Title: IMMUNOGLOBULIN CONJUGATES OF AUTOANTIGENS AND THEIR USE IN THE PREVENTION OF DISEASE

Abstract: Conjugates comprising a disease-relevant autoantigen (proinsulin protein chain) and an isologous immunoglobulin are disclosed. Such conjugates demonstrate efficacy in the treatment of autoimmune diseases, such as type I diabetes.
Immunoglobulin Conjugates of Autoantigens and their Use in the Prevention of Disease

Background of the Invention

1. Field of the Invention

The present invention relates to novel therapeutics comprising conjugated autoantigens for the study, treatment, and prevention of disease.

2. Background of the Related Art

Tolerance refers to the immunological non-reactivity of the body's immune system towards an antigen. Tolerance can occur towards self antigens but can also be induced to non-self antigens. When an antigen induces tolerance, it is described as a tolerogen. Factors that affect whether an antigen will cause an immune reaction or tolerance include, for example, the physical form of the antigen, the route of administration of the antigen, the dosage of the antigen, the age of the subject, and the differentiation stage of the cells.

Immunological tolerance to self-antigens is vital to the proper functioning of the mammalian immune system. In autoimmune diseases such as, for example, multiple sclerosis, rheumatoid arthritis or diabetes, there is a failure of the proper regulation of tolerance. Improved treatment methods for re-establishing tolerance are therefore desirable in the treatment of autoimmune diseases.
Insulin-dependent diabetes mellitus (IDDM; type I diabetes) is one of the most commonly occurring metabolic disorders in the world and provides a good example of an autoimmune disease. IDDM is caused by an autoimmune response that results in the T lymphocyte-mediated destruction of the insulin-producing beta-cells of the pancreas. Castaris et al., Ann. Rev. Immunol. 8:647-679 (1990). Studies directed at identifying the autoantigen(s) responsible for beta-cell destruction have generated several candidates, including poorly characterized islet cell antigens (ICA) (Bottazzo et al., Lancet 2: 1279-83, 1974), insulin (Palmer et al., Science 222: 1337-39, 1983), glutamic acid decarboxylase (GAD) (Baekkeskov et al., Nature 298: 167-69, 1982; Baekkeskov et al., Nature 347: 151-56, 1990), and a 64 kD islet cell antigen that is distinct from GAD and that which yields 37 kD and 40 kD fragments upon trypsin-digestion (Christie et al., Diabetes 41: 782-87, 1992). Unfortunately, however, preventative measures based on this knowledge have been slow to develop.

Treatment protocols are further complicated by the fact that, although the general mechanism by which IDDM occurs is known, IDDM becomes clinically evident only after the vast majority (approximately 80% or more) of the pancreatic beta-cells have been irrevocably destroyed and the individual becomes dependent upon an exogenous source of insulin. In other words, at the time that the disease becomes clinically evident, the autoimmune response is well established and has already caused irreparable damage to the insulin-producing pancreatic tissue.
Because autoimmune-induced pancreatic damage is far progressed by the time that clinical symptoms of IDDM become evident, successful treatment of the autoimmune response ideally should be initiated well before the patient begins to exhibit overt symptoms of Type 1 diabetes and requires insulin replacement for his or her own lost capability to produce insulin.


Antibodies to the 40 kD, and more particularly the 37 kD, ICA fragments are detected when clinical onset of IDDM is imminent and are found to be closely associated with IDDM development (Christie et al., Diabetes 41: 782-87, 1992). Diabetic sera containing antibodies specific to the 40 kD fragment were recently found to bind to the intracellular domain of the protein tyrosine phosphatase, IA-2/ICA512 (Lu et al., Biochem. Biophys. Res. Comm. 204: 930-36, 1994; Lan et al., DNA Cell Biol. 13: 505-14, 1994; Rabin et al., J. Immunol. 152: 3183-88, 1994; Payton et al., J. Clin. Invest. 96: 1506-11, 1995; and Passini et al., Proc. Natl. Acad. Sci. USA 92: 9412-16, 1995). Antibodies specific to the 37 kD fragment are thought to bind either to a posttranslational in vivo modification of IA-2/ICA512 or
a different, but probably related, protein precursor
(Passini et al., ibid.).

ICA 512 was initially isolated as an
autoantigen from an islet cell cDNA library, and was
subsequently shown to be related to the receptor-linked
protein tyrosine phosphatase family (Rabin et al.,
ibid.). ICA 512 was later found to be identical to a
mouse and human protein tyrosine phosphatase, IA-2,
isolated from brain and insulinoma cDNA libraries (Lu et
al., ibid.; and Lan et al., ibid.).

Detection of Type 1 diabetes-associated
autoantigens in prediabetic individuals has been shown to
be useful as a predictive marker of IDDM, especially
detection of combinations of autoantigens, genotypes such
as HLA DR and HLA DQ, and loci such as the polymorphic
region in the 51 flanking region of the insulin gene; see
for example, Bell et al., (Diabetes 33:176-83, 1984);
Sheehy et al., (J. Clin. Invest. 83:830-35, 1989); and
Bingley et al., (Diabetes 43: 1304-10, 1994).

Prior to the onset of clinical symptoms of
IDDM but after the onset of the IDDM-associated
autoimmune response, attempts have been made to control
the established diverse autoreactive T cell population,
thereby effectively inhibiting progression of the
disease. For example, immunosuppressants and antibodies
which are specifically directed against autoimmune T
cells may be useful for delaying the onset of disease.
However, such treatments lack specificity and often
significantly debilitate immune system function.

Moreover, immunotherapeutics directed at blocking T cell-
receptor/major histocompatibility complex (MHC)
interactions can be highly specific, but may also be
confounded by the complexity of the autoreactive T cell
population and the genetic diversity of MHCs within the patient population.

Once the clinical symptoms of IDDM become evident, numerous different therapies have been employed for treating the debilitating effects of the disease. For example, by far the most commonly employed therapy for the clinical symptoms of IDDM is exogenous insulin replacement. However, while insulin replacement therapy allows most IDDM patients to lead somewhat normal lives, insulin replacement is also imperfect and does not completely restore metabolic homeostasis. As a result, severe complications including dysfunctions of the eye, kidney, heart, and other organs are common in diabetic patients undergoing insulin replacement therapy.

Another common treatment for the clinical symptoms of IDDM is pancreatic or beta-islet cell transplantation. However, the insulin-producing beta-cells of transplanted tissues are often rapidly destroyed by the same autoimmune response which had previously destroyed the patient's own pancreatic tissue. Therefore, the use of immunosuppressants after transplantation is common, carrying with it the adverse side effects described above.

Accordingly, in addition to the urgent need for improved methods for the early diagnostic identification of persons who are at risk for developing the clinical symptoms of IDDM and for monitoring the progression of the autoimmune response in those at risk persons, there is also an urgent need for improved methods for therapeutically treating those persons who already exhibit clinical symptoms of the disease. The same problem exists for many autoimmune diseases. Specifically, there exists a need for methods and
compounds effective in inhibiting the autoimmune mechanism underlying the disease (for example, T cell-mediated mechanisms). Ideally, for example with respect to IDDM, such treatments would guide the immune system back to a healthy state where the treatment could be discontinued without the return of T lymphocyte-mediated destruction of the insulin-producing beta-cells of the pancreas.

The autoimmune response underlying IDDM is thought to be mediated by proinflammatory T helper 1 (Th1) cells; cells that are known to secrete interferon-gamma (IFN-gamma) and promote the production of murine IgG2a isotype antibodies that are directed against pancreatic beta-cell-associated autoantigens. In contrast to Th1 cells, T helper 2 (Th2) cells are known to secrete interleukin-4 (IL-4) and interleukin-5 (IL-5) and promote the production of murine IgG1 isotype antibodies directed against pancreatic beta-cell-associated autoantigens. It has been shown in an animal model of human IDDM, the nonobese diabetic (NOD) mouse, that a pathogenic Th1 response to the beta-cell-associated autoantigen glutamic acid decarboxylase (GAD) arises at 4 weeks of age, concurrent with the onset of insulitis in these animals (Kaufman et al., Nature 366:69-72 (1993)). GAD is a mammalian protein which serves to catalyze the rate-limiting step in the synthesis of gamma-aminobutyric acid (GABA), a major inhibitory neurotransmitter of the mammalian central nervous system (Spink et al., J. Neurochem. 40:1113-1119 (1983), Huang et al., Proc. Natl. Acad. Sci. U.S.A. 87:8491-8495 (1990), Kobayashi et al., Neurosci. 7:2768-2772 (1987), Chang et al., J. Neurosci. 8:2123-2130 (1988), Bu et al., Proc. Natl. Acad. Sci. U.S.A. 89:2115-2119 (1992), Karlsen et al., Diabetes
41:1355-1359 (1992) and U.S. Pat. No. 5,475,086, issued Dec. 12, 1995, all hereby incorporated by reference). The GAD protein is present on various tissues and exists in multiple isoforms, one of which is GAD65, an antigen found to be associated with pancreatic beta-cells.

Subsequent to the anti-GAD Th1 response described above, T-cell autoimmunity appears to spread to other beta-cell antigens such as a 65 kD heat shock protein (hsp65), insulin B-chain, carboxypeptidase H and peripherin in a cascade of autoimmune responses that ultimately leads to IDDM (Kaufman et al., supra and Tisch et al., Nature 366:72-75 (1993)).

GAD expression or suppression in beta cells”, *Science* 284:1183 (1997)) in antigen presenting cells (APCs), pituitary cells or pancreatic β-cells has been shown to prevent NOD mice from diabetes.

A main challenge, however, remains in (1) deciphering the role of individual antigens and epitopes within these autoantigens in the initiation and amplification of the autoimmune reaction; and (2) providing derivations of such antigens or epitopes in forms which allow for the greatest efficacy. Succeeding in this challenge will allow for the development of immunotherapy strategies using autoantigens or peptides to induce immune tolerance and prevent the development of, for example, type 1 diabetes in susceptible individuals.

For example, in vivo half-life of a candidate autoantigen has been demonstrated to be one possible factor in the efficacy of the autoantigen in the treatment and prevention of, for example, IDDM.

Accordingly, autoantigen derivations or formulations which exhibit increased half-life provide an exciting area of exploration. Additionally, alternative modes of presentation of the autoantigen may enhance the response to the autoantigen and, in doing so, increase the efficacy of the treatment.

Proinsulin is a predominant β-cell protein and is the only candidate autoantigen for which expression is largely restricted to β-cells. Insulin and proinsulin have been defined as targets for autoantibodies (Dubois-Laforge, D. et al., “T-cell response to proinsulin and insulin in type 1 and pretype 1 diabetes”, *J. Clin. Immunol.* 19, 127-34 (1999), Lucassen,A.M. et al., “Susceptibility to insulin
(1996)) while proinsulin I expression is restricted to β-cells. Proinsulin I may be converted into insulin more rapidly than proinsulin II. In the NOD mouse, protection from disease has been observed by injecting insulin or the insulin B chain (Muir, A. et al., "Insulin immunization of nonobese diabetic mice induces a protective insulitis characterized by diminished intraislet interferon-gamma transcription", J.Clin.Invest 95:628 (1995)) or the insulin B chain peptide B9-23 (Daniel, D. and D. R. Wegmann, "Protection of nonobese diabetic mice from diabetes by intranasal or subcutaneous administration of insulin peptide B-(9-23)" Proc.Natl.Acad.Sci.U.S.A 93:956 (1996), Hutchings, P. and A. Cooke, "Protection from insulin dependent diabetes mellitus afforded by insulin antigens in incomplete Freund's adjuvant depends on route of administration", J.Autoimmun. 11:127 (1998)). Protection was however, incomplete, consisting of delayed development of diabetes rather than definitive protection from disease development. Such incomplete protection may be a result of the formulation or derivation or presentation of the peptide. In other words, investigating methods of conjugation which may alter the presentation or half life of the autoantigen may result in improved compositions for protection from disease. For example, haptens (Borel (1989), in "Concepts in Immunopathology", Cruse and Lewis (Eds.), 7:145-161, Karger, Basel; Sehon 91982), Prog. Allergy 32: 161-202) and proteins or protein fragments can be covalently linked to a carrier molecule naturally tolerated by the host, such as an isologous immunoglobulin, in order to induce unresponsiveness in the host to these proteins (Fillion et al. (1980), Cell Immunol. 54:115-128; Borel and Borel (1990), J. Immunol.
Methods 126:159-168). It is this conjugation that is thought to provide the proper presentation to cause tolerance.

It would be, therefore, of great interest to develop an autoantigen formulation that can exert protective effects against the development of autoimmune diseases, for example, IDDM and does not have the limitations of the prior art compounds and formulations. In that respect, demonstration of the efficacy of the technique in the treatment of an autoimmune disease, for example IDDM, would provide proof of concept for the treatment of a range of autoimmune diseases.

Recently, a method of preparing conjugates comprising autoantigens has been described by Borel et al. (EP 1119335 A1 and United States Patent Application No. 20010007755, hereby incorporated by reference). Borel et al. describe a method of preparing a conjugate comprising a first and second polypeptide, the method comprising (a) incubating the first polypeptide in the presence of a heterobifunctional crosslinker comprising an N-hydroxysuccinimide ester group and a maleimide group linked via a polyethylene oxide spacer; (b) removing excess heterobifunctional crosslinker; and (c) incubating the reaction product of step (b) with the second polypeptide, wherein the second polypeptide comprises at least one sulfhydryl group. In their method, the polyethylene oxide spacer consists of from 1 to 10 monomer units. In such conjugates, the first polypeptide can be an immunoglobulin or a structurally related fragment thereof and the second polypeptide can be an autoantigen or an immunologically equivalent fragment thereof. Such conjugates demonstrate improved efficacy through increased half-life and improved presentation of
antigen. Borel et al. proceed to demonstrate the use of such conjugates in the treatment of allergies. However, Borel et al. do not disclose the embodiments that are described herein.

Brief Summary of the Invention

The need for compounds capable of treating autoimmune disease is satisfied by the embodiments of the instant invention which relate in general to conjugates for use in the treatment of autoimmune disease.

For example, proinsulin peptide conjugates exert protective effects against the development of type 1 diabetes in the NOD mouse and do not exhibit the limitations of compounds of the prior art. These conjugates establish the efficacy of similar conjugates in the treatment of a range of autoimmune diseases.

The formulation of the conjugate of the peptide comprises a peptide relevant to autoimmune disease and an isologous immunoglobulin. The peptide chosen for this study was proinsulin II B chain peptide 9-23 (proinsulin peptide 33-47) due to its striking protective effect in the NOD mouse and to its complete homology with an equivalent peptide of the human proinsulin B chain. As will be described in detail herein, injection of a conjugate of B chain peptide 9-23 (proinsulin II peptide 33-47) with a mouse immunoglobulin prevents the development of type 1 diabetes in female NOD mice.
Figure 1: Protection against spontaneous autoimmune type 1 diabetes in NOD mice by tolerogenic Ins9.23/Ig conjugates.

Shown is the incidence of type 1 diabetes in NOD mice untreated (control) and treated with either Ins9-23/Ig or OVA/Ig.

Upper panel: Treatment from week 4 to week 13 (first experiment) resulting in delayed onset of type 1 diabetes.

Lower panel: Treatment from week 4 to week 18 (second experiment) resulting in delayed onset and permanent protection; in this second experiment, the difference between Ins9-26/Ig group and OVA/Ig group was highly significant (p<0.006).

Figure 2: Histological analysis of the pancreases of 10-week-old female NOD mice treated with either Ins9-23/Ig (left column) or OVA/Ig (right column) from 4 to 8 weeks of age.

Detailed Description of the Invention

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In the case of conflict, the present
specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

As used herein, the term “reagent” includes, for example, peptides, immunoglobulins and antibodies (or fragments thereof).

As used herein, the term “antibody” as used in this disclosure refers to both polyclonal and monoclonal antibody. The ambit of the term deliberately encompasses not only intact immunoglobulin molecules, but also such fragments and derivatives of immunoglobulin molecules (such as single chain Fv constructs, diabodies, and fusion constructs) as may be prepared by techniques known in the art, and retaining a desired antibody binding specificity.

Reagents of the invention, and individual moieties or analogs and derivatives thereof, can be chemically synthesized. A variety of protein synthesis methods are common in the art, including synthesis using a peptide synthesizer. See, e.g., Peptide Chemistry, A Practical Textbook, Bodansky, Ed. Springer-Verlag, 1988; Merrifield, Science 232: 241-247 (1986); Barany, et al, Intl. J. Peptide Protein Res. 30: 705-739 (1987); Kent, Ann. Rev. Biochem. 57:957-989 (1988), and Kaiser, et al, Science 243: 187-198 (1989). The peptides are purified so that they are substantially free of chemical precursors or other chemicals using standard peptide purification techniques. The language "substantially free of chemical precursors or other chemicals" includes preparations of peptide in which the peptide is separated from chemical precursors or other chemicals that are involved in the synthesis of the peptide. In one embodiment, the language "substantially free of chemical precursors or other
chemicals" includes preparations of peptide having less than about 30% (by dry weight) of chemical precursors or non-peptide chemicals, more preferably less than about 20% chemical precursors or non-peptide chemicals, still more preferably less than about 10% chemical precursors or non-peptide chemicals, and most preferably less than about 5% chemical precursors or non-peptide chemicals.


Introduction of covalent cross-links into a peptide sequence can conformationally and topographically constrain the peptide backbone. This strategy can be used to develop peptide analogs of reagents with increased potency, selectivity and stability. A number of other methods have been used successfully to introduce conformational constraints into peptide sequences in order to improve their potency, receptor selectivity and biological half-life. These include the use of (i) Cα-methylamino acids (see, e.g., Rose, et al., Adv. Protein Chem. 37: 1-109 (1985); Prasad and Balaram, CRC


(iii) α,β-unsaturated amino acids (see, e.g., Bach and Gerasch, Biopolymers, 25: 5175-S192 (1986); Singh, et al., Biopolymers, 26: 819-829 (1987)). These and many other amino acid analogs are commercially available, or can be easily prepared. Additionally, replacement of the C-terminal acid with an amide can be used to enhance the solubility and clearance of a peptide.

Alternatively, a reagent may be obtained by methods well-known in the art for recombinant peptide expression and purification. A DNA molecule encoding the protein reagent can be generated. The DNA sequence is known or can be deduced from the protein sequence based on known codon usage. See, e.g., Old and Primrose, Principles of Gene Manipulation 3rd ed., Blackwell Scientific Publications, 1985; Wada et al., Nucleic Acids Res. 20: 2111-2118(1992). Preferably, the DNA molecule includes additional sequence, e.g., recognition sites for restriction enzymes which facilitate its cloning into a suitable cloning vector, such as a plasmid. The invention provides the nucleic acids comprising the coding regions, non-coding regions, or both, either alone or cloned in a recombinant vector, as well as oligonucleotides and related primer and primer pairs corresponding thereto. Nucleic acids may be DNA, RNA, or a combination thereof. Nucleic acids encoding the reagent may be obtained by any method known within the art (e.g., by PCR amplification using synthetic primers hybridisable to the 3'- and 5'-termini of the sequence and/or by cloning from a cDNA or genomic library using an oligonucleotide sequence
specific for the given gene sequence, or the like). Nucleic acids can also be generated by chemical synthesis.

Any of the methodologies known within the relevant art regarding the insertion of nucleic acid fragments into a vector may be used to construct expression vectors that contain a chimeric gene comprised of the appropriate transcriptional/translation control signals and reagent-coding sequences. Promoter/enhancer sequences within expression vectors may use plant, animal, insect, or fungus regulatory sequences, as provided in the invention.

A host cell can be any prokaryotic or eukaryotic cell. For example, the peptide can be expressed in bacterial cells such as E. coli, insect cells, fungi or mammalian cells (such as Chinese hamster ovary cells (CHO) or COS cells). Other suitable host cells are known to those skilled in the art. In one embodiment, a nucleic acid encoding a reagent is expressed in mammalian cells using a mammalian expression vector. Examples of mammalian expression vectors include pCDM8 (Seed (1987) Nature 329:840) and pMT2PC (Kaufman et al. (1987) EMBO J 6: 187-195). Furthermore, transgenic animals containing nucleic acids that encode PDGF may also be used to express peptides of the invention.

The host cells, can be used to produce (i.e., over-express) peptide in culture. Accordingly, the invention further provides methods for producing the peptide using the host cells of the invention. In one embodiment, the method comprises culturing the host cell of invention (into which a recombinant expression vector encoding the peptide has been introduced) in a suitable medium such that peptide is produced. The method further

An "isolated" or "purified" recombinant peptide or biologically active portion thereof is substantially free of cellular material or other contaminating proteins from the cell or tissue source from which the peptide of interest is derived. The language "substantially free of cellular material" includes preparations in which the peptide is separated from cellular components of the cells from which it is isolated or recombinantly produced. In one embodiment, the language "substantially free of cellular material" includes preparations of peptide having less than about 30% (by dry weight) of peptide other than the desired peptide (also referred to herein as a "contaminating protein"), more preferably less than about 20% of contaminating protein, still more preferably less than about 10% of contaminating protein, and most preferably less than about 5% contaminating protein. When the peptide or biologically active portion thereof is recombinantly produced, it is also preferably substantially free of culture medium, i.e., culture medium represents less than about 20%, more preferably less than about 10%, and most preferably less than about 5% of the volume of the peptide preparation.

The invention also pertains to variants of a reagent that function as either agonists (mimetics) or as antagonists. Variants of a reagent can be generated by mutagenesis, e.g., discrete point mutation. An agonist of a reagent can retain substantially the same, or a subset of, the biological activities of the naturally occurring form of the reagent. An antagonist of the reagent can
inhibit one or more of the activities of the naturally occurring form of the reagent by, for example, competitively binding to the receptor. Thus, specific biological effects can be elicited by treatment with a variant with a limited function. In one embodiment, treatment of a subject with a variant having a subset of the biological activities of the naturally occurring form of the reagent has fewer side effects in a subject relative to treatment with the naturally occurring form of the reagent.

Preferably, the analog, variant, or derivative reagent is functionally active. As utilized herein, the term "functionally active" refers to species displaying one or more known functional attributes of a full-length reagent. "Variant" refers to a reagent differing from naturally occurring reagent, but retaining essential properties thereof. Generally, variants are overall closely similar, and in many regions, identical to the naturally occurring reagent.

Variants of the reagent that function as either agonists (mimetics) or as antagonists can be identified by screening combinatorial libraries of mutants of the reagent for peptide agonist or antagonist activity. In one embodiment, a variegated library of variants is generated by combinatorial mutagenesis at the nucleic acid level and is encoded by a variegated gene library. A variegated library of variants can be produced by, for example, enzymatically ligating a mixture of synthetic oligonucleotides into gene sequences such that a degenerate set of potential sequences is expressible as individual peptides, or alternatively, as a set of larger fusion proteins (e.g., for phage display) containing the set of sequences therein. There are a variety of methods

Derivatives and analogs of the reagent or individual moieties can be produced by various methods known within the art. For example, the polypeptide sequences may be modified by any of numerous methods known within the art. See e.g., Sambrook, et al., 1990. Molecular Cloning: A Laboratory Manual, 2nd ed., (Cold Spring Harbor Laboratory Press; Cold Spring Harbor, NY). Manipulations can include by glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, linkage to an antibody molecule or other cellular reagent, and the like. Any of the numerous chemical modification methodologies known within the art may be utilized including, but not limited to, specific chemical cleavage by cyanogen bromide, trypsin, chymotrypsin, papain, V8 protease, NaBH₄, acetylation, formylation, oxidation, reduction, metabolic synthesis in the presence of tunicamycin, etc.

Derivatives and analogs may be full length or other than full length, if said derivative or analog contains a modified nucleic acid or amino acid, as
described infra. Derivatives or analogs of the reagent include, but are not limited to, molecules comprising regions that are substantially homologous in various embodiments, of at least 30%, 40%, 50%, 60%, 70%, 80%, 90% or preferably 95% amino acid identity when: (i) compared to an amino acid sequence of identical size; (ii) compared to an aligned sequence in that the alignment is done by a computer homology program known within the art (e.g., Wisconsin GCG software) or (iii) the encoding nucleic acid is capable of hybridizing to a sequence encoding the aforementioned peptides under stringent (preferred), moderately stringent, or non-stringent conditions. See, e.g., Ausubel, et al., Current Protocols in Molecular Biology, John Wiley and Sons, New York, NY, 1993.

Derivatives of the reagent may be produced by alteration of their sequences by substitutions, additions or deletions that result in functionally-equivalent molecules. One or more amino acid residues within the reagent may be substituted by another amino acid of a similar polarity and net charge, thus resulting in a silent alteration. Conservative substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. Polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. Positively charged (basic) amino acids include arginine, lysine and histidine. Negatively charged (acidic) amino acids include aspartic acid and glutamic acid.
In one embodiment, the invention provides a conjugate comprising an autoantigen, or fragment thereof, and an isologous immunoglobulin. In preferred embodiments, the autoantigen may be the acetylcholine receptor, insulin, the insulin receptor, myelin basic protein or an immunologically equivalent fragment of these proteins.

In one embodiment, the invention provides a conjugate comprising a peptide or protein auto-antigen which has been implicated in disease and an immunoglobulin. In a preferred embodiment, the disease is type 1 diabetes.

According to one aspect of the present invention, there is provided a conjugate comprising a B chain peptide comprising amino acids 9-23 (proinsulin II peptide 33-47) and an isologous immunoglobulin.

In a preferred embodiment, the conjugate is represented by formula I:

Formula I

\[ \text{IgG} \quad \text{Peptide 9-23} \]

In a preferred embodiment of the invention, the B chain peptide is a human B chain peptide and the immunoglobulin is human IgG.

According to another aspect of the present invention, there is provided a method for preventing the
development of type 1 diabetes in a mammal by administering a therapeutically effective regimen comprising the conjugates of the invention. In a preferred embodiment, the conjugate is the conjugate of formula 1.

In a preferred embodiment of the invention, the development of type 1 diabetes is prevented in a human.

According to another aspect of the present invention, there is provided a method for inducing tolerance to insulin in a mammal by administering a therapeutically effective regimen comprising the conjugate of formula 1.

In a preferred embodiment of the invention, tolerance is induced in a human.

According to another aspect of the present invention, there is provided a method for preparing the conjugates comprising:

(a) incubating an immunoglobulin in the presence of a heterobifunctional crosslinker comprising an N-hydroxysuccinimide ester group and a maleimide group linked via a polyethylene oxide spacer;

(b) removing excess heterobifunctional crosslinker to yield a reaction product; and

(c) incubating the reaction product with a B chain peptide comprising amino acids 9-23, wherein the B chain peptide comprises at least one sulphydryl group.

In a preferred embodiment of the invention, the conjugate is prepared using human B chain peptide and human IgG.
The present invention is even further directed to methods of inhibiting TH1 activity, comprising administering an effective amount of a conjugated of the invention. For example, one can inhibit TH1 activity in mammalian tissue by administering a compound of formula I or a pharmaceutically acceptable formulation thereof such that Fc IgG receptors are contacted.

The activity of the inventive compounds as inhibitors of TH1 activity may be measured by any of the methods available to those skilled in the art, including in vivo and in vitro assays.

Administration of the conjugates of the invention, or their pharmaceutically acceptable formulations, may be performed according to any of the accepted modes of administration available to those skilled in the art. Illustrative examples of suitable modes of administration include, but are not limited to nasal, parenteral, transdermal, subcutaneous etc.

A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, e.g., intravenous, intradermal, subcutaneous, oral (e.g., inhalation), transdermal (topical), transmucosal, and rectal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as
ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates, and agents for the adjustment of tonicity such as sodium chloride or dextrose. The pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, N.J.) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for
example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound (e.g., chimeric peptide) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle that contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, methods of preparation are vacuum drying and freeze-drying that yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Solid or liquid pharmaceutically acceptable carriers, diluents, vehicles, or excipients may be employed in the pharmaceutical compositions. The carrier or diluent may include a suitable prolonged-release material. When a liquid carrier is used, the preparation may be in the form of a nonaqueous or aqueous liquid suspension.

A dose of the pharmaceutical composition contains at least a therapeutically effective amount of the active compound (i.e., a compound of formula I or a pharmaceutically acceptable formulation thereof) and preferably is made up of one or more pharmaceutical dosage units. The selected dose may be administered to a
mammal, for example, a human patient, in need of treatment mediated by inhibition of TH1 activity, by any known method of administering the dose.

A "therapeutically effective amount" is intended to mean that amount of a compound of formula I that, when administered to a mammal in need thereof, is sufficient to effect treatment for disease conditions. The amount of a given compound of, for example, formula I that will correspond to a "therapeutically effective amount" will vary depending upon factors such as the particular compound, the disease condition and the severity thereof, the identity of the mammal in need thereof, but it can nevertheless be readily determined by one of skill in the art.

"Therapeutically effective regimen" as used herein and in the claims, refers to a combination of dosage amount and such continuing administration of such dosage amounts as will be determinable by one of skill in the art for the patient being treated and the result being sought.

"Treating" or "treatment" is intended to mean at least the mitigation of a disease condition in a mammal, such as a human, that is alleviated by the inhibition of the progression of IDDM:

(a) prophylactic treatment in a mammal, particularly when the mammal is found to be predisposed to having the disease condition but not yet diagnosed as having it;

(b) inhibiting the disease condition; and/or

(c) alleviating, in whole or in part, the disease condition.
The inventive compounds may be prepared by employing the techniques available in the art using starting materials that are readily available.

Preferably, the inventive conjugates are prepared by the methods of the present invention, including the general methods described herein.

Other features of the invention will become apparent in the course of the following description of exemplary embodiments which are given for illustration of the invention and are not intended to be limiting thereof.

Examples

Preparation of tolerogenic IgG conjugates with Insulin peptide 9-23 (Ins9-23/Ig) and a reference peptide (Ovalbumine peptide, Ova/Ig).

Insulin peptide 9-23 (Ins9-23) and an irrelevant control peptide (peptide 323-339 of Ovalbumin, OVA) to which a cysteine were added at N terminus were covalently linked to mouse IgG2B as described in the European Patent application EP 118335A1 with the exception that DMF was used instead of ethanol to dissolve the crosslinker and activation of IgG with the crosslinker was done at pH 7.0 with a reaction time of 10 minutes instead of 30 minutes.

Animals.

NOD mice were bred under specific-pathogen-free conditions and checked every 6 months for bacterial, viral and parasitic infections. Cumulative incidence of
spontaneous type 1 diabetes in female NOD mice in the colony during the 30 month period in which the present experiments were performed was 0% at 4 and 8 weeks of age, 0.3-1.1% at 11 weeks, 9.9-11.7% at 14 weeks and 27.2-32.3% at 17 weeks. Diabetes incidence reached a plateau of 82.5-87.2% at 39-42 weeks of age. Peri-insulitis was detected in 100% of mice at 4 weeks of age (peri-insulitis detected in 16% of islets). Insulitis was detected in 88% of female mice by 12 weeks of age. Mice were monitored for glucosuria (Glucostest, Boehringer Mannheim, Germany) twice a week. Glycaemia was evaluated in glucosuric mice using test strips and a colorimetric assay (Haemoglucotest and Reflolux F, Boehringer Mannheim). Diabetes was diagnosed as persistent hyperglycemia above 350 mg/dl at a 48 h interval. Animals were fed standard chow and tap water ad libitum.

Organization of the tolerization experiments.

First experiment: Two groups of 15 female NOD mice were injected weekly by the intravenous route with either proinsulin II peptide 9-23 conjugate (Ins9-23/Ig) or control Ovalbumin peptide 323-339 conjugate (OVA/Ig). Injections were performed starting at 4 weeks of age up to 13 weeks of age. Treated mice received a total of 10 injections. The conjugate dose was 0.5 mg/mouse for each injection. A group of 15 female NOD remained untreated and provided a further control group.

Second experiment: Two groups of 18 female NOD mice were injected weekly by the intravenous route with either Ins9-23/Ig or control OVA/Ig. Injections were performed starting at 4 weeks of age up to 18 weeks of age. Treated mice received a total of 15 injections. The conjugate dose was 0.5 mg/mouse for each injection. A
group of 19 female NOD remained untreated and provided a further control group.

**Evaluation of insulinitis.**

Two groups of 5 female NOD mice were injected weekly by the intravenous route from 4 weeks of age up to 8 weeks of age with either Ins9-23/Ig or control OVA/Ig. The conjugate dose was 0.5mg/mouse for each injection. Pancreases were collected from anesthetized mice at 10 weeks of age and fixed in 4% formaldehyde and further processed for histological evaluation. The extent of insulinitis was scored on tissue sections and graded: 0, normal islet; 1, peri-insulitis (infiltrating cells at the periphery of the islet); 2, insulitis (invasive infiltrate); 3, extensive and destructive insulitis. A mean of 38 ± 7 islets per pancreas was analysed in each animal.

**STATISTICAL ANALYSIS**

Incidence of diabetes was compared between groups using Kaplan-Meier estimates.

**Results**

**Prevention of type 1 diabetes in NOD female mice by injection of Ins9-23/Ig.**

In a first experiment, treatment of NOD mice from 4 weeks of age up to 13 weeks of age by weekly i.v. injection of Ins9-23/Ig conjugate delayed the onset of type 1 diabetes as compared with control Ova/Ig conjugate treated mice or control untreated mice (Figure 1); at 22 weeks of age, the incidence of diabetes was 27%, 54% and 67% respectively in the three experimental groups.
However, treatment with Ins9-23/Ig from week 4 to week 13 could not prevent the incidence of diabetes after cessation; indeed, after week 26 the percentage of diabetic animals was the same in all three groups.

In a second experiment, we tested whether a treatment of longer duration could improve the therapeutic efficacy of Ins9-23/Ig and lead to a more sustained prevention of autoimmune diabetes development in treated mice. Treatment of female NOD mice from 4 up to 18 weeks of age with Ins9-23/Ig caused a strong and lasting protection against diabetes development as compared to control mice treated with OVA/Ig (p < 0.006). The incidence of diabetes in mice treated with OVA/Ig was comparable to that of age matched control mice, which remained untreated along the whole experiment (Figure 1).

Effect of Ins9-23/Ig on insulitis.

Histological analysis of the pancreases of 10-week-old female NOD mice treated with either Ins9-23/Ig or OVA/Ig from 4 to 8 weeks of age showed no significant difference between two groups (Figure 2). The percentage of normal islets, islets with a peri-islet infiltrate and islets with intra-islet lymphocyte infiltration was 82.6%, 10.6% and 6.8% in Ins9-23/Ig treated mice and 75%, 11% and 14% in Ova/Ig treated mice, respectively. There were no significant differences between the two groups.
Claims:

1. A conjugate comprising any portion of the proinsulin protein chain and an isologous immunoglobulin.

2. A conjugate comprising any IDDM-relevant antigen and an isologous immunoglobulin.

3. The conjugate according to claim 2, wherein the IDDM-relevant antigen is insulin, proinsulin or GAD protein.

4. A conjugate comprising a disease-relevant auto-antigen or immunologically relevant fragment thereof and an isologous immunoglobulin.

5. A conjugate comprising any therapeutic protein or peptide and an isologous immunoglobulin.

6. A conjugate comprising a B chain peptide comprising amino acids 9-23 (proinsulin II peptide 33-47) and an isologous immunoglobulin.

7. The conjugate of claim 6 where the B chain peptide and the immunoglobulin are human.

8. A method for preventing or treating the development of disease in a mammal by administering a therapeutically effective regimen comprising the conjugate of any one of claims 1-7.
9. The method of claim 8 wherein the mammal is a human, the conjugate is the conjugate of claim 6 and the disease is IDDM.

10. A method for inducing tolerance to insulin in a mammal by administering a therapeutically effective regimen comprising the conjugate of claim 6 or 7.

11. The method of claim 10 wherein the mammal is human and the conjugate is the conjugate of claim 7.

12. A method for preparing the conjugates according to any one of claims 1-7, the method comprising:

(d) incubating an immunoglobulin in the presence of a heterobifunctional crosslinker comprising an N-hydroxysuccinimide ester group and a maleimide group linked via a polyethylene oxide spacer;

(e) removing excess heterobifunctional crosslinker to yield a reaction product; and

(f) incubating the reaction product with an autoantigen peptide comprising at least one sulphydryl group.

13. The method of claim 12, wherein the autoantigen peptide is a B chain peptide comprising amino acids 9-23.
Figure 1

First Experiment

- OVA/Ig
- Ins9-23/Ig
- control

percentage of diabetic mice

weeks

Second Experiment

- Ins9-23/Ig
- OVA/Ig
- control

percentage of diabetic mice

weeks

$p < 0.006$
## INTERNATIONAL SEARCH REPORT

### A. CLASSIFICATION OF SUBJECT MATTER

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

### B. FIELDS SEARCHED

**Minimum documentation searched** (classification system followed by classification symbols)

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

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

EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE, CHEM ABS Data

### C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<td>WO 91/08773 A (BLOOD RES CENTER) 27 June 1991 (1991-06-27)</td>
<td>1-5, 8, 9, 12</td>
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<td>X</td>
<td>Further documents are listed in the continuation of box C.</td>
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**Special categories of cited documents:**

**A** document defining the general state of the art which is not considered to be of particular relevance

**E** earlier document but published on or after the international filing date

**L** 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 when the document is taken alone

**Y** document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

**A** document member of the same patent family

**Date of the actual completion of the international search**

25 November 2003

**Date of mailing of the international search report**

24 O3, 2004

**Name and mailing address of the ISA**

European Patent Office, P.B. 5818 Patentieten 2 NL - 2280 HV Rijswijk
Tel: (+31-70) 340-0240, Tx: 31 651 epc nl,
Fax: (+31-70) 340-3016

**Authorized officer**

Vadot, P
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### Box I  Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

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

1. **x** Claims Nos.; because they relate to subject matter not required to be searched by this Authority, namely:
   - Although claims 8-11 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. **x** Claims Nos.; because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
   - see FURTHER INFORMATION sheet PCT/ISA/210

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

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

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

- see additional sheet

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

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

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

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

   - 2-5, 8, 10, 12 (partially), 1, 6, 7, 9, 11, 13

**Remark on Protest**
- □ The additional search fees were accompanied by the applicant's protest.
- □ No protest accompanied the payment of additional search fees.
Continuation of Box I.2

Claims Nos.: -

Present claims 1-7 relate to an extremely large number of possible products/methods. Support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT is to be found, however, for only a very small proportion of the products/methods claimed. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Consequently, the search has been carried out for those parts of the claims which appear to be supported and disclosed, namely the examples.

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guideline C-VI, 8.5), should the problems which led to the Article 17(2) declaration be overcome.
FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 2,3,4,5,8,10,12 (partially), 1,6,7,9,11,13

   A conjugate comprising any portion of the proinsulin protein chain, and more specifically, B chain peptide comprising amino acids 9-23 and an isologous immunoglobulin. A method of preparing thereof, and for preventing or treating the development of the related disease (diabetes type 1).

   ---

2. claims: 2,3,4,5,8,10,12 (partially)

   A conjugate comprising any IDDM-relevant antigen, more specifically insulin, and an isologous immunoglobulin. A method of preparing thereof and for preventing or treating the development of the related disease.

   ---

3. claims: 2,3,4,5,8,10,12 (partially)

   A conjugate comprising any IDDM-relevant antigen, more specifically GAD protein, and an isologous immunoglobulin. A method of preparing thereof and for preventing or treating the development of the related disease.

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