fusion Protein by Western Blot

Once clones were obtained using the immunoscreening technique described above, beta-galactosidase-fusion proteins were produced. Fusion of the beta cell autoantigen nucleotide sequence with the beta galactosidase nucleotide sequence is believed to be essential for efficient transcription and translation in a procaryotic cell host, such as E. coli.

Beta-galactosidase-fusion proteins were produced as follows. Cell lysates from several clones were collected from plates after soaking with phage buffer (10 mM Tris, pH 7.5, 10 mM $MgSO_4$, 100 mM NaCl), or were prepared from the lysogen lysate, as described in Maniatis, et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, p. 254, (1982). The lysates were separated on a 7.5% SDS-PAGE gel and electrotransferred to the nitrocellulose paper according to the methods of Burnette, Anal. Biochem., 112:195-203 (1981) and Towbin et al, Proc. Natl. Acad. Sci.USA, 76:4350-4354 (1979). The fusion proteins consisting of the beta galactosidase gene and the gene for the beta cell autoantigen were detected with the DM monoclonal antibody or with mouse anti-beta galactosidase monoclonal antibodies. Most clones tested

thus far contained a protein detectable using the DM monoclonal antibody. Except for one clone, the protein bands observed were larger than the intact beta-galactosidase. In addition to a top band, other protein bands, which were presumably degradation products of beta-galactosidase, also were detected by anti-beta galactosidase monoclonal antibodies.

The eighteen positive clones were identified at low stringency conditions as described above. These clones were further characterized at high stringency conditions as follows: E. coli strain Y1090, which was infected with approximately 5 to 200 phage forming units (pfu), was plated on an 82 mm TYE agar plate and overlaid with IPTG-treated nitrocellulose paper as described previously. The nitrocellulose papers, which absorbed the E. coli lysate, were then incubated with TBS containing 1% skim milk and 10 micrograms/ml of DM monoclonal antibody for 4 hours or overnight at room temperature. The filter papers were washed twice with TBS-0.5% Tween 20 and twice with TBS for five minutes each and then incubated with goat anti-mouse IgM-horseradish peroxidase conjugate (1:1000 dilution) for 2 hours. The nitrocellulose papers were washed under the same conditions as described above and developed with DBA (3,3'-diaminobenzidine) substrate. Among the 18 low stringent positive clones, three clones -- numbers 4, 14, and 18 -- still showed specific immunoreactivity under the high stringent washing condition using 0.5% Tween 20.

The cDNA inserts comprising sequences coding for the beta cell autoantigen, and in particular, the immunodominant epitope specifically reactive with the DM monoclonal antibody and the anti-64 kd autoantibody, were isolated from recombinant phage clones 4 and 14 with EcoRI digestion and cloned into the EcoRI site of plasmid pUC13. Restriction mapping was accomplished

using standard techniques as specified in Maniatis, et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, (1982). The restriction map for plasmid pUH14, which is a subclone of clone 14, is illustrated in Figure 1. The map indicates a DNA insert sequence comprising about 1400 bases. Each terminus is marked by an EcoRI site. Two Pst restriction sites are located at about 180 bases and at about 670 bases respectively from one of the EcoRI termini. Located at about 1000 bases and at about 1200 bases from this EcoRI terminus are, respectively, a HindIII site and a SacI site.

The fusion protein that clone 14 produced had a molecular weight of approximately 110-115 kd which is slightly larger than the authentic beta-galactosidase as judged by the mobility of the immunoreactive band on the Western blot.

Clone 4 contained an approximately 0.75 kb cDNA insert. The fusion protein that clone 4 produced had a molecular weight of approximately 120-130 kd as determined by immunoblot using either DM monoclonal antibody or anti-beta galactosidase monoclonal antibody.

These results suggest: (1) the selected clones produced fusion proteins recognized by both the DM monoclonal antibody and anti-beta galactosidase, and (2) the epitope recognized by the DM monoclonal antibody resides in the C-terminus of the cloned fusion protein, and, therefore, resides in the C-terminus of the gene bearing the epitope.

It is apparent that many modifications and variations of this invention as set forth above may be made without departing from the spirit and scope. The specific embodiments described are given by way of example only, and the invention is limited only by the terms of the appended claims.

CLAIMS

What is claimed is:

1. A monoclonal antibody produced by hybridoma
5 cell line ATCC No. HB 10502, and functional equivalents thereof, said monoclonal antibody specifically reacting with a beta cell autoantigen of approximately 64,000 daltons associated with the presence of insulin dependent diabetes mellitus and capable of causing
10 antibody-dependent complement mediated cytoxicity of cells expressing beta cell autoantigen.

2. The monoclonal antibody of Claim 1 wherein said antibody recognizes and binds the same
15 immunodominant epitope on the autoantigen as human islet cell autoantibodies such that the monoclonal antibody and the autoantibodies are capable of excluding each other from binding to the same immunodominant epitope.

20 3. The monoclonal antibody of Claim 1 wherein the antibody is produced by recombinant means.

4. The monoclonal antibody of Claim 3 wherein the recombinant means comprises an <u>in vivo</u> recombinant gene
25 expression system, the gene expression system constructed so as to express a DNA sequence sufficient to code for the specific antibody, the DNA sequence being derived at least in part from hybridoma cell line ATCC No. HB 10502 and encoding at least a portion of an
30 immunoglobulin molecule.

5. The monoclonal antibody of Claim 4 wherein the <u>in vivo</u> recombinant gene expression system is selected from the group consisting of procaryotic cells, yeast
35 cells, or mammalian cells.

6. The monoclonal antibody of Claim 5 wherein the procaryotic cells are E. coli cells.

7. Hybridoma cell line ATCC No. HB 10502.

5

8. A method for preventing destruction of pancreatic islet cells in vivo comprising introducing the monoclonal antibody of Claim 1 into a subject to produce anti-id antibodies specifically reactive with
10 the monoclonal antibodies, the anti-id antibodies reacting with autoantibodies produced by the subject in response to the beta cell autoantigen, thereby preventing destruction of said islet cells by said autoantibodies.

15

9. A composition for treating insulin dependent diabetes mellitus in vivo comprising a therapeutically effective amount of the monoclonal antibody of Claim 1 in association with a pharmaceutically acceptable
20 carrier vehicle.

10. The monoclonal antibody of Claim 1 which is of class IgM.

25 11. The monoclonal antibody of Claim 11 which is a murine antibody.

12. The monoclonal antibody of Claim 1 which is a chimeric mouse-human antibody.

30

13. An anti-idiotype antibody produced by using the monoclonal antibody of Claim 1.

14. The anti-idiotype monoclonal antibody of Claim
35 13 wherein the antibody is produced by recombinant means.

15. The antibody of Claim 14 wherein the recombinant means comprises a recombinant gene expression system situated in E. coli bacteria, the gene expression system constructed so as to express a DNA sequence sufficient to code for the specific antibody, the DNA sequence being derived at least in part from hybridoma cell line ATCC No. HB 10502 and encoding at least a portion of an immunoglobulin molecule.

16. A beta cell autoantigen associated with insulin dependent diabetes mellitus having a molecular weight of approximately 64,000 as determined by polyacrylamide gel electrophoresis, wherein the autoantigen has specific reactivity with at least one antibody selected from the group consisting of the monoclonal antibody of Claim 1 and autoantibodies specifically reactive with human beta cell autoantigen.

17. The autoantigen of Claim 16 wherein the autoantigen is purified from a mammalian cell line characterized by a high concentration of the autoantigen.

18. The autoantigen of Claim 17 wherein the cell line is human epidermoid carcinoma cells (HEp2).

19. The autoantigen of Claim 17 wherein the cell line is rat insulinoma cells.

20. The autoantigen of Claim 17 wherein the cell line is derived from neuronal tissue.

21. The autoantigen of Claim 20 wherein the cell line is human neuroblastoma.

22. The autoantigen of Claim 20 wherein the cell line is human glioblastoma.

23. The autoantigen of Claim 16 wherein the autoantigen is produced by recombinant means.

24. The autoantigen of Claim 23 wherein the recombinant means comprises a recombinant gene expression system situated in a bacterial or non-bacterial cell, the gene expression system constructed so as to express a DNA sequence sufficient to code for at least the immunodominant epitope of the autoantigen, the DNA sequence being derived at least in part from a cell that produces beta cell autoantigen, the cell selected from the group consisting of pancreatic beta cells, human epidermoid carcinoma cells, insulinoma cells, and neuronal cells.

25. The autoantigen of Claim 24 wherein the neuronal cells are selected from the group consisting of human neuroblastoma cells and glioblastoma cells.

26. A method for detecting insulin dependent diabetes mellitus in a sample by detecting human islet cell autoantibodies by reacting the autoantigen of Claim 16 labeled for detection with a sample and detecting reaction of the autoantigen with the autoantibodies present in the sample.

27. The method of Claim 26 wherein the autoantigen is labeled with a label selected from the group consisting of a radionuclide, an enzyme, a fluorescent agent and a chromophore.

28. The method of Claim 26 wherein the specific reaction between the autoantigen and the autoantibody

can be detected with a conjugate of a detectable label and a specific binding partner of the autoantibody.

29. The method of Claim 28 wherein the label is selected from the group consisting of a radionuclide, an enzyme, a fluorescent agent and a chromophore.

30. A method for detecting insulin dependent diabetes mellitus in a sample by detecting beta cell autoantigen which comprises reacting the monoclonal antibody of Claim 1 labeled for detection with a sample and detecting reaction of the monoclonal antibody with the autoantigen present in the sample, the autoantigen having a molecular weight of approximately 64,000 daltons as determined by polyacrylamide gel electrophoresis.

31. The method of Claim 30 wherein the sample is pancreatic islet tissue.

32. The method of Claim 30 wherein the antibody is labeled with a label selected from the group consisting of a radionuclide, an enzyme, a fluorescent agent and a chromophore.

33. A cDNA library comprising cDNA sequences coding for beta cell autoantigen recognized by the monoclonal antibody of Claim 1 having a determinant site containing an immunodominant epitope associated with insulin dependent diabetes mellitus and said antigen having a molecular weight of approximately 64,000 daltons as determined by polyacrylamide gel electrophoresis and capable of being recognized and bound by anti-64 kd autoantibody reactive with beta cell autoantigen.

34. The cDNA library of Claim 33 wherein the cDNA sequences coding for the beta cell autoantigen of human pancreatic cells are obtained from a cell line.

5 35. The cDNA library of Claim 34 wherein the cell line is of non-pancreatic origin.

36. The cDNA library of Claim 34 wherein the cell line is selected from the group consisting of pancreatic 10 beta cells, human epidermoid carcinoma cells, insulinoma cells, and neuronal cells.

37. The cDNA library of Claim 36 wherein the neuronal cells are selected from the group consisting of 15 neuroblastoma cells and glioblastoma cells.

38. The cDNA library of Claim 34 wherein the cell line is human epidermoid carcinoma cells.

20 39. A recombinant DNA sequence of at least about 1300 bases coding for an autoantigen having specific reactivity with at least one antibody selected from the group consisting of the monoclonal antibody of Claim 1 and autoantibodies specifically reactive with beta cell 25 autoantigen, and substantially purified from other DNA sequences associated with cells producing the autoantigen.

40. The DNA sequence of Claim 39 wherein the cells 30 producing the autoantigen are non-pancreatic in origin.

41. The DNA sequence of Claim 39 wherein the cells are selected from the group consisting of pancreatic beta cells, human epidermoid carcinoma cells, 35 insulinoma cells, and neuronal cells.

42. The DNA sequence of Claim 39 having termini marked by EcoRI restriction sites and incorporating two Pst restriction sites, a HindIII site, and a SacI site.

5 43. The DNA sequence of Claim 39 having the sequence contained in pUH14.

44. A method of immunotherapy for the treatment of insulin dependent diabetes mellitus comprising the step 10 of removing circulating beta cell autoantigen autoantibodies reactive with beta cell autoantigen characterized by a molecular weight of approximately 64,000 daltons and specifically reactive with the monoclonal antibodies of Claim 1, using ligands 15 specifically reactive with said circulating autoantibodies present in the serum of a subject.

45. The method of Claim 44 wherein the ligands comprise plasma membrane bound protein autoantigen of 20 human islet beta cells having a molecular weight of approximately 64,000 daltons.

46. The method of Claim 44 wherein the ligands comprise anti-id antibodies specifically recognizing and 25 binding autoantibody reactive with beta cell autoantigen present in the serum.

47. A method of treating a patient suffering from insulin dependent diabetes mellitus comprising the steps 30 of:

(a) obtaining the plasma of a patient to be treated;

(b) contacting the plasma with immobilized anti-idiotypic antibodies reactive with autoantibodies 35 that bind to an autoantigen characterized by a molecular weight of approximately 64,000 daltons associated with

the pathogenesis of insulin dependent diabetes mellitus, the autoantigen specifically reactive with the monoclonal antibody of Claim 1, under conditions to form a complex of the anti-idiotypic antibodies with the
5 autoantibodies;

(c) removing the anti-idiotypic antibody\autoantibody complex; and

(d) returning the plasma, having said autoantibodies removed, to the patient whereby
10 destruction of the pancreatic islet beta cells is reduced.

48. A method of immunotherapy for the treatment of insulin dependent diabetes mellitus by destroying
15 lymphocytes in body fluids that destroy islet cells comprising the step of contacting said lymphocytes with an effective amount of anti-idiotypic antibodies reactive with autoantibody against beta cell autoantigen having a molecular weight of approximately 64,000
20 daltons and specifically reactive with the monoclonal antibodies of Claim 1.

49. The method of Claim 48 wherein said anti-idiotypic antibodies are conjugated with a toxin.

25

50. The method of Claim 49 wherein said toxin is selected from the group consisting of abrin, diphtheria toxin A chains, ricin A chains, and gelanin.

30 51. A method of immunotherapy for the treatment of insulin dependent diabetes mellitus by destroying lymphocytes in body fluids that destroy islet cells comprising the step of contacting said lymphocytes with an effective amount of beta cell autoantigen reactive
35 with anti-64 kd autoantibodies.

52. The method of Claim 51 wherein the step of contacting the lymphocytes comprises contacting the lymphocytes with an effective amount of beta cell autoantigen conjugated with a toxin.

5

53. The method of Claim 52 wherein said toxin is selected from the group consisting of abrin, diphtheria toxin A chains, ricin A chains, and gelanin.

10 54. The method of Claim 48 wherein the lymphocytes are T-lymphocytes and B-lymphocytes capable of reacting with beta cell autoantigen of approximately 64,000 daltons molecular weight associated with insulin dependent diabetes mellitus.

15

55. A vaccine for use in immunization to prevent insulin dependent diabetes mellitus comprising a monoclonal antibody produced by hybridoma cell line ATCC No. HB 10502 in a pharmaceutical carrier, the monoclonal
20 antibody specifically reactive with a protein autoantigen of human pancreatic islet beta cells, causing the human to produce anti-id antibodies which react with autoantibodies reactive with beta cell autoantigen, thereby reducing destruction by the
25 autoantibodies of the islet beta cells.

56. The vaccine of Claim 55 comprising peptide fragments containing at least a substantial portion of the idiotope of the monoclonal antibody of Claim 1 in a
30 carrier such that the peptide fragments are capable of binding the determinant site on a protein autoantigen of human pancreatic islet beta cells of approximately 64,000 daltons molecular weight.

35 57. A method for immunizing against insulin dependent diabetes mellitus comprising administering a

vaccine selected from the group consisting of the vaccine of Claim 55 and the vaccine of Claim 56.

58. The method of Claim 57 wherein the vaccine is administered to a subject who produces islet cell autoantibodies.

59. The method of Claim 57 wherein the vaccine is administered to a subject likely to begin producing islet cell autoantibodies, the subject being a first degree relative of a proband with insulin dependent diabetes mellitus.

60. An immunoassay kit for diagnosing insulin dependent diabetes mellitus by detecting the presence of anti-64 kd autoantibody comprising the autoantigen of Claims 16 or 23 and a conjugate of a detectable label and a specific binding partner of the autoantibody.

61. The immunoassay kit of Claim 60 wherein the specific binding partner is anti-IgG conjugated to a label capable of producing a detectable signal.

62. The immunoassay kit of Claim 61 wherein the label is selected from the group consisting of a radionuclide, an enzyme, a fluorescent agent and a chromophore.

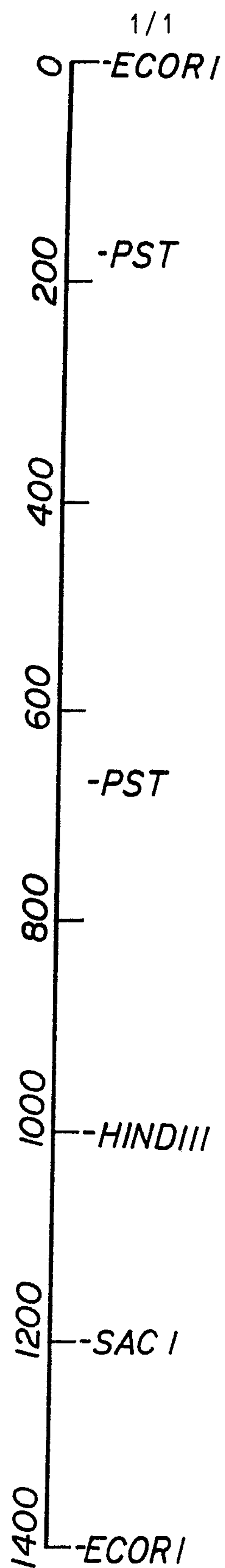

FIG. 1

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/07052

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

US: 530/350,387; 435/7.21; 424/85.8; 536/27; 604/6

IPC(5): C07K 3/00; G01N 33/567; A61K 39/00; C07H 19/00; A61M 37/00

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
US	530/350,387; 435/7.21, 965; 424/85.8; 536/27; 604/6; 436/811

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

APS Dialog

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X Y	WO,A, 90/07117 (Atkinson et al.) 28 June 1990, see pages 35-38 and the claims.	16,26,27,33,60 1-15,17-25, 28-32,34-59 61,62
X Y	EP,A, 0,383,129 (Rabin) 22 August 1990, see pages 2-10.	16,26,27,60 1-15,17-25 28-59,61,62
Y	Annual Review of Immunology, Volume 4, issued 1986, Gaulton et al., "Idiotypic Mimicry of Biological Receptors," pages 255-280, see page 254, lines 3-17 and pages 271-274	8,13-15, 46-59

* Special categories of cited documents: [10]

"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" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" 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

"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.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
16 January 1992	10 FEB 1992
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Donna C. Wortman

Form PCT/ISA/210 (second sheet) (Rev.11-87)