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Review

Dissecting the Role of Peptides in the Immune Response: Theory, Practice and the Application to Vaccine Design

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Abstract: Analytical biochemistry and synthetic peptide based chemistry have helped to reveal the pivotal role that peptides play in determining the specificity, magnitude and quality of both humoral (antibody) and cellular (cytotoxic and helper T cell) immune responses. In addition, peptide based technologies are now at the forefront of vaccine design and medical diagnostics. The chemical technologies used to assemble peptides into immunogenic structures have made great strides over the past decade and assembly of highly pure peptides which can be incorporated into high molecular weight species, multimeric and even branched structures together with non-peptidic material is now routine. These structures have a wide range of applications in designer vaccines and diagnostic reagents. Thus the tools of the peptide chemist are exquisitely placed to answer questions about immune recognition and along the way to provide us with new and improved vaccines and diagnostics. Copyright © 2003 European Peptide Society and John Wiley & Sons, Ltd.

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ANTIGEN PROCESSING AND THE ROLE OF PEPTIDES IN T CELL RECOGNITION

Although it had been long understood that antigen or fragments of antigen dictate the magnitude and specificity of immune responses, it was not until the solution of the three dimensional structure of a complex between an antigenic fragment and a cell surface receptor responsible for displaying the peptide to immune effector cells, that the pivotal role of peptides in directing the T cell response was fully appreciated. The structure of a class I human leukocyte antigen (HLA) molecule revealed for the first time the unique structure of the receptor's antigen binding cleft and the presence of electron density within the cleft [1,2] indicated the presence of a short antigen-derived peptide that was the focus of antigen-specific T cell receptors present on the

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BIOGRAPHIES

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Tony Purcell is a Research Fellow in the Department of Microbiology and Immunology at the University of Melbourne where he leads a team that focuses on epitope discovery and fundamental investigations into peptide ligand selection by HLA molecules and the structural basis of T cell recognition. Originally trained in peptide chemistry and separation sciences, he moved into the field of molecular immunology in 1994. His work has involved employing high-resolution peptide separation and biophysical techniques such as mass spectrometry to address questions of biological significance in the immune response of humans and mice to various pathogens, tumours and self-antigens recognized during organ specific autoimmune disease. Most recently he has taken on a management role and manages ImmunoID, an immune proteomics and imaging facility within the Department of Microbiology and Immunology. He has published a number of papers on the role of peptides in immune recognition and has particular interests in identifying killer T cell epitopes in autoimmune disease, cancer and antiviral immunity.

David Jackson

David Jackson is a Principal Research Fellow with the National Health and Medical Research Council of Australia and also Program Leader of the Cooperative Research Centre for Vaccine Technology. His initial training was in biochemistry and viral immunology and from these backgrounds it was a logical step for him to become involved in the molecular design of vaccines. His current activities centre around the design of totally synthetic and self-adjuvanting vaccines for the control of viral and bacterial diseases as well as cancer and also of vaccines that can be used to control normal physiological processes such as reproduction.

surface of responding T cells [3–5]. Not only did this structure reveal the basis of MHC restriction, which underlies the phenomenon of immune recognition, but it also explained why certain tissue types exhibited differential responses to pathogenic challenge; the discontinuous array of polymorphic amino acids encoded by different HLA alleles is brought into close proximity in three dimensions in this hitherto undescribed fold. This results in clustering of the polymorphic amino acids throughout the antigen binding cleft and consequently alters peptide binding specificity impacting on the nature and success of an immune response. Since these early studies a tremendous amount of information has been obtained concerning the role of peptides in shaping the immune response, many of these advances have involved research at the interface of peptide chemistry, analytical biochemistry and molecular immunology.

The Cytotoxic T Cell Response

The initial response towards pathogenic challenge in a naïve host involves generic and non-specific mechanisms facilitated by the innate immune response. A second, highly specific response towards the pathogen occurs shortly afterwards via the adaptive immune response. Two arms of adaptive immunity contribute to the eradication of pathogens; these are cellular cytotoxic responses and antibody production and involve CD8+ and CD4+ T lymphocytes. It is the cells that express the CD8 co-receptor that are responsible for the cytotoxic response, principally through recognition of HLA class I molecules complexed to antigenic peptides. It is through these cytotoxic T lymphocyte (CTL) responses that virally infected cells, tumour cells and sometimes even normal healthy cells are destroyed, clearing the virus or eradicating tumour cells from the host. In the case of normal tissue destruction the result is overt autoimmune disease such as that observed in the destruction of pancreatic β-cells in the pancreas in type 1 diabetes.

The structure of HLA class I molecules is now well defined and consists of a polymorphic heavy chain, a monomorphic light chain (β-2 microglobulin) and an antigenic peptide (see Figure 1A). The heavy chain forms three extracellular domains (α1, α2 domains that together form the peptide binding cleft and the membrane proximal α3 domain which is linked to a transmembrane domain and a short cytoplasmic tail). The peptide-binding cleft is composed of an eight stranded anti-parallel β-pleated sheet floor bounded by helices from the α1 and α2 domains. This peptide binding groove measures approximately 30Å in length and 12Å in width at the centre and accommodates an antigenic peptide typically

8–11 amino acid residues in length. This antigen-binding cleft is the focus for the majority of HLA polymorphisms, which in turn determine the antigen specificity of different allelic forms of HLA molecules. The groove contains several conserved depressions or pockets (denoted A–F) that vary in composition and stereochemistry depending on the allele. The A and F pockets are located at either end of the antigen binding cleft and contain conserved residues involved in hydrogen-bonding interactions with the *N* and *C*-termini of the bound peptide respectively. These interactions effectively close off each end of the peptide binding cleft encapsulating the termini of the bound peptide. The A pocket is frequently shallow, whilst the stereochemistry of the F pocket can vary significantly and contributes both to conserved interactions with the *C*-termini of the bound peptide ligand as well as to the specificity of the last amino acid residue of the peptide. The B, C, D and E pockets contribute to the specificity of the central portion of the bound peptide. Our understanding of the correlation of binding specificity and HLA polymorphism has come both from structural studies of class I molecules that bind to different peptide antigens and from the biochemical analysis of peptides that are bound by different class I molecules. The rules that determine the binding between peptide ligands and class I molecules are sufficiently well adhered to that a number of algorithms exist which predict whether a peptide sequence is a candidate for binding.

Peptide antigen is generated predominantly in the cytoplasm through the action of a multi-catalytic protease structure known as the proteasome. The proteasome can exist in several different forms, which engender different proteolytic activities and consequently produce a different array of peptide precursors for transport into the lumen of the ER. Transport of these peptides occurs in an energy-dependent manner through a member of the ABC cassette transporter family known as TAP (transporter associated with antigen processing). The loading of these peptides into the binding cleft of nascent class I molecules involves a complex interplay of multiple ER-resident chaperones [6–9]. This sequence of events is represented in Figure 2a. Initially, nascent HLA class I heavy chain (hc) is targeted to the ER and is stabilized by interacting with the chaperones Grp78 and calnexin. Once β2-microglobulin (β2m) associates with the class I hc, calnexin is exchanged for another ER resident chaperone, calreticulin. The association of the

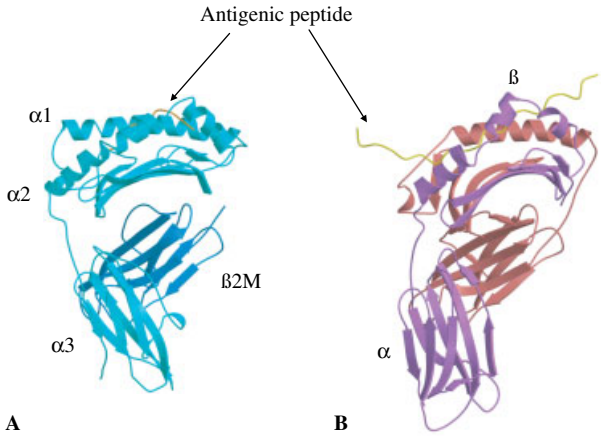


Figure 1 Three-dimensional structures of class I and class II HLA molecules: (A) The structure of class I HLA B8 complexed to a peptide from the EBNA 3A antigen (FLRGRAYGL) solved to 2.3 Å [187] highlighting α-helices and β-sheets, the domain structures and the Cα backbone of the peptide. (B) A class II HLA molecule: The structure of HLA DR2 bound to an immunodominant peptide from myelin basic protein [188] highlighting α and β chains as well as the backbone of the peptide ligand.

class I heterodimer with calreticulin is also associated with the recruitment of other members of the peptide loading complex (PLC) including tapasin and ERp57. ERp57 is a thiol oxidoreductase [10,11] involved in assuring the correct disulphide bonding of the nascent class I hc [12–14] and tapasin is a 48 kDa glycoprotein that bridges peptide receptive class I heterodimers to the TAP heterodimer [15–18]. Co-localization of the PLC to the TAP facilitates the loading of peptides into the antigen binding cleft of the class I molecules. In addition to a bridging function, tapasin is thought to stabilize the peptide receptive state of the class I complex independently of TAP-association [19] and enhances both expression and translocation via TAP [19–21]. Tapasin also retains empty MHC class I molecules and prevents their premature release from the ER [22,23]. These and perhaps additional functions of tapasin are believed to engender a functional editing of the peptide repertoire of class I molecules to ensure optimal peptide display at the cell surface, a notion supported by recent biochemical studies [24,25]. Once loaded with a suitable peptide cargo, the class I molecule is released from the ER, traverses the Golgi network and is ultimately transported to the cell surface, where the complex is scrutinized by CD8+ T cells.

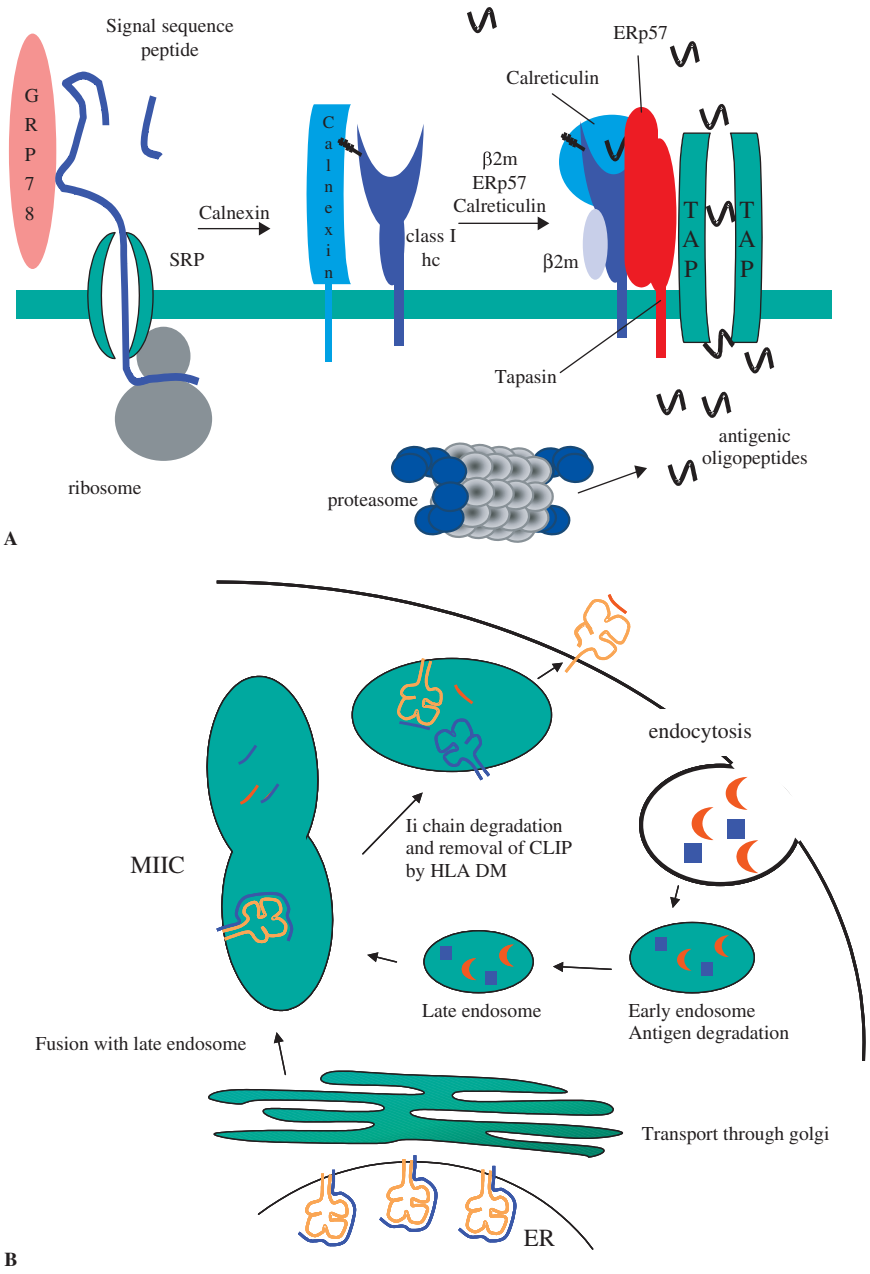


Figure 2 (A) Class I antigen processing pathway. Nascent class I hc is co-translationally inserted into the ER via the signal recognition pore (SRP) and initially stabilized by interacting with the chaperone Grp78 (known also as BiP). In human class I molecules a single amino acid residue on the hc (Asn86) bears a *N*-linked glycan, which is recognized by calnexin. Upon further folding, disulphide bond formation and association with β2m, the class I heterodimer exchanges calnexin with the chaperone calreticulin. At this stage the oxidoreductase ERp57 and tapasin are recruited to the peptide loading complex (PLC). Tapasin co-localizes the PLC to the TAP allowing peptides generated in the cytoplasm by the proteasome to be translocated into the lumen of the ER. Following peptide loading, the class I molecules dissociate from the PLC and are then transported to the cell surface where they are scrutinized by CTL. (B) Class II antigen processing pathway. Exogenous antigen is taken up by endocytosis and degraded in the early and late endosomes. The late endosome (containing antigenic peptides) fuses with class II rich transport vesicles (containing class II $\alpha\beta$ heterodimers associated with Ii) to form the MIIC compartment. In the MIIC, HLA DM catalyses the removal of Ii derived peptide (CLIP) from the antigen binding cleft of the $\alpha\beta$ heterodimers facilitating loading with antigenic peptides. This mature class II complex is then transported to the cell surface for scrutiny by CD4+ T helper cells.

The Humoral Response

In conjunction with a robust cytotoxic response, the production of antibodies are essential to the clearance of many pathogenic agents. Antibodies also recognize and bind to relatively short peptide sequences but in this case the peptide sequences are recognized in the context of the intact antigen and frequently display conformational dependence for binding. This means that unless a relatively short peptide sequence can be assembled that resembles the conformation of the corresponding sequence in the native protein, antigenic activity will be lost. Antibody production is controlled by the interaction between CD4+ T helper cells and antigen specific B cells that express immunoglobulin on their cell surface. T helper cells recognize HLA class II molecules that are expressed constitutively on specialized antigen presenting cells (APC) such as B cells, dendritic cells (DCs) and macrophages. Class II HLA molecules acquire antigen via a pathway that is mechanistically and physically distinct to that of class I HLA molecules.

Class II molecules are composed of two polymorphic polypeptide chains (α and β) forming a $\alpha\beta$ heterodimer, which like class I molecules combine to form a binding cleft that accommodates antigenic peptide (see Figure 1B). Class II molecules are inserted co-translationally into the lumen of the ER where they associate with invariant chain (Ii), a chaperone that provides a scaffold for the assembly of nascent class II heterodimers [26]. Class II $\alpha\beta$-heterodimers are unstable in the absence of bound antigenic peptide and the Ii stabilizes their structure in preparation for their exit from the ER. A consequence of this stabilization by Ii is the prevention of premature binding of exogenous peptides to class II molecules within the ER. The invariant chain is also important in trafficking nascent class II molecules to the endocytic route by virtue of a sorting signal located in the amino terminal region of the Ii [27]. For antigenic peptides to be able to bind to class II molecules that are resident in the endocytic compartment, Ii must first be degraded to allow the peptide antigens access to the peptide binding cleft. Cathepsin S mediates the proteolysis of the Ii-MHC complex [28] leaving a portion of the Ii (residues 81–104) bound to class II heterodimers. These class II-associated Ii peptides (known as CLIP) demonstrate promiscuous binding to MHC class II alleles and occlude the peptide binding cleft of these molecules [29,30]. In order to displace CLIP from the class II binding site antigenic peptides must have a higher binding affinity than CLIP. This peptide exchange is catalysed by another MHC-encoded gene product HLA DM [30,31] and the whole process is modulated in B cells by the HLA DO molecule [30,32,33]. Antigenic peptides are loaded in specialized intracellular compartments designated MIIC (MHC class II compartments) and form stable class II heterodimers which are transferred to the cell surface. The class II processing pathway is summarized in Figure 2b.

The mode of binding and repertoire of peptide ligands bound by HLA class II molecules has also been analysed by biochemical methods such as peptide elution and x-ray crystallographic studies [34–39], and differs in several ways from the binding of peptides to class I molecules. Peptides that bind to MHC class II molecules are typically longer than class I ligands and tend to be around 13 amino acids in length but can be considerably longer. The hydrogen-bonding events that close the peptide binding cleft of class I molecules are not apparent in class II molecules, allowing the termini of the bound class II peptide to project out of the ends of the cleft. The bound peptide is retained in the cleft of class II molecules by interactions between the side chains of the peptide ligand, the specificity determining pockets of the class II molecule and a conserved hydrogen bonding network between the peptide backbone and non-polymorphic amino acids of the antigen binding cleft. Like class I molecules, polymorphic amino acid residues also line the pockets of the binding cleft and both structural and biochemical studies indicate that amino acid side chains at residues 1, 4, 6 and 9 of the class II-bound peptide interact with these pockets conferring allelic specificity [34]. It has also been suggested that the binding of ligands to HLA class II molecules is more promiscuous than the binding of peptides to HLA class I molecules, making it more difficult to define anchor residues and to predict which peptides will be able to bind particular MHC class II molecules.

EPITOPE IDENTIFICATION

A sound understanding of the underlying mechanisms of determinant selection by class I and class II HLA molecules provides a framework to apply synthetic peptide based approaches to vaccine design, molecular diagnostics and fundamental immunological studies. The identification of the vast majority of physiologically relevant epitopes derived from pathogens, tumours and tissues targeted by

aberrant autoimmune responses, however, remain undefined. Coupled with the extensive polymorphism exhibited by HLA molecules this creates a challenge for the incorporation of minimal peptide epitopes into immunotherapeutics and diagnostics. Thus, a combination of bioinformatics, analytical biochemistry and peptide based validation studies needs to be applied to identify useful lead compounds for subsequent exploitation by peptide chemistry.

Identification of Binding Motifs and Subsequent Epitope Prediction

The biochemical analysis of peptides eluted from mature class I or class II molecules [40] has led to an appreciation of allelic polymorphism and its influence on ligand specificity. Listings of binding motifs for the common HLA class I and class II molecules are now conveniently web based (see for example [41,42]), although the class II binding motifs remain more loosely defined. These binding motifs describe the amino acids located at critical positions along the sequence of the antigenic peptide that are responsible for making highly conserved and energetically important contacts with pockets in the binding cleft of the class I and class II molecules. Many of these motifs have been defined by pool Edman sequencing [43] of peptides isolated from the surface of APC (as described later). Nevertheless, rather than providing a panacea for vaccine design, the use of these binding motifs to predict candidate T cell determinants from the sequence of pathogenic microorganisms has been disappointing. This approach is successful in *de novo* prediction of T cell epitopes in 50–70% of cases but there are numerous examples of atypical ligands possessing non-motif based sequences, post-translationally modified ligands or of the failure of antigen processing to liberate the candidate oligopeptides [44–53]. A further consideration in vaccine design that relates more to induction of effector responses rather than to ligand selection *per se*, is the observation that many T cell responses are focused on one or two immunodominant peptides during infection [54]. The focused nature of the T cell response has led to an appreciation of the complex relationships that exist between antigen presentation and the generation of T cell responses [54–63]. Moreover, the participation of so few epitopes from a viral genome hamper predictive studies since markers of immunogenicity must take into account not just peptide binding but the abundance, time of expression, correct processing and transport of the epitope as well as the available T cell repertoire. Despite these potential pitfalls, epitope prediction remains a popular first screening method to identify candidate T cell determinants for subsequent biological validation. Frequently, therefore predictive algorithms are combined with binding assays to confirm experimentally that the predicted ligands bind to the targeted HLA molecule [64,65]. A more comprehensive approach that allows assessment of natural processing and presentation of candidate epitopes involves the direct biochemical analysis of class I or class II ligands.

Identification of Naturally Processed and Presented T Cell Epitopes by Direct Biochemical Analysis

Several different approaches have been used to isolate HLA-bound peptides directly from cells; these include analysis of peptides contained within acidified cell lysates [66–68], isolation of peptides directly from the cell surface [69,70] and immunoaffinity purification of the HLA–peptide complexes from detergent solubilized cell lysates [43,71]. Each approach has advantages, with the latter providing the best chance of epitope identification due to the additional specificity of the immunoaffinity chromatography step and subsequent simplification of the range of cellular peptides isolated. However, each method shares common features and the assumptions that (i) upon cell lysis, peptides bound to HLA molecules are protected from intracellular and extracellular proteolysis by virtue of the fact that the peptide is bound to the HLA receptor and (ii) acid treatment dissociates bound peptides from the HLA complexes.

In the first approach peptides are extracted from whole cell lysates following treatment with an aqueous acid solution such as 1% trifluoroacetic acid (TFA). The presence of TFA also aids in the precipitation of larger proteins leaving a complex mixture of intracellular and extracellular peptides, a proportion of which were bound to and protected from proteolysis by HLA molecules. Typically these preparations are fractionated by RP-HPLC and screened with an immunological assay such as a T cell reporter assay which confirms the presence of a particular T cell epitope and also allows quantitation of known T cell epitopes in different cell types [66–68]. In some circumstances the peptides are amenable to *de novo* sequencing of individual components

of the fractionated material by mass spectrometry (MS). Figure 3a demonstrates an example of this approach, where a determinant from chicken ovalbumin (^{257}SIINFEKL^{264}) was identified in an acid eluate of a murine thymoma that expresses chicken ovalbumin as a transfected gene product and surrogate tumour associated antigen. The epitope was identified in a fraction issuing from RP-HPLC by virtue of recognition by a T cell hybridoma GA4.2 specific for the SIINFEKL determinant. Titration of such fractions allows relative efficiencies of antigen presentation to be assessed.

An alternative to the acid lysis method utilizes a non-lytic approach for recovering cell surface associated peptides. The cells are washed in an isotonic buffer containing citrate at pH 3.3; the acidic nature of this buffer facilitates dissociation of HLA-bound peptides from the cell surface without affecting cell

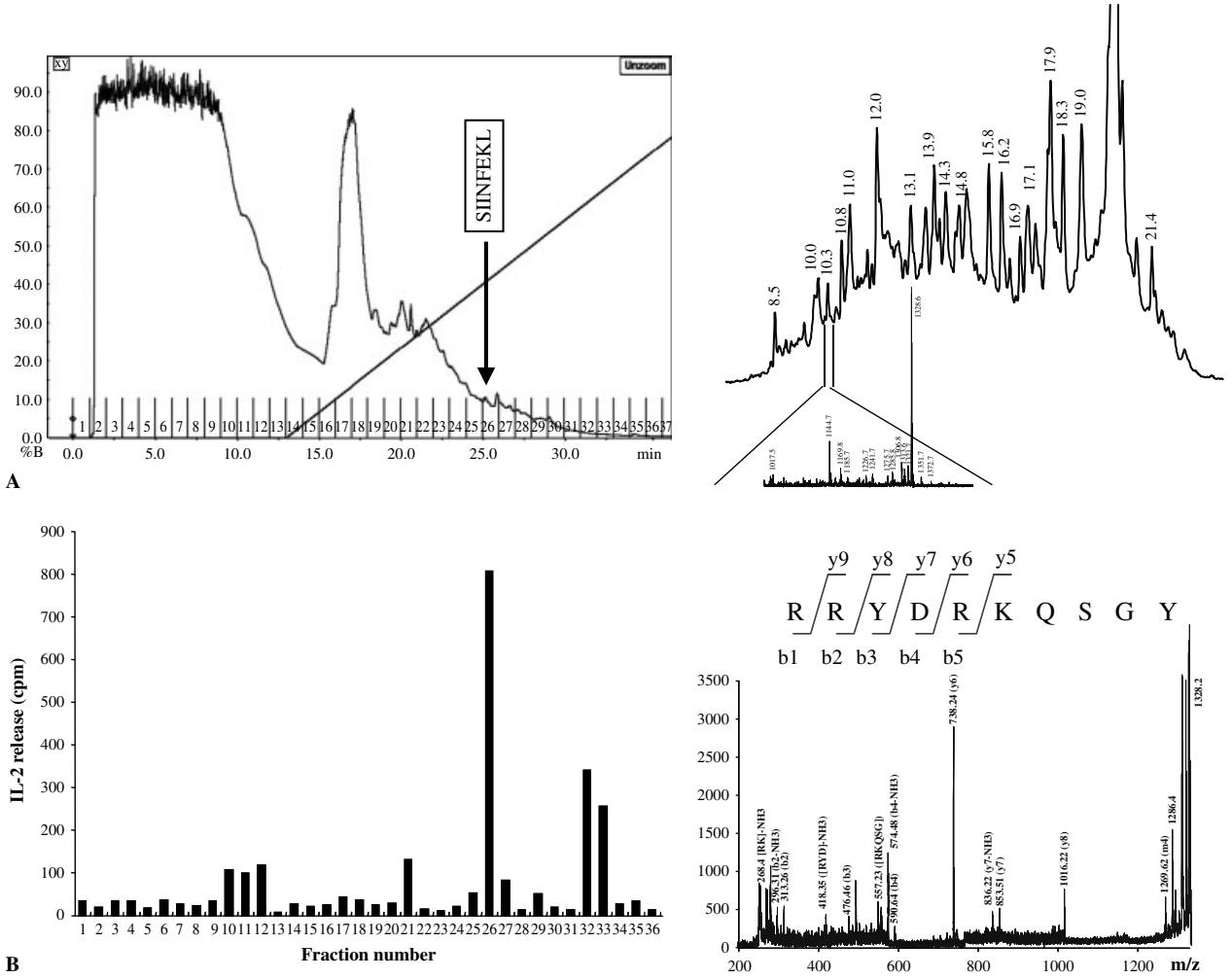


Figure 3 Direct isolation of class I molecule bound peptide ligands. (a) Identification of T cell epitopes from acid lysates of cells using a combination of T cell recognition and RP-HPLC retention behaviour. The upper panel shows a RP-HPLC chromatogram of peptides isolated from an acid lysate of 1×10^9 EG7 cells separated on a C2/C18 μRPC column (Pharmacia Biotech, Uppsala, Sweden) using a linear gradient of 0–60% acetonitrile over 20 min at a flow rate of 200 µl/min. The arrow highlights the retention time of synthetic SIINFEKL peptide chromatographed under identical conditions. The lower panel shows the concordance of fractions able to stimulate IL-2 production by the GA4.2 T cell hybridoma and the chromatographic migration of SIINFEKL. (b) Immunoaffinity purification and multi-dimensional RP-HPLC simplify complex mixtures of peptide eluates and allow sequencing of individual species by tandem MS. The upper panel shows a RP-HPLC chromatogram of peptides isolated from immunoaffinity purified HLA B*2705 molecules. The middle panel shows a MALDI-TOF mass spectrum of an individual fraction and the lower panel the subsequent identification of the dominant ion by tandem MS.

viability [69]. The great advantage of this technique is that the same cells may be harvested daily in an iterative approach for obtaining HLA-bound material. Although the specificity of this process is somewhat better for HLA-bound material than it is from whole cell lysates, some form of biological assay is again necessary to locate the peptide(s) of interest prior to attempting more definitive biochemical characterization. This is particularly relevant when the epitope(s) is unknown and some form of *de novo* sequencing is required to identify the peptide(s). The use of immunoaffinity chromatography dramatically improves the specificity of the peptide extraction process. The use of appropriate monoclonal antibodies allows isolation of a single HLA allele and some antibodies can even select a subpopulation of HLA molecules with defined molecular or functional properties [72,73]. The use of immunoaffinity chromatography to isolate specific MHC molecules provides the most appropriate material for identifying individual peptide ligands restricted by a known MHC allele. Furthermore, in all the approaches discussed, the complexity of the eluates/lysates can be reduced by using cell lines that express reduced numbers of HLA alleles. For example, homozygous lymphoblastoid cell lines express a more limited number of HLA class I or class II alleles, whilst mutant cell lines such as C1R express very low levels of endogenous class I molecules but support a high level expression of a single transfected class I molecules [74]. The simplified array of HLA molecules present on the surface of such cell lines make them very attractive for examining endogenous peptides presented by individual class I alleles under normal physiological conditions [75–78] or during infection [79,80]. Following additional RP-HPLC based fractionation, individual species can be analysed by mass spectrometry to examine the molecular diversity of bound peptides and also to sequence individual peptide epitopes using tandem MS technologies [81,82]. Figure 3b shows an example of a second dimension separation of peptides eluted from immunoaffinity purified HLA B*2705 molecules and the identification of a prominent peptide [24] by tandem MS.

In Vitro Techniques to Identify B and T Cell Determinants

B cell epitope discovery. The antigen-binding domains of an antibody are resistant to proteolysis and consequently confer protection against degradation of bound antigen, particularly in the immediate vicinity of the epitope [83,84]. This means that an epitope can be generated *in situ*, whilst bound to antibody, by exposing the complex to proteases. Because non-denaturing conditions are used until the epitope is eluted from the antibody, 'conformational' as well as 'linear' epitopes can be identified [85–87]. This approach can utilize monoclonal antibodies of single epitopic specificity as well as polyclonal anti-sera containing clinically relevant antibodies. The use of parallel treatments with proteases of different specificities such as exopeptidases (e.g. carboxypeptidase Y, amino-peptidase M) and endoproteases (e.g. trypsin, lysyl endopeptidase, etc.) further refine the epitope. This procedure produces antibody–epitope complexes that after separation of the individual components are amenable to analysis by HPLC and MS. Determination of the mass of the epitope, knowledge of antigen sequence and the specificity of the protease(s) used will allow identification of the precise region of the antigen that was bound by antibodies. Alternatively, because antibodies recognize particular shapes that may be mimicked by chemically unrelated molecules (mimotopes), peptide or phage display libraries can also be used to identify mimics of natural epitopes. Thus, in biopanning studies, the antibody is used to extract individual components from peptide or phage display libraries allowing subsequent identification of the mimotope [88].

Epitope extraction for the identification of CTL epitopes. Identification of immunogenic oligopeptides by epitope extraction techniques can provide rapid identification of HLA-restricted T cell epitopes. CTL epitopes can be identified following exposure of nascent HLA class I hc and β2m to mixtures or libraries of short peptides derived from the sequence of a specific antigen. After refolding of the class I molecules within this environment, potential CTL epitopes are captured. Peptides that are extracted in this way from the peptide library can then be eluted from the refolded complexes and analysed by MS leading to the identification of all potential class I ligands for any antigen of known sequence. This approach does not bias the epitope search for known binding motifs and is amenable to high throughput, using multiple alleles to maximize haplotype coverage. This form of epitope extraction has been used to define class I binding motifs in combination with pool Edman sequencing [89–91] and this technique was recently modified to allow for the identification of individual peptides using RP-HPLC and sensitive MS approaches [64].

Peptide libraries can be constructed of octamers through to 15 mers which are derived from the primary sequences of antigens and in some cases, peptide varimers (nested sets of *N*-terminally truncated peptides) can be employed to remove length biases [92]. The libraries may also consist of overlapping peptides with a single amino acid sliding window to improve resolution of the epitope [93]. The use of synthetic peptide libraries also affords the opportunity to include post-translational modifications (e.g. phospho-amino acids and non-genetically encoded amino acid changes). To minimize competition between ligands, sub-libraries containing approximately 10–20 peptides can be assembled with solubilized class I hc ectodomain and β2m and refolded on an analytical scale (~20 nmol peptide). The remaining insoluble material is removed by microcentrifugation and the refolded complexes washed and collected by ultrafiltration. Bound peptides are acid dissociated from class I complexes and collected following ultrafiltration. The peptide eluate is finally concentrated, desalted and analysed by MS. Comparison of input sub-library to the eluted peptides reveals binders and non-binders, as well as providing quantitative information concerning the relative efficiencies of extraction. The subsequent synthesis and assembly of these potential epitopes into appropriate immunogenic structures can then be investigated for antigenic and immunogenic activity.

PEPTIDE BASED DIAGNOSTICS

For many years the identification and quantitation of antigen specific T cells *ex vivo* has been fraught with technical difficulties. The assays almost invariably relied on a functional readout with quantitation extrapolated from a titration of effector cells and concomitant loss of detectable antigen specific responses. Whilst the use of a synthetic peptide determinant simplified matters and removed requirements for intracellular antigen processing, the functional assays remained laborious and demonstrated poor reproducibility in all but the most specialized laboratories. In 1996, Altman and colleagues devised an ingenious way to identify peptide specific CD8+ T cells using HLA tetramers [94], these studies were subsequently followed with studies using tetramers of class II molecules to identify peptide specific CD4+ T cells [95–97]. Remarkably, studies with tetramers revealed a considerable underestimate of the number of antigen specific T cells that are involved in immune responses [98] and consequently revolutionized the quantitation of antigen specific effector cells. HLA tetramers are prepared by generating recombinant, soluble class I or class II molecules that are complexed to a single peptide. In the case of class I tetramers the refolding technology described for epitope extraction is exploited to produce class I hc-β2m-peptide complexes from material that is typically produced in bacterial expression systems as insoluble inclusion bodies. The class I hc is engineered to contain a substrate peptide for the BirA biotin ligase enzyme allowing site-specific biotinylation of the *C*-terminus of the class I complex. These monomers are subsequently mixed stoichiometrically with fluorophore tagged streptavidin to create a tetramer of HLA-peptides with a fluorescent tag. This reagent can be used to stain peptide specific T cells using standard flow cytometric protocols to enumerate antigen specific T cells and can also be used to isolate cells using the monomer attached to a multivalent matrix or by sorting tetramer positive cells in a high proficiency cell sorter. The great advantage of tetramer identification is that it can be combined with other phenotypic analysis using conventional monoclonal antibody staining to sort for effector cells, memory cells, activated cells, resting cells, etc. of defined antigen specificity. Figure 4 demonstrates the utility of tetramers in quantitating *in vitro* expansion of CD8+ T cells specific for an Epstein-Barr virus determinant from the EBNA-3 antigen, and is compared with a comparable functional assay in which IFN-γ elicited by antigen specific $CD8^+$ T cells is detected intracellularly by flow cytometry.

Incorporation of multivalent peptide constructs in various geometries (as discussed below) into routine diagnostic procedures also promised to improve the specificity and sensitivity of such techniques. This has been known for some time in simple ELISA-based (enzyme linked immunosorbent assay) systems where the use of tetravalent MAP (multiple antigenic peptides) constructs improves the accessibility of the immobilized peptide monomers with concomitant improvements in specificity and sensitivity [99,100].

TOTALLY SYNTHETIC PEPTIDE-BASED VACCINES

It is clear from the preceding discussion that peptides play a central role in the immune recognition of antigens. It would seem logical therefore that

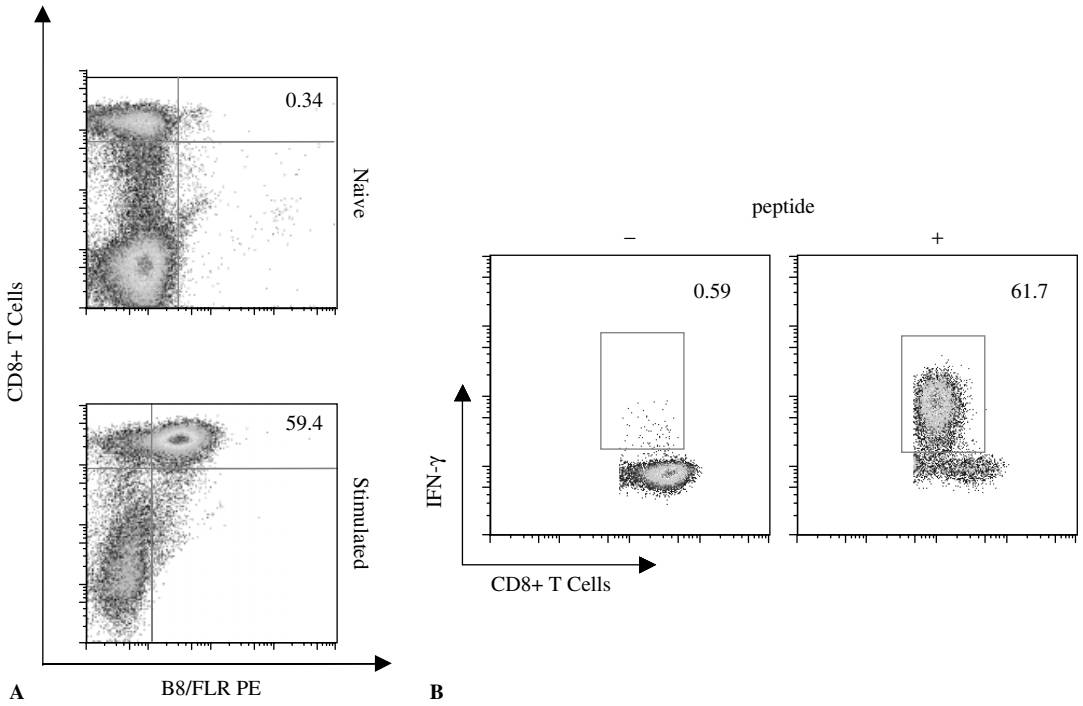


Figure 4 Identification and quantitation of Epstein-Barr virus (EBV) specific T cells using HLA tetramers and functional assays based on peptide recognition. (a) The identification of CD8+ HLA B8 restricted EBV specific T cells recognizing the FLRGRAYGL determinant using HLA B8/FLR tetramer staining of naïve and peptide stimulated PBMCs. EBV specific CD8+ T cells appear in the upper right hand quadrant of the profiles where it can be seen that peptide stimulation increased antigen specific cells from 0.3% to 59.4%. (b) The identification of CD8+ HLA B8 restricted EBV specific T cells recognizing the FLRGRAYGL determinant using intracellular IFN-γ staining. Using the same peptide stimulation as in (a), an increase in antigen specific cells from 0.6% to 61.7% was observed when intracellular IFN-γ staining was performed.

given the techniques that are available to identify epitopes recognized by antibodies and by immune T cells, peptide or epitope-based vaccines should be available. There are, however, no totally synthetic peptide-based vaccines available on the market. There are several reasons why such vaccines are not presently available, but the major underlying reason is that the form in which the epitope-based vaccine is administered determines how it will be processed by the immune system and ultimately whether it will be capable of inducing an appropriate immune response. We are now only just discovering how to administer peptides to elicit useful or appropriate immune responses *in vivo* and the rules that we are learning will hopefully lead to a new generation of vaccines.

Peptide epitopes, whether recognized by antibody or immune cells, are the end result of the complex sequence of processing events that an intact antigen undergoes. Administration of an epitope taken out of the context of the whole antigen will not follow the same itinerary of processing events and consequently only very rarely do simple epitopes elicit appropriate immune responses. In the case of antibody recognition, the biggest obstacle to induction of antibody of relevant specificity is conformational integrity of the peptide representing the epitope; in order to induce antibody that will recognize the parent antigen, the epitope needs to possess a conformation that is similar to that assumed by the same sequence within the native antigen. In some cases relatively short peptide sequences will adopt conformations that mimic those assumed by the peptide sequence within the native antigen but in most cases a relatively short sequence of amino acids (10–40 in length) will rarely result in a peptide that folds into the correct conformation. Thus, while it is a relatively simple matter to elicit antibodies to a peptide, the resulting antibodies are unlikely to cross-react with the parent antigen. A number of approaches have been taken to induce peptides to fold correctly but any approach where conformational elements are incorporated requires some knowledge of the structure of the

native antigen. CTL on the other hand have little or no requirement for conformational integrity and simple nine-residue epitopes are able to induce cytotoxic responses if administered in the presence of an adjuvant. The problem with this otherwise encouraging finding is that such simple epitopes need to be administered in usually toxic adjuvants and often the immune response induced is short lived (see below).

In addition to the epitopes that are recognized by the antibodies and cytotoxic T lymphocytes, a successful immunogen must also contain epitopes that are accessible to specific receptors present on *helper* T lymphocytes. These are the cells that are necessary to provide the signals required for lymphocytes to differentiate. Helper T cell epitopes (T_H-epitopes) are a subset of epitopes present in antigens that are bound by class II MHC and recognized by CD4+ T helper lymphocytes. In this way the T cell, presented with an antigenic epitope in the context of an MHC molecule, can be activated and provide the necessary signals for the lymphocyte to differentiate. Because there is little or no requirement for conformational integrity in epitopes recognized by class II MHC molecules, functional synthetic peptides (8–15 amino acids in length) representing helper T cell epitopes are easily made.

For a molecule to be able to induce an immune response therefore it must contain two basic elements, a T_H-epitope and an epitope that will either induce specific antibody or a specific CTL response. The traditional approach to vaccine design in which peptide epitopes are used is to covalently couple the antibody or CTL epitope to a carrier protein, which provide a source of T_H-epitopes. The proteins widely used are tetanus toxoid, diphtheria toxoid, keyhole limpet haemocyanin as well as proteins such as ovalbumin and bovine serum albumin. For experimental purposes these materials are adequate but because the conjugation methods usually make use of relatively non-specific or non-selective chemistries the products are not well defined and do not lend themselves to rigorous quality control on a batch to batch basis. Another limitation with carrier proteins is their cost and availability; the manufacture of tetanus toxoid for use in a conjugate vaccine would introduce a significant and additional cost factor to production. Other limitations to the carrier protein conjugation approach include the peptide load that can be coupled and the dose of carrier that can be safely administered [101]. Although carrier molecules can lead to the induction of strong immune responses they are also associated with undesirable effects such as suppression of the anti-peptide antibody response because the carrier protein itself usually induces an antibody response that competes with the anti-peptide response [102–104]. Thus, to avoid the problems associated with the use of carrier proteins the use of defined T_H-epitopes has been investigated [105,106]. Moreover, promiscuous T_H-epitopes have been identified that are not to a restricted to a single MHC haplotype [107–110] and may therefore serve as a source of T cell help in-out bred populations.

Antibody is of limited benefit in the recovery from some, particularly viral, diseases and in these cases and many types of cancer, the immune system responds with CTL. Like helper T cells, CTL are first activated by interaction with APC bearing their specific peptide epitope presented on the cell surface, this time in association with MHC class I rather than class II molecules. Once activated the CTL can engage a target cell bearing the same peptide/class I complex and cause its lysis. It is also becoming apparent that helper T cells play a role in this process; before the APC is capable of activating the CTL it must first receive signals from the helper T cell to up-regulate the expression of the necessary co-stimulatory molecules [111]. Thus, like antibody-inducing vaccines, optimal CTL-inducing vaccines require both a CTL-epitope and a T_H-epitope.

The following assessment of synthetic peptide-based vaccines reviews our own work that has focused on totally synthetic epitope-based vaccines in which antibody or CTL responses are required. The review highlights the fact that *totally synthetic* vaccines can be designed and assembled which are capable of inducing antibody or CTL and demonstrates advantages that peptides have to offer in the field of vaccine design. Our studies into synthetic peptide-based vaccines that require the induction of antibodies have centred around the 10-residue peptide hormone luteinizing hormone releasing hormone (LHRH), a hormone that controls ovulation in females and sperm production in males. Our investigations into the design of CTL epitope-based vaccine design have concerned influenza and the influenza virus. The animal model that we use allows protection from disease to be assessed following vaccination as well as measurement of many of the parameters, such as immunological memory, which are important in the design of any vaccine.

Some Rules for Peptide-based Vaccine Design

Juxtaposition of individual epitopes and the molecular geometry of immunogens. A large number of investigators have shown that inoculation with synthetic peptide-based immunogens can result in the production of high titres of antibodies and that in some cases these antibodies can have a biological effect. During our efforts to assemble totally synthetic peptide-based vaccines we investigated the effects on immunogenicity by altering the T_H-epitope, increasing the copy number of B-cell epitopes and by comparing the presentation of T_H-epitopes either in linear or in branched geometries. We found that in general peptides that incorporated T_H-epitopes and B cell epitopes into branched conformations were better immunogens. The resulting antibodies were not only specific for the peptide but also for the native antigen from which the epitope was derived and that these antibodies were of higher titre than those induced by corresponding linear constructs [112]. Increasing the copy number of the B-cell epitopes also proved to be an advantage in terms of increasing antibody titres. Additionally we obtained evidence that the processing events for linear and branched immunogens are not the same.

The events that must occur in order to generate an antibody response include the uptake of antigen by antigen-presenting cells and the subsequent activation of naive CD4+ T helper cells by these APC. These antigen processing and presentation functions are most efficiently carried out by DCs and B cells. Following their activation by antigen presenting cells T cells are then able to deliver the appropriate signals that initiate B-cell differentiation and lead to antibody production. In order to understand the mechanisms that results in improved immunogenicity of branched versus linear peptide-based vaccines, we studied the ability of purified DCs to present chemically similar but geometrically different synthetic peptide antigens to T cells [113]. The results of this study demonstrated that the enhanced ability of branched immunogens to induce antibody production is paralleled by their more efficient presentation by DCs and B cells, (Figure 5A) and also by their resistance to degradation by serum proteases (Figure 5B). Although an increased susceptibility of linear peptides to proteolysis in serum could result in a shorter biological half life which could reduce the chances of encountering an APC and decreased antibody production downstream, this did not seem to be the only factor that was responsible for the superiority of branched peptides, since these branched constructs were presented more efficiently to T cells even in the absence of serum. It seems possible therefore that the unusual structure of branched immunogens contributes to the increased efficiency of antigen handling by APC and to the enhanced antibody levels observed.

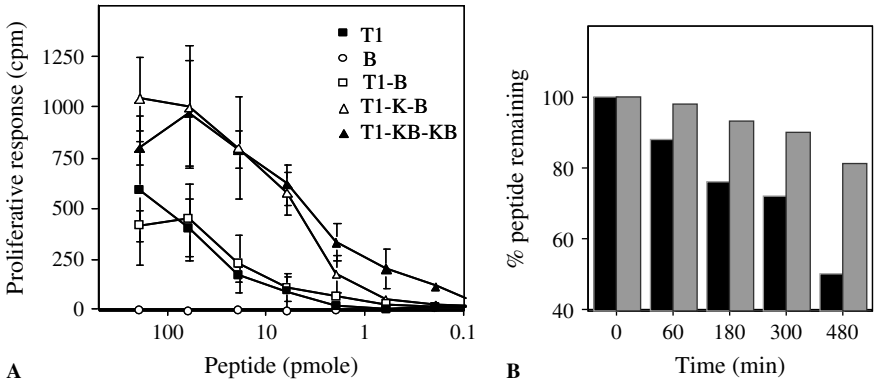


Figure 5 (A) Proliferation of T cell clone 4.51 to the synthetic peptide based constructs bearing the T-cell determinant T1 in response to antigens of different geometries presented by 50 CD8- dendritic cells. The proliferative response to the B cell epitope alone is also shown. Counts are expressed as a mean cpm of triplicate samples measured by direct incorporation of ^{3}H-thymidine. Error bars represent the standard deviation of the mean. (B) Serum stability of branched and linear peptides. The columns represent the percentage of intact linear (solid) or branched (grey) peptides remaining at the indicated times following exposure to serum as a source of proteases. These values were obtained by analysing the peptide content of serum-treated samples by HPLC, following precipitation of high molecular weight material in TFA. The area under the peak containing intact peptide in each sample was compared with the area under the corresponding peak at time zero to determine the percentage peptide remaining at various time intervals.

These *in vitro* studies demonstrated that DCs were far more potent APC than B cells; as few as 50 DC per culture achieved responses of a similar magnitude as did 10 000 B cells. The splenic DC isolated for this work are relatively mature with high levels of class II molecule expression and have been shown to be able to process complex antigens within the first 24 h of culture [114]. It is likely that linear and branched peptides delivered to DC are capable of accessing both macropinocytosis and direct binding to class II molecules on the cell surface for presentation to T cells. Although B cells are also capable of pinocytosis this is far less efficient than the macropinocytosis of DC and it is likely that the major pathway for peptide uptake in naive B cells is via interaction with class II molecules on the cell surface [115]. Indeed, evidence is now emerging that different APCs possess unique combinations of mechanisms for antigen uptake [116,117]. Thus, peptides that are generated from branched constructs and subsequently presented to T cells may well be different to those derived from linear peptides by virtue of different antigen uptake mechanisms and/or differential resistance to the proteases involved in the processing events. The fact that antigens can be exposed to a variety of processing pathways may explain the different efficiencies of presentation to T cells and the resulting differences in the immunogenicity of branched and linear peptides observed in this study.

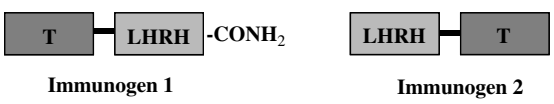
Whatever the mechanism, the stimulation of T cells is clearly affected by the geometry and stability of the antigen and also the type of APC involved in antigen uptake. The correlation of increased protease stability and presentation efficiency of branched immunogens with their enhanced ability to induce antibody responses *in vivo* [112] demonstrates that structural aspects of an immunogen are important to consider in the design of synthetic peptide-based vaccines. Clearly, the processing events that lead to successful immune responses are sequence-dependent but at another level other physical characteristics of an immunogen such as geometry are also important. The contributions that the *form* of an antigen, i.e. its physical attributes, have on its ability to induce an immune response is a reflection of the accessibility of sequences of amino acids within the epitope that are important recognition sites for receptors and proteases encountered throughout the processing pathway. Furthermore, the ability of APCs to capture antigen on first encounter has a major affect on levels of expression of essential molecules such as MHC class II molecules [117] and this will affect the vigour of the ensuing response. The physical as well as chemical (i.e. protein sequence) properties of an antigen must influence downstream mechanisms and even the type of APC that becomes involved in antigen uptake and presentation. We are beginning to compile a picture that indicates that particular structural features such as geometry can determine the efficiency of a resulting immune response.

Vaccines that elicit antibody. The findings described above provided useful general rules with which to start to assemble synthetic peptide-based vaccine candidates and our initial studies [118] involved the assembly of a variety of simple linear and branched structures which had LHRH as the antibody epitope of interest. It was demonstrated

Table 1 Antobody Titres and Reproductive Capability of Female Mice following Inoculation with Peptide Vaccines

Antigen	Ab titre (log_{10})	Pregnancies	Ab titre (log_{10})	Pregnancies	Ab titre (log_{10})	Pregnancies
	2 weeks following secondary immunization		12 weeks following secondary immunization		24 weeks following secondary immunization	
Immunogen 1	5.6	0/5	4.0	0/5	4.1	0/4
Immunogen 2	2.7	0/5	2.0	3/5	2.2	ND[a]
Saline control 1	2.6	2/5	2.0	3/3	2.2	2/2

[a] ND, not determined



Immunogen 1 **Immunogen 2**

that a linear tandem synthetic peptide composed of a T_H-epitope when synthesized *N*-terminal to LHRH was able to induce high titres of antibody that prevented pregnancy in female mice (Table 1). It was also shown that the orientation of the T_H-epitope and the B cell epitope influenced the efficacy of the vaccine with far less antibody being elicited if LHRH was attached to the helper T cell epitope through its *C*-terminus. The importance of epitope orientation in inducing biologically effective antibodies has been reported before [119–124] and in the case of LHRH it is thought to be an advantage to leave the biologically active *C*-terminus of the hormone free [125]. For this reason, presumably, involvement of the *C*-terminus of LHRH in peptide linkage to a T_H-epitope reduces LHRH specific antibody titres [118].

As discussed above, one of the major problems associated with coupling peptides to proteins is the phenomenon of carrier-induced epitope suppression [102,103] in which antibodies are elicited to the carrier protein in preference to the target peptide epitope. It is likely therefore that use of a T_H-epitope that induces little or no antibody against itself would be an advantage. In our study although the best vaccine candidate, Immunogen 1 (Table 1), also induced antibodies directed against the helper epitope, the titres were less than those against LHRH. Furthermore following the second inoculation, the T_H-epitope specific antibody titre did not increase despite a ten-fold increase in anti-LHRH antibody titre. This finding is in contrast to observations with LHRH–protein conjugates where high titres against the carrier are usually found and which increase with time at the expense of antibodies against LHRH. The ability of a T_H-epitope to provide help without diverting antibody responses towards itself should be regarded as one of the biggest advantages of using defined T_H-epitopes in vaccines.

Chemoselective ligation as a means of assembling epitope based vaccines. In some situations the use of short peptide sequences as epitopes for induction of antibody is not sufficient and longer sequences that are able to mimic the conformation of the native antigen are needed. Although recently developed chemistries used in peptide assembly allow relatively long peptides to be made, difficult sequences often haunt would be synthesizers and yields are concomitantly low. A solution to the problem is to assemble component epitopes of the final vaccine and then ligate these to produce the final structure. This approach not only has the potential of allowing different epitopes to be mixed and matched but also allows for an 'off the shelf' approach in which different immunogenic modules can be ligated in various orientations to provide the highest yield and the most immunogenic vaccine. A number of elegant methods for the assembly of long synthetic peptides using such a modular approach have been described including the formation of thioether bonds [126], oxime bonds [127], disulphide bonds [128], thioester bonds [129], thiazolidine bonds [130] and hydrazone bonds [131] between separately synthesized peptide modules. Each method results in different linkages being created at the conjugation site and some reports concerning the reaction rates and the different chemical stabilities of some of these linkages have also been published [132,133]. Careful study of the influence of the different linkages used to generate peptide constructs on their stability and subsequent biological activity not only gives an indication of the best way of obtaining the highest yield and purity of product but also provides knowledge on the quality of the immune response that is elicited. We undertook to compare the immunogenicity of a vaccine candidate based on a synthetic T_H-epitope and LHRH.

Because epitope orientation has a profound effect on the specificity of the antibody elicited and also on the magnitude of the immune response that result from epitope-based vaccine candidates and because a free *C*-terminus of LHRH within the peptide constructs is important for the efficacy of a LHRH-based vaccines, two series of constructs were made in which the orientation of the T_H-epitope was changed but in which the *C*-terminus of LHRH was always kept free. For example, in the constructs **T-B**, **T-S-B, T-oxm-B** and **T-S-S-B**, the T_H-epitope is ligated through its *C*-terminus to the *N*-terminus of the B cell epitope (i.e. C_T-N_B), providing an orientation that is similar to that of the parent peptide **T-B**. In other constructs, e.g. **B-S-T, B-oxm-T** and **B-S-S-T**, the *N*-terminus of the T_H-epitope is connected to the *N*-terminus of the B cell epitope, i.e. N_T-N_B (Figure 6).

Our findings [134] demonstrated that with this particular combination of epitopes, thioether bond formation gave the highest yield of final material and also showed that the chemistry did not detract from the immunogenic activity when compared with the tandem synthesized **T-B** sequence (Figure 7). The yields of vaccine produced using oxime chemistry, **T-oxm-B and B-oxm-T** were lower than when thioether bond formation was used and the immunogenic activity was slightly less when compared with

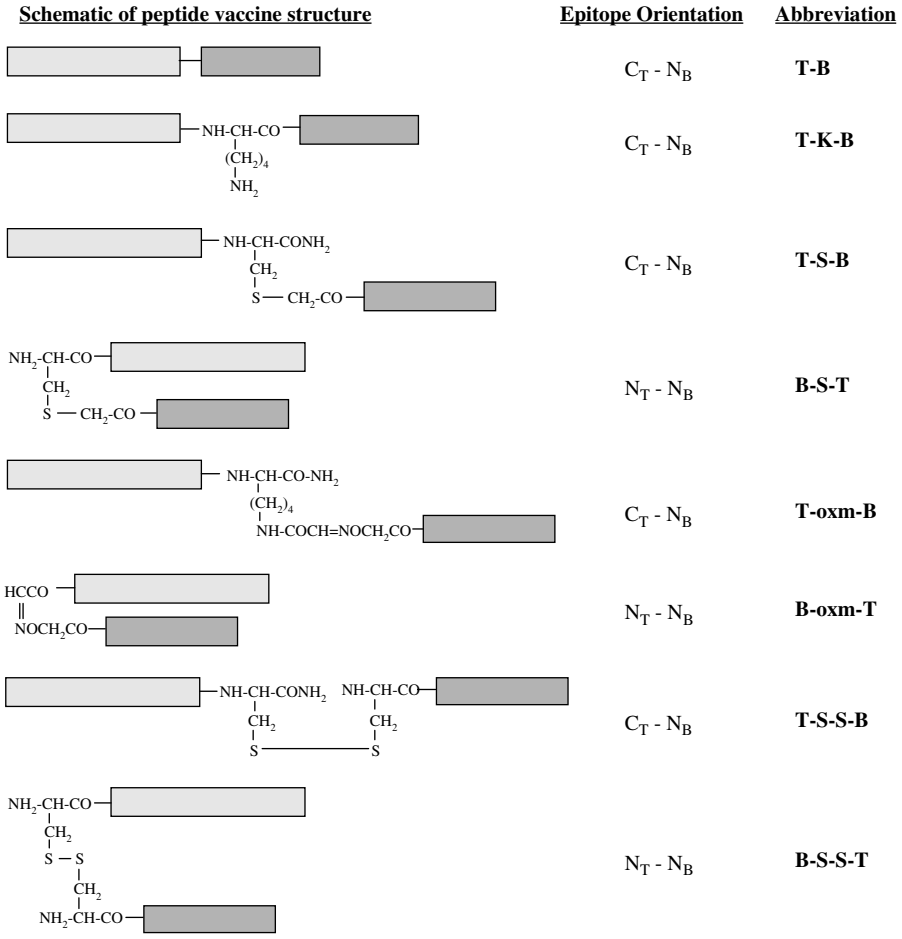


Figure 6 Structures of the synthetic peptide-based vaccines assembled by chemoselective ligation. The dark grey box represents the B cell epitope component of each of the vaccines and the light grey box represents the T helper cell epitope. The chemical linkages resulting from each of the different ligation strategies and the orientation of the epitope are shown. Abbreviations of the structures are shown at the right of the diagram. In the constructs T-S-B, T-oxm-B and T-S-S-B the T helper cell epitope is ligated through its *C*-terminus to the *N*-terminus of the B cell epitope, here abbreviated as C_T-N_B, providing an orientation that is similar to that of the parent peptide T-B. In other constructs B-S-T, B-oxm-T and B-S-S-T the T helper cell epitope is connected to the *N*-terminus of the B cell epitope through its *N*-terminus, here abbreviated as N_T-N_B.

the thioether conjugates. The vaccines in which a disulphide bond was used to attach the two epitopes, **T**-**S**-**S**-**B** and **B**-**S**-**S**-**T** resulted in the lowest yield and also the weakest immunogens. Studies on serum stability of peptide immunogens have shown that the enhanced immune response induced by more stable peptide immunogens could be due to the longer circulation times of intact immunogens [113,135,136] perhaps as a consequence of resistance to the predominant degradation mechanism, which is exopeptidase-catalysed cleavage [135]. The different vaccines may exhibit different half-lives *in vivo* affecting their persistence and consequently immunogenicity. The fact that vaccines conjugated by a disulphide bond were less immunogenic vaccines could be due to the fact that disulphide bonds occur naturally in physiological systems and may be less persistent *in vivo* than the more physiologically unusual thioether and oxime bonds. Another observation from these studies was that connection of the T_H-epitope via its *N*-terminus or its *C*-terminus to the *N*-terminus of LHRH had little influence on resulting immunogenicity.

Self adjuvanting peptide vaccines. Poor immunogenicity in the absence of an adjuvant is a problem that confronts many, if not all, soluble protein

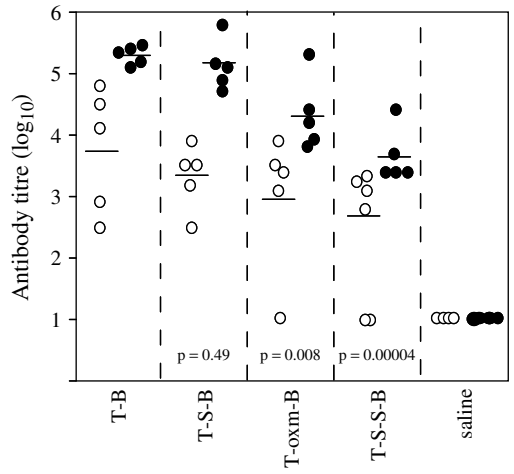


Figure 7 Titres of anti-LHRH antibodies in individual mice elicited following inoculation with peptide vaccines produced by chemoselective ligation of individual epitopes. Titres of antibody were determined 4 weeks after the initial dose (open symbols) and 2 weeks following a second dose (closed symbols) of vaccine. The bars represent the mean of the antibody titres of each group. The results of a paired two tailed *t*-test between the titres obtained following the second dose of each vaccine and the 'parent' vaccine, T-B, are shown as p values. All peptides were inoculated in Freund's complete adjuvant.

and peptide-based vaccines. The problem is compounded by the fact that many of the adjuvants that are currently available for experimental use are either too toxic for use in humans and animals or are ineffective. Lipids have been shown to be capable of adjuvanting otherwise weak immunogens and show none of the harmful side effects of other adjuvant formulations [137–141]. During peptide synthesis, lipids are easily attached to the peptide chain using established chemistries. The resulting lipopeptide constructs are self-adjuvanting [142–145] and have the advantage of being totally synthetic and lending themselves to rigorous quality control. There have been a number of successful applications of lipopeptides as immunogens used to generate antibody and cellular responses [138,143,146] and several different lipids have been used for these purposes. Tripalmitoyl-S-glyceryl cysteine (Pam3Cys) is a synthetic version of the *N*-terminal moiety of Braun's lipoprotein, a protein that spans the inner and outer membranes of Gram-negative bacteria and is capable of stimulating virus-specific CTL responses [147]. Pam3Cys has also been shown to elicit protective antibodies against foot-and-mouth disease when coupled to appropriate synthetic epitopes [143]. Pam2Cys, which is an analogue of Pam3Cys, has also been synthesized [148] and shown to correspond to the lipid moiety of MALP-2, a macrophage-activating lipopeptide isolated from mycoplasma [149–151]. Pam2Cys has only two ester-bound palmitic acids and a free amino group, properties that should lead to improved water-solubility of Pam2Cys-containing compared with Pam3Cys-containing constructs. It has also been reported that Pam2Cys is a more potent stimulator of splenocytes [148] and macrophages [150,151] than is Pam3Cys.

These physical, chemical and immunological properties of Pam2Cys make it an attractive candidate for adjuvanting synthetic peptide vaccine candidates and its potential was investigated by examining the immunogenicity of LHRH-based peptide vaccines in which Pam2Cys was used to provide adjuvanting capability to the vaccine. We were also able to compare the effects on immunogenicity when lipid was attached at different positions within the peptide sequence, either in the middle of the molecule or at the *N*-terminus. The results of these experiments in which antibody titre was determined (Figure 8A) showed that the most effective vaccines contained the Pam2Cys moiety and had this lipid attached at the centre of the molecule. Furthermore, in this configuration the vaccines were far more soluble than when the lipid was attached at the *N*-terminus. The relative ability of the lipopeptides to induce an antibody response in the absence of external adjuvant was reflected by their ability to upregulate the surface expression of MHC class II molecules on immature DCs (Figure 8B). These results demonstrate that the composition and position within peptide vaccines of self-adjuvanting lipid groups can influence the ability to induce the maturation of DC and in turn the magnitude of the resulting antibody response. Of particular importance is the fact that the antibody titres achieved with the totally synthetic self-adjuvanting lipopeptides were equal to those achieved with peptide in Freund's complete adjuvant, which is viewed as the bench mark for all adjuvants.

The problem of valency. Many of the perceived limitations of peptide-based vaccines centre around the small size, low copy number and low immunogenicity of peptide-based immunogens. As noted above, these problems have traditionally been addressed by conjugating peptide epitopes to carrier proteins, a procedure that not only provides T_H-epitopes but can also provide multivalent display of epitopes to

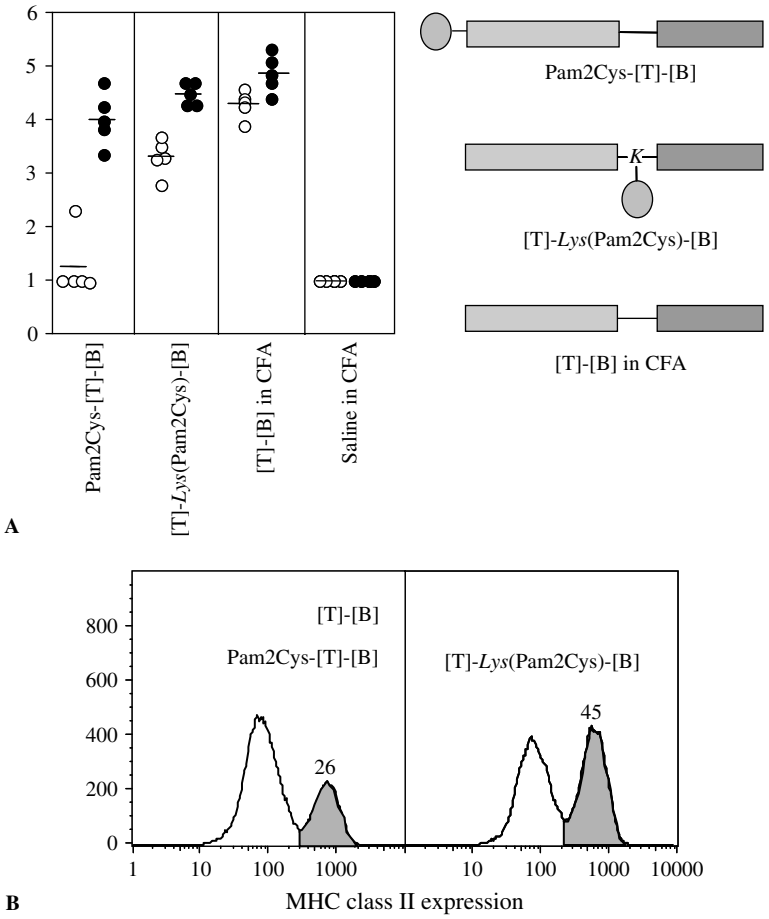


Figure 8 (A) Immunogenicity of peptide and lipopeptide immunogens based on LHRH. The lipopeptides used were Pam2Cys-[T]-[B] in which the Pam2Cys lipid moiety is attached at the *N*-terminus of the peptide and [T]-*Lys*(Pam2Cys)-[B] in which the Pam2Cys lipid moiety is attached to the epsilon amino group of a lysine residue situated between the T cell epitope [T] and the B cell epitope [B] at the approximate centre of the molecule. The peptide-based vaccines were administered in saline for both primary (open circles) and secondary (closed circles) inoculations. The non-lipidated peptides [T]-Lys-[B] and [T]-[B] were administered in complete Freund's adjuvant for the primary and in incomplete Freund's adjuvant for the secondary doses. The dose of vaccine used was 20 nmole in all cases. Control groups of animals received saline only emulsified in CFA for priming and saline only emulsified in IFA for secondary inoculations. (B) Ability of peptide and lipopeptide to cause the maturation of DCs. For each group, 8×10^4 DCs were exposed to 4.5 fmole of lipopeptide and incubated overnight. The cells were collected and the degree of maturation determined by measuring the expression of MHC class II molecules by flow cytometry. The result is a representative of four independent experiments. The result obtained with the non-lipidated peptide is equivalent to that obtained with medium alone indicating a spontaneous maturation rate of 26%.

the immune system. Carrier induced epitope suppression and difficulties encountered in quality control are not the only limitations of this method, the coupling procedure can also have deleterious effects on the integrity of the peptide epitopes [152].

Pathogens often display multiple serotypes such that antibodies or T cells directed to a neutralizing epitope on strain A are not able to recognize the corresponding epitope on strain B. This means that vaccines directed at pathogens, which occur in multiple serotypes, need to contain antigens representing the predominant serotypes that are likely to be encountered. An elegant solution to the problem of presenting multiple antigenic determinants to the immune system was first described by Tam and colleagues [121,153,154]. They assembled multiple peptides onto a branched oligolysine support such that a dendrimer of epitopes was produced. We and others [155,156] have synthesized peptides on solid phase supports

which contain an acid stable linker but cross-links within the support which are cleaved on exposure to TFA allowing the generation of a long single chain polyamide to which multiple copies of peptide are attached. There are, however, limitations to the degree of purity that can be achieved with these products and although the problems of purity have been addressed using ligation strategies [127,132,157], there are restrictions on the number of different epitopes that can be incorporated using any of these approaches.

Realizing the necessity for quality control in vaccine design and assembly and also the capabilities that ligation strategies offer in terms of purification of individual peptides before assembly into the final construct, we developed a modular method for the incorporation of very large numbers of the same or different purified epitopes into covalent structures [158,159]. The technique depends upon acylation with the acryloyl (CH_2=CH–) group of the *N*-terminus of the peptide, while still attached to the solid phase support and with side chain protecting groups intact. Following cleavage, deprotection and purification, the same or different peptides can be incorporated into a polymer using free radical initiation of chain elongation. This process is analogous to the polymerization of acrylamide into polyacrylamide gels. Individual peptides are assembled into polymers in which the peptide determinants form side chains pendant from an alkane backbone (Figure 9). The method allows purification of the individual determinants, avoids errors inherent in long sequential syntheses, allows multiple copies of the same or different peptide epitopes to be incorporated into a single polymeric structure and permits polymers of predictable size to be produced [160]. A proof of principle study [111] has demonstrated that this approach provides a solution to the problem of vaccines that require multiple serotypes.

Vaccines that elicit CTL. The clearance of most viral infections and the control of some cancers depend upon the induction of CD8+ cytotoxic T

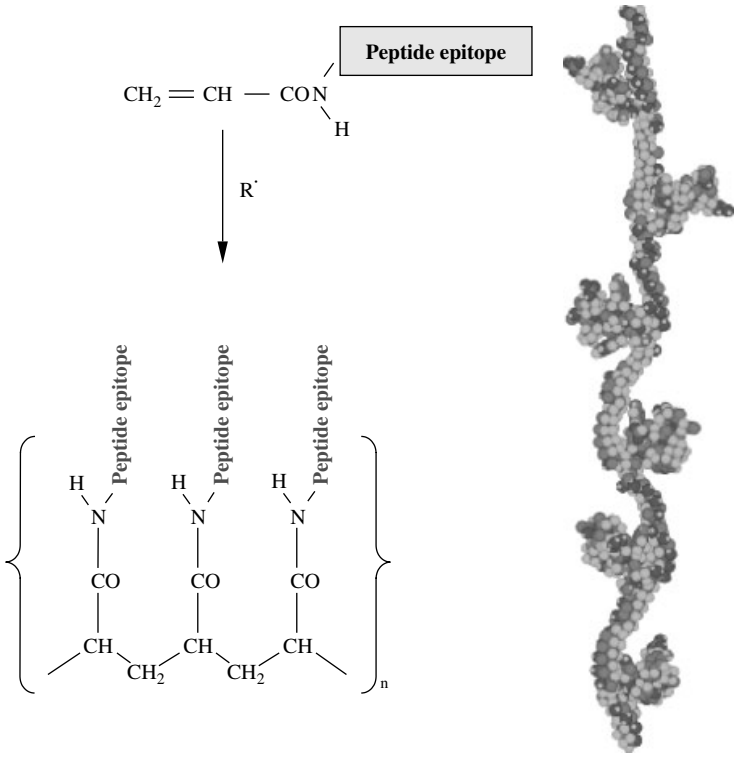


Figure 9 Schematic of the assembly of synthetic peptide-based polymers. Peptides are assembled in the normal way on solid phase supports and then acylated at the *N*-terminus with acryloyl chloride. Following removal of the peptide from the support and concomitant removal of the side chain protecting groups, the peptide epitopes are purified and then polymerized by exposure to free radical. The space filling molecular model at the right of the figure is a representation of a portion of a polymer molecule in which acryloyl LHRH has been co-polymerized with acrylamide in a 1 : 10 ratio such that each LHRH epitopes is separated from its neighbour along the polymer backbone by 10 acrylamide residues.

cells. Vaccines consisting of inactivated virus or soluble proteins do not induce cytotoxic responses because the antigens involved are not able to enter the cell cytoplasm to gain access to the endogenous class I processing pathway [161]. Strategies that can potentially overcome this problem include the use of live viral vectors [162–164], ISCOM [165–167] and liposome [168,169] based approaches and DNA vaccines [170]. Each of these techniques lead to transport into or expression of antigen within the cytoplasm that can then enter the class I processing pathway.

The induction of CTL with peptides can be achieved with oil-based adjuvants [171–175] but lipopeptides in which lipid is covalently attached to a peptide-based vaccine can also induce these responses [141,147,176]. Cytolytic responses in non-human primates [177] as well as in human volunteers [178,179] have also been reported using lipopeptides.

Because the rewards for designing efficient self-adjuvanting peptide-based vaccines that induce CTL responses are so high — the possibility of anti-viral and anti-cancer vaccines for example, there is enormous interest in finding methods for inducing CTL using peptide-based strategies. Despite this interest and the efforts that have been made to achieve effective peptide-based vaccines which can provide protective responses, few reports [173,174,180–182] describe effective T cell-mediated viral clearing responses and other workers describe either no or little benefit from anti-viral responses induced by inoculation with peptides [172,183,184]. Thus, there is an apparent dissociation in the ability to induce CTL and to achieve effective viral clearing responses using synthetic peptide-based immunogens [185].

In an attempt to design an effective vaccine against influenza, a disease that requires a CTL response for successful resolution, we used the design features that we had developed in our studies with antibody-inducing vaccines. The resulting synthetic peptide construct contained a T_H-epitope in branched geometry with a nine-residue CTL epitope that was covalently attached to a lipid [186]. We were able to show not only that this CTL epitope-based vaccine had the capacity to significantly impact on the rate of viral clearance in a challenge model but also that the longevity of the lipopeptide-induced response contrasted sharply with the short-lived responses induced by non-lipidated peptides. The data reported here also highlighted the lack of correlation between measurable CD8+ T cell activity, a parameter usually measured in assessing the efficacy of a CTL epitope-based vaccine and protection from infection. Despite equivalent cytolytic and IFNγ-secreting CD8+ T cell responses in both lipopeptide and non-lipidated peptide-primed mice, only those receiving lipopeptides were protected. This study represents the first demonstration of CTL-mediated immunity induced by a synthetic lipopeptide immunogen that is capable of enhanced clearance of pulmonary influenza following challenge with infectious virus, in addition, it represents one of the few reports describing protective responses induced by a T-cell epitope-based vaccine of any type for a viral disease.

CONCLUDING REMARKS

It is clear that the outcome of the processing events to which antigens are exposed is determined in part by the amino acid sequence of the antigen. The successful extraction and eventual presentation to recognition elements of the immune system will occur only if the *context* of an epitope is appropriate and it is that context which is the issue for designers of epitope or peptide-based vaccines. The studies that we have reviewed here appear to point to some generic rules that can be applied to peptide vaccines.

- Molecules with unusual geometries, particularly branched geometries, are presented more efficiently by dendritic cells to CD4+ T cells.
- Synthetic helper T-cell determinants can provide strong help with little or no evidence of epitope suppression.
- Orientation of B cell epitopes can be important in determining antibody specificity, the orientation of the helper T cell epitope seems not to be important.
- Incorporation of lipids into simple epitope-based structures confers adjuvanting effects which are applicable to vaccines required to induce antibody or cytotoxic T cells.
- Underlying all these advances in synthetic peptide based vaccines, a major hurdle still resides in the identification of minimal T cell epitopes. This is best accomplished using a combination of strategies that include analytical biochemistry and recent advances in mass spectrometry, bioinformatics and predictive algorithms and experimental validation studies that incorporate simple linear synthetic peptides.

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