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(54) Title: METHODS FOR TREATMENT OF DIABETES USING PEPTIDE ANALOGUES OF INSULIN

(57) Abstract

The present invention is directed toward peptide analogues of insulin (B) chain that are generally derived from peptides comprising residues (9 to 23) of the native (B) chain sequence. The analogues are altered from the native sequence at position (12, 13, 15) and/or (16), and may be additionally altered at position (19) and/or other positions. Pharmaceutical compositions containing these peptide analogues are provided. The peptide analogues are useful for treating and inhibiting the development of diabetes.

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METHODS FOR TREATMENT OF DIABETES USING PEPTIDE ANALOGUES OF INSULIN

TECHNICAL FIELD

The present invention relates generally to peptide analogues of insulin, and more specifically to methods for treating diabetes using peptide analogues derived from residues 9-23 of human insulin B chain.

BACKGROUND OF THE INVENTION

Insulin dependent diabetes mellitus (IDDM) is an organ specific autoimmune disease affecting close to a million people in different age groups in the United States. The disease is characterized by extensive destruction of the insulin producing beta cells in the pancreatic islets and dysregulation of glucose metabolism leading to frank diabetes. The defining feature of IDDM is the lymphocytic infiltration of the islets. Among the invading cells, T cells appear to be one of the major mediators of autoimmune destruction.

Type I diabetes is further characterized by increased levels of antibodies to various islet associated antigens, including insulin, GAD65, GAD67 and ICA512. These antibodies can be detected much before frank disease, and an immune response to such antigens can be used as a predictor for impending diabetes in patients with susceptible genetic (HLA) haplotypes.

Currently, patients are dependent on insulin injections to maintain normoglycemia. Insulin is a polypeptide hormone consisting of two disulfide-linked chains, an A chain consisting of 21 amino acid residues and a B chain of 30 residues. While administration of insulin provides significant benefits to patients suffering from diabetes, the short serum half-life of insulin creates difficulties for maintaining proper dosage. The use of insulin also can result in a variety of hypoglycemic side-effects and the generation of neutralizing antibodies.

In view of the problems associated with existing treatments of diabetes, there is a compelling need for improved treatments that are more effective and are not

associated with such disadvantages. The present invention exploits the use of peptide analogues which antagonize a T cell response to insulin to effectively treat diabetes, while further providing other related advantages.

SUMMARY OF THE INVENTION

The present invention provides compounds and methods for treating and preventing diabetes. Within certain aspects, the present invention provides peptide analogues comprising residues 9 to 23 of human insulin B chain (SEQ ID NO:2), wherein the peptide analogue differs in sequence from native human insulin B chain residues 9 to 23 due to substitutions at between 1 and 4 amino acid positions. Such substitutions may be made at one or more residues selected from the group consisting of residues 12, 13, 15 and 16, with or without additional substitutions at other residues. Within certain preferred embodiments, such substitutions may occur at two or three amino acid residues within residues 9 to 23 of insulin B chain. Substitutions may also occur at residue 19. Substitutions are preferably non-conservative, and analogues wherein residue 12, 13, 15, 16 and/or 19 are altered (to, for example, alanine) are preferred. Analogues further comprising residue 24 of insulin B chain are also preferred. In certain other embodiments, the peptide analogues comprise no more than 18 residues, no more than 16 residues or no more than 15 residues of human insulin B chain.

Within further embodiments, the peptide analogues consist essentially of residues 9 to 23 or 9 to 24 of human insulin B chain (SEQ ID NO:2), wherein the peptide analogue differs in sequence from native human insulin B chain residues 9 to 23 due to substitutions at between 1 and 4 amino acid positions, and wherein at least one substitution occurs at a residue selected from the group consisting of residues 12, 13, 15 and 16.

Within further aspects, pharmaceutical compositions are provided, comprising a peptide analogue as described above in combination with a physiologically acceptable carrier or diluent.

The present invention further provides methods for treating and/or inhibiting the development of diabetes, comprising administering to a patient a therapeutically effective amount of a pharmaceutical composition as described above.

These and other aspects of the invention will become evident upon reference to the following detailed description and attached drawings. In addition, various references are set forth below which describe in more detail certain procedures or compositions. These references are incorporated herein by reference in their entirety as if each were individually noted for incorporation.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the amino acid sequence of residues 9-23 of insulin B chain (SEQ ID NO:2).

Figure 2 is a graph showing the proliferative response (measured in cpm) of a NOD mouse T cell clone to a native insulin B chain (9-23) peptide in the presence of varying amounts of representative peptide analogues, in which different residues are substituted with alanine, as indicated.

Figure 3 is a graph showing the proliferative response (measured in cpm) of a NOD mouse T cell clone to a native insulin B chain (9-23) peptide in the presence of varying amounts of the representative peptide analogue in which amino acids at positions 16 and 19 are substituted with alanine (NBI-6024; indicated by squares). For comparison the proliferative response in the presence of an unrelated control peptide derived from myelin basic protein (NBI-5096; indicated by circles) is also shown. The response is shown as mean CPM $\pm$ SEM of triplicate cultures.

Figures 4-6 are histograms illustrating the proliferative response (measured in cpm) of T cell lines from different diabetic patients to the native B chain (9-23) peptide or to representative peptide analogues. Peripheral blood mononuclear cells were isolated from diabetic patients and cultured in the presence of insulin B chain (9-23) peptide. After three rounds of restimulation with insulin B chain (9-23), 1×10^5 T-cells and 7×10^4 irradiated autologous PBMCs were added to each well in a round bottom 96-well plate in complete medium. Cells were cultured for 5 days with NBI-

6024 (insulin B chain 9-23 with alanine substitutions at positions 16 and 19), insulin B chain (9-23) or medium only. On day 4, the cells were pulsed with ^{3}H-thymidine and re-cultured for an additional 18 hours. The cultures were then harvested, counted using liquid scintillation, and the data was expressed as the mean counts per minute (cpm) of replicate samples $\pm$ standard error of the mean (sem).

Figure 7 is a histogram illustrating the proliferative response (measured in cpm) of a T cell line from a diabetic patient to the native B chain (9-23) peptide or to representative peptide analogues containing alanine substitutions as indicated. Peripheral blood mononuclear cells were isolated from diabetic patients and cultured in the presence of insulin B chain (9-23) peptide. After three rounds of restimulation with insulin B chain (9-23), $1x10^5$ T-cells and 7×10^4 irradiated autologous PBMCs were added to each well in a round bottom 96-well plate in complete medium. Cells were cultured for 5 days with analogue, insulin B chain (9-23) or medium only (BKG), as indicated. On day 4, the cells were pulsed with ^{3}H-thymidine and re-cultured for an additional 18 hours. The cultures were then harvested, counted using liquid scintillation, and the data was expressed as the mean counts per minute (cpm) of replicate samples $\pm$ standard error of the mean (sem).

Figure 8 is a graph showing the percent of female NOD mice that were diabetic following nine weekly treatments with representative peptide analogues. Ten mice each were treated subcutaneously beginning on day 24 with peptide analogues of the B chain (9-23) containing alanine substitutions at residue 12 (open triangles), 13 (squares) or 16 (solid triangles). All of the mice treated with a control peptide, neurotensin (circles), became diabetic.

Figure 9 is a graph showing the same data as in Figure 8, but contrasting only the A13 analogue-treated group with the control peptide (neurotensin)-treated group.

Figure 10 is a graph showing the percent of NOD mice that were diabetic following 13 weekly treatments with a representative peptide analogue. Ten mice were treated subcutaneously beginning on day 24 with 400 μg of neurotensin (squares), B

chain (9-23) {diamonds} or a peptide analogue of the B chain (9-23) containing an alanine substitution at residue 13 (triangles).

Figure 11 is a graph showing the effect of representative peptide analogues on the incidence of diabetes in NOD mice. Four week old female NOD mice (n=9) were treated subcutaneously with 20 mg/kg of NBI-6024 ($A^{16,19}$) at weekly intervals for 12 weeks, followed by every other week until 35 weeks of age. The control group (n=10) consisted of untreated animals. Mice with blood glucose greater than 200 mg/dL at two consecutive time points were considered to be diabetic. The data are expressed as the percent of non-diabetic over the 35-week study. The log-rank test was used to assess whether the results of the two treatment groups were significantly different. The percent of NOD mice that were diabetic following treatments with NBI-6024 ($A^{16,19}$) is indicated at the various time points by squares, and the percent of mice that were diabetic in the control group is indicated by circles.

Figure 12 is a graph showing the effect of representative peptide analogues on the incidence of diabetes in NOD mice. Four week old female NOD mice (n=13-15) were treated subcutaneously with 20 mg/kg of NBI-6024 ($A^{16,19}$) or NBI-6201 (a control peptide, neurotensin) at weekly intervals for 12 weeks, followed by every other week until 35 weeks of age. An additional control group (n=8) consisted of untreated animals. Mice with blood glucose greater than 200 mg/dL at two consecutive time points were considered to be diabetic. The data are expressed as the percent of non-diabetic over the 35-week study. The log-rank test was used to assess whether the results of the two treatment groups were significantly different. The percent of NOD mice that were diabetic following treatments with NBI-6024 ($A^{16,19}$) is indicated at the various time points by squares, the percent that were diabetic following treatment with the neurotensin peptide is shown by triangles, and the percent of untreated mice that were diabetic is indicated by circles.

Figures 13A-13D are graphs illustrating the immunogenicity of representative peptide analogues containing B chain residues 9-23 with an alanine substitution at residue 12 (Figure 13A), residue 13 (Figure 13B), residue 15 (Figure 13C) or residue 16 (Figure 13D). NOD mice were injected 2-4 times subcutaneously

with the peptide analogue in soluble form before assaying the proliferative response of lymph node cells to varying concentrations of either the peptide analogue or native insulin B chain (9-23) peptide as indicated. Proliferative response was assessed by determining the amount of radioactive thymidine incorporated in the cells (plotted as mean counts per minute (CPM) of triplicate culture wells) by counting in a liquid scintillation counter following completion of the culture period.

Figures 14A-14F are graphs showing the immunogenicity of six different peptide analogues in the NOD mice. Peptide analogues with two alanine substitutions (A12,13; A12,15; A12,16; A13,15; A13,16 and A15,16, as indicated) were injected in NOD mice and after 10 days their lymph node cells were used in a proliferation assay using different concentrations of the immunizing peptide as stimulators. Proliferative response was assessed by determining the amount of radioactive thymidine incorporated in the cells (plotted as mean counts per minute (CPM) of triplicate culture wells) by counting in a liquid scintillation counter following completion of the culture period.

Figures 15A-15D are graphs illustrating the immunogenicity of representative double substituted peptide analogs of insulin B chain (9-23). The following peptides were tested for their ability to elicit an immune response in NOD mice: A12,19 (Figure 15A); A13,19 (Figure 15B); A15,19 (Figure 15C); A16,19 (Figure 15D). Proliferative response as counts per minute of the draining lymph node cells is shown in response to the immunizing analogue and also to the native insulin B chain (9-23) peptide.

Figure 16 is a graph showing the ability of a series of triple substituted peptides to evoke a T cell proliferative response in NOD mice. Mice were immunized separately with representative peptide analogues containing the following combinations of substitutions: A12,13,19; A12,15,19; A12,16,19; A13,15,19; or A13,16,19; A15,16,19. Lymph node cells were then used in a proliferation assay, and the response to each of the immunizing peptides at different concentrations is shown.

Figures 17A and 17B are graphs showing the ability of a double substituted peptide ($A^{16,19}$) to evoke an immune response. Five female NOD mice were immunized subcutaneously with 20 mg/kg of soluble NBI-6024 on days 1, 6 and 12.

On day 15, the mice were sacrificed, the inguinal lymph node cells removed and cultured in the presence of varying concentrations (0-50 μM) of either NBI-6024 (Figure 17A) or insulin B chain (9-23) peptide (Figure 17B). The extent of T-cell proliferation was determined using ^{3}H-thymidine incorporation. The response is expressed as mean CPM ± SEM of triplicate cultures.

Figure 18 is a histogram showing a comparison of the cytokines produced by immune cells induced by $A^{16,19}$ (NBI-6024) peptide in the presence or absence of adjuvant. Groups of NOD mice were immunized with the NBI-6024 alone or emulsified with CFA. The cytokines IL-2 and IL-4, as indicated, were measured at 25 μM of NBI-6024 and expressed as pg/mL after subtracting the background values.

DETAILED DESCRIPTION OF THE INVENTION

Prior to setting forth the present invention, it may be helpful to an understanding thereof and to provide definitions of certain terms that are used herein.

"Insulin B chain" refers to a 30 amino acid polypeptide present as one of the two disulfide-linked polypeptides that make up insulin. The sequence of human insulin B chain is provided in SEQ ID NO:1, and the sequence of residues 9-23 of human B chain is provided in Figure 1 and SEQ ID NO:2.

"Peptide analogues" of the insulin B chain comprise at least 15 amino acid residues derived from residues 9-23 of human insulin B chain (SEQ ID NO:2), with at least one difference in amino acid sequence between the analogue and the native B chain. Within a peptide analogue, at least one difference in amino acid sequence occurs at residue 12, 13, 15 and/or 16. In addition, residue 19 may be substituted, and other alterations are possible. Preferably, a peptide analogue contains between 1 and 4 substitutions within residues 9-23, relative to a native insulin B chain (9-23) sequence, although a greater number of substitutions (*e.g.*, 5 or 6) may be possible. Additional residues derived from insulin B chain may be included, up to the full 30 residues of native B chain, preferably up to a total of 25 residues, more preferably up to a total of 16 or 18 residues of the peptide analogue. Within a preferred embodiment, residue 24 of insulin B chain is also included in the peptide analogue. Sequences that are not

derived from insulin B chain may, but need not, be present at the amino and/or carboxy terminus of the peptide analogue. Such sequence(s) may be used, for example, to facilitate synthesis, purification or solubilization of the peptide analogue.

Unless otherwise indicated, a named amino acid refers to the L-form. An L-amino acid residue within the native peptide sequence may be altered to any one of the 20 L-amino acids commonly found in proteins, any one of the corresponding D-amino acids, rare amino acids, such as 4-hydroxyproline or hydroxylysine, or a non-protein amino acid, such as β-alanine or homoserine. Also included with the scope of the present invention are analogues comprising amino acids that have been altered by chemical means such as methylation (*e.g.*, α-methylvaline); amidation of the C-terminal amino acid by an alkylamine such as ethylamine, ethanolamine or ethylene diamine; and/or acylation or methylation of an amino acid side chain function (*e.g.*, acylation of the epsilon amino group of lysine).

"Residue 12," "residue 13," "residue 15," "residue 16" and "residue 19" (also called position 12, position 13, position 15, position 16 and position 19, respectively) refer to amino acids 12, 13, 15, 16 and 19 of insulin B chain as displayed in Figure 1. More specifically, the numbering system for these residues relates to the amino acid position within the native human protein, regardless of the length of the peptide analogue or the amino acid position within the analogue. Peptide analogues having an alanine substitution at residues 12, 13, 15 or 16 are referred to as the A12, A13, A15 or A16 analogues, respectively.

Peptide Analogues of Insulin B Chain

As noted above, the present invention provides peptide analogues comprising at least residues 9-23 of human insulin B chain and including an alteration of the naturally occurring L-valine at position 12, L-glutamate at position 13, L-leucine at position 15 and/or L-tyrosine at position 16, to another amino acid. In one embodiment, peptide analogues contain additional alterations of one to three L-amino acids at positions 12, 13, 15, 16 and/or 19 of insulin B chain. Preferably, the peptide

analogues contain two or three alterations in which one of the substituted residues is at position 19.

The portion of a peptide analogue that is derived from insulin B chain is typically 15-30 residues in length, preferably 15-18 residues in length, and more preferably 15-16 residues in length. Particularly preferred peptide analogues contain 15 amino acids derived from insulin B chain.

As noted above, peptide analogues comprising any amino acid alteration(s) at the positions recited above are within the scope of this invention. Preferred peptide analogues contain non-conservative substitutions (*i.e.*, alterations to amino acids having differences in charge, polarity, hydrophobicity and/or bulkiness). Particularly preferred analogues contain alterations of one or more residues to alanine.

Peptide analogues may be synthesized by standard chemistry techniques, including automated synthesis. In general, peptide analogues may be prepared by solid-phase peptide synthesis methodology which involves coupling each protected amino acid residue to a resin support, preferably a 4-methyl-benzhydrylamine resin, by activation with dicyclohexylcarbodiimide to yield a peptide with a C-terminal amide. Alternatively, a chloromethyl resin (Merrifield resin) may be used to yield a peptide with a free carboxylic acid at the C-terminus. Side-chain functional groups may be protected as follows: benzyl for serine and threonine; cyclohexyl for glutamic acid and aspartic acid; tosyl for histidine and arginine; 2-chlorobenzyloxycarbonyl for lysine; and 2-bromobenzyloxycarbonyl for tyrosine. Following coupling, the t-butyloxycarbonyl protecting group on the alpha amino function of the added amino acid may be removed by treatment with trifluoroacetic acid followed by neutralization with di-isopropyl ethylamine. The next protected residue is then coupled onto the free amino group, propagating the peptide chain. After the last residue has been attached, the protected peptide-resin is treated with hydrogen fluoride to cleave the peptide from the resin and deprotect the side chain functional groups. Crude product can be further purified by gel filtration, HPLC, partition chromatography or ion-exchange chromatography, using well known procedures.

Peptide analogues within the present invention (a) should not stimulate NOD mouse T cell clones specific to the native insulin B chain (9-23) peptide (SEQ ID NO:2), or should stimulate such clones at a level that is lower than the level stimulated by the native peptide; (b) should not stimulate insulin B chain (9-23) specific human T cells from patients; (c) should be immunogenic in the NOD mouse; (d) should reduce the incidence of diabetes in NOD mice and (e) may inhibit a response of T cell clones specific to the native insulin B chain (9-23) peptide (SEQ ID NO:2). Thus, candidate peptide analogues may be screened for their ability to treat diabetes by assays measuring T cell proliferation, immunogenicity in NOD mice and the effect on the incidence of the disease in NOD mice. Certain representative assays for use in evaluating candidate peptide analogues are discussed in greater detail below. Those analogs that satisfy the above criteria are useful therapeutics.

Candidate peptide analogues may initially be tested for the ability to stimulate T cells specific to the native insulin B chain (9-23) peptide (SEQ ID NO:2) (from clonal cell lines or isolated from patients). Such tests may be performed using a direct proliferation assay in which native B chain (9-23) reactive T cell lines or T cells isolated from patients are used as target cells. T cell lines may generally be established, using well known techniques, from lymph nodes taken from rats injected with B chain (9-23). Lymph node cells may be isolated and cultured for 5 to 8 days with B chain (9-23) and IL-2. Viable cells are recovered and a second round of stimulation may be performed with B chain (9-23) and irradiated splenocytes as a source of growth factors. After 5 to 6 passages in this manner, the proliferative potential of each cell line is determined. To perform a proliferation assay, B chain (9-23)-reactive T cell lines may be cultured for three days with various concentrations of peptide analogues and irradiated, autologous splenocytes. After three days, 0.5-1.0 μCi of [^{3}H]-thymidine is added for 12-16 hours. Cultures are then harvested and incorporated counts determined. Mean CPM and standard error of the mean are calculated from triplicate cultures. Peptide analogues yielding results that are less than three standard deviations of the mean response with a comparable concentration of B chain (9-23) are considered non-

stimulatory. Peptide analogues which do not stimulate proliferation at concentrations of less than or equal to 20-50 μM are suitable for further screenings.

Candidate peptides that do not stimulate B chain (9-23) specific T cells, and preferably inhibit a response of such T cells *in vitro*, are further tested for their immunogenicity in the NOD mouse. Briefly, groups of NOD mice may be immunized with 100-400μg of the candidate peptides subcutaneously in mannitol acetate buffer three times within a period of 10-15 days. Following the last immunization, lymph node cells and/or spleen cells may be used in a proliferation assay in which different concentrations of the immunizing peptide are cultured with the cells for 3-4 days. The last 18 hours of culture may be performed with tritiated thymidine. Cells may then be harvested and counted in a scintillation counter, and the proliferative response may be expressed as CPM ± SEM. Candidate peptides that induce a proliferation that is at least 2-fold higher than the background (no antigen) at 25μM of the peptide are considered to be immunogenic. Alternatively, the candidate peptide analogue is considered immunogenic if it elicits a proliferative response following immunization of the NOD mice in complete Freund's adjuvant. The draining lymph node cells or spleen cells, when cultured in the presence of the immunizing analogue, should induce a proliferation that is at least 2-fold higher than the background (no antigen) at 25μM of the peptide.

Candidate peptides that can inhibit proliferation by B chain (9-23) are further tested for the ability to reduce the incidence of diabetes in NOD mice. Briefly, peptides may be administered to NOD mice in soluble form or emulsified with, for example, incomplete Freund's adjuvant (IFA). Typically, weekly administration of about 400 μg of peptide is sufficient. The incidence of diabetes in the treated mice, as well as in untreated or control mice, is then evaluated by weekly monitoring of blood glucose levels. A value of 200 mg/dl or more of blood glucose on two consecutive occasions is generally considered indicative of the appearance of diabetes. Peptide analogues should result in a statistically significant decrease in the percent of NOD mice afflicted with diabetes within a monitoring period of up to about 25 weeks.

As noted above, peptide analogues may also inhibit the response of B chain (9-23) specific human T cells *in vitro*. Such inhibition may be measured by a competition assay in which candidate peptide analogues are tested for the ability to inhibit T cell proliferation induced by native B chain (9-23) (SEQ ID NO:2). In such an assay, antigen presenting cells are first irradiated and then incubated with the competing peptide analogue and the native B chain (9-23) peptide. T cells are then added to the culture. Various concentrations of candidate peptide analogues are included in cultures which may be incubated for a total of 4 days. Following the incubation period, each culture is pulsed with, for example, 1 μCi of [^{3}H]-thymidine for an additional 12-18 hours. Cultures may then be harvested on fiberglass filters and counted as above. Mean CPM and standard error of the mean can be calculated from data determined in triplicate cultures. Peptide analogues that reduce proliferation by at least 25% at a concentration 20-50 μM are preferred.

Treatment and Prevention of Diabetes

As noted above, the present invention provides methods for treating and preventing Type I diabetes by administering to the patient a therapeutically effective amount of a peptide analogue of insulin B chain as described herein. Diabetic patients suitable for such treatment may be identified by criteria accepted in the art for establishing a diagnosis of clinically definite diabetes. Such criteria may include, but are not limited to, low (less than tenth or first percentile of controls) first phase insulin secretion following an intravenous glucose tolerance test (IVGTT) or the persistence of high titer antibodies to islet antigens such as insulin, GAD65 and/or ICA512.

Patients without clinically definite diabetes who may benefit from prophylactic treatment may generally be identified by any predictive criteria that are accepted in the art. Patients who are not frankly diabetic may be predicted to develop diabetes in the coming years (1-5yrs) based upon the following criteria: i) family history - first degree relatives are automatically in the high risk group unless they have a protective HLA allele; ii) genetic make-up - *i.e.*, the presence or absence of an HLA allele that is associated with a high risk of diabetes (*e.g.*, DR3/4; DQ8); iii) presence or

absence of high titer autoantibodies in their blood to any or all of the antigens: insulin, GAD65 and/or ICA 512; and iv) intravenous glucose tolerance test (IVGTT): low first-phase insulin secretion, usually defined as below the tenth or first percentile of normal controls, typically precedes the development of type I diabetes by 1-5 years. In general, several of the above criteria may be considered. For example, the chances of developing diabetes in 5 years for a first degree relative of an individual with diabetes are estimated to be: 100% for relatives with all 3 autoantibodies listed above; 44% for relatives with 2 antibodies; 15% for relatives with one antibody; and 0.5% for relatives with no antibodies. Among 50 first degree relatives of patients with Type I diabetes followed to the onset of diabetes, 49/50 expressed one or more of the above listed autoantibodies.

Effective treatment of diabetes may be determined in several different ways. Satisfying any of the following criteria, or other criteria accepted in the art, evidences effective treatment. Criteria may include, but are not limited to, delay in developing frank hyperglycemia, lowered frequency of hyperglycemic events and/or prolongation of normal levels of C-peptide in the blood of the patients.

Peptide analogues of the present invention may be administered either alone, or as a pharmaceutical composition. Briefly, pharmaceutical compositions of the present invention may comprise one or more of the peptide analogues described herein, in combination with one or more pharmaceutically or physiologically acceptable carriers, diluents or excipients. Such compositions may comprise buffers such as neutral buffered saline, phosphate buffered saline and the like, carbohydrates such as glucose, mannose, sucrose or dextrans, mannitol, proteins, polypeptides or amino acids such as glycine, antioxidants, chelating agents such as EDTA or glutathione, adjuvants (*e.g.*, aluminum hydroxide) and preservatives. In addition, pharmaceutical compositions of the present invention may also contain one or more additional active ingredients, such as, for example, sustained delivery systems or other immunopotentiators.

Compositions of the present invention may be formulated for the manner of administration indicated, including for example, for oral, nasal, venous, intracranial,

intraperitoneal, subcutaneous, or intramuscular administration. Within other embodiments of the invention, the compositions described herein may be administered as part of a sustained release implant. Within yet other embodiments, compositions of the present invention may be formulated as a lyophilizate, utilizing appropriate excipients which provide stability as a lyophilizate and/or following rehydration.

Pharmaceutical compositions of the present invention may be administered in a manner appropriate to the disease to be treated (or prevented). The quantity and frequency of administration will be determined by such factors as the condition of the patient, and the type and severity of the patient's disease. Within particularly preferred embodiments of the invention, the peptide analogue may be administered at a dosage ranging from 0.1 to 100 mg/kg, although appropriate dosages may be determined by clinical trials. Patients may be monitored for therapeutic effectiveness by delay in progression to frank diabetes and sustained use of insulin for maintaining normoglycemia as described above.

The following examples are offered by way of illustration and not by way of limitation.

EXAMPLE 1

Preparation of Peptides

This Example illustrates the synthesis of representative peptide analogues.

Peptides were synthesized by solid phase methodology on a peptide synthesizer (Beckman model 990). Peptides with an amidated carboxyl-terminus were prepared with a p-methylbenzhydrylamine resin (MBHA resin); for peptides with a free carboxyl-terminus, a Merrifield resin coupled with the appropriately protected amino acid was used. Both resins were obtained from Bachem Fine Chemicals (Torrance, CA). Derivatized amino acids (Bachem Fine Chemicals) used in the synthesis were of the L-configuration unless specified otherwise, and the N-alpha-amino function protected exclusively with the t-butyloxycarbonyl group. Side-chain functional groups were protected as follows: benzyl for serine and threonine; cyclohexyl for glutamic acid and aspartic acid; tosyl for histidine and arginine; 2-chlorobenzyloxycarbonyl for lysine and 2-bromobenzyloxycarbonyl for tyrosine. Coupling of the carboxyl-terminal amino acid to the MBHA resin was carried out with dicyclohexylcarbodiimide and the subsequent amino acids were coupled with dicyclohexylcarbodiimide according to Ling et al. (*Proc. Natl. Acad. Sci. USA 81*:4302, 1984). After the last amino acid was incorporated, the t-butyloxycarbonyl protecting group was removed and the peptide-resin conjugate treated with a mixture of 14 ml hydrofluoric acid (HF), 1.4 ml anisole, and 0.28 ml methylethyl sulfide per gram of resin conjugate at -20°C for 0.5 hr and at 0°C for 0.5 hr. HF was removed in vacuum at 0°C, and the resulting peptide and resin mixture was washed twice with diethyl ether and twice with chloroform and diethyl ether alternately. The peptide was extracted five times with 2 M acetic acid, and the extract lyophilized. The lyophilized product was first purified on a column of Sephadex G-25 fine (Pharmacia-LKB, Piscataway, NJ) developed in 30% acetic acid to remove the truncated fragments and inorganic salts (Ling et al., 1984). Next, peptides were further purified by CM-32 carboxymethylcellulose cation-exchange chromatography (Ling et al., 1984). Final purification was achieved by partition chromatography on Sephadex G-25 fine (Ling et al., 1984). Alternatively, the crude peptide could be

purified by preparative HPLC on a Biotage KP-100 gradient HPLC system. The synthetic product was characterized by amino acid analysis, mass spectrometric analysis and reversed-phase HPLC.

5 EXAMPLE 2

Long-term T cell lines

This Example illustrates the preparation of long-term insulin-specific NOD T cell lines.

Insulin specific NOD T cell lines were established by culturing 10 lymphocytes isolated from islet-infiltrating populations by *in vitro* stimulation with either porcine insulin at 25 μg/ml and irradiated NOD islet cells in the presence of irradiated NOD spleen cells as antigen presenting cells and cytokines. To obtain the infiltrating lymphocytes the following procedures were performed (*see* Wegmann et al., *Eur. J. Immunol. 24*:1853, 1994): the pancreas from the NOD mouse was digested with 15 collagenase and individual islets were isolated manually. The infiltrating lymphocytes were then obtained by mild trypsin digestion of the islets. The insulin specific T cell lines or clones were propagated by serial stimulation in the presence of NOD spleen cells, porcine insulin and lymphokines. Clones were obtained by limiting dilution of the B chain (9-23) specific T cell lines in the presence of the antigen presenting cells 20 and porcine insulin at 25 μg/ml. Wells with a growing population of cells following limiting dilution were expanded in appropriate medium, and after one cycle of growth were tested for reactivity to the B chain (9-23) peptide of insulin by evaluating the proliferative response.

25 EXAMPLE 3

Effect of Peptide Analogues on Proliferation of Insulin-specific NOD T cell Clones

This Example illustrates the effect of representative peptide analogues on T cell proliferation.

Insulin B chain (9-23) (SEQ ID NO:2) specific mouse (NOD) T cell 30 clones were isolated from infiltrated islets as described in Example 2. Peptide

analogues with single alanine substitutions were prepared as described in Example 1. The effect of each analogue on T cell proliferation was then evaluated using an assay performed in 96-well flat bottom microtiter plates (*see* Daniel et al., *Eur. J. Immunol. 25* :1056, 1995). Briefly 25,000 T cell clones along with 1 million irradiated NOD spleen cells were cultured in the presence of 50 μg/ml of insulin B chain 9-23 peptide or any of the alanine substituted peptides listed below in triplicate sets. The plates were incubated for a total of 72 hours in 7% carbon dioxide atmosphere with a pulse of 1μCi/well of tritiated thymidine for the last 6-8 hours of culture. Cells were harvested on a glass fiber filter and the associated radioactivity was counted in a liquid scintillation counter. Results are expressed as mean counts per minute of triplicate wells.

The data obtained from five separate T cell clones showed either a lack of proliferation or significantly reduced proliferation (relative to the 9-23 native peptide of insulin B chain; SEQ ID NO:2) in the presence of the following alanine substituted analogues: A12, A13, A15, A16, A17, and A18. These data are presented in Tables 1 and 2, below.

Table 1
Response (cpm) of Insulin Specific NOD T Cell Clones

Modified Position	Native Residue	Substitution	T cell clone	
			PD6-4.3	PD12-2.40
9	S	A	12861	42234
10	H	A	12507	1409
11	L	A	14148	2594
12	V	A	8292	671
13	E	A	142	519
14	A	none		
15	L	A	161	1422
16	Y	A	98	539
17	L	A	553	19321
18	V	A	234	44785
19	C	A	7678	34212
20	G	A	2440	38685
21	E	A	91	39087
22	R	A	6555	51722
23	G	A	14304	75441
no antigen			163	682
Native 9-23			10463	32221

Table 2
Response (cpm) of Insulin Specific Murine, NOD T Cell Clones

Modified Position	Native Residue	Substitution	T Cell Clone		
			PD12-4.4	PD12-4.29	PD12-4.34
9	S	A	1000	18422	259
10	H	A	823	15484	356
11	L	A	474	18416	190
12	V	A	1129	15041	194
13	E	A	373	891	179
14	A	none			
15	L	A	675	809	191
16	Y	A	779	636	202
17	L	A	332	1460	4360
18	V	A	225	1193	721
19	C	A	4295	6054	689
20	G	A	1323	13736	466
21	E	A	7900	4904	773
22	R	A	1313	12635	1555
23	G	A	3228	18422	791
no antigen			350	789	231
Native 9-23			10000	14820	3614

Table 3 shows the response of four different NOD derived T cell clones to the double alanine substituted peptide analog A16, A19 (NBI-6024; 16Y>A/19C>A). NOD T cell clones were incubated in the presence of 50 μM of either the native B chain (9-23) peptide or NBI-6024. The data in Table 3 represent the mean of triplicate sample ± standard error of the mean. Within Table 3, S.I. (Stimulation Index) = proliferation (cpm) in the presence of the peptide/proliferation (cpm) in medium alone. These data show a significant response when the cells were cultured with the native B chain (9-23)

peptide, but little or no proliferation over medium only (background) in the presence of NBI-6024.

Table 3

Proliferative Response of Insulin B Chain (9-23) Specific Murine T cell Clones to 50μM of B Chain (9-23) or the Analogue $A^{16,19}$ (NBI-6024)

T Cell Clone	Exp. No.	Medium Only	Insulin B Chain (9-23)		NBI-6024 ($A^{16,19}$)	
			Mean cpm±sem	S.I.	Mean cpm±sem	S.I.
PD12-2.35	1	688 ± 227	120,886 ± 7,171	175.7	841 ± 88	1.22
	2	493 ± 20	100,521 ± 1,581	203.89	452 ± 179	0.91
PD12-2.40	1	170 ± 8	16,730 ± 3,835	98.4	272 ± 34	1.16
	2	1,834 ± 638	176,359 ± 36,306	96.16	1,863 ± 451	1.01
PD12-4.1	1	215 ± 17	28,593 ± 4664	132.99	566 ± 30	2.63
PD12-4.9	1	9,111 ± 1,889	45,541 ± 5,222	4.99	12,313 ± 1,372	1.35
	2	7,202 ± 2,773	65,624 ± 4,979	9.1	6,171 ± 725	0.85

EXAMPLE 4

Antagonism of T cell Proliferation Assay

This Example illustrates the inhibition of the response of B chain (9-23) specific mouse T cell clones to the insulin B chain (9-23) peptide by representative peptide analogues.

Peptide analogues of B chain (9-23) containing alanine substitutions at residue 12, 13, 15 or 16 or the doubly substituted peptide at positions 16 and 19 ($A^{16,19}$; NBI-6024) were prepared as described in Example 1. T cell antagonism was detected by evaluating the ability of the peptide analogues to inhibit T cell proliferation induced by native B chain (9-23) (SEQ ID NO:2). In this assay, antigen presenting cells were first irradiated and then incubated with the competing peptide analogue and the native B chain (9-23) peptide. T cells were then added to the culture. Various concentrations of candidate peptide analogues were included in cultures which were incubated for a total

of 4 days. Following this incubation period, each culture was pulsed with 1 μCi of [^{3}H]-thymidine for an additional 12-18 hours. Cultures were then harvested on fiberglass filters and counted as above. Mean CPM and standard error of the mean were calculated from data determined in triplicate cultures. The results, shown in Figure 2, indicate that the peptide analogues containing alanine substitutions at residue 12, 13 or 16 are capable of attenuating the response of the pathogenic insulin B chain (9-23) T cells.

The ability of the doubly substituted peptide to inhibit insulin-dependent proliferation by T cells is shown in Table 4 and Figure 3. Within Table 4, the control peptide, NBI-5096, is an unrelated peptide from myelin basic protein. The percent inhibition was calculated as: (1-experimental cpm/insulin peptide cpm) x 100%.

Table 4
Inhibition of Insulin B Chain (9-23) Peptide Response in Two Murine NOD T Cell Clones by a Peptide Analogue

Clone	Conditions	CPM ± SEM	% Inhibition
PD12-2.35	Medium Only	213 ± 17	
	B(9-23) 5μM	7,840 ± 528	
	B(9-23) 5μM + 10 μM NBI-6024	4,441 ± 626	43.0
	B(9-23) 5μM + 50 μM NBI-6024	1,389 ± 218	82.0
	B(9-23) 5μM + 10 μM NBI-5096	10,089 ± 1,113	N/A
	B(9-23) 5μM + 50 μM NBI-5096	10,125 ± 887	N/A
PD12-2.40	Medium Only	305 ± 13	
	B(9-23) 5μM	9,149 ± 1,062	
	B(9-23) 5μM + 10 μM NBI-6024	6,379 ± 1,485	30.0
	B(9-23) 5μM + 50 μM NBI-6024	4,305 ± 941	52.9
	B(9-23) 5μM + 10 μM NBI-5096	12,336 ± 1,556	N/A
	B(9-23) 5μM + 50 μM NBI-5096	17,988 ± 584	N/A

N/A = Not applicable as no inhibition was observed.

The ability of NBI-6024 to block the B chain (9-23) peptide-induced stimulation of NOD derived T clones suggests that the alterations at positions 16 and 19 of the native insulin B chain (9-23) peptide did not alter the ability of the analogue to be recognized by the pathogenic T cells. Moreover, these results indicate that the analogue also binds to the MHC with sufficient affinity to allow for recognition by the insulin B chain (9-23)-specific T cell.

EXAMPLE 5

Effect of Peptide Analogues on Proliferation of T cell Lines and Clones from Diabetic Patients

This Example illustrates the lack of stimulation of T cell lines and clones derived from diabetic patients by representative peptide analogues.

Peptide analogues of B chain (9-23) containing alanine substitutions at residues 13, 15, 16 or 17 or the doubly substituted alanine analog $A^{16, 19}$ (NBI-6024) were prepared as described in Example 1. T cell lines from diabetic patients were prepared by isolating lymphocytes from the blood of the patient by subjecting the blood to a density gradient separation. Isolated lymphocytes were then cultured in the presence of the insulin B chain (9-23) peptide (10μM) and recombinant human IL-2 in the presence of 5-10% of autologous serum and irradiated autologous peripheral blood lymphocytes in culture medium. Four to five days later cells were harvested and the cycle repeated for 2 or 3 times.

Proliferation of the T cell line, in response to the native B chain (9-23) peptide (SEQ ID NO:2) or to the peptide analogs, was measured by culturing 25,000 to 100,000 T cells in the presence of 50,000-200,000 irradiated autologous PBLs and different concentrations of the insulin B chain (9-23) peptide or a peptide analogue in triplicate cultures. Following 4-5 days of culture, including the last 18 hours with radioactive thymidine, cells were harvested and the associated radioactivity was counted in a liquid scintillation counter. Results are expressed as mean counts per minute for each of the peptide analogues tested.

The results, shown in Figures 4-7, indicate that T cell lines and clones that proliferate in response to the native insulin B chain (9-23) peptide (SEQ ID NO:2) are not stimulated by the peptide analogues. The results from these patients and others are summarized in Table 5.

Table 5
Proliferative Response of Patient PBLs to Native Insulin Peptide or the Analogue NBI-6024

Patient Number	Patient ID	Stimulation Index*	
		Insulin B (9-23) [50 μM]	NBI-6024 [50 μM]
1	100	9.9	0.9
2	200	5.3	1.2
3	400	7.8	1.0
4	500	2.1	0.9
5	600	5.8	1.6
6	700	3.2	1.5
7	900	2.6	0.9
8	1100	3.7	0.8

*Stimulation Index = CPM with antigen/CPM with medium alone (no antigen)

The results clearly demonstrate that cells from diabetic patients that are responsive to the insulin B chain (9-23) peptide do not respond to the altered peptide ligand, NBI-6024 which has substitutions at position 16 and 19. We have also determined that the APL NBI-6024 binds with similar affinity to DQ8 antigens. Thus, the absence of stimulation of the diabetic patient's T cells by NBI-6024 is not due to any incompatibility of the peptide with the presenting MHC molecules, but is more likely due to altered recognition by the B chain (9-23)-specific T cells.

EXAMPLE 6

Reduction of Incidence of Diabetes in NOD Mice

This Example illustrates the ability of representative peptide analogs to prevent diabetes in NOD mice.

The NOD mouse spontaneously develops diabetes beginning around 3 months of age (Makino *et al.*, *in* Current Topics in Clinical and Experimental Aspects

of Diabetes Mellitus, Sakamoto et al., eds., p. 25-32 (Elsevier, Amsterdam, 1985)). The disease is preceded by cellular infiltration into the pancreas of T cells beginning even by one month of age. Soluble peptide analogues of B chain (9-23) containing alanine substitutions at residues 12, 13 or 16 were administered subcutaneously to NOD mice at weekly intervals. 400 μg of each peptide were administered at each treatment to ten animals. Following 9 treatments, the percent of mice in each treatment group that had become diabetic was evaluated by measuring blood glucose levels using a glucometer at weekly intervals. A reading of more than 200mg/dl of blood glucose on two consecutive observations was considered indicative of frank diabetes.

As shown in Figure 8, treatment with each of the alanine-substituted analogues resulted in a marked reduction in the incidence of diabetes. The data for the A13 substituted analogue is also shown in Figure 9.

In another experiment, B chain (9-23), the A13 substituted analogue or neurotensin (as a control) was administered subcutaneously to NOD mice at weekly intervals. 400 μg of each peptide were administered at each treatment to ten animals. Following 13 treatments, the percent of mice in each treatment group that had become diabetic was evaluated as described above. As shown in Figure 10, the B chain (9-23) peptide reduced the incidence of diabetes. This reduction was more pronounced for the A13 substituted analogue.

To determine the ability of the double substituted peptide $A^{16,19}$ (NBI-6024) to control the development of diabetes in the NOD mice, the peptide was administered to animals at an early age. Thus, female mice (n=9, approximately 4 weeks old) were treated subcutaneously with 20mg/kg (400 μg/mouse) of NBI-6024 for twelve weeks and then every other week until Week 35. Beginning at 9-10 weeks of age, mice were then monitored weekly for hyperglycemia, measuring the blood glucose levels. As a control, a group of 10 female mice was left untreated. The results from this experiment are shown in Figure 11. As can be seen, treatment with NBI-6024 significantly reduced the incidence of diabetes by about 60-70%, compared to the untreated group, ($p < 0.004$).

The observations were then confirmed and extended in a second experiment. Here, animals (n=13-15) were treated with either NBI-6024 or an unrelated peptide, neurotensin, NBI-6201 as described above. An additional group (n=8) was left untreated. As shown in Figure 12, treatment with 20mg/kg of the altered peptide NBI-6024 resulted in a reduced incidence of diabetes compared to either the neurotensin treated or untreated group.

These results demonstrate that the altered peptide ligand NBI-6024, designed around insulin B chain (9-23) peptide was capable of conferring protection to animals at risk to develop diabetes spontaneously. It is likely that T cells that recognize other pancreas antigens are present in these animals, yet they too appear to be regulated by the insulin APL. The timing of administration was approximately at the same time that autoreactive lymphocytes begin to infiltrate the pancreas and initiate the destructive process. These results offer hope that early intervention with this APL may prove useful in delaying or preventing the onset of Type I diabetes in people.

EXAMPLE 7

Immunogenicity of Representative Peptide Analogues

This Example illustrates the immunogenicity of representative peptide analogues in NOD mice.

Groups of 3-4 NOD mice were immunized with 100-400μg of peptide analogues subcutaneously in mannitol acetate buffer three times within a period of 10-15 days. Following the last immunization, lymph node cells and/or spleen cells were used in a proliferation assay in which different concentrations of the immunizing peptide were cultured with the cells for 3-4 days. The last 18 hours of culture included tritiated thymidine. Cells were harvested and counted in a scintillation counter and the response is expressed as CPM ± SEM. These results, shown in Figure 13-16, indicate that these representative peptide analogues have the ability to bind to the mouse MHC molecules and be recognized by the corresponding T cells.

The ability of the double substituted peptide NBI-6024 ($A^{16,19}$) to induce a cellular immune response in NOD mouse strain was next determined. Two female NOD mice were immunized with 10mg/kg NBI-6024 either as an aqueous suspension or, as a control, emulsified in complete Freund's adjuvant (CFA). On Day 8, three days following the last injection, the mice were sacrificed, the spleen and inguinal lymph node cells were removed and pooled, and a single-cell suspension was prepared. Cells were cultured in the presence of varying concentrations (0-25 μM) of NBI-6024. The ability of these lymphoid cells to proliferate in response to NBI-6024 was measured *in vitro* by [^{3}H]-thymidine incorporation.

The results are presented in Table 6, in which the response is expressed as mean CPM ± SEM of triplicate cultures. Lymph node cells isolated from mice immunized with the analogue in CFA showed a strong proliferative response to challenge with the immunizing analogue in a dose-dependent manner (Table 6). These results indicate that alterations made in the native insulin B chain (9-23) sequence at positions 16 and 19 have not affected the ability of the peptide to bind the NOD disease-associated MHC haplotype molecule and, more importantly, did not hinder recognition by T cells.

Table 6

Proliferative Response of Lymph Node Cells to NBI-6024 from NOD Mice Immunized with NBI-6024 in CFA

NBI-6024 (μM)	Proliferative Response (CPM ± SEM)
0	2,445 ± 137
1	140,061 ± 7,289
5	187,711 ± 2,548
25	218,149 ± 4,462

In addition, both the spleen and inguinal lymph nodes cells isolated from soluble administrated peptide exhibited a strong proliferative response to the APL when

challenged *in vitro* with NBI-6024 (Table 7 and Figures 17A and 17B). Even more impressive was the finding that NBI-6024-derived lymphocytes from mice immunized with the soluble peptide also responded to Insulin B chain (9-23). This cross-reactive feature was not seen with CFA-emulsified peptide. This ability of the soluble peptide to induce a cross-reactive response may be desirable in controlling diabetes, as it may help to mobilize the protective NBI-6024-specific T cells to the pathogenic target tissue.

Table 7
Proliferative Response to NBI-6024 or Native Insulin B-chain (9-23) of T cells from NOD Mice Immunized with Soluble NBI-6024

NBI-6024 Induced T Cell Line	Peptide Conc. [μM]	CPM ± SEM NBI-6024	CPM ± SEM Insulin B(9-23)
Mouse # 1	0	19,130 ± 2191	19,130 ± 2191
	1	48,319 ± 1918	16,870 ± 4469
	5	160,673 ± 2269	21,292 ± 3216
	25	268,005 ± 11198	33,317 ± 3619
Mouse #2	0	21,588 ± 2326	21,588 ± 2326
	1	54,519 ± 5666	17,262 ± 602
	5	126,123 ± 13851	19,648 ± 2169
	25	202,707 ± 8125	30,612 ± 3557
Mouse #3	0	24,006 ± 2803	24,006 ± 2803
	1	64,239 ± 9493	25,825 ± 3841
	5	140,836 ± 11778	58,567 ± 2737
	25	240,278 ± 15015	113,366 ± 515

To determine the type of T cells produced following soluble administration of NBI-6024, the culture supernatants from immune lymphoid cells were removed 48 hours following the initiation of culture and the levels of various cytokines measured using standard ELISA technology. Strikingly, the cytokine production profile of T cells from mice immunized with soluble NBI-6024 produced the Th2 cytokines, interleukin-4 (Figure 18) and interleukin-5 (Table 8), and not the Th1-derived

interleukin-2. Within Table 8, values are expressed in pg/ml as mean of triplicates ± SEM. As a control, NBI-6024 emulsified with CFA did induce the expected Th1-cytokine profile (IL-2) from immune T cells upon *in vitro* stimulation.

Table 8
Cytokine Response of Soluble NBI-6024 Induced T cells Cultured with NBI-6024

NBI-6024 [mM]	Cytokine (pg/ml)		
	IL-2	IL-4	IL-5
0	< 15 pg/ml	134 ± 0	1,814 ± 332
1	< 15 pg/ml	684 ± 92	9,999 ± 503
5	< 15 pg/ml	1,653 ± 51	23,496 ± 684
25	< 15 pg/ml	2,102 ± 85	28,062 ± 141

The ability of the soluble subcutaneous administration of NBI-6024 to induce Th2-like cells is a desirable feature, as such cells are associated with recovery from diabetes and other organ-specific autoimmune diseases (Sarvetnick, *J. Exp. Med. 184*:1597-1600, 1996; Shaw *et al.*, 1997; Balasa *et al.*, *J. Exp. Med. 186*:385-391, 1997). These Th2-derived cytokines have strong anti-inflammatory activities that suppress development of pro-inflammatory cytokine-secreting auto-reactive Th1 cells that mediate disease.

From the foregoing, it will be evident that although specific embodiments of the invention have been described herein for the purpose of illustrating the invention, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the present invention is not limited except as by the appended claims.

CLAIMS

What is claimed is:

1. A peptide analogue comprising residues 9 to 23 of human insulin B chain, wherein the peptide analogue differs in sequence from native human insulin B chain residues 9 to 23 due to substitutions at between 1 and 4 amino acid positions, and wherein at least one substitution occurs at a residue selected from the group consisting of residues 12, 13, 15 and 16.

2. The peptide analogue of claim 1, wherein the peptide analogue has a sequence that differs from native human insulin B chain at two amino acid residues.

3. The peptide analogue of claim 1, wherein the peptide analogue has a sequence that differs from native human insulin B chain at three amino acid residues.

4. The peptide analogue of claim 1, wherein an amino acid substitution occurs at residue 19.

5. The peptide analogue of claim 1, wherein at least one amino acid substitution is non-conservative.

6. The peptide analogue of claim 1, wherein residue 12 is substituted.

7. The peptide analogue of claim 6, wherein residue 12 is an alanine residue.

8. The peptide analogue of claim 1, wherein residue 13 is substituted.

9. The peptide analogue of claim 8, wherein residue 13 is an alanine residue.

10. The peptide analogue of claim 1, wherein residue 15 is substituted.

11. The peptide analogue of claim 10, wherein residue 15 is an alanine residue.

12. The peptide analogue of claim 1, wherein residue 16 is substituted.

13. The peptide analogue of claim 12, wherein residue 16 is an alanine residue.

14. The peptide analogue of any one of claims 6-13, wherein residue 19 is substituted.

15. The peptide analogue of claim 14, wherein residue 19 is an alanine residue.

16. The peptide analogue of claim 1, further comprising residue 24 of human insulin B chain.

17. The peptide analogue of claim 1, wherein the peptide analogue comprises no more than 18 residues of human insulin B chain.

18. The peptide analogue of claim 1, wherein the peptide analogue comprises no more than 16 residues of human insulin B chain.

19. The peptide analogue of claim 1, wherein the peptide analogue comprises no more than 15 residues of human insulin B chain.

20. A peptide analogue consisting essentially of residues 9 to 23 of human insulin B chain, wherein the peptide analogue differs in sequence from native human insulin B chain residues 9 to 23 due to substitutions at between 1 and 4 amino acid positions, and wherein at least one substitution occurs at a residue selected from the group consisting of residues 12, 13, 15 and 16.

21. A peptide analogue consisting essentially of residues 9 to 24 of human insulin B chain, wherein the peptide analogue differs in sequence from native human insulin B chain residues 9 to 23 due to substitutions at between 1 and 4 amino acid positions, and wherein at least one substitution occurs at a residue selected from the group consisting of residues 12, 13, 15 and 16.

22. A pharmaceutical composition comprising a peptide analogue according to any one of claims 1-19 in combination with a physiologically acceptable carrier or diluent.

23. A method for inhibiting the development of diabetes, comprising administering to a patient a therapeutically effective amount of a pharmaceutical composition according to claim 22.

24. A method for treating diabetes, comprising administering to a patient a therapeutically effective amount of a pharmaceutical composition according to claim 22.

25. A peptide analogue comprising residues 9 to 23 of human insulin B chain, wherein the peptide analogue differs in sequence from native human insulin B chain residues 9 to 23 due to substitutions at residues 16 and 19.

26. A pharmaceutical composition comprising a peptide analogue according to claim 25 in combination with a physiologically acceptable carrier or diluent.

27. A method for inhibiting the development of diabetes, comprising administering to a patient a therapeutically effective amount of a pharmaceutical composition according to claim 26.

INSULIN B 9-23

TCR

9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
S	H	L	V	E	A	L	Y	L	V	C	G	E	R	G

I-A^{97}

Fig. 1

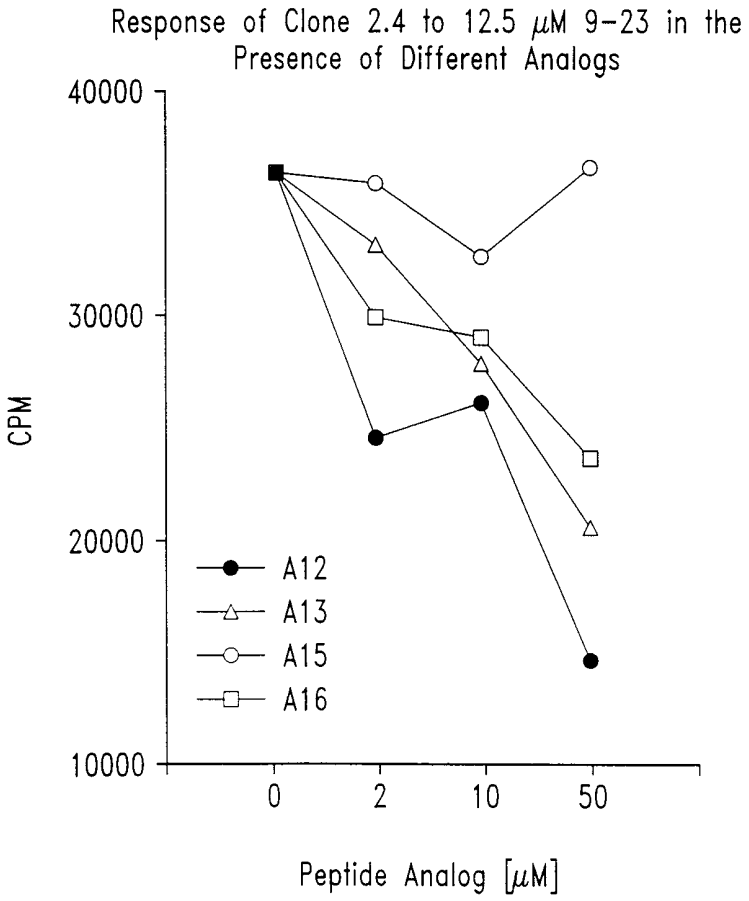


Fig. 2

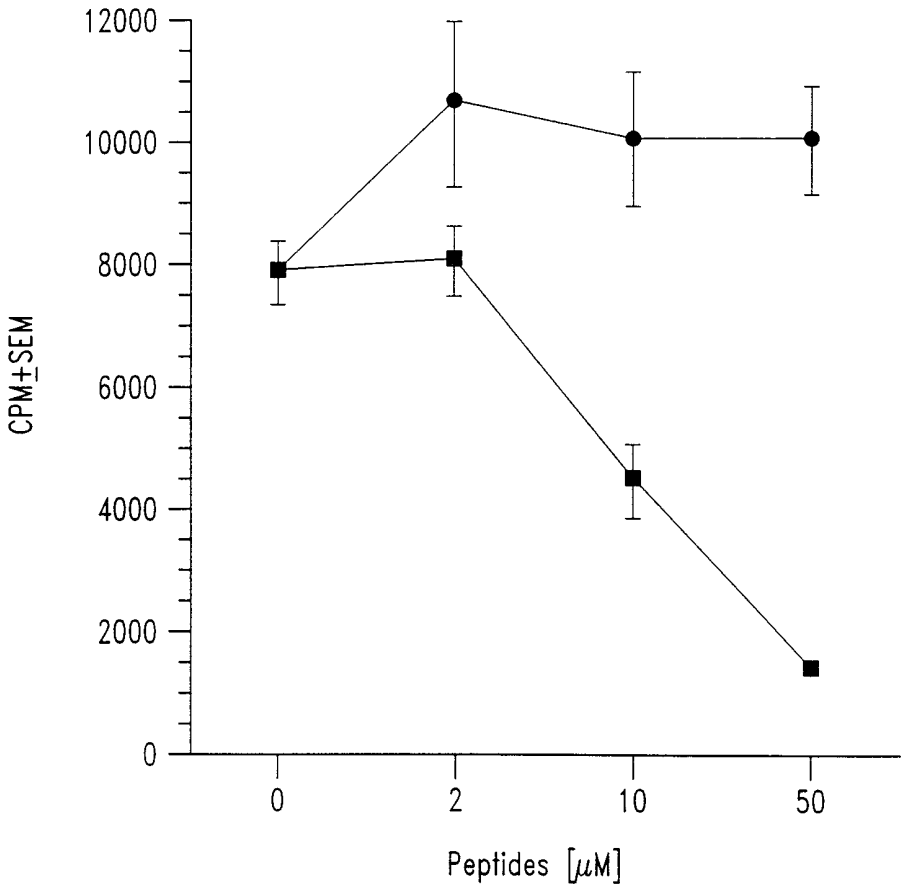
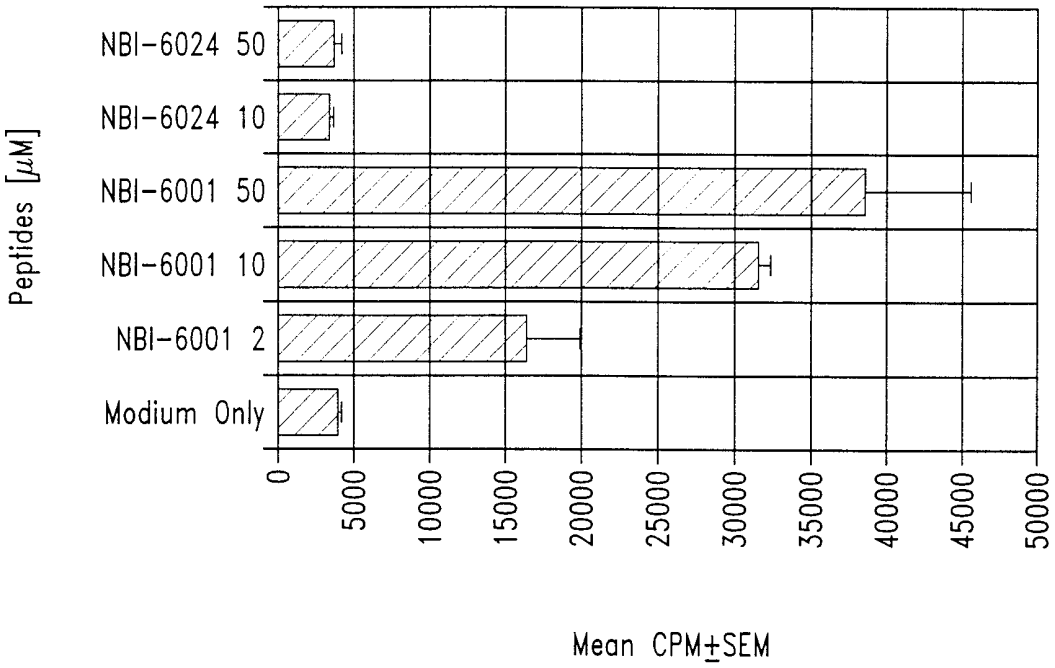


Fig. 3


Peptides [μM]
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NBI-6024 10
NBI-6001 50
NBI-6001 10
NBI-6001 2
Modium Only
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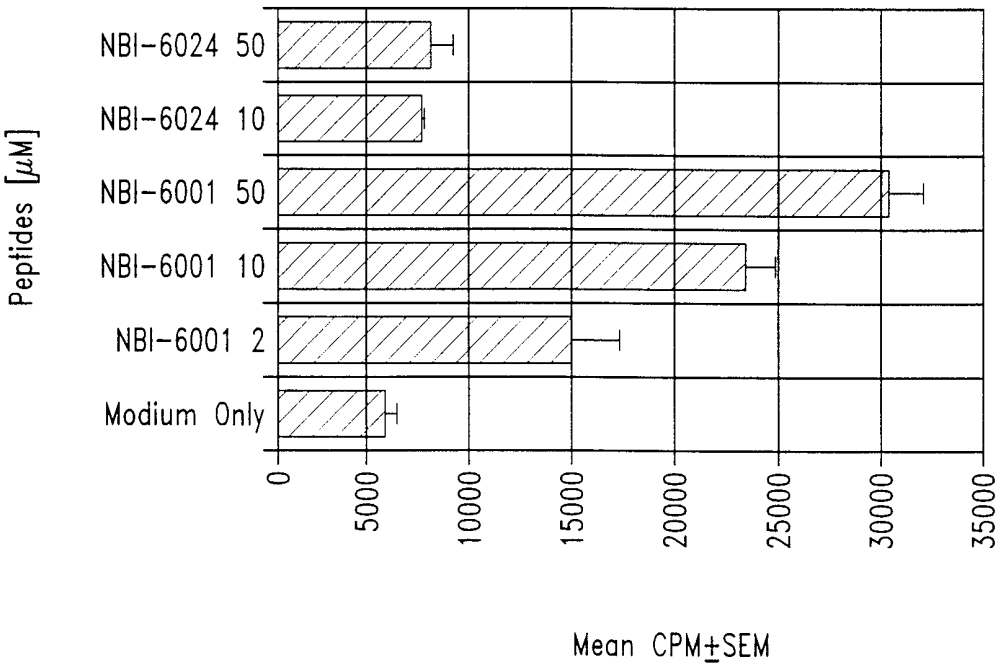
5000
10000
15000
20000
25000
30000
35000
40000
45000
50000
Mean CPM±SEM

Fig. 4


Peptides [μM]
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NBI-6024 10
NBI-6001 50
NBI-6001 10
NBI-6001 2
Modium Only
0
5000
10000
15000
20000
25000
30000
35000
Mean CPM±SEM

Fig. 5

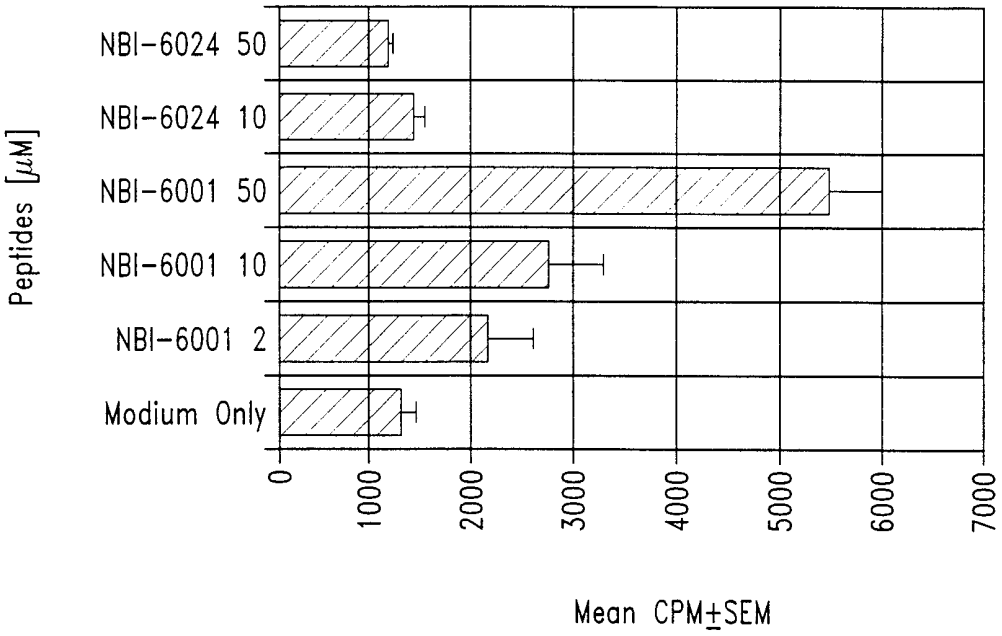
NBI-6024 50
NBI-6024 10
NBI-6001 50
NBI-6001 10
NBI-6001 2
Modium Only
Peptides [μM]
0
1000
2000
3000
4000
5000
6000
7000
Mean CPM±SEM

Fig. 6

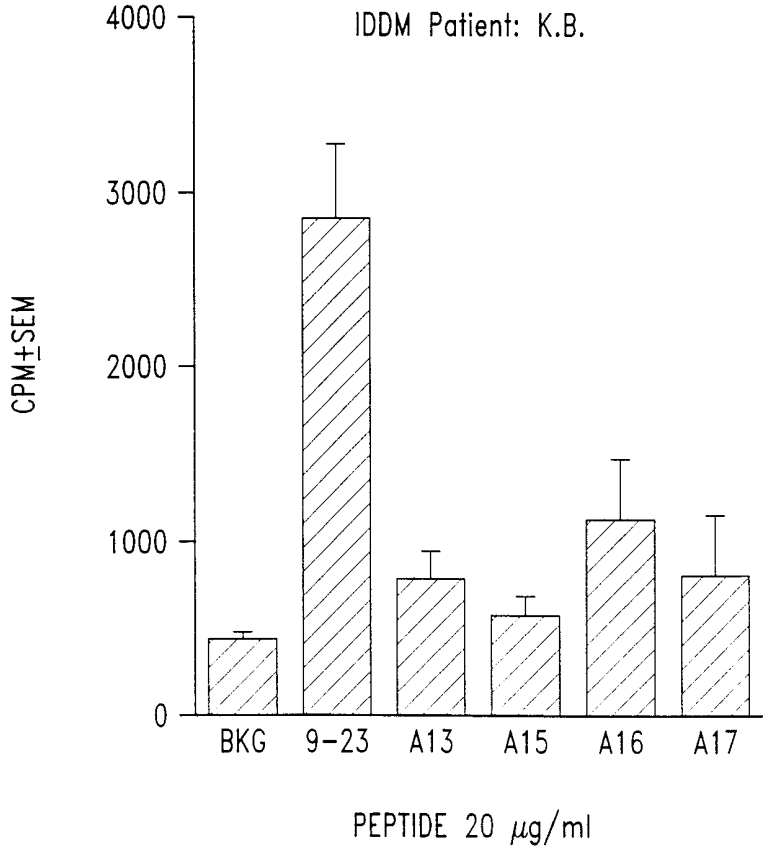
IDDM Patient: K.B.
4000
3000
2000
1000
0
CPM±SEM
BKG
9-23
A13
A15
A16
A17
PEPTIDE 20 μg/ml

Fig. 7

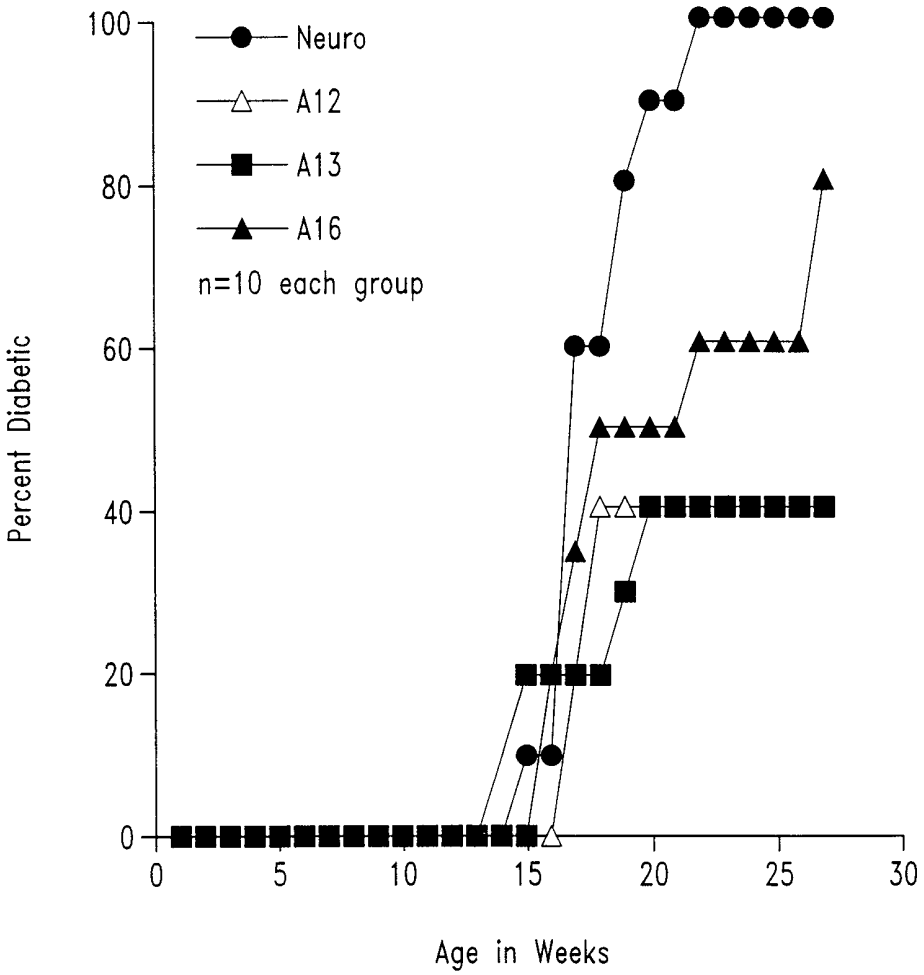
Neuro
A12
A13
A16
n=10 each group
Percent Diabetic
100
80
60
40
20
0
0
5
10
15
20
25
30
Age in Weeks

Fig. 8

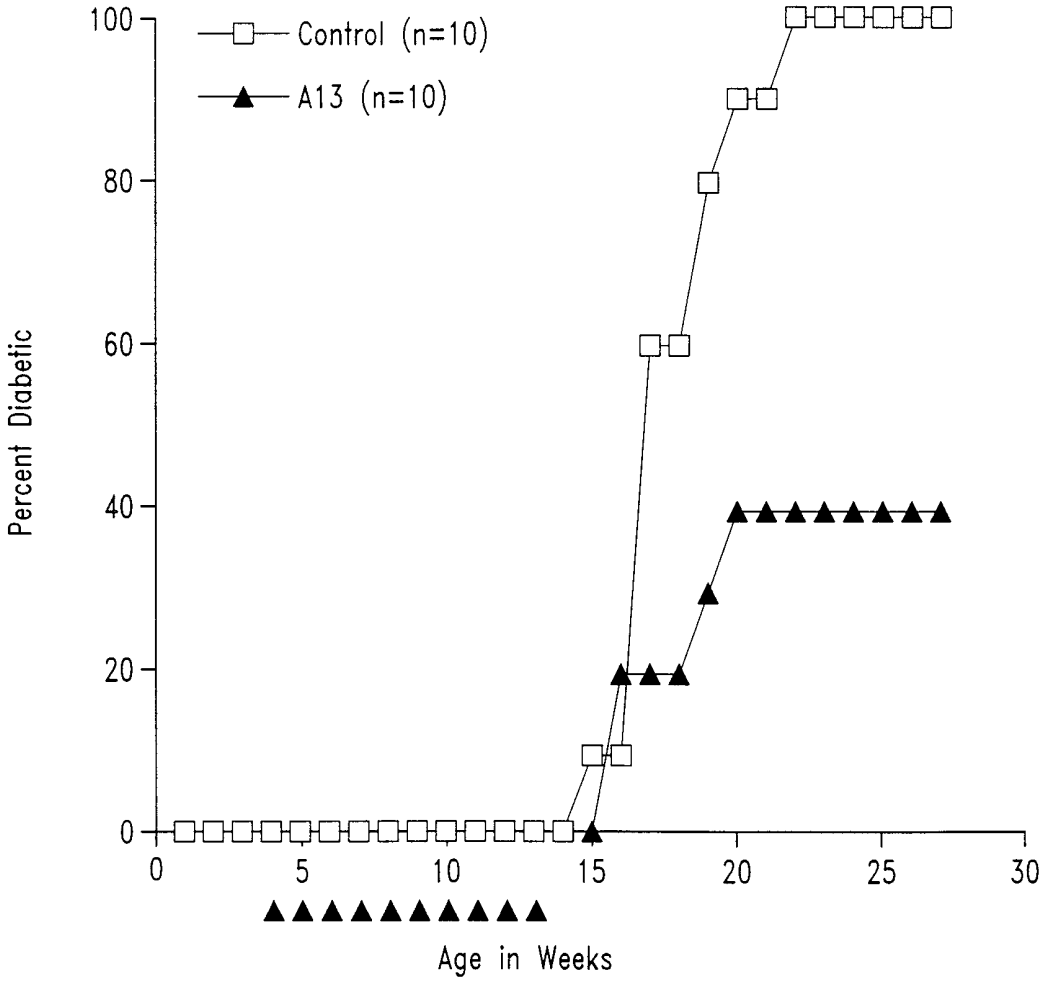
Control (n=10)
A13 (n=10)
Percent Diabetic
100
80
60
40
20
0
0
5
10
15
20
25
30
Age in Weeks

Fig. 9

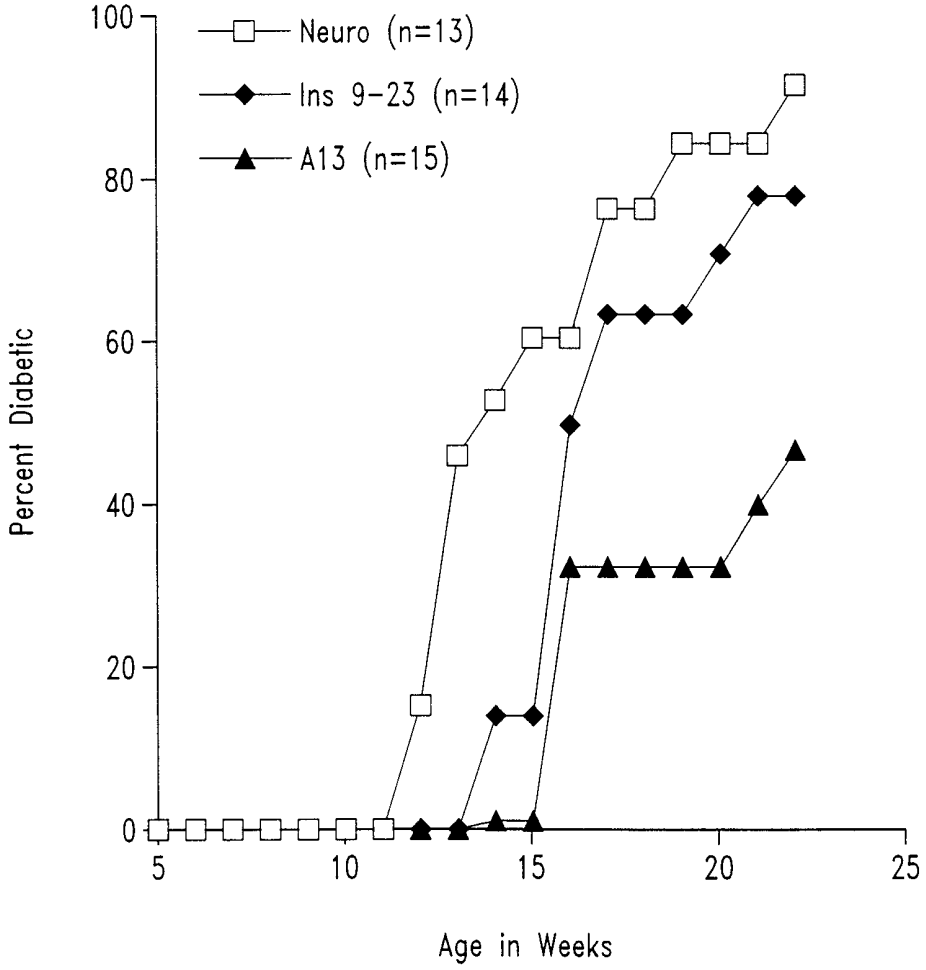
Neuro (n=13)
Ins 9-23 (n=14)
A13 (n=15)
Percent Diabetic
100
80
60
40
20
0
5
10
15
20
25
Age in Weeks

Fig. 10

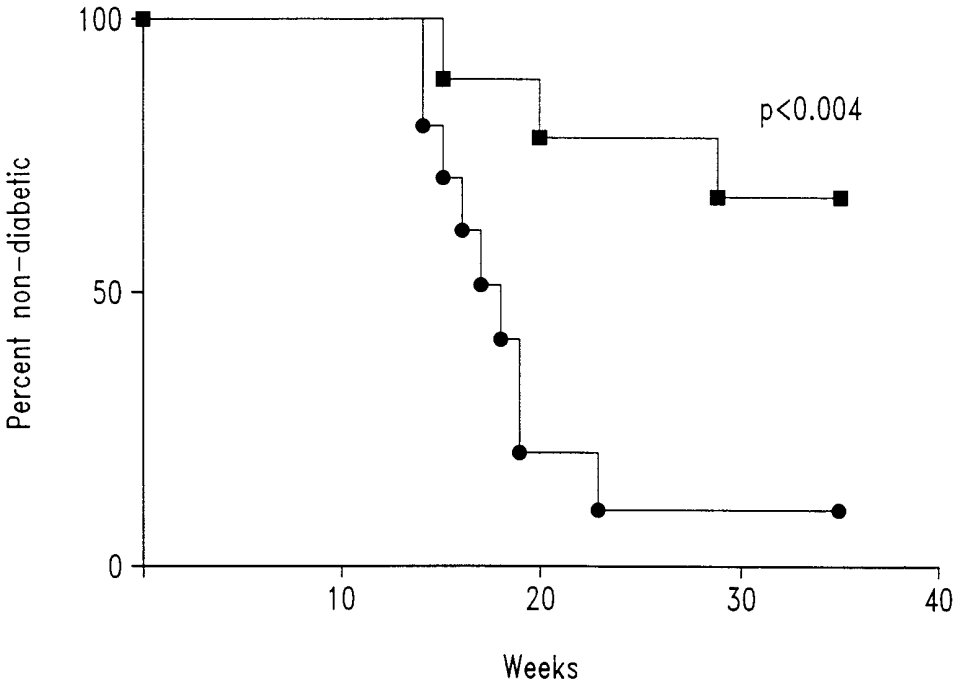
100
50
0
Percent non-diabetic
p<0.004
10
20
30
40
Weeks

Fig. 11

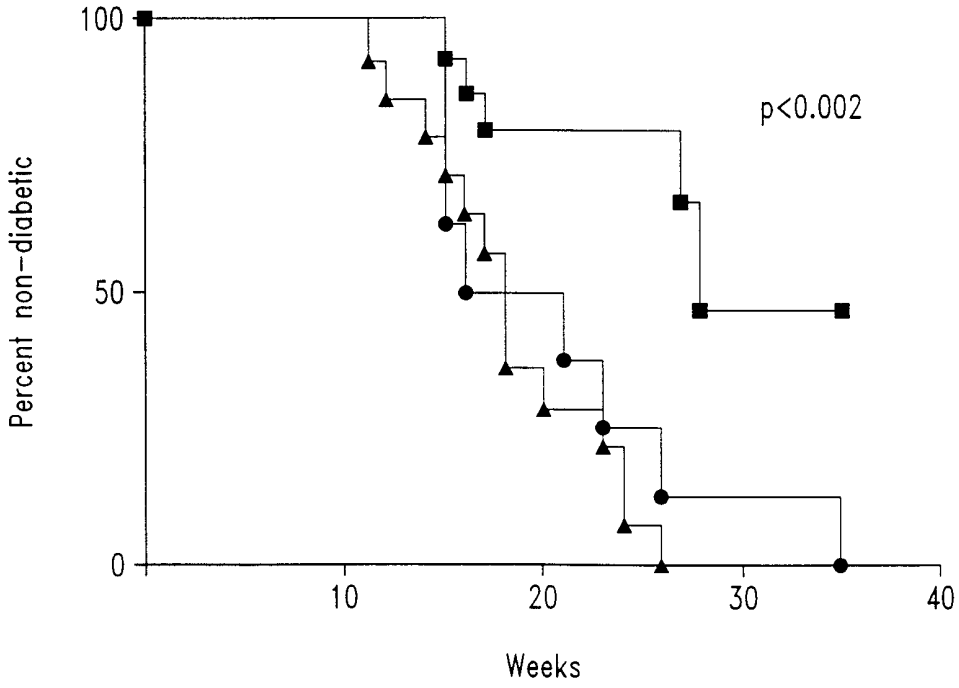
100
50
0
Percent non-diabetic
p<0.002
10
20
30
40
Weeks

Fig. 12

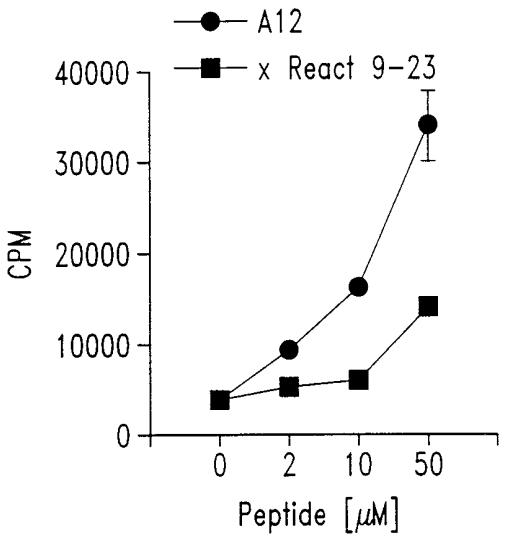

Fig. 13A

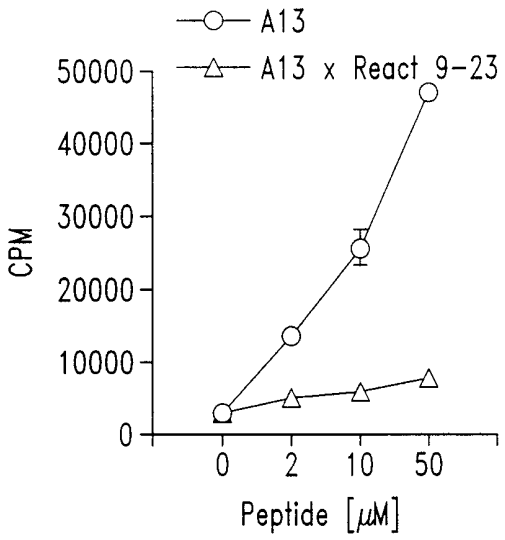

Fig. 13B

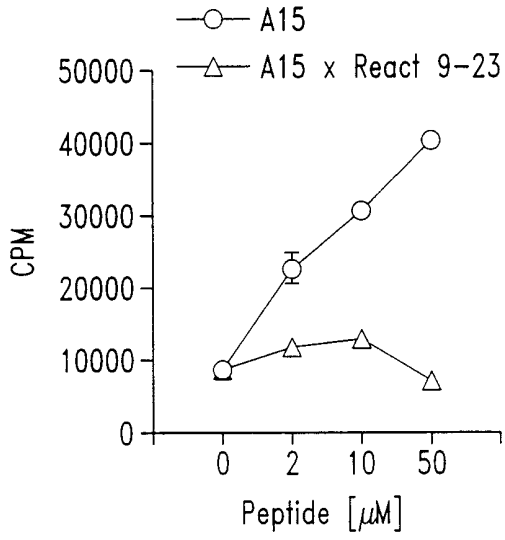

Fig. 13C

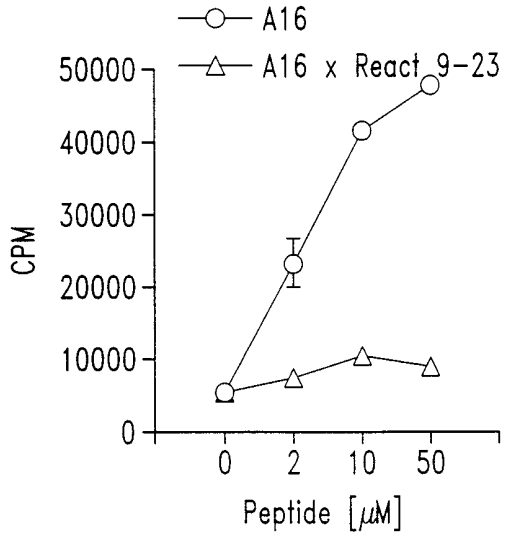

Fig. 13D

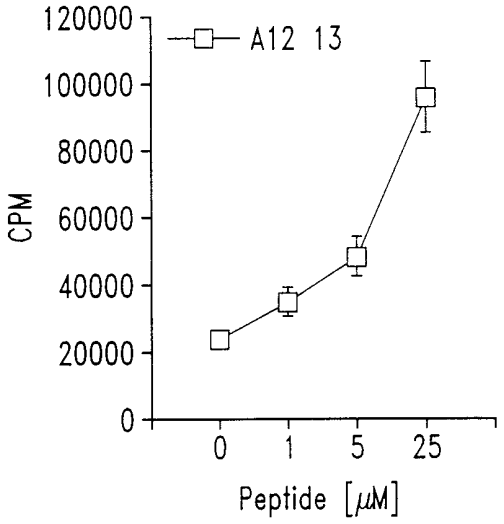

Fig. 14A

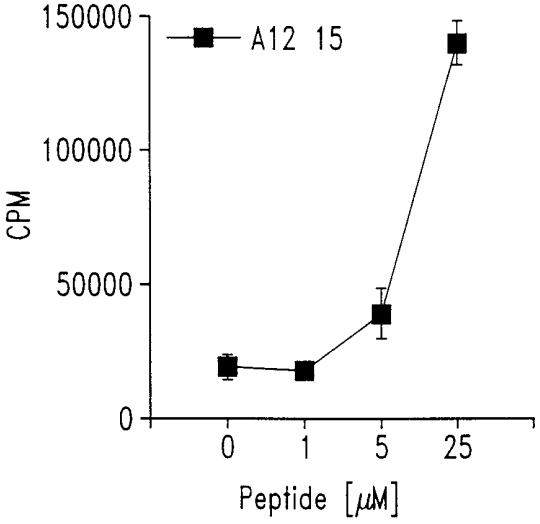

Fig. 14B

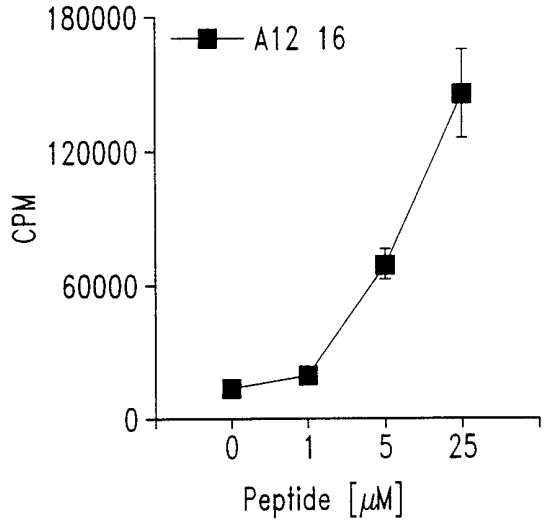

Fig. 14C

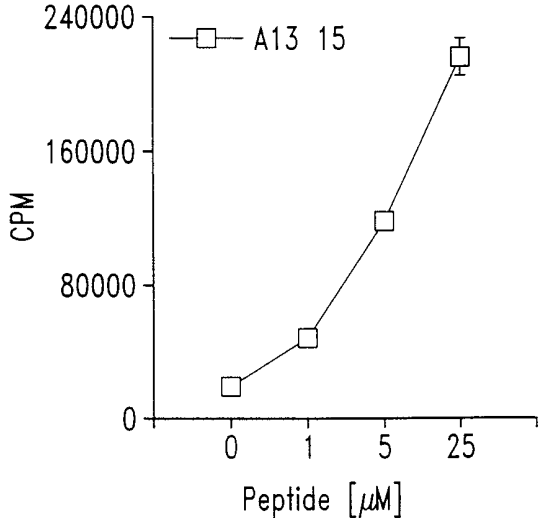

Fig. 14D

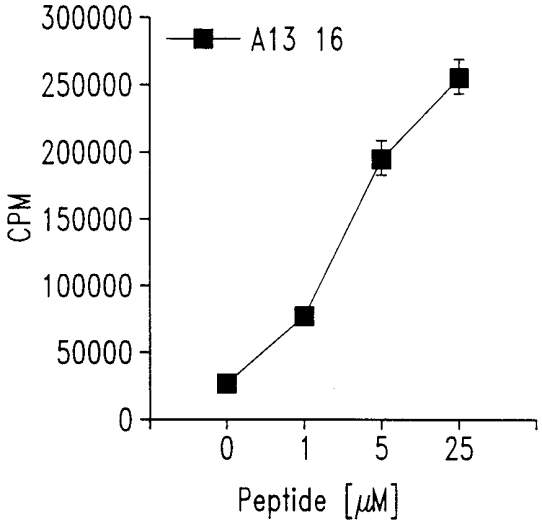

Fig. 14E

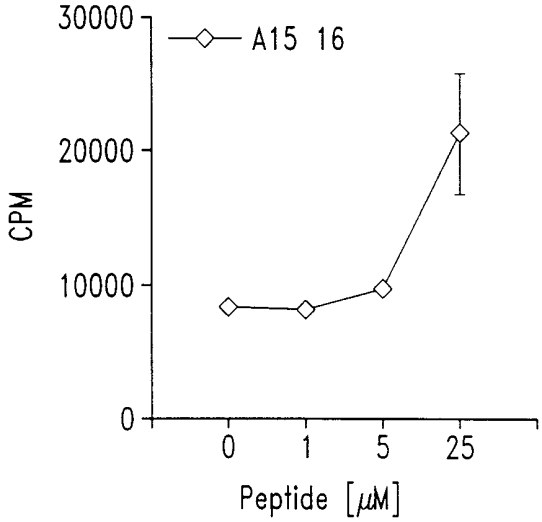

Fig. 14F

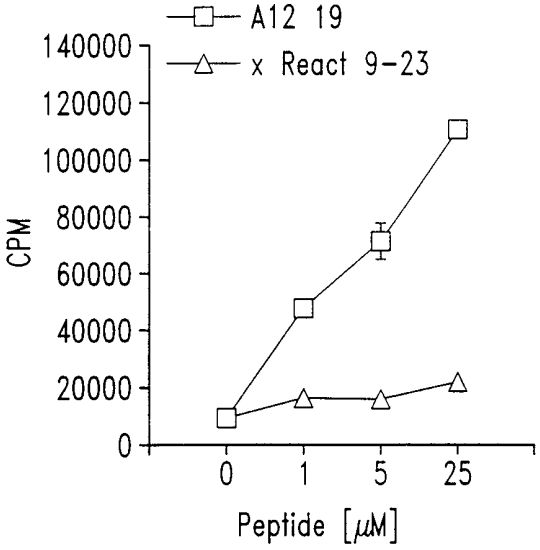

Fig. 15A

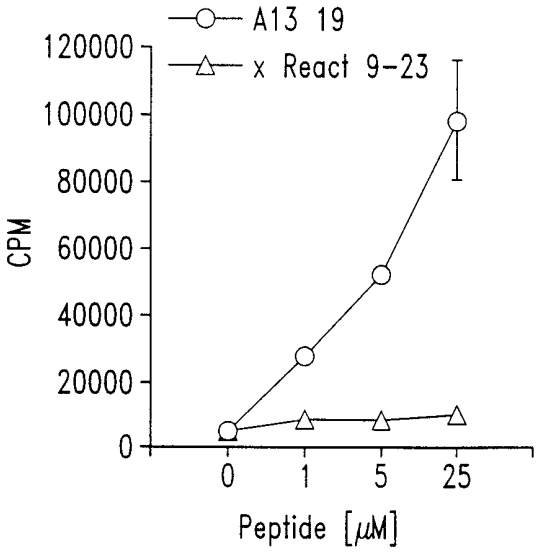

Fig. 15B

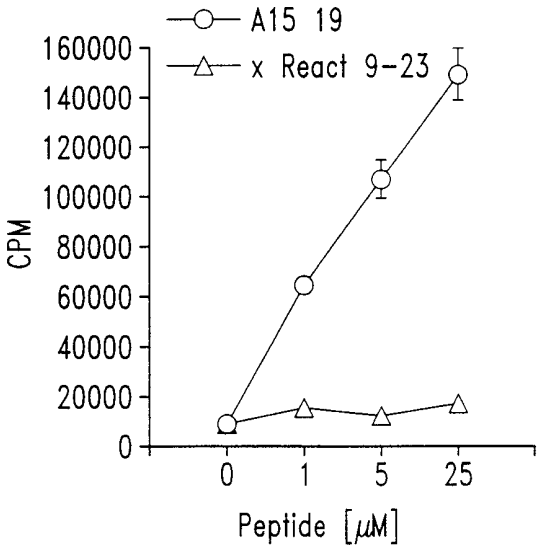

Fig. 15C

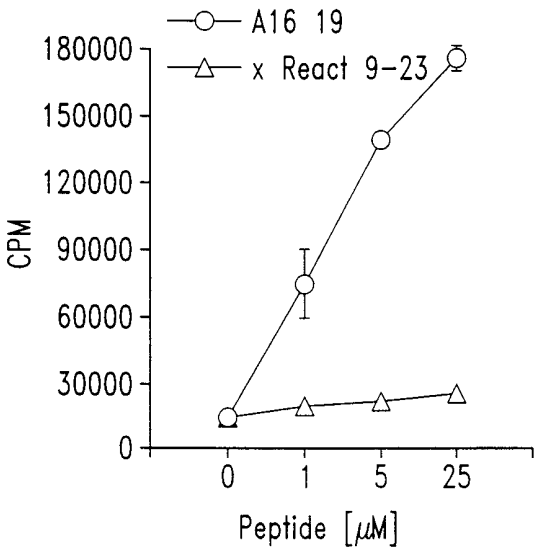

Fig. 15D

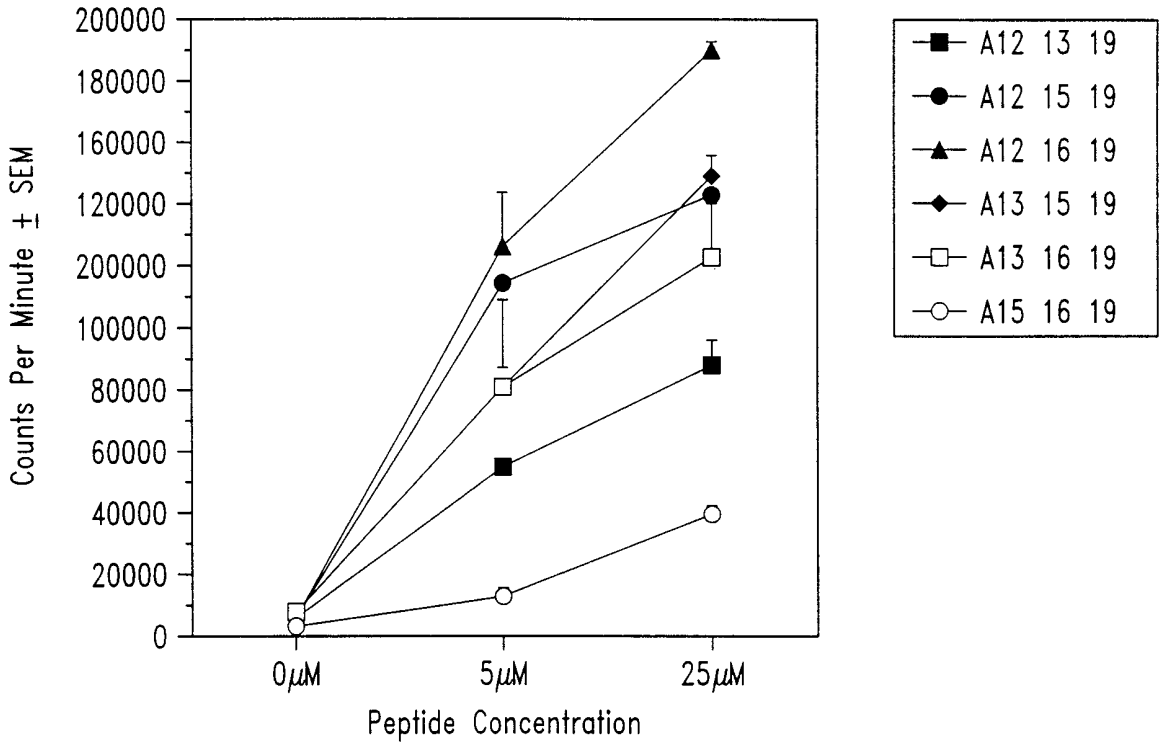
200000
180000
160000
120000
200000
100000
80000
60000
40000
20000
0
Counts Per Minute ± SEM
0μM
5μM
25μM
Peptide Concentration
A12 13 19
A12 15 19
A12 16 19
A13 15 19
A13 16 19
A15 16 19

Fig. 16

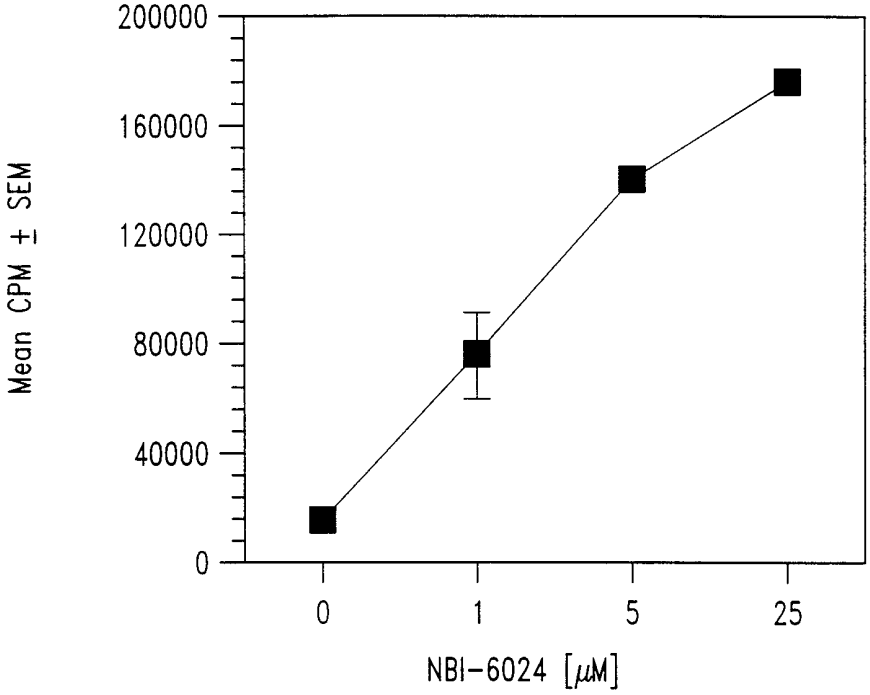

Fig. 17A

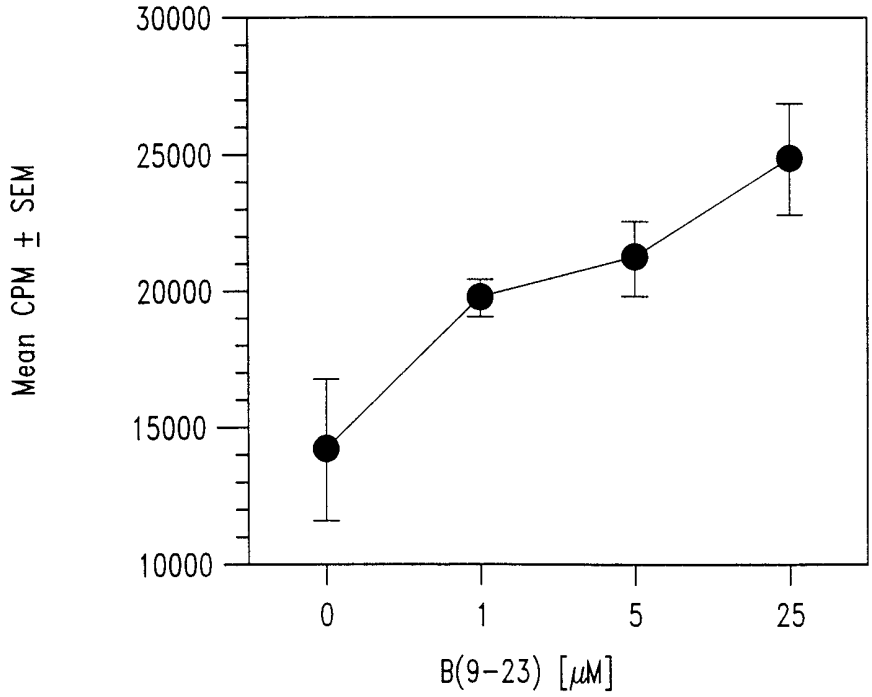

Fig. 17B

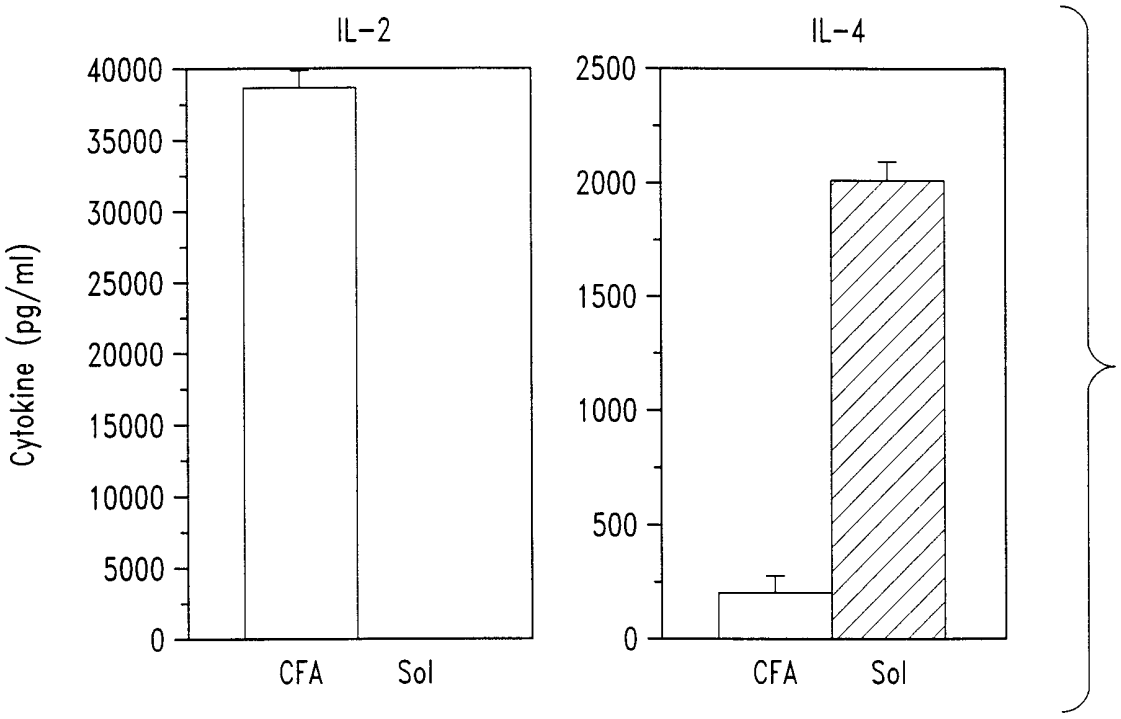
IL-2
IL-4
Cytokine (pg/ml)
40000
35000
30000
25000
20000
15000
10000
5000
0
2500
2000
1500
1000
500
0
CFA
Sol
CFA
Sol

Fig. 18

SEQUENCE LISTING

<110> Neurocrine Biosciences, Inc.

<120> METHODS FOR TREATMENT OF DIABETES USING PEPTIDE
ANALOGUES OF INSULIN

<130> 690068.448PC

<140> PCT
<141> 1999-02-23

<160> 2

<170> PatentIn Ver. 2.0

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<213> Homo sapiens

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<223> Residues 9-23 of human insulin B chain

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1 5 10 15

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 99/03915

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/62 A61K38/28

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

B. FIELDS SEARCHED

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

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	C. KRISTENSEN ET AL.: "Alanine Scanning Mutagenesis of Insulin" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 20, 16 May 1997, pages 12978-12983, XP002106057 BALTIMORE, MD US see page 12981, left-hand column, last paragraph - page 12983, left-hand column, paragraph 2; table I ---	1,5-9, 12,13, 16,22,24
X	WO 97 02043 A (NOVONORDISK AS ;DYRBERG THOMAS (DK); WORSAAE ANNE (DK)) 23 January 1997 see page 14, line 9 - line 12; claims ---	1,5,6, 22-24

-/--

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

[X] Patent family members are listed in annex.

° Special categories of cited documents :

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"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.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
15 June 1999	08/07/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl. Fax: (+31-70) 340-3016	Fuhr, C

1

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INTERNATIONAL SEARCH REPORT

In ational Application No
PCT/US 99/03915

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 04307 A (LILLY CO ELI ;BREMS DAVID NETTLESHIP (US); BAKAYSA DIANE LEE (US)) 15 February 1996 see claims; example 2 ---	1,5,8, 22-24
A	D. DANIEL AND D.R. WEGMANN: "Protection of nonobese diabetic mice from diabetes by intranasal or subcutaneous administration of insulin peptide B-(9-23)" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 93, January 1996, pages 956-960, XP002106058 WASHINGTON US see page 958, right-hand column, paragraph 2 - page 960, left-hand column, last paragraph ---	1,17-20, 22-24
A	M. POLANSKI ET AL.: "Oral Administration of the Immunodominant B-chain of Insulin Reduces Diabetes in a Co-transfer Model of Diabetes in the NOD Mouse and is Asociated with a Switch from Th1 to Th2 Cytokines" JOURNAL OF AUTOIMMUNITY, vol. 10, no. 4, August 1997, pages 339-346, XP002106059 see page 342, left-hand column, paragraph 2 - right-hand column, paragraph 1 see page 343, right-hand column, paragraph 1 - page 344, right-hand column, last paragraph -----	1,17-20, 22-24

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int .tional Application No
PCT/US 99/03915

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9702043 A	23-01-1997	AU 6299596 A	05-02-1997
		EP 0835129 A	15-04-1998
WO 9604307 A	15-02-1996	US 5559094 A	24-09-1996
		AU 3236895 A	04-03-1996
		CA 2196569 A	15-02-1996
		EP 0781295 A	02-07-1997
		JP 10503523 T	31-03-1998