Novel compounds and Process

The present invention relates to a novel chemical process for the covalent conjugation of disulphide bridge cyclised peptides to immunogenic carrier molecules by thio-ether linkages to form vaccine immunogens. In particular, the novel chemistry involves reacting a thiolated carrier with a cyclic peptide containing a disulphide bridge, which cyclic peptide (herein a disulphide bridge cyclised peptide) has attached to it, usually *via* a linker, a reactive group capable of forming thio-ether bonds with the carrier. The invention further relates to activated peptide intermediates of the process, medicaments produced by the process, pharmaceutical compositions containing the medicaments, and the use of the pharmaceutical compositions in medicine. The process of the present invention is particularly useful for the preparation of highly pure immunogens for vaccines, comprising disulphide bridge cyclised peptides. Also novel immunogens are provided, based on peptides derived from the sequence of human IgE, which are useful in the immunotherapy of allergy. Accordingly, the invention relates also to a process for conjugation of IgE disulphide bridge cyclised peptides to carriers, immunogens produced by the process and vaccines and pharmaceutical compositions comprising them and their use in the treatment of allergy.

Immunogens comprising short peptides are becoming increasingly common in the field of vaccine prophylaxis or therapy. In many disease states it is often possible, and desirable, to design vaccines comprising a short peptide rather than a large protein. Peptides which may be used as immunogens may be the full length native protein, for example human peptidic hormones, or may be fragments of a larger antigen derived from a given pathogen, or from a large self-protein. For example, short peptides of IgE may be used for prophylaxis of allergy, whereas the use of IgE itself as the immunogen may induce anaphylactic shock.

It has previously been thought that amongst the problems associated with the peptide approach to vaccination, is the fact that peptides *per se* are poor immunogens. Generally the sequences of the peptides chosen are such that they include a B-cell epitope to provide a target for the generation of anti-peptide antibody responses, but because of their limited size rarely encompass sufficient T-cell epitopes in order to provide the necessary cytokine help in the

induction of strong immune responses following priming and boosting applications of the vaccine.

Strategies to overcome this problem of immunogenicity include the linking of the peptide to large highly immunogenic protein carriers. The carrier proteins contain a large number of peptidic T-cell epitopes which are capable of being loaded into MHC molecules, thereby providing bystander T-cell help, and/or alternatively the use of strong adjuvants in the vaccine formulation. Examples of these highly immunogenic carriers which are currently commonly used for the production of peptide immunogens include the Diptheria and Tetanus toxoids (DT and TT respectively), Keyhole Limpet Haemocyanin (KLH), and the purified protein derivative of Tuberculin (PPD).

Peptides used in a particular vaccine immunogen are often chosen such that they generate an antibody response to the location site of that peptide in the context of the full length native protein. Thus, in order to generate antibodies that bind to such chosen locations, the peptide in the immunogen must assume substantially the same shape as it would exist if it was confined by the flanking regions of the full length native protein. However, merely conjugating a linear peptide sequence, by conventional chemistry, to a carrier protein rarely achieves this goal. This is because such an immunogen presents the linear peptide with too much conformational freedom, such that the peptide may adopt a loose structure that either is not well recognised by the immune system, or may be entirely different to the conformation adopted by the peptide in the context of the flanking regions of the full length native protein.

In order to overcome this conformational freedom problem, it is known to design peptides in a constrained manner, by chemical interactions between two distant amino acid residues, such that the peptide is held in a curved structure which closely resembles the curve in which the peptide would be held by the flanking sequences in the full length native protein (US 5,939,383; Hruby *et al.*, 1990, *Biochem J.*, 268, 249-262). To do this it is most common to incorporate two cysteine residues into the peptide sequence between which the desired intramolecular disulphide bridge forms after gentle oxidation of a dilute solution of the peptide.

The cyclised peptide thus formed is commonly conjugated to a protein carrier to form an immunogen by one of several chemistry methods. Examples of known chemistries include conjugation of amino groups between the peptide and carrier by amino reactive agents such as glutaraldehyde or formaldehyde; or condensing carboxyl groups and amino groups with carbodiimide reagents or alternatively by converting n-terminal α-hydroxy groups to aldehydes by an oxidation reaction and conjugating this group to an amino or oxamino moiety. However, each of these chemistries has disadvantages, including a need for relatively harsh oxidative reaction conditions, poor controllability at industrial levels, formation of polymers, or not being suitable for peptides that contain specific internal amino acids (especially: Lysine, Aspartic acid, Glutamic acid, Tryptophan, Tyrosine or Serine) that could also interfere with the chemistry in an inappropriate manner.

It is common, therefore, to use thio-ether linkage to conjugate peptides to protein carriers. The most common method to achieve this conjugation is to add a moiety with a terminal thiol group onto the peptide, most commonly by adding a cysteine, and then to react the reactive thiol group with a maleimide-derivatised protein carrier (Friede *et al.*, 1994, Vaccine, 12, 791-797), for a schematic summary see FIG 1.

However, in the case of peptides containing an internal disulphide bond this commonly preferred peptide chemistry may have problems because of the posibility of internal disulphide rearrangement, or external rearrangement of disulphide bonds between between two adjacent peptides. In some cases the presence of a third cysteine causes unwanted interference with the disulphide bond, and a thiol-disulfide exchange can occur such that the resultant intermediate cyclised peptide product is a mixture of three possible disulphide bridge cyclised peptides (reassortant intermediates, see FIG 2), or may additionally comprise peptide dimers or polymers.

In the case of conjugation of these peptide intermediates to a maleimide activated carrier protein, each of the reassortant intermediates is equally reactive with the reactive carrier protein, and as such they will all conjugate to the carrier. As a result, the purity of the desired product is decreased, and use of this mixture of immunogens may result in immune responses that may not, or only weakly, cross react with the epitope on the full native protein that the peptide was intended to mimic. In order to overcome these problems several authors have

replaced the disulphide bond stabilised cyclic peptides, by thio-ether bonds. For example, in Ivanov et al., 1995, Bioconjugate Chemistry, 6, 269-277, one cystein is replaced by a trifunctional bromoacetyl-derivitised amino acid, thus permiting cyclisation via a non-reversible thioether bond. In such thio-ether cyclised peptides, however, the resulting peptide is fundamentally different to the original disulphide-cyclised peptide, and has a different structure which may not resemble the disulphide-cyclised peptide. Hence antibodies formed against the thio-ether cyclised peptide may not recognise the parent peptide as efficiently as antibodies formed against the disulphide-cyclised peptides.

The present invention overcomes the problems of forming a thio-ether linkage between a disulphide cyclised peptide and a carrier by providing a chemistry that does not use a terminal thiol containing group on the cyclised peptide, but instead uses another reactive group on the peptide, which may then be reacted with a thiolated carrier protein to form a thio-ether bond.

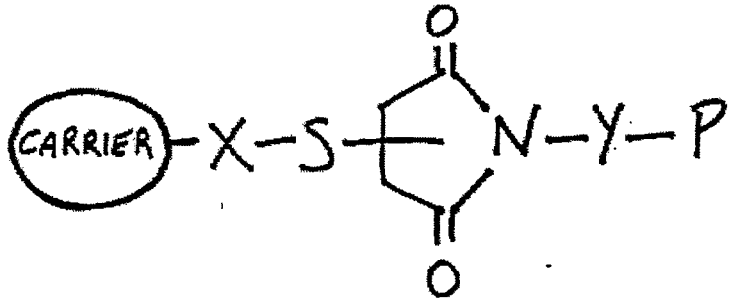
Therefore, in the present invention, there is provided a process for the manufacture of a vaccine immunogen comprising conjugating a disulphide bridge cyclised peptide to an immunogenic carrier comprising, (a) adding to a disulphide cyclised peptide a moiety comprising a reactive group which is capable of forming thio-ether linkages with thiol bearing carriers, and (b) reacting the activated cyclised peptide thus formed with a thiol bearing immunogenic carrier.

The process of the present invention overcomes the problems of internal and external disulphide rearrangement, and in addition provides conjugated products wherein the disulphide cyclised peptides are in the desired conformation. In a preferred process of the present invention, a peptide is synthesised containing two cysteine residues which are allowed to form a disulphide bridge, followed by the addition of the reactive group. The activated peptide, thus obtained, is then reacted with the thiol bearing carrier.

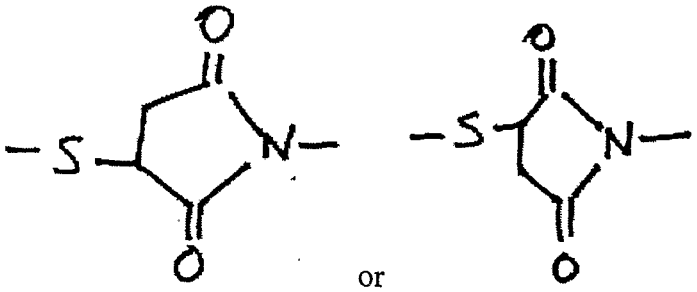
The reactive groups that are suitable for use in the present invention include any group which is capable forming thio-ether linkages with thiolated carriers. As which will be apparent to the man skilled in the art, preferred reactive groups may be selected from active imides, especially maleimides, haloalkyl groups such as iodoalkyl or bromoalkyl groups. Preferably the

bromoalkyl group is a bromoacetyl group. The use of maleimide to link linear peptides to thiolated polymer is described in Van Dijk-Wolthius *et al.*, 1999, Bioconjugate Chemistry, 10, 687-692. Use of bromoacetyl groups to link peptides to carriers is described in Ivanov et al., 1995, Bioconjugate Chemistry, 6, 269-277 and US 5,444,150. Conjugation of proteins to thiolated solid phase supports for diagnostic assays is described in EP 0 396 116 A.

It is a particularly preferred aspect of the present invention when the process uses maleimide as the reactive group. Accordingly, a preferred process for conjugating a disulphide bridge cyclised peptide to a carrier comprises, (a) adding to a disulphide cyclised peptide a moiety comprising a maleimide group, and (b) reacting the activated cyclised peptide thus formed with a thiol bearing carrier. The product of this process (A conjugate suitable for use in a vaccine) forms an aspect of the present invention, and has the formula (I):



wherein, Carrier is a carrier molecule, X is either a linker or a bond, Y is either a linker or a bond, and P is a disulphide bridge cyclised peptide. When X is a bond, it should be understood that the carrier is directly linked to the sulphur atom S. Similarly, when Y is a linker it should be understood that the disulphide bridge cyclised peptide is linked directly to the nitrogen atom N. A "linker" refers to a suitable linker group. When X is a linker group an example is the group $-NHCO(CH_2)_2-$. When Y is a linker group, an example is $-(CH_2)_3-$CONH-. It will also be clear to the man skilled in the art, that Formula (I) covers conjugates where the sulphur atom (S) is joined onto the imide ring to either of the two adjacent non-carbonyl carbon atoms, such that the conjugate may comprise the following structures:



Forming an aspect of the invention is the intermediate to the process of the present invention, which is a disulphide cyclised peptide which bears a reactive group which is capable forming thio-ether linkages with thiolated carriers. Preferably said intermediate comprises a disulphide bridge cyclised peptide linked to an active imide group, in particular a maleimide group. The high purity of the final conjugated product derives from the fact that any internal or external rearrangement that occurs between the disulphide bridge and the thio-ether reactive group is irreversible, and consequently these reassortant intermediates are not reactive with the thiolated carrier protein. Only the activated peptide intermediates that have the disulphide bridge at the desired location (i.e. between the cysteines present in the peptide) with the free reactive group participate in the conjugation reaction with the thiolated carrier, thereby forming a conjugate of extremely high purity which contains cyclised peptides of the desired conformation.

Preferred maleimide derivatisation reagents are gamma-maleimidobutyric acid N-hydroxysuccinimide ester (GMBS, Molecular Formula: $C_{12}H_{12}N_2O_6$, Fujiwara, K., et al., J. Immunol. Meth., 45, 195-203 (1981), Tanimori, H., et al., J. Pharmacobiodyn., 4, 812-819 (1981); H. Tanimori, et al., J. Immunol. Methods *62*, 123 (1983); M.D. Partis, et al., J. Prot. Chem. *2*, 263 (1983); L. Moroder, et al., Biopolymers *22*, 481 (1983); S. Hashida, et al., J. Appl. Biochem. *6*, 56 (1984); S. Inoue, et al., Anal. Lett. *17*, 229 (1984); E. Wünsch, et al., Biol. Chem. Hoppe-Seyler *366*, 53 (1985)) , which can be purchased from the Sigma or Pierce companies. It will be recognised that many maleimide-derivitisation reagents exist and can be used, and the addition of the maleimide group to the cyclised peptide can be performed during peptide synthesis using reagents compatible with organic synthesis, or after peptide synthesis using reagents commonly used for derivitising peptides and proteins with maleimide groups.

The process, intermediates and products of the present invention are preferably used in the manufacture of immunogens for use in vaccines. The peptides for conjugation may be selected from any antigen against which is desired to create an immune response. The peptide may be derived from a pathogen, such as a virus, bacterium, parasite such as a worm etc.

Equally the peptide may be selected from a self protein, for example in the vaccine therapy of cancer or allergy.

In an allergic response, the symptoms commonly associated with allergy are brought about by the release of allergic mediators, such as histamine, from immune cells into the surrounding tissues and vascular structures. Histamine is normally stored in mast cells and basophils, until such time as the release is triggered by interaction with allergen specific IgE. The role of IgE in the mediation of allergic responses, such as asthma, food allergies, atopic dermatitis, type-I hypersensitivity and allergic rhinitis, is well known. On encountering an antigen, such as pollen or dust mite allergens, B-cells commence the synthesis of allergen specific IgE. The allergen specific IgE then binds to the FcεRI receptor (the high affinity IgE receptor) on basophils and mast cells. Any subsequent encounter with allergen leads to the triggering of histamine release from the mast cells or basophils, by cross-linking of neighbouring IgE/ FcεRI complexes (Sutton and Gould, Nature, 1993, 366: 421-428; EP 0 477 231 B1).

IgE, like all immunoglobulins, comprises two heavy and two light chains. The ε heavy chain consists of five domains: one variable domain (VH) and four constant domains (Cε1 to Cε4). The molecular weight of IgE is about 190,000 Da, the heavy chain being approximately 550 amino acids in length. The structure of IgE is discussed in Padlan and Davis (Mol. Immunol., 23, 1063-75, 1986) and Helm et al., (2IgE model structure deposited 2/10/90 with PDB (Protein Data Bank, Research Collabarotory for Structural Bioinformatics; http:\pdb-browsers.ebi.ac.uk)). Each of the IgE domains consists of a squashed barrel of seven anti-parallel strands of extended (β-) polypeptide segments, labelled a to f, grouped into two β-sheets. Four β-strands (*a,b,d* & *e*) form one sheet that is stacked against the second sheet of three strands (*c,f* & *g*) (see FIG 8). The shape of each β-sheet is maintained by lateral packing of amino acid residue side-chains from neighbouring anti-parallel strands within each sheet (and is further stabilised by main-chain hydrogen-bonding between these strands). Loops of residues, forming non-extended (non-β-) conformations, connect the anti-parallel β-strands, either within a sheet or between the opposing sheets. The connection from strand *a* to strand *b* is labelled as the *A-B* loop, and so on. The *A-B* and *d-e* loops belong topologically to the four-stranded sheet, and loop *f-g* to the three-stranded sheet. The interface between the pair of opposing sheets provides the hydrophobic interior of the globular domain. This water-

inaccessible, mainly hydrophobic core results from the close packing of residue side-chains that face each other from opposing β-sheets.

In the past, a number of passive or active immunotherapeutic approaches designed to interfere with IgE-mediated histamine release mechanism have been investigated. These approaches include interfering with IgE or allergen/IgE complexes binding to the FcεRI or FcεRII (the low affinity IgE receptor) receptors, with either passively administered antibodies, or with passive administration of IgE derived peptides to competitively bind to the receptors. In addition, some authors have described the use of specific peptides derived from IgE in active immunisation to stimulate histamine release inhibiting immune responses.

Therefore, in order to be effective, the peptide vaccines need to be able to mimic specific sites of IgE very efficiently. The preferred immunogens of the present invention, therefore, are based on peptides derived from IgE and which are capable of triggering an immune response which inhibits histamine release from basophils.

Much work has been carried out to identify specific anti-IgE antibodies which do have some beneficial effects against IgE-mediated allergic reaction (WO 90/15878, WO 89/04834, WO 93/05810). Attempts have also been made to identify epitopes recognised by these useful antibodies, to create peptide mimotopes of such epitopes and to use those as immunogens to produce anti-IgE antibodies.

WO 97/31948 describes an example of this type of work, and further describes IgE peptides from the Cε3 and Cε4 domains conjugated to carrier molecules for active vaccination purposes. These immunogens may be used in vaccination studies and are said to be capable of generating antibodies which subsequently inhibit histamine release *in vivo* . In this work, a monoclonal antibody (BSW17) was described which was said to be capable of binding to IgE peptides contained within the Cε3 domain which are useful for active vaccination purposes.

EP 0 477 231 B1 describes immunogens derived from the Cε4 domain of IgE (residues 497-506, also known as the Stanworth decapeptide), conjugated to Keyhole Limpet Haemocyanin (KLH) used in active vaccination immunoprophylaxis. WO 96/14333 is a continuation of the work described in EP 0 477 231 B1.

Other approaches are based on the identification of peptides derived from Cε3 or Cε4, which themselves compete for IgE binding to the high or low affinity receptors on basophils or mast cells (WO 93/04173, WO 98/24808, EP 0 303 625 B1, EP 0 341 290).

Accordingly in a preferred aspect of the present invention the process, peptide intermediates, immunogens and vaccines, comprise a peptide selected from human IgE. Preferably the disulphide bridge cyclised peptides used in the present invention are designed from the group of peptides listed in table 1. The peptides in table 1, reflect a specific area of the IgE molecule against which it is desired to generate an immune response. The peptides, therefore, constitute a starting point from which a cyclised peptide may be designed, and accordingly they either do not contain a cysteine residue, or contain a single cysteine, or contain two cysteines which may not form a disulphide bridge. Suitable peptides for use in the process or immunogens of the present invention may be designed by the addition of at least one cysteine residue to the following peptides:

Table 1, IgE peptides suitable to be cyclised and used in the process of the present invention

Peptide sequence	SEQ ID NO.
EDGQVMDVD	1
STTQEGEL	2
SQKHWLSDRT	3
GHTFEDSTKK	4
GGGHFPPT	5
PGTINI	6
FTPPT	7
CLEDGQVMDVDLL	8
LLDVDMVQGDELC	9
WLEDGQVMDVDLC	10
QVMDVDL	11
LEDGQVMDVD	12
CSTTQEGELA	13
TTQEGE	14
CSQKHWLSDRT	15
TYQGHTFEDSTKKCADSNPRGV	16
GGHFPP	17
CCVADPETQMTPSSEMF	18
CCVADPETQMTPSSEMF	19
CCVTDVQTTNMDVPAGQ	20
TCCVTDIPPPDYEQSLG	21
CCESDIPLNELHALADP	22
CCKSDIPSPVTQFNTMK	23
CCQSDVPHQPGINDLHV	24
CCMSDTPDISRLPVPDS	25
CCMSDSPADPNRGLPIW	26
CCLSDDAPTLPVRR	27
CCITDVPQGVMYKGSPD	28
ECKVDGQLSDSPLLRNN	29

CCMTDDPMDPNSTWAIR	30
CCMTDDPMYTNSTWAIR	31
CCVDDTPNSGLAMRVSK	32
CCEVDDFPTHHPGWTLR	33
SCNLNHQSCDIPPVKQI	34
CCMADQELDLGHNAANA	35
CCVMDLELASGF	36
CCVMDIEVRGSA	37
CCQRDVELVFGS	38
CCRADFEVGNGG	39
CCVSDEPAGVRD	40
GAGWQEKDKELR	41
GAMTAGQLSDLP	42
VAGGQVVDRELK	43
KAGEQAMDMELR	44
RGRNQIMDLEI	45
QIDRQITDTLL	46
REQQISDVPRV	47
CQAMDAEILNQV	48
GQMMDTELLNR	49
SMEGQVRDIQV	50
YQQRDLELLAE	51
SMGQKVDRELV	52
SMGQEVDRELV	53
AENDQMVDWEI	54
GGWQESDIPGR	55
GGWQEKDKELR	56
HCCRIDREVSGA	57
DCDWINPPDPPHFWKDT	58
DALDERAWRARA	59
RASGKPVNHSTRKEEKQRNGTL	60
GTRDWIEGE	61
PHLPRALMRSTTKTSGPRA	62
PEWPGSRDKRT	63
EQKDE	64
LSRPSPFDLFIRKSPTITC	65
WLHNEVQLPDARHSTTQPRKT	66
CRASGKPVNHSTRKEEKQRNGLL	67
GKPVNHSTGGC	68
GKPVNHSTRKEEKQRNGC	69
CGKPVNHSTRKEEKQRNGLL	70
RASGKPVNHSTGGC	71
CGTRDWIEGLL	72
CGTRDWIEGETL	73
GTRDWIEGETGC	74
CHPHLPRALMLL	75
CGTHPHLPRALM	76
THPHLPRALMRSC	77

GPHLPRALMRSSSC	78
APEWPGSRDKRTC	79
APEWPGSRDKRTLAGGC	80
CGGATPEWPGSRDKRTL	81
CTRKDRSGPWEPA	82
CGAEWEQKDEL	83
AEWEQKDEFIC	84
GEQKDEFIC	85
CAEGEQKDEL	86
LFIRKS	87
PSKGTVN	88
LHNEVQLPDARHSTTQPRKTKGS	89
SVNPGK	90
CPEWPGCRDKRTG	91
TPEWPGCRDKRCG	92
DPEWPGSRDKKGSC	93
DWPGSRDKRKGSC	94
DATPEWPGSRDKRTLKGSC	95

Accordingly examples of peptides listed in table 1, which have been modified to be specific disulphide bridge cyclised peptides suitable for the present invention are listed in table 2.

Table 2, modified cyclic peptides.

Peptide sequence	SEQ ID NO.
CLEDGQVMDVDLC	96
CFINKQMADLELCPRE	97
CFMNKQLADLELCPRE	98
CLEDGQVMDVDLCPREAAEGDK	99
CLEDGQVMDVDLCGGSSGGP	100
CLEDGQVMDVDCPREAAEGDK	101
KCREVWLGESETIMDCE	102
ACREVWLGESETIMDCD	103
SCREVWLGESETVMDCG	104
NCQDLMLREDAGCWSKM	105
DCEEPMCSPVLLQQLKL	106
CFINKQMADLELC	107
CFMNKQLADLELC	108
KCREVWLGESETIMDC	109
HCQQVFFPQDYLWCQRG	110
SCREVWLGGSEMIMDCE	111
ECNQNLSGSLRHVDLNC	112
DCEEPMCSPVLLQKLKP	113
SCREVWLGGSEMIMDCE	114
RCDQQLPRDSYTFCMMS	115
SCPAFPREGDLCAPPTV	116

FCPEPICSPPLSRMTLS	117
VCDECVSRELAL	118
WCLEPECAPGLL	119
VCDECVSRELAL	120
DCLSKGQMADLC	121
SCQGREVRRECW	122
WCREVWLGESETIMDCE	123
ACREVWLGESETIMDCD	124
GCAEPKCWQALHQKLKP	125
ECRGPNMQMQDHCPTTD	126
QCNAVLEGLQMVDHCWN	127
HCKNEFKKGQWTYSCSD	128
QCRQFVMNQSEKEFGQC	129
NCFMNKQLADLELCPRE	130
SCAYTAQRQCSDVPNPG	131
GCFMNKQMADLELCPRTAA	132
ACFMNKQMADLELCPRVAA	133
GCFINKQLADLELCPRVAA	134
GCFMNKQLADWELCPRAAA	135
ECFMNKQLADSELCPRVAA	136
GCFMNKQLADPELCPREAE	137
GCFMNKQLVDLELCPRGAA	138
GCFMNKQLADLELCPREAA	139
GCFMNKQQADLELCPRGAA	140
GCFINKQMADLELCPREAA	141
CLEDGQVMDVDCPREAAEGD	142
CLEDGQVMDVDLCPREAAEGD	143
QCNAVLEGLQMVDHCWN	144
ECLKIEQQCADIVEIPR	145
SCAYTAQRQCSDVPNPG	146
ECRGPNMQMQDHCPTTD	147
ECLVYGQMADCAAGGWP	148
QCRQFVMNQSEKEFGQC	149
HCKNEFKKGQWTYSCSD	150
CAPGMGCWESVK	151
SCREVWLGGSEMIMDCE	152
SCPAFPREGDLCAPPTV	153
FCPEPICSPPLSRMTLS	154
ECNQNLSGSLRHVDLNC	155
RCDQQLPRDSYTFCMMS	156
HCQQVFFPQDYLWCQRG	157
DCEEPMCSPVLLQKLKP	158
NCQDQMLREDAGCWSKI	159
HCEEPEYSPATRVFCGR	160
ACFSRNGQVTDVPHSCY	161
KCPTYPKPNDRCLWPVP	162
YCPKYPLEGDCLLDNDY	163
RCEEWLCIPPAPAFAPP	164

TCGQSELRCASLETHHV	165
NCNDNPMLDCMPAWSS	166
SCQGREVRRECW	167
VCDECVSRELAL	168
WCLEPECAPGLL	169
DCLSKGQMADLC	170
VCDECVSRELAL	171
GCPTWPRVGDHC	172
RCQSARVVPECW	173
SCAPSGDCGYKG	174
GCPMWPQPDDEC	175
ECPRWPLMGDGC	176
GCQVGELVWCRE	177
QCVRDGTRKVCM	178
TCLVDRQESDVC	179
DCVVDGDRLVCL	180
RCEQGALRCVGE	181
VCPPGWKNLGCN	182
MCQGWEIVSECW	183
ADGAGCFMNKQMADLELCPREAAEA	184
ADGAGCFMNKQMADLELCPRTAAEA	185
ADGAACFMNKQMADLELCPRVAAEA	186
ADGAGCFINKQLADLELCPRVAAEA	187
ADGAGCFINKQLADLELCPREAAEA	188
ADGAGCFMNKQLADLEMCPRDDAEA	189
ADGAGCFMNKQLADPELCPREAEEA	190
ADGAGCFMNKQLVDLELCPRGAAEA	191
ADGAGCFMNNQLADWELCPRAAAEA	192
ADGAGCFMNKQMADWEMCPRAAAEA	193
ADGAGCFMNKQQADLELCPRGAAEA	194
ADGAECFMNKQLADSELCPRVAAEA	195
ADGAGCFMNKQLADLELCPREAAEA	196
ADGAGCFINMQMADQELCPRAAAEA	197
ADGAGCFINKQMSDFELCPREAGEA	198
ADGAGCFINKQMADLELCTREAAEA	199
ADGAGCFINKQMADLELCPRQAAEA	200
ADGAGCFINNQMADLELCPRGGAEA	201
ADGAGCFINKQMADWELCPREGAEA	202
ADGAGCFINKQMADLELCPSQAAEA	203
ADGAGCFINKQMADLELCPREGAEA	204
ADGAGCFINKQMADSELCPREPAEA	205
ADGAGCFIKKQMADLELCPREAWEA	206
ADGAECFINKQMADRELCAREVAEA	207
ADGAGCFIDKQMADLELCPRAAAEA	208
ADGAGCFINKQMADLELCRREAGEA	209
ADGAGCFKNKQMVDSELCARQAAEA	210
ADGAGCFQNKQMADLELCPREAAEA	211
ADGAECFINKQRADLELCPGEAAEA	212

ADGAGCFINKQMADSELCPAAAAEA	213
ADGAGCFINRQMADPELCPREAAEA	214
ADGAGCFIEKQMADMELCQARAAEA	215
ADGAGCFINKQMADWELCPREAAEA	216
ADGAGCFINNQMADLELCPREAAEA	217
ADGAGCFIEKQMADMELCQRETAEA	218
ADGAGCFINKQMADMELCPREAAEA	219
ADGAGCFINKQMADLELCPREAAEA	220
ADGAGCFRNKQMADLELCPREAAEA	221
ADGAGCFINKQMADLELCPARAAEA	222
ADGAGCFINRQLADMELCSRGAAEA	223
ADGAECFINRQMADLELCGREAAEA	224
ADGAGCFISPQLADWKRCMREAAEA	225
ADGAGCSIHTQMADWERCLREGAEA	226
ADGAGCSIHRQMADWERCLREGAEA	227
CSSCDGGGHKPPTIQC	228
CLQSSCDGGGHFPPTIQLLC	229
APCWPGSRDCRTLAG	230
ACPEWPGSRDRCTLAG	231
CATPEWPGSRDKRTLCG	232
CATPEWPGSRDKRTCG	233
TPCWPGSRDKRCG	234
CSRPSPFDLFIRKSPTITC	235
CSRPSPFDLFIRKSPTIC	236
CSRPSPFDLFIRKSPTC	237
CSRPSPFDLFIRKSPC	238
CRPSPFDLFIRKSPC	239
CRPSPFDLFIRKSPTC	240
CRPSPFDLFIRKSPTIC	241
CRPSPFDLFIRKSPTITC	242
CPSPFDLFIRKSPTITC	243
CPSPFDLFIRKSPTIC	244
CPSPFDLFIRKSPTC	245
CPSPFDLFIRKSPC	246
CYAFATPEWPGSRDKRTLAC	247
CYAFATPEWPGSRDKRTLC	248
CYAFATPEWPGSRDKRTC	249
CYAFATPEWPGSRDKRC	250
CAFATPEWPGSRDKRC	251
CAFATPEWPGSRDKRTC	252
CAFATPEWPGSRDKRTLC	253
CAFATPEWPGSRDKRTLAC	254
CFATPEWPGSRDKRTLAC	255
CFATPEWPGSRDKRTLC	256
CFATPEWPGSRDKRTC	257
CFATPEWPGSRDKRC	258
CTWSRASGKPVNHSTRC	259
CTWSRASGKPVNHSTC	260

CTWSRASGKPVNHSC	261
CTWSRASGKPVNHC	262
CWSRASGKPVNHC	263
CWSRASGKPVNHSC	264
CWSRASGKPVNHSTC	265
CWSRASGKPVNHSTRC	266
CSRASGKPVNHSTRC	267
CSRASGKPVNHSTC	268
CSRASGKPVNHSC	269
CSRASGKPVNHC	270
CQWLHNEVQLPDARHSC	271
CQWLHNEVQLPDARHC	272
CQWLHNEVQLPDARC	273
CQWLHNEVQLPDAC	274
CWLHNEVQLPDAC	275
CWLHNEVQLPDARC	276
CWLHNEVQLPDARHC	277
CWLHNEVQLPDARHSC	278
CLHNEVQLPDARHSC	279
CLHNEVQLPDARHC	280
CLHNEVQLPDARC	281
CLHNEVQLPDAC	282
CPSPFDLFIRKSPCGSK	283
CPSPFDLFIRKSPTCGSK	284
FAGCSRASGKPVNHCGAAEG	285
FAGCSRASGKPVNHSCGAAEG	286
FAGCSRASGKPVNHSTCGAAEG	287
FAGCSRASGKPVNHSTRCGAAEG	288
CSRASGKPVNHCGSK	289
CSRASGKPVNHSCGSK	290
CSRASGKPVNHSTCGSK	291
FAGCFATPEWPGSRDKRCGAAEG	292
FAGCFATPEWPGSRDKRTCGAAEG	293
FAGCFATPEWPGSRDKRTLCGAAEG	294
FAGCFATPEWPGSRDKRTLACGAAEG	295
CPEWPGSRDKRCGSK	296
CWPGSRDKRCGSK	297
CPEWPGSRDKRCGAAEG	298
FAGCLHNEVQLPDACGAAEG	299
FAGCLHNEVQLPDARCGAAEG	300
FAGCLHNEVQLPDARHCGAAEG	301
FAGCLHNEVQLPDARHSCGAAEG	302
FAGCLHNEVQLPDASGAAEG	303
CPEWPGSRDRCGSK	304
CWPGSRDRRCGSK	305
CDSNPRGVSAADSNPRGVSC	306
CLVVDLAPSKGTVNC	307
CKQRNGTLC	308

CEEKQRNGTLTVC	309
CHPHLPRC	310
CTHPHLPRAC	311
CVTHPHLPRALC	312
CRVTHPHLPRALMC	313
CXRVTHPHLPRALMRC	314
CQXRVTHPHLPRALMRSC	315
CYQXRVTHPHLPRALMRSTC	316
CPEWPGSRDKRC	317
CRQRNGTLC	318
CEERQRNGTLTVC	319
CMRVTHPHLPRALMRC	320
CQMRVTHPHLPRALMRSC	321
CYQMRVTHPHLPRALMRSTC	322
ACPEWPGSRDRCTLAG	323
GGCLEDGQVMDVDC	324
CLEDGQVMDCGSK	325
CLEDGQVMDVDLCGSK	326
CLEDGQVMDVDLCPREAAEGDK	327
CLEDGQVMDVDLCGGSSGGK	328

Immunogens produced by the process of the present invention which may incorporate the modified peptides of table 1, or the cyclic peptides of table 2, form a preferred aspect of the present invention. Mimotopes which have the same characteristics as these peptides, and immunogens comprising such mimotopes which generate an immune response which cross-react with the IgE epitope in the context of the IgE molecule, also form part of the present invention. The meaning of mimotope is defined as an entity which is sufficiently similar to the native IgE peptides listed in tables 1 or 2, so as to be capable of being recognised by antibodies which recognise the native IgE peptide; (Gheysen, H.M., et al., 1986, Synthetic peptides as antigens. Wiley, Chichester, Ciba foundation symposium 119, p130-149; Gheysen, H.M., 1986, Molecular Immunology, 23,7, 709-715); or are capable of raising antibodies, when coupled to a suitable carrier, which antibodies cross-react with the native IgE epitope.

The preferred peptides to be used in the process or immunogens of the present invention mimic the surface exposed regions of the IgE structure, however, within those regions the dominant aspect is thought by the present inventors to be those regions within the surface exposed area which correlate to a loop structure. The structure of the domains of IgE are described in "Introduction to protein Structure" (page 304, 2nd Edition, Branden and Tooze, Garland Publishing, New York, ISBN 0 8153 2305-0) and take the form a β-barrel

made up of two opposing anti-parallel β-sheets (see FIG. 8). The immunogens may comprise a disulphide bridge cyclised peptide which is a sequence derived from a loop of the IgE domains. Preferred examples of this are the A-B loop of Cε3, the A-B loop of Cε4, the C-D loop of Cε3, the C-D loop of Cε4, the A-B loop of Cε2 and the C-D loop of Cε2.

Peptide mimotopes of the above-identified IgE epitopes may be designed for a particular purpose by addition, deletion or substitution of elected amino acids. Thus, the peptides of the present invention may be modified for the purposes of ease of conjugation to a protein carrier. For example, it may be desirable for some chemical conjugation methods to include a terminal cysteine to the IgE epitope. In addition it may be desirable for peptides conjugated to a protein carrier to include a hydrophobic terminus distal from the conjugated terminus of the peptide, such that the free unconjugated end of the peptide remains associated with the surface of the carrier protein. This reduces the conformational degrees of freedom of the peptide, and thus increases the probability that the peptide is presented in a conformation which most closely resembles that of the IgE peptide as found in the context of the whole IgE molecule. For example, the peptides may be altered to have an N-terminal cysteine and a C-terminal hydrophobic amidated tail. Alternatively, the addition or substitution of a D-stereoisomer form of one or more of the amino acids may be performed to create a beneficial derivative, for example to enhance stability of the peptide. Those skilled in the art will realise that such modified peptides, or mimotopes, could be a wholly or partly non-peptide mimotope wherein the constituent residues are not necessarily confined to the 20 naturally occurring amino acids. In addition, these may be cyclised by techniques known in the art to constrain the peptide into a conformation that closely resembles its shape when the peptide sequence is in the context of the whole IgE molecule. A preferred method of cyclising a peptide comprises the addition of a pair of cysteine residues to allow the formation of a disulphide bridge.

Further, those skilled in the art will realise that mimotopes or immunogens of the present invention may be larger than the above-identified epitopes, and as such may comprise the sequences disclosed herein. Accordingly, the mimotopes of the present invention may consist of addition of N and/or C terminal extensions of a number of other natural residues at one or both ends. The peptide mimotopes may also be retro sequences of the natural IgE sequences, in that the sequence orientation is reversed; or alternatively the sequences may be entirely or at least in part comprised of D-stereo isomer amino acids (inverso sequences). Also, the peptide sequences may be retro-inverso in character, in that the sequence orientation

is reversed and the amino acids are of the D-stereoisomer form. Such retro or retro-inverso peptides have the advantage of being non-self, and as such may overcome problems of self-tolerance in the immune system (for example P14c).

Alternatively, peptide mimotopes may be identified using antibodies which are capable themselves of binding to the IgE epitopes of the present invention using techniques such as phage display technology (EP 0 552 267 B1). This technique, generates a large number of peptide sequences which mimic the structure of the native peptides and are, therefore, capable of binding to anti-native peptide antibodies, but may not necessarily themselves share significant sequence homology to the native IgE peptide. This approach may have significant advantages by allowing the possibility of identifying a peptide with enhanced immunogenic properties (such as higher affinity binding characteristics to the IgE receptors or anti-IgE antibodies, or being capable of inducing polyclonal immune response which binds to IgE with higher affinity), or may overcome any potential self-antigen tolerance problems which may be associated with the use of the native peptide sequence. Additionally this technique allows the identification of a recognition pattern for each native-peptide in terms of its shared chemical properties amongst recognised mimotope sequences.

Alternatively, peptide mimotopes may be generated with the objective of increasing the immunogenicity of the peptide by increasing its affinity to the anti-IgE peptide polyclonal antibody, the effect of which may be measured by techniques known in the art such as (Biocore experiments) . In order to achieve this the peptide sequence may be electively changed following the general rules:

* To maintain the structural constraints, prolines and glycines should not be replaced
* Other positions can be substituted by an amino acid that has similar physicochemical properties.

As such, each amino acid residue can be replaced by the amino acid that most closely resembles that amino acid. For example, A may be substituted by V, L or I, as described in the following table 3.

Original residue	**Exemplary substitutions**	**Preferred substitution**
A	V, L, I	V
R	K, Q, N	K
N	Q, H, K, R	Q
D	E	E
C	S	S

Q	N	N
E	D	D
G	A	A
H	N, Q, K, R	N
I	L, V, M, A, F	L
L	I, V, M, A, F	I
K	R, Q, N	R
M	L, F, I	L
F	L, V, I, A, Y,W	W
P	A	A
S	T	T
T	S	S
W	Y, F	Y
Y	W, F, T, S	F
V	I, L, M, F, A	L

The present invention, therefore, provides a process for the manufacture of a vaccine and novel immunogens comprising disulphide bridge cyclised peptides conjugated by the process of the present invention, and the use of the immunogens in the manufacture of pharmaceutical compositions for the prophylaxis or therapy of disease. Preferably the process and the immunogens of the present invention are used in vaccines for the immunoprophylaxis or therapy of allergies.

It is envisaged that the peptides used in the process of present invention will be of a small size. Peptides, therefore, should be less than 100 amino acids in length, preferably shorter than 75 amino acids, more preferably less than 50 amino acids, and most preferable within the range of 4 to 25 amino acids long.

The most preferred peptides for use in the processes and conjugates of the present invention are SEQ ID NO.s 99, 304, 305, 306, 307, 308, 309, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 323, 324, 325, 326, 327, and 328.

The types of immunogenic carriers used in the immunogens of the present invention will be readily known to the man skilled in the art. The preferred function of the carrier is to provide cytokine help in order to help induce an immune response against the IgE peptide. A non-exhaustive list of carriers which may be used in the present invention include: Keyhole limpet Haemocyanin (KLH), serum albumins such as bovine serum albumin (BSA), inactivated bacterial toxins such as tetanus or diptheria toxins (TT and DT), or recombinant fragments thereof (for example, Domain 1 of Fragment C of TT, or the translocation domain

of DT), or the purified protein derivative of tuberculin (PPD). Alternatively, the process may be used to conjugate the cyclic peptides directly to liposome carriers, which may additionally comprise carriers capable of providing T-cell help. Preferably the ratio of peptides to carrier is in the order of 1:1 to 20:1, and preferably each carrier should carry between 3-15 peptides.

In an embodiment of the invention a preferred carrier is Protein D from *Haemophilus influenzae* (EP 0 594 610 B1). Protein D is an IgD–binding protein from *Haemophilus influenzae* and has been patented by Forsgren (WO 91/18926, granted EP 0 594 610 B1). In some circumstances, for example in recombinant immunogen expression systems it may be desirable to use fragments of protein D, for example Protein D 1/3rd (comprising the N-terminal 100-110 amino acids of protein D (GB 9717953.5)).

Peptides can be readily prepared using the 'Fmoc' procedure, utilising either polyamide or polyethyleneglycol-polystyrene (PEG-PS) supports in a fully automated apparatus, through techniques well known in the art (techniques and procedures for solid phase synthesis are described in 'Solid Phase Peptide Synthesis: A Practical Approach' by E. Atherton and R.C. Sheppard, published by IRL at Oxford University Press (1989)) followed by acid mediated cleavage to leave the linear, deprotected, modified peptide. This peptide can be readily oxidised and purified to yield the disulphide-bridge modified peptide, using methodology outlined in 'Methods in Molecular Biology, Vol. 35: Peptide Synthesis Protocols (ed. M.W. Pennington and B.M. Dunn), chapter 7, pp91-171 by D. Andreau et al.

Alternatively, the peptides may be produced by recombinant methods, including expressing nucleic acid molecules encoding the mimotopes in a bacterial or mammalian cell line, followed by purification of the expressed mimotope. Techniques for recombinant expression of peptides and proteins are known in the art, and are described in Maniatis, T., Fritsch, E.F. and Sambrook et al., *Molecular cloning, a laboratory manual*, 2nd Ed.; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989).

The amount of protein in each vaccine dose is selected as an amount which induces an immunoprotective response without significant adverse side effects in typical vaccinees. Such amount will vary depending upon which specific immunogen is employed and how it is presented. Generally, it is expected that each dose will comprise 1-1000 μg of protein, preferably 1-500 μg, more preferably 1-100 μg, of which 1 to 50μg is the most preferable range. An optimal amount for a particular vaccine can be ascertained by standard studies

involving observation of appropriate immune responses in subjects. Following an initial vaccination, subjects may receive one or several booster immunisations adequately spaced.

Vaccines of the present invention, may advantageously also include an adjuvant. Suitable adjuvants for vaccines of the present invention comprise those adjuvants that are capable of enhancing the antibody responses against the immunogen. Adjuvants are well known in the art (Vaccine Design – The Subunit and Adjuvant Approach, 1995, Pharmaceutical Biotechnology, Volume 6, Eds. Powell, M.F., and Newman, M.J., Plenum Press, New York and London, ISBN 0-306-44867-X). Preferred adjuvants for use with immunogens of the present invention include aluminium or calcium salts (for example hydroxide or phosphate salts). Preferred adjuvants for use with immunogens of the present invention include: aluminium or calcium salts (hydroxide or phosphate), oil in water emulsions (WO 95/17210, EP 0 399 843), or particulate carriers such as liposomes (WO 96/33739). Immunologically active saponin fractions (*e.g.* Quil A) having adjuvant activity derived from the bark of the South American tree Quillaja Saponaria Molina are particularly preferred. Derivatives of Quil A, for example QS21 (an HPLC purified fraction derivative of Quil A), and the method of its production is disclosed in US Patent No.5,057,540. Amongst QS21 (known as QA21) other fractions such as QA17 are also disclosed. 3 De-O-acylated monophosphoryl lipid A is a well known adjuvant manufactured by Ribi Immunochem, Montana. It can be prepared by the methods taught in GB 2122204B. A preferred form of 3 De-O-acylated monophosphoryl lipid A is in the form of an emulsion having a small particle size less than 0.2μm in diameter (EP 0 689 454 B1).

Adjuvants also include, but are not limited to, muramyl dipeptide and saponins such as Quil A, bacterial lipopolysaccharides such as 3D-MPL (3-O-deacylated monophosphoryl lipid A), or TDM. As a further exemplary alternative, the protein can be encapsulated within microparticles such as liposomes, or in non-particulate suspensions of polyoxyethylene ether (UK Patent Application No. 9807805.8). Particularly preferred adjuvants are combinations of 3D-MPL and QS21 (EP 0 671 948 B1), oil in water emulsions comprising 3D-MPL and QS21 (WO 95/17210, PCT/EP98/05714), 3D-MPL formulated with other carriers (EP 0 689 454 B1), or QS21 formulated in cholesterol containing liposomes (WO 96/33739), or immunostimulatory oligonucleotides (WO 96/02555). Alternative adjuvants include those described in WO 99/52549.

The vaccines of the present invention will be generally administered for both priming and boosting doses. It is expected that the boosting doses will be adequately spaced, or preferably given yearly or at such times where the levels of circulating antibody fall below a desired level. Boosting doses may consist of the peptide in the absence of the original carrier molecule. Such booster constructs may comprise an alternative carrier or may be in the absence of any carrier.

In a further aspect of the present invention there is provided an immunogen or vaccine as herein described for use in medicine.

Preferably, the vaccine preparation of the present invention may be used to protect or treat a mammal susceptible to, or suffering from allergies, by means of administering said vaccine via systemic or mucosal route. These administrations may include injection *via* the intramuscular, intraperitoneal, intradermal or subcutaneous routes; or *via* mucosal administration to the oral/alimentary, respiratory, genitourinary tracts. A preferred route of administration is via the transdermal route, for example by skin patches. Accordingly, there is provided a method for the treatment of allergy, comprising the administration of a peptide, immunogen, or ligand of the present invention to a patient who is suffering from or is susceptible to allergy.

Vaccine preparation is generally described in New Trends and Developments in Vaccines, edited by Voller et al., University Park Press, Baltimore, Maryland, U.S.A. 1978. Conjugation of proteins to macromolecules is disclosed by Likhite, U.S. Patent 4,372,945 and by Armor et al., U.S. Patent 4,474,757.

The present invention is illustrated by but not limited to the following examples.

Example 1, *Conjugation of disulphide cyclised peptide to a carrier, by conjugating a maleimide activated peptide to thiolated Protein D or BSA as a carrier.*

In the present example, a maleimide derivatised cyclic peptide is reacted with a thiol bearing carrier. The thiol group being generated on either Protein D (PD) or BSA as the carrier by reduction of the SPDP derivative of the carrier.

N-Succinimidyl 3-(2-pyridyldithio)propionate (SPDP) is a heterobifunctional cross-linking agent which under mild conditions, reacts by its NHS-ester group with amino groups of the protein (Fig; 3) (Hermanson G.T. Bioconjugate Techniques, 1996). NHS-ester crosslinking reactions are most commonly performed in phosphate, bicarbonate/carbonate and borate buffers. Other buffers can be used provided they do not contain primary amines. Treatment of a SPDP modified protein with DTT (Dithiothreitol, or another disulfide-reducing agent) releases the pyridine-2-thione leaving group and forms a free sulfhydryl (Fig 3A). The reaction is generally performed with 25 mM DTT at pH 4.5 to avoid the reduction of the protein's S-S bonds. For protein not containing S-S bonds, the DTT reduction may be performed at pH 7-9. The reaction between a maleimide group added on the peptide and the sulfhydryl groups present on the carrier produces the immunogen of the present invention (Fig. 3B). The maleimide-activated peptide was obtained by reaction between the peptide (P) and a heterobifunctionnal cross-linking reagent like GMBS (gamma-maleimidobutyric acid N-hydroxysuccinimide ester).

Methods

SPDP modified protein

BSA (Pierce) is dissolved at a concentration of 10 mg/ml in 50 mM sodium phosphate, 0.15 M NaCl, pH 7.2. SPDP was dissolved at a concentration of 6.2 mg/ml in DMSO (makes a 20 mM stock solution). A sufficient quantity of the stock solution of SPDP was then added to the protein to be modified (for BSA, a 15 fold molar excess of SPDP over protein, and for PD, a 25 fold molar excess). After one hour at room temperature, the modified protein was purified from reaction by products by dialysis against 50 mM sodium phosphate, 10 mM EDTA pH 6.8 or by gel filtration. The sample is applied on a desalting column (Sephadex G25) equilibrated with phosphate buffer pH 6.8 (or 100 mM sodium acetate, 0.15 M NaCl, 1 mM EDTA pH 4.5 if S-S containing proteins are to be reduced in the next step). Fractions of 1 ml are collected and monitored by adsorbance at 280 nm. Fractions containing SPDP modified protein are pooled.

The number of thiopyridyl groups introduced in BSA is estimated spectrophotometrically: transfer 200 µl of modified BSA in a spectrophotometer cuvette and add 200 µl of 50 mM mercaptoethanol in 100 mM phosphate buffer, pH 7. Measure absorbance at 343 nm before

and after addition of mercaptoethanol. Evaluate the quantity of thiopyridone liberated using $A_{343\ nm}$= 8000 $M^{-1}cm^{-1}$.

Use of DTT to cleave disulfide-containing cross-linking agents

DTT was added to a final concentration of 1-10 mM. Incubate for 2 h at room temperature. For removal of excess of DTT, gel filtration using Sephadex G-25 was used. To maintain the stability of the exposed sulfhydryl groups, 10 mM EDTA was included in the chromatography buffer (100 mM sodium phosphate pH 6.8). The presence of oxidized DTT can be monitored during elution by measuring the absorbance at 280 nm.

Maleimide modified peptide

Peptide was dissolved in 100 mM sodium phosphate pH 6.8. GMBS (Pierce) was then added to the peptide sample. A 2.5-fold molar excess of the cross-linker over the peptide was used. After 1 hr at room temperature, reaction by-products were removed by gel filtration using a sephadex G-10 (100 mM sodium phosphate pH 6.8). Fractions of 1 ml were collected and monitored by adsorbance at 280 nm. Presence of maleimide group was demonstrated by Ellman's reaction.

Reaction between SPDP modified protein and maleimide activated peptide

An excess of maleimide activated peptide (about 22 fold molar excess of maleimide activated peptide over the protein) was added to the SPDP modified protein and was agitated during 1 hr at room temperature followed by three dialysis against 100 mM Na phosphate pH 6.8. After filtration through 0.2 μm pore size (millipore filter), protein content was estimated by Lowry.

Results

1. Obtention of the SPDP modified protein

Several assays were conducted with different concentrations of SPDP using BSA or PD as carrier.

1.a Assays on PD

The number of thiopyridyl groups introduced was estimated spectrophotometrically by evaluation of thiopyridone liberated after addition of mercaptoethanol. Several assays were realized using PD at a concentration of 6.6 mg/ml or 10 mg/ml. results At least 14 thiopyridyl groups could be introduced on PD (Fig. 4). However, at a concentration of 10 mg/ml of PD only 4-5 thiopyridyl groups could be introduced on PD (Fig. 5). Indeed, precipitation of PD was observed when assays to obtain more thiopyridyl groups were carried out. However, this precipitation is partially induced by DMSO used to dissolve SPDP (6.2 mg/ml). This problem could be resolved by using the water-soluble sulfo-LC-SPDP (Sulfosuccinimidyl 6-[2-pyridyldithio)-propionamido]hexanoate)

1.b Assays on BSA

A maximum of 8 to 10 thiopyridyl groups can be added on BSA. A higher thiopyridyl number can be obtained if a 20 fold molar excess of SPDP over BSA was used (Fig. 6). However, a slight clouding was then observed during the reaction resulting in a lower yield of SPDP modified BSA.

Assays of reduction of pyridyl disulfide with DTT were carried out in sodium acetate pH 4.5 (to avoid reduction of native disulphide bonds) or in phosphate buffer (for SPDP modified PD). Efficacy of DTT was determined by release of pyridine-2-thione.

2. Conjugation of constrained p15 peptides

Five constrained peptides were conjugated to the BSA using the chemistry described hereabove:

Original sequence: EDGQVMDVD (SEQ ID NO. 1)

p15a: GGCLEDGQVMDVDC (SEQ ID NO. 324)

p15b: Ac-CLEDGQVMDCGSK-NH_2 (SEQ ID NO. 325)

p15c: Ac-CLEDGQVMDVDLCGSK-NH_2 (SEQ ID NO. 326)

p15d: Ac-CLEDGQVMDVDLCPREAAEGDK-NH_2 (SEQ ID NO. 327)

p15e: Ac-CLEDGQVMDVDLCGGSSGGK-NH_2 (SEQ ID NO. 328)

The resulting conjugates were soluble and were characterized by SDS-PAGE (Coomassie blue-staining) (Fig. 7).

3. Conjugation of constrained p14 peptides

Three constrained peptides were conjugated:

Original sequence: PEWPGSRDKRT (SEQ ID NO.63)

p14e: ACPEWPGSRDRCTLAG-NH_2 (SEQ ID NO.323)

p14f: Ac-CPEWPGSRDRCGSK-NH_2 (SEQ ID NO.304)

p14i: Ac-CWPGSRDRRCGSK-NH_2 (SEQ ID NO.305)

The resulting conjugates were soluble and were characterized by SDS-PAGE (coomassie blue-staining and western blot) (Fig. 7B, lane 7, Fig. 8 and Fig.9).

4. Thiol-disulfide exchange

Compounds containing a disulfide group are able to participate in disulfide exchange reactions with another thiol. The disulfide exchange process involves attack of the thiol at the disulfide, breaking the S-S bond, with subsequent formation of a new mixed disulfide constituting a portion of the original disulfide compound. If the thiol is present in excess, the mixed disulfide can go on to form a symmetrical disulfide consisting entirely of the thiol reducing agent. If the thiol is not present in large excess, the mixed disulfide product is the end result.

In order to test if a disulfide interchange could be observed during the reaction between BSA-SH and the maleimide activated disulfide bridge cyclised peptide, a reaction between BSA-SH and the unmodified p14i peptide was realized in the same coupling conditions (buffer, pH, ratio peptide/ carrier and temperature). After 1 hour, the sample was dialyzed or applied on a desalting column (sephadex G25) equilibrated with phosphate buffer pH 6.8. The resulting product was analyzed on SDS-PAGE (coomassie blue staining) (Fig. 10). A positive control was included resulting from the reaction between SPDP-modified BSA and p14a peptide (AcAPEWPGSRDKRTLAGGC) in which disulfide interchange occurs (Fig. 3A). The resulting conjugate was purified by dialysis or by gel filtration.

No increase of the molecular size was seen for the product resulting of the reaction between BSA-SH and p14i (Fig. 10A: Lane 9). Moreover, no protein was detected with the mAb 31

(Fig. 1B: lane 9) suggesting the absence of disulfide interchange during the reaction at least in the conditions used for the coupling.

Conclusions

The combination of two chemistries was used to conjugate constrained peptides to a carrier. Soluble conjugates with 6 to 8 peptides on the carrier were obtained and were characterized by SDS-PAGE with antibodies against p14. The resulting conjugates were principally obtained by the reaction between the GMBS activated peptide and BSA-SH and not by disulfide interchange as confirmed by Western-blot. These results demonstrate that these chemistries can be used to conjugate constrained peptides to a carrier. In the above examples the maleimide was added to the peptide via reaction of maleimide-N-hydroxysuccinimide ester reagents with a lysine side-chain or with a N-terminal amino group. It is clear that alternative methods of adding the maleimide group can be readily conceived: notably for peptides containing a lysine within the epitope, the maleimide can be added during peptide synthesis prior to final deprotection of the side-chains and cleavage of the peptide.

Example 2, *Immune response induced by different disulphide bridged peptide-BSA conjugates.*

To evaluate the immunogenicity of the conjugates produced in Example 1, 10 mice per group were immunised intramuscularly (IM) on days 0, 14 and 28 with 25 μg of conjugate mixed with AS2 adjuvant (oil/water emulsion, 3D-MPL, QS21). The serologic response for the P14 peptides was analysed by ELISA on days 28 and 42 (14 post III). The results are shown below in Table 4.

Table 4. *IgG response against P14 peptides, day 14 post III.*

Peptide conjugate	IgG anti-peptide responses (midpoint titre)	Average.	st.deviation.	geomean

P14e	3151	3224	3051		2873	4647	1461	4227	3821	2345	3200	963	3051
P14f	67086	36031	74838	56496	51304	92885	92868	113041	89155	101502	77521	24519	73541
P14I	85882	39268	39460	57276	50834	54664	62263	36621	26202	28989	48146	17926	45336

Immune response induced by different P15-BSA conjugates.

The P15 peptide conjugates produced in Example 1 were also used to immunise 10 mice per group,intramuscularly (IM) on days 0, 14 and 28 with 25 μg of conjugate mixed with AS2 adjuvant (oil/water emulsion, 3D-MPL, QS21). Anti peptide and anti-IgE antibody responses are shown in Table 5 (14 days post III). Very homogenous responses were obtained with all cyclic P15 peptides. Anti-IgE antibody responses were assayed by comparison with a monoclonal antibody, mAb11, which is known to recognise the P15 target site (c-d loop of Cε2) and inhibit histamine release in the Human Basophil Assay, the levels of anti-IgE were subsequently expressed as μg/ml mAb11 equivalents.

Table 5, *Immune response by cyclic P15 –BSA conjugates.*

BSA conjugate	anti-peptide (midpoint titre)			anti-IgE (μg/ml or mAb11 equivalent)		
	average	St Dev.	geomean	average	St Dev.	geomean
P15b	11169	10766	8385	70	104	35
P15c	66452	10917	65685	200	64	189

P15d	35118	11601	32801	174	168	111
P15e	57432	16589	55207	129	68	113

Human Basophil Assays

Two types of assay were performed with human basophils (HBA), one to determine the anaphylactogenicity of the vaccine induced antibodies, consisting of adding the antibodies to isolated PBMC; and a second to measure the inhibition of *Lol P I* (a strong allergen) triggered histamine release by pre-incubation of the HBA with the vaccine induced antibodies.

Blood was collected by venepuncture from 4 allergic donors into tubes containing 0.1 volumes 2.7% EDTA, pH 7.0. It is then diluted 1/2 with an equal volume of HBH medium containing 0.1% human serum albumin (HBH/HSA). The resulting cell suspension was layered over 50% volume Ficoll-Paque and centrifuged at 400g for 30 minutes at room temperature. The peripheral blood mononuclear cell (PBMC) layer at the interface is collected and the pellet is discarded. The cells are washed once in HBH/HSA, counted, and re-suspended in HBH/HSA at a cell density of 2.0×10^6 per ml. 100µl cell suspension are added to wells of a V-bottom 96-well plate containing 100µl diluted test sample or vaccine induced antibody. Each test sample is tested at a range of dilutions with 6 wells for each dilution. Well contents are mixed briefly using a plate shaker, before incubation at 37°C for 30 minutes with shaking at 120 rpm.

For each serum dilution 3 wells are triggered by addition of 10µl *Lol p I* extract (final dilution 1/10000) and 3 wells have 10µl HBH/HSA added for assessment of anaphylactogenicity. Well contents are again mixed briefly using a plate shaker, before incubation at 37°C for a further 30 minutes with shaking at 120 rpm. Incubations are terminated by centrifugation at 500g for 5 min. Supernatants are removed for histamine assay using a commercially available histamine EIA measuring kit (Immunotech). Control wells containing cells without test sample are routinely included to determine spontaneous and triggered release. Wells containing cells + 0.05% Igepal detergent are also included to determine total cell histamine.

The results are expressed as following:

Anaphylactogenesis assay

Histamine release due to test samples =

% histamine release from test sample treated cells – % spontaneous histamine release.

Blocking assay

The degree of inhibition of histamine release can be calculated using the formula:

% inhibition

$$= 1 - \frac{\text{(histamine release from test sample treated cells*)}}{\text{(histamine release from antigen stimulated cells*)}} \times 100$$

Values corrected for spontaneous release.

Results

The results of the histamine release activity of the P15 disulphide bridge cyclised peptides conjugated to the BSA carriers using the chemistry of the present invention are shown in FIGs 11 to 14.

FIG 11, A and B, show the histamine release blocking activity of antiserum induced by P15c, P15d and P15e; in comparison with the positive controls: 1079 BSA, PT11 and mAb005, and the negative controls BSA-BAL (activated carrier alone), anti-BSA, non-specific isotype controls (IgG1 and IgG2b); also shown are the data produced for spontanteous release of histamine, and histamine release after triggering with allergen, and total histamine content of the cells (released by detergent).

FIG 12, A and B, show the histamine release blocking activity of antiserum induced by P15c compared to the same controls as in FIG 11, with the addition of a further positive control 1079 HBC, and one additional negative control HBC wt.

FIG 13 shows the anaphylactogenicity of the same test samples (antiserum added to HBA in the absence of allergen) as described for FIG 11 (P15c, P15d and P15e). FIG 14 shows the anaphylactogenicity of the same test samples as described for FIG 12.

In summary, P15 c, P15d and P15e induced antisera that inhibited histamine release from human basophils after triggering with allergen, without the antiserum being anaphylactogenic themselves.

Claims

1. A process for the manufacture of a vaccine immunogen comprising conjugating a disulphide bridge cyclised peptide to an immunogenic carrier comprising, (a) adding to a disulphide cyclised peptide a moiety comprising a reactive group which is capable of forming thio-ether linkages with thiol bearing carriers, and (b) reacting the activated cyclised peptide thus formed with a thiol bearing immunogenic carrier.

2. A process as claimed in claim 1 wherein the reactive group capable of forming thio-ether linkages with thiol bearing carriers is a maleimide group.

3. A process as claimed in claim 1 wherein the disulphide bridge cyclised peptide is derived from human IgE.

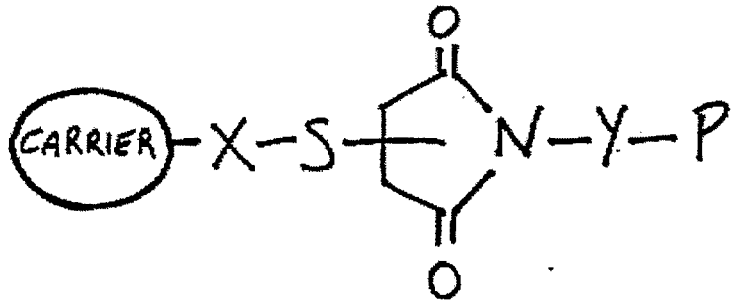
4. A process as claimed in claim 3, wherein the human IgE peptide is selected from any one of SEQ ID NOs. 1 to 328.

5. A process as claimed in claim 1, wherein the carrier is selected from Haemophilus Influenzae Protein D, BSA, Keyhole limpet Haemocyanin (KLH), serum albumins such as bovine serum albumin (BSA), inactivated bacterial toxins such as tetanus or diptheria toxins (TT and DT), or recombinant fragments thereof (for example, Domain 1 of Fragment C of TT, or the translocation domain of DT), or the purified protein derivative of tuberculin (PPD).

6. A disulphide bridge cyclised IgE peptide maleimide derivative.

7. Use of a peptide derivative as claimed in claim 6, in the manufacture of a medicament for the treatment of allergy.

8. A conjugate suitable for use in a vaccine, of formula (I):



wherein, Carrier is an immunogenic carrier molecule, X is either a linker or a bond, Y is either a linker or a bond, and P is a disulphide bridge cyclised peptide.

9. A conjugate as claimed in claim 8 wherein P is selected from the following group SEQ ID NO.s 99, 304, 305, 306, 307, 308, 309, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 323, 324, 325, 326, 327, and 328.

10. A vaccine composition comprising the product of the process claimed in any one of claims 1 to 5, and a suitable adjuvant or carrier.

11. A vaccine composition comprising a conjugate as claimed in claim 8 or 9, and a suitable adjuvant or carrier.

12. A vaccine as claimed in claim 10 or 11, wherein the vaccine is an allergy vaccine.

13. A conjugate as claimed in claim 8 for the treatment of allergy.