Letters in Peptide Science, **10:** 561–569, 2003.
KLUWER/ESCOM

Optimisation of peptide-based cytotoxic T-cell determinants using non-natural amino acids

Andrew I. Webb[1], Marie-Isabel Aguilar[1] & Anthony W. Purcell[2]*
[1]*Department of Biochemistry and Molecular Biology, Monash University, Victoria 3800, Australia;* [2]*Department of Microbiology and Immunology, University of Melbourne, Victoria 3010, Australia*
*(*Author for correspondence, e-mail: apurcell@unimelb.edu.au, Fax: +613 93471540, Tel: +613 83449911)*

Received 5 February 2004; Accepted 4 March 2004

Key words: cytotoxic T-cells, epitope engineering, fixed anchor substitution, heteroclitic peptides, peptidomimetic, vaccines

Summary

In this brief review we describe the methods that our group and others have developed in incorporating non-natural amino acids into peptide antigens, principally to increase protease resistance, for potential use in peptide-based vaccines. Peptide-based vaccination has the potential to generate protective immunity without the need for *in situ* antigen synthesis or further proteolytic processing of the antigen. The ability to deliver minimal T cell epitopes to the effector cells of the immune system also minimises unwanted side effects and simplifies clinical monitoring. The major hurdle in designing successful peptide-based vaccines resides in issues surrounding the delivery and stability of the peptide immunogen, as it is the form in which the epitope is delivered that will determine how it will be processed by the immune system and ultimately whether it will be capable of inducing an appropriate immune response. Thus, one of the confounding issues with peptide-based vaccines is their poor bioavailability, which is predominantly due to proteolysis and oxidative damage of the 'naked' peptide. Strategies that stabilise peptide epitopes promise to overcome the current problems and make peptide-based immunogens more applicable in vaccine design.

Introduction

Vaccination aims to generate pathogen specific responses by stimulating the adaptive immune system. The two branches of adaptive immunity, the cellular cytotoxic responses and antibody production, contribute to the eradication of pathogens [1]. This process involves complex interplay between $CD8^+$ and $CD4^+$ T lymphocytes. It is cells that express the CD8 co-receptor that are responsible for the cytotoxic response, primarily through recognition of major histocompatibility (MHC) class I molecules complexed to antigenic peptides (epitopes) [2]. Cytotoxic T lymphocytes (CTL) destroy 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 autoimmune disease [3]. Advances in immune imaging and dissection of the immune response have lead to a detailed knowledge of the antigens and the epitopes that are recognised by the adaptive immune system [4]. This knowledge can be harnessed in epitope-specific immunotherapy of a variety of human diseases. There are several advantages of peptide-based approaches that make them attractive as immunotherapeutics. The totally synthetic nature of a peptide-based vaccine is devoid of infectious material which may compromise many live or attenuated vaccines. In addition, there is no risk of reversion to or formation of adverse reassortants

that may lead to virulence of live attenuated vaccines. Likewise, there is no potential for genetic integration or recombination, which is a potential problem with DNA vaccination [5]. Apart from these safety advantages, many pathogens are difficult or impossible to culture by conventional methods, making mass production of attenuated or inactivated virus vaccines impractical. Selection of epitopes from the whole antigen also allows deleterious sequences to be removed. Such sequences may be oncogenic [6] or implicated in autoimmune diseases, for example the M protein of group A streptococci [7, 8]. Moreover, the production of chemically defined peptides can be carried out economically on a large scale, with the resultant peptides easily analysed for purity and fidelity of sequence using well established analytical techniques such as liquid chromatography and mass spectrometry, facilitating quality control and ultimately approval by regulatory authorities. Peptide preparations can subsequently be stored freeze-dried dispensing with the need to maintain a ''cold chain'' in storage, transport and distribution.

The ''designability'' of peptide-based vaccines also makes them extremely versatile vaccine components. For example, multiple antigenic determinants from a number of pathogens, or multiple antigenic epitopes from the same pathogen can be incorporated into multivalent structures using peptide monomers [9, 10]. In addition, a variety of chemical modifications can be implemented during peptide synthesis, allowing peptides to be modified in ways not easily achieved using other techniques and improving their overall performance as immunogens. For example, lipid, carbohydrate and phosphate groups can be readily introduced in a controlled manner to improve immunogenicity, stability and solubility [11]. Moreover, nature can be improved upon and amino acid residues can be modified or substituted with an array of naturally occurring and non-natural amino acid residues to generate mimotopes that are more potent immunogens or possess more appropriate *in vivo* characteristics. Recent studies of cyclic peptides in experimental allergic encephalomyelitis (EAE) [12, 13] and the non-obese diabetic mice [14] have provided promising results in $CD4^+$ mediated immunity. The backbones of cyclic peptides are conformationally constrained that enable mimicry of the native protein structure and have been shown to elicit better immune responses than their linear counterparts [15]. The constrained nature of the peptide also provides a barrier to protease attack [12]. However, cyclic peptides are not applicable to the design of MHC class I restricted analogues, as the free termini are essential for binding within the cleft [16].

Dissection of immune responses against several pathogens has revealed that the immune response does not react to all possible epitopes encoded by the pathogen genome, but rather focuses on a few epitopes and in some cases only a single immunodominant epitope [17]. Although the mechanism for such focused immune responses and the benefit to the host of such a restricted response is unclear, 'immunodominant' responses have been observed in a large variety of infectious diseases [18–27]. These determinants result in a large proportion of CTLs with restricted epitope specificities dictating the clearance of the pathogen. The fact that many common human pathogens are cleared by one or two T-cell specificities is encouraging for peptide-based vaccine development.

In some cases of chronic viral infection and cancers, the natural primary CTL response in conjunction with innate immune response is not effective in resolving the infection/tumour. In these instances the response induced is not optimal and can be improved upon by isolating the individual components of the response and further optimizing them. Epitope modification in the past has mostly consisted of mutating fixed anchor substitutions to improve MHC binding and heteroclitic substitutions that mutate exposed residues subtly altering T-cell receptor (TcR) interactions. These types of modifications have provided a good indication of the scope available for optimization, in some cases analogues have been able to significantly enhance CTL activation [28]. More recently other, more subtle modifications such as backbone and termini modifications have been incorporated to enhance the bioavailability of these immunogens. Increasing bioavailability is important as once taken out of the context of the full length native antigen, synthetic peptides are readily degraded and cleared from the body. Incorporating protease resistance also allows other possible routes of administration not normally available for naked peptides to be used. Such alternate routes of administration my also increase patient compliance. Thus, in situations where the natural immune response is not capable of eliminating

infection there exists an opportunity to further optimize and enhance crucial components of this response. There are additional advantages to using epitope-based vaccines including safety, ability to focus the immune response, and to rationally engineer increased immunogenicity and protease resistance into relatively simple chemical entities. This brief review will discuss the current direction and methods for modifying peptide epitopes for inclusion in vaccines directed at eliciting CTL responses.

The cytotoxic T cell response

The highly specific CTL response is primarily achieved via the direct recognition of an antigenic peptide complexed to a class I MHC molecule on the surface of target cells via the clonally distributed TcR. The structure of MHC class I molecules are well defined and consist of a non-covalent complex of a polymorphic heavy chain, a monomorphic light chain (β-2 microglobulin) and an antigenic peptide. The heavy chain has three extracellular domains (α1–3) that form a platform for binding and presentation of an antigenic peptide to CTL. 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 cleft measures approximately 30 Å in length and 12 Å in width at the center and accommodates a peptide typically 8–11 amino acid residues in length. The cleft is the focus of the majority of MHC polymorphisms, which in turn dictates the peptide binding specificity of different allelic forms of class I molecules due to the presence of several conserved depressions or pockets that vary in composition and stereochemistry depending on the allele. Our understanding of how HLA polymorphism impacts on peptide binding specificity 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 [16].

Peptide antigen is generated 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 [29]. Transport of these peptides occurs in an energy-dependent manner through a member of the ATP binding 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 is orchestrated by multiple ER-resident chaperones [28, 30–32]. This process of events ultimately leads to the loading of the class I molecules with a suitable peptide cargo, allowing the class I molecule to be released from the ER, traverse the Golgi network and be transported to the cell surface, where the complex is scrutinized by $CD8^+$ T cells.

Applications for peptide-based vaccines exist in a wide variety of cancers and infectious diseases. The potential for vaccine development is directly related to the ability to characterize the naturally expressed epitopes that are or can potentially be targeted by the immune system. Several mechanisms exist for the determination of new epitopes, such as identification through predictive binding algorithms (such as SYFPEITHI and MHCPEP) [33, 34]. These and related algorithms are based on the fact that peptides bind MHC molecules in an extended conformation and the binding energy of a candidate epitope can be approximated by a linear polynomial function that calculates the contribution each amino acid residues found at different positions along the candidate sequence. Using this method the probability that a given epitope will bind to a given MHC molecule can be approximated for many MHC molecules [33]. Although, recent findings [35–40] have shown that MHC molecules can also bind non-canonical anchor motif peptides, which would not be picked up by these more conventional studies. Another method involves the use of an antigen peptide library, where the whole antigen sequence is made in short overlapping peptides. This library of peptides is then used to scan the peripheral T-cell repertoire in search of peptide specific CTL using assays of T-cell function [41]. Once potential targets are identified the natural presentation of these epitopes needs to be confirmed. This can be achieved by creating T-cell lines against known peptides that will kill natural target cells (i.e. tumour cells or virally infected cells). More rigorous methods for epitope identification exist such as the isolation of peptides from tumor or infected cells which are subsequently characterized by LC-MS techniques [42]. Existing immune responses in patients can

also be harnessed to identify potential antigens, such as the serological analysis of antibodies (SEREX) in cancer patients to discover new protein antigens that may also contain new T-cell epitopes [43]. Despite the ready availability of these experimental tools, epitope identification still remains a limiting factor in generation of peptide-based vaccines. As discussed earlier, MHC molecules are extremely polymorphic, with several hundred HLA molecules known to exist. The frequency of these alleles differ between different ethnic groups and are highly varied which complicates attempts to design vaccines of widespread application. However, although different HLA molecules are associated with different peptide binding patterns, many variants can be classified into a relatively small number of HLA 'supertypes' [44]. A supertype is defined by a group of HLA molecules having related peptide binding motifs, and overlapping binding repertoires. For class I molecules, approximately 90% of known HLA molecules can be categorized into eight or nine major HLA supertypes. This may simplify epitope selection requiring inclusion of only a few epitopes from different supertypes to allow broad coverage of a high proportion of the population and avoiding ethnic bias.

In stark contrast to the enormous heterogeneity found within HLA molecules, proteases and peptidases have remained relatively homologous throughout the human population. Mechanisms within the body have evolved to efficiently digest and degrade any non-essential proteins into their re-usable subunits. It is this highly efficient process that severely reduces the potential for peptide-based vaccine approaches. The need for improving the bioavailability of these epitope-based vaccines becomes essential. Increased stability against proteases has several potential advantages such as reducing the relative dose amount of vaccine, increased immunogenicity and potential alternate routes of administration not available to natural peptides. There lies an enormous potential to develop oral vaccination to stimulate protective CTL [45]. In addition to this, oral administration would permit readily available vaccination on a widespread basis for those patients with limited resources due to economic or geo-political constraints. It would also circumvent health risks associated with the use of syringes and the expense of disposable supplies, both of which are serious issues in developing countries.

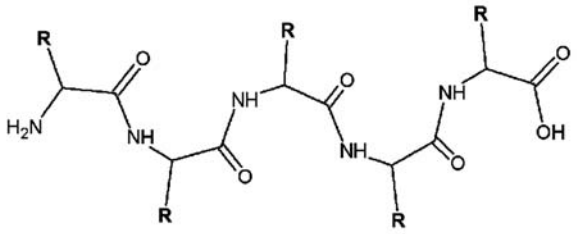
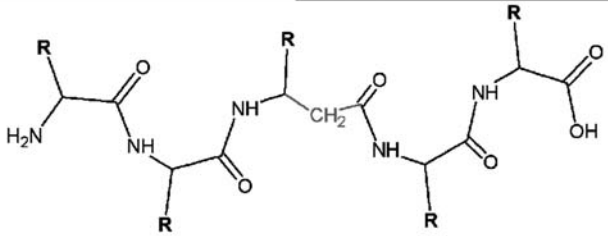
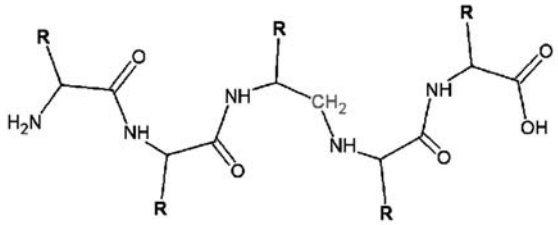
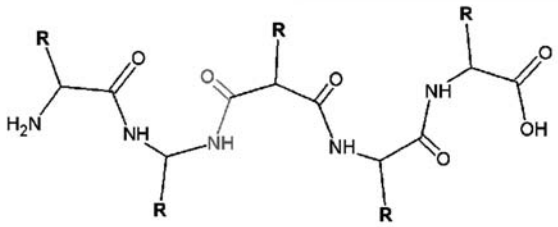
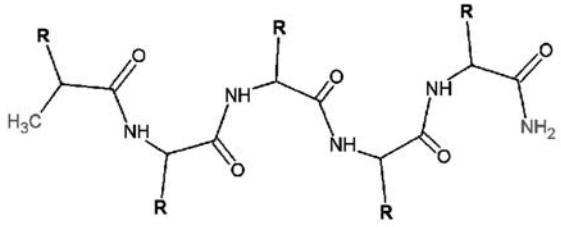
Approaches to optimizing MHC class I-restricted epitopes

In circumstances where the immune system fails to mount an effective immune response to the natural immunogen, there is an opportunity to optimize epitope(s) to enhance their immunogenicity. Epitope modifications have typically consisted of MHC anchor substitutions, where sub-optimal anchor residues are substituted to provide higher binding affinities [27]. More recently, analogues with substitutions not occurring at MHC anchor residues that have the capacity to induce hyperstimulation of T cells, generating more potent responses have been coined heteroclitic analogues [46]. Heteroclitic analogues are of interest in the development of vaccines as they can achieve higher magnitudes of immune responses or may induce the same response at lower concentrations. This may be explained by increased stability of the MHC-peptide complex and increased avidity and dwell time of the TCR-peptide/MHC complex at the immunological synapse [47]. It has also been reported that in some experimental models, heteroclitic antigens have been able to break T-cell tolerance [48], potentially assisting in development of anti-tumour immunity which frequently involves self antigens. More recently it has been reported that many potent heteroclitic analogues generally do not involve major MHC anchor or TcR contact residues directly, but involve minor alterations in the overall complex recognized by the TcR, resulting in increased affinity of the TcR-peptide/MHC interactions [44]. Whilst these approaches can produce better vaccine components, they have until recently been constrained to naturally occurring amino acid substitutions (which has recently been reviewed by Sette and colleagues) [46]. For the remainder of this review we will focus on attempts to introduce non-natural amino acid analogues into peptide epitopes, an approach that promises not only to improve class I binding and TcR avidity but also to introduce favorable biophysical properties to the epitope such as protease resistance and ultimately oral stability.

Several studies have explored alternative modifications that not only provide subtle conforma-

tional changes to the peptide/MHC structure, but also incorporate resistance against proteases. Results from our work and others [49, 50] have shown that incorporating β-amino acids into epitopes can increase the binding affinity of the mimetic for the MHC molecule relative to the wild type peptide. The side chains of β-amino acids are identical to their parent α-amino acid, which is of particular importance to maintaining similar properties of the natural epitope. This modification of the backbone by introducing a methylene moiety (Table 1) results in the complete resistance of

Table 1. Non-natural amino acid modifications for potential use in peptide-based vaccine design (**R** = sidechain)

Modification	Structure	
Normal α-peptide		
β-amino acid substitution		Guichard *et al*. [31] Reinelt *et al*. [32] Webb *et al*. [33]
Backbone reduction		Guichard *et al*. [39] Stemmer *et al*. [37] Calbo *et al*. [40] Quesnel *et al*. [56]
Partial retro-inversion		Guichard *et al*. [41] Ostankovitch *et al*. [42]
N-terminal methylation and C-terminal amidation		Marschutz *et al*. [45]

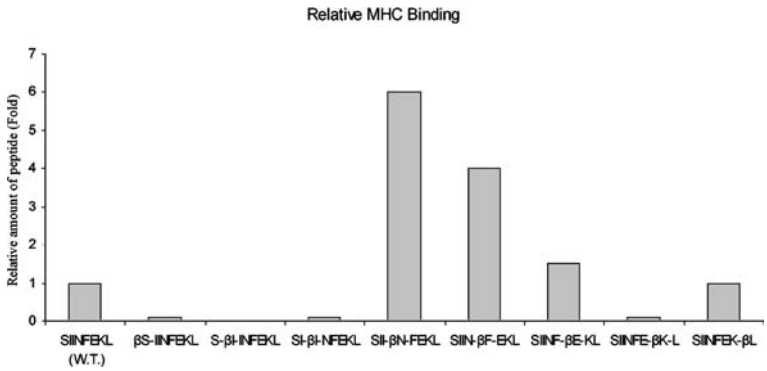


Figure 1. Binding of peptides in RMA-S stabilization assay. Cold induced MHC molecules were stabilised by the binding peptide when returned to 37 °C, stabilised surface MHC/Peptide complexes were measured by flow cytometry [33]. Results are shown as relative comparison to wild type binding (i.e., concentration of peptide needed to induce 50% maximal response). The Ovalbumin$_{(257–264)}$ peptide SIINFEKL was systematically substituted at each position with the corresponding β-amino acid. Substitutions at position **4**, **5**, and **6** resulted in substantially better binders.

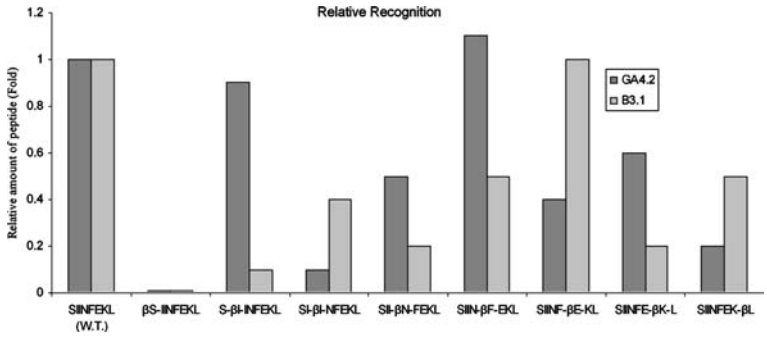


Figure 2. Recognition of selected analogs by two SIINFEKL specific T cell hybridomas. T cell activation measured by relative IL-2 secretion detected via an IL-2 bioassay [33]. Results are shown as the relative amount of peptide required to produce 50% maximal IL-2 secretion by the wild type peptide. Analogues displayed a varied spectrum of recognition by the two T cell hybridomas which did not correlate to MHC affinity. Substitutions at positions **2**, **5** maintained full recognition by Ga4.2 clone, while a substitution at position **6** maintained full recognition by the B3. 1 clone.

peptides solely composed β-amino acids to proteolytic degradation [51]. Furthermore, it has been shown in other systems that even single amino acid substitutions for the corresponding residue can have dramatic effects on the overall stability of the entire peptide [52, 53]. In the example shown in Figure 1, the dominant ovalbumin derived epitope in C57BL/6 mice, SIINFEKL, underwent a β-amino acid scan and the resultant single amino acid substituted set of peptides was tested for binding to the murine H-2K^b class I MHC molecule. Substitution at the N-terminus of the peptide resulted in decreased binding affinity to the K^b molecule. Moreover, a differential pattern of T-cell recognition was observed when CTL clones expressing different TcRs restricted by the H2-K^b molecule and specific to the wild type peptide were tested for their ability to recognize the β-amino acid substituted analogues (Figure 2). This suggests that each T-cell clone recognised the peptide/MHC complex in slightly different ways and subsequently differentially tolerated the subtle conformational changes in the β-amino acid substituted analogues. The effect was dependent on the position of the substitution and exhibited a subtle correlation with stability of the K^b/peptide complex. As some of the analogues (position **2** and **5** for GA4.2, and position **6** for B3.1) were recognised equally as well as the wild type peptide, we would predict that these analogues would maintain a high level cross-reactivity when used to prime *in-vivo*. We have also shown that modifications such

as these not only induce small changes in TcR reactivity but can significantly increase their resistance to proteolytic degradation [54]. The use of β–amino acids represents a systematic approach suited for the successive development of epitope modification.

Other methods incorporating backbone modifications have been utilized by several groups to achieve protease resistance and maintain T-cell cross-reactivity. For example, Stemmer *et al.* [55], showed that reducing the peptide bond from the natural amine bonds (CO-NH) to a aminomethylene (CH_2-NH) maintains similar MHC-binding and T cell recognition by a LCMV-GP33 specific clone. When assessed *in-vivo* this analogue exhibited substantial increases in protease resistance. Furthermore, in contrast with immunization with native GP33, three injections with this analogue in saline induced significant antiviral protection. More recently Quesnel *et al.* [56] systematically replaced each bond of the melanoma associated MART-$1_{(27–35)}$ epitope with its aminomethylene surrogate. All analogues showed increased resistance to proteolysis, with two analogues binding more efficiently to the HLA-A2 molecule. These analogues were also recognised by a melanoma specific T cell clone. Prior to this, Guichard *et al.* [57] showed that a panel of reduced peptide bond analogues could bind to soluble recombinant class I molecules but the relative binding affinities were three to ten fold lower than the parent peptide. This discrepancy may relate to the fact that the binding of MART-1 peptide and HLA-A2 is not optimal and can be improved upon. In a paper by Calbo *et al.* [58], they showed that the tumour associated CW3 epitope restricted by H-$2K^d$, when reduced at the seventh peptidic bond could maintain strong binding and increased serum half life by two-fold. Furthermore, when this analogue was administered into DBA/2 mice it induced protective immunity against a subsequent challenge with tumour cells expressing the parent epitope. Thus modifications in the backbone of antigenic peptides can decrease protease susceptibility while preserving immunogenicity. Such peptide modifications may prove useful in the development of new therapeutic tools aimed at eradicating pathogens and tumours.

Another method for incorporating protease resistance into epitopes has been employed by Guichard *et al.* [59] that involves replacing two successive amino acids residues with a two substituted melonate derivative and *gem*-diaminoalkyl residue, otherwise known as partial retro-inversion (Table 1). In this case an influenza virus matrix protein peptide ($M_{(58–66)}$) was modified at each successive bond. Broad differences were observed in the capacity of the various analogues to bind their cognate class I molecule, HLA-A2, with only one analogue modified at the first peptide bond exhibiting superior binding affinity relative to the unmodified peptide. More recently Ostankovitch *et al.* [60] described using partially modified retro-inverso pseudopeptides to modulate the cytokine secretion profile of a panel of influenza virus specific CTL. The modification again involved replacing the N-terminal residue with the corresponding gem-diaminoalkyl residue which resulted in a two-fold increase the serum half-life, while maintaining comparative reactivity with three wild-type specific clones. Additionally, influenza infected cells were lysed by $CD8^+$ CTL raised by the retro-inverso analogue. More interestingly though the analogue appeared to stimulate synthesis of TNF-α, which was not observed in the response to the wild type peptide. Furthermore, this analogue was also much more potent in stimulating effector cells for the production of IFN-γ. These findings have considerable implications for peptide-based immunotherapy, as the production of TNF-α and IFN-γ are known to be involved in the response to viral infections and in the eradication of tumours [61]. Other groups have reported modifying the N-and C-terminal ends of a peptide to prevent its degradation by exopeptidases [62]. Marschütz *et al.* [63] significantly improved stability of the ovalbumin CTL epitope SIINFEKL by chemically modifying the N- and C-terminus of the peptide. The modifications at the N- and C-terminus involve N-methylation and C-amidation respectively. Such modifications when used in conjunction with a mucoadhesive drug carrier matrix allowed up to 50% of the modified peptide to remain after 3 h in the native intestinal mucosa, whereas the unmodified peptide was totally degraded. This study demonstrates that the stability of a model-CTL epitope can be greatly enhanced by combining the advantages of two different strategies. On the one hand, it was shown that the N- and C-terminal modifications were sufficient to protect from proteolysis, but did not influence the ability to elicit immune responsiveness. However, it was shown

that these modifications were not able to protect against luminally secreted endopeptidases, in fact terminal modification tended to enhance peptide degradation by these enzymes. It was proposed that this might be a result of the terminal modification altering the steric contacts of the peptide structure, subsequently making the cleavage sights more accessible to the enzyme. This enhanced cleavage interior to the terminal modifications destroys the structure of the minimal epitope resulting in complete loss of antigenicity. Epitope modifications such as these may benefit from combination of other strategies, such as the incorporation of β-amino acids to protect central regions of the peptide against protease attack.

The field of non-natural amino-acid vaccine development is still in its infancy. While there is a substantial amount of promising *in vitro* data, it is yet to be made clear whether these results will be reproducible *in vivo*. Future work will inevitably involve highly specific reagents to further probe immune responses to resolve important underlying factors such as the kinetics of responses, as well as the quality of the cross-reactive CTLs generated. However, it is clear that vaccines of the future will require a systematic approach to tailor the desired immune response, involving more than just a single approach to combat the enormous diversity found within any given population. Basic research in these key areas will likely uncover many interesting issues relating to T cell receptor recognition, repertoire selection and possibly T cell signaling events. Our current work aims to characterize the *in vivo* immunogenicity of single β-amino acid substitutions in a model mouse epitope. This work will involve detailed analysis of the T-cell repertoire raised by the modified antigen in comparison to the natural response.

Conclusions

By systematically isolating the immunodominant components of the immune response it is possible to further optimize and enhance these for potential use in vaccines. Typical modifications consisting of backbone and N- and C-terminal modification can only be made synthetically and could not be produced by any other strategy such as DNA vaccines or recombinant antigens. It has been shown that small modifications such as these are well tolerated, in some cases improving the binding affinity of the peptide for the MHC cleft. These modifications have also provided significant improvements to the protease resistance of the peptide antigens, which not only increases bioavailability, but may potentially allow alternate routes of administration. The variety of methods utilized in the design of such optimsied epitopes is providing a promising approach to the treatment of chronic viral infections and tumours.

References

1. Doherty, P.C. and Christensen, J.P., Annu. Rev. Immunol., 18 (2000) 561.
2. Butz, E.A. and Bevan, M.J., Immunity, 8 (1998) 167.
3. Huseby, E.S., Liggitt, D., Brabb, T., Schnabel, B., Ohlen, C. and Goverman, J., J. Exp. Med., 194 (2001) 669.
4. Radvanyi, L., Int. Arch. Allergy. Immunol., (2004).
5. Donnelly, J., Berry, K. and Ulmer, J.B., Int. J. Parasitol., 33 (2003) 457. Review.
6. Moss, D.J., Schmidt, C., Elliott, S., Suhrbier, A., Burrows, S. and Khanna, R., Adv. Cancer Res., 69 (1996) 213.
7. Kotb, M., Courtney, H.S., Dale, J.B. and Beachey, E.H., J. Immunol., 142 (1989) 966.
8. Pruksakorn, S., Currie, B., Brandt, E., Phornphutkul, C., Hunsakunachai, S., Manmontri, A., Robinson, J.H., Kehoe, M.A., Galbraith, A. and Good, M.F., Int. Immunol., 6 (1994) 1235.
9. Jackson, D.C., Purcell, A.W., Fitzmaurice, C.J., Zeng, W. and Hart, D.N., Curr. Drug. Targets., 3 (2002) 175.
10. Purcell, A.W., Zeng, W., Mifsud, N.A., Ely, L.K., Macdonald, W.A. and Jackson, D.C., J. Pept. Sci., 9 (2003) 255.
11. Oseroff, C., Sette, A., Wentworth, P., Celis, E., Maewal, A., Dahlberg, C., Fikes, J., Kubo, R.T., Chesnut, RW. and Grey, H.M., Alexander J., Vaccine. 16 (1998) 823.
12. Tselios, T., Apostolopoulos, V., Daliani, I., Deraos, S., Grdadolnik, S., Mavromoustakos, T., Melachrinou, M., Thymianou, S., Probert, L., Mouzaki, A. and Matsoukas, J.J., Med. Chem., 45 (2002) 275.
13. Tselios, T., Daliani, I., Deraos, S., Thymianou, S., Matsoukas, E., Troganis, A., Gerothanassis, I., Mouzaki, A., Mavromoustakos, T., Probert L. and Matsoukas, J., Bioorg. Med. Chem. Lett., 10 (2000) 2713.
14. Berezhkovskiy, L., Pham, S., Reich, E.P. and Deshpande, S., J. Pept. Res., 54 (1999) 112.
15. Leonetti, M., Cotton, J., Leroy, S., Mourier, G. and Menez, A., J. Immunol., 155 (1995) 210.
16. Purcell, A.W., Mol. Immunol., 37 (2000) 483–492. Review.
17. Yewdell, J.W. and Bennink, J.R., Annu. Rev. Immunol., 17 (1999) 51. Review.

18. Rudolf, M.P., Man, S., Melief, C.J., Sette, A. and Kast, W.M., Clin. Cancer Res., 7(3 Suppl) (2001) 788s.
19. Wizel, B., Starcher B.C., Samten, B., Chroneos, Z., Barnes, P.F., Dzuris, J., Higashimoto, Y., Appella, E. and Sette, A., J. Immunol., 169(5) (2002) 2524.
20. Kather, A., Ferrara, A., Nonn, M., Schinz, M., Nieland, J., Schneider, A., Durst, M. and Kaufmann, A.M., Int. J. Cancer, 104(3) (2003) 345.
21. Caccamo, N., Milano, S., Di Sano, C., Cigna, D., Ivanyi, J., Krensky, A.M., Dieli, F. and Salerno, A.J., Infect Dis., 186(7) (2002) 991.
22. Akiyama, Y., Maruyama, K., Mochizuki, T., Sasaki, K., Takaue, Y. and Yamaguchi, K., Immunol. Lett., 83(1) (2002) 21.
23. Wang, Q.J., Huang, X.L., Rappocciolo, G., Jenkins, F.J., Hildebrand, W.H., Fan, Z., Thomas, E.K. and Rinaldo, C.R., Jr. ,Blood, 99(9) (2002) 3360.
24. Frankenberg, N., Pepperl-Klindworth, S., Meyer, R.G. and Plachter, B., Virology, 295(2) (2002) 208.
25. Micheletti, F., Monini, P., Fortini, C., Rimessi, P., Bazzaro, M., Andreoni, M., Giuliani, M., Traniello, S., Ensoli, B. and Gavioli, R., Immunology, 106(3) (2002) 395.
26. Altfeld, M.A., Livingston, B., Reshamwala, N., Nguyen, P.T., Addo, M.M., Shea, A., Newman, M., Fikes, J., Sidney, J., Wentworth, P., Chesnut, R., Eldridge, R.L., Rosenberg, E.S., Robbins, G.K, Brander, C., Sax, P.E., Boswell, S., Flynn, T., Buchbinder, S., Goulder, P.J., Walker, B.D., Sette, A. and Kalams, S.A., J. Virol., 75(3) (2001) 1301.
27. Currier, J.R., deSouza, M., Chanbancherd, P., Bernstein, W., Birx, D.L. and Cox, J.H., J. Virol., 76(10) (2002) 4971.
28. Zhao, R. and Collins, E.J., Arch. Immunol, Ther. Exp. (Warsz), 49 (2001) 271. Review.
29. Niedermann, G., Curr. Top. Microbiol. Immunol., 268 (2002) 91–136. Review.
30. Cresswell, P., Bangia, N., Dick, T. and Diedrich, G., Immunol. Rev., 172 (1999) 21.
31. Momburg, F. and Tan, P., Mol. Immunol. 39 (2002) 217.
32. Williams, A., Peh, C.A. and Elliott, T., Tissue Antigens, 59 (2002) 3.
33. Rammensee, Friede, Stevanovic. Immunogenetics, 41 (1995) 178.
34. Brusic, V., Rudy, G., Kyne, A.P. and Harrison, L.C., Nucleic Acids Research, 26(1) (1998) 368.
35. Apostolopoulos, V., Karanikas, V., Haurum, J.S. and McKenzie, I.F., J. Immunol., 159 (1997) 5211.
36. Apostolopoulos, V., Haurum, J.S. and McKenzie, I.F., Eur. J. Immunol., 27 (1997) 2579.
37. Apostolopoulos, V., Pietersz, G.A. and McKenzie, I.F., Curr. Opin. Mol. Ther., 1 (1999) 98. Review.
38. Daser, A., Urlaub, H. and Henklein, P., 31 (1994) 331.
39. Mandelboim, O., Bar-Haim, E., Vadai, E., Fridkin, M. and Eisenbach, L., J. Immunol., 159 (1997) 6030.
40. Apostolopoulos, V., Yu, M., Corper, A.L., Teyton, L., Pietersz, G.A., McKenzie I.F., Wilson, I.A. and Plebanski M., J Mol Biol. ,318 (2002) 1293.
41. Rubio-Godoy, V., Dutoit, V., Zhao, Y., Simon, R., Guillaume, P., Houghten, R., Romero, P., Cerottini, JC., Pinilla, C. and Valmori, D., Immunol. 169(10) (2002) 5696.
42. Purcell, A.W., Methods. Mol. Biol., 251 (2004) 291.
43. Sahin, U., Türeci, Ö. and Pfreudschuh, M., Curr. Opin. Immunol., 9 (1997) 709.
44. Sette, A., Newman, M., Livingston, B., McKinney, D., Sidney, J., Ishioka, G., Tangri, S., Alexander, J., Fikes, J. and Chesnut, R., Tissue Antigens., 59(6) (2002) 443. Review.
45. Mestecky, J. and McGhee, J.R., Adv Immunol 40 (1987) 167.
46. Sette, A. and Fikes, J., Curr. Opin. Immunol., 15 (2003) 461.
47. Slansky, J.E., Rattis, F.M., Boyd, L.F., Fahmy, T., Jaffee, E.M., Schneck, J.P., Margulies, D.H. and Pardoll, D.M., Immunity., 13(4) (2000) 529.
48. Wang, R., Wang-Zhu, Y., Gabaglia, C.R., Kimachi, K. and Grey, H.M., J. Exp. Med., 190(7) (1999) 983.
49. Guichard, G., Zerbib, A., Le Gal, F.A., Hoebeke, J., Connan, F., Choppin, J., Briand, J.P. and Guillet, J.G., J. Med. Chem., 43(20) (2000) 3803.
50. Reinelt, S., Marti, M., Dedier, S., Reitinger, T., Folkers, G., de Castro, J.A. and Rognan, D., J. Biol. Chem., 276(27) (2001) 24525.
51. Seebach, D. and Matthews, J.L., Chem. Commun., (1997) 2015.
52. Steer, D., Lew, R., Perlmutter, P., Smith, A.I. and Aguilar, M.I., Biochemistry, 41(35) (2002) 10819.
53. Steer, D.L., Lew, R.A., Perlmutter, P., Smith, A.I. and Aguilar, M.I., Curr. Med. Chem., 9(8) (2002) 811. Review.
54. Aguilar, M., Purcell, A., Webb, A., Perlmutter, P. and Stewart, K., European Peptide Symposium. Sorrento, Italy. S238, P-C53, (2002).
55. Stemmer, C., Quesnel, A., Prevost-Blondel, A., Zimmermann, C., Muller, S., Briand, J.P. and Pircher, H., J. Biol. Chem., 274(9) (1999) 5550.
56. Quesnel, A., Zerbib, A., Connan, F., Guillet, J.G., Briand, J.P. and Choppin, J., J. Pept. Sci., 7(3) (2001) 157.
57. Guichard, G., Calbo, S., Muller, S., Kourilsky, P., Briand, J.P. and Abastado, J.P., J. Biol. Chem., 270(44) (1995) 26057.
58. Calbo, S., Guichard, G., Muller, S., Kourilsky, P., Briand, J.P. and Abastado, J.P., J. Immunother., 23(1) (2000) 125.
59. Guichard, G., Connan, F., Graff, R., Ostankovitch, M., Muller, S., Guillet, J.G., Choppin, J. and Briand, J.P., J. Med. Chem., 39(10) (1996) 2030.
60. Ostankovitch, M., Guichard, G., Connan, F., Muller, S., Chaboissier, A., Hoebeke, J., Choppin, J., Briand, J.P. and Guillet, J.G., J. Immunol., 161(1) (1998) 200.
61. Webby, R.J., Andreansky, S., Stambas, J., Rehg, J.E., Webster, R.G., Doherty, P.C. and Turner, S.J., Proc. Natl. Acad. Sci. U.S.A., 100(12) (2003) 7235.
62. Beck, A., Bussat, M.C., Klinguer-Hamour, C., Goetsch, L., Aubry, J.P., Champion, T., Julien, E., Haeuw, J.F., Bonnefoy, J.Y. and Corvaia, N., J. Pept. Res., 57(6) (2001) 528.
63. Marschutz, M.K., Zauner, W., Mattner, F., Otava, A., Buschle, M. and Bernkop-Schnurch, A., Peptides, 23(10) (2002) 1727.