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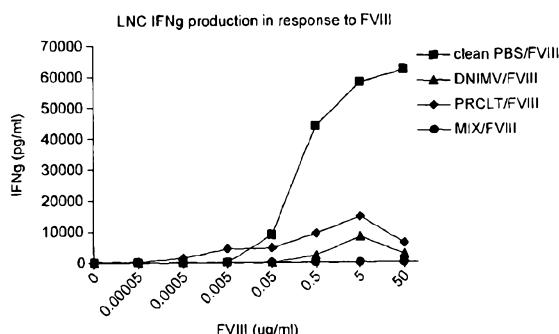


FIG. 8

(57) Abstract: The present invention provides peptides at least partly derivable from FVIII which are capable of binding to an MHC class II molecule without further antigen processing and being recognised by a factor VIII specific T cell. In particular, the present invention provides a peptide comprising or consisting of the sequence EDNIMVTFRNQASR. The present invention also relates to the use of such a peptide for the prevention or suppression of inhibitor antibody formation in haemophilia A and/or acquired haemophilia.

PEPTIDE

FIELD OF THE INVENTION

The present invention relates to a peptide. In particular, it relates to peptides at least the core sequence of which is derivable from factor VIII (FVIII). The peptides can be used to reduce or prevent factor VIII inhibitor antibody formation, for example in haemophilia A treatment and acquired haemophilia.

BACKGROUND TO THE INVENTION

HAEMOPHILIA

Haemophilia belongs to a group of inheritable blood disorders that includes haemophilia A, haemophilia B (Christmas disease) and Von Willebrand's disease.

In haemophilia, the blood's ability to clot is severely reduced because an essential clotting factor is partly or completely missing, resulting in increased bleeding time.

Haemophilia A is a deficiency of the clotting factor VIII, whereas Haemophilia B is a deficiency of clotting factor IX. In both diseases, the faulty gene is found on the X chromosome, so the conditions are X-linked. Haemophilia A is five times more common than haemophilia B.

Haemophilia is a lifelong inherited genetic condition, which affects females as carriers and males who inherit the condition. About a third of new diagnoses are where there is no previous family history. It appears world-wide and occurs in all racial groups. About 6,000 people are affected with haemophilia in the UK.

Haemophiliacs bleed for a prolonged period following injury. External injuries such as cuts and grazes do not usually pose serious problems: it is often possible to stop bleeding by applying a degree of pressure and covering the affected area (e.g with a plaster).

The main problem is internal bleeding into joints, muscles and soft tissues, which can occur spontaneously. Internal bleeding, such as haemorrhages into the brain, is very difficult to manage and can be fatal. Repeated bleeding in the joints causes acute pain and can cause arthritis and/or long-term joint damage leading to disability.

Treatment for haemophilia is usually by replacement of the missing clotting factor. In mild or moderate haemophilia injections may be given at the time a bleed occurs (on-demand therapy). However, in severe haemophilia regular prophylactic injections are given to help the blood to clot and minimise the likelihood of long term joint damage.

A potentially serious complication of coagulation factor replacement therapy for haemophilia A is the development of antibodies that neutralise the procoagulant function of factor VIII. Factor VIII inhibitors occur in approximately 25% of those with severe haemophilia A. Since patients with congenital haemophilia A can be genetically deficient in FVIII, the synthesis of inhibitors is an alloimmune response to the foreign protein administered to prevent or treat bleeding episodes.

CD4+ T cells play a central role in the immune response to FVIII. After being taken up by antigen-presenting cells (APCs), FVIII undergoes proteolytic degradation into peptide fragments (Reding *et al* (2006) Haemophilia 12(supp 6) 30-36). These peptides are then presented on the surface of the APC in association with MHC class II molecules. This complex is then recognised by the T cell receptor of a CD4+ cell specific for FVIII. In the presence of the appropriate costimulatory signals, this recognition ultimately causes the CD4+ cell to direct the synthesis of antibodies by B cells.

The incidence of inhibitor formation initially increases with the number of factor VIII treatments, but appears to plateau after 50-100 exposure days. Inhibitor formation is much more common in severe haemophilia than in moderate or mild disease and some molecular defects, most clearly large deletions and nonsense mutations in the factor VIII light chain, appear to predispose to inhibitor formation. Parameters such as the concentration, type (purified or recombinant) of replacement factor, and treatment history may also affect the likelihood of antibody production.

The management of haemophilia patients with inhibitors is an ongoing challenge. Immune tolerance induction (ITI) using a desensitization technique is successful in some patients with alloantibodies against factor VIII. This therapeutic approach requires ongoing exposure to factor replacement therapy, so is a long-term strategy.

Although ITI can be successful, a significant proportion (about 30%) of patients fail to respond to ITI. Patients with high inhibitor titres are much less likely to respond to treatment. Another significant contributing factor is age at the start of commencing ITI, with greatly decreased success rates when the patient is older than 20 (Hay *et al* (2005) *Seminars in Thrombosis and Hemostasis* 32:15-21)

When ITI therapy is unsuccessful, the inhibitor generally persists for life, and because such patients are usually high-responders, it is necessary to treat episodes of bleeding with FVIII bypassing products, such as activated prothrombin complex concentrates (FEIBATM), and recombinant-activated FVII. However, the use of such agents is associated with adverse events such as disseminated intravascular coagulation, acute myocardial infarction, pulmonary embolus and thromboses (Acharya and DiMichele (2006) *Best Practice & Research Clinical Haematology* 19:51-66).

Immunosuppressive therapy is sometimes used for patients who fail to respond to ITI. Treatment includes administration of immunosuppressive drugs such as cyclophosphamide, prednisone, azathioprine and cyclosporine which non-specifically target the immune system. These treatments can have side-effects associated with general immunosuppression.

There is renewed interest on selective B cell depletion using RituximabTM, a humanised monoclonal antibody to B cell CD20 antigen. However, infusion reactions, serum sickness and opportunistic infections have occurred in some children treated with this drug (DiMichele (2007) *J Thromb Haemost* 5:143-50).

ACQUIRED HAEMOPHILIA

Acquired haemophilia is a rare autoimmune condition which affects between 1 and 4 people in every million. In this condition, subjects who are not born with haemophilia

develop antibodies against one of the clotting factors such as factor VIII. It is thought that pregnancy and autoimmune diseases such as rheumatoid arthritis and cancer may increase the risk of developing acquired haemophilia. Although there are differences in the underlying immune mechanisms leading to their production, the clinical manifestations of FVIII inhibitors produced in response to coagulation factor replacement therapy and those produced in acquired haemophilia are similar.

Acquired haemophiliac patients have a mortality rate that approaches 25%, partly because of the association of acquired inhibitors with severe bleeding complications. The therapy of acquired autoantibody inhibitors is based primarily on the need to control or prevent acute hemorrhagic complications, which frequently are life and limb threatening and secondarily to eradicate the autoantibody to restore normal coagulation.

Some bleeds associated with low titre autoantibody inhibitors (< 5 Bethesda Units) may be treated effectively with FVIII concentrates administered at high doses. Porcine FVIII concentrate was formerly considered a critical first-line therapy for acquired hemophilia-related bleeding since it was the only replacement therapy that provided an opportunity to actually measure post-infusion FVIII coagulation activity levels in the laboratory. The product was removed from the marketplace in 2004 because of contamination of the porcine plasma pools by porcine parvovirus. Now, "bypassing" agents are most commonly used, but potential risks of thrombogenicity exist and there is only about 80% efficacy for each product. Plasma exchange via plasmapheresis and extracorporeal immunoabsorption may be necessary to temporarily reduce the inhibitor titer enough for bypassing agents or FVIII replacement to provide adequate hemostasis.

Eradication of autoantibody inhibitors depends on immunosuppressive measures, such as: (1) administration of corticosteroids with 30%–50% efficacy in 3–6 weeks; (2) use of cytotoxic and myelosuppressive chemotherapeutic agents, e.g., cyclophosphamide, cyclosporine, 2-chlorodeoxyadenosine; (3) immunomodulation with intravenous immunoglobulin; and (4) selective B-lymphocyte depletion with rituximab. RituximabTM responders may require concurrent use of steroids and relapses may respond to retreatment.

Thus, all currently available methods for reducing alloantibody production associated with haemophilia A treatment, and autoantibody production in acquired haemophilia,

have shortcomings. There is therefore a need for improved methods to address the issue of anti-FVIII antibodies in haemophilia A and acquired haemophilia.

The present inventors have found that it is possible to prevent FVIII inhibitor antibody formation by pre-tolerising the patient with FVIII-derived peptides.

SUMMARY OF ASPECTS OF THE INVENTION

The first aspect of the present invention, therefore, relates to a peptide, at least part of the sequence of which is derivable from FVIII, which is capable of inducing or restoring tolerance to FVIII.

In a first embodiment, the present invention provides a peptide comprising one of the following FVIII-derived sequences:

GTLMVFFGNVDSSGI
TQTLHKFILLFAVFD
SLYISQFIIMYSLDG
PPIIARYIRLHPTHY
PPLLTRYLRIHPQSW
MHTVNGYVNRSLPGL
LGQFLLFCHISSHQH
DTLLIIFKNQASRPY
PRCLTRYYSSFVNME
TENIQRFLPNPAGVQ
DNIMVTFRNQASRPY
RYLRIHPQSWVHQIA

with one or more of the following modifications:

- (i) removal of one or more hydrophobic amino acid(s);
- (ii) replacement of one or more hydrophobic amino acid(s) with charged hydrophilic amino acid(s); and
- (iii) insertion of a charged amino acid at one or both terminus(i)

which modified peptide is capable of binding to an MHC class II molecule without further antigen processing and being recognised by a factor VIII specific T cell.

The "parent" (unmodified) peptide may be PRCLTRYYSSFVNME or DNIMVTFRNQASRPY

In a second embodiment the present invention provides a peptide comprising the sequence

$X(aa)_n$ -core sequence -(aa)_m
wherein X is a charged hydrophilic residue;
aa is an amino acid;
n is an integer between 0 and 5;
m is an integer between 0 and 5; and
the "core sequence" is selected from the following group of FVIII-derived peptides: LYISQFIIM

FIIMYSLDG

IARYIRLHP

LIIFKNQAS

LTRYYSSFV

MVTFRNQAS

LRIHPQSWV

which peptide is capable of binding to an MHC class II molecule without further antigen processing and being recognised by a factor VIII specific T cell.

For example, the peptide may comprise the sequence

XdNIMVTFRNQAS.

In a third embodiment, the present invention provides a peptide comprising the sequence:

$Y(aa)_n$ -core sequence-(aa)_m Z
wherein Y and Z are charged amino acids having reverse polarity;
aa is an amino acid;
n is an integer between 0 and 5;
m is an integer between 0 and 5; and
the "core sequence" is selected from the following group of FVIII-derived peptides:

LYISQFIIM

FIIMYSLDG

IARYIRLHP

LIIFKNQAS

LTRYYSSFV

MVTFRNQAS

LRIHPQSWV

which peptide is capable of binding to an MHC class II molecule without further antigen processing and being recognised by a factor VIII specific T cell.

For example, the peptide may comprise the sequence:

YDNIMVTFRNQASZ

In the third embodiment, for example, Y may be a positively charged amino acid and Z may be a negatively charged amino acid. Alternatively, Y may be a negatively charged amino acid and Z may be a positively charged amino acid.

A charged, hydrophilic amino acid may, for example be D, E, K, H or R. A positively charged amino acid may, for example be K, H or R. A negatively charged amino acid may, for example be D or E.

The peptide of the first aspect of the invention may, for example, comprise or consist of the sequence EDNIMVTFRNQASR.

In a second aspect, the present invention provides a composition, such as a pharmaceutical composition comprising one or more peptide(s) of the first aspect of the invention. The composition may comprise a plurality of peptides wholly or partly derivable from FVIII which are capable of inducing or restoring tolerance to FVIII.

The composition may be in the form of a kit, in which the plurality of peptides are provided separately for separate, subsequent, sequential or simultaneous administration.

The peptide or a composition of the invention may be for use in suppressing, reducing, or preventing the development of factor VIII inhibitor antibodies.

The present invention also provides the use of such a peptide or composition in the manufacture of a medicament to suppress, reduce or prevent the development of factor VIII inhibitor antibodies.

The present invention also provides a method for suppressing, preventing or reducing the development of Factor VIII inhibitor antibodies in a subject, which comprises the step of administration of such a peptide or composition to the subject.

The subject may be deficient in FVIII. In particular the subject may have haemophilia A, and may be, or be about to, undergo factor VIII replacement therapy.

Alternatively the subject may have, or be at risk from contracting, acquired haemophilia.

Factor VIII inhibitors are found more frequently in individuals expressing HLA-DR2. The subject treated by the method of the invention may therefore be HLA-DR2 positive.

DESCRIPTION OF THE FIGURES

Figure 1: Recall responses for lymph node cells (LNC) from FVIII+DR2+ mice primed with rhFVIII/CFA

- a) LNC proliferation to FVIII peptides 1-6
- b) LNC proliferation to FVIII peptides 7-12
- c) LNC proliferation to FVIII peptides 1, 3 and 11

Figure 2: Representative examples of FVIII+DR2+ T cell hybridoma clones specific for FVIII-derived peptides

Figure 3: Recall responses for LNC from FVIII-DR2+ mice primed with rhFVIII/CFA

Figure 4: Representative examples of FVIII-DR2+ T cell hyridoma clones specific for FVIII-derived peptides

Figure 5: FVIII-/- clones specific for a) DNIMV and b) PRCLT

Figure 6: Recall responses for LNC to FVIII for FVIII+DR2+ mice treated 3x i.p. with peptide prior to priming with rhFVIII/CFA.

Figure 7: Determination of the range of peptide epitopes capable of functions as apitopes using FVIII-DR2+ T cell hybridoma clones specific for FVIII-derived overlapping peptides. The original peptide is termed 0. One amino acid shift towards the N-terminal is -1, two amino acid shifts towards the N-terminal is -2 etc. One shift towards the C-terminal is +1 etc.

Figure 8: Lymph node cell IFN-gamma production in response to FVIII for FVIII-DR2+ mice treated with FVIII-derived peptides PRCLT, DNIMV or a mixture of both of these.

Figure 9: Responses from naive or tolerised mice stimulated with either EDNIMVTFRNQASR (EDNIMV) or a control peptide (DNIMV).

DETAILED DESCRIPTION

PEPTIDE

The present invention relates to a peptide.

The term "peptide" is used in the normal sense to mean a series of residues, typically L-amino acids, connected one to the other typically by peptide bonds between the α -amino and carboxyl groups of adjacent amino acids. The term includes modified peptides and synthetic peptide analogues.

The peptide of the present invention may be made using chemical methods (Peptide Chemistry, A practical Textbook. Mikos Bodansky, Springer-Verlag, Berlin.). For example, peptides can be synthesized by solid phase techniques (Roberge JY *et al* (1995) *Science* 269: 202-204), cleaved from the resin, and purified by preparative high performance liquid chromatography (e.g., Creighton (1983) *Proteins Structures And Molecular Principles*, WH Freeman and Co, New York NY). Automated synthesis may

be achieved, for example, using the ABI 431 A Peptide Synthesizer (Perkin Elmer) in accordance with the instructions provided by the manufacturer.

The peptide may alternatively be made by recombinant means, or by cleavage of the peptide from factor VIII followed by modification of one or both ends. The composition of a peptide may be confirmed by amino acid analysis or sequencing (e.g., the Edman degradation procedure).

For practical purposes, there are various other characteristics which the peptide may show. For example, it is important that the peptide is sufficiently stable *in vivo* to be therapeutically useful. The half-life of the peptide *in vivo* may be at least 10 minutes, 30 minutes, 4 hours, or 24 hours.

The peptide may also demonstrate good bioavailability *in vivo*. The peptide may maintain a conformation *in vivo* which enables it to bind to an MHC molecule at the cell surface without due hindrance.

CORE RESIDUES

In an adaptive immune response, T lymphocytes are capable of recognising internal epitopes of a protein antigen. Antigen presenting cells (APC) take up protein antigens and degrade them into short peptide fragments. A peptide may bind to a major histocompatibility complex (MHC) class I or II molecule inside the cell and be carried to the cell surface. When presented at the cell surface in conjunction with an MHC molecule, the peptide may be recognised by a T cell (via the T cell receptor (TCR)), in which case the peptide is a T cell epitope.

An epitope is thus a peptide derivable from an antigen which is capable of binding to the peptide-binding groove of a MHC class I or II molecule and be recognised by a T cell.

The minimal epitope is the shortest fragment derivable from an epitope, which is capable of binding to the peptide-binding groove of a MHC class I or II molecule and being recognised by a T cell. For a given immunogenic region, it is typically possible

to generate a "nested set" of overlapping peptides which act as epitopes, all of which contain the minimal epitope but differ in their flanking regions.

By the same token, it is possible to identify the minimal epitope for a particular MHC molecule:T cell combination by measuring the response to truncated peptides. For example if a response is obtained to the peptide comprising residues 1-15 in the overlapping library, sets which are truncated at both ends (i.e. 1-14, 1-13, 1-12 etc. and 2-15, 3-15, 4-15 etc.) can be used to identify the minimal epitope.

The present invention provides peptides comprising a "core residue" sequence of FVIII which selected from the following list:

LYISQFIIM

FIIMYSLDG

IARYIRLHP

LIIFKNQAS

LTRYYSSFV

MVTFRNQAS

LRIHPQSWV

These core residue sequences were predicted using HLA-DR2 binding algorithms to represent or comprise the minimal epitope for each region, as shown in the Examples.

APITOPES

The present inventors have previously determined that there is a link between the capacity of a peptide to bind to an MHC class I or II molecule and be presented to a T cell without further antigen processing, and the peptide's capacity to induce tolerance *in vivo* (WO 02/16410). If a peptide is too long to bind the peptide binding groove of an MHC molecule without further processing (e.g. trimming), or binds in an inappropriate conformation then it will not be tolerogenic *in vivo*. If, on the other hand, the peptide is of an appropriate size and conformation to bind directly to the MHC peptide binding groove and be presented to a T cell, then this peptide can be predicted to be useful for tolerance induction.

It is thus possible to investigate the tolerogenic capacity of a peptide by investigating whether it can bind to an MHC class I or II molecule and be presented to a T cell without further antigen processing *in vitro*.

The peptides of the present invention are apitopes (Antigen Processing-Independent epiTOPES) in that they are capable of binding to an MHC class II molecule and stimulating a response from factor VIII specific T cells without further antigen processing. Such apitopes can be predicted to cause tolerance to FVIII, following the rule-based method described in WO 02/16410.

A peptide of the present invention may be any length that is capable of binding to an MHC class I or II molecule without further processing. Typically, the peptide of the present invention is capable of binding MHC class II.

Peptides that bind to MHC class I molecules are typically 7 to 13, more usually 8 to 10 amino acids in length. The binding of the peptide is stabilised at its two ends by contacts between atoms in the main chain of the peptide and invariant sites in the peptide-binding groove of all MHC class I molecules. There are invariant sites at both ends of the groove which bind the amino and carboxy termini of the peptide. Variations in peptide length are accommodated by a kinking in the peptide backbone, often at proline or glycine residues that allow the required flexibility.

Peptides which bind to MHC class II molecules are typically between 8 and 20 amino acids in length, more usually between 10 and 17 amino acids in length, and can be longer (for example up to 40 amino acids). These peptides lie in an extended conformation along the MHC II peptide-binding groove which (unlike the MHC class I peptide-binding groove) is open at both ends. The peptide is held in place mainly by main-chain atom contacts with conserved residues that line the peptide-binding groove.

PEPTIDE SEQUENCES

The first embodiment of the invention relates to a peptide comprising one of the following FVIII-derived sequences:

GTLMVFFGNVDSSGI

TQTLHKFILLFAVFD
SLYISQFIIMYSLDG
PPIIARYIRLHPTHY
PPLLTRYLRIHPQSW
MHTVNGYVNRSLPGL
LGQFLLFCHISSHQH
DTLLIIFKNQASRPY
PRCLTRYYSSFVNME
TENIQRFLPNPAGVQ
DNIMVTFRNQASRPY
RYLRIHPQSWVHQIA

with one or more of the following modifications:

- removal of one or more hydrophobic amino acid(s);
- replacement of one or more hydrophobic amino acid(s) with charged hydrophilic amino acid(s); and
- insertion of a charged amino acid at one or both terminus(i)

which modified peptide is capable of binding to an MHC class II molecule without further antigen processing and being recognised by a factor VIII specific T cell.

A list of standard amino acids, together with their side chain polarity, charge and hydropathy index is given in Table 1.

Table 1

Amino Acid	3-Letter	1-Letter	Side chain polarity	Side chain charge (pH 7)	Hydropathy index
Alanine	Ala	A	nonpolar	neutral	1.8
Arginine	Arg	R	polar	positive	-4.5
Asparagine	Asn	N	polar	neutral	-3.5
Aspartic acid	Asp	D	polar	negative	-3.5
Cysteine	Cys	C	nonpolar	neutral	2.5
Glutamic acid	Glu	E	polar	negative	-3.5
Glutamine	Gln	Q	polar	neutral	-3.5
Glycine	Gly	G	nonpolar	neutral	-0.4
Histidine	His	H	polar	positive	-3.2
Isoleucine	Ile	I	nonpolar	neutral	4.5
Leucine	Leu	L	nonpolar	neutral	3.8
Lysine	Lys	K	polar	positive	-3.9

Methionine	Met	M	nonpolar	neutral	1.9
Phenylalanine	Phe	F	nonpolar	neutral	2.8
Proline	Pro	P	nonpolar	neutral	-1.6
Serine	Ser	S	polar	neutral	-0.8
Threonine	Thr	T	polar	neutral	-0.7
Tryptophan	Trp	W	nonpolar	neutral	-0.9
Tyrosine	Tyr	Y	polar	neutral	-1.3
Valine	Val	V	nonpolar	neutral	4.2

Hydrophobic amino acids include: G, C, M, A, P, I, L, V and the aromatic amino acids F and W. Hydrophobic amino acids may be removed from the ends of the sequence or within the sequence.

Charged hydrophilic amino acids include: K, R, D, H and E. Hydrophobic amino acids may be exchanged with charged hydrophilic amino acids the ends of the sequence or within the sequence.

One or more charged amino acid(s) may be inserted at the N-terminus of the sequence. Advantageously, a positively charged amino acid is inserted or substituted at one terminus and a negatively charged amino acid is inserted/substituted at the other terminus in order to create a charge dipole.

Modification of the parent sequence should not significantly impair binding of the peptide to the peptide binding groove of an MHC molecule, its capacity to be recognised by a T cell, or its capacity to act as an epitope (bind to an MHC molecule and be presented to a T cell without further antigen processing). This may be readily tested using known antigen presentation assays and T-cell hybridomas.

The second embodiment of the invention relates to a peptide comprising the sequence

$X(aa)_n$ -core sequence -(aa)_m

wherein X is a charged hydrophilic residue;

aa is an amino acid;

n is an integer between 0 and 5;

m is an integer between 0 and 5; and

the "core sequence" is selected from the following group of FVIII-derived peptides: LYISQFIIM

FIIMYSLDG

IARYIRLHP

LIIFKNQAS

LTRYYSSFV

MVTFRNQAS

LRIHPQSWV

which peptide is capable of binding to an MHC class II molecule without further antigen processing and being recognised by a factor VIII specific T cell.

The sequence (aa)_n or (aa)_m can be any sequence of between 4 and 5 amino acids. For example, the peptide may have the sequence:

XDNIMVTFRNQAS

in which case, n=3 and m=0.

The third embodiment of the present invention relates to a peptide comprising the sequence:

Y(aa)_n-core sequence-(aa)_mZ

wherein Y and Z are charged amino acids having reverse polarity;

aa is an amino acid;

n is an integer between 0 and 5;

m is an integer between 0 and 5; and

the "core sequence" is selected from the following group of FVIII-derived peptides:

LYISQFIIM

FIIMYSLDG

IARYIRLHP

LIIFKNQAS

LTRYYSSFV

MVTFRNQAS

LRIHPQSWV

which peptide is capable of binding to an MHC class II molecule without further antigen processing and being recognised by a factor VIII specific T cell.

For example, for the peptide EDNIMVTFRNQASR,

Y=E;

(aa)_n = DNI

m=0; and

Z=R

The peptide may comprise the core residue sequence, together with additional flanking sequences at the N and/or C terminal end ((aa)_n and (aa)_m respectively), provided that the resulting peptide is capable of binding to an MHC class II molecule without further antigen processing.

The flanking N and/or C terminal sequences may be derivable from the sequences flanking the core residue sequences in human FVIII.

APIPS

Various antigen processing independent presentation systems (APIPS) are known, including:

- a) fixed APC (with or without antibodies to CD28);
- b) Lipid membranes containing Class I or II MHC molecules (with or without antibodies to CD28); and
- c) purified natural or recombinant MHC in plate-bound form (with or without antibodies to CD28).

All of these systems are capable of presenting antigen in conjunction with an MHC molecule, but are incapable of processing antigen. In all these systems the processing function is either absent or disabled. This makes it possible to investigate whether a peptide can bind to an MHC class I or II molecule and be presented to a T cell without further antigen processing.

The use of fixed APC to investigate T cell responses is well known in the art, for example in studies to investigate the minimal epitope within a polypeptide, by measuring the response to truncated peptides (Fairchild *et al* (1996) *Int. Immunol.*

8:1035-1043). APC may be fixed using, for example formaldehyde (usually paraformaldehyde) or glutaraldehyde.

Lipid membranes (which may be planar membranes or liposomes) may be prepared using artificial lipids or may be plasma membrane/microsomal fractions from APC.

In use, the APIPS may be applied to the wells of a tissue culture plate. Peptide antigens are then added and binding of the peptide to the MHC portion of the APIPS is detected by addition of selected T cell lines or clones. Activation of the T cell line or clone may be measured by any of the methods known in the art, for example via ³H-thymidine incorporation or cytokine secretion.

FACTOR VIII

The core sequence of peptide of the invention is derivable from factor VIII. One or both flanking sequences ((aa)_n and (aa)_m) may also be derivable from factor VIII.

Factor VIII participates in the intrinsic pathway of blood coagulation; factor VIII is a cofactor for factor IXa which, in the presence of Ca+2 and phospholipids, converts factor X to the activated form Xa.

The factor VIII gene produces two alternatively spliced transcripts. Transcript variant 1 encodes a large glycoprotein, isoform a, which circulates in plasma and associates with von Willebrand factor in a noncovalent complex. This protein undergoes multiple cleavage events. Transcript variant 2 encodes a putative small protein, isoform b, which consists primarily of the phospholipid binding domain of factor VIIIc. This binding domain is essential for coagulant activity.

The complete 186,000 base-pair sequence of the human factor VIII gene was elucidated in the mid 1980s (Gitschier *et al* (1984) *Nature* 312:326-330). At the same time, DNA clones encoding the complete 2351 amino acid sequence were used to produce biologically active factor VIII in cultured mammalian cells (Wood *et al* (1984) *Nature* 312:330-337). The complete 2,351 amino acid sequence for human factor VIII is given in SEQ ID No. 1.

The core residues of peptide of the present invention are derivable from factor VIII. Optionally the flanking sequence(s) may also be derivable from factor VIII if they are the same as the sequences flanking the core sequence in the native FVIII polypeptide. This sequence may be obtainable or obtained from cleavage of the factor VIII sequence.

SOLUBILITY

The peptide of the first embodiment of the invention is a modified form of one of the following peptides:

GTLMVFFGNVDSSGI
TQTLHKFILLFAVFD
SLYISQFIIMYSLDG
PPIIARYIRLHPTHY
PPLLTRYLRIHPQSW
MHTVNGYVNRSLPGL
LGQFLLFCHISSHQH
DTLLIIFKNQASRPY
PRCLTRYYSSFVNME
TENIQRFLPNPAGVQ
DNIMVTFRNQASRPY
RYLRIHPQSWVHQIA

These peptides have already been shown to act as apitopes and be tolerogenic *in vivo* (See examples and International patent application No PCT/GB2008/003996).

It has since come to light that solubility is an important consideration in peptide-mediated tolerance induction. Solubility may be improved by one or more of the following:

- (i) removal of one or more hydrophobic residues;
- (ii) addition/substitution to add one or more charged hydrophilic residues
- (iii) placing positively and negatively charged amino acids at either end to create a charge dipole.

The modified peptide may be more soluble than the parent (unmodified) peptide. The modified peptide may have 2, 3, 4, or 5-fold greater solubility than the parent peptide. The peptide may be soluble at concentrations of up to 0.5 mg/ml, 1 mg/ml, or 5 mg/ml.

TOLERANCE

T cell epitopes play a central role in the adaptive immune response to any antigen, whether self or foreign. The central role played by T cell epitopes in hypersensitivity diseases (which include allergy, autoimmune diseases and transplant rejection) has been demonstrated through the use of experimental models. It is possible to induce inflammatory or allergic diseases by injection of synthetic peptides (based on the structure of T cell epitopes) in combination with adjuvant.

By contrast, it has been shown to be possible to induce immunological tolerance towards particular antigens by administration of peptide epitopes in soluble form. Administration of soluble peptide antigens has been demonstrated as an effective means of inhibiting disease in experimental autoimmune encephalomyelitis (EAE – a model for multiple sclerosis (MS)) (Metzler and Wraith (1993) *Int. Immunol.* 5:1159-1165; Liu and Wraith (1995) *Int. Immunol.* 7:1255-1263; Anderton and Wraith (1998) *Eur. J. Immunol.* 28:1251-1261); and experimental models of arthritis, diabetes, and uveoretinitis (reviewed in Anderton and Wraith (1998) as above). This has also been demonstrated as a means of treating an ongoing disease in EAE (Anderton and Wraith (1998) as above).

Tolerance is the failure to respond to an antigen. Tolerance to self antigens is an essential feature of the immune system, when this is lost, autoimmune disease can result. The adaptive immune system must maintain the capacity to respond to an enormous variety of infectious agents while avoiding autoimmune attack of the self antigens contained within its own tissues. This is controlled to a large extent by the sensitivity of immature T lymphocytes to apoptotic cell death in the thymus (central tolerance). However, not all self antigens are detected in the thymus, so death of self-reactive thymocytes remains incomplete. There are thus also mechanisms by which tolerance may be acquired by mature self-reactive T lymphocytes in the peripheral

tissues (peripheral tolerance). A review of the mechanisms of central and peripheral tolerance is given in Anderton *et al* (1999) (*Immunological Reviews* **169**:123-137).

In haemophilia A, patients have a defect in the factor VIII gene. This means that factor VIII is not recognised as a "self" antigen by the immune system. When factor VIII is administered during coagulation factor replacement therapy, therefore, an alloimmune response is generated to the foreign protein, leading to the production of FVIII inhibitor antibodies.

The peptides of the present invention are capable of inducing tolerance to factor VIII such that when FVIII is administered therapeutically, it does not induce an immune response and FVIII inhibitors do not develop.

Acquired haemophilia is an autoimmune disease in which tolerance to factor VIII breaks down. In this case, peptides of the present invention may be administered to reinstate tolerance to this self protein and curtail the pathogenic immune response.

Tolerance may result from or be characterised by the induction of anergy in at least a portion of CD4+ T cells. In order to activate a T cell, a peptide must associate with a "professional" APC capable of delivering two signals to T cells. The first signal (signal 1) is delivered by the MHC-peptide complex on the cell surface of the APC and is received by the T cell via the TCR. The second signal (signal 2) is delivered by costimulatory molecules on the surface of the APC, such as CD80 and CD86, and received by CD28 on the surface of the T cell. It is thought that when a T cell receives signal 1 in the absence of signal 2, it is not activated and, in fact, becomes anergic. Anergic T cells are refractory to subsequent antigenic challenge, and may be capable of suppressing other immune responses. Anergic T cells are thought to be involved in mediating T cell tolerance.

Without wishing to be bound by theory, the present inventors predict that peptides which require processing before they can be presented in conjunction with MHC molecules do not induce tolerance because they have to be handled by mature antigen presenting cells. Mature antigen presenting cells (such as macrophages, B cells and dendritic cells) are capable of antigen processing, but also of delivering both signals 1

and 2 to a T cell, leading to T cell activation. Apitopes, on the other hand, will be able to bind class II MHC on immature APC. Thus they will be presented to T cells without costimulation, leading to T cell anergy and tolerance.

Of course, apitopes are also capable of binding to MHC molecules at the cell surface of mature APC. However, the immune system contains a greater abundance of immature than mature APC (it has been suggested that less than 10% of dendritic cells are activated, Summers *et al.* (2001) *Am. J. Pathol.* **159**: 285-295). The default position to an apitope will therefore be anergy/tolerance, rather than activation.

The induction of tolerance to FVIII can be monitored *in vivo* by looking for a reduction in the level of:

- (i) FVIII inhibitory antibodies;
- (ii) CD4+ T cells specific for FVIII
- (iii) B cells capable of secreting FVIII inhibitory antibodies

by techniques known in the art.

It has been shown that, when tolerance is induced by peptide administration, the capacity of antigen-specific CD4+ T cells to proliferate is reduced. Also, the production of IL-2, IFN- γ and IL-4 production by these cells is down-regulated, but production of IL-10 is increased. Neutralisation of IL-10 in mice in a state of peptide-induced tolerance has been shown to restore completely susceptibility to disease. It has been proposed that a population of regulatory cells persist in the tolerant state which produce IL-10 and mediate immune regulation (Burkhart *et al* (1999) *Int. Immunol.* **11**:1625-1634).

The induction of tolerance can therefore also be monitored by various techniques including:

- (a) the induction of anergy in CD4+ T cells (which can be detected by subsequent challenge with FVIII *in vitro*);
- (b) changes in the CD4+ T cell population, including
 - (i) reduction in proliferation;
 - (ii) down-regulation in the production of IL-2, IFN- γ and IL-4; and
 - (iii) increase in the production of IL-10.

As used herein, the term "tolerogenic" means capable of inducing tolerance.

COMPOSITION

The present invention also relates to a composition, such as a pharmaceutical composition comprising one or more peptide(s) according to the invention.

The peptide may comprise a plurality of peptides, for example, two, three, four, five or six peptides.

The composition of the present invention may be for prophylactic or therapeutic use.

When administered for prophylactic use, the composition may reduce or prevent the generation of an immune response to FVIII. The level of immune response is less than would have been obtained in the patient had not been treated with the composition. The term "reduce" indicates that a partial reduction in immune response is observed, such as a 50%, 70%, 80% or 90% reduction in the response that would have been observed in the patient had not been treated with the composition (or in the response observed in an untreated patient over the same time-frame). The term "prevent" indicates that no appreciable immune response to FVIII is observed.

When administered for therapeutic use, the composition may suppress an already ongoing immune response to FVIII. The term "suppress" indicates a reduction in the level of an on-going immune response, compared to the level before peptide treatment, or the level which would have been observed at the same time point had the treatment not been given.

Treatment with the composition of the present invention may cause a reduction in levels of any or all of the following:

- (i) FVIII inhibitory antibodies:
- (ii) CD4+ T cells specific for FVIII
- (iii) B cells secreting FVIII inhibitory antibodies.

Detection of all these factors can be carried out by techniques known in the art, such as ELISA, FACS etc.

Treatment with the composition of the present invention may also or alternatively cause anergy in CD4+ T cells specific for FVIII. Anergy can be detected by for example subsequent challenge with FVIII *in vitro*.

It is important to bear in mind that not all immune responses to FVIII are pathogenic. Non-inhibitory anti-FVIII antibodies may be found in haemophilia patients without inhibitors (Moreau *et al* (2000) Blood 95:3435-41) and approximately 15% of healthy blood donors (Algiman *et al* (1992) 89:3795-9).

FVIII inhibitors may be detected by the Nijmegen modification of the clotting Bethesda assay, in which the ability of the patient's plasma to inactivate FVIII in normal plasma is tested. A Bethesda unit is defined as the amount of antibody that neutralizes 50% of plasma FVIII activity, and titres of 0.6BU or greater suggest the presence of antibody.

Inhibitors are generally classified as low titre if the level is <5 BU and high titre if ≥ 5 BU.

The level of circulating FVIII inhibitory antibodies may be reduced to 90%, 75%, 50%, 20%, 10% 5% of the level of antibodies which would have been observed had the patient not received treatment.

The level of circulating FVIII inhibitory antibodies may be reduced to 5, 4, 3, 2, 1 or 0.5 BU.

The peptides and composition of the invention may increase the amount or proportion of therapeutically administered FVIII which is available to aid clotting in a patient. This is due to the reduction in FVIII inhibitors which may effectively remove a proportion of FVIII from exerting its therapeutic function. The peptide or composition

of the invention may increase the amount of available FVIII by, for example, 10%, 25%, 50% 75% or 100%.

The peptides and composition of the invention may thus reduce the amount of FVIII which needs to be administered to aid clotting in a patient.

FORMULATION

The composition may be prepared as an injectable, either as liquid solution or suspension; solid form suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The preparation may also be emulsified, or the peptides encapsulated in liposomes. The active ingredients may be mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline (for example, phosphate-buffered saline), dextrose, glycerol, ethanol, or the like and combinations thereof.

In addition, if desired, the composition may contain minor amounts of auxiliary substances such as wetting or emulsifying agents and/or pH buffering agents. Buffering salts include phosphate, citrate, acetate. Hydrochloric acid and/or sodium hydroxide may be used for pH adjustment. For stabilisation, disaccharides may be used such as sucrose or trehalose.

If the composition comprises a plurality of peptides, the relative ratio of the peptides may be approximately equal. Alternatively the relative ratios of each peptide may be altered, for example, to focus the tolerogenic response on a particular sub-set of autoreactive T-cells or if it is found that one peptide works better than the others in particular HLA types.

After formulation, the composition may be incorporated into a sterile container which is then sealed and stored at a low temperature, for example 4°C, or it may be freeze-dried.

Conveniently the composition is prepared as a lyophilized (freeze dried) powder. Lyophilisation permits long-term storage in a stabilised form. Lyophilisation

procedures are well known in the art, see for example <http://www.devicelink.com/ivdt/archive/97/01/006.html>. Bulking agents are commonly used prior to freeze-drying, such as mannitol, dextran or glycine.

The composition may be administered in a convenient manner such as by the oral, intravenous (where water soluble), intramuscular, subcutaneous, sublingual, intranasal, intradermal or suppository routes or implanting (e.g. using slow release molecules).

The composition may advantageously be administered via intranasal, subcutaneous or intradermal routes.

The peptide and composition of the invention may be used to treat a human subject. The subject may have haemophilia A, in particular severe haemophilia A. The subject may be genetically deficient in FVIII. The subject may have acquired haemophilia. The subject may have inhibitory anti-FVIII antibodies.

The subject may be undergoing or about to undergo coagulant replacement therapy with FVIII.

The subject may be undergoing or about to undergo gene therapy with the FVIII gene.

The subject may be an HLA-haplotype which is associated with a predisposition to develop inhibitory anti-FVIII alloantibodies or autoantibodies. The subject may express HLA-DR2. Methods for determining the HLA haplotype of an individual are known in the art.

Typically, a physician will determine the actual dosage which will be most suitable for an individual subject and it will vary with the age, weight and response of the particular patient.

In a preferred embodiment a "dose escalation" protocol may be followed, where a plurality of doses is given to the patient in ascending concentrations. Such an approach has been used, for example, for phospholipase A2 peptides in immunotherapeutic

applications against bee venom allergy (Müller *et al* (1998) *J. Allergy Clin Immunol.* 101:747-754 and Akdis *et al* (1998) *J. Clin. Invest.* 102:98-106).

KITS

Conveniently, if the composition comprises a plurality of peptides, they may be administered together, in the form of a mixed composition or cocktail. However, there may be circumstances in which it is preferable to provide the peptides separately in the form of a kit, for simultaneous, separate, sequential or combined administration.

The kit may also comprise mixing and/or administration means (for example a vapouriser for intranasal administration; or a syringe and needle for subcutaneous/intradermal dosing). The kit may also comprise instructions for use.

The pharmaceutical composition or kit of the invention may be used to treat and/or prevent a disease.

In particular, the composition/kit may be used to treat and/or prevent haemophilia A or acquired haemophilia.

HAEMOPHILIA A

Hemophilia A (classic hemophilia), is caused by the deficiency of Factor VIII.

Hemophilia A has an estimated incidence of 1 in 10,000 males, while hemophilia B is estimated to occur in one in 40,000 males. Approximately 1 woman in 5,000 is a carrier for hemophilia A, and 1 in 20,000 is a carrier of hemophilia B.

Hemophilia is typically divided into three classes: severe, moderate and mild, based on the level of clotting factor in the blood. In severe hemophilia, there is less than 1 percent of normal clotting factor. The degree of severity tends to be consistent from generation to generation.

Contrary to popular belief, minor cuts and wounds do not usually present a threat to hemophiliacs. Rather, the greatest danger comes from spontaneous bleeding that may

occur in joints and muscles. This is most prone to occur during years of rapid growth, typically between the ages of 5 and 15 years.

Repeated spontaneous bleeding in joints may cause arthritis, and adjacent muscles become weakened. Pressure on nerves caused by the accumulation of blood may result in pain, numbness, and temporary inability to move the affected area.

Haemophilia A is usually diagnosed with a blood test to determine the effectiveness of clotting and to investigate whether the levels of clotting factors are abnormal.

The development of purified clotting factors in the 1970s, isolated from donated blood, significantly improved the long-term outlook for hemophiliacs. Mild to moderate haemophiliacs can use treatment with FVIII on an *ad hoc* basis, whereas severe haemophiliacs may require regular, indefinite treatment.

Previously, patients were given factor VIII concentrates pooled from thousands of plasma donations. This lead to significant problems of contamination with viral pathogens, particularly the human immunodeficiency virus and the hepatitis viruses. Monoclonal antibody purification techniques, heat inactivation, and virucidal detergent treatments have rendered plasma-derived concentrates relatively safe.

Recombinant DNA technology has now provided a series of synthetic products, such as RecombinateTM and KogenateTM. Kogenate is made using baby hamster kidney cells expressing human factor VIII. The resulting factor is highly purified, eliminating any possibility of transmission of virus from plasma.

The peptide or composition of the present invention may be administered before and/or during factor VIII replacement therapy.

Hemophilia A is an ideal disease target for gene therapy since i) it is caused by a mutation in a single identified gene, ii) a slight increase in clotting factor levels *in vivo* can convert severe hemophilia into milder disease, and iii) current replacement therapies are considered suboptimal. Also, there is a wide range of safety if there is an "overshoot" of desired level of coagulation activity.

Unfortunately, to date the promise of gene therapy as a cure for haemophilia has not been realized, primarily because of difficulties in finding a gene delivery system which is sufficiently non-immunogenic to allow for long term expression of the clotting factor.

The peptides of the present invention would also be suitable for tolerising a subject prior to gene therapy with factor VIII and/or managing FVIII inhibitor formation in a patient following gene therapy.

ACQUIRED HAEMOPHILIA

Acquired haemophilia is characterised by the presence of autoantibody inhibitors against FVIII in individuals with previously normal coagulation. It is a rare condition, with an estimated incidence of 1–3 per million population per year. The mortality rate associated with acquired autoantibody inhibitors approaches 25% versus the substantially lower risk of death in those with alloantibodies.

Compared to alloantibody inhibitor patients, acquired hemophilia is characterized by: (1) a more severe bleeding pattern; (2) higher incidence in older population; (3) occurrence in conjunction with identifiable underlying autoimmune diseases, lymphoproliferative or solid tumor malignancies, pregnancy, and use of certain antibiotics such as penicillin and sulfonamides in approximately 50% of cases; and (4) in vitro inhibitor activity that follow a type II pharmacokinetic pattern with incomplete neutralization of the targeted clotting factor activity by the autoantibody, typically resulting in residual factor VIII levels ranging between 2%–18% in patient plasma.

The peptide or composition of the present invention may be administered to a patient with acquired haemophilia, or to a patient believed to be at risk of developing acquired haemophilia due to, for example:

- i) imminent treatment with, for example penicillin or a sulfonylurea
- ii) progression of a tumour or other malignancy
- iii) imminent or early pregnancy.

The invention will now be further described by way of Examples, which are meant to serve to assist one of ordinary skill in the art in carrying out the invention and are not intended in any way to limit the scope of the invention.

EXAMPLES

Example 1: Selection of HLA-DR2 Factor VIII peptides

A series of FDVIII 15mer peptides were compared using three HLA-DR binding algorithms:

SYFPEITHI (<http://www.syfpeithi.de/home.htm>)

ProPred (<http://www.imtech.res.in/raghava/propred/>) and

and IEDB (<http://www.immuneepitope.org/home.do>).

Peptides were selected which were predicted to be HLA-DR2-binding by more than one of the programmes and flanking sequences were designed for the predicted core residues (table 2).

TABLE 2

Peptide No	FVIII First AA	Sequence in single amino acid code	Also referred to herein as:
1	2140	GTLMVFFGNVDSSGI	GTLMV
2	0208	TQTLHKFILLFAVFD	TQTLH
3	2114	SLYISQFIIIMYSLDG	SLYIS
4	2161	PPIIARYIRLHPTHY	PPIIA
5	2318	PPLLTRYLRIHPQSW	PPLLT
6	250	MHTVNGYVNRSLPGL	MHTVN
7	322	LGQFLLFCHISSHQH	LGQFL
8	478	DTLLIIFKNQASRPY	DTLLI
9	545	PRCLTRYYSSFVNME	PRCLT
10	607	TENIQRFLPNPAGVQ	TENIQ
11	1788	DNIMVTFRNQASRPY	DNIMV
12	2322	RYLRIHPQSWVHQIA	RYLRI

Example 2: Investigating the response of HLA-DR2 restricted cells from factor VIII immunised mice to peptides

HLA-DR2 transgenic mice were immunised with human factor VIII in adjuvant. Draining lymph node cells were collected and restimulated *in vitro* with different concentrations of the 12 peptides from table 2. The results are shown in Figure 1.

HLA-DR2 restricted cells from factor VIII immunised mice clearly respond strongly to peptide DNIMV (1st amino acid 1788). There are also responses to peptides PRCLT (545) and PPIIA (2161).

Example 3: Investigating the response of T cells from HLA-DR2 mice to peptides

HLA-DR2 mice were first immunised with factor VIII in adjuvant. Spleen cells from immune mice were restimulated *in vitro* with factor VIII and the resulting lymphoblasts were fused with the BW5147 thymoma using polyethylene glycol.

T-cell hybridomas were selected in HAT medium and the hybridomas cloned and tested for their response to factor VIII. The hybridomas were then screened for their response to the 12 predicted peptides. Of the 27 hybridomas screened, 11 responded to DNIMV, 3 to PRCLT and 3 to PPIIA, although the response to PPIIA was weaker and less specific. The response of two hybridomas specific for DNIMV and PRCLT is shown in Figure 2.

Example 4 - Investigating the response of lymph node cells from FVIII-DR2+ mice to peptides

HLA-DR2 transgenic mice were crossed with factor VIII deficient mice to create a model of haemophilia expressing the human HLA class II MHC molecule.

These FVIII-DR2+ animals were immunised with factor VIII in adjuvant. Draining lymph nodes were isolated and tested for their response to the peptide panel. As shown in Figure 3, these cells responded well to PRCLT and DNIMV. There was a weak response to GTLMV and significant response to RYLRI.

Example 5 - Investigating the response of T cells from HLA-DR2 mice to peptides

Factor VIII deficient mice expressing HLA-DR2 were immunised with factor VIII in adjuvant. Spleen cells from the immunised mice were restimulated *in vitro* with factor VIII and the resulting lymphoblasts were fused with BW5147, as described above. T-cell hybridomas were screened for their response to the 12 predicted peptides. Yet again, the majority of hybridomas responded to peptides DNIMV and PRCLT. Of 19 hybridomas specific for factor VIII, 10 responded to DNIMV, 6 to PRCLT, 1 to PPIIA, 1 to SLYIS and 1 to DTLLI. Examples of responses by these hybridomas are shown in Figure 4.

Based on these experiments it is clear that two peptides DNIMV (first amino acid number 1788) and PRCLT (first amino acid 545) constitute the immunodominant T-cell epitopes in the HLA-DR2 restricted T-cell response to human factor VIII.

Example 6 - DNIMV and PRCLT behave as apitopes

In order to be an apitope, a peptide must be capable of binding to an MHC class I or II molecule without further antigen processing (i.e. trimming) and be presented to a T cell. In the present case, the capacity of peptides to be presented by fixed APC was investigated.

Mgar cells were either fresh or fixed with 1% paraformaldehyde. Clones were tested for antigenic specificity by culturing 100 μ l of hybridoma cells with 5x10⁴ Mgar cells in the presence and absence of 20 μ g/ml rhFVIII or peptide epitopes overnight. Supernatants were then collected and assessed for IL-2 production by ELISA. The fact that rhFVIII must be presented by live Mgar cells demonstrates that the intact protein requires antigen processing to be presented. Peptides DNIMV and PRCLT, on the other hand, are presented by both live and fixed Mgar cells indicating that these peptides function as apitopes (Figure 5).

Example 7 - Determination of the range of peptide epitopes capable of functioning as apitopes

The range of peptide epitopes capable of functioning as apitopes in the sequences surrounding DNIMV, PRCLT and the other peptides was identified by preparing panels of overlapping peptides (shown on pages 36-37) and screening these using the T-cell hybridomas using the same method as Example 5 (Figure 7).

Example 8 - DNIMV and PRCLT induce tolerance to whole factor VIII protein

HLA-DR2 transgenic mice were treated with either of the two soluble peptides, or PBS as a control, prior to immunisation with factor VIII in adjuvant. Draining lymph nodes were isolated and the cells restimulated *in vitro* with factor VIII protein in order to assess the immune status of the mice. As shown in Figure 6, treatment of mice with either DNIMV or PRCLT led to a substantial suppression of the immune response to factor VIII.

Example 9 - Investigation of whether DNIMV and PRCLT able to induce tolerance in the factor VIII knockout mouse

It was known from Example 8 that these two peptides are able to prevent the immune response to factor VIII in mice expressing endogenous factor VIII. The experiment was repeated with FVIII-DR2+ animals to determine whether these peptides also prevent the immune response to factor VIII in factor VIII deficient mice.

Example 10 – Investigation of whether DNIMV and PRCLT in combination are able to induce tolerance in the factor VIII knockout mouse

The two peptides which were shown to individually reduce the immune response to factor VIII in factor VIII deficient mice in Example 9 were combined. As shown in Figure 8, treatment of mice with both DNIMV and PRCLT led to a substantial suppression of the immune response to factor VIII, as shown by the decrease in IFN-gamma production. IFN-gamma is the major class switch lymphokine required for neutralising antibodies in the mouse. The effect demonstrated was greater than that observed using either peptide alone.

Example 11: The induction of tolerance using a modified peptide

The peptide DNIMV is partly, but not completely soluble. In order to improve the solubility of the peptide, a modified version was designed with the following sequence: EDNIMVTFRNQASR.

This is extended at the N-terminus to add a charged hydrophilic residue. It is also a truncated at the C-terminus to remove the proline and tyrosine residues. Furthermore by placing positively and negatively charged amino acids at either end of the peptide a charge dipole is created reported to increase solubility.

The modified peptide is more soluble than DNIMV and sufficiently soluble to allow intranasal peptide delivery. In order to assess the induction of tolerance using this peptide epitope, FVIII deficient mice were taken and half treated with the modified EDNIMV peptide (referred to as 'tolerised' in Figure 9). The mice were then immunised with DNIMV in CFA and 10 days later draining lymph nodes were collected and stimulated *in vitro* with either DNIMV or EDNIMV. Figure 9 shows the results for responses from either naïve or tolerised mice stimulated with either DNIMV or EDNIMV *in vitro*.

The results demonstrate that EDNIMV is able to recall an immune response from mice immunised with DNIMV. Furthermore, they demonstrate very clearly that mice tolerized with EDNIMV fail to mount an immune response to DNIMV *in vivo* as revealed by the lack of response to either DNIMV or EDNIMV *in vitro* after priming with DNIMV in CFA.

Example 12: EDNIMV induces tolerance to whole factor VIII protein

The experiment described in Example 8 is repeated for the modified peptide EDNIMV to demonstrate that EDNIMV is able to suppress the immune response to the whole factor VIII protein.

Methods**(i) Recall responses for DR2+ mice primed with rhFVIII**

HLA-DR2+ murine MHC class II null mice were immunised with 40 μ g rhFVIII emulsified in Complete Freunds Adjuvant supplemented with 400 μ g heat-killed *M.tuberculosis* H37Ra, subcutaneously at the base of the tail. 10 days later the mice were sacrificed and the draining lymph nodes removed. Single cell suspensions were prepared and lymphocytes incubated at 4-5x10⁵ cells per well in 96-well flat bottomed plates for 72 hours with the indicated concentrations of peptide or control antigens before pulsing with 0.5 μ Ci/well tritiated thymidine for a further 16 hours. Plates were then frozen before cells were harvested onto glass filter mats and radioactive incorporation measured using a liquid scintillation β -counter

(ii) FVIII peptide specificity of T cell hybridomas generated from DR2+ mice

HLA-DR2+ murine MHC class II null mice were immunised as above. On day 10 draining lymph nodes were removed and lymphocytes cultured at 2.5x10⁶ cells/ml, 1ml/well in 24 well plates in the presence of 20 μ g/ml rhFVIII for 3 days. Following this stimulation, lymphocytes were recovered, washed and fused with TCR α β ⁻ BW fusion partner cells at a ratio of 4 BW cells to 1 lymphocyte, using polyethylene glycol as described by Nelson et al (1980) *PNAS* 77(5):2866. Fused cells were carefully washed and then plated out in flat bottomed 96 well plates for 2 days before the addition of HAT medium to select for T cell hybridomas. Cells were monitored for growth and approximately 10 days after fusions were performed, individual clones were selected and transferred to 24 well plates in HAT medium. Clones were maintained in HAT medium for at least 2 weeks before being weaned into HT medium and then complete medium. Clones were tested for antigenic specificity by culturing 100 μ l of hybridoma cells with 5x10⁴ Mgar cells in the presence and absence of 20 μ g/ml rhFVIII overnight. Supernatants were then collected and assessed for IL-2 production by ELISA, with clones producing IL-2 in response to rhFVIII being considered positive for FVIII-specificity. To investigate the repertoire of predicted FVIII peptides FVIII-specific clones were again tested for IL-2 production, following overnight incubation with 20 μ g/ml of each of the 12 peptides.

(iii) Recall responses for FVIII-/- mice primed with rhFVIII

The same method was followed as for (i), except the mice were FVIII-deficient, HLA-DR2+ and murine MHC class II null.

(iv) FVIII peptide specificity of T cell hybridomas generated from FVIII-/- mice

The same method was followed as for (ii), except the mice were FVIII-deficient and HLA-DR2+.

(v) Tolerisation of FVIII-specific responses in DR2+ mice by pre-treatment with immunodominant FVIII peptides

HLA-DR2+ murine MHC class II null mice were treated 3 times with 100µg of DNIMV, PRCLT or PPIIA dissolved in PBS, or the equivalent volume of PBS alone. Peptides were administered intraperitoneally, with 3-4 days between each dose. Following the final administration, mice were primed with rhFVIII emulsified in complete Freunds adjuvant as for (i). 10 days later, draining lymph nodes were recovered and lymphocytes subsequently cultured *in vitro* with rhFVIII, or each of the tolerising peptides as well as control antigens, for 72 hours before the addition of tritiated thymidine as for (i).

(vi) Tolerisation of FVIII-specific responses in DR2+ mice by pre-treatment with a combination immunodominant FVIII peptides

HLA-DR2+ murine MHC class II null mice were treated 3 times with DNIMV, PRCLT or a combination of both DNIMV and PRCLT dissolved in PBS, or the equivalent volume of PBS alone. Peptides were administered intraperitoneally, over 8 days. Following the final administration, mice were primed with rhFVIII emulsified in complete Freunds adjuvant as for (i). 10 days later, draining lymph nodes were recovered and lymphocytes subsequently re-stimulated *in vitro* with rhFVIII. The supernatants were then collected and IFN-gamma was measured.

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in

connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular immunology or related fields are intended to be within the scope of the following claims.

The reference in this specification to any prior publication (or information derived from it), or to any matter which is known, is not, and should not be taken as an acknowledgment or admission or any form of suggestion that that prior publication (or information derived from it) or known matter forms part of the common general knowledge in the field of endeavour to which this specification relates.

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

SEQ ID No.1

1 mqielstcff lcllrcfcfa trrylgave lswdymqsdI gelpvdarfp prvpksfpfn
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121 gvsywkaeeg aeyddqtsqr ekeeddkvfpq gshtywqvl kengpmasdp lcltysylsh
181 vdlvkdlnsg ligallvcre gslakektqt lhkfillfav fdegkswhse tknslmqdrd
241 aasarawpkm htvngyvnr sIpglighrk svywhvigmg ttpevhslf eghflvrnh
301 rqaIeispi tfltaqtllm dlqqlflch isshqhdgme ayvkvdscpe epqlrmknne
361 eaedydddlt dsemdvrvfd ddnspsfqi rsvakkhpkt wwhyiaaeee dwdyaplvia
421 pddrsyksqy lnngpqrigr kykkvrfmay tdefktrae iqhesgilgp llygevgdtl
481 liifknqasr pyniyphgit dvrplysrlI pkvgvhkldf pilpgeifky kwvtvvedgp
541 tksdprcltr yyssfvnmer dIasgligpl licykesvdq rgnqimsdkr nvilfsvfde
601 nrswlyteni qrflpnpagv qIedpefqas nimhsingyv fdslqlsvcl hevaywyls
661 igaqtdflsv ffsgytfkhk mvyedtltf pfsgetvfmI menplglwlg chnsdfrnrg
721 mtallkvssc dkntgdyed syedisayll sknnaieprs fsqnsrhpst rkqfnatti
781 pendiektdp wfahrtppmk iqnvsddll mllrqspIph glsIstdqea kyetsddps
841 pgaidsnnsI semthfrpql hhsgdmvftp esglqlrIine klgtaatel kkldfkvsst
901 snnlistips dIlaagtdnt sIsgppsmIv hydsqldtlI fgkkssplte sggplslsee
961 nndskllesg lmnqsesswg knvsstesgr lfkgkrahgp alltkdnalf kvsisllktn
1021 ktsnnnsatnr kthidgpsI ienspsvwqn ilesdtefkk vtplihdrml mdknatalrI
1081 nhmsnktss knmemvqqkk egiippdaqn pdmsffkmlf lpesarwiqr thgknslnsg
1141 qgpIpkqlvs lgpeksvegq nIseknkvv vkggefIkdv glkemvfppss rnlfltnldn
1201 lhen nthnqe kkiqeeiekk etIeqenvlI pqihtvtgkI nfrmknIfls trqnvegsyd
1261 gayapvlqdf rslndstnrt kktafskk geeenleglg nqtkqiveky acttrispt
1321 sqqnfvtqrs kralkqfrlp leetelekri ivddtstqws knmkhltpst ltqidyneke
1381 kgaitqspls dcltrhsip qanrsplia kvssfpsirp iyltrvlfqd nsshIpaasy
1441 rkkdsgvqes shflqgakkn nIslalittle mtgdqrevgs lgtIatnsvt ykkventvlp
1501 kpdlpktskI vellpkvhiy qkdlfpIets ngspghldlv egssllqgIeg aikwneanrp
1561 gkvplrvat essakpskl ldplawdnhy gtqipkeewk sqekspekta fkkkdtIlsI
1621 nacesnhaia ainegqnkpe ievtwakqgr terIcsqnpp vIkrhqreit rtIqlsdqee
1681 idyddtisve mkkedfdIyd edenqsprsf qkkrhyfia averIwdygI sssphvIrn
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1801 pysfyslIsI yeedqrqgae prknfvkpne tktyfwkvqh hmaptkdefd ckawayfsdv
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1981 vftvrkkeey kmalynlypg vfetvemIps kagiwrvecl igeIhagms tIflvysnkc
2041 qtplgmasgh irdfqitasg qygqwapkla rlhysgsina wstkepfsI kvdIlapmIi
2101 hgiktqgarq kfsslyisqf iimysldgkk wqtygrnsg tImlvfgnvd ssgikhnifn
2161 ppiIaryirl hptysirst lrmewmgcdl nscsmplgme skaisdaqit assyftnmfa
2221 twspskarlh lqgrsnawrp qvnnpkewlq vdfqktrmkvt gvtqgvksI ltsmyvkefI
2281 issqdghqw tIffqngkvk vfqgnqdsft pvnslppl ltrylrihpq swvhqialrm
2341 evlgceaqlI y

Overlapping peptide panels prepared in Example 7**Overlapping set for DTLLIIFKNQASRPY**

1.	473-488	YGEVGDTLLIIFKNQ
2.	474-489	GEVGDTLLIIFKNQA
3.	475-490	EVGDTLLIIFKNQAS
4.	476-491	VGDTLLIIFKNQASR
5.	477-492	GDTLLIIFKNQASRP
6.	478-493	DTLLIIFKNQASRPY
7.	479-494	TLLIIFKNQASRPYN
8.	480-495	LLIIFKNQASRPYNI
9.	481-496	LIIFKNQASRPYNTY
10.	482-497	IIFKNQASRPYNTYP
11.	483-498	IFKNQASRPYNTYPH

Overlapping set for PRCLTRYYSSFVNME

1.	540-554	PTKSDPRCLTRYYSS
2.	541-555	TKSDPRCLTRYYSSF
3.	542-556	KSDPRCLTRYYSSFV
4.	543-557	SDPRCLTRYYSSFVN
5.	544-558	DPRCLTRYYSSFVN
6.	545-559	PRCLTRYYSSFVNME
7.	546-560	RCLTRYYSSFVNMER
8.	547-561	CLTRYYSSFVNMERD
9.	548-562	LTRYYSSFVNMERDL
10.	549-563	TRYYSSFVNMERDLA
11.	550-564	RYYSSFVNMERDLAS

Overlapping set for DNIMVTFRNQASRPY

1. 1783-1797	RAEVEDNIMVTFRNQ
2. 1784-1798	AEVEDNIMVTFRNQA
3. 1785-1799	EVEDNIMVTFRNQAS
4. 1786-1800	VEDNIMVTFRNQASR
5. 1787-1801	EDNIMVTFRNQASRP
6. 1788-1802	DNIMVTFRNQASRPY
7. 1789-1803	NIMVTFRNQASRPYS
8. 1790-1804	IMVTFRNQASRPYSF
9. 1791-1805	MVTFRNQASRPYSFY
10. 1792-1806	VTFRNQASRPYSFYS
11. 1793-1807	TFRNQASRPYSFYSS

Overlapping set for SLYISQFIIMYSLDG

1. 2109-2123	RQKFSSLYISQFIIM
2. 2110-2124	QKFSSLYISQFIIMY

3. 2111-2125	KFSSLYISQFIIMYS
4. 2112-2126	FSSLYISQFIIMYSL
5. 2113-2127	SSLYISQFIIMYSLD
6. 2114-2128	SLYISQFIIMYSLDG
7. 2115-2129	LYISQFIIMYSLDGK
8. 2116-2130	YISQFIIMYSLDGKK
9. 2117-2131	ISQFIIMYSLDGKKW
10. 2118-2132	SQFIIMYSLDGKKWQ
11. 2119-2133	QFIIMYSLDGKKWQT

Overlapping set for PPIIARYIRLHPTHY

1. 2156-2170	HNIFNPPPIIARYIRL
2. 2157-2171	NIFNPPPIIARYIRLH
3. 2158-2172	IFNPPPIIARYIRLHP
4. 2159-2173	FNPPPIIARYIRLHPT
5. 2160-2174	NPPPIIARYIRLHPTH
6. 2161-2175	PPIIARYIRLHPTHY
7. 2162-2176	PIIARYIRLHPTHYS
8. 2163-2177	IIARYIRLHPTHYSI
9. 2164-2178	IARYIRLHPTHYSIR
10. 2165-2179	ARYIRLHPTHYSIRS
11. 2166-2180	RYIRLHPTHYSIRST

Overlapping set for RYLRRIHPQSWVHQIA

1. 2317-2331	PPLLTRYLRIHPQSW
2. 2318-2332	PLLTRYLRIHPQSWV
3. 2319-2333	LLTRYLRIHPQSWVH
4. 2320-2334	LTRYLRIHPQSWVHQ
5. 2321-2335	TRYLRIHPQSWVHQI
6. 2322-2336	RYLRIHPQSWVHQIA
7. 2323-2337	YLRRIHPQSWVHQIAL
8. 2324-2338	LRIHPQSWVHQIALR
9. 2325-2339	RIHPQSWVHQIALRM
10. 2326-2340	IHPQSWVHQIALRME
11. 2327-2341	HPQSWVHQIALRMEV

CLAIMS

1. An isolated peptide which consists of the sequence EDNIMVTFRNQASR.
2. A composition comprising a plurality of peptides, including the peptide according to claim 1.
3. A peptide according to claim 1, or a composition according to claim 2, for use in suppressing or preventing the production of factor VIII inhibitor antibodies *in vivo*.
4. The use of a peptide according to claim 1, or a composition according to claim 2, in the manufacture of a medicament to suppress or prevent the production of factor VIII inhibitor antibodies *in vivo*.
5. A method for suppressing or preventing the production of factor VIII inhibitor antibodies in a subject, which comprises the step of administration of a peptide according to claim 1, or a composition according to claim 2, to the subject.
6. A method for treating haemophilia in a subject which comprises the step of administration of a peptide according to claim 1, or a composition according to claim 2, to the subject.
7. A method according to claim 5 or 6, wherein the subject has haemophilia A, and is undergoing, or is about to undergo, factor VIII replacement therapy.
8. A method according to claim 5 or 6, wherein the subject has, or is at risk from contracting, acquired haemophilia.
9. A method according to any of claims 5 to 8, wherein the subject is HLA-DR2.
10. A peptide according to claim 1, composition according to claim 2, or method according to any one of claims 5-9 substantially as hereinbefore described with reference to the Examples and/or Figures.

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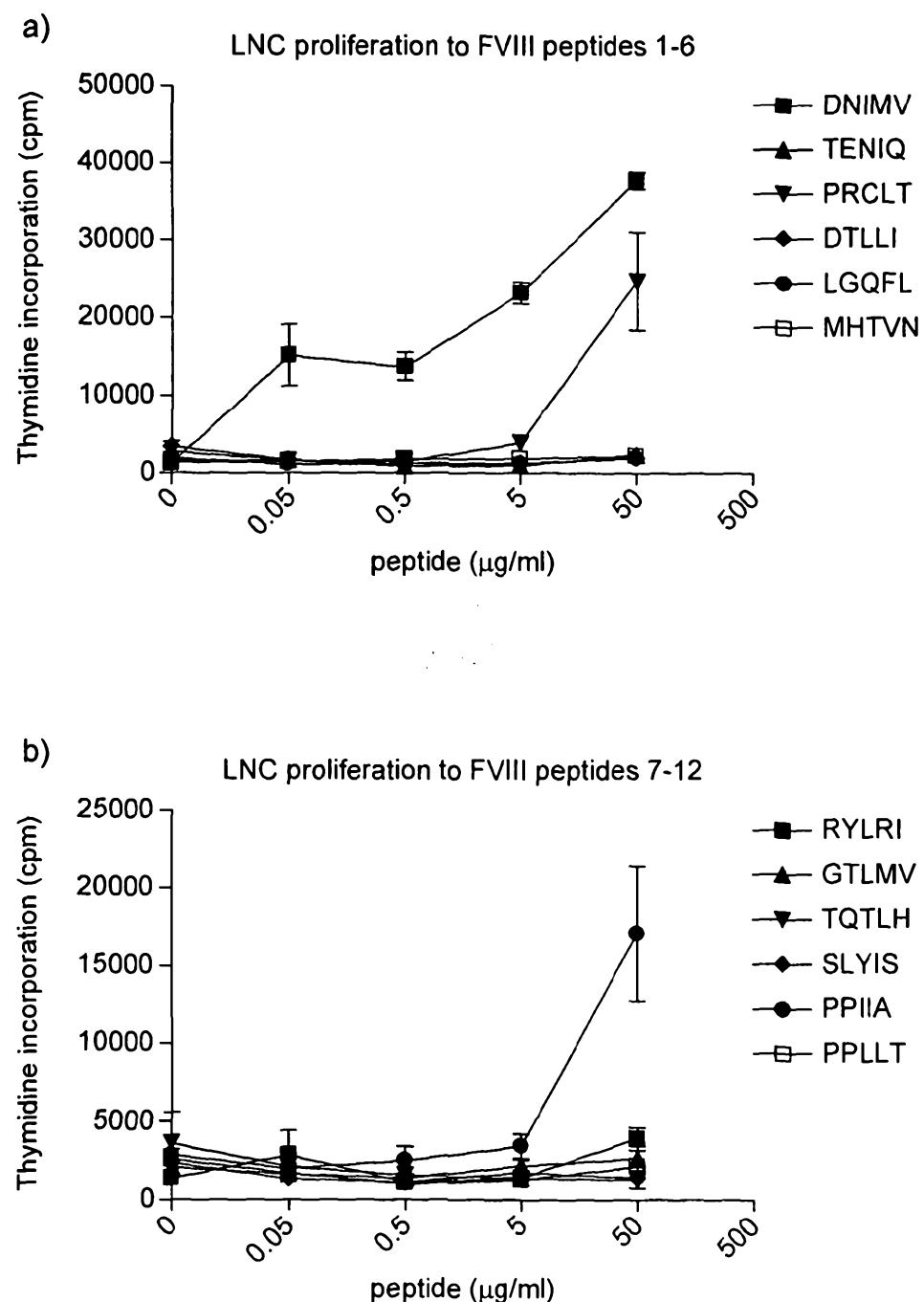


FIG. 1

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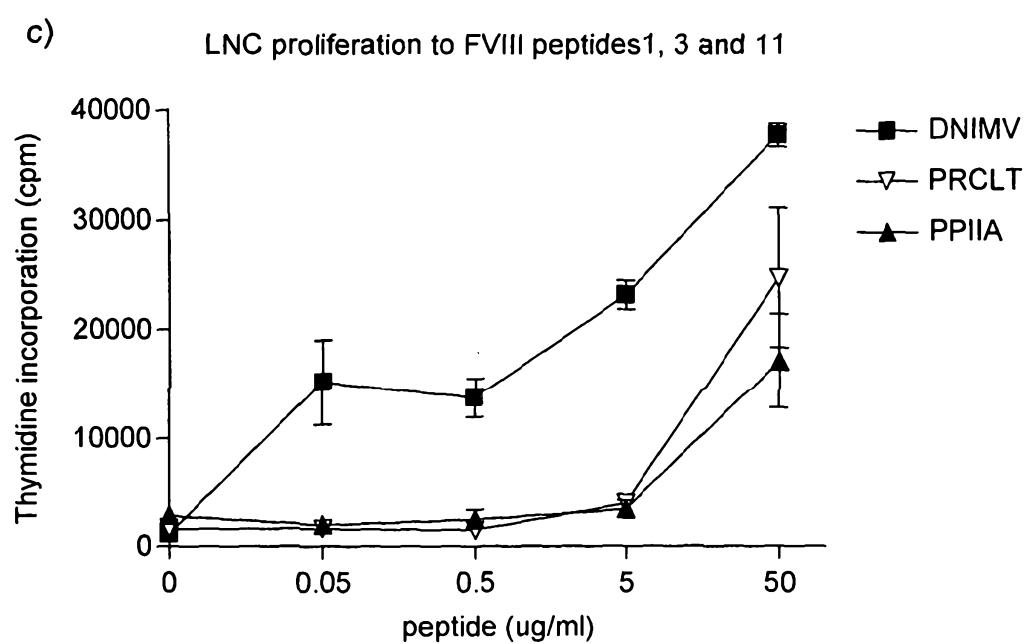


FIG. 1 (cont'd)

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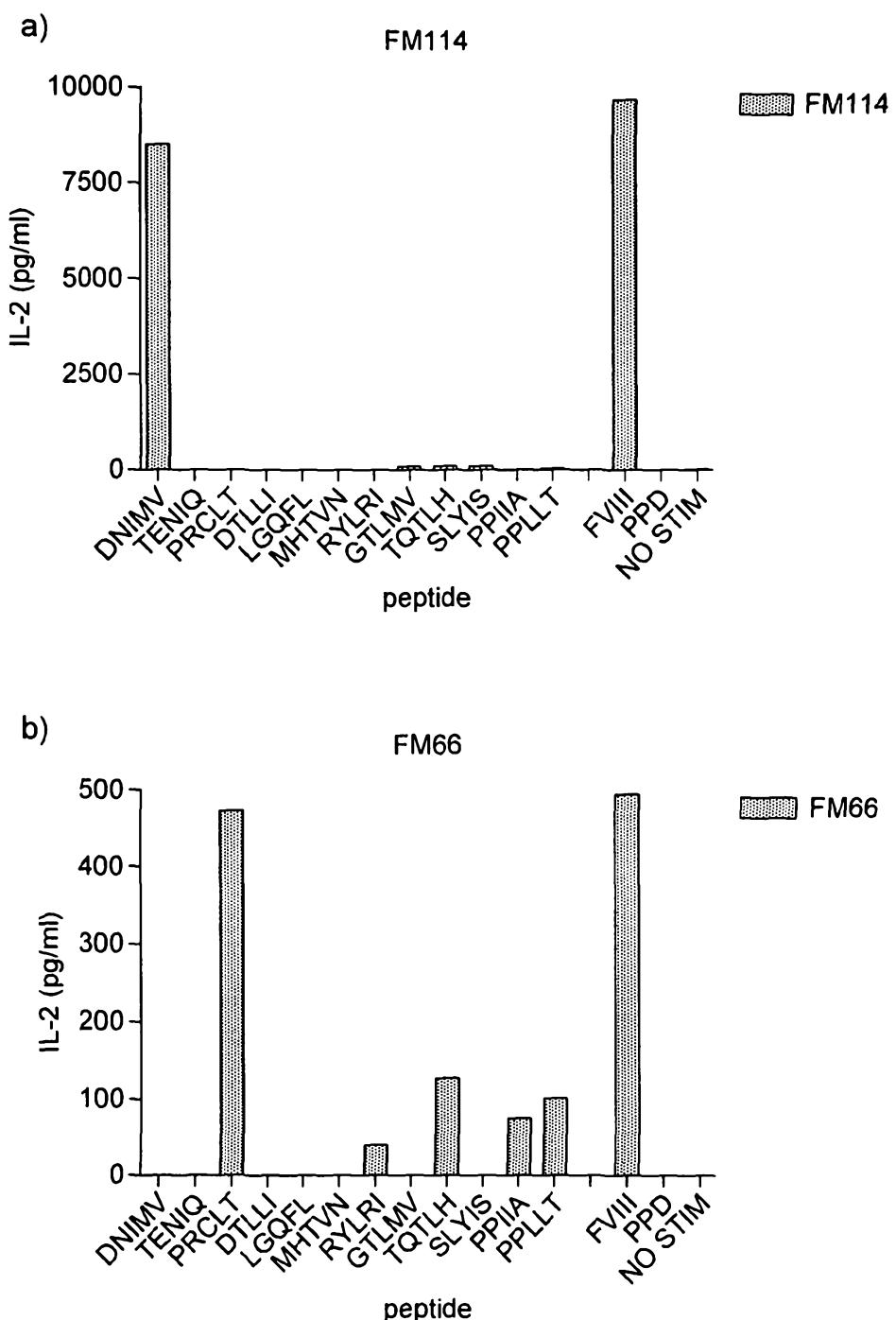


FIG. 2

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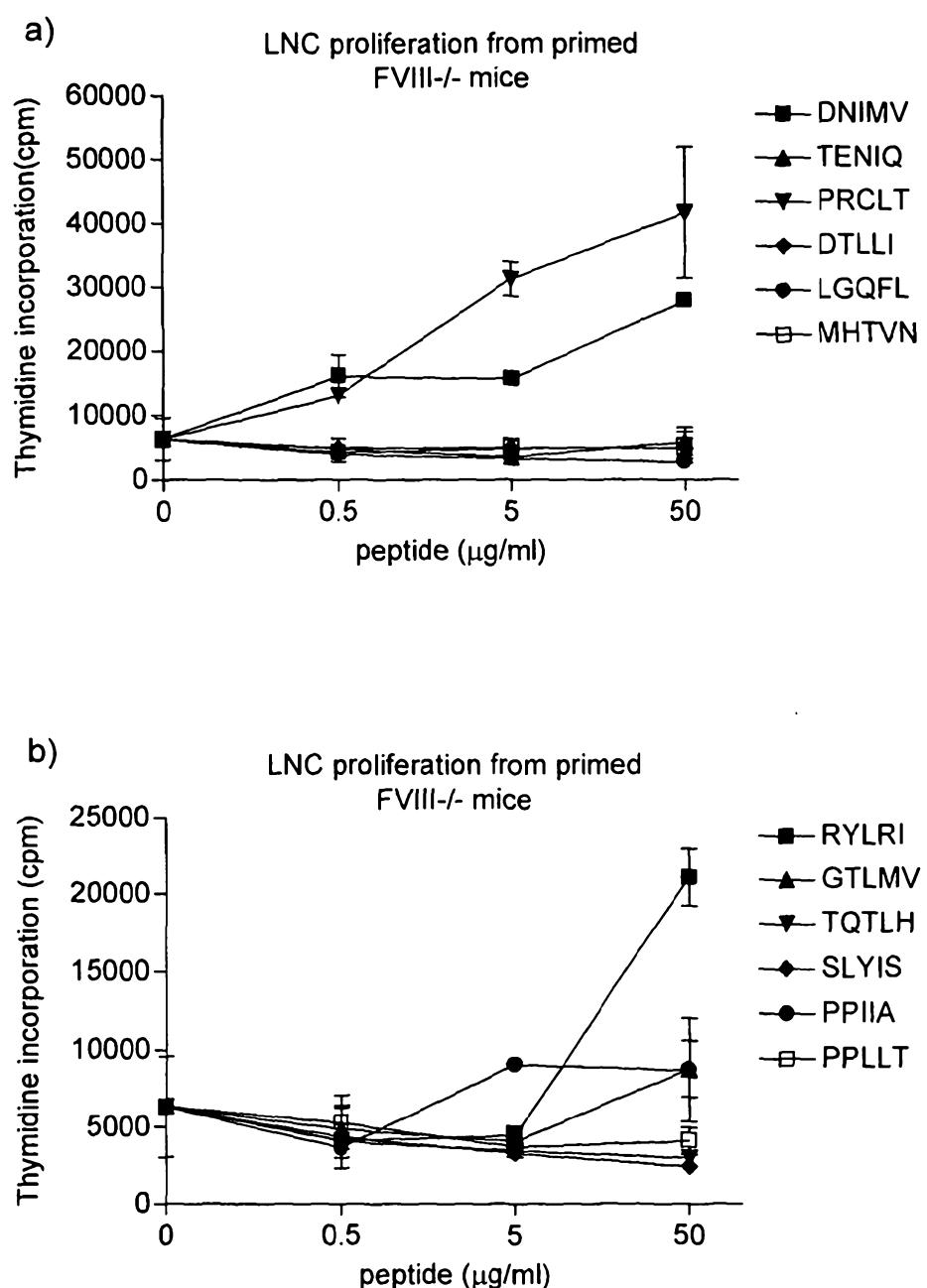


FIG. 3

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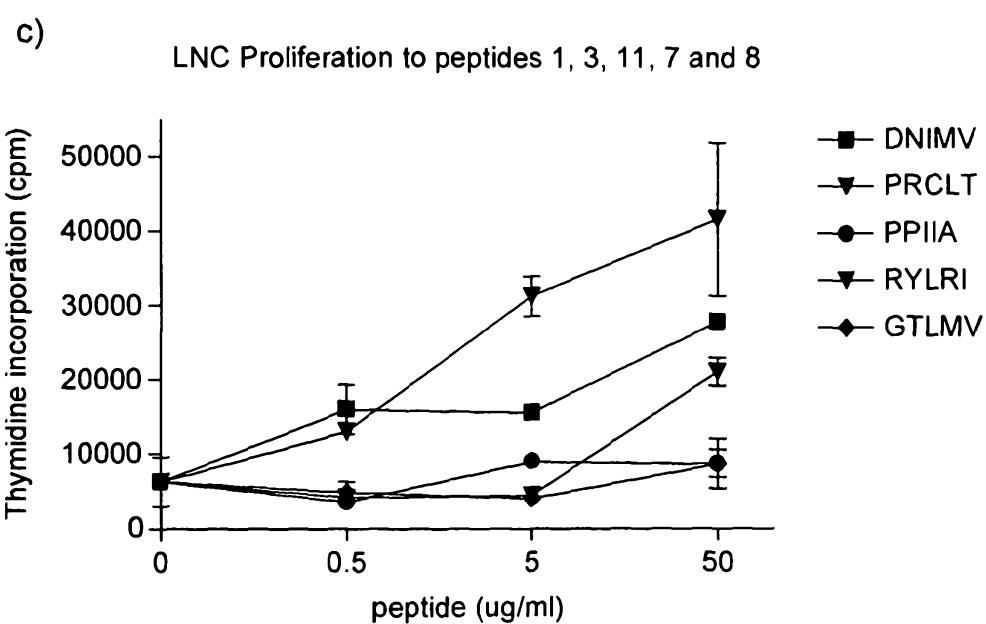


FIG. 3(cont'd)

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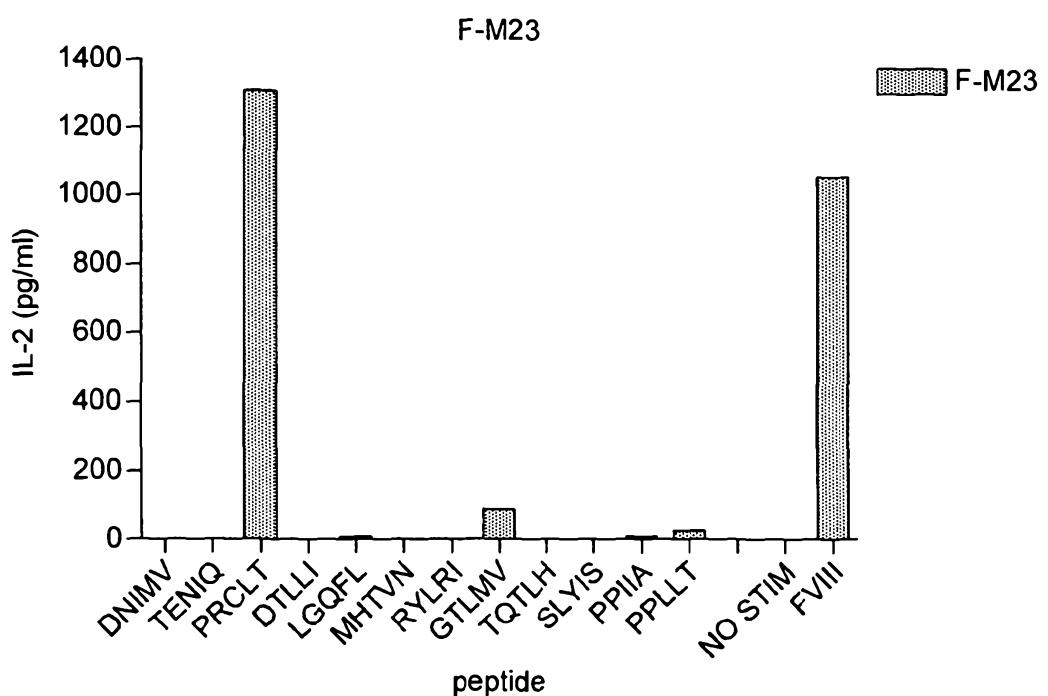
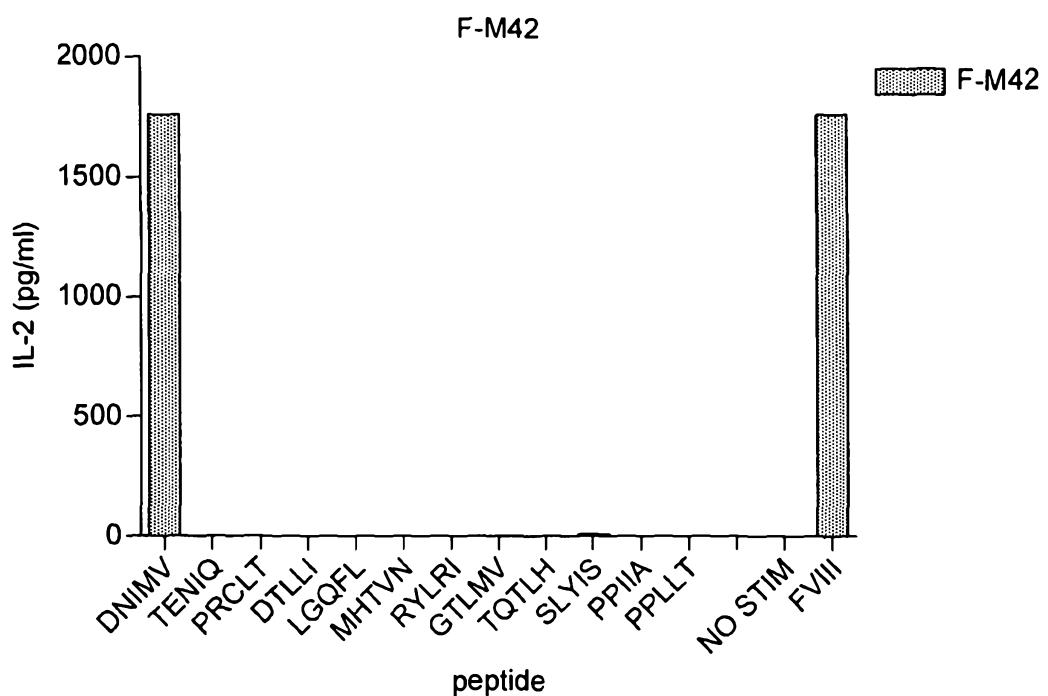
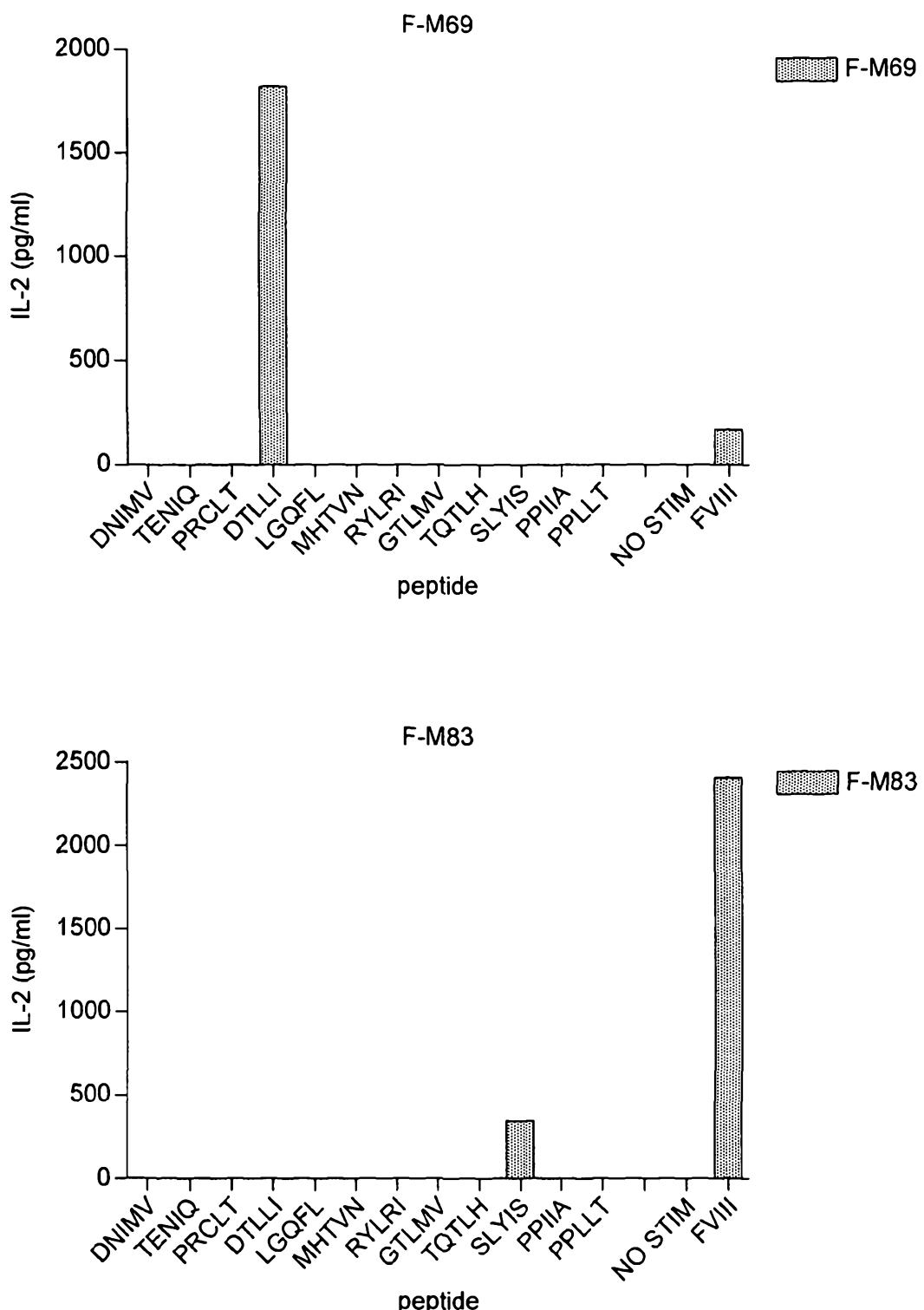
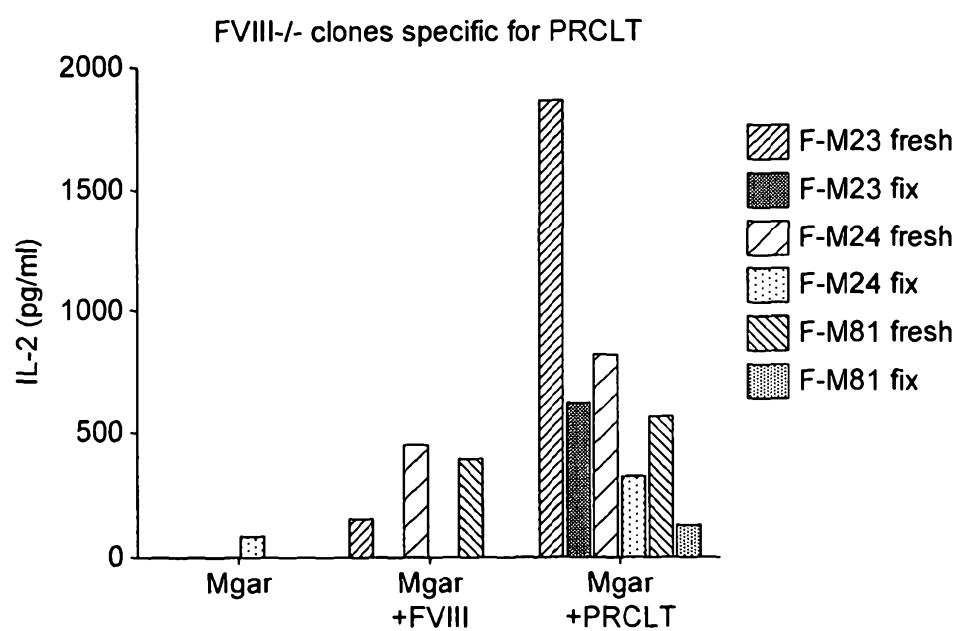
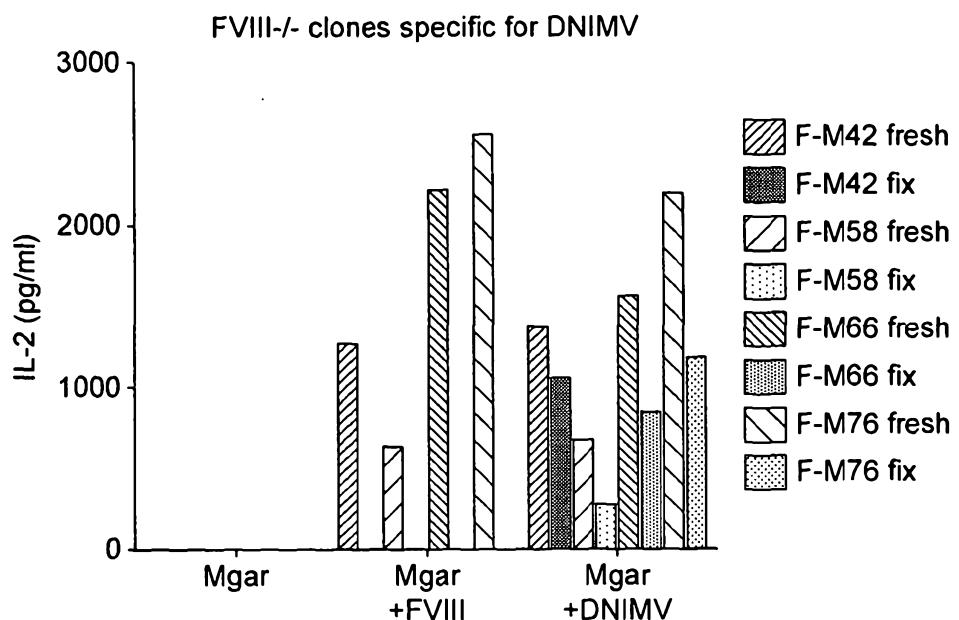


FIG. 4

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FIG. 4_(cont'd)

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**FIG. 5**

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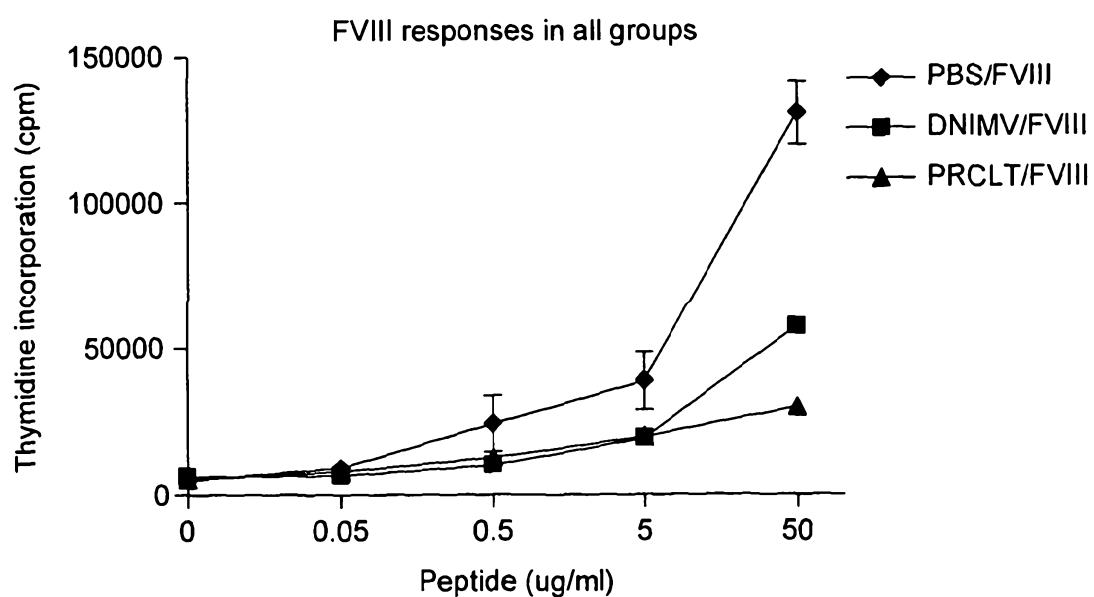


FIG. 6

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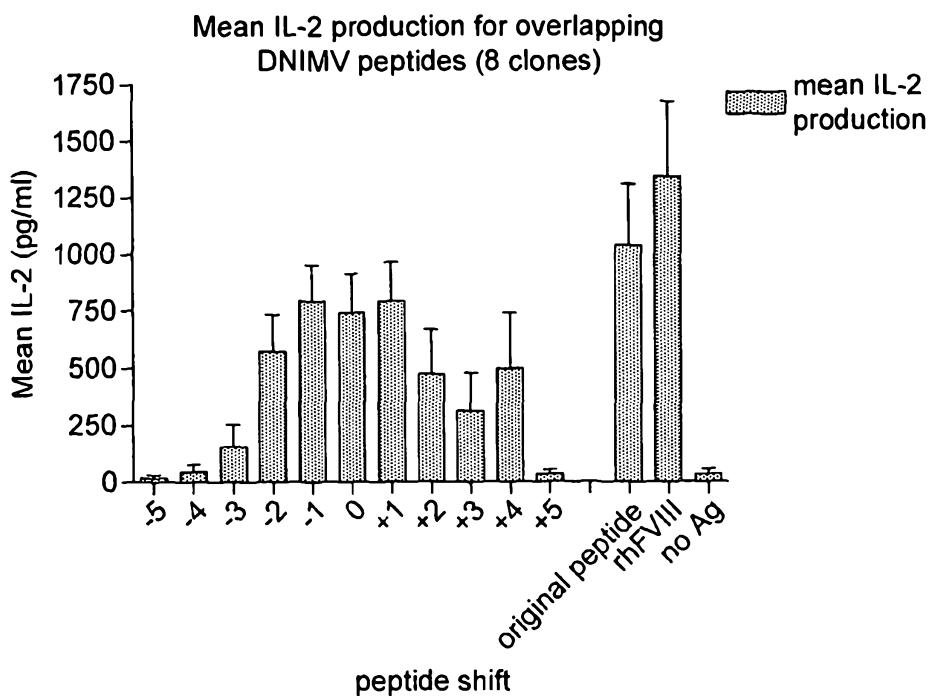


FIG. 7a

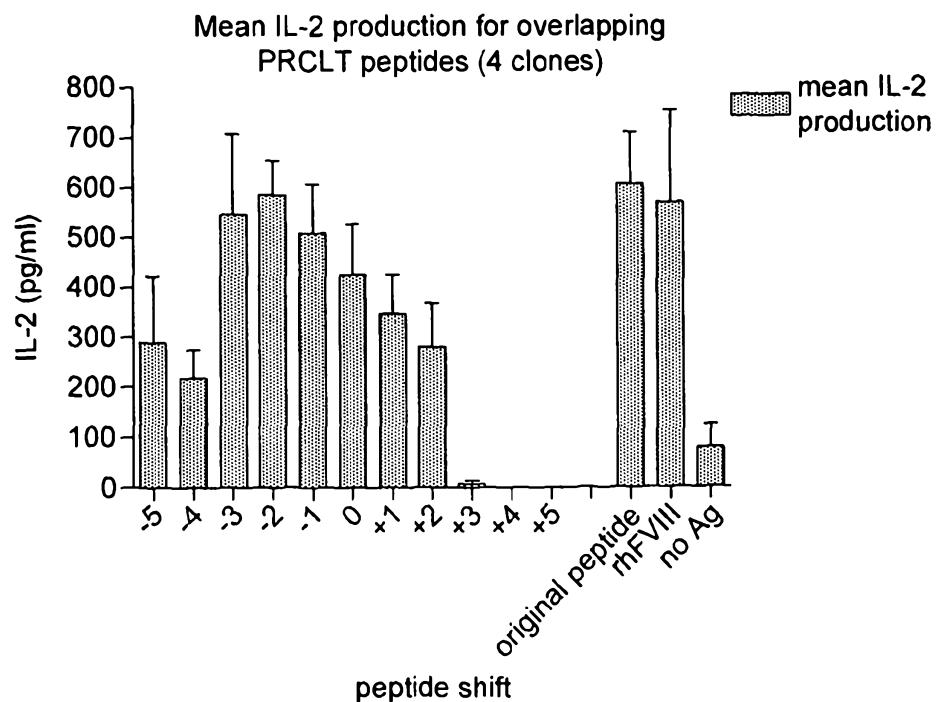


FIG. 7b

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Mean IL-2 production for overlapping peptides
PPIIA, DTLLI, SLYIS and RYLRI

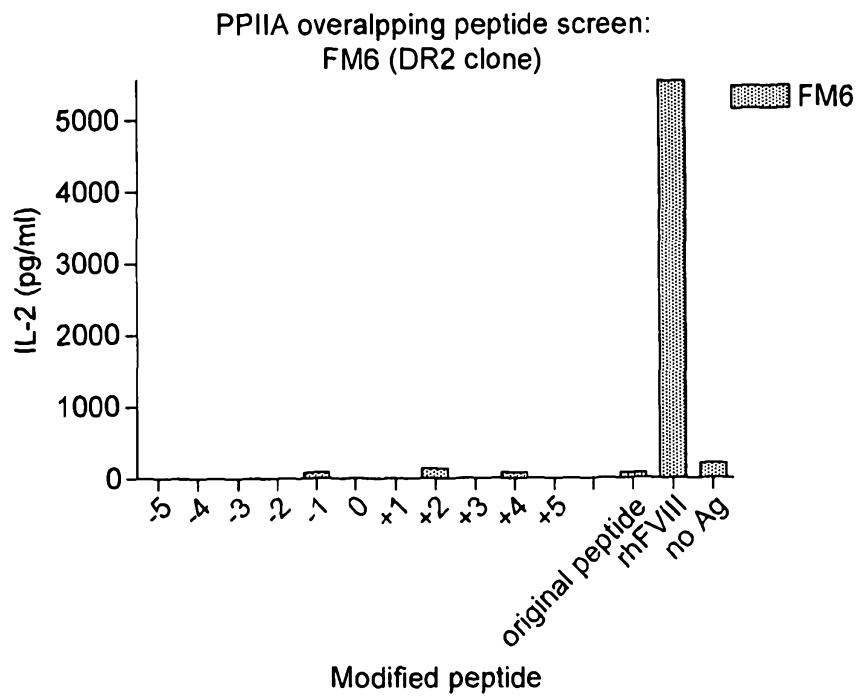
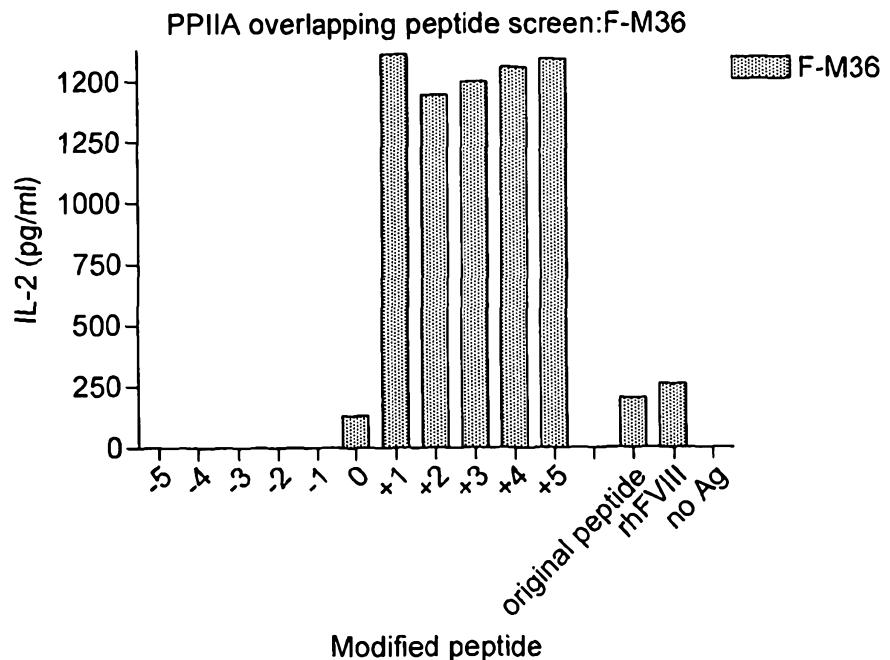


FIG. 7c

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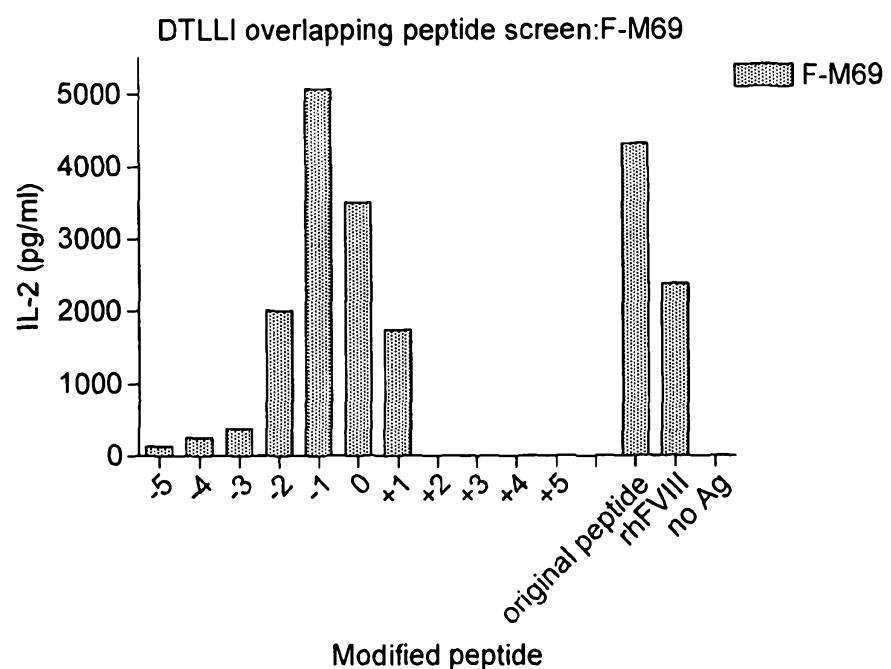
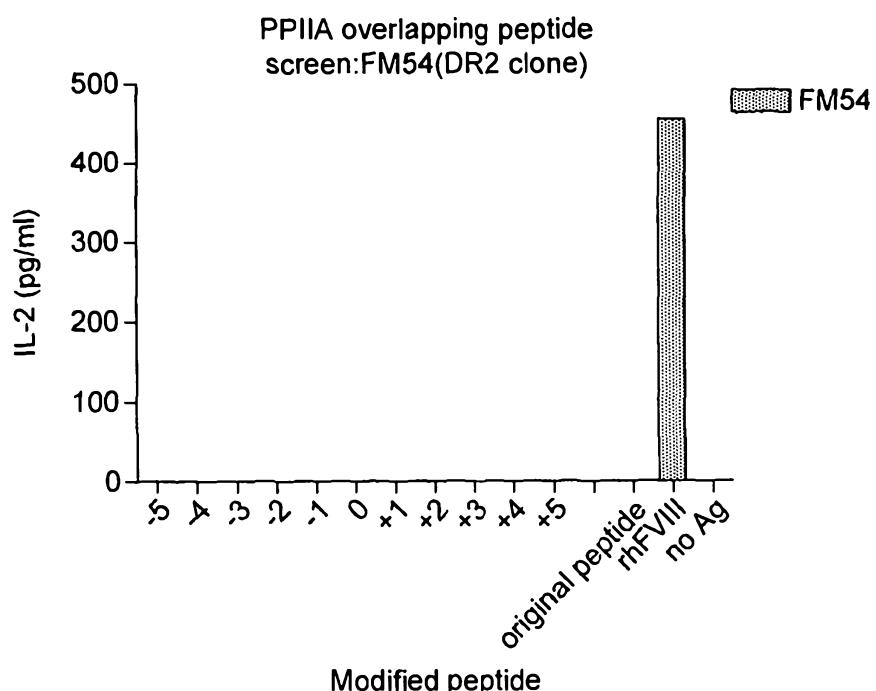


FIG. 7 (cont'd)

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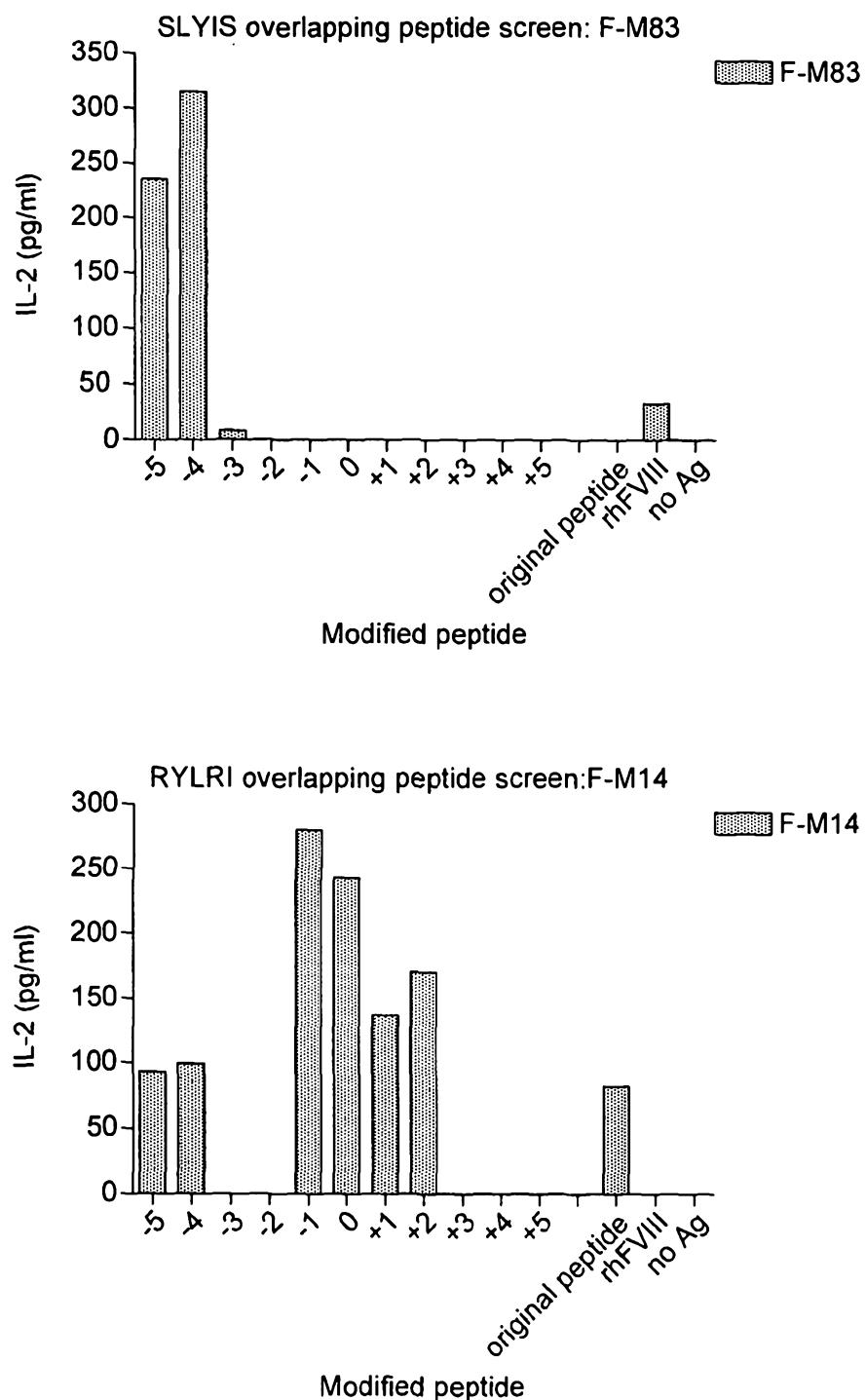


FIG. 7 (cont'd)

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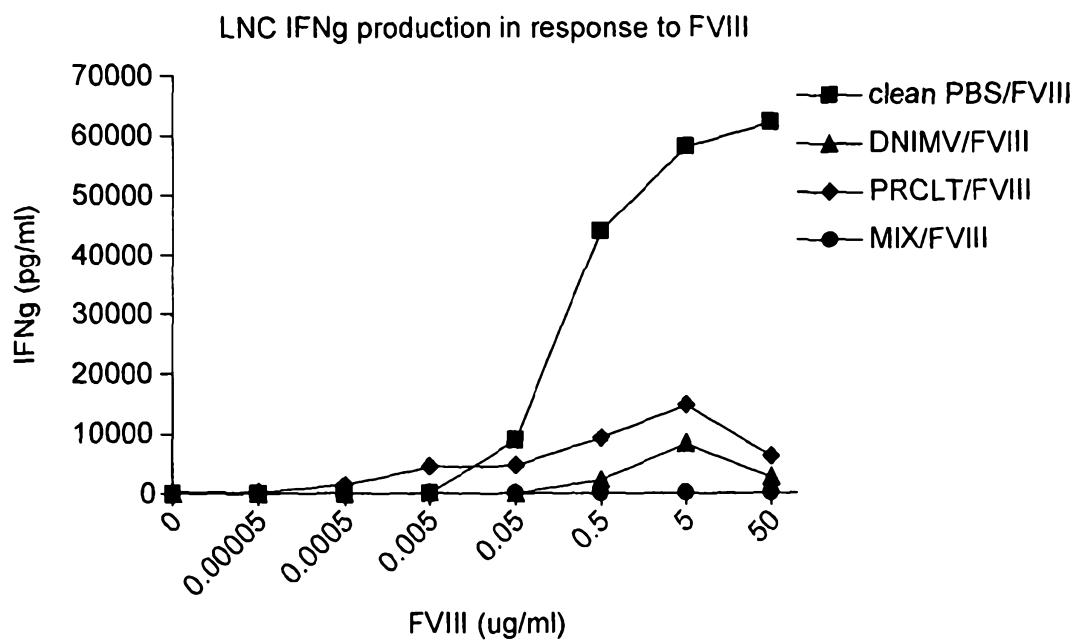


FIG. 8

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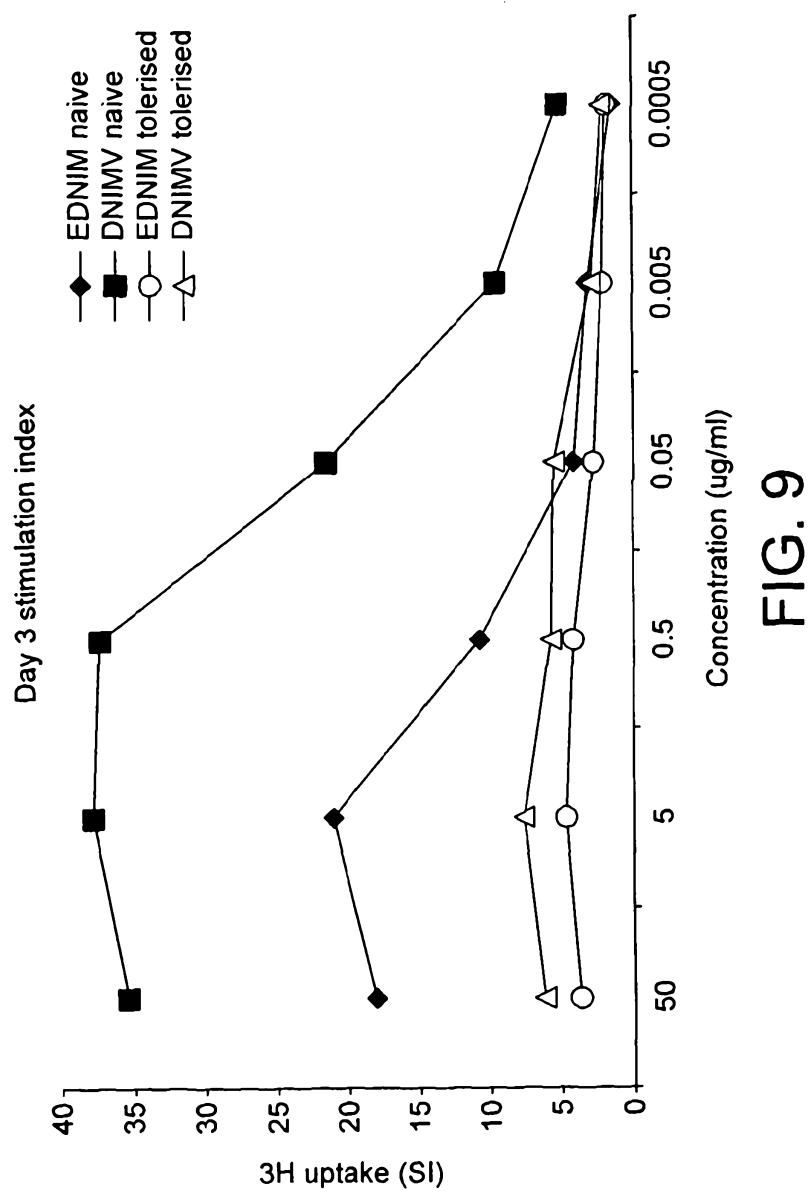


FIG. 9

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- 1 -

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<140> PCT/GB2010/000997

<141> 2010-05-17

<150> GB 0908515.0

<151> 2009-05-18

<160> 83

<170> PatentIn version 3.5

<210> 1

<211> 2351

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20 25 30

Trp Asp Tyr Met Gln Ser Asp Leu Gly Glu Leu Pro Val Asp Ala Arg
35 40 45

Phe Pro Pro Arg Val Pro Lys Ser Phe Pro Phe Asn Thr Ser Val Val
50 55 60

Tyr Lys Lys Thr Leu Phe Val Glu Phe Thr Asp His Leu Phe Asn Ile
65 70 75 80

Ala Lys Pro Arg Pro Pro Trp Met Gly Leu Leu Gly Pro Thr Ile Gln
85 90 95

Ala Glu Val Tyr Asp Thr Val Val Ile Thr Leu Lys Asn Met Ala Ser
100 105 110

His Pro Val Ser Leu His Ala Val Gly Val Ser Tyr Trp Lys Ala Ser
115 120 125

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- 2 -

Glu Gly Ala Glu Tyr Asp Asp Gln Thr Ser Gln Arg Glu Lys Glu Asp
130 135 140

Asp Lys Val Phe Pro Gly Gly Ser His Thr Tyr Val Trp Gln Val Leu
145 150 155 160

Lys Glu Asn Gly Pro Met Ala Ser Asp Pro Leu Cys Leu Thr Tyr Ser
165 170 175

Tyr Leu Ser His Val Asp Leu Val Lys Asp Leu Asn Ser Gly Leu Ile
180 185 190

Gly Ala Leu Leu Val Cys Arg Glu Gly Ser Leu Ala Lys Glu Lys Thr
195 200 205

Gln Thr Leu His Lys Phe Ile Leu Leu Phe Ala Val Phe Asp Glu Gly
210 215 220

Lys Ser Trp His Ser Glu Thr Lys Asn Ser Leu Met Gln Asp Arg Asp
225 230 235 240

Ala Ala Ser Ala Arg Ala Trp Pro Lys Met His Thr Val Asn Gly Tyr
245 250 255

Val Asn Arg Ser Leu Pro Gly Leu Ile Gly Cys His Arg Lys Ser Val
260 265 270

Tyr Trp His Val Ile Gly Met Gly Thr Thr Pro Glu Val His Ser Ile
275 280 285

Phe Leu Glu Gly His Thr Phe Leu Val Arg Asn His Arg Gln Ala Ser
290 295 300

Leu Glu Ile Ser Pro Ile Thr Phe Leu Thr Ala Gln Thr Leu Leu Met
305 310 315 320

Asp Leu Gly Gln Phe Leu Leu Phe Cys His Ile Ser Ser His Gln His
325 330 335

Asp Gly Met Glu Ala Tyr Val Lys Val Asp Ser Cys Pro Glu Glu Pro
340 345 350

Gln Leu Arg Met Lys Asn Asn Glu Glu Ala Glu Asp Tyr Asp Asp Asp

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355

360

365

Leu Thr Asp Ser Glu Met Asp Val Val Arg Phe Asp Asp Asp Asn Ser
370 375 380

Pro Ser Phe Ile Gln Ile Arg Ser Val Ala Lys Lys His Pro Lys Thr
385 390 395 400

Trp Val His Tyr Ile Ala Ala Glu Glu Asp Trp Asp Tyr Ala Pro
405 410 415

Leu Val Leu Ala Pro Asp Asp Arg Ser Tyr Lys Ser Gln Tyr Leu Asn
420 425 430

Asn Gly Pro Gln Arg Ile Gly Arg Lys Tyr Lys Lys Val Arg Phe Met
435 440 445

Ala Tyr Thr Asp Glu Thr Phe Lys Thr Arg Glu Ala Ile Gln His Glu
450 455 460

Ser Gly Ile Leu Gly Pro Leu Leu Tyr Gly Glu Val Gly Asp Thr Leu
465 470 475 480

Leu Ile Ile Phe Lys Asn Gln Ala Ser Arg Pro Tyr Asn Ile Tyr Pro
485 490 495

His Gly Ile Thr Asp Val Arg Pro Leu Tyr Ser Arg Arg Leu Pro Lys
500 505 510

Gly Val Lys His Leu Lys Asp Phe Pro Ile Leu Pro Gly Glu Ile Phe
515 520 525

Lys Tyr Lys Trp Thr Val Thr Val Glu Asp Gly Pro Thr Lys Ser Asp
530 535 540

Pro Arg Cys Leu Thr Arg Tyr Tyr Ser Ser Phe Val Asn Met Glu Arg
545 550 555 560

Asp Leu Ala Ser Gly Leu Ile Gly Pro Leu Leu Ile Cys Tyr Lys Glu
565 570 575

Ser Val Asp Gln Arg Gly Asn Gln Ile Met Ser Asp Lys Arg Asn Val
580 585 590

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Ile Leu Phe Ser Val Phe Asp Glu Asn Arg Ser Trp Tyr Leu Thr Glu
595 600 605

Asn Ile Gln Arg Phe Leu Pro Asn Pro Ala Gly Val Gln Leu Glu Asp
610 615 620

Pro Glu Phe Gln Ala Ser Asn Ile Met His Ser Ile Asn Gly Tyr Val
625 630 635 640

Phe Asp Ser Leu Gln Leu Ser Val Cys Leu His Glu Val Ala Tyr Trp
645 650 655

Tyr Ile Leu Ser Ile Gly Ala Gln Thr Asp Phe Leu Ser Val Phe Phe
660 665 670

Ser Gly Tyr Thr Phe Lys His Lys Met Val Tyr Glu Asp Thr Leu Thr
675 680 685

Leu Phe Pro Phe Ser Gly Glu Thr Val Phe Met Ser Met Glu Asn Pro
690 695 700

Gly Leu Trp Ile Leu Gly Cys His Asn Ser Asp Phe Arg Asn Arg Gly
705 710 715 720

Met Thr Ala Leu Leu Lys Val Ser Ser Cys Asp Lys Asn Thr Gly Asp
725 730 735

Tyr Tyr Glu Asp Ser Tyr Glu Asp Ile Ser Ala Tyr Leu Leu Ser Lys
740 745 750

Asn Asn Ala Ile Glu Pro Arg Ser Phe Ser Gln Asn Ser Arg His Pro
755 760 765

Ser Thr Arg Gln Lys Gln Phe Asn Ala Thr Thr Ile Pro Glu Asn Asp
770 775 780

Ile Glu Lys Thr Asp Pro Trp Phe Ala His Arg Thr Pro Met Pro Lys
785 790 795 800

Ile Gln Asn Val Ser Ser Asp Leu Leu Met Leu Leu Arg Gln Ser
805 810 815

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- 5 -

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Glu Thr Phe Ser Asp Asp Pro Ser Pro Gly Ala Ile Asp Ser Asn Asn
835 840 845

Ser Leu Ser Glu Met Thr His Phe Arg Pro Gln Leu His His Ser Gly
850 855 860

Asp Met Val Phe Thr Pro Glu Ser Gly Leu Gln Leu Arg Leu Asn Glu
865 870 875 880

Lys Leu Gly Thr Thr Ala Ala Thr Glu Leu Lys Lys Leu Asp Phe Lys
885 890 895

Val Ser Ser Thr Ser Asn Asn Leu Ile Ser Thr Ile Pro Ser Asp Asn
900 905 910

Leu Ala Ala Gly Thr Asp Asn Thr Ser Ser Leu Gly Pro Pro Ser Met
915 920 925

Pro Val His Tyr Asp Ser Gln Leu Asp Thr Thr Leu Phe Gly Lys Lys
930 935 940

Ser Ser Pro Leu Thr Glu Ser Gly Gly Pro Leu Ser Leu Ser Glu Glu
945 950 955 960

Asn Asn Asp Ser Lys Leu Leu Glu Ser Gly Leu Met Asn Ser Gln Glu
965 970 975

Ser Ser Trp Gly Lys Asn Val Ser Ser Thr Glu Ser Gly Arg Leu Phe
980 985 990

Lys Gly Lys Arg Ala His Gly Pro Ala Leu Leu Thr Lys Asp Asn Ala
995 1000 1005

Leu Phe Lys Val Ser Ile Ser Leu Leu Lys Thr Asn Lys Thr Ser
1010 1015 1020

Asn Asn Ser Ala Thr Asn Arg Lys Thr His Ile Asp Gly Pro Ser
1025 1030 1035

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Leu Leu Ile Glu Asn Ser Pro Ser Val Trp Gln Asn Ile Leu Glu
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Ser Asp Thr Glu Phe Lys Lys Val Thr Pro Leu Ile His Asp Arg
1055 1060 1065

Met Leu Met Asp Lys Asn Ala Thr Ala Leu Arg Leu Asn His Met
1070 1075 1080

Ser Asn Lys Thr Thr Ser Ser Lys Asn Met Glu Met Val Gln Gln
1085 1090 1095

Lys Lys Glu Gly Pro Ile Pro Pro Asp Ala Gln Asn Pro Asp Met
1100 1105 1110

Ser Phe Phe Lys Met Leu Phe Leu Pro Glu Ser Ala Arg Trp Ile
1115 1120 1125

Gln Arg Thr His Gly Lys Asn Ser Leu Asn Ser Gly Gln Gly Pro
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Ser Pro Lys Gln Leu Val Ser Leu Gly Pro Glu Lys Ser Val Glu
1145 1150 1155

Gly Gln Asn Phe Leu Ser Glu Lys Asn Lys Val Val Val Gly Lys
1160 1165 1170

Gly Glu Phe Thr Lys Asp Val Gly Leu Lys Glu Met Val Phe Pro
1175 1180 1185

Ser Ser Arg Asn Leu Phe Leu Thr Asn Leu Asp Asn Leu His Glu
1190 1195 1200

Asn Asn Thr His Asn Gln Glu Lys Lys Ile Gln Glu Glu Ile Glu
1205 1210 1215

Lys Lys Glu Thr Leu Ile Gln Glu Asn Val Val Leu Pro Gln Ile
1220 1225 1230

His Thr Val Thr Gly Thr Lys Asn Phe Met Lys Asn Leu Phe Leu
1235 1240 1245

Leu Ser Thr Arg Gln Asn Val Glu Gly Ser Tyr Asp Gly Ala Tyr

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Ala Pro Val Leu Gln Asp Phe Arg Ser Leu Asn Asp Ser Thr Asn		
1265	1270	1275
Arg Thr Lys Lys His Thr Ala His Phe Ser Lys Lys Gly Glu Glu		
1280	1285	1290
Glu Asn Leu Glu Gly Leu Gly Asn Gln Thr Lys Gln Ile Val Glu		
1295	1300	1305
Lys Tyr Ala Cys Thr Thr Arg Ile Ser Pro Asn Thr Ser Gln Gln		
1310	1315	1320
Asn Phe Val Thr Gln Arg Ser Lys Arg Ala Leu Lys Gln Phe Arg		
1325	1330	1335
Leu Pro Leu Glu Glu Thr Glu Leu Glu Lys Arg Ile Ile Val Asp		
1340	1345	1350
Asp Thr Ser Thr Gln Trp Ser Lys Asn Met Lys His Leu Thr Pro		
1355	1360	1365
Ser Thr Leu Thr Gln Ile Asp Tyr Asn Glu Lys Glu Lys Gly Ala		
1370	1375	1380
Ile Thr Gln Ser Pro Leu Ser Asp Cys Leu Thr Arg Ser His Ser		
1385	1390	1395
Ile Pro Gln Ala Asn Arg Ser Pro Leu Pro Ile Ala Lys Val Ser		
1400	1405	1410
Ser Phe Pro Ser Ile Arg Pro Ile Tyr Leu Thr Arg Val Leu Phe		
1415	1420	1425
Gln Asp Asn Ser Ser His Leu Pro Ala Ala Ser Tyr Arg Lys Lys		
1430	1435	1440
Asp Ser Gly Val Gln Glu Ser Ser His Phe Leu Gln Gly Ala Lys		
1445	1450	1455
Lys Asn Asn Leu Ser Leu Ala Ile Leu Thr Leu Glu Met Thr Gly		
1460	1465	1470

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Asp Gln Arg Glu Val Gly Ser Leu Gly Thr Ser Ala Thr Asn Ser
1475 1480 1485

Val Thr Tyr Lys Lys Val Glu Asn Thr Val Leu Pro Lys Pro Asp
1490 1495 1500

Leu Pro Lys Thr Ser Gly Lys Val Glu Leu Leu Pro Lys Val His
1505 1510 1515

Ile Tyr Gln Lys Asp Leu Phe Pro Thr Glu Thr Ser Asn Gly Ser
1520 1525 1530

Pro Gly His Leu Asp Leu Val Glu Gly Ser Leu Leu Gln Gly Thr
1535 1540 1545

Glu Gly Ala Ile Lys Trp Asn Glu Ala Asn Arg Pro Gly Lys Val
1550 1555 1560

Pro Phe Leu Arg Val Ala Thr Glu Ser Ser Ala Lys Thr Pro Ser
1565 1570 1575

Lys Leu Leu Asp Pro Leu Ala Trp Asp Asn His Tyr Gly Thr Gln
1580 1585 1590

Ile Pro Lys Glu Glu Trp Lys Ser Gln Glu Lys Ser Pro Glu Lys
1595 1600 1605

Thr Ala Phe Lys Lys Asp Thr Ile Leu Ser Leu Asn Ala Cys
1610 1615 1620

Glu Ser Asn His Ala Ile Ala Ala Ile Asn Glu Gly Gln Asn Lys
1625 1630 1635

Pro Glu Ile Glu Val Thr Trp Ala Lys Gln Gly Arg Thr Glu Arg
1640 1645 1650

Leu Cys Ser Gln Asn Pro Pro Val Leu Lys Arg His Gln Arg Glu
1655 1660 1665

Ile Thr Arg Thr Thr Leu Gln Ser Asp Gln Glu Glu Ile Asp Tyr
1670 1675 1680

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Asp Asp Thr Ile Ser Val Glu Met Lys Lys Glu Asp Phe Asp Ile
1685 1690 1695

Tyr Asp Glu Asp Glu Asn Gln Ser Pro Arg Ser Phe Gln Lys Lys
1700 1705 1710

Thr Arg His Tyr Phe Ile Ala Ala Val Glu Arg Leu Trp Asp Tyr
1715 1720 1725

Gly Met Ser Ser Ser Pro His Val Leu Arg Asn Arg Ala Gln Ser
1730 1735 1740

Gly Ser Val Pro Gln Phe Lys Lys Val Val Phe Gln Glu Phe Thr
1745 1750 1755

Asp Gly Ser Phe Thr Gln Pro Leu Tyr Arg Gly Glu Leu Asn Glu
1760 1765 1770

His Leu Gly Leu Leu Gly Pro Tyr Ile Arg Ala Glu Val Glu Asp
1775 1780 1785

Asn Ile Met Val Thr Phe Arg Asn Gln Ala Ser Arg Pro Tyr Ser
1790 1795 1800

Phe Tyr Ser Ser Leu Ile Ser Tyr Glu Glu Asp Gln Arg Gln Gly
1805 1810 1815

Ala Glu Pro Arg Lys Asn Phe Val Lys Pro Asn Glu Thr Lys Thr
1820 1825 1830

Tyr Phe Trp Lys Val Gln His His Met Ala Pro Thr Lys Asp Glu
1835 1840 1845

Phe Asp Cys Lys Ala Trp Ala Tyr Phe Ser Asp Val Asp Leu Glu
1850 1855 1860

Lys Asp Val His Ser Gly Leu Ile Gly Pro Leu Leu Val Cys His
1865 1870 1875

Thr Asn Thr Leu Asn Pro Ala His Gly Arg Gln Val Thr Val Gln
1880 1885 1890

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Glu Phe Ala Leu Phe Phe Thr Ile Phe Asp Glu Thr Lys Ser Trp
1895 1900 1905

Tyr Phe Thr Glu Asn Met Glu Arg Asn Cys Arg Ala Pro Cys Asn
1910 1915 1920

Ile Gln Met Glu Asp Pro Thr Phe Lys Glu Asn Tyr Arg Phe His
1925 1930 1935

Ala Ile Asn Gly Tyr Ile Met Asp Thr Leu Pro Gly Leu Val Met
1940 1945 1950

Ala Gln Asp Gln Arg Ile Arg Trp Tyr Leu Leu Ser Met Gly Ser
1955 1960 1965

Asn Glu Asn Ile His Ser Ile His Phe Ser Gly His Val Phe Thr
1970 1975 1980

Val Arg Lys Lys Glu Glu Tyr Lys Met Ala Leu Tyr Asn Leu Tyr
1985 1990 1995

Pro Gly Val Phe Glu Thr Val Glu Met Leu Pro Ser Lys Ala Gly
2000 2005 2010

Ile Trp Arg Val Glu Cys Leu Ile Gly Glu His Leu His Ala Gly
2015 2020 2025

Met Ser Thr Leu Phe Leu Val Tyr Ser Asn Lys Cys Gln Thr Pro
2030 2035 2040

Leu Gly Met Ala Ser Gly His Ile Arg Asp Phe Gln Ile Thr Ala
2045 2050 2055

Ser Gly Gln Tyr Gly Gln Trp Ala Pro Lys Leu Ala Arg Leu His
2060 2065 2070

Tyr Ser Gly Ser Ile Asn Ala Trp Ser Thr Lys Glu Pro Phe Ser
2075 2080 2085

Trp Ile Lys Val Asp Leu Leu Ala Pro Met Ile Ile His Gly Ile
2090 2095 2100

Lys Thr Gln Gly Ala Arg Gln Lys Phe Ser Ser Leu Tyr Ile Ser

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2105	2110	2115
Gln Phe Ile Ile Met Tyr Ser	Leu Asp Gly Lys	Trp Gln Thr
2120	2125	2130
Tyr Arg Gly Asn Ser Thr Gly	Thr Leu Met Val Phe	Phe Gly Asn
2135	2140	2145
Val Asp Ser Ser Gly Ile Lys	His Asn Ile Phe Asn	Pro Pro Ile
2150	2155	2160
Ile Ala Arg Tyr Ile Arg Leu	His Pro Thr His Tyr	Ser Ile Arg
2165	2170	2175
Ser Thr Leu Arg Met Glu Trp	Met Gly Cys Asp Leu	Asn Ser Cys
2180	2185	2190
Ser Met Pro Leu Gly Met Glu	Ser Lys Ala Ile Ser	Asp Ala Gln
2195	2200	2205
Ile Thr Ala Ser Ser Tyr Phe	Thr Asn Met Phe Ala	Thr Trp Ser
2210	2215	2220
Pro Ser Lys Ala Arg Leu His	Leu Gln Gly Arg Ser	Asn Ala Trp
2225	2230	2235
Arg Pro Gln Val Asn Asn Pro	Lys Glu Trp Leu Gln	Val Asp Phe
2240	2245	2250
Gln Lys Thr Met Lys Val Thr	Gly Val Thr Thr Gln	Gly Val Lys
2255	2260	2265
Ser Leu Leu Thr Ser Met Tyr	Val Lys Glu Phe Leu	Ile Ser Ser
2270	2275	2280
Ser Gln Asp Gly His Gln Trp	Thr Leu Phe Phe Gln	Asn Gly Lys
2285	2290	2295
Val Lys Val Phe Gln Gly Asn	Gln Asp Ser Phe Thr	Pro Val Val
2300	2305	2310
Asn Ser Leu Asp Pro Pro Leu	Leu Thr Arg Tyr Leu	Arg Ile His
2315	2320	2325

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Pro Gln Ser Trp Val His Gln Ile Ala Leu Arg Met Glu Val Leu
2330 2335 2340

Gly Cys Glu Ala Gln Asp Leu Tyr
2345 2350

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Thr Gln Thr Leu His Lys Phe Ile Leu Leu Phe Ala Val Phe Asp
1 5 10 15

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Ser Leu Tyr Ile Ser Gln Phe Ile Ile Met Tyr Ser Leu Asp Gly
1 5 10 15

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Pro Pro Ile Ile Ala Arg Tyr Ile Arg Leu His Pro Thr His Tyr
1 5 10 15

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<211> 15
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Pro Pro Leu Leu Thr Arg Tyr Leu Arg Ile His Pro Gln Ser Trp
1 5 10 15

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<213> Homo sapiens

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Met His Thr Val Asn Gly Tyr Val Asn Arg Ser Leu Pro Gly Leu
1 5 10 15

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<212> PRT

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Leu Gly Gln Phe Leu Leu Phe Cys His Ile Ser Ser His Gln His
1 5 10 15

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<213> Homo sapiens

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Asp Thr Leu Leu Ile Ile Phe Lys Asn Gln Ala Ser Arg Pro Tyr
1 5 10 15

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Pro Arg Cys Leu Thr Arg Tyr Tyr Ser Ser Phe Val Asn Met Glu
1 5 10 15

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Thr Glu Asn Ile Gln Arg Phe Leu Pro Asn Pro Ala Gly Val Gln
1 5 10 15

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Asp Asn Ile Met Val Thr Phe Arg Asn Gln Ala Ser Arg Pro Tyr
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Arg Tyr Leu Arg Ile His Pro Gln Ser Trp Val His Gln Ile Ala
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Gly Thr Leu Met Val Phe Phe Gly Asn Val Asp Ser Ser Gly Ile
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Leu Tyr Ile Ser Gln Phe Ile Ile Met
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Phe Ile Ile Met Tyr Ser Leu Asp Gly
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Ile Ala Arg Tyr Ile Arg Leu His Pro
1 5

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Leu Ile Ile Phe Lys Asn Gln Ala Ser
1 5

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<212> PRT
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Leu Thr Arg Tyr Tyr Ser Ser Phe Val
1 5

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Met Val Thr Phe Arg Asn Gln Ala Ser
1 5

<210> 20
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Leu Arg Ile His Pro Gln Ser Trp Val
1 5

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<400> 21

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1 5 10

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<222> (1)...(1)

<223> Xaa is a charged amino acid with reverse polarity to Xaa at
position 14

<220>

<221> MISC_FEATURE

<222> (14)...(14)

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position 1

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Xaa Asp Asn Ile Met Val Thr Phe Arg Asn Gln Ala Ser Xaa
1 5 10

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Glu Asp Asn Ile Met Val Thr Phe Arg Asn Gln Ala Ser Arg
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<211> 15

<212> PRT

<213> Homo sapiens

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Tyr Gly Glu Val Gly Asp Thr Leu Leu Ile Ile Phe Lys Asn Gln
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<213> Homo sapiens

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Gly Glu Val Gly Asp Thr Leu Leu Ile Ile Phe Lys Asn Gln Ala
1 5 10 15

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Glu Val Gly Asp Thr Leu Leu Ile Ile Phe Lys Asn Gln Ala Ser
1 5 10 15

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<213> Homo sapiens

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Val Gly Asp Thr Leu Leu Ile Ile Phe Lys Asn Gln Ala Ser Arg
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<213> Homo sapiens

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Gly Asp Thr Leu Leu Ile Ile Phe Lys Asn Gln Ala Ser Arg Pro
1 5 10 15

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<213> Homo sapiens

<400> 29

Thr Leu Leu Ile Ile Phe Lys Asn Gln Ala Ser Arg Pro Tyr Asn
1 5 10 15

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<212> PRT

<213> Homo sapiens

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<400> 30

Leu Leu Ile Ile Phe Lys Asn Gln Ala Ser Arg Pro Tyr Asn Ile
1 5 10 15

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<213> Homo sapiens

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Leu Ile Ile Phe Lys Asn Gln Ala Ser Arg Pro Tyr Asn Ile Tyr
1 5 10 15

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<212> PRT

<213> Homo sapiens

<400> 32

Ile Ile Phe Lys Asn Gln Ala Ser Arg Pro Tyr Asn Ile Tyr Pro
1 5 10 15

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<213> Homo sapiens

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Ile Phe Lys Asn Gln Ala Ser Arg Pro Tyr Asn Ile Tyr Pro His
1 5 10 15

<210> 34

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<213> Homo sapiens

<400> 34

Pro Thr Lys Ser Asp Pro Arg Cys Leu Thr Arg Tyr Tyr Ser Ser
1 5 10 15

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Thr Lys Ser Asp Pro Arg Cys Leu Thr Arg Tyr Tyr Ser Ser Phe
1 5 10 15

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Lys Ser Asp Pro Arg Cys Leu Thr Arg Tyr Tyr Ser Ser Phe Val
1 5 10 15

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<400> 37

Ser Asp Pro Arg Cys Leu Thr Arg Tyr Tyr Ser Ser Phe Val Asn
1 5 10 15

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Asp Pro Arg Cys Leu Thr Arg Tyr Tyr Ser Ser Phe Val Asn Met
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Arg Cys Leu Thr Arg Tyr Tyr Ser Ser Phe Val Asn Met Glu Arg
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Cys Leu Thr Arg Tyr Tyr Ser Ser Phe Val Asn Met Glu Arg Asp
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<210> 41
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Leu Thr Arg Tyr Tyr Ser Ser Phe Val Asn Met Glu Arg Asp Leu
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Thr Arg Tyr Tyr Ser Ser Phe Val Asn Met Glu Arg Asp Leu Ala
1 5 10 15

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Arg Tyr Tyr Ser Ser Phe Val Asn Met Glu Arg Asp Leu Ala Ser
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Arg Ala Glu Val Glu Asp Asn Ile Met Val Thr Phe Arg Asn Gln
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Ala Glu Val Glu Asp Asn Ile Met Val Thr Phe Arg Asn Gln Ala
1 5 10 15

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<210> 46
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Glu Val Glu Asp Asn Ile Met Val Thr Phe Arg Asn Gln Ala Ser
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Val Glu Asp Asn Ile Met Val Thr Phe Arg Asn Gln Ala Ser Arg
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Glu Asp Asn Ile Met Val Thr Phe Arg Asn Gln Ala Ser Arg Pro
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Asn Ile Met Val Thr Phe Arg Asn Gln Ala Ser Arg Pro Tyr Ser
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Ile Met Val Thr Phe Arg Asn Gln Ala Ser Arg Pro Tyr Ser Phe
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<212> PRT
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Met Val Thr Phe Arg Asn Gln Ala Ser Arg Pro Tyr Ser Phe Tyr
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Val Thr Phe Arg Asn Gln Ala Ser Arg Pro Tyr Ser Phe Tyr Ser
1 5 10 15

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<400> 53

Thr Phe Arg Asn Gln Ala Ser Arg Pro Tyr Ser Phe Tyr Ser Ser
1 5 10 15

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Arg Gln Lys Phe Ser Ser Leu Tyr Ile Ser Gln Phe Ile Ile Met
1 5 10 15

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Gln Lys Phe Ser Ser Leu Tyr Ile Ser Gln Phe Ile Ile Met Tyr
1 5 10 15

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<400> 56

Lys Phe Ser Ser Leu Tyr Ile Ser Gln Phe Ile Ile Met Tyr Ser
1 5 10 15

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<213> Homo sapiens

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Phe Ser Ser Leu Tyr Ile Ser Gln Phe Ile Ile Met Tyr Ser Leu
1 5 10 15

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<400> 58

Ser Ser Leu Tyr Ile Ser Gln Phe Ile Ile Met Tyr Ser Leu Asp
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Leu Tyr Ile Ser Gln Phe Ile Ile Met Tyr Ser Leu Asp Gly Lys
1 5 10 15

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Tyr Ile Ser Gln Phe Ile Ile Met Tyr Ser Leu Asp Gly Lys Lys
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Ile Ser Gln Phe Ile Ile Met Tyr Ser Leu Asp Gly Lys Lys Trp
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Ser Gln Phe Ile Ile Met Tyr Ser Leu Asp Gly Lys Lys Trp Gln
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Gln Phe Ile Ile Met Tyr Ser Leu Asp Gly Lys Lys Trp Gln Thr
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His Asn Ile Phe Asn Pro Pro Ile Ile Ala Arg Tyr Ile Arg Leu
1 5 10 15

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Asn Ile Phe Asn Pro Pro Ile Ile Ala Arg Tyr Ile Arg Leu His
1 5 10 15

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<400> 66

Ile Phe Asn Pro Pro Ile Ile Ala Arg Tyr Ile Arg Leu His Pro

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1 5 10 15

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Phe Asn Pro Pro Ile Ile Ala Arg Tyr Ile Arg Leu His Pro Thr
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Asn Pro Pro Ile Ile Ala Arg Tyr Ile Arg Leu His Pro Thr His
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Pro Ile Ile Ala Arg Tyr Ile Arg Leu His Pro Thr His Tyr Ser
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Ile Ile Ala Arg Tyr Ile Arg Leu His Pro Thr His Tyr Ser Ile
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Ile Ala Arg Tyr Ile Arg Leu His Pro Thr His Tyr Ser Ile Arg
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<210> 72
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Ala Arg Tyr Ile Arg Leu His Pro Thr His Tyr Ser Ile Arg Ser
1 5 10 15

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Arg Tyr Ile Arg Leu His Pro Thr His Tyr Ser Ile Arg Ser Thr
1 5 10 15

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Pro Pro Leu Leu Thr Arg Tyr Leu Arg Ile His Pro Gln Ser Trp
1 5 10 15

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Pro Leu Leu Thr Arg Tyr Leu Arg Ile His Pro Gln Ser Trp Val
1 5 10 15

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<400> 76

Leu Leu Thr Arg Tyr Leu Arg Ile His Pro Gln Ser Trp Val His
1 5 10 15

<210> 77

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<211> 15
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Leu Thr Arg Tyr Leu Arg Ile His Pro Gln Ser Trp Val His Gln
1 5 10 15

<210> 78
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<400> 78

Thr Arg Tyr Leu Arg Ile His Pro Gln Ser Trp Val His Gln Ile
1 5 10 15

<210> 79
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Tyr Leu Arg Ile His Pro Gln Ser Trp Val His Gln Ile Ala Leu
1 5 10 15

<210> 80
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Leu Arg Ile His Pro Gln Ser Trp Val His Gln Ile Ala Leu Arg
1 5 10 15

<210> 81
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Arg Ile His Pro Gln Ser Trp Val His Gln Ile Ala Leu Arg Met
1 5 10 15

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Ile His Pro Gln Ser Trp Val His Gln Ile Ala Leu Arg Met Glu
1 5 10 15

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<212> PRT

<213> Homo sapiens

<400> 83

His Pro Gln Ser Trp Val His Gln Ile Ala Leu Arg Met Glu Val
1 5 10 15