Compounds in which autoantigen, analogs of said autoantigen, peptide fragments of said autoantigen, and analogs of said peptide are chemically conjugated to fatty acids in various forms. Such derivatives effectively modulate the immune responses in an autoantigen–specific way and are therefore useful for autoimmune diseases, such as juvenile diabetes, multiple sclerosis, rheumatoid arthritis, and many others.
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CHEMICAL DERIVATIVES OF AUTOANTIGENS AND AUTOIMMUNE-SUPPRESSIVE PEPTIDES AND PHARMACEUTICAL COMPOSITION CONTAINING THE SAME

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is based on and claims priority to Provisional Application No. 60/072,702, filed January 27, 1998, Provisional Application No. 60/090,677, filed June 25, 1998, and Provisional Application No. 60/104,663, filed October 16, 1998.

FIELD OF THE INVENTION

The invention relates to the fatty acid acylated conjugates of an autoantigen, fatty acid acylated conjugates of immunologically reactive peptide fragments of said autoantigen, fatty acid acylated conjugates of analogs of said autoantigen, and fatty acid acylated conjugates of analogs of said peptide fragments, and to their medical use.

BACKGROUND

Autoimmune diseases are characterized by an abnormal immune response involving either cells or antibodies, that are in either case directed against normal autologous tissues. Autoimmune diseases in mammals can generally be classified in one of two different categories: cell-mediated disease (i.e. T
cells) or antibody-mediated disorders. Antibody-mediated production is usually T-cell dependent. Non-limiting examples of cell-mediated autoimmune diseases include multiple sclerosis, rheumatoid arthritis, autoimmune thyroiditis, insulin-dependent diabetes mellitus (IDDM), autoimmune uveoretinitis. Non-limiting examples of antibody-mediated autoimmune disorders include myasthenia gravis and systemic lupus erythematosus (or SLE). An autoimmune reaction is one mounted by the body against its own antigens (known as "autoantigens"). It is widely believed that a specific class of active T cells lymphocytes is produced which recognizes autoantigens.

The current treatments for both categories of autoimmune diseases involve administration of drugs which non-specifically suppress the immune response. Examples of such drugs are methotrexate, cyclophosphamide, Imuran (azathioprine) and cyclosporin A. Steroid compounds such as prednisone and methylprednisilone are also employed in many instances. These drugs have limited efficacy against both cell- and antibody-mediated autoimmune diseases. Use of such drugs is limited by virtue of their toxic side effects and also because they induce "global" immuno-suppression in a patient receiving prolonged treatment with the drug, e.g. the normal protective immune response to pathogenic microorganisms is down-regulated thereby increasing the risk of infections caused by these pathogens. A further drawback is that there is an increased risk that malignancies will develop in patients receiving prolonged global immuno-suppression.

Administration of autoantigens have been found to effectively suppress autoimmune-like disease in rodents. For example, insulin and myelin basic protein (MBP) peptides were given to rodents by injection to prevent Type I diabetes and suppress

An acetyl derivative of MBP (1-11) fragment at the N-terminal amino group was reportedly effective in preventing and treating experimental autoimmune encephalomyelitis (EAE) (an MS model in rodent) in rodents (Eur. J. Immunol. (1994) 24: 2104-2109) while the non-modified MBP (1-11), though not recognized by encephalitogenic T cells (Nature (1986) 324:258-260), was not never tested in preventing and treating MS.
It is not known whether acetylation improves efficacy of MBP (1-11) in treating MS.

From the studies of the cell wall components of bacteria, lipopeptides with three fatty acids conjugated to a thio-ether complex containing cysteine and glycerol were prepared and reportedly shown to elicit antibody response (immunogenic property) (Tetrahedron (45) 6331-6360, 1989). The thio-ether complex linking three fatty acids is very bulky and its safety in humans is not known. A further damaging mechanism exerted by this type of construct is that eliciting antibody response will be harmful to autoimmune disease because antibody production toward an autoantigen of an internal vital organ will cause precipitation of antibodies in, and fatally damage, the organ. Obviously, this type of construct using fatty acids via a thio-ether complex is not adverse for autoimmune diseases, for these diseases occur due to abnormal immunological reactions including antibody production and T cell-mediated destruction.

A palmityl derivative of herpes simplex virus peptide at the lysine amino group, when injected intraperitoneally, reportedly enhanced the effect of eliciting antibody production and cytotoxic T cells (J. Virol. (64) 680-685, 1990). Reportedly, immunoadjuvants are not required for such derivatives to induce immunological defensive attacks, humoral and cellular attack (Peptide in Immunology (1996), John Wiley & Sons, New York). In a study using intraperitoneal injection with Freund’s complete adjuvant, a construct of palmitic acid-cystine-serine-peptide, was shown to promote the cytotoxic T cell (CTL) responses against a Plasmodium berghei circumsporozoite protein CTL epitope (J. Immunol. Methods, (182) 219-226, 1995). The complex of spacer and palmitic acid, S-[2,3-bis(palmityloxy)-(2-RS)-
propyl]-N-palmitoyl- (R) -cysteine) is abbreviated as tripam-C. It is reported that the cystine-serine spacer is required to elicit cytotoxic T cell responses. Again, antibody and cytotoxic T cell responses are both detrimental to autoimmune diseases.

Interestingly, BSA (bovine serum albumin) conjugated heavily to median chain fatty acids was found to induce delayed type hypersensitivity when injected into the guinea pig (J. Immunol. (114) 1518-1522, 1975). This publication does not provide any useful instruction as to how to treat and prevent autoimmune diseases using fatty acid conjugates.

To improve the delivery of bioactive peptides to cells, a construct using tromethamine or ethanolamine is disclosed in US Pat No 5,583,198 for linking three fatty acid to a peptide. It is described that these constructs will enhance the immunogenic properties and the lipopeptides disclosed have mitogenic effect on B cells and macrophages. Tromethamine is an emulsifying agent and can enhance the interaction of fat and water, and may thus serve as a linker to facilitate delivery of peptides. However, these compounds are exogenous compounds to the human body. Their toxicity in humans at the dose required for achieving the efficacy of peptides is not defined. Further, as stated above, eliciting antibody production are harmful and undesirable for autoimmune diseases. This prior art does not provide any useful instruction for modulating the immune system and ameliorating autoimmune diseases using fatty acid conjugates.

Another linker, cysteiny1 2-pyridyl disulfide, was described by Ekrami et al. (FEBS Letters (1995) 371: 283-286), for linking palmitic acid and protein and peptide. This prior art does not provide useful
instruction for treating autoimmune diseases. Further, it is not known whether such linker is safe for the human body.

A construct of linking fatty acid, myristic acid, to amino acid or other compound is disclosed in U.S. Patent Nos. 5,744,631 and 5,599,947, and such construct is claimed to be a good substrate for N-myristoyl-transferase and/or its acyl coenzyme and useful as anti-viral and anti-fungal agents. This prior art does not provide useful instruction for treating autoimmune diseases using fatty acid conjugates.

Fatty acid conjugates of a cyclic lactam α-melanotropin seemed to increase the potency of melanotropin when applied to the skin. It was believed that such conjugates are sequestered in the cell membrane and continue to stimulate the receptor signal transduction (J. Med. Chem. (1992) 35:118-123). It is not known whether this construct can be applied to autoantigen and its autoimmune-suppressive peptides to enhance their immuno-suppressive effects for treating autoimmune diseases.

It is disclosed in US Pat No 5,502,039 that O-derivatized alginic acid using short chain fatty acids has the enhanced immunogenic and antigenic responses against alginic acid, a large polyacid. Again, this prior art does not provide useful instruction for treating autoimmune diseases using fatty acid conjugates.

In the broad definition of derivatives in U.S. Patent No. 5,594,100 and U.S. Patent No. 5,475,086, the ester derivatives on the carboxyl group using ethyl or methyl groups or others are described for autoimmune-suppressive insulin B-chain (9-23) and GAD65. O-alkyl or acyl derivatives formed on the
hydroxyl group of insulin B-chain (9-23) and GDA_{65} are also listed. In these patents, there is no clear definition regarding the specific structural characteristics of such acyl derivatives for effectively inducing immuno-suppressive response for the prophylactic treatment of IDDM. These patents do not provide useful instruction on the specific structural characteristics of O-acyl and ester derivatives necessary to enhance the suppression of the onset of IDDM and to achieve better therapeutic effect than non-modified insulin B-chain (9-23) and GAD_{65} peptides in suppressing the onset and progressive deterioration of IDDM. These patents do not provide useful instruction as to whether O-acylation and ester derivatives can be applied to other autoantigens and their autoimmune-suppressive peptides for a better treatment for other autoimmune diseases. Furthermore, these patents do not specify the use of fatty acid-acylated autoantigen, fatty acid-acylated autoimmune-suppressive peptides of said autoantigen, fatty acid-acylated conjugates of analogs of said autoantigen, fatty acid-acylated conjugates of analogs of said peptide, for a better attenuation of clinical manifestation of autoimmune diseases.

In U.S. Patent No, 5,571,499, 5,641,473, 5,571,500, and 5,645,820, aerosol administration and inhalation of autoantigens for multiple sclerosis and rheumatoid arthritis is described. Analogs in these patents are defined as “compounds which mimic the autoimmune-suppressive activity of the autoantigen in its ability to suppress or alleviate the symptoms of the disease.” However, no clear instruction is provided on the specific structures of these compounds to use to achieve a better therapeutic effect for MS or RA.
In prior art publications, fatty acid derivatives of pharmacological active peptides were used for enhancing absorption of pharmacologically active peptides from the intestine to the blood circulation, from the circulation to the brain, for increasing resistance to intestinal and plasma enzymatic degradation, and for improving physical properties. However, the results are somewhat disappointing. For example, fatty acid derivatives of thyrotropin-releasing hormone (TRH) and tetragastrin reportedly lost partial pharmacological activities (Pharm. Res. (1993) 10:1488-1492; J. Pharm. Pharmacol. (1992) 44:717-721). Lauric acid reportedly made no further improvement on the pharmacological activity of tetragastrin as compared to acetic acid when administered intravenously (Pharm. Res. (1993) 10:1488-1492). Conjugation with lauric acid improved absorption of TRH into the plasma by increasing resistance to plasma enzymes (J. Pharm. Pharmacol. (1992) 44:717-721). Fatty acid acylated conjugates of desmopressin (dDAVP) were synthesized to increase systemic absorption (Pharm. Res. (1993) 10: 68-74). It was concluded that pivalate ester (a branched C5 alkyl chain) with high stability against chymotrypsin activity is the most desirable and esters with a straight chain fatty acid are not desirable due to their rapid degradation by chymotrypsin. The prior art does not provide any useful instruction for treating autoimmune diseases using fatty acid conjugates.

Though insulin is used to treat both IDDM as well as insulin independent diabetes mellitus by injection and is claimed to be an autoanitgen in IDDM and useful for the prophylactic treatment of this disease (US Pat. No 5,643,868), the prior art does not disclose that fatty acid-acylated insulin can enhance the efficacy of prophylactic treatment of insulin in IDDM through the enhancement of the
induction of immunological tolerance and arrest of T cell-mediated attack.

U.S. Pat. 5,631,347 discloses a method for processing N-palmitoyl Lys \(^\text{829}\) human insulin in the production process and eliminate adverse gelling problem of the conjugated insulin, and describes such conjugate is useful for administration by injection for treating diabetes by its hypoglycemic effect. This prior art does not disclose that fatty acid-acylated insulin can achieve better clinical attenuation of IDDM before the onset of IDDM through the induction of immunological tolerance and arrest of T cell-mediated attack.

With an attempt to increase oral absorption, fatty acid derivatives of insulin have been tested. Acylated insulin with two palmitic acids, though with higher lipophilic index, disappointingly showed no hypoglycemic effect (J. Pharm. Sci. (1995) 84: 682-687). Caproyl derivatives of insulin had no hypoglycemic effect either when administered via the rat large intestine (J. Pharm. Sci. (1995) 84: 682-687). These publications were aimed to increasing the hypoglycemic effect of insulin by conjugation to fatty acid. However, these publications failed to disclose that fatty acid derivatives of insulin can modulate the immune system to arrest the T-cell mediated attack of insulin-secreting cells and are useful for the prophylactic treatment and attenuation of clinical manifestation of IDDM prior to the onset of disease.

The prior art does not provide any useful specific instruction about the structural characteristics of fatty acid-acylated conjugates of autoantigen, fatty acid acylated conjugates of immunologically reactive peptide fragments of said autoantigen, fatty acid acylated conjugates of
analogs of said autoantigen, and fatty acid acylated conjugates of analogs of said peptide fragments, for better therapeutic benefit of ameliorating autoimmune diseases.

The prior art has not discovered that fatty acid-acylated autoantigen, fatty acid-acylated autoimmune-suppressive peptide fragments of said autoantigen, fatty acid-acylated conjugates of analogs of said peptide and fatty acid-acylated conjugates of analogs of said autoantigen are useful for better modulation of the immune system and better clinical attenuation of autoimmune diseases.

The present invention is the only invention disclosed so far which provides specific instruction to ensure a clinically useful efficacy by enhancing secretion of inhibitory cytokines and suppressing secretion of inflammatory cytokines, by inducing regulatory memory T cells, via fatty acid-acylated conjugates of autoantigen, fatty acid-acylated conjugates of auto-immune suppressive peptide fragments of said autoantigen, fatty acid-acylated conjugates of analogs of said peptide, and fatty acid-acylated conjugates of analogs of said autoantigens.

The present invention is the only invention disclosed so far which provides the specific structural characteristics of fatty acid-acylated conjugates of autoantigen, fatty acid-acylated conjugates of auto-immune suppressive peptide fragments of said autoantigen, fatty acid-acylated conjugates of analogs of said peptide, and fatty acid-acylated conjugates of analogs of said autoantigen for detecting and ameliorating autoimmune diseases.
DESCRIPTION OF THE DRAWINGS

Figure 1. compares the clinical EAE severity scores of SJL mice treated with phosphate-buffer saline (PBS) (n=3), or PLP (139-151) (n=4), or lauric acid-conjugated PLP (139-151) (BA 103) (n=5) at days 1, 8, and 15 post immunization. At day 0, SJL mice were immunized s. c. using PLP (139-151) (100 μg)/ CFA. Post immunization treatment was given intra-nasally at a dose of PLP (50 μg) or BA 103 at an equal amount in PBS.

Figure 2 compares the clinical EAE severity scores of SJL mice treated with PBS (n=3), or PLP (139-151) (n=4), or oleic acid-conjugated PLP (139-151) (BA 104) (n=5) at days 1, 8, and 15 post immunization. At day 0, SJL mice were immunized s. c. using PLP (139-151) (100 μg)/ CFA. Post immunization treatment was given intra-nasally at a dose of PLP (50 μg) or conjugated peptide at an equal molar amount in PBS.

Figure 3 compares the clinical EAE severity scores of SJL mice treated with PBS (n=3), or PLP (139-151) (n=4), or acetic acid-conjugated PLP (139-151) (BA 102) (n=4) at days 1, 8, and 15 post immunization. At day 0, SJL mice were immunized s. c. using PLP (139-151) (100 μg)/ CFA. Post immunization treatment was given intra-nasally at a dose of PLP (50 μg) or conjugated peptide at an equal amount in PBS.

Figure 4 compares the clinical EAE severity scores of SJL mice treated with PBS (n=5), or PLP (139-151) (n=5), or lauric acid-conjugated PLP (139-151) (BA 103) (n=5) at days 1, 3, 5, and 7 post immunization. At day 0, SJL mice were immunized s. c. using PLP (139-151) (100 μg)/ CFA. Post immunization treatment was given intra-nasally at a
dose of PLP (50 μg) or conjugated peptide at an equal amount in PBS.

Figure 5 compares the clinical EAE severity scores of SJL mice treated with PBS (n=5), or PLP (139-151) (n=5), or butyric acid-conjugated PLP (139-151) (BA 105) (n=5) at days 1, 8, 15, and 25 post immunization. At day 0, SJL mice were immunized s. c. using PLP (139-151) (100 μg)/ CFA. Post immunization treatment was given intra-nasally at a dose of PLP (50 μg) or conjugated peptide at an equal amount in PBS.

Figure 6 compares the clinical EAE severity scores of SJL mice treated with PBS (n=5), or PLP (139-151) (n=5), or octanoic acid-conjugated PLP (139-151) (BA 106) (n=5) at days 1, 8, 15, and 25 post immunization. At day 0, SJL mice were immunized s. c. using PLP (139-151) (100 μg)/ CFA. Post immunization treatment was given intra-nasally at a dose of PLP (50 μg) or conjugated peptide at an equal amount in PBS.

Figure 7 shows the T cell proliferative responses to PBS, PLP (139-151), acetyl-Tyr-PLP (139-151) using T cells from draining lymph nodes of PLP(139-151) primed SJL mice.

Figure 8. ELISOTSPOT analysis for interleukin 4 (IL-4) of draining lymph node, inguinal and poplineal, cells (LNC) obtained from different treatment groups of SJL mice at day 41 post-immunization. LNC cells from PBS-treated mice, BA 101(PLP (139-159))–treated mice, and BA 105–treated mice were stimulated in culture for 24 hr with PLP (139-151) (50 μg). In a group of wells, Con A was added to maximize the stimulation to validate the in vitro system. The data are the ratio of the number of IL-4 secreting cells from each treatment group versus
the control and expressed as mean ± S.D. of three experiments for each treatment group. The control is the culture without PLP(139-151) using LNC from PBS-treated group.

Figure 9. ELIOTSPOT analysis for interferon γ (INF-γ) of draining lymph node, inguinal and poplineal, cells (LNC) obtained from different treatment groups of SJL mice at day 62 post-immunization. LNC cells from PBS (phosphate-buffered saline)-treated mice, BA 101 (PLP (139-159)-treated mice, BA 102-treated mice, and BA 103-treated mice were stimulated in culture for 24 hr with PLP (139-151) (50 µg). In a group, Con A was added to maximize the stimulation. The data are the ratio of the number of INF-γ secreting cells from each treatment group versus the control and expressed mean ± S.D. of three experiments for each treatment group. The control is the culture without PLP(139-151) using LNC from PBS-treated group.

Figure 10. ELIOTSPOT analysis for transforming growth factor β (TGF-β) of draining lymph node, inguinal and poplineal, cells (LNC) obtained from different treatment groups of SJL mice at day 41 post-immunization (Figure 10a) and at day 62 post-immunization (Figure 10b). LNC cells from PBS-treated mice, BA 101 (PLP (139-159)-treated mice, BA 102-treated mice, BA 103-treated mice, and BA 105-treated mice were stimulated in culture for 24 hr with PLP (139-151) (50 µg). In a group, Con A was added to maximize the stimulation. The data are the ratio of the number of TGF-β secreting cells from each treatment group versus the control and expressed mean ± S.D. of three experiments for each treatment group. The control is the culture without PLP(139-151) using LNC from the PBS-treated group.
Figure 11. ELIOTSPOT analysis for tumor necrosis factor α (TNF-α) of draining lymph node, inguinal and poplineal, cells (LNC) obtained from different treatment groups of SJL mice at day 41 post-immunization. LNC cells from PBS-treated mice, BA 101 (PLP (139-159))-treated mice, BA 103-treated mice, BA 105-treated mice were stimulated in culture for 24 hr with PLP (139-151) (50 μg). In a group, Con A was added to maximize the stimulation. The data are the ratio of the number of TNF-α secreting cells from each treatment group versus the control and expressed mean ± S.D. of three experiments for each treatment group. The control is the culture without PLP(139-151) using LNC from the PBS-treated group.

**SUMMARY OF THE INVENTION**

According to the present invention, administration of one or more agents selected from the group consisting of fatty acid-acylated conjugates of autoantigens specific for the autoimmune disease, fatty acid-acylated conjugates of disease-suppressive peptides of said autoantigen, fatty acid-acylated conjugates of analogs of said peptides, and fatty acid-acylated conjugates of analogs of said autoantigen, the autoimmune disease is remarkably alleviated.

According to the present invention, said conjugate enhances the in vitro proliferation of T cells, providing a means to more effectively identify those who have cell-mediated attack yet without any clinical manifestations.

The conjugate is formed via a covalent bond between the carboxyl group of fatty acid and a free functional group of amino acid residue of autoantigen, between the carboxyl group of fatty acid and a free functional group of amino acid residue of disease-suppressive peptides of said autoantigen,
between the carboxyl group of fatty acid and a free functional group of amino acid residue of analogs of said peptide, between the carboxyl group of fatty acid and a free functional group of analog of said amino acid residue of said peptide analog, between the carboxyl group of fatty acid and a free functional group of amino acid residue or analogs of said amino acid of analogs of said autoantigen.

The conjugate uses one or more saturated and non-saturated fatty acids. The conjugate fatty acids containing at least 2 carbons.

Particularly relevant is the use of said conjugate in the diagnosis and treatment of patients having, or at risk of having, autoimmune diseases. Not only is it possible to use said fatty acid conjugates to more effectively ameliorating people with autoimmune disease, but it is also now possible to more accurately classify patients with such autoimmune diseases as insulin-dependent diabetes mellitus.

**DETAILED DESCRIPTION OF THE INVENTION**

In mammals, an autoimmune disease occurs when the immune system cannot or does not distinguish between exogenous (foreign) substances within the mammal and autologous tissues or substance. As a result, the immune system treats autologous tissues and substances (self antigens) as if they were foreign and evokes the proliferative immune defense that is initiated against exogenous (foreign) tissues or invading organisms. In essence, the normal immune system begins a proliferative response against autologous tissues. As employed herein, the term "mammal" refer to a life form which has an immunoregulatory system.
According to the invention, therefore, there is provided a conjugate of (1) autoantigen and (2) one or more fatty acids.

According to the invention, the fatty acids include any fatty acids containing at least 2 carbon atoms, saturated fatty acids, and non-saturated fatty acids.

The “conjugate” of (1) and (2) includes products comprising (1) bond to (2) by an effective means for covalent bonding. As employed herein, the term “acyl derivative” refers to any of said conjugate.

As employed herein, the term “autoantigen” refers to any substances normally found within a mammal that (i) is not recognized as part of the mammal itself by the lymphocytes or antibodies of that mammal, (ii) is attacked by the immuno-regulatory system of the mammal as though such antigen were a foreign substance and (iii) acts to downregulate the arm of the immune system that is responsible for causing a specific autoimmune disease. The term autoantigen also includes antigenic substances which induce conditions having the symptoms of an autoimmune disease when administered to mammals. As employed herein, the terms “autoantigen” and “autoimmune-suppressive protein” are used interchangeably.

As used herein the term “autoimmune suppressive peptide” or “disease-suppressive peptide” includes any peptides or polypeptides containing partial amino acid sequences or moieties of autoantigens and possessing the ability to suppress or prevent an autoimmune response upon administration. Such fragments need not possess the autoantigenic properties of the entire autoantigen. By way of non-limiting example, when MBP is administered

As employed herein the term “analogs” of such autoantigens or peptide fragments thereof refers to compounds that are structurally related to autoantigens or their autoimmune-suppressive peptides and which possess the same biological activity, i.e. the ability to eliminate or suppress the autoimmune response, upon administration. By way of example, the term includes peptides having amino acid sequences which differ from the amino acid sequence of the autoantigen or disease suppressive peptides thereof by one or more amino acid residues (while still retaining the autoimmune-suppressive activity of the autoantigen or fragment) as well as compounds or compositions which mimic the autoimmune-suppressive activity of the autoantigen in its ability to suppress or alleviate the symptoms of the disease. One example is tissue from an organ that is the target of attack by an arm of the immune system in an autoimmune disease, e.g. the pancreas in diabetes or the white matter of the central nervous system in MS.

As used herein the term “autoimmune-disease suppressive agent” or autoimmune suppressive agent refers to a compound or composition which can be administered to suppress, prevent or delay the clinical onset or manifestation of a specific autoimmune disease. The term includes antoantigens, autoimmune-suppressive peptide of autoantigen and analogs of said peptide thereof as defined above.
As employed herein the term "mucosal immune tissues" include mucosal immune tissues present in the intestinal, nasal, tracheal, colonic, rectal, buccal, and vaginal mucosa tissues. As employed herein the term "non-parenteral delivery" means delivery via the oral, nasal, tracheal, colonic, rectal, buccal, and vaginal mucosa tissues. The standard pharmaceutical formulations for administration via these routes are well described in the art. Examples include aerosols for delivery to the trachea and nasal sprays for delivery to the nasal mucosal tissue. As employed herein the term "intestine" includes the small intestine, the caecum, the colon, and the rectum.

As used herein the term "acylating" or "acylation" means the introduction of one or more acyl groups covalently bonded to a functional group of autoantigen or autoimmune suppressive peptide of said autoantigen or analogs of said autoantigen or analogs of said peptides. Such a functional group is present in, or added to, said amino acid residue or amino acid analog of said amino acid residue. In the preferred embodiment, the acyl groups are from fatty acids. For acylation, typical attachment sites for fatty acid chains in peptides, proteins, protein analogs, and peptide analogs re tyrosine (acylation of the phenolic hydroxyl), serine (acylation of methyl hydroxyl), cysteine (acylation of the side chain sulfhydryl), lysine (acylation of the ε amine) and the N-terminus (acylation of the α amine). In the preferred embodiment, acylation is through the free hydroxyl group or the N-terminal α amine or free ε amine of comprising amino acid residues or free sulfhydryl group of autoantigen or autoimmune-suppressive peptides or their analogs.

As used herein the term "fatty acid" means saturated or unsaturated C_2-C_21 fatty acids as well as high molecular weight fatty acids. The saturated fatty acids include (but not limited to) acetic acid,
proprionic acid, butyric acid, caproic acid, caprylic acid, decanoic acid (capric acid), lauric acid, myristic acid, palmitic acid and stearic acid. The non-saturated fatty acids generally have the formula of CₙH₂ₙ₊₁COOH, and include (but are not limited to) oleic acid, linoleic acid, and arachidonic acid. The high molecular weight fatty acids include (but are not limited to) lignoceric acid(CH₅-(CH₂)₇-COOH), cerebroptic acid(CH₅-(CH₂)₂₁-CH(OH)-COOH, nervonic acid(CH₃-(CH₂)₇-CH=CH-(CH₂)₁₃-COOH) and oxynerveonic acids (CH₃-(CH₂)₇-CH=CH-(CH₂)₁₂-CH(OH)-COOH). Most preferably, the fatty acids are saturated fatty acids, unsaturated fatty acids and high molecular weight fatty acids, including, acetic acid, proprionic acid, butyric acid, caproic acid, caprylic acid, capric acid, lauric acid, myristic acid, palmitic acid, stearic acid, oleic acid, linoleic acid, arachidonic acid, lignoceric acid, cerebroptic acid, nervonic acid and oxynerveonic acid.

The α-amine group of N-terminal amino acid residue can be from any amino acids including alanine, isoleucine, leucine, methionine, phenylalanine, proline, tryptophan, valine, arginine, aspartic acid, asparagine, cysteine, glutamic acid, glutamine, glycine, histidine, lysine, serine, threonine, tyrosine, homocysteine, cysteinesulfinic acid, homoserine, ornithine, citrulline, argininosuccinic acid, and organic compounds containing both amino and carboxyl groups including but not limited to α-aminohexadecanoic acid and ε-amino caproic acid. The N-terminal amino acid residue or organic compound used is either present in, or added to, autoantigen or autoimmune-suppressive peptides of said autoantigen or analogs of said autoantigen or analogs of said peptide.

The free hydroxyl group can be present in an amino acid residue of, or be introduced to via the N-
terminus or C-terminus of, an autoantigen or its autoimmune-suppressive peptides or analogs of said autoantigen and said peptide. Amino acid residues containing such free hydroxyl group include but are not limited to tyrosine, serine, threonine, hydroxylsine, 4-hydroxyproline, and homoserine. One or more hydroxyl groups can be introduced by adding the above-mentioned amino acids, glycolic acid or lactic acid to the autoantigen or its autoimmune-suppressive peptide or analogs of said autoantigen and peptide through an amide linkage to an amino acid residue. In general, any compounds, which contain a free carboxyl group or amino group for amide bond formation with an amino acid residue as well as a free hydroxyl group for ester linkage, can be added to the autoimmune-suppressive protein or its peptide fragments to introduce a free hydroxyl group. Such compounds include but are not limited to tyrosine, serine, threonine, hydroxylsine, 4-hydroxyproline, homoserine, glycolic acid, lactic acid, gluconic acid, saccharic acid, mevalonic acid and glycuronic acid. Such compounds can be linked to an autoantigen or autoimmune-suppressive peptide of autoantigen or analogs of said autoantigen and said peptide via amide linkage, leaving one or more free hydroxyl group available for ester linkage.

In the preferred embodiment, acylation to form a conjugate is accomplished by one or more ester linkage between the free hydroxyl group of a tyrosine residue and the carboxyl group of a fatty acid, or by one or more amide linkage between an N-terminal α or ε amine group (either primary or secondary) and the carboxyl group of a fatty acid, or by a mixture of such ester and amide linkage. In the still preferred embodiment, acyl derivatives are obtained by utilizing the free hydroxyl group on a tyrosine residue or the amine group (α or ε) on the N-terminal amino acid residue, which is either intrinsically
present or covalently introduced to the autoantigen or autoimmune-suppressive peptide or analogs of said autoantigen and peptide via the N-terminus or C-terminus.

5

In accordance with the present invention, the fatty acid conjugate of autoantigen or autoimmune-suppressive peptide of autoantigen or analogs of said autoantigen and said peptide has at least one fatty acid attached via covalent bond(s).

10

Techniques of forming the ester linkage involving peptides or proteins are well known in the art (J. Med. Chem. (1977) 20:1435; Pharm. Res. (1993) 10:68-74). The esters reported involve an alkyl chain of C2-C8. The same principle can be extended to acyl derivatives involving fatty acids with longer acyl chains. The solid phase synthesis or polymer-supported synthesis of peptides are well known in the art, and are widely used in commercial production of peptides (Advances Organic Chemistry, 3rd, Jerry March, Wiley & Sons, NY). The present invention is not limited to any particular way of preparing any intermediates involved in the process of forming the acyl derivatives.


30 Once the acylation reaction is complete, the reaction typically is diluted with water. The acylated autoantigen or acylated autoimmune-suppressive peptide of said autoantigen or acylated analogs of said autoantigen or acylated analogs of said peptide is placed in a properly buffered aqueous solution for further processing. Such processing particularly includes purification by standard
chromatographic methods such as reverse phase or hydrophobic chromatography, concentration by crossflow filtration, solvent exchange by ultrafiltration and the like (Pharm. Res. (1993) 10; 68-74; J. Pharm. Sci. (1995) 84;682-687). All peptides will be purified by semi-preparative HPLC and structures will be confirmed by amino acid analysis and mass spectroscopy and, when appropriate, by $^1$H and $^{13}$C NMR.

After purification and enrichment steps, the aqueous solution of the purified fatty acid-acylated autoantigen or autoimmune-suppressive peptide or analogs of said autoantigen and said peptide, can be processed to recover the soluble acyl derivative as a powder. In the broad practice of the present invention, any procedure for recovering the acylated autoantigen or autoimmune-suppressive peptide or analogs of said autoantigen and said peptide as a powder, including lyophilization (freeze drying), crystallization or precipitation techniques, can be used. The present invention is not limited to the way of isolating and recovering the acylated protein and peptide in powder form.

The present invention is applicable to administration by injection means, oral (including colonic and rectal), nasal, tracheal, vaginal and buccal administration, and intrathymic injection, and administration to the tissue targeted by the autoimmune attack. When administered, the acyl derivatives can be formulated in any standard pharmaceutical dosage forms, such as solution, pellet, tablet, capsule, spray, aerosol, cream, granule, insert, patches, matrix and mucoadhesive formulations.

For oral administration, premature lumenal degradation should be prevented. Intestinal esterases and proteolytic enzymes secreted by the
pancreas can act on the acyl derivative of autoantigen or autoimmune-suppressive peptide or analogs of said autoantigen and said peptide, targeting the ester and amide bonds, respectively.

These enzymes in general require a pH higher than 7 for optimal activities and lose activities at acidic pH. The distal small intestine has much lower lumenal concentration of digestive enzymes. Therefore, the luminal premature degradation of acyl conjugate of autoantigen or autoimmune-suppressive peptide or analogs of said autoantigen and said peptide by esterases or by proteolytic enzymes can be minimized by delivering to the distal ileum and by co-administering any agents which are non-toxic and can reduce intestinal pH or pH at any munoal surface to be lower than 5.5. Such agents are any organic acids present in food or of pharmaceutical grade or used as food additives. Such organic acids include, but are not limited to, citric acid, malic acid, poly(acrylic acid), lactic acid, glycolic acid, Carbopol 971P, Carbopol 974P, Carbopol 934P, and EDTA. These acids can be incorporated using standard pharmaceutical formulation dosage forms and formulating procedures.

For oral administration, it is preferred that the acyl derivative of the autoantigen or autoimmune-suppressive peptide or analogs of said autoantigen or analogs of said peptide is delivered by formulation approaches to the human ileum or colon because the concentration of luminal pancreatic enzymes are much less. Such formulation approaches are well known in the art. For example, U.S. Patent No. 5,443,841 and U.S. Patent No. 55,78,323 disclose a system of proteinoid microspheres consisting of proteinoids having 2 to 20 amino acid residues. Based on the compositions of the amino acids, these microspheres are able to deliver proteins or peptides only in the distal intestine when the majority of the comprising
amino acids are basic amino acids which become ionized at the distal intestinal pH, which is higher than 7. Proteinoid microspheres which are selectively soluble under alkaline pH environments, such as the distal portion of the intestine, are based on base-soluble proteinoids. A base-soluble proteinoid consists of a majority of basic amino acids in its composition. Hence, proteinoids thus designed can protect peptides and proteins from gastrointestinal proteolysis and start release only in the ileum. Such exemplified preparation reportedly increases the pharmacological effect of insulin on reducing blood glucose level by protecting insulin from lumenal degradation, thus increasing the amount of insulin reaching the circulation.

Another example is Eudragit polymers which can be employed to manipulate the release of drugs at different pH. Eudragit L-100 is an acrylic copolymer based on methacrylic acid and ethylacrylate, which dissolves at pH above 5-6, and Eudragit S is an acrylic copolymer based on methacrylic acid and methylmethacrylate which dissolves at pH higher than 7. There are other polymers derived from this family for a precise control of release at a certain pH for a certain intestinal site. The combination of these Eudragit polymers in the coating can manipulate the drug to be released in a desired intestinal environment (Published in "Practical Course in Lacquer Coating, by Rohm Tech Inc., in 1989).

Delivery to the colon can be achieved by azo polymer or other type coatings which allows only release in the colon by utilizing colonic bacterial enzymes. Further, mucoadhesive coating can be employed to any pharmaceutical dosage forms to facilitate the anchor of a solid dosage form on the mucosal surface. Delivery to the rectum can be achieved by wax-based or hydrogel-based
suppositories. Pharmaceutical practice in making suppositories is well developed. Delivery to the vaginal can be achieved by cream, gel and suppositories.

5 For aerosol administration or nasal spray or buccal spray, the acyl derivative of the autoantigen or autoimmune-suppressive peptide or analogs of said autoantigen and said peptide can be administered as a dry powder or in a solution. Dry aerosol in the form of finely divided solid particles that are not dissolved or suspended in a liquid are also useful in the practice of the present invention. The acyl derivative of the autoantigen or autoimmune-suppressive peptides of said autotantigen or analogs of said autoantigen or analogs of said peptide may be in the form of powder and comprises finely divided particles having an average particle size of between about 1 and 5 μm, preferably between 2 and 3 μm. Finely divided particles may be prepared by pulverization or screen filtration or lyophilization. The particles may be administered by inhaling a predetermined dose of the finely divided material, which can be in the form of a powder.

The pharmaceutical formulation for the present invention may be administered in the form of an aerosol spray using, for example, a nebulizer such as those described in U.S. Pat. Nos. 4,624,251, 3,703,173, 3,561,444, and 4,635,627. The aerosol material is inhaled by the subject to be treated.

30 Other systems of aerosol delivery, such as the pressurized metered dose inhaler (MDI) and the dry powder inhaler as disclosed in Newman, S. P. in Aerosols and the Lung (Clarke, S.W. and Davis, D. eds, pp 197-224, Butterworths, London, England, 1984) can be used when practicing the present invention. Aerosol delivery systems of the type disclosed herein are available from numerous commercial sources.
including Fisons Corporation (Bedford, Mass.), Schering Corp. (Kenilworth, N.J.), American Pharmaseal Co., (Valencia, CA), and Dura Pharmaceutical Inc. (San Diego, CA).

The present invention can be applied for the treatment and prevention of a variety of autoimmune diseases. Autoantigen and its suppressive peptide thus employed herein include, but are not limited to, self-antigens and nonself-antigens implicated in autoimmune diseases, and their autoimmune-suppressive fragments, such as insulin, insulin B-chain (9-23), insulin A-chain (7-21), glutamic acid decarboxylase, heat shock protein 65, heat shock protein 60, carboxypeptidase H, ICA 512/IA, peripherin, 38 kD antigen, Gm2-1, and ICA-69 for juvenile diabetes, type II collagen and its effective disease-suppressive fragments for rheumatoid arthritis, myelin basic protein, proteolipid protein, myelin oligodendrocyte glycoprotein and their effective disease-suppressive peptide fragments for multiple sclerosis, thyroglobulin for autoimmune thyroiditis, acetylcholine receptor for myasthenia Gravis, haptenized colonic proteins for Colitis, alloantigen and MHC peptide for transplantation, heptenized colonic proteins for colitis, S-antigen and interphotoreceptor binding protein (IRBP) for autoimmune uveoretinitis, DNA for systemic lupus erythematosus, haptenized colonic proteins for colitis, myosin for myocarditis, α-Fodrin for Sjogren’s syndrome, bovine serum albumin, and many others implicated in autoimmune diseases. The autoantigens effective for the treatment of individual autoimmune disease models also include, but are not limited to, liver extract for chronic active hepatitis, adrenal gland extract for adrenalitis, muscle extract for polymyositis, hematopoietic cells for autoimmune hemolytic anemia,
heart extract for rheumatic carditis, and skin cell extract for scleroderma.

The autoimmune diseases include, but are not limited to, colitis, herpes simplex virus-induced autoimmune diseases (including but not limited to herpes stromal keratitis), systemic lupus erythematosus, dermatomyositis, Sydenham's chorea, rheumatoid arthritis, rheumatic fever, thrombocytopenic purpura, polyglandular syndromes, bullous pemphigoid, diabetes mellitus, henoch-schonlein purpura, post-streptococcal nephritis, systemic lupus erythematosus, erythema nodosum, Takayasu's arteritis, myasthenia gravis, Addison's disease, multiple sclerosis, sarcoidosis, ulcerative colitis, erythema multiforme, IgA nephropathy, polyarteritis nodosa, ankylosing spondylitis, goodpasture's syndrome, thromboangiitis obliterans, Sjogren's syndrome, primary biliary cirrhosis, thyrotoxicosis, scleroderma, myocarditis, chronic active hepatitis, polymyositis/dermatomyositis, polychondritis, pemphigus vulgaris, idiopathic thrombocytopenia, Wegener's granulomatosis, membranous nephropathy, amyotrophic lateral sclerosis, tabes dorsalis, giant cell arteritis/polymyalgia, pernicious anemia, rapidly progressive glomerulonephritis, and fibrosing alveolitis.

Immunoadjuvants are known in literature regarding their enhancing effects on immunological responses and can be included in the formulation. They include, but are not limited to, liposaccharides and lipid A.

The dose of fatty-acid acylated autoantigen or autoimmune-suppressive peptide of autoantigen or analogs of said autoantigen and said peptide ranges from 0.01 mg/kg to 30 mg/kg. In the preferred embodiment, the dose
ranges from 0.1 mg to 25 mg/kg. The dosing frequency
ranges from once a day, twice a week, once a week ,once
every other week, or once a month depending on the
disease.

The following examples are given to further
illustrate the present invention.

**EXAMPLE 1:**

**Preparation of acylated PLP(139-159) fragment**

The procedures of peptide synthesis is well
known in the art using solid phase peptide synthesis
with Fmoc protection of the α amine group and
appropriate side chain protecting groups (J. Med.
Advances Organic Chemistry, 3rd, Jerry March, Wiley &
Sons, NY). Automated synthesizers are available from
many commercial sources such as Applied Biosystems
synthesizers (models ABI 431 and 433) or Rainin
Symphony multiple peptide synthesizer, or PerSeptive
BioSystems Pioneer.

The amino acid sequence of the PLP (139-151) is
His-Ser-Leu-Gly-Lys-Trp-Leu-Gly-His-Pro-Asp-Lys and
it was prepared using the following procedure. The
Fmoc and Boc protected amino acids were obtained
Advances ChemTech and Bachem. Fmoc-His (Trt), Fmoc-
Ser(tBu), Fmoc-Leu, Fmoc-Gly, Fmoc-Lys(Boc), Fmoc-
Trp, Fmoc-Leu, Fmoc-Gly, Fmoc-His (Trt), Fmoc-Pro,
Fmoc-Asp(tBu), Fmoc-Lys(Boc) were sequentially
coupled to Phe-PEF-PS resin (PerSeptive Biosystems)
on ABI 431 Peptide Synthesizer using ABI FastMoc
chemistry. The final protected product is a 13
amino acid peptide resin, Fmoc-His(Trt)-Ser(tBu)-Leu-
Gly-Lys(Boc)-Trp-Leu-Gly-His(Trt)-Pro-Asp(tBu)-
Lys(Boc)-Phe-PEG-PS resin. The resin was dried
under vacuum and then incubated at room temperature
in 20% v/v piperidine in N-methyl pyrrolidone for 25
min. This procedure removed the N-terminal Fmoc.
The deprotected resin was washed first with N-methylpyrrolidone, then dichloromethane/methanol, and finally dichloromethane and dried under vacuum. The deprotected resin was then incubated in a mixture of trifluoroacetic acid, water and ethanedithiol (9.5 ml: 0.25 ml: 0.25 ml) for one and half hours. The resin was removed using filtration and washed with trifluoroacetic acid and dichloromethane. The final filtrate was rotovapped to 2 ml. The peptide was precipitated using cold ether and the suspension was left at 4° C overnight. Crude product was obtained using filtration and dried under vacuum, and then it was dissolved in 2 ml methanol, diluted with HPLC solvent 1 (0.1% v/v trifluoroacetic acid in water) and run through the 15-20 micro semi-prep column.

Elution was done using a linear gradient from 5% to 35% of HPLC solvent 2 (0.1% v/v trifluoroacetic acid in acetonitrile) in 30 min. The peak eluted at 25 min was collected in fractions using semi-prep 4312. The fraction with the highest purity under the isocratic HPLC at 19% HPLC solvent 2 was rotovapped and lyophilized to PLP (139-151).

<table>
<thead>
<tr>
<th>Table 1. <strong>Synthesis of Acyl derivatives of PLP (139-159) peptide</strong></th>
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<tr>
<td><strong>25</strong> PLP (139-159) peptide is acylated at the N-terminal added tyrosine residues with an n-acetic acid. PLP (139-159) peptide acylated at the N-terminal added tyrosine residues with an n-oleic acid. PLP (139-159) peptide is acylated at the N-terminal added tyrosine residues with an n-butanoic acid.</td>
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A. PLP (139-159) peptide is acylated at the N-terminal added tyrosine residues with an n-caproic acid.

The synthesis of the above-mentioned fatty acid acylated conjugates of PLP (139-151) described are briefly described in the following. N-Boc-Tyrosine
(Ac) was coupled to Fmoc-His(Trt)-Ser(tBu)-Leu-Gly-Lys(Boc)-Trp-Leu-Gly-His(Trt)-pro-Asp(tBu)-Lys(Boc)-Phe-PEG-PS-resin using the deprotection and cleavage procedures described above. The crude acetylated peptide was then dissolved in methanol (4 ml) and HPLC solvent 1 (50 ml) and a 15-20 micron semi-prep column was used to purify the peptide using a linear gradient from 5% to 35% HPLC solvent 2 in 60 min. The portion eluted at 47 min was analyzed using isocratic HPLC using 22% HPLC solvent 2 and demonstrated to have high purity > 99%. The fraction eluted at 47 min was dried, rotovapped and lyophilized. Acetyl-Tyr-His-Ser-Leu-Gly-Lys-Trp-Leu-Gly-His-Pro-Asp-Lys was thus obtained.

Boc-Tyr with no side chain protection was coupled to Fmoc-His(Trt)-Ser(tBu)-Leu-Gly-Lys(Boc)-Trp-Leu-Gly-Lys(Boc)-Trp-Leu-Gly-His(Trt)-Pro-Asp(tBu)-Lys(Boc)-Phe-PEG-PS-resin as described above. Boc-Tyr-His(Trt)-Ser(tBu)-Leu-Gly-Lys(Boc)-Trp-Leu-Gly-His(Trt)-Pro-Asp(tBu)-Lys(Boc)-Phe-PEG-PS-resin then reacted with 6 fold excess of diisopropylethylamine and lauroryl chloride in dichloromethane under N2 gas on a Vega 250C Peptide Synthesizer for 19 hours. Deprotection and cleavage was done as described above. The crude lipopeptide was then put in 5 ml of dimethylformamide/80% acetic acid (9:1 v/v) and diluted with 50 ml HPLC solvent 1, and then purified with a 15-20 micron semi-prep column and a linear gradient system of 10% to 50% HPLC solvent 2 in 50 min. The fraction eluted at 70 min was then analyzed using isocratic HPLC with 40% HPLC solvent 2. The purest fractions were rotovapped and lyophilized to yield lauryl-Tyr-His-Ser-Leu-Gly-Lys-Trp-Leu-Gly-His-Pro-Asp-Lys with purity greater than 98%. Oleyl-Tyr-His-Ser-Leu-Gly-Lys-Trp-Leu-Gly-His-Pro-Asp-Lys, butyryl-Tyr-His-Ser-Leu-Gly-Lys-Trp-Leu-Gly-His-Pro-Asp-Lys, and caproyl-Tyr-His-Ser-Leu-Gly-Lys-Trp-Leu-Gly-His-Pro-Asp-Lys were all
synthesized using similar procedures described above.

**EXAMPLE 2**

*Suppression of multiple sclerosis by acyl derivatives of PLP (139-151)*

Female SJL mice, 6-8 weeks of age, were obtained from the National Cancer Institute (Frederick, MD). Immunizations are done on day 0 (d0) and day 7 (d7) in the posterior flank at two sites with 0.15ml/site subcutaneous injection of 100 μg of PLP (139-151) in an emulsion of Complete Freund's adjuvant H37Ra(CFA) (Difco Laboratories) and PBS (1:1 v/v). There were five mice in each group. Individual groups received a nasal dose of 50 or 0 (control) μg of PLP (139-151) and equal molar amount of fatty acid acylated conjugates of PLP (139-151). The nasal solution was prepared in PBS, and the nasal dosing was administered at days 1, 8, 15, or at days 1, 8, 15, 25 or at days 1, 3, 5, 7 through two nostrils of each mouse (10 μl for each nostril) using a PE-10 tubing connected to a 25 μl Hamilton syringe. All clinical neurological disorders are monitored daily and the symptom manifestation is scored as the following: 4+, full hind limb paralysis; 3+ partial hind limb paralysis; 2+ ataxia; 1+ flaccid tail and 0, normal.

Re-challenge can be done after day 60 to observe better clinical attenuation of EAE in SJL mice. The results would hint at the induction by acylated conjugates of PLP (139-151) of regulatory memory T cells which act to suppress the clinical symptoms of EAE.

As shown in Figure 1, in the acute phase of EAE, the mice treated with lauric acid-conjugated PLP (139-151) had lower clinical scores than those treated PBS and entered remission sooner than those treated with PLP(130-151). By highest extent, lauric acid-conjugated PLP (139-151)-treated mice exhibited almost no sign of relapse between days 22-60, in
comparison to very severe episodes of relapse in the PBS-treated group and some mild episodes in the PLP (139-151) treated group. Upon re-challenge with antigen on day 62, very little response was seen with the lauric acid-conjugated PLP (139-151)-treated group.

As shown in Figure 2, oleic acid-conjugated PLP (139-151)-treated mice had lower clinical score than PBS-treated mice and PLP (139-151)-treated mice and had no sign of relapse between days 20-35, in comparison to severe episodes of relapse in the PBS-treated mice and mild episodes of relapse in the PLP(131-159)-treated mice.

As shown in Figure 3, acetic acid-conjugated PLP(139-151)-treated mice had much lower clinical scores in the acute phase than PBS-treated and PLP(139-151)-treated mice, and entered remission much earlier than the PLP(139-151)-treated and PBS-treated mice. Further, the acetic acid-conjugated PLP (139-151)-treated mice had little remission while PBS-treated group had very serious relapse and the PLP(139-151)-treated mice had mild relapse.

As shown in Figure 4, in a separate experiment, the lauric acid-conjugated PLP (139-151)-treated mice had lower clinical scores during the acute phase than the PBS-treated and PLP(139-151)-treated mice, entered remission much earlier and had no sign of relapse than the other two groups.

As shown in Figure 5, the butyric acid-conjugated PLP(139-151)-treated mice had later onset of EAE and lower clinical scores than the PBS-treated mice and the PLP (139-151)-treated mice.

As shown in Figure 6, the octanoic acid-conjugated PLP (139-151)-treated mice had later onset
of EAE and lower clinical scores than the PBS-treated mice and the PLP (139-151)-treated mice.

**EXAMPLE 3**

**T cell proliferation responses to acetyl-Tyr-PLP(139-159)**

Female SJL mice, 6-8 weeks of age, were obtained from the National Cancer Institute (Frederick, MD). Immunizations are done on days 0 and 7 (d7) in the posterior flank at two sites with 0.15ml/site subcutaneous injection of 100 μg of PLP (139-151) in an emulsion of Complete Freund's adjuvant H37Ra(CFA) (Difco Laboratories) and phosphate buffered saline (PBS) in 1:1 ratio. After the mice were challenged with the PLP (139-151) and CFA at day 0 and day 7, draining lymph node (inguinal and popliteal) and spleen cells were harvested from 3 PLP(139-151) primed mice on day 16. The lymph node cells are washed and suspended in RPMI 1640 medium containing 2% fresh SJL mouse serum, 2 mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, 25 mM HEPES, and 5 X 10^{-3} M 2-ME. Spleen cells were irradiated at 2000 rad. Spleen cells were plated at 105 cells/well in 50 ml complete RPMI. Lymph node cells were plated at 105 cells/well in 100 ml complete RPMI with spleen cells. Peptides were dissolved in 25 ml complete RPMI and added to appropriate wells (Costar Corp., Cambridge, MA). PLP (139-151) was tested at five concentrations: 5 μg/ml, 10 μg/ml, 25 μg/ml, 40 μg/ml and 50μg/ml and acetyl-Tyr-PLP(139-151) was tested at the same concentration. The culture condition was 37°C in 5% CO2. After 3 days culture, [\(^{3}\)H]thymidine (0.25 μCi in 25 μl) was added and the culture was harvested 18h later and counted using a beta-plate reader. The average of [\(^{3}\)H]thymidine uptake was obtained from triplicate wells. The results are shown in Figure 7. Acetyl-Tyr-PLP(139-151) showed better mean T cell proliferative responses than the unmodified peptide, PLP(139-151) at several
concentrations. Higher T cell proliferative responses will enable more precise identification of a subject whose T cells are reactive to self proteins in an vital organ.

**EXAMPLE 4.**

ELISPOT analysis of cytokine-secreting PLP (139-151)-responding T cells prepared from the draining lymph nodes of PLP (139-151)-challenged mice that received fatty acid-conjugated PLP (139-151) intra-nasally

**Table 2: Organization of animal groups for ELISPOT analysis.**

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Mice</th>
<th>Nasal Dosing Protocol</th>
<th>Day after immunization begins in which draining lymph nodes were taken</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Vehicle</td>
<td>Antigen</td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td>PBS</td>
<td>control</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>PBS</td>
<td>PLP (139-151) (50μg)</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>PBS</td>
<td>acetyl-Tyr-PLP (139-151)</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>PBS</td>
<td>lauryl-Tyr-PLP (139-151)</td>
</tr>
</tbody>
</table>

On day 62 after the mice were immunized with PLP (139-151), the mice were sacrificed and the draining lymph nodes (poplineal and inguinal) were obtained. The T cell suspensions from individual groups were prepared and mixed with irradiated spleen cells that were prepared from the PBS-treated mice in a ratio of 1:2 with PLP (139-151) (50 μg/ml) or PLP (139-151) (25 μg/ml) or Con A. The cell mixture was incubated for 24 hr in a humidified atmosphere containing 5% CO₂. The cells were then transferred to 96-well microtiter plates that were each coated with a monoclonal antibody directed to mouse interferon gamma (10 μg/ml) (PharMingen, San Diego, CA) or mouse tumor necrosis factor-alpha (TNF-alpha) (PharMingen, San Diego, CA) (10 μg/ml) or mouse transforming growth factor beta (TGF-beta) (R&D System, Minneapolis, MN) (5 μg/ml) for 24hr.

The cells were then removed from the wells by washing several times with 0.25% Tween 20 (v/v) in PBS. The secondary antibodies to individual cytokines were then added to the 96-
well microtiter plate at the concentrations of 4 μg/ml for interferon gamma, 1 μg/ml for TGF-beta and 1 μg/ml for TNF-alpha. The the cytokine-secreting spots in the wells were visualized using the alkaline phosphatase substrate, 5-bromo-4-chloro-3-indolyl phosphate (Sigma). The Con A-treated wells had very high density of cytokine-secreting spots, serving as an indicator of a successful experiment.

In another experiment, ELISPOT analysis was performed to determine PLP (139-151)-responding T cells that secrete interleukin-4, TGF-beta, and TNF-alpha. The concentrations of primary and secondary antibodies for interleukin-4 were 10 μg/ml and 0.6 μg/ml. respectively. The organization of animal groups are summarized in Table 3.

Table 3: Organization of animal groups for ELISPOT analysis.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Mice</th>
<th>Nasal Dosing Protocol</th>
<th>Day after immunization begins in which draining lymph nodes were taken</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5</td>
<td>PBS none</td>
<td>1, 8, 15, 25</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>PBS PLP (139-151) (50μg)</td>
<td>' '</td>
</tr>
<tr>
<td>3</td>
<td>5</td>
<td>PBS lauryl-Tyr-PLP (139-151)</td>
<td>' '</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>PBS oleyl-Tyr-PLP (139-151)</td>
<td>' '</td>
</tr>
</tbody>
</table>

The results of ELISPOT analysis for interleukin 4 is summarized in Figure 8, interferon gamma in Figure 9, TGF-beta in Figure 10, and TNF-alpha in Figure 11. The results indicate that the lauryl-Tyr-PLP (139-151)-treated mice and butanyl-Tyr-PLP (139-151)-treated mice had significantly higher frequency of PLP (139-151) responding T cells secreting interleukin 4 than the PBS-treated mice (Figure 8). The acetyl-Tyr-PLP (139-151)-treated mice and lauryl-Tyr-PLP (139-151)-treated mice had significantly lower frequency of PLP (139-151) responding T cells secreting interferon gamma than the PBS treated mice (Figure 9).
The lauryl-Tyr-PLP (139-151)-treated mice had significantly lower frequency of PLP (139-151) responding T cells secreting TNF-alpha as compared to the PBS-treated and PLP (139-151)-treated mice. The oleyl-Tyr-PLP(139-151)-treated mice had slightly lower frequency of PLP (139-151) responding T cells secreting TNF-alpha as compared to the PBS-treated and PLP (139-151)-treated mice (Figure 11). The acetyl-Tyr-PLP (139-151)-treated mice, lauryl-Tyr-PLP (139-151)-treated mice, oleyl-Tyr-PLP (139-151)-treated mice all had higher frequency of PLP (139-151) responding T cells secreting TGF-beta (Figure 10).

The interferon gamma and TNF-alpha are inflammatory cytokines while interleukin-4 and TGF-beta are both suppressive cytokines. The results suggest that the fatty acid-conjugated PLP (139-151) are able to stimulate T cells that secrete suppressive cytokines while suppress T cells that secrete inflammatory cytokines.

EXAMPLE 5
Preparation of acylated insulin B-chain

Several acyl derivatives of insulin B-chain (9-26) are prepared using FMOC chemistry and solid phase peptide synthesizers as described in EXAMPLE 1, and they are listed in Table 4. The amino acid sequence of insulin B-chain is Glu-Val-Lys-Gln-His-Leu-Cys-Gly-Ser-His-Leu-Val-Glu-Ala-Leu-Tyr-Leu-Val-Cys-Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Met-Ser (see, for example, U.S. Patent No. 5,594,100).

Table 4: Acyl derivatives of insulin B-chain
Insulin B-chain (9-26) is acylated at the 26th tyrosine residue with an n-oleic acid.
Insulin B-chain (9-26) is acylated at the 26th tyrosine residue with an n-caprylic acid.
A. Insulin B-chain (9-26) is acylated at the 26th tyrosine residue with an n-lauric acid.

EXAMPLE 6:
Oral delivery of acylated insulin B-chain (9-26) for suppressing the onset of insulin-dependent diabetes mellitus

There are a total of 20 NOD (non-obese diabetic) mice from the Jackson lab (Bar Harbor, Maine) in each of the groups at the commencement of the experiment. At 4 weeks of age, mice are given orally compound E. Each mouse received two doses a week until 35 weeks old. The mice were fed either phosphate-buffered saline (control) or 10 microgram, 100 microgram or 1 gram of compound E in phosphate-buffered saline. The mice were gavaged with an 18 gauge ball-point needle. Beginning at 12 weeks the urine is collected and tested weekly for the presence of glucose using Glucosuria test tape (Eli Lilly, Indianapolis, IN). If the mice had more than two positive (the presence of sugar in the urine) test on the glucose urine test, a serum sample is taken and tested for blood glucose level (Beckman glucose analyzer). The mice were declared diabetic and sacrificed if the blood glucose was above 220 or higher.

EXAMPLE 7

Inhalation of acylated insulin B-chain (9-26) for the suppression of insulin-dependent diabetes mellitus

The NOD mice are given compound E in aerosol using a nebulizer (American Pharmosel Co., Valencia, CA.). All the procedures and experimental details are as described in example 6 except the dosing route and dose. The doses are 0 and 1 mg. The animals are retained in airtight cages, into which the aerosol is dispensed. The amount of material per unit of area can thus be determined and the results quantified in terms of unit of aerosol material per unit volume of cage area. The nebulizer is attached to an air pressure outlet delivering the equivalent of 7.4 liters of oxygen (the amount of oxygen used in the hospital for nebulization). The nebulizer produces droplets of spray having a diameter of between 0.3 micrometers and about 0.5 micrometers in diameter. One mg of compound E is dispersed in 5 ml phosphate-buffered
saline and nebulized over a 15 min period per cage. During nebulization, a fine mist is created in the cage and the mice move about freely. All other procedures are as described in Example 6.

EXAMPLE 8

T cell proliferation responses to acylated insulin B-chain (9-26)

Groups of NOD mice are immunized in the hind foot pads by a subcutaneous inoculation of compound E (50 μg) in complete Freund's adjuvant. Ten days later, the lymph nodes draining the sites of foot pad injection (popliteal, inguinal and periaortic) are collected and tested for the proliferative response to derivatized compound E as compared to the control peptide (Con A) or insulin B-chain (9-26). Lymphocytes are seeded in 96-well microtiter plates (Costar Corp., Cambridge, MA), 2 X 10⁵ cells in 0.2 ml of HL-1 medium (Ventrex) with 2% autologous serum for 72 hr. The culture medium also contains 2 mM glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin. The compound E (experimental) or insulin B-chain (9-26) (positive control) or ConA (negative control) is added at a concentration of 5 mM/ml in triplicate. The culture condition is at 37° C in 5% CO₂, and the culture period is 72 hr. During the last 16 hr of the 72 culture period, 1 mCi [³H] thymidine is added per well. The incorporation of radio-labeled thymidine is measured by liquid scintillation counting. The ratio of the antigen-driven thymidine incorporation to the background incorporation in the absence of antigen is used to determine the degree of stimulation in T cell proliferation.
EXAMPLE 9

Preparation of acylated Tyr-GP(66-88) fragment

Several acyl derivatives of guinea pig myelin basic protein 66-88 fragment with a tyrosine added to the N-terminus, GP (66-88)-Tyr, are prepared using the FMOC chemistry and solid phase peptide synthesizers as described in EXAMPLE 1. The amino acid sequence of guinea pig myelin basic protein 68-88 fragment (GP MBP 68-88) is Tyr-Gly-Ser-Leu-Pro-Gln-Lys-Ser-Gln-Arg-Ser-Gln-Asp-Glu-Asn-Pro-Val-Val-His-Phe-Phe (J. Immunol., 155, 1599-1605 (1995)).

Table 5: Acyl derivatives of GP-MBP 66-88

<table>
<thead>
<tr>
<th>Description</th>
<th>Tyrosine Acylation</th>
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</thead>
<tbody>
<tr>
<td>Tyr-GP-MBP (66-88) is acylated at the N-terminal added</td>
<td>Tyr with two n-caproic acids.</td>
</tr>
<tr>
<td>Tyr-GP-MBP (66-88) is acylated at the N-terminal added</td>
<td>Tyr with two n-caprylic acids.</td>
</tr>
<tr>
<td>Tyr-GP-MBP (66-88) is acylated at the N-terminal added</td>
<td>Tyr with two n-capric acids.</td>
</tr>
</tbody>
</table>

EXAMPLE 10

Suppression of multiple sclerosis by acyl derivatives of GP-MBP

Experimental autoimmune encephalomyelitis (EAE) is induced in Lewis rats (Charles River, Wilmington, Mass), aged 6-8 weeks, and experimental procedures are as described previously (J. Immunol. (1995) 155: 1599-1605). There are six rats in each group. Individual groups received a dose of 5, 1 or 0 (control) mg/ml of compound I suspended in phosphate-buffered saline. There are four feedings over an 8-day period and three days after the 4th feeding, rats received an intradermal injection in both hind foot pads of guinea pig myelin basic protein (GP-MBP) (25 µg) combined with Mycobacterium tuberculosis Jamaica strain (4 mg/ml). All clinical neurological disorders are monitored daily and the symptom manifestation is scored as the following: 4+, full hind lumb paralysis; 3+ partial hind limb paralysis; 2+ ataxia;
1+ flaccid tail and 0, normal. Observation ends 21 day after induction of EAE and rats are sacrificed.

EXAMPLE 11
All procedures are as described in EXAMPLE 10 except that compound I is given using a nebulizer.
EXAMPLE 12

Chick type II collagen 190-200 fragment is extended by adding a tyrosine at the C-terminus of the 190-200 fragment and is also acylated with various fatty acids using the FMOC chemistry and solid phase peptide synthesizers as described in EXAMPLE 1. These conjugates are listed in Table 6. The amino acid sequence of the 190-200 fragment is GPRGESGTBGS.

Table 6. Acyl derivatives of Tyr-chick type II collagen

190-200

Collagen (190-200)-Tyr is acylated at the 12th tyrosine residue with an n-lauric acid.
Collagen (190-200)-Tyr is acylated at the 12th tyrosine residue with an n-palmityl acid.
A. Collagen (190-200)-Tyr is acylated at the 12th tyrosine residue with an n-stearic acid.

EXAMPLE 13

Compound K are given to rats at doses of 0.1, 1 and 5 mg per animal. Adjuvant arthritis, which is used as the disease model for rheumatoid arthritis, is induced by injecting Freund's complete adjuvant in the base of the tail. There are six rats in each experimental group and the rats are treated with compound K administered in 1 ml PBS orally by gavage on days, -8, -6, -4, -2, 0, +2,+ 4, + 6, and +8, relative to the induction of arthritis. The clinical arthritis score is described on a 0-4 scale for each of four paws of the rats according to J. Exp. Med. 146: 857, 1997 as follows: 4, joint deformity; 3, severe swelling; 2, redness plus mild swelling; 1, redness only; 0, normal. Each day, the rats are evaluated for their clinical arthritis score. Individual scores of four paws are added together for the score of each animal. The mean clinical score of each treated group is determined by summing the total score of all animals in the group and dividing the total score by the number of rats in the group.
EXAMPLE 14

All procedures are as described in EXAMPLE 13 except that the doses are 0.01, 0.1, and 1 mg and that compound K is given using a nebulizer.

EXAMPLE 15

Preparation of acylated GAD peptide

Several acyl derivatives of GAD peptides are prepared using the FMOC chemistry and solid phase peptide synthesizers as described in EXAMPLE 1, and these conjugates are listed in Table 7.

Table 7: Acyl derivatives of GAD peptides

GAD peptide analog, Tyr-Phe-Pro-Glu-Val-Lys-Glu-Lys-Gly, is acylated at N-terminal Tyr residue with two n-caproic acids.

GAD peptide analog, Tyr-Phe-Pro-Glu-Val-Lys-Glu-Lys-Gly, is acylated at N-terminal Tyr residue with two n-caprylic acids.

GAD peptide analog, Tyr-Phe-Pro-Glu-Val-Lys-Glu-Lys-Gly, is acylated at N-terminal Tyr residue with two n-lauric acids.

EXAMPLE 16

Nasal delivery of acylated GAD peptide for suppressing the onset of insulin-dependent diabetes mellitus

There are a total of 20 NOD (non-obese diabetic mice) from the Jackson lab (Bar Harbor, Maine) in each of the groups at the commencement of the experiment. At 4 weeks of age, mice are given nasally compound N. Each mouse received two doses a week until 35 weeks old. The mice are fed either PBS (control) or 10 microgram, 100 microgram or 1 gram of compound N in PBS. The mice are gavaged with an 18 gauge ball-point needle. Beginning at 12 weeks the urine is collected and tested weekly for the presence of glucose using Glucosuria test tape (Eli Lilly, Indianapolis, IN). If the mice has more than two positive (the presence of sugar in the urine) on the glucose urine test, a serum sample is taken and tested for blood glucose
level (Beckman glucose analyzer). The mice are declared diabetic and sacrificed if the blood glucose is above 220 or higher.

EXAMPLE 17

5 Inhalation of acylated GAD peptide for the suppression of insulin-dependent diabetes mellitus

The NOD mice are given compound N in aerosol using a nebulizer (American Pharmosel Co., Valencia, CA.). All the procedures and experimental details are as described in EXAMPLE 16 except the dosing route and dose. The doses are 0 and 250 µg. The animals are retained in airtight cages, into which the aerosol is dispensed. The amount of material per unit of area can thus be determined and the results quantified in terms of unit of aerosol material per unit volume of cage area. The nebulizer is attached to an air pressure outlet delivering the equivalent of 7.4 liters of oxygen (the amount of oxygen used in the hospital for nebulization). The nebulizer produces droplets of spray having a diameter of between 0.3 micrometers and about 0.5 micrometers in diameter. One mg of compound N is dispersed in 5 ml phosphate-buffered saline and nebulized over a 15 min period per cage. During nebulization, a fine mist is created in the cage and the mice move about freely. All other procedures are as described in EXAMPLE 6.

EXAMPLE 18

T cell proliferatation responses to acylated GAD peptide

Groups of NOD mice are immunized in the hind foot pads by a subcutaneous inoculation of compound N (50 µg) in complete Freund's adjuvant. Ten days later, the lymph nodes draining the sites of foot pad injection (popliteal, inguinal and periaortic) are collected and tested for the proliferative response to derivatized compound N as compared to the control peptide (Con A) or nonmodified GAD peptide. Lymphocytes are seeded in 96-well microtiter plates (Costar Corp., Cambridge, MA), 2 X 10^5 cells in 0.2 ml of HL-1 medium (Ventrex) with 2% autologous serum for
72 hr (21). The culture medium also contains 2 mM glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin. The compound N (experimental) or non-modified GAD peptide (positive control) or ConA (negative control) is added at a concentration of 5 mM/ml in triplicate. The culture condition is at 37°C in 5% CO₂, and the culture period is 72 hr. During the last 16 hr of the 72 culture period, 1 mCi [³H] thymidine is added per well. The incorporation of radio-labeled thymidine is measured by liquid scintillation counting. The ratio of the antigen-driven thymidine incorporation to the background incorporation in the absence of antigen is used to determine the degree of stimulation in T cell proliferation.
WHAT IS CLAIMED IS

1. A conjugate comprising an agent and at least one fatty acid, wherein said agent is selected from the group consisting of an autoantigen, an immune-reactive peptide fragment of autoantigen, an analog of said autoantigen, and an analog of said immune-reactive peptide fragment.

2. The conjugate of Claim 1 wherein said conjugate stimulates in vitro proliferation of mammalian T lymphocytes.

3. The conjugate of Claim 1 wherein said fatty acid is selected from the group consisting of saturated fatty acids, unsaturated fatty acids, and high molecular weight fatty acids containing more than 20 carbon atoms.

4. The conjugate of Claim 1 wherein said agent is selected from the group consisting of myelin basic protein, an autoimmune-suppressive peptide derived from myelin basic protein, an analog of myelin basic protein, and an analog of said peptide.

5. The conjugate of Claim 1 wherein said agent is selected from the group consisting of proteolipoprotein, an autoimmune-suppressive peptide derived from proteolipoprotein, an analog of proteolipoprotein and an analog of said peptide.

6. The conjugate of Claim 1 wherein said agent is selected from the group consisting of glutamic acid decarboxylase, an autoimmune-suppressive peptide derived from glutamic acid decarboxylase, an analog of glutamic acid decarboxylase, and an analog of said peptide.

7. The conjugate of Claim 1 wherein said agent is selected from the group consisting of insulin, an autoimmune-suppressive peptide derived from insulin, an analog of insulin, and an analog of said peptide.
8. The conjugate of Claim 1 wherein said agent is selected from the group consisting of type II collagen, an autoimmune-suppressive peptide derived from of type II collagen, an analog of type II collagen, and an analog of said peptide.

9. A method of Claim 1 for ameliorating an autoimmune disease in a mammal, said method comprising the step of administering to the mammal a therapeutic amount of a conjugate comprising an agent and at least one fatty acid.

10. The method of Claim 9 wherein said agent is selected from the group consisting of an autoantigen, an immune-reactive peptide fragment of autoantigen, an analog of said autoantigen, and an analog of said immune-reactive peptide fragment.

11. The method of Claim 9 wherein said autoimmune disease is multiple sclerosis.

12. The method of Claim 9 wherein said autoimmune disease is juvenile diabetes.

13. The method of Claim 9 wherein said autoimmune disease is rheumatoid arthritis.

14. The method of Claim 9 wherein said agent is administered to tissue that is the target of autoimmune attack.

15. The method of Claim 9 wherein said administration is selected from the group consisting of injections, intra-thymus injection, buccal delivery, nasal delivery, pulmonary delivery, vaginal delivery, colonic delivery, rectal delivery, distal small intestinal delivery and oral delivery.
16. A method for the diagnosis and treatment of the autoimmune disease juvenile diabetes, comprising using an effective amount of a conjugate comprising an agent and at least one fatty acids.

17. The method of Claim 16 wherein said conjugate is selected from the group consisting of fatty acid-conjugated insulin A-chain, fatty acid-conjugated insulin B-chain, fatty acid-conjugated autoimmune-suppressive peptide fragments of insulin B-chain, fatty acid-conjugated autoimmune-suppressive peptide fragments of insulin A-chain, fatty acid-acylated autoimmune-suppressive peptide fragment analogs of insulin B-chain, fatty acid-acylated autoimmune-suppressive peptide fragment analogs of insulin A-chain, fatty acid-acylated glutamic acid decarboxylase, fatty acid-acylated autoimmune-suppressive peptide fragments of glutamic acid decarboxylase, and fatty acid-conjugated autoimmune-suppressive peptide fragment analogs of glutamic acid decarboxylase.

18. The conjugate of Claim 16 wherein said fatty acid is selected from the group consisting of saturated fatty acids, non-saturated fatty acids, and high molecular weight fatty acids containing more than 20 carbon atoms.

19. The method of Claim 16 wherein said administration is selected from the group consisting of injections, intra-thymus injection, buccal delivery, nasal delivery, pulmonary delivery, vaginal delivery, colonic delivery, rectal delivery, distal small intestinal delivery and oral delivery.

20. A method for the diagnosis and treatment of multiple sclerosis in a mammal, comprising using an effective amount of a conjugate comprising an agent conjugated to at least one fatty acids.
21. The method of Claim 20 wherein said conjugate is selected from the group consisting of fatty acid-acylated myelin basic protein, fatty acid-acylated autoimmune-suppressive peptide fragments of myelin basic protein, fatty acid-conjugated autoimmune-suppressive peptide fragment analogs of myelin basic protein, fatty acid-acylated proteolipoprotein, fatty acid-acylated autoimmune-suppressive peptide fragments of proteolipoprotein, fatty acid-conjugated autoimmune-suppressive peptide fragment analogs of proteolipoprotein.

22. The conjugate of Claim 20 wherein said fatty acid is selected from the group consisting of saturated fatty acids, non-saturated fatty acids, and high molecular weight fatty acids containing more than 20 carbon atoms.

23. The method of Claim 20 wherein said administration is selected from the group consisting of injections, intra-thymus injection, buccal nasal delivery, pulmonary delivery, vaginal delivery, colonic delivery, rectal delivery, distal small intestinal delivery and oral delivery.

24. A method for the diagnosis and treatment of rheumatoid arthritis in a mammal, comprising using an effective amount of a conjugate comprising an agent conjugated to at least one fatty acids.

25. The method of Claim 24 wherein said conjugate is selected from the group consisting of fatty acid-acylated type II collagen, fatty acid-acylated collagen, fatty acid-acylated autoimmune-suppressive peptide fragments of type II collagen, fatty acid-acylated autoimmune-suppressive peptide fragments of collagen, fatty acid-conjugated autoimmune-suppressive peptide fragment analogs of type II collagen, fatty acid-conjugated autoimmune-suppressive peptide fragment analogs of collagen.
26. The conjugate of Claim 24 wherein said fatty acid is selected from the group consisting of saturated fatty acids, non-saturated fatty acids, and high molecular weight fatty acids containing more than 20 carbon atoms.

27. The method of Claim 24 wherein said administration is selected from the group consisting of injections, intra-thymus injection, buccal nasal delivery, pulmonary delivery, vaginal delivery, colonic delivery, rectal delivery, distal small intestinal delivery and oral delivery.
Figure 1. Lauric acid-conjugated PLP (139-151) reduces EAE severity scores in SJL mice to a greater extent than non-conjugated PLP (139-151)
Figure 2. Oleic acid-conjugated PLP (139-151)-reduces EAE severity scores in SJL mice to a greater extent than non-conjugated PLP (139-151).
Figure 3. Acetic acid-conjugated PLP (139-151) reduces EAE severity scores in SJL mice to a greater extent than non-conjugated PLP (139-151).
Figure 4. Lauric acid-conjugated PLP (139-151) reduces EAE severity scores in SJL mice to a greater extent than nonconjugated PLP (139-151)
Figure 5. Butyric acid-conjugated PLP (139-151) reduces EAE severity scores in SJL mice to a greater extent than non-conjugated PLP (139-151).
Figure 6. Octanoic acid-conjugated PLP (139-151) reduces EAE severity scores in SJL mice to a greater extent than non-conjugated PLP (139-151).
Figure 7. Proliferative response in PLP (139-151) primed SJL mice.
Figure 8. Plot of ELISPOT analysis of IL-4-secreting T cells
Figure 9. Plot of ELISPOT analysis of IFN-gamma secreting cells

Ratio of frequency of spot-forming cells relative to the non PNP control

PBS  BA 101  BA 102  BA 103
Figure 10a. Plot of ELISPOT analysis for TGF-beta-secreting cell

Ratio of frequency of spot-forming cells relative to the non-PLP control

- PBS
- BA 101
- BA 103
- BA 105
Figure 10b. Plot of ELISPOT analysis for TGF-beta-secreting cell

Ratio of frequency of spot-forming cells relative to the non-PUP control

PBS  BA 101  BA 102  BA 103
Figure 11. Plot of ELISPOT analysis of TNF-alpha-secreting cell
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(s) : A61K 35/12, 38/02, 39/00, 49/00; C07K 14/00, 14/78, 17/00
US CL : 414/185.1, 193.1, 283.1; 530/345, 402, 403

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)

U.S. : 414/185.1, 193.1, 283.1; 530/345, 402, 403

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

NONE

Electronic database consulted during the international search (name of database and, where practicable, search terms used)

MEDLINE, SCISearch, BIOSIS, DERWENT WORLD PATENT, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category*</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
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</table>

[ ] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* 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 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
  "*" document member of the same patent family

Date of the actual completion of the international search: 02 APRIL 1999

Date of mailing of the international search report: 12 MAY 1999

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks

Box PCT
Washington, D.C. 20231
Facsimile No. (703) 305-3230

Authorized officer
Martha Lubet
Telephone No. 703 308 0196

JOYCE BRIDGES
PARALEGAL SPECIALIST
CHEMICAL MATRIX
### INTERNATIONAL SEARCH REPORT

#### C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
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<th>Relevant to claim No.</th>
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<tbody>
<tr>
<td>Y</td>
<td>US 5,284,935 A (CLARK et al.) 08 February 1994, see entire document, especially column 11, lines 14-36.</td>
<td>1-4, 9-27</td>
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<td>Y</td>
<td>US 5,512,447 A (BAEKKESKOV et al.) 30 April 1996, see entire document.</td>
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<tr>
<td>Y</td>
<td>US 5, 571,499 A (HAFLER et al.) 05 November 1996, see entire document.</td>
<td>1-4, 9-27</td>
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<td>Y</td>
<td>US 5,256,641 A (YATVIN et al.) 26 October 1993, see entire document.</td>
<td>1-4, 4-27</td>
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<td>Y</td>
<td>US 5,674,978 A (TOBIN et al.) 07 October 1997, see entire document.</td>
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</table>
INTERNATIONAL SEARCH REPORT

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

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

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

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

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

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

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

Please See Extra 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.:
   1-4, 9-27

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

Remark on Protest ☐ The additional search fees were accompanied by the applicant’s protest.
☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)∗
BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-4, drawn to a conjugate comprising a fatty acid and a myelin basic protein antigen, peptide or analog.

Group II, claim(s) 1-3 and 5, drawn to a conjugate comprising a fatty acid and a proteolipoprotein antigen, peptide or analog.

Group III, claim(s) 1-3 and 6, drawn to a conjugate comprising a fatty acid and a glutamic acid decarboxylase antigen, peptide or analog.

Group IV, claim(s) 1-3 and 7, drawn to a conjugate comprising a fatty acid and insulin antigen, peptide or analog.

Group V, claim(s) 1-3 and 8 drawn to a conjugate comprising a fatty acid and collagen antigen, peptide or analog.

Group VI, claim(s) 9-10, 11, 14, 15, 20-23, drawn to a method of ameliorating an multiple sclerosis by administering a conjugate comprising a fatty acid and a myelin basic protein antigen, peptide or analog.

Group VII, claim(s) 9-10, 11, 14, 15, 20-23, drawn to a method of ameliorating an multiple sclerosis by administering a conjugate comprising a fatty acid and a proteolipoprotein antigen, peptide or analog.

Group VIII, claim(s) 9-10, 12, 14-19, drawn to a method of ameliorating an juvenile diabetes by administering a conjugate comprising a fatty acid and a glutamic acid decarboxylase antigen, peptide or analog.

Group IX, claim(s) 9-10, 12, 14-19, drawn to a method of ameliorating juvenile diabetes by administering a conjugate comprising a fatty acid and insulin antigen, peptide or analog.

Group X, claim(s) 9-10, 13, 14, 15, 24-27 drawn to a method of ameliorating rheumatoid arthritis by administering a conjugate comprising a fatty acid and collagen antigen, peptide or analog.

Group XI, claim(s) 20-23, drawn to a method of diagnosing an multiple sclerosis by using a conjugate comprising a fatty acid and a myelin basic protein antigen, peptide or analog.

Group XII, claim(s) 20-23, drawn to a method of diagnosing an multiple sclerosis by using a conjugate comprising a fatty acid and a proteolipoprotein antigen, peptide or analog.

Group XIII 24-27 drawn to a method of diagnosing rheumatoid arthritis by using a conjugate comprising a fatty acid and collagen antigen, peptide or analog.

The inventions listed as Groups I-XIII do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The technical feature linking Groups I-XIII appears to be that they relate to conjugate of a autoantigen to fatty acid.

Cohen WO 90/10449 teach a conjugate comprising an immune-reactive peptide of an heat shock protein autoantigen conjugated to a fatty acid and that such a conjugate could be used to induce tolerance to the autoantigen and treat diabetes.

Smiluck et al. (PNAS 88:9633, 1991) teach that myelin basic protein peptide Acl-11 induced experimental autoimmune encephalomyelitis and that immunization with an analog of Acl-11 prevents EAE. Therefore it would be obvious to one with skill in the art to make a conjugate comprising a MBP peptide or analog such as the ones taught by Smiluck et al. and a fatty acid with the expectation that such a conjugate could be used to downregulate the immune response to MBP.
Therefore the technical feature linking the inventions of groups I-XII does not constitute a special technical feature as defined by PCT Rule 13.2, as it does not define a contribution over the prior art.

The special technical feature of Groups I is considered to be a conjugate of fatty acid to myelin basic protein or analog of myelin basic protein.

The special technical feature of Groups II is considered to be conjugate of fatty acid to proteolipoprotein or analog of proteolipoprotein.

The special technical feature of Groups III is considered to be conjugate of fatty acid to glutamic acid decarboxylase or analog of glutamic acid decarboxylase.

The special technical feature of Groups IV is considered to be conjugate of fatty acid to insulin or analog of insulin.

The special technical feature of Groups V is considered to be conjugate of fatty acid to collagen or an analog of collagen.

The inventions of each of groups I-V have distinct biochemical and functional properties.

Groups VI-X are methods of using each of the inventions of Groups I-V to ameliorate a particular autoimmune disease.

Groups XI-XIII are drawn to methods of using each of the inventions of Groups I-II and V to diagnose a particular autoimmune disease.

Accordingly, Groups I-XIII are not so linked by the same or a corresponding special technical feature as to for a single general inventive concept.