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(54) Title: T-CELL EPITODES IN CARBOXYPEPTIDASE G2

(57) Abstract: The invention in particular relates to the modification of a bacterial enzyme carboxypeptidease G2 (CPG2) to result in CPG2 proteins that are substantially non-immunogenic or less immunogenic than any non-modified counterpart when used in vivo. The present invention relates also to T-cell epitope peptides derived from said non-modified protein by means of which it is possible to create modified CPG2 variants with reduced immunogenicity. These polypeptides are suitable particularly for therapeutic use in humans.

T-CELL EPITOPES IN CARBOXYPEPTIDASE G2

FIELD OF THE INVENTION

The present invention relates to polypeptides to be administered especially to humans and in particular for therapeutic use. The polypeptides are modified polypeptides whereby the modification results in a reduced propensity for the polypeptide to elicit an immune response upon administration to the human subject. The invention in particular relates to the modification of a bacterial enzyme carboxypeptidease G2 (CPG2) to result in CPG2 proteins that are substantially non-immunogenic or less immunogenic than any non-modified counterpart when used *in vivo*. The invention relates furthermore to T-cell epitope peptides derived from said non-modified protein by means of which it is possible to create modified CPG2 variants with reduced immunogenicity.

BACKGROUND OF THE INVENTION

There are many instances whereby the efficacy of a therapeutic protein is limited by an unwanted immune reaction to the therapeutic protein. Several mouse monoclonal antibodies have shown promise as therapies in a number of human disease settings but in certain cases have failed due to the induction of significant degrees of a human anti-murine antibody (HAMA) response [Schroff, R. W. et al (1985) *Cancer Res.* 45: 879-885; Shawler, D.L. et al (1985) *J. Immunol.* 135: 1530-1535]. For monoclonal antibodies, a number of techniques have been developed in attempt to reduce the HAMA response [WO 89/09622; EP 0239400; EP 0438310; WO 91/06667]. These recombinant DNA approaches have generally reduced the mouse genetic information in the final antibody construct whilst increasing the human genetic information in the final construct. Notwithstanding, the resultant "humanized" antibodies have, in several cases, still elicited an immune response in patients [Issacs J.D. (1990) *Sem. Immunol.* 2: 449, 456; Rebello, P.R. et al (1999) *Transplantation* 68: 1417-1420].

Antibodies are not the only class of polypeptide molecule administered as a therapeutic agent against which an immune response may be mounted. Even proteins of human origin and with the same amino acid sequences as occur within humans can still induce an immune response in humans. Notable examples amongst others include the therapeutic use of granulocyte-macrophage colony stimulating factor [Wadhwa, M. et al (1999) *Clin. Cancer Res.* 5: 1353-1361] and interferon alpha 2 [Russo, D. et al (1996) *Bri. J. Haem.*

94: 300-305; Stein, R. et al (1988) *New Engl. J. Med.* 318: 1409-1413]. In such situations where these human proteins are immunogenic, there is a presumed breakage of immunological tolerance that would otherwise have been operating in these subjects to these proteins.

This situation is different where the human protein is being administered as a replacement therapy for example in a genetic disease where there is a constitutional lack of the protein such as can be the case for diseases such as hemophilia A, Christmas disease, Gauchers disease and numerous other examples. In such cases, the therapeutic replacement protein may function immunologically as a foreign molecule from the outset, and where the individuals are able to mount an immune response to the therapeutic, the efficacy of the therapy is likely to be significantly compromised.

Irrespective of whether the protein therapeutic is seen by the host immune system as a foreign molecule, or if an existing tolerance to the molecule is overcome, the mechanism of immune reactivity to the protein is the same. Key to the induction of an immune response is the presence within the protein of peptides that can stimulate the activity of T-cells via presentation on MHC class II molecules, so-called "T-cell epitopes". Such T-cell epitopes are commonly defined as any amino acid residue sequence with the ability to bind to MHC Class II molecules. Implicitly, a "T-cell epitope" means an epitope which when bound to MHC molecules can be recognized by a T-cell receptor (TCR), and which can, at least in principle, cause the activation of these T-cells by engaging a TCR to promote a T-cell response.

MHC Class II molecules are a group of highly polymorphic proteins which play a central role in helper T-cell selection and activation. The human leukocyte antigen group DR (HLA-DR) are the predominant isotype of this group of proteins however, isotypes HLA-DQ and HLA-DP perform similar functions. In the human population, individuals bear two to four DR alleles, two DQ and two DP alleles. The structure of a number of DR molecules has been solved and these appear as an open-ended peptide binding groove with a number of hydrophobic pockets which engage hydrophobic residues (pocket residues) of the peptide [Brown et al *Nature* (1993) 364: 33; Stern et al (1994) *Nature* 368: 215]. Polymorphism identifying the different allotypes of class II molecule contributes to a wide diversity of different binding surfaces for peptides within the

peptide binding grove and at the population level ensures maximal flexibility with regard to the ability to recognize foreign proteins and mount an immune response to pathogenic organisms.

An immune response to a therapeutic protein proceeds via the MHC class II peptide presentation pathway. Here exogenous proteins are engulfed and processed for presentation in association with MHC class II molecules of the DR, DQ or DP type. MHC Class II molecules are expressed by professional antigen presenting cells (APCs), such as macrophages and dendritic cells amongst others. Engagement of a MHC class II peptide complex by a cognate T-cell receptor on the surface of the T-cell, together with the cross-binding of certain other co-receptors such as the CD4 molecule, can induce an activated state within the T-cell. Activation leads to the release of cytokines further activating other lymphocytes such as B cells to produce antibodies or activating T killer cells as a full cellular immune response.

T-cell epitope identification is the first step to epitope elimination, however there are few clear cases in the art where epitope identification and epitope removal are integrated into a single scheme. Thus WO98/52976 and WO00/34317 teach computational threading approaches to identifying polypeptide sequences with the potential to bind a sub-set of human MHC class II DR allotypes. In these teachings, predicted T-cell epitopes are removed by the use of judicious amino acid substitution within the protein of interest. However with this scheme and other computationally based procedures for epitope identification [Godkin, A.J. et al (1998) *J. Immunol.* 161: 850-858; Sturniolo, T. et al (1999) *Nat. Biotechnol.* 17: 555-561], peptides predicted to be able to bind MHC class II molecules may not function as T-cell epitopes in all situations, particularly, *in vivo* due to the processing pathways or other phenomena. In addition, the computational approaches to T-cell epitope prediction have in general not been capable of predicting epitopes with DP or DQ restriction.

Equally, *in vitro* methods for measuring the ability of synthetic peptides to bind MHC class II molecules, for example using B-cell lines of defined MHC allotype as a source of MHC class II binding surface and may be applied to MHC class II ligand identification [Marshall K.W. et al. (1994) *J. Immunol.* 152:4946-4956; O'Sullivan et al (1990) *J. Immunol.* 145: 1799-1808; Robadey C. et al (1997) *J. Immunol* 159: 3238-3246].

However, such techniques are not adapted for the screening multiple potential epitopes to a wide diversity of MHC allotypes, nor can they confirm the ability of a binding peptide to function as a T-cell epitope.

Recently techniques exploiting soluble complexes of recombinant MHC molecules in combination with synthetic peptides have come into use [Kern, F. et al (1998) *Nature Medicine* 4:975-978; Kwok, W.W. et al (2001) *TRENDS in Immunol.* 22:583-588]. These reagents and procedures are used to identify the presence of T-cell clones from peripheral blood samples from human or experimental animal subjects that are able to bind particular MHC-peptide complexes and are not adapted for the screening multiple potential epitopes to a wide diversity of MHC allotypes.

Biological assays of T-cell activation provide a practical option to providing a reading of the ability of a test peptide/protein sequence to evoke an immune response. Examples of this kind of approach include the work of Petra et al using T-cell proliferation assays to the bacterial protein staphylokinase, followed by epitope mapping using synthetic peptides to stimulate T-cell lines [Petra, A.M. et al (2002) *J. Immunol.* 168: 155-161]. Similarly, T-cell proliferation assays using synthetic peptides of the tetanus toxin protein have resulted in definition of immunodominant epitope regions of the toxin [Reece J.C. et al (1993) *J. Immunol.* 151: 6175-6184]. WO99/53038 discloses an approach whereby T-cell epitopes in a test protein may be determined using isolated sub-sets of human immune cells, promoting their differentiation *in vitro* and culture of the cells in the presence of synthetic peptides of interest and measurement of any induced proliferation in the cultured T-cells. The same technique is also described by Stickler et al [Stickler, M.M. et al (2000) *J. Immunotherapy* 23:654-660], where in both instances the method is applied to the detection of T-cell epitopes within bacterial subtilisin. Such a technique requires careful application of cell isolation techniques and cell culture with multiple cytokine supplements to obtain the desired immune cell sub-sets (dendritic cells, CD4+ and or CD8+ T-cells) and is not conducive to rapid through-put screening using multiple donor samples.

As depicted above and as consequence thereof, it would be desirable to identify and to remove or at least to reduce T-cell epitopes from a given in principal therapeutically valuable but originally immunogenic peptide, polypeptide or protein. One of these therapeutically valuable molecules is carboxypeptidase G2 (herein abbreviated to CPG2).

CPG2 is a bacterial enzyme (EC number 3.4.17.11) originally isolated from a Pseudomonas species strain RS-16. The enzyme has broad substrate specificity and catalyses the release of C-terminal glutamate residues from a range of different N-acyl groups. The gene encoding the enzyme has been characterized [Minton, N.P. et al (1984) *Gene* 31: 1-3] and the crystal structure of the protein resolved [Roswell, S. et al (1997) *Structure* 5: 337-347]. The enzyme has been used previously in an antibody-directed enzyme prodrug therapeutic (ADEPT) strategy for the treatment of cancer and has also been adopted for use in experimental gene-directed enzyme prodrug therapy (GDEPT). In the ADEPT approach, the enzyme molecule is linked to a targeting moiety such as an antibody or fragment of an antibody retaining the antigen binding specificity [Napier, M.P. et al (2000) *Clinical Cancer Res.* 6: 765-772]. The linkage to the antibody may be via chemical cross-linker or the antibody CPG2 enzyme may by be linked as a fusion protein expressed from a recombinant host organism such as E.*coli*.

The present invention is therefore concerned with the enzyme CPG2, and the amino acid sequence of the wild-type form of the protein as depicted in single letter code is as follows:

QKRDNVLFQAATDEQPAVIKTLEKLVNIETGTGDAEGIAAAGNFLEAELKNLGFTVTRSKSAGLV
VGDNIVGKIKGRGGKNLLLMSHMDTVYLKGILAKAPFRVEGDKAYGPGIADDKGGNAVILHTLKL
LKEYGVRDYGTITVLFNTDEEKGSFGSRDLIQEEAKLADYVLSFEPTSAGDEKLSLGTSGIAYVQ
VNITGKASHAGAAPELGVNALVEASDLVLRTMNIDDKAKNLRFNWTIAKAGNVSNIIPASATLNA
DVRYARNEDFDAAMKTLEERAQQKKLPEADVKVIVTRGRPAFNAGEGGKKLVDKAVAYYKEAGGT
LGVEERTGGGTDAAYAALSGKPVIESLGLPGFGYHSDKAEYVDISAIPRRLYMAARLIMDLGAGK

Despite the availability of therapeutic quantities of CPG2 fusion proteins there is a need for enhancement of the *in vivo* characteristics when administered to the human subject. In this regard, it is highly desired to provide CPG2 with reduced or absent potential to induce an immune response in the human subject. Such proteins would expect to display an increased circulation time within the human subject. The present invention provides for modified forms of CPG2 proteins that are expected to display enhanced properties *in vivo*.

Others have provided CPG2 molecules [WO88/07378] including modified CPG2 [US,6,004,550] but none of these teachings recognize the importance of T cell epitopes to

the immunogenic properties of the protein nor have been conceived to directly influence said properties in a specific and controlled way according to the scheme of the present invention.

It is a particular objective of the present invention to provide modified CPG2 proteins in which the immune characteristic is modified by means of reduced numbers of potential T-cell epitopes.

SUMMARY AND DESCRIPTION OF THE INVENTION

The present invention provides for modified forms of CPG2, in which the immune characteristic is modified by means of reduced numbers of potential T-cell epitopes. The invention discloses sequences identified within the CPG2 primary sequence that are potential T-cell epitopes by virtue of MHC class II binding potential. This disclosure specifically pertains the mature CPG2 protein of 390 amino acid residues.
The present invention discloses the major regions of the CPG2 primary sequence that are immunogenic in man and thereby provides the critical information required to conduct modification to the sequences to eliminate or reduce the immunogenic effectiveness of these sites.

In one embodiment, synthetic peptides comprising the immunogenic regions can be provided in pharmaceutical composition for the purpose of promoting a tolerogenic response to the whole molecule.
In a further embodiment CPG2 molecules modified within the epitope regions herein disclosed can be used in pharmaceutical compositions.

In summary the invention relates to the following issues:

- using a panel of synthetic peptides in a naïve T-cell assay to map the immunogenic region(s) of CPG2;
- using a panel of CPG2 protein variants in a naïve T-cell assay to select variants displaying minimal immunogenicity *in vitro*;
- using a panel of synthetic CPG2 peptide variants in a naïve T-cell assay to select peptide sequences displaying minimal immunogenicity *in vitro*;

- using biological assays of T-cell stimulation to select a CPG2 protein variant which exhibits a stimulation index of less than the index recorded for a wild-type CPG2 molecule and preferably less than 2.0 in a naïve T-cell assay;
- CPG2 derived peptide sequences originally found to have a stimulation index of greater than 1.8 and preferably greater than 2.0 in a naïve T-cell assay and wherein the peptide is modified to a minimum extent and tested in the naïve T-cell assay and found to have a reduced stimulation index;
- CPG2 derived peptide sequences sharing 100% amino acid identity with the wild-type protein sequence and able to evoke a stimulation index of 1.8 or greater and preferably greater than 2.0 in a naïve T-cell assay;
- an accordingly specified CPG2 peptide sequence modified to contain less than 100% amino acid identity with the wild-type protein sequence and evoking a stimulation index of less than 2.0 when tested in a T-cell assay;
- a CPG2 molecule containing modifications such that when tested in a T-cell assay evokes a reduced stimulation index in comparison to a non modified protein molecule;
- a CPG2 molecule in which the immunogenic regions have been mapped using a T-cell assay and then modified such that upon re-testing in a T-cell assay the modified protein evokes a stimulation index smaller than the parental (non-modified) molecule and most preferably less than 2.0;
- a modified molecule having the biological activity of CPG2 and being substantially non-immunogenic or less immunogenic than any non-modified molecule having the same biological activity when used *in vivo*;
- an accordingly specified CPG2 molecule, wherein said loss of immunogenicity is achieved by removing one or more T-cell epitopes derived from the originally non-modified molecule;
- an accordingly specified CPG2 molecule, wherein said loss of immunogenicity is achieved by reduction in numbers of MHC allotypes able to bind peptides derived from said molecule;
- an accordingly specified CPG2 molecule, wherein one T-cell epitope is removed;
- an accordingly specified CPG2 molecule, wherein said originally present T-cell epitopes are MHC class II ligands or peptide sequences which show the ability to stimulate or bind T-cells via presentation on class II;

- an accordingly specified molecule, wherein said peptide sequences are selected from the group as depicted in Table 1 or Table 2;
- an accordingly specified molecule, wherein the molecule contains one or more amino acid substitutions selected from the group as depicted in Table 4 or Table 5;
- an accordingly specified molecule, wherein 1 – 9 amino acid residues, preferably one amino acid residue in any of the originally present T-cell epitopes are altered;
- an accordingly specified molecule, wherein the alteration of the amino acid residues is substitution, addition or deletion of originally present amino acid(s) residue(s) by other amino acid residue(s) at specific position(s);
- an accordingly specified molecule, wherein, if necessary, additionally further alteration usually by substitution, addition or deletion of specific amino acid(s) is conducted to restore biological activity of the molecule;
- an accordingly specified molecule wherein alteration is conducted at one or more residues from any or all of the string of contiguous residues of sequences (A) - (K) as below wherein said sequences are derived from the CPG2 wild-type sequence where using single letter code;

  A. = VGKIKGRGGKNLLLMSHMDTVYLKGILAK;
  B. = KAYGPGIADDKGGNAVILHTLKLLKEYG;
  C. = LFNTDEEKGSFGSRDLIQEEA;
  D. = KLADYVLSFEPTSAGDEKLSLGTSG;
  E. = VNITGKASHAGAAPELGVNALVEASDL;
  F. = KAKNLRFNWTIAKAGNVSNIIPASATLNAD;
  G. = ADVKVIVTRGRPAFNAGEGGKKLVDKA;
  H. = KKLVDKAVAYYKEAGG;
  I. = YKEAGGTLGVEERTGGG;
  J. = TDAAYAALSGKPVIESLGLPGFGY
  K. = LEKLVNIETGTGDAE;

- a peptide molecule comprising at least 9 consecutive residues from any of the sequences (A) - (K) above;
- a peptide molecule of above sharing greater than 90% amino acid identity with any of the peptide sequences derived from (A) - (K);
- a peptide molecule of above sharing greater than 80% amino acid identity with any of the peptide sequences derived from (A) - (K) above;

- a peptide molecule containing a sequence element identical or substantially homologous with one or more of the sequences (A) - (K) above;
- peptide sequences as above able to bind MHC class II;
- a pharmaceutical composition comprising any of the peptides or modified peptides of above having the activity of binding to MHC class II
- a DNA sequence or molecule which codes for any of the specified modified molecules as defined above and below;
- a pharmaceutical composition comprising a modified molecule having the biological activity of CPG2;
- a pharmaceutical composition as defined above and / or in the claims, optionally together with a pharmaceutically acceptable carrier, diluent or excipient;
- a method for manufacturing a modified molecule having the biological activity of CPG2 as defined herein comprising the following steps: (i) determining the amino acid sequence of the polypeptide or part thereof; (ii) identifying one or more potential T-cell epitopes within the amino acid sequence of the protein by any method including determination of the binding of the peptides to MHC molecules using *in vitro* or *in silico* techniques or biological assays; (iii) designing new sequence variants with one or more amino acids within the identified potential T-cell epitopes modified in such a way to substantially reduce or eliminate the activity of the T-cell epitope as determined by the binding of the peptides to MHC molecules using *in vitro* or *in silico* techniques or biological assays; (iv) constructing such sequence variants by recombinant DNA techniques and testing said variants in order to identify one or more variants with desirable properties; and (v) optionally repeating steps (ii) – (iv);
- an accordingly specified method, wherein step (iii) is carried out by substitution, addition or deletion of 1 – 9 amino acid residues in any of the originally present T-cell epitopes;
- an accordingly specified method, wherein the alteration is made with reference to an homologous protein sequence and / or *in silico* modeling techniques;
- a peptide sequence consisting of at least 9 consecutive amino acid residues of a 13mer T-cell epitope peptide as specified above and its use for the manufacture of CPG2 having substantially no or less immunogenicity than any non-modified molecule and having the biological activity of CPG2 when used *in vivo*;

- a CPG2 molecule of the following structure:

X^0QKRDNVLFQAATDEQPAVIKTLEKLVNIETGTGDAEGIAAAGNFLEAELKNLGFTVTRSKS
AGLVVGDNIVGKX^1KGRGGKNLX^2LX^3SHMDTVYX^4KGILAKAPFRVEGDKAX^5GPGX^6ADDKGG
NAX^7IX^8HT$X^9X^{10}X^{11}X^{12}$KEYGVRDYGTITVX^{13}FNTDEEKGSFGSRDLX^{14}QEEAKLADY$X^{15}X^{16}$
SX^{17}EPTSAGDEKX^{18}SLGTSGIAYVQVNITGKASHAGAAPEX^{19}GX^{20}NA$X^{21}X^{22}$EASDLVLRTM
NIDDKAKNX^{23}RFNX^{24}TX^{25}AKAG$X^{26}X^{27}$S$X^{28}X^{29}X^{30}$PASATX^{31}NADVRYARNEDFDAAMKTLE
ERAQQKKLPEADX^{32}KX^{33}IVTRGRPAFNAGEGGK$X^{34}X^{35}X^{36}$DKAX^{37}A$X^{38}X^{39}$KEAGGTLGVEER
TGGGTDAAYAALSGKPVIESLGLPGFGYHSDKAEYVDISAIPRRLYMAARLIMDLGAGK

wherein X^0 is optionally a targeting moiety such as an antibody domain;
X^1 is I, T; X^2 is L, A; X^3 is M, K; X^4 is L, I; X^5 is Y, T; X^6 is I, A; X^7 is V, A;
X^8 is L, A; X^9 is L, T, A; X^{10} is K, T; X^{11} is L, M, A; X^{12} is L, T, A; X^{13} is L, G, T;
X^{14} is I, T, A; X^{15} is V, A; X^{16} is L, T, G; X^{17} is F, A; X^{18} is L, A; X^{19} is L, A;
X^{20} is V, A; X^{21} is L, A; X^{22} is V, T, A; X^{23} is L, A; X^{24} is W, A, H; X^{25} is I, A;
X^{26} is N, T, A; X^{27} is V, T; X^{28} is N, T, A; X^{29} is I, T, A; X^{30} is I, T, A;
X^{31} is L, T, A, I; X^{32} is V, A; X^{33} is V, A; X^{34} is K, T; X^{35} is L, A; X^{36} is V, A;
X^{37} is V, S, T, A; X^{38} is Y, T, A; X^{39} is Y, S, T, A;
and whereby simultaneously X1 = I, X2 = L, X3 = M, X4 = L, X5 = Y, X6 = I, X7 = V, X8 = L, X9 = L, X10 = K, X11 = L, X12 = L, X13 = L, X14 = I, X15 = V, X16 = L, X17 = F, X18 = L, X19 = L, X20 = V, X21 = L, X22 = V, X23 = L, X24 = W, X25 = I, X26 = N, X27 = V, X28 = N, X29 = I, X30 = I, X31 = L, X32 = V, X33 = V, X34 = K, X35 = L, X36 = V, X37 = V, X38 = Y and X39 = Y are excluded.

The term "T-cell epitope" means according to the understanding of this invention an amino acid sequence which is able to bind MHC class II, able to stimulate T-cells and / or also to bind (without necessarily measurably activating) T-cells in complex with MHC class II.

The term "peptide" as used herein and in the appended claims, is a compound that includes two or more amino acids. The amino acids are linked together by a peptide bond (defined herein below). There are 20 different naturally occurring amino acids involved in the biological production of peptides, and any number of them may be linked in any order to form a peptide chain or ring. The naturally occurring amino acids employed in the biological production of peptides all have the L-configuration. Synthetic peptides can be prepared employing conventional synthetic methods, utilizing L-amino acids, D-amino acids, or various combinations of amino acids of the two different configurations. Some peptides contain only a few amino acid units. Short peptides, e.g., having less than ten

amino acid units, are sometimes referred to as "oligopeptides". Other peptides contain a large number of amino acid residues, e.g. up to 100 or more, and are referred to as "polypeptides". By convention, a "polypeptide" may be considered as any peptide chain containing three or more amino acids, whereas a "oligopeptide" is usually considered as a particular type of "short" polypeptide. Thus, as used herein, it is understood that any reference to a "polypeptide" also includes an oligopeptide. Further, any reference to a "peptide" includes polypeptides, oligopeptides, and proteins. Each different arrangement of amino acids forms different polypeptides or proteins. The number of polypeptides–and hence the number of different proteins–that can be formed is practically unlimited.

"Alpha carbon (Cα)" is the carbon atom of the carbon-hydrogen (CH) component that is in the peptide chain. A "side chain" is a pendant group to Cα that can comprise a simple or complex group or moiety, having physical dimensions that can vary significantly compared to the dimensions of the peptide.

The invention may be applied to any CPG2 species of molecule with substantially the same primary amino acid sequences as those disclosed herein and would include therefore CPG2 molecules derived by genetic engineering means or other processes and may not contain 390 amino acid residues. CPG2 proteins such as identified from other sources including different strains of Pseudomonas and other organisms have in common many of the peptide sequences of the present disclosure and have in common many peptide sequences with substantially the same sequence as those of the disclosed listing. Such protein sequences equally therefore fall under the scope of the present invention.

The invention is conceived to overcome the practical reality that soluble proteins introduced with therapeutic intent in man trigger an immune response resulting in development of host antibodies that bind to the soluble protein. The present invention seeks to address this by providing CPG2 □proteins with altered propensity to elicit an immune response on administration to the human host. According to the methods described herein, the inventors have discovered the regions of the CPG2 molecule comprising the critical T-cell epitopes driving the immune responses to this protein.

The general method of the present invention leading to the modified CPG2 comprises the following steps:

(a) determining the amino acid sequence of the polypeptide or part thereof;

(b) identifying one or more potential T-cell epitopes within the amino acid sequence of the protein by any method including determination of the binding of the peptides to MHC molecules using *in vitro* or *in silico* techniques or biological assays;

(c) designing new sequence variants with one or more amino acids within the identified potential T-cell epitopes modified in such a way to substantially reduce or eliminate the activity of the T-cell epitope as determined by the binding of the peptides to MHC molecules using *in vitro* or *in silico* techniques or biological assays. Such sequence variants are created in such a way to avoid creation of new potential T-cell epitopes by the sequence variations unless such new potential T-cell epitopes are, in turn, modified in such a way to substantially reduce or eliminate the activity of the T-cell epitope; and

(d) constructing such sequence variants by recombinant DNA techniques and testing said variants in order to identify one or more variants with desirable properties according to well known recombinant techniques.

The identification of potential T-cell epitopes according to step (b) can be carried out according to methods described previously in the art. Suitable methods are disclosed in WO 98/59244; WO 98/52976; WO 00/34317 and may be used to identify binding propensity of CPG2-derived peptides to an MHC class II molecule.

Another very efficacious method for identifying T-cell epitopes by calculation is described in the Example 1 which is a preferred embodiment according to this invention.

The results of an analysis according to step (b) of the above scheme and pertaining to the CPG2 protein sequence is presented in Table 1.

Table 1: *Peptide sequences in CPG2 with potential human MHC class II binding activity.*

DNVLFQAATDEQP,	NVLFQAATDEQPA,	VLFQAATDEQPAV,
PAVIKTLEKLVNI,	AVIKTLEKLVNIE,	KTLEKLVNIETGT,
EKLVNIETGTGDA,	KLVNIETGTGDAE,	VNIETGTGDAEGI,
EGIAAAGNFLEAE,	GNFLEAELKNLGF,	NFLEAELKNLGFT,
AELKNLGFTVTRS,	KNLGFTVTRSKSA,	LGFTVTRSKSAGL,
FTVTRSKSAGLVV,	AGLVVGDNIVGKI,	GLVVGDNIVGKIK,
LVVGDNIVGKIKG,	DNIVGKIKGRGGK,	NIVGKIKGRGGKN,
GKIKGRGGKNLLL,	KNLLLMSHMDTVY,	NLLLMSHMDTVYL,
LLLMSHMDTVYLK,	LLMSHMDTVYLKG,	SHMDTVYLKGILA,
DTVYLKGILAKAP,	TVYLKGILAKAPF,	VYLKGILAKAPFR,
KGILAKAPFRVEG,	GILAKAPFRVEGD,	APFRVEGDKAYGP,
FRVEGDKAYGPGI,	KAYGPGIADDKGG,	PGIADDKGGNAVI,

NAVILHTLKLLKE, AVILHTLKLLKEY, VILHTLKLLKEYG,
HTLKLLKEYGVRD, LKLLKEYGVRDYG, KLLKEYGVRDYGT,
KEYGVRDYGTITV, YGVRDYGTITVLF, RDYGTITVLFNTD,
GTITVLFNTDEEK, ITVLFNTDEEKGS, TVLFNTDEEKGSF,
VLFNTDEEKGSFG, GSFGSRDLIQEEA, RDLIQEEAKLADY,
DLIQEEAKLADYV, AKLADYVLSFEPT, ADYVLSFEPTSAG,
DYVLSFEPTSAGD, YVLSFEPTSAGDE, LSFEPTSAGDEKL,
EKLSLGTSGIAYV, LSLGTSGIAYVQV, SGIAYVQVNITGK,
IAYVQVNITGKAS, AYVQVNITGKASH, VQVNITGKASHAG,
VNITGKASHAGAA, PELGVNALVEASD, LGVNALVEASDLV,
NALVEASDLVLRT, ALVEASDLVLRTM, SDLVLRTMNIDDK,
DLVLRTMNIDDKA, LVLRTMNIDDKAK, RTMNIDDKAKNLR,
MNIDDKAKNLRFN, KNLRFNWTIAKAG, LRFNWTIAKAGNV,
FNWTIAKAGNVSN, WTIAKAGNVSNII, GNVSNIIPASATL,
SNIIPASATLNAD, NIIPASATLNADV, ATLNADVRYARNE,
ADVRYARNEDFDA, VRYARNEDFDAAM, EDFDAAMKTLEER,
AAMKTLEERAQQK, KTLEERAQQKKLP, KKLPEADVKVIVT,
ADVKVIVTRGRPA, VKVIVTRGRPAFN, KVIVTRGRPAFNA,
VIVTRGRPAFNAG, PAFNAGEGGKKLV, KKLVDKAVAYYKE,
KLVDKAVAYYKEA, KAVAYYKEAGGTL, VAYYKEAGGTLGV,
AYYKEAGGTLGVE, GTLGVEERTGGGT, LGVEERTGGGTDA,
AAYAALSGKPVIE, AALSGKPVIESLG, KPVIESLGLPGFG,
PVIESLGLPGFGY, ESLGLPGFGYHSD, LGLPGFGYHSDKA,
PGFGYHSDKAEYV, FGYHSDKAEYVDI, AEYVDISAIPRRL,
EYVDISAIPRRLY, VDISAIPRRLYMA, SAIPRRLYMAARL,
RRLYMAARLIMDL, RLYMAARLIMDLG, LYMAARLIMDLGA

A further important technical approach for the detection of T-cell epitopes is via biological T-cell proliferation assay. For the detection of T-cell epitopes within the CPG2 molecule a particularly effective method is to test overlapping peptides derived from the CPG2 sequence so as to test the entire CPG2 sequence, or alternatively to test a sub-set of CPG2 peptides such as all or some of those listed in Table 1. The synthetic peptides are tested for their ability to evoke a proliferative response in human T-cells cultured *in vitro*. This type of approach can be conducted using naïve human T-cells taken from healthy donors. The inventors have established that in the operation of such an assay, a stimulation index equal to or greater than 2.0 is a useful measure of induced proliferation.

The stimulation index is conventionally derived by division of the proliferation score measured (e.g. counts per minute if using ^{3}H-thymidine incorporation) to the test (poly) peptide by the proliferation score measured in cells not contacted with a test (poly)peptide. A suitable method of this type is detailed in Example 2. Results from this assay are presented in Table 2 and FIGURE 1 where are listed CPG2 derived peptide sequences shown by the method of example 2 to evoke a proliferative response in human T-cells.

Table 2: *CPG2 peptide sequences able to stimulate human T-cells in vitro.*

Peptide ID #	Peptide Sequence	Residue #
1	QKRDNVLFQAATDEQ	1
8	LEKLVNIETGTGDAE	22
17	LKNLGFTVTRSKSAG	49
23	GDNIVGKIKGRGGKN	67
25	KIKGRGGKNLLLMSH	73
26	GRGGKNLLLMSHMDT	76
28	LLLMSHMDTVYLKGI	82
29	MSHMDTVYLKGILAK	85
32	KGILAKAPFRVEGDK	94
37	AYGPGIADDKGGNAV	109
38	PGIADDKGGNAVILH	112
39	ADDKGGNAVILHTLK	115
40	KGGNAVILHTLKLLK	118
41	NAVILHTLKLLKEYG	121
42	ILHTLKLLKEYGVRD	124
43	TLKLLKEYGVRDYGT	127
48	ITVLFNTDEEKGSFG	142
49	LFNTDEEKGSFGSRD	145
50	TDEEKGSFGSRDLIQ	148
52	SFGSRDLIQEEAKLA	154
55	EEAKLADYVLSFEPT	163
56	KLADYVLSFEPTSAG	166
58	LSFEPTSAGDEKLSL	172
59	EPTSAGDEKLSLGTS	175
60	SAGDEKLSLGTSGIA	178

63	GTSGIAYVQVNITGK	187
66	VNITGKASHAGAAPE	196
68	ASHAGAAPELGVNAL	202
69	AGAAPELGVNALVEA	205
70	APELGVNALVEASDL	208
77	IDDKAKNLRFNWTIA	229
78	KAKNLRFNWTIAKAG	232
79	NLRFNWTIAKAGNVS	235
81	TIAKAGNVSNIIPAS	241
82	KAGNVSNIIPASATL	244
83	NVSNIIPASATLNAD	247
97	ADVKVIVTRGRPAFN	289
100	GRPAFNAGEGGKKLV	298
101	AFNAGEGGKKLVDKA	301
102	AGEGGKKLVDKAVAY	304
103	GGKKLVDKAVAYYKE	307
104	KLVDKAVAYYKEAGG	310
108	AGGTLGVEERTGGGT	322
112	GGTDAAYAALSGKPV	334
116	KPVIESLGLPGFGYH	346
124	SAIPRRLYMAARLIM	370
127	AARLIMDLGAGK	379

Where multiple potential epitopes are identified and in particular where a number of peptide sequences are found to be able to stimulate T-cells in a biological assay, cognizance may also be made of the structural features of the protein in relation to its propensity to evoke an immune response via the MHC class II presentation pathway. For example where the crystal structure of the protein of interest is known the crystallographic B-factor score may be analyzed for evidence of structural disorder within the protein, a parameter suggested to correlate with the proximity to the biologically relevant immunodominant peptide epitopes [Dai G. et al (2001) *J. Biological Chem.* 276: 41913-41920]. Such an analysis when conducted on the CPG2 crystal structure [PDB ID: 1CG2, Rowsell, S. et al (1997), *Structure* 5: 337] suggests a high likelihood for multiple immunodominant epitopes with at least 11 discrete zones mapping to the medial position of areas with above average B-factor scores in at least three of the four individual chains

in the CPG2 modeled crystal structure. Of the these 11 areas, 9 mapped to the N-terminal boundary of peptides shown to evoke a proliferative response in the naïve T-cell assay of example 2.

This data taken together with the data for the numbers of naïve donors responding to particular peptides enables a predicted ranking of the most immunodominant regions of the molecule. It is however recognized that in practice, each of these regions are considered immunogenic in man and therefore require modification under the scheme of the invention. Accordingly, with reference to the above defined sequence strings (A) – (K), sequences may be ranked in the order (A), {(B), (E)}, {(D), (G), (F), (H)}, {(I), (J), (K)}; where (A) is considered the most immunogenic sequence within the molecule. Equal ranking is ascribed to those sequences in brackets. These regions have been listed in Table 3 as the major epitope regions A-H and the lesser epitope regions I and J. This distinction has been drawn based on the frequency of responding donors in the naïve T-cell assay, whereby for the so called "lesser epitope" regions, T-cells from one donor of the panel where shown to respond to the synthetic peptide representative of this region of sequence. By contrast, peptides mapping to the major epitope regions were for the most part stimulatory to T-cells from multiple donors. The donor panel used was representative of a wide repertoire of different MHC class II allotypes.

Table 3: *CPG2 Epitope regions*

Major Epitope Region	Sequence	Residue #
A	VGKIKGRGGKNLLLMSHMDTVYLKGILAK	71-99
B	KAYGPGIADDKGGNAVILHTLKLLKEYG	108-135
C	LFNTDEEKGSFGSRDLIQEEA	145-165
D	KLADYVLSFEPTSAGDEKLSLGTSG	166-190
E	VNITGKASHAGAAPELGVNALVEASDL	196-222
F	KAKNLRFNWTIAKAGNVSNIIPASATLNAD	232-261
G	ADVKVIVTRGRPAFNAGEGGKKLVDKA	289-315
H	KKLVDKAVAYYKEAGG	309-324
Lesser Regions		
I	YKEAGGTLGVEERTGGG	319-235
J	TDAAYAALSGKPVIESLGLPGFGY	236-259
K	LEKLVNIETGTGDAE	25-39

In practice a number of variant CPG2 proteins will be produced and tested for the desired immune and functional characteristic. The variant proteins will most preferably be

produced by the widely known methods of recombinant DNA technology although other procedures including chemical synthesis of CPG2 fragments may be contemplated. Suitable methods for the construction and expression of CPG2 proteins including a modified CPG2 protein are provided in the Examples 3 – 5.

The invention relates to CPG2 analogues in which substitutions of at least one amino acid residue have been made at positions resulting in a substantial reduction in activity of or elimination of one or more potential T-cell epitopes from the protein. It is most preferred to provide CPG2 molecules in which amino acid modification (e.g. a substitution) is conducted within the most immunogenic regions of the parent molecule. The major preferred embodiments of the present invention comprise CPG2 molecules for which any of the MHC class II ligands are altered such as to eliminate binding or otherwise reduce the numbers of MHC allotypes to which the peptide can bind. The inventors have discovered and herein disclose, the immunogenic regions of the CPG2 molecule in man. It is understood that under certain circumstances additional regions of sequence to those disclosed herein can become immunogenic epitopes, for example in the event of infection with a pathogen expressing a protein or peptide with a similar sequence to that of the present case. In any event, it will be critical for the sequence element to act as an MHC class II ligand and in principle therefore any of the sequences disclosed in Table 1 can be considered immunogenic epitopes under the scope of the present invention.

For the elimination of T-cell epitopes, amino acid substitutions are preferably made at appropriate points within the peptide sequence predicted to achieve substantial reduction or elimination of the activity of the T-cell epitope. In practice an appropriate point will preferably equate to an amino acid residue binding within one of the pockets provided within the MHC class II binding groove.

It is most preferred to alter binding within the first pocket of the cleft at the so-called P1 or P1 anchor position of the peptide. The quality of binding interaction between the P1 anchor residue of the peptide and the first pocket of the MHC class II binding groove is recognized as being a major determinant of overall binding affinity for the whole peptide. An appropriate substitution at this position of the peptide will be for a residue less readily accommodated within the pocket, for example, substitution to a more hydrophilic residue. Amino acid residues in the peptide at positions equating to binding within other pocket regions within the MHC binding cleft are also considered and fall under the scope of the present.

It is understood that single amino acid substitutions within a given potential T-cell epitope are the most preferred route by which the epitope may be eliminated. Combinations of substitution within a single epitope may be contemplated and for example can be particularly appropriate where individually defined epitopes are in overlap with each other. Moreover, amino acid substitutions either singly within a given epitope or in combination within a single epitope may be made at positions not equating to the "pocket residues" with respect to the MHC class II binding groove, but at any point within the peptide sequence. Substitutions may be made with reference to an homologues structure or structural method produced using *in silico* techniques known in the art and may be based on known structural features of the molecule according to this invention. All such substitutions fall within the scope of the present invention.

Amino acid substitutions other than within the peptides identified above may be contemplated particularly when made in combination with substitution(s) made within a listed peptide. For example a change may be contemplated to restore structure or biological activity of the variant molecule. Such compensatory changes and changes to include deletion or addition of particular amino acid residues from the CPG2 polypeptide resulting in a variant with desired activity and in combination with changes in any of the disclosed peptides fall under the scope of the present.

A particularly preferred set of mutations within the CPG2 molecule contemplated under the scheme of the present are listed in Table 4. Such substitutions are selected using the computational methods of Example 1. Each of the substitutions map to the epitope regions defined herein above and listed also as Table 3.

Table 4: *Substitutions in CPG2*

Epitope Region	CPG2 Peptide	Substitution within CPG2 peptide		
A	DNIVGKIKGRGGK	WT Residue	I	G
		Position	3	11
		Substitution	C,G,P,D,E,H,K,N,Q,R,S,T	I,P,T
A	NIVGKIKGRGGKN	WT Residue	V	G
		Position	3	11
		Substitution	A,C,D,E,G,H,K,N,P,Q,R,S,T	T

Epitope Region	CPG2 Peptide	Substitution within CPG2 peptide				
A	GKIKGRGGKNLLL	WT Residue	I	G	L	
		Position	3	8	11	
		Substitution	A,C,D,E,H,K,N,P,Q,R,S,T	H,P	C,D,E,G,H,I,K,M,N,P,Q,R,S,T,V,W	
A	NLLLMSHMDTVYL	WT Residue	L	S	M	D	V
		Position	3	6	8	9	11
		Substitution	A,C,D,E,F,G,H,I,K,M,N,P,Q,R,S,T,V,W,Y	P	D,E,H,K,N,P,Q,R,,S,T	P	T
A	LLMSHMDTVYLKG	WT Residue	M	V	L		
		Position	3	9	11		
		Substitution	A,C,D,E,G,H,K,N,P,Q,R,S,T	P	I,P,T,W	P	
A	DTVYLKGILAKAP	WT Residue	V	I	L		
		Position	3	8	9		
		Substitution	A,C,D,E,G,H,K,N,P,Q,R,S,T	P,W,Y	P		
A	TVYLKGILAKAPF	WT Residue	Y				
		Position	3				
		Substitution	A,C,G,M,P				
B	KAYGPGIADDKGG	WT Residue	Y	G	A	D	K
		Position	3	6	8	9	11
		Substitution	A,C,D,E,G,H,K,M,N,P,Q,R,S,T,W	P	F,L,W,Y	P	I,P,W
B	PGIADDKGGNAVI	WT Residue	I	D	G	G	A
		Position	3	6	8	9	11
		Substitution	A,C,D,E,G,H,K,M,N,P,Q,R,S,T,W	P	H,P,Q,S	D,H,K,N,P,Q,R,S,T	D,E,H,K,N,P,Q,R,S,T
B	NAVILHTLKLLKE	WT Residue	V	H	K		
		Position	3	6	9		

Epitope Region	CPG2 Peptide	Substitution within CPG2 peptide		
		Substitution	A,C,D,E,G,H,K,M,N,P,Q,R,S,T,W	P	P,T
B	VILHTLKLLKEYG	WT Residue	L		
		Position	3		
		Substitution	A,C,D,E,G,H,K,N,P,Q,R,S,T		
B	HTLKLLKEYGVRD	WT Residue	L		
		Position	3		
		Substitution	A,C,D,E,G,H,K,N,P,Q,R,S,T		
B	LKLLKEYGVRDYG	WT Residue	L	V	
		Position	3	9	
		Substitution	A,C,D,E,G,H,K,M,N,P,Q,R,S,T,W,Y	P	
B	KLLKEYGVRDYGT	WT Residue	L		
		Position	3		
		Substitution	A,C,D,E,G,H,K,N,P,Q,R,S,T		
C	TVLFNTDEEKGSF	WT Residue	L	G	
		Position	3	11	
		Substitution	A,C,D,E,G,H,K,M,N,P,Q,R,S,T,W	D,E,H,K,N,P,Q,R,S,T,W	
C	RDLIQEEAKLADY	WT Residue	L		
		Position	3		
		Substitution	P		
C	DLIQEEAKLADYV	WT Residue	I	L	D
		Position	3	9	11
		Substitution	A,C,D,E,G,H,K,N,P,Q,R,S,T	C,D,E,G,H,K,N,P,Q,R,S,T	T

Epitope Region	CPG2 Peptide	Substitution within CPG2 peptide				
D	DYVLSFEPTSAGD	WT Residue	V	F	A	
		Position	3	6	11	
		Substitution	A,C,G,P,W,Y	P	P,W	
D	YVLSFEPTSAGDE	WT Residue	L			
		Position	3			
		Substitution	T,Q,R			
D	LSFEPTSAGDEKL	WT Residue	F	T	A	G	E
		Position	3	6	8	9	11
		Substitution	A,C,G,P	P	P,W	E,N,P,Q,R,S,T	P,T,W
D	EKLSLGTSGIAYV	WT Residue	L				
		Position	3				
		Substitution	A,C,G,P,W				
E	PELGVNALVEASD	WT Residue	L	N	V	A	
		Position	3	6	9	11	
		Substitution	A,C,D,E,F,G,H,I,K,M,N,P,Q,R,S,T,V,W,Y	P	E,H,K,N,PD,E,H,N,P,Q,R,S,T	,Q,S,T	
E	LGVNALVEASDLV	WT Residue	V	L	A	D	
		Position	3	6	9	11	
		Substitution	A,C,D,E,G,H,K,N,P,Q,R,S,T	A,C,D,E,G,H,I,K,M,N,P,Q,R,S,T,V,W,Y	D,E,H,K,N,P,Q,R,S,T	P	
E	NALVEASDLVLRT	WT Residue	L				
		Position	3				
		Substitution	A,C,D,E,G,H,K,N,P,Q,R,S,T				
F	KNLRFNWTIAKAG	WT Residue	L	I	K		
		Position	3	9	11		
		Substitution	A,C,G,M,P,W	P	T		
F	FNWTIAKAGNVSN	WT Residue	W	A	A	G	
		Position	3	6	8	9	
		Substitution	A,C,G,P	C,D,H,K,N,P,Q,R,S,T	D,E,H,K,N,P,Q,W,Y	P	

Epitope Region	CPG2 Peptide	Substitution within CPG2 peptide				
F	WTIAKAGNVSNII	WT Residue	I	A	N	V	N
		Position	3	6	8	9	11
		Substitution	A,C,D,E,G,H,K,M,N,P,Q,R,S,T,W	D,E,H,K,N,P,Q	F,L,P,W	G,P	P,T,W
F	GNVSNIIPASATL	WT Residue	V	I	A	A	
		Position	3	6	9	11	
		Substitution	A,C,D,E,G,H,K,N,P,Q,R,S,T,W	P	P	P	
F	SNIIPASATLNAD	WT Residue	I	A			
		Position	3	8			
		Substitution	A,C,D,E,G,H,K,N,P,Q,R,S,T	H			
F	NIIPASATLNADV	WT Residue	I	L	A		
		Position	3	9	11		
		Substitution	A,C,D,E,G,H,K,N,P,Q,R,S,T	D,E,H,K,N,P,Q,R,S,T	P,I,Q,S,T,V,W		
G	KKLPEADVKVIVT	WT Residue	L				
		Position	3				
		Substitution	A,C,D,E,G,H,K,N,P,Q,R,S,T				
G	PAFNAGEGGKKLV	WT Residue	F	G	G	G	K
		Position	3	6	8	9	11
		Substitution	A,C,G,M,P,W	P	H,P,S	D,E,H,K,N,P,Q,R,S,T	P,T,W
H	KKLVDKAVAYYKE	WT Residue	L				
		Position	3				
		Substitution	A,C,D,E,G,H,K,N,P,Q,R,S,T				
H	KLVDKAVAYYKEA	WT Residue	V				
		Position	3				

Epitope Region	CPG2 Peptide	Substitution within CPG2 peptide				
		Substitution	A,C,D,E,G,H,K,N,P,Q,R,S,T			
H	KAVAYYKEAGGTL	WT Residue	V	Y	E	A	G
		Position	3	6	8	9	11
		Substitution	A,C,D,E,G,H,K,N,P,Q,R,S,T	P	H	P,T	I,P,T,S,W,Y
H	VAYYKEAGGTLGV	WT Residue	Y	G	G	L	
		Position	3	8	9	11	
		Substitution	A,C,D,E,G,H,K,N,P,Q,R,S,T	D,E,H,N,P,Q	H,P	A,C,D,E,F,G,H,I,K,M,N,P,Q,R,S,T,V,W	
H	AYYKEAGGTLGVE	WT Residue	Y	A	G	G	
		Position	3	6	8	11	
		Substitution	A,C,D,E,G,H,K,M,N,P,Q,R,S,T	D,E,H,K,N,P,Q,R,S,T	D,E,F,H,L,N,P,Q,W,Y	P,T	
I	GTLGVEERTGGGT	WT Residue	L	E	R	T	G
		Position	3	6	8	9	11
		Substitution	A,C,D,E,F,G,H,I,K,M,N,P,Q,R,S,T,V,W,Y	P	H,P	P	D,E,H,K,N,P,Q,R,S,T
J	AALSGKPVIESLG	WT Residue	L	V	I	S	
		Position	3	8	9	11	
		Substitution	A,C,D,E,G,H,K,M,N,P,Q,R,S,T,W,Y	D,E,F,H,K,N,P,Q,S,T,W,Y	D,E,H,K,N,P,Q,R,S,T	T	
J	KPVIESLGLPGFG	WT Residue	V	L	G		
		Position	3	9	11		
		Substitution	D,E,H,K,N,P,Q,S,T	P	T		

It is recognized that other known methods could be applied and which may result in definition of particular alternative substitutions. In all events, an especially desired substitution, will be one which can satisfy the parallel objectives of retaining functional activity in the molecule and yet disrupt the ability of the peptide sequence within the locale to act as a ligand for one or more human MHC class II molecules and or cease to stimulate a cognate T-cell receptor. One known and applicable scheme could involve random mutagenesis of the epitope regions disclosed herein and selection of enzymatically functional variants. The selected variant may then be passed to an independent second screen for immunological analysis. A convenient immunological screen for example would be a T-cell proliferation assay using synthetic peptides of the variant sequence and human T-cells or T-cell lines cultured *in vitro*.

A further method may exploit molecular modeling techniques to select *in silico* substitutions compatible with the parallel dual objectives outlined above. The structural model of the CPG2 molecule may be examined using any suitable software package and highly desired substitutions may be selected. Examples of such especially preferred substitutions are provided in Table 5; the substitutions are considered broadly accommodated within the CPG2 structure and variant CPG2 molecules containing any of these listed substitutions are to be considered as preferred embodiments of the present invention.

Table 5: *Preferred substitutions in CPG2*

	Epitope	Wt Residue & #	Substitution
1	A1	I 74	T
2	A2	L 83	A or G
3	A3	M 85	K
4	A4	L 93	I
5	B1	Y 110	T
6	B2	I 114	A or G
7	B3	V 123	A or G
8	B4	L 125	A or G
9	B5	L 128	T, A
10	B6	K 129	T
11	B7	L 130	M, A
12	B8	L 131	T, A

13	C1	L 145	G or T
14	C2	I 161	T, A
15	D1	V 171	A / G
16	D2	L 172	T or G or R
17	D3	F 174	A or G
18	D4	L 184	A or G
19	E1	L 211	A or G
20	E2	V 213	A or (D,E,G,K,N,Q,S,T,)
21	E3	L 216	A
22	E4	V 217	T, A
23	F1	L 236	A or G
24	F2	W 240	A / G (D,K,N,Q,S,T)
25	F3	I 242	A or T
26	F4	N 247	T, A
27	F5	V 248	T or (H, N,Q, S)
28	F6	N 250	T, A
29	F7	I 251	T, A
30	F8	I 252	T, A
31	F9	L 258	T, A, I
32	G1	V 291	A or G
33	G2	V 293	A or G
34	G3	K 310	T
35	H1	L 311	A or G
36	H2	V 312	A or G
37	H3	V 316	S, T, A
38	H4	Y 318	T, A
39	H5	Y 319	S, T, A

Accordingly, a particularly preferred modified CPG2 molecule is given by the structure below:

X^0QKRDNVLFQAATDEQPAVIKTLEKLVNIETGTGDAEGIAAAGNFLEAELKNLGFTVTRSKSA
GLVVGDNIVGKX^1KGRGGKNLX^2LX^3SHMDTVYX^4KGILAKAPFRVEGDKAX^5GPGX^6ADDKGGNA
X^7IX^8HT$X^9$$X^{10}$$X^{11}$$X^{12}$KEYGVRDYGTITV$X^{13}$FNTDEEKGSFGSRDL$X^{14}$QEEAKLADY$X^{15}$$X^{16}SX^{17}$
EPTSAGDEKX^{18}SLGTSGIAYVQVNITGKASHAGAAPEX^{19}GX^{20}NA$X^{21}$$X^{22}$EASDLVLRTMNIDDK
AKNX^{23}RFNX^{24}TX^{25}AKAG$X^{26}$$X^{27}SX^{28}$$X^{29}$$X^{30}$PASAT$X^{31}$NADVRYARNEDFDAAMKTLEERAQQK

KLPEADX^{32}KX^{33}IVTRGRPAFNAGEGGK$X^{34}X^{35}X^{36}$DKAX^{37}A$X^{38}X^{39}$KEAGGTLGVEERTGGGTDA
AYAALSGKPVIESLGLPGFGYHSDKAEYVDISAIPRRLYMAARLIMDLGAGK

wherein X^0 is optionally a targeting moiety such as an antibody domain;

X^1 is I, T; X^2 is L, A; X^3 is M, K; X^4 is L, I; X^5 is Y, T; X^6 is I, A; X^7 is V, A; X^8 is L, A;
X^9 is L, T, A; X^{10} is K, T; X^{11} is L, M, A; X^{12} is L, T, A; X^{13} is L, G, T; X^{14} is I, T, A;
X^{15} is V, A; X^{16} is L, T, G; X^{17} is F, A; X^{18} is L, A; X^{19} is L, A; X^{20} is V, A; X^{21} is L, A;
X^{22} is V, T, A; X^{23} is L, A; X^{24} is W, A, H; X^{25} is I, A; X^{26} is N, T, A; X^{27} is V, T;
X^{28} is N, T, A; X^{29} is I, T, A; X^{30} is I, T, A; X^{31} is L, T, A, I; X^{32} is V, A; X^{33} is V, A;
X^{34} is K, T; X^{35} is L, A; X^{36} is V, A; X^{37} is V, S, T, A; X^{38} is Y, T, A; X^{39} is Y, S, T, A;
and whereby simultaneously X^1 = I, X^2 = L, X^3 = M, X^4 = L, X^5 = Y, X^6 = I, X^7 = V, X^8 = L, X^9 = L, X^{10} = K, X^{11} = L, X^{12} = L, X^{13} = L, X^{14} = I, X^{15} = V, X^{16} = L, X^{17} = F, X^{18} = L, X^{19} = L, X^{20} = V, X^{21} = L, X^{22} = V, X^{23} = L, X^{24} = W, X^{25} = I, X^{26} = N, X^{27} = V, X^{28} = N, X^{29} = I, X^{30} = I, X^{31} = L, X^{32} = V, X^{33} = V, X^{34} = K, X^{35} = L, X^{36} = V, X^{37} = V, X^{38} = Y and X^{39} = Y are excluded.

Modified CPG2 proteins according to the above structure are an embodiment of the present invention. A modified CPG2 protein has been produced and has demonstrated reduced ability to elicit a proliferative response in human T-cells cultured *in vitro* (detailed in Example 6). Such data is concordant with a modified CPG2 protein having a reduced immunogenic potential *in vivo*.

It is recognized that the native CPG2 enzyme forms a homodimer and requires zinc ions for activity. It is an object of the present invention to produce a modified CPG2 molecule which contains a reduced number of T-cell epitopes or sequences able to bind to MHC class II or able to bind to a T-cell in association with an MHC class II molecule and which also preferably is able to form a homodimer and bind zinc ions.

Whilst it is recognized that the native CPG2 enzyme forms a homodimer and requires zinc ions for activity, it is most desired to provide a modified CPG2 molecule in which there is a reduced or absent potential to induce an immune response upon administration to a human subject and said modified CPG2 molecule with such a desired property may be compromised in its ability to form a homodimer and or bind zinc ions but retain a degree of enzyme activity with respect to its ability to convert a prodrug to an active drug. Such a molecule falls under the scope of the present invention.

A modified CPG2 molecule of the present invention may not form a homodimer and sequence modification resulting in a monomeric CPG2 but with a desired enzymatic activity and having none or at least a reduced number of T-cell epitopes or sequences able to bind to MHC class II or able to bind to a T-cell in association with an MHC class II molecule is equally also a desired object of the present invention.

It is recognized that a modified CPG2 molecule that is not able to form a homodimer of itself and may not have the desired enzymatic activity as monomer in solution could none the less have activity restored in part or in totality should two or more molecules of the modified CPG2 be brought into proximity. Such a molecule should it also have a reduced number of T cell epitopes or sequences able to bind to MHC class II or able to bind to a T cell in association with an MHC class II molecule equally falls under the scope of the present.

Such a situation where two of more modified CPG2 molecules are brought into proximity to thereby re-constitute an enzymatic activity could be achieved by genetic engineering means for example by fusion of the CPG2 molecules to domains from a second protein able to facilitate or engage in dimeric or other degrees of binding interaction. Examples of such domains include antibody constant regions such as the Fc domain of IgG or another immunoglobulin isotypes. Further examples include antibody V-region domains or the b-zip motif exemplified in proteins such as FOS and JUN. Other recognized protein domains may equally be contemplated. It may also be expected that a protein linker domain connecting two CPG2 moieties as a single recombinant fusion protein may also achieve the effect of bringing together into suitable proximity said CPG2 moieties.

The formation of modified CPG2 complexes with non-protein compounds including synthetic water soluble polymers such as hydroxypropylmethacrylamide or polystyrene-co-maleic acid or others may also be contemplated. Equally liposomal or carbohydrate preparations may be considered and conjugated with a modified CPG2 for the purpose of restoring or providing a degree of enzyme activity to the complex which otherwise would not be present were said CPG2 moieties not in close or forced proximity.

It is an objective of the present invention to produce a modified CPG2 molecule with a reduced or absent potential to induce an immune response upon administration to a

human host. Within this objective it is also desired to retain the functional activity of the molecule with respect to its ability to catalyse the conversion of a prodrug to an active drug. It is preferred that the active drug is at least one order of magnitude more toxic to the desired target cell than the prodrug and it is most preferred that the active drug will be greater than one order of magnitude more toxic. Suitable prodrugs include nitrogen mustard prodrugs and other compounds as those described in WO88/07378; WO89/10140; WO90/02729; WO91/03460; EP-A-540263; WO94/02450; WO95/02420; WO95/03830 or US,6,004,550 which are incorporated herein by reference. Any other compound able to undergo conversion by the modified CPG2 of the present invention and able to achieve a suitable toxicity profile may be contemplated for use in combination with the modified CPG2 of the present invention.

In as far as this invention relates to modified CPG2, compositions containing such modified CPG2 proteins or fragments of modified CPG2 proteins and related compositions should be considered within the scope of the invention. In another aspect, the present invention relates to nucleic acids encoding modified CPG2 entities. In a further aspect the present invention relates to methods for therapeutic treatment of humans using the modified CPG2 proteins. In this aspect the modified CPG2 protein may be linked with an antibody molecule or fragment of an antibody molecule. The linkage may be by means of a chemical cross-linker or the CPG2-antibody may be produced as a recombinant fusion protein. The fusion molecule may contain the modified CPG2 domain with antibody domain orientated towards the N-terminus of the fusion molecule although the opposite orientation may be contemplated.

Desired antibody specificities for linkage to the modified CPG2 molecule of the present include those directed towards the carcinoembryonic antigen as exemplified by numerous antibodies including MFE23 [Chester, K.A. et al (1994) *Lancet* 343: 455], A5B7 [WO92/010159], T84.66 [US,5,081,235] MN-14 [Hansen, H.J. et al (1993) *Cancer* 71: 3478-3485], COL-1 [US,5,472,693] and others. Other desired specificities include antibodies directed to non-internalizing antigens and this may include antigens such as the 40kDa glycoprotein antigen as recognized by antibody KS1/4 [Spearman et al (1987) *J. Pharmacol. Exp. Therapeutics* 241: 695-703] and other antibodies. Other antigens such as the epidermal growth factor receptor (HER1) or related receptors such as HER2 may be selected including anti-GD2 antibodies such as antibody 14.18 [US,4,675,287; EP 0

192 657], or antibodies to the prostate specific membrane antigen [US,6,107,090], the IL-2 receptor [US,6,013,256], the A33 antigen [Heath, J.K. et al (1997) *Proc. Natl, Acad. Sci U.S.A.* 94: 469-474], the Lewis Y determinant, mucin glycoproteins or others may be contemplated.

In all instances where a modified CPG2 protein is made in fusion with an antibody sequence it is most desired to use antibody sequences in which T cell epitopes or sequences able to bind MHC class II molecules or stimulate T cells or bind to T cells in association with MHC class II molecules have been removed.

A further embodiment of the present invention, the modified CPG2 protein may be linked to a non-antibody protein yet a protein able to direct a specific binding interaction to a particular target cell. Such protein moieties include a variety of polypeptide ligands for which there are specific cell surface receptors and include therefore numerous cytokines, peptide and polypeptide hormones and other biological response modifiers. Prominent examples include such proteins as vascular epithelial growth factor, epidermal growth factor, heregulin, the interleukins, interferons, tumor necrosis factor and other protein and glycoprotein molecules. Fusion proteins of these and other molecules with CPG2 of the present invention may be contemplated and may comprise the modified CPG2 moiety in either the N-terminal or C-terminal orientation with respect to the protein ligand domain. Equally, chemical cross-linking of the purified ligand to the modified CPG2 protein may be contemplated and within the scope of the present invention.

In a further embodiment the modified CPG2 protein of the present invention may be used as a complex containing a water soluble polymer such as hydroxypropylmethacrylamide or other polymers where the modified CPG2 protein is in covalent attachment to the polymer or in a non-covalent binding interaction with the polymer. Such an embodiment may additionally include an antigen binding domain such as and antibody or a fragment of an antibody in combination with the polymer CPG2 complex.

In a further embodiment of the present invention the gene for the modified CPG2 enzyme may itself be used as a therapeutic entity such as the gene directed enzyme prodrug strategy and may include linkage to a tissue specific promoter sequence within the vector

or may be expressed from a promoter of viral origin within the vector or the vector itself may be of viral origin or able to be packaged within viral particles able to infect cells. The invention will now be illustrated but not limited, by the following examples. The examples refer to the following drawings:

FIGURE 1 provides a listing of the synthetic peptides used in the naïve T-cell proliferation assay according to the method of EXAMPLE 2. ID# = the identification number of each peptide tested; Donor # = identifies donor PBMC cultures in which a stimulation index of >1.95 was scored for the particular peptide; Sequence = the peptide sequence in single letter code.

FIGURE 2 provides a plots depicting the results of an immunogenicity assay using naïve human PBMC cultured in the presence of differing concentrations of wild-type or modified CPG2 protein. The graphs show the results from two responsive donor PBMC samples. Panel A = donor #1. Panel B = donor #16. SI = stimulation index.

EXAMPLE 1

Identification of potenital MHC class II ligands in the CPG2 protein sequence by computational means.

The scheme for the analysis of peptide sequences with potential to act as MHC class II binding ligands has been described in detail previously [WO 02/069232]. A software tool using this procedure has been developed and applied to the analysis of the CPG2 protein sequence. In brief, the software was built from a number of elements including a library of model MHC class II molecules, produced to cover a wide number of allotypic variants extant in the human population and a library of peptide backbone structures, produced to encompass theoretical and known backbone conformations. Using these elements, a large data set was generated based on the results of docking each backbone conformation within each model MHC allotype binding groove. The data set encompasses also the best side-chain conformation for all possible amino acids at the given position. The interatomic distances between the peptide side-chains (in the optimum conformation) and the MHC protein are stored in this dataset. A test peptide from the protein of interest is analyzed by adding its sequence of side-chains to all backbones and then retrieving the data sets for the optimum side-chain conformations and thus calculating a "peptide score" for each backbone. The best score is selected for display and the process repeated for each of the available MHC model structures.

The algorithm has been applied to the analysis of the entire CPG2 protein sequence. The analysis identifies multiple 13mer peptide sequences which are potential T-cell epitopes by their being predicted MHC class II ligands for one or more allotypes. These peptides are shown in table 1 wherein the peptide sequences are depicted using single letter code.

EXAMPLE 2

Identification of T-cell epitopes using synthetic peptides and naïve human PBMC in vitro proliferation assays.

The interaction between MHC, peptide and T-cell receptor (TCR) provides the structural basis for the antigen specificity of T-cell recognition. T-cell proliferation assays test the binding of peptides to MHC and the recognition of MHC/peptide complexes by the TCR. *In vitro* T-cell proliferation assays of the present example, involve the stimulation of peripheral blood mononuclear cells (PBMCs), containing antigen presenting cells (APCs) and T-cells. Stimulation is conducted *in vitro* using synthetic peptide antigens, and in some experiments whole protein antigen. Stimulated T-cell proliferation is measured using ^{3}H-thymidine (^{3}H-Thy) and the presence of incorporated ^{3}H-Thy assessed using scintillation counting of washed fixed cells.

Donated cells were obtained from the National Blood Service (Addenbrooks Hospital, Cambridge, UK). Ficoll-paque was obtained from Amersham Pharmacia Biotech (Amersham, UK). Serum free AIM V media for the culture of primary human lymphocytes and containing L-glutamine, 50□g/ml streptomycin, 10□g/ml gentomycin and 0.1% human serum albumin was from Gibco-BRL (Paisley, UK). Synthetic peptides were obtained from Eurosequence (Groningen, The Netherlands) and Babraham Technix (Cambridge, UK).

Erythrocytes and leukocytes were separated from plasma and platelets by gentle centrifugation of buffy coats. The top phase (containing plasma and platelets) was removed and discarded. Erythrocytes and leukocytes were diluted 1:1 in phosphate buffered saline (PBS) before layering onto 15ml ficoll-paque (Amersham Pharmacia, Amersham UK). Centrifugation was done according to the manufacturers recommended conditions and PBMCs were harvested from the serum+PBS/ficoll paque interface. PBMCs were mixed with PBS (1:1) and collected by centrifugation. The supernatant was removed and discarded and the PBMC pellet re-suspended in 50ml PBS. Cells were again pelleted by centrifugation and the PBS supernatant discarded. Cells were re-

suspended using 50ml AIM V media and at this point counted and viability assessed using trypan blue dye exclusion. Cells were again collected by centrifugation and the supernatant discarded. Cells were re-suspended for cryogenic storage at a density of $3x10^7$ per ml. The storage medium was 90%(v/v) heat inactivated AB human serum (Sigma, Poole, UK) and 10%(v/v) DMSO (Sigma, Poole, UK). Cells were transferred to a regulated freezing container (Sigma) and placed at –70°C overnight. When required for use, cells were thawed rapidly in a water bath at 37°C before transferring to 10ml pre-warmed AIM V medium.

The tissue types for all PBMC samples were assayed using a commercially available reagent system (Dynal, Wirral, UK). Assays were conducted in accordance with the suppliers recommended protocols and standard ancillary reagents and agarose electrophoresis systems. The tissue types of the panel of 20 donor PBMC samples selected for the CPG2 epitope analysis are identified in table 6 (below) and were chosen to provide a wide spectrum of allotypes.

Table 6 *Tissue types of donor panel*

No	DR typing
1	DRB1*01, DRB1*08
2	DRB1*13, DRB1*0103 and DRB3
3	DRB1*03, DRB1*14, DRB3
4	DRB1*01, DRB1*03, DRB3
5	DRB1*01, DRB1*04, DRB4*01
6	DRB1*01, DRB1*07, DRB4*01
7	DRB1*03, DRB1*04, DRB3, DRB4*01
8	DRB1*07, DRB1*15 and DRB4*01, DRB5
9	DRB1*01, DRB1*11 and DRB3
10	DRB1*04, DRB1*08, DRB4
11	DRB1*03, DRB1*15, DRB3, DRB5
12	DRB1*04 AND DRB1*08, 11 OR 13, DRB3, DRB4*01
13	DRB1*04, DRB1*16, DRB4*01, DRB5
14	DRB1*12, DRB1*15, DRB3, DRB5
15	DRB1*01, DRB1*04, DRB4*01

16	DRB1*03, DRB1*09, DRB3, DRB4*01
17	DRB1*13, DRB1*15, DRB3, DRB5
18	DRB1*10, DRB1*13, DRB3
19	DRB1*11, DRB1*15, DRB3, DRB5
20	DRB1*13, DRB3

PBMC were stimulated with protein and peptide antigens in a 96 well flat bottom plate at a density of $2x10^5$ PBMC per well. PBMC were incubated for 7 days at 37°C before pulsing with ^{3}H-Thy (Amersham-Phamacia, Amersham, UK). For the present study, synthetic peptides (15mers) spanning the entire CPG2 sequence and including an additional C-terminal 6-His tag were produced. Each peptide overlapped each successive peptide in the sequence by 12 residues, i.e., each peptide incremented from the next in the sequence by 3 residues. The peptide sequences and identification numbers are shown in FIGURE 1. Each peptide was screened individually against PBMC's isolated from 20 naïve donors. Two control peptides C32 (PKYVKQNTLKLAT) and C49 (KVVDQIKKISKPVQH) that have previously been shown to be immunogenic and a potent non-recall antigen KLH were used in each donor assay. Peptides were dissolved in DMSO to a final concentration of 10mM, these stock solutions were then diluted 1/500 in AIM V media (final concentration 20μM). Peptides were added to a flat bottom 96 well plate to give a final concentration of 2 and 10μM in a 100μl. The viability of thawed PBMC's was assessed by trypan blue dye exclusion, cells were then re-suspended at a density of $2x10^6$ cells/ml, and 100μl ($2x10^5$ PBMC/well) was transferred to each well containing peptides. Triplicate well cultures were assayed at each peptide concentration. Plates were incubated for 7 days in a humidified atmosphere of 5% CO_2 at 37°C. Cells were pulsed for 18-21 hours with 1μCi ^{3}H-Thymidine per well before harvesting onto filter mats. CPM values were determined using a Wallac microplate beta top plate counter (Perkin Elmer). Results were expressed as stimulation indices (SI), where SI = CPM Test Peptide/CPM untreated control.

Mapping T cell epitopes in the CPG2 sequences using the naïve T cell proliferation assay resulted in the identification of several immunogenic regions. Peptides with significant stimulation indices in individual donors are listed in Figure 1. The results of the naïve T-cell proliferation assay can be used to compile an epitope map of the CPG2 protein. In general, in compilation of such a map, an SI > 1.95 is taken as a positive response.

EXAMPLE 3

Production of CPG2 gene.

The original sequence of CPG2 was taken from that of Pseudomonas sp. Strain RS-16 (gene bank accession no. AE002078). The protein sequence of 390 amino acids was back-translated to give a DNA sequences of 1170 nucleotides. Back-translation was done using commercially available software (DNAstar, Madison, WI, USA) and the sequence compiled based on the most frequently used codons for E.coli. The sequence was used to design a set of 24 synthetic oligonucleotides. The oligonucleotides ranged in size from 50 to 83 nucleotides in length and were designed to have overlapping temini of 19 to 25 nucleotides. The gene was designed also to have an Asc I site at the 5' end and a Sac I site at the 3' end to allow cloning into a plasmid vector. The oligonucleotides are listed in table 7.

Table 7: *Synthetic oligonucleotide sequences*

Name	Sequence
OL549	CAGAAACGTGACAACGTTCTGTTCCAGGCTGCTACCGACGAACAGCCGGCTGTTATCAA AACCCTGGAAAAAC
OL550	GAAGTTACCAGCAGCAGCGATACCTTCAGCGTCACCGGTACCGGTTTCGATGTTAACCA GTTTTTCCAGGGTTTTGATAAC
OL551	GTATCGCTGCTGCTGGTAACTTCCTGGAAGCTGAACTGAAAAACCTGGGTTTCACCGTT ACCCGTTCTAAATCTGCTGGTC
OL552	CAGCAGGTTTTTACCACCACGACCTTTGATTTTACCAACGATGTTGTCACCAACAACCA GACCAGCAGATTTAGAACGGG
OL553	CGTGGTGGTAAAAACCTGCTGCTGATGTCTCACATGGACACCGTTTACCTGAAAGGTAT CCTGGCTAAAGCTC
OL554	GTCGTCAGCGATACCCGGACCGTAAGCTTTGTCACCTTCAACACGGAACGGAGCTTTAG CCAGGATACCTTTC
OL555	GTCCGGGTATCGCTGACGACAAAGGTGGTAACGCTGTTATCCTGCACACCCTGAAACTG CTGAAAGAATACGGTGTTC
OL556	GAACCGAAAGAACCTTTTTCTTCGTCGGTGTTGAACAGAACGGTGATGGTACCGTAGTC ACGAACACCGTATTCTTTCAGCAG
OL557	GAAGAAAAAGGTTCTTTCGGTTCTCGTGACCTGATCCAGGAAGAAGCTAAACTGGCTGA CTACGTTCTGTCTTTCG
OL558	CTGAACGTAAGCGATACCAGAGGTACCCAGAGACAGTTTTTCGTCACCAGCAGAGGTCG GTTCGAAAGACAGAACGTAGTCAG
OL559	CTCTGGTATCGCTTACGTTCAGGTTAACATCACCGGTAAAGCTTCTCACGCTGGTGCTG CTCCGGAACTGGGTGTTAACGCTC

OL560	GTTTTTAGCTTTGTCGTCGATGTTCATGGTACGCAGAACCAGGTCAGAAGCTTCAACCA GAGCGTTAACACCCAGTTCCG
OL561	CATCGACGACAAAGCTAAAAACCTGCGTTTCAACTGGACCATCGCTAAAGCTGGTAACG TTTCTAACATCATCCCG
OL562	CATAGCAGCGTCGAAGTCTTCGTTACGAGCGTAACGAACGTCAGCGTTCAGGGTAGCAG AAGCCGGGATGATGTTAGAAACG
OL563	GAAGACTTCGACGCTGCTATGAAAACCCTGGAAGAACGTGCTCAGCAGAAAAAACTGCC GGAAGCTGACGTTAAAG
OL564	CTTCACCAGCGTTGAAAGCCGGACGACCACGGGTAACGATAACTTTAACGTCAGCTTCC GGCAG
OL565	CGGCTTTCAACGCTGGTGAAGGTGGTAAAAAACTGGTTGACAAAGCTGTTGCTTACTAC AAAGAAGCTGGTGGTAC
OL566	GACAGAGCAGCGTAAGCAGCGTCGGTACCACCACCGGTACGTTCTTCAACACCCAGGGT ACCACCAGCTTCTTTGTAGTAAG
OL567	GCTGCTTACGCTGCTCTGTCTGGTAAACCGGTTATCGAATCTCTGGGTCTGC
OL568	CGTATTCAGCTTTGTCAGAGTGGTAACCGAAACCCGGCAGACCCAGAGATTCGATAAC
OL569	CACTCTGACAAAGCTGAATACGTTGACATCTCTGCTATCCCGCGTCGTCTGTACATGGC TGCTC
OL570	TTTACCAGCACCCAGGTCCATGATCAGACGAGCAGCCATGTACAGACGAC
OL571	AAAAAAGAGCTCTTTACCAGCACCCAGGTCCATGATC
OL572	AAAAGGCGCGCCGCAGAAACGTGACAACGTTCTGTTCCAG

The gene was assembled by polymerase chain reaction (PCR). A set of four different PCR mixes (A, B, C, D) were compiled featuring different sets of olignucleotides as identified below. In all instances the primers driving the reaction were present in higher concentration within each mix (50pmol), and are shown below underlined:

Mix A) OL549 + OL550 + OL551 + OL552

Mix B) OL553 + OL554 + OL555 + OL556 + OL557 + OL558

Mix C) OL559 + OL560 + OL561 + OL562 + OL563 + OL564

Mix D) OL565 + OL566 + OL567 + OL568 + OL569 + OL570

The complete CPG2 gene was formed in a joining reaction using the purified products of the above reactions and driven by flanking primers OL571 and OL572 to introduce the cloning sites. The PCRs were conducted using hi fidelity polymerase (Promega, Southampton, UK) and a reaction buffer supplied with the enzyme. The reactions were cycled using a thermal cycler running the program detailed below:

PCR cycling conditions:

Stage	Step	Temperature (°C)	Time (sec)	No of Cycles
1	1	94	120	1

2	1	94	15	10
2	2	45-60 (gradient)	60	10
2	3	72	45	10
3	1	94	15	25
3	2	60	30	25
3	3	72	45	25
4	1	72	420	1
4	2	4	hold	1

The assembled CPG2 gene was subcloned into pGEM and the sequence confirmed by sequence analysis. To test the activity of CPG2, cells containing the CPG2 gene were plated out on to LB agar containing 0.1% folic acid (folic acid was dissolved in 1M NaOH to make a 10% stock). These were then incubated at 37°C, colonies that had CPG2 activity were identified by a yellow halo. The halo is the pteroic acid that is precipitated when the folic acid is hydrolyzed.

EXAMPLE 4

Site-directed Mutagenesis of CPG2 gene

The cloned active CPG2 gene was used as a template for the development of mutated variants of the gene using the QuickChange™ Site-Directed Mutagenesis Kit (Stratagene, LaJolla, CA). A high fidelity thermostable polymerase is used to extend pairs of oligonucleotide primers, which are complementary to opposite strands of the vector and contain the desired mutation. Incorporation of the primers results in a mutated vector containing staggered nicks. Parental DNA is digested using DpnI endonuclease which is specific for methylated and hemimethylated DNA and the nicked vector DNA incorporating the desired mutations is then transformed into competent E.coli cells. Sixteen pairs of oligonucleotide primers designed to introduce a point mutation each in the template, were used. Oligonucleotide sequences are shown in Table 8.

Table 8: *Oligonucleotide primers used to introduce mutations to the CPG2 template gene. The sequence, length and the mutation introduced is shown.*

	Name	Substitutions	Primer Sequence	Length
1	OL573	L93I (A4)	CCGTTTACATAAAAGGTA	18
2	OL574	L128T	ACACCACGAAACTGCTG	17

		(B5)		
3	OL575	K129T (B6)	CCCTGACACTGCTGAAAG	18
4	OL576	L130M (B7)	CTGAAAATGCTGAAAGAA	18
5	OL577	L131T (B8)	GAAACTGACGAAAGAATA	18
6	OL578	I161T (C2)	ACCTGACCCAGGAAGAA	17
7	OL579	V217T (E4)	CGCTCTGACTGAAGCCTC	18
8	OL580	N247T (F4)	AAGCTGGAACCGTTTCTA	18
9	OL581	N250T (F6)	AACGTTTCTACCATCATC	18
10	OL582	I251T (F7)	GTTTCTAACACCATCCC	17
11	OL583	I252T (F8)	TAACATCACCCCGGCTTC	18
12	OL584	L258T (F9)	TCTGCTACCACGAACGCT	18
13	OL585	K310T (G3)	GTGGTAAAACACTGGTTG	18
14	OL586	V316S (H3)	ACAAAGCATCTGCTTACT	18
15	OL587	Y318T (H4)	TGTTGCTACCTACAAAGA	18
16	OL588	Y319S (H5)	TTGCTTACTCCAAAGAA	17
1	OL589	L93I (A4) R	TACCTTTTATGTAAACGG	18
2	OL590	L128T (B5) R	CAGCAGTTTCGTGGTGT	17
3	OL591	K129T (B6) R	CTTTCAGCAGTGTCAGGG	18
4	OL592	L130M	TTCTTTCAGCATTTTCAG	18

		(B7) R		
5	OL593	L131T (B8) R	TATTCTTTCGTCAGTTTC	18
6	OL594	I161T (C2) R	TTCTTCCTGGGTCAGGT	17
7	OL595	V217T (E4) R	GAGGCTTCAGTCAGAGCG	18
8	OL596	N247T (F4) R	TAGAAACGGTTCCAGCTT	18
9	OL597	N250T (F6) R	GATGATGGTAGAAACGTT	18
10	OL598	I251T (F7) R	GGGATGGTGTTAGAAAC	17
11	OL599	I252T (F8) R	GAAGCCGGGGTGATGTTA	18
12	OL600	L258T (F9) R	AGCGTTCGTGGTAGCAGA	18
13	OL601	K310T (G3) R	CAACCAGTGTTTTACCAC	18
14	OL602	V316S (H3) R	AGTAAGCAGATGCTTTGT	18
15	OL603	Y318T (H4) R	TCTTTGTAGGTAGCAACA	18
16	OL604	Y319S (H5) R	TTCTTTGGAGTAAGCAA	17

EXAMPLE 5

Expression and purification of recombinant CPG2

Mutant CPG2 genes were cloned into an expression vector utilizing the AscI/SacI restriction sites flanking the coding region. Ligation mixtures were used to transform E.coli cells. Several ampicillin resistant clones were selected on LB plates containing 50-100μg/ml ampicillin. These were analyzed for the presence and orientation of the recombinant insert and to ensure that it is in frame with the His-tag. After verification of the correct orientation, cells were grown at 37°C, induced for expression and harvested by centrifugation. Expression was confirmed by analysis of lyzed cell samples in SDS-

PAGE gels and stained with Coomassie Blue. Expression was scaled up to 50ml cell cultures for subsequent purification of recombinant protein.

The 6xHis tagged CPG2 protein was purified using ProBond™ Purification System (Invitrogen, Carlsbad, CA) and protocols recommended by the supplier. Briefly, cells were harvested from 50ml cultures by centrifugation, re-suspended in Binding Buffer and lyzed. Lysates were allowed to bind to Purification Column resin under gentle agitation for 60 minutes. Resin was washed and recombinant protein was eluted using Elusion Buffer. Samples were analyzed using SDS-PAGE and Coomassie staining as previously. The concentration of the purified protein was determined using spectrophotometry.

EXAMPLE 6

Demonstration of reduced immunogenic potential in modified CPG2 protein using human PBMC in vitro proliferation assay.

A modified protein was prepared according to the method of examples 3 - 5. The modified protein contained multiple substitutions from the wild-type sequence. Positive control protein was wild-type CPG2.

For T cell proliferation assays $4x10^6$ PBMC (per well) from healthy donors were incubated with unmodified and modified antibodies in 2ml bulk cultures (in 24 well plates). Each donor culture was treated with modified and unmodified CPG2 at 5 and 50μg/ml. In addition an untreated control bulk culture was maintained enabling stimulation indexes to be determined. At days 5, 6, 7 and 8 cells for each bulk culture were gently agitated and 50μl samples removed in triplicate for determination of the proliferation index. The 50μl sample aliquots were each transferred to 3 wells of a U-bottom 96 well plate. Fresh AIM V media (130μl) was added to each of the 96 wells. Cells were pulsed (for 18-21 hours) with 1μCi [^{3}H]Thymidine per well diluted in a total volume of 20ul AIM V media. The total volume for each culture was 200μl. CPM values were collected using a beta-plate reader and the stimulation index for each time point determined as per Example 2.

The SI was plotted for each time point and treatment. In responsive donors, treatment with wild-type CPG2 results in a significant proliferative response with a peak at day 7. In the same donors, treatment with the modified CPG2 composition of the present invention does not result in a significant proliferative response. This results indicate a

reduced immunogenic potential in the modified CPG2 protein. Plots from responsive donors #1 and #16 are provided in FIGURE 2.

Patent Claims

1. A modified bacterial enzyme carboxypeptidease G2 (CPG2) being substantially non-immunogenic or less immunogenic than any non-modified CPG2 having essentially the same biological specificity when used in vivo, and comprising specific amino acid residues having alterations compared with the non-modified parental enzyme, wherein said alterations cause a reduction or an elimination of one or more of T-cell epitope sequences, which act in the parental enzyme as MHC class II binding ligands and stimulate T-cells.

2. A modified CPG2 molecule according to claim 1, wherein said alterations are made at one or more positions within following strings of contiguous amino acid residues from the CPG2 wild-type sequence:

A = VGKIKGRGGKNLLLMSHMDTVYLKGILAK;
B = KAYGPGIADDKGGNAVILHTLKLLKEYG;
C = LFNTDEEKGSFGSRDLIQEEA;
D = KLADYVLSFEPTSAGDEKLSLGTSG;
E = VNITGKASHAGAAPELGVNALVEASDL;
F = KAKNLRFNWTIAKAGNVSNIIPASATLNAD;
G = ADVKVIVTRGRPAFNAGEGGKKLVDKA;
H = KKLVDKAVAYYKEAGG;
I = YKEAGGTLGVEERTGGG;
J = TDAAYAALSGKPVIESLGLPGFGY
K = LEKLVNIETGTGDAE;

3. A modified CPG2 molecule according to claim 1, wherein said T-cell epitope sequences are 13mer or 15mer peptides and are selected from any of Table 1 or Table 2.

4. A modified CPG2 molecule according to any of the claims 1 to 3, wherein said alterations are substitutions of 1 - 9 amino acid residues.

5. A modified CPG2 molecule of claim 3, wherein said substitutions are selected from any of Table 4 or Table 5.

6. A modified CPG2 molecule according to any of the claims 1 to 5, comprising one or more further alterations of amino acid residues, wherein said alterations are conducted to restore biological activity of the molecule.

7. A molecule having the biological activity of bacterial enzyme carboxypeptidease G2 (CPG2) and the following amino acid sequence:

X^0QKRDNVLFQAATDEQPAVIKTLEKLVNIETGTGDAEGIAAAGNFLEAELKNLGFTVT
RSKSAGLVVGDNIVGKX^1KGRGGKNLX^2LX^3SHMDTVYX^4KGILAKAPFRVEGDKAX^5GP
GX^6ADDKGGNAX^7IX^8HT$X^9$$X^{10}$$X^{11}$$X^{12}$KEYGVRDYGTITV$X^{13}$FNTDEEKGSFGSRDL$X^{14}$Q
EEAKLADY$X^{15}$$X^{16}SX^{17}$EPTSAGDEK$X^{18}$SLGTSGIAYVQVNITGKASHAGAAPEX^{19}GX^{20}
NA$X^{21}$$X^{22}$EASDLVLRTMNIDDKAKN$X^{23}RFNX^{24}TX^{25}$AKAG$X^{26}$$X^{27}SX^{28}$$X^{29}$$X^{30}$PASAT$X^{31}$
NADVRYARNEDFDAAMKTLEERAQQKKLPEADX^{32}KX^{33}IVTRGRPAFNAGEGGK$X^{34}$$X^{35}$
X^{36}DKAX^{37}A$X^{38}$$X^{39}$KEAGGTLGVEERTGGGTDAAYAALSGKPVIESLGLPGFGYHSDKA
EYVDISAIPRRLYMAARLIMDLGAGK,

wherein X^0 is optionally a targeting moiety such as an antibody domain and X^1 is I, T; X^2 is L, A; X^3 is M, K; X^4 is L, I; X^5 is Y, T; X^6 is I, A; X^7 is V, A; X^8 is L, A; X^9 is L, T, A; X^{10} is K, T; X^{11} is L, M, A; X^{12} is L, T, A; X^{13} is L, G, T; X^{14} is I, T, A; X^{15} is V, A; X^{16} is L, T, G; X^{17} is F, A; X^{18} is L, A; X^{19} is L, A; X^{20} is V, A; X^{21} is L, A; X^{22} is V, T, A; X^{23} is L, A; X^{24} is W, A, H; X^{25} is I, A; X^{26} is N, T, A; X^{27} is V, T; X^{28} is N, T, A; X^{29} is I, T, A; X^{30} is I, T, A; X^{31} is L, T, A, I; X^{32} is V, A; X^{33} is V, A; X^{34} is K, T; X^{35} is L, A; X^{36} is V, A; X^{37} is V, S, T, A; X^{38} is Y, T, A; X^{39} is Y, S, T, A;

and whereby simultaneously

X^1 = I, X^2 = L, X^3 = M, X^4 = L, X^5 = Y, X^6 = I, X^7 = V, X^8 = L, X^9 = L, X^{10} = K, X^{11} = L, X^{12} = L, X^{13} = L, X^{14} = I, X^{15} = V, X^{16} = L, X^{17} = F, X^{18} = L, X^{19} = L, X^{20} = V, X^{21} = L, X^{22} = V, X^{23} = L, X^{24} = W, X^{25} = I, X^{26} = N, X^{27} = V, X^{28} = N, X^{29} = I, X^{30} = I, X^{31} = L, X^{32} = V, X^{33} = V, X^{34} = K, X^{35} = L, X^{36} = V, X^{37} = V, X^{38} = Y and X^{39} = Y are excluded.

8. A modified CPG2 enzyme according to any of the claims 1 to 7, wherein when tested as a whole protein in a biological assay of induced cellular proliferation of human T-cells exhibits a stimulation index smaller than the parental enzyme tested in parallel using cells from the same donor wherein said index is taken as the value of cellular proliferation scored following stimulation by the protein and divided by the value of cellular proliferation scored in control cells not in

receipt of protein and wherein cellular proliferation is measured by any suitable means.

9. A DNA sequence coding for a CPG2 molecule as defined in any of the claims 1 to 8.

10. A pharmaceutical composition comprising a modified CPG2 molecule of any of the preceding claims, optionally together with a pharmaceutically acceptable carrier, diluent or excipient.

11. A peptide molecule consisting of 9 – 15 consecutive amino acid residues, having a potential MHC class II binding activity and created from the primary sequence of non-modified CPG2 enzyme, whereby said peptide molecule has a stimulation index of at least 1.8 to 2 in a biological assay of cellular proliferation wherein said index is taken as the value of cellular proliferation scored following stimulation by a peptide and divided by the value of cellular proliferation scored in control cells not in receipt peptide and wherein cellular proliferation is measured by any suitable means.

12. A peptide molecule according to claim 11, wherein said stimulation index is greater than 2.

13. A peptide molecule of claim 11 or 12 selected from the group of contiguous T-cell epitope sequences as depicted in Table 1 or Table 2.

14. A modified peptide molecule deriving from the peptide molecule of any of the claims 11 to 13 by amino acid substitution, having a reduced or absent potential MHC class II binding activity expressed by a stimulation index of less than 2, whereby said index is taken as the value of cellular proliferation scored following stimulation by a peptide and divided by the value of cellular proliferation scored in control cells not in receipt peptide and wherein cellular proliferation is measured by any suitable means.

15. A modified peptide molecule of claim 14, wherein said stimulation index is less than 2.

16. Use of a peptide according to any of the claims 11 to 15 for the manufacture of a modified CPG2 enzyme having substantially no or less immunogenicity than any non-modified parental enzyme when used *in vivo*.

17. Use of a peptide according to any of the claims 11 to 13 for the purpose of vaccination of patients to reduce immunogenicity to CPG2 *in vivo*.

18. A DNA sequence coding for a peptide of any of the claims 11 to 15.

19. Pharmaceutical composition comprising a peptide of any of the claims 11 to 15, optionally together with a pharmaceutically acceptable carrier, diluent or excipient.

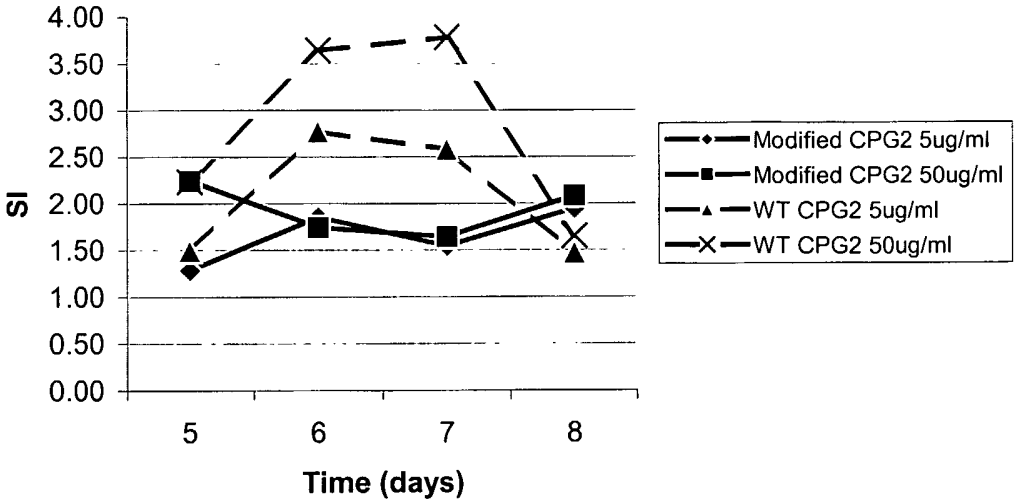
FIGURE 1

ID#	Donor #	Sequence
1	5	QKRDNVLFQAATDEQ
2		DNVLFQAATDEQPAV
3		LFQAATDEQPAVIKT
4		AATDEQPAVIKTLEK
5		DEQPAVIKTLEKLVN
6		PAVIKTLEKLVNIET
7		IKTLEKLVNIETGTG
8	5	LEKLVNIETGTGDAE
9		LVNIETGTGDAEGIA
10		IETGTGDAEGIAAAG
11		GTGDAEGIAAAGNFL
12		DAEGIAAAGNFLEAE
13		GIAAAGNFLEAELKN
14		AAGNFLEAELKNLGF
15		NFLEAELKNLGFTVT
16		EAELKNLGFTVTRSK
17	18	LKNLGFTVTRSKSAG
18		LGFTVTRSKSAGLVV
19		TVTRSKSAGLVVGDN
20		RSKSAGLVVGDNIVG
21		SAGLVVGDNIVGKIK
22		LVVGDNIVGKIKGRG
23	5	GDNIVGKIKGRGGKN
24		IVGKIKGRGGKNLLL
25	1, 6, 16, 18	KIKGRGGKNLLLMSH
26	1, 9	GRGGKNLLLMSHMDT
27		GKNLLLMSHMDTVYL
28	9	LLLMSHMDTVYLKGI
29	1, 18, 19	MSHMDTVYLKGILAK
30		MDTVYLKGILAKAPF
31		VYLKGILAKAPFRVE
32	1	KGILAKAPFRVEGDK
33		LAKAPFRVEGDKAYG
34		APFRVEGDKAYGPGI
35		RVEGDKAYGPGIADD
36		GDKAYGPGIADDKGG
37	5, 10	AYGPGIADDKGGNAV
38	10	PGIADDKGGNAVILH
39	1	ADDKGGNAVILHTLK
40	1, 18	KGGNAVILHTLKLLK
41	1, 18	NAVILHTLKLLKEYG
42	1, 18	ILHTLKLLKEYGVRD
43	18	TLKLLKEYGVRDYGT
44		LLKEYGVRDYGTITV
45		EYGVRDYGTITVLFN
46		VRDYGTITVLFNTDE
47		YGTITVLFNTDEEKG
48	10	ITVLFNTDEEKGSFG
49	10, 18	LFNTDEEKGSFGSRD
50	10	TDEEKGSFGSRDLIQ
51		EKGSFGSRDLIQEEA
52	10	SFGSRDLIQEEAKLA
53		SRDLIQEEAKLADYV
54		LIQEEAKLADYVLSF
55	1	EEAKLADYVLSFEPT
56	5, 7	KLADYVLSFEPTSAG
57		DYVLSFEPTSAGDEK
58	7	LSFEPTSAGDEKLSL
59	5, 7	EPTSAGDEKLSLGTS
60	7, 15	SAGDEKLSLGTSGIA
61		DEKLSLGTSGIAYVQ
62		LSLGTSGIAYVQVNI
63	4	GTSGIAYVQVNITGK
64		GIAYVQVNITGKASH

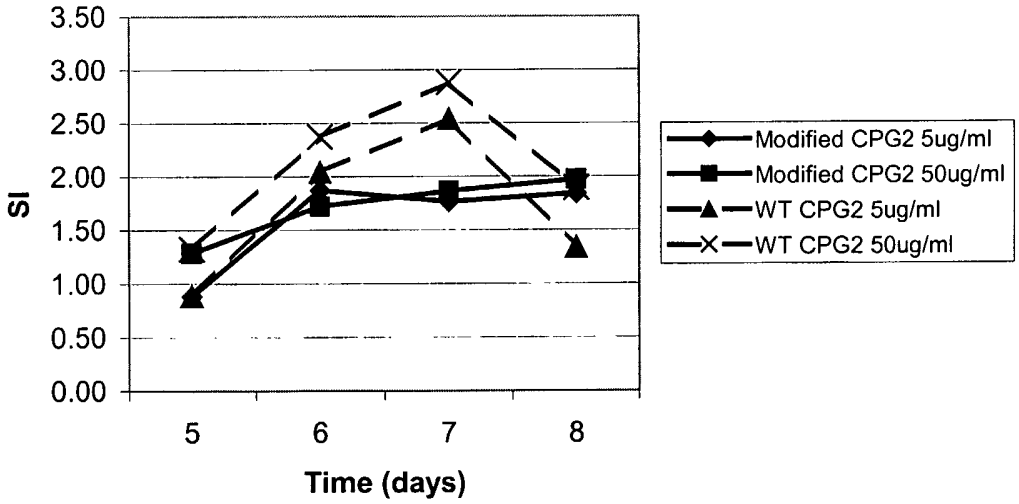
ID#	Donor #	Sequence
65		YVQVNITGKASHAGA
66	9, 16	VNITGKASHAGAAPE
67		TGKASHAGAAPELGV
68	18	ASHAGAAPELGVNAL
69	16	AGAAPELGVNALVEA
70	9, 16, 20	APELGVNALVEASDL
71		LGVNALVEASDLVLR
72		NALVEASDLVLRTMN
73		VEASDLVLRTMNIDD
74		SDLVLRTMNIDDKAK
75		VLRTMNIDDKAKNLR
76		TMNIDDKAKNLRFNW
77	18	IDDKAKNLRFNWTIA
78	1, 18	KAKNLRFNWTIAKAG
79	10, 18	NLRFNWTIAKAGNVS
80		FNWTIAKAGNVSNII
81	18	TIAKAGNVSNIIPAS
82	1	KAGNVSNIIPASATL
83	18	NVSNIIPASATLNAD
84		NIIPASATLNADVRY
85		PASATLNADVRYARN
86		ATLNADVRYARNEDF
87		NADVRYARNEDFDAA
88		VRYARNEDFDAAMKT
89		ARNEDFDAAMKTLEE
90		EDFDAAMKTLEERAQ
91		DAAMKTLEERAQQKK
92		MKTLEERAQQKKLPE
93		LEERAQQKKLPEADV
94		RAQQKKLPEADVKVI
95		QKKLPEADVKVIVTR
96		LPEADVKVIVTRGRP
97	1, 3, 18	ADVKVIVTRGRPAFN
98		KVIVTRGRPAFNAGE
99		VTRGRPAFNAGEGGK
100	3	GRPAFNAGEGGKKLV
101	16	AFNAGEGGKKLVDKA
102	9	AGEGGKKLVDKAVAY
103	18	GGKKLVDKAVAYYKE
104	16	KLVDKAVAYYKEAGG
105		DKAVAYYKEAGGTLG
106		VAYYKEAGGTLGVEE
107		YKEAGGTLGVEERTG
108	5	AGGTLGVEERTGGGT
109		TLGVEERTGGGTDAA
110		VEERTGGGTDAAYAA
111		RTGGGTDAAYAALSG
112	3	GGTDAAYAALSGKPV
113		DAAYAALSGKPVIES
114		YAALSGKPVIESLGL
115		LSGKPVIESLGLPGF
116	3	KPVIESLGLPGFGYH
117		IESLGLPGFGYHSDK
118		LGLPGFGYHSDKAEY
119		PGFGYHSDKAEYVDI
120		GYHSDKAEYVDISAI
121		SDKAEYVDISAIPRR
122		AEYVDISAIPRRLYM
123		VDISAIPRRLYMAAR
124	18	SAIPRRLYMAARLIM
125		PRRLYMAARLIMDLG
126		LYMAARLIMDLGAGK
127	11	AARLIMDLGAGKHHH
128		LIMDLGAGKHHHHHH

FIGURE 2

A: Donor #1



B: Donor #16



INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 02/13351

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K38/48 C12N9/48 C12N15/57

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N A61K

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

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
MEDLINE, EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	US 2002/090709 A1 (SHARMA SURINDER K ET AL) 11 July 2002 (2002-07-11) the whole document	1-10
P,X	SPENCER DANIEL I R ET AL: "A strategy for mapping and neutralizing conformational immunogenic sites on protein therapeutics." PROTEOMICS. GERMANY MAR 2002, vol. 2, no. 3, March 2002 (2002-03), pages 271-279, XP009009265 ISSN: 1615-9853 the whole document	1-10

-/--

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

[X] Patent family members are listed in annex.

° Special categories of cited documents :

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

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

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

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

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

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 April 2003	15/05/2003

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

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 02/13351

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	NAPIER M P ET AL: "Antibody-directed enzyme prodrug therapy: efficacy and mechanism of action in colorectal carcinoma." CLINICAL CANCER RESEARCH: AN OFFICIAL JOURNAL OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH. UNITED STATES MAR 2000, vol. 6, no. 3, March 2000 (2000-03), pages 765-772, XP002238264 ISSN: 1078-0432 the whole document ---	1-10
Y	MOOLA Z B ET AL: "Erwinia chrysanthemi L-asparaginase: epitope mapping and production of antigenically modified enzymes." THE BIOCHEMICAL JOURNAL. ENGLAND 15 SEP 1994, vol. 302 (Pt 3), 15 September 1994 (1994-09-15), pages 921-927, XP000918634 ISSN: 0264-6021 the whole document ---	1-10
P,A	WO 02 069232 A (MERCK PATENT GMBH ;CARTER GRAHAM (GB); JONES TIM (GB); CARR FRANCI) 6 September 2002 (2002-09-06) -----	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 02/13351

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

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

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

2. ☒ Claims Nos.: 11-19
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
see FURTHER INFORMATION sheet PCT/ISA/210

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

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

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

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

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

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

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

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Claims Nos.: 11-19

Present claims 11-19 relate to an extremely large number of possible compounds. Support within the meaning of Article 6 PCT and disclosure within the meaning of Article 5 PCT is to be found, however, for none of the compounds claimed. Claims 11-19 relate to a multitude of structurally undefined peptides defined by an unusual parameter which is not suitable to compare the subject-matter claimed with what is set out in the prior art. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible.

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 02/13351

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2002090709 A1	11-07-2002	NONE	
WO 02069232 A	06-09-2002	WO 02062832 A2	15-08-2002
		WO 02062375 A1	15-08-2002
		WO 02077034 A2	03-10-2002
		WO 02062843 A2	15-08-2002
		WO 02062842 A1	15-08-2002
		WO 02062833 A2	15-08-2002
		WO 02066058 A1	29-08-2002
		WO 02069232 A2	06-09-2002