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Stabilising peptides of a sequential epitope of the Measles Virus Haemagglutinin protein and the implications for antigenicity and immunogenicity

Stabilisierung von Peptiden eines sequenziellen Epitops des Masernvirus Haemagglutinin-Protein und deren Auswirkungen auf Antigenität und Immunogenität

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List of Abbreviations

aa	amino acid
ABC	ammonium bicarbonate buffer
AFU	arbitrary fluorescence units
ANOVA	analysis of variance
APC	antigen presenting cell
BCE	B cell epitope
Boc	*tert*-butyloxycarbonyl
CFA	complete Freund adjuvant
ct	cholera toxin
ctb	cholera toxin B subunit
DCM	dichloromethane
DHB	2,5-Dihydroxybenuoic acid
DIC	*N*,*N*'-diisopropylcarbodiimide
DIPEA	*N*,*N*-diisopropylethylamine
DMEM	Dulbecco's modified Eagle's medium
DMF	*N*,*N*-dimethylformamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTP	diphtheria-tetanus-pertussin vaccine
DTT	dithiothreitol
EDC	*N*-ethyl-*N*'-[3-dimethylaminopropyl]carbodiimide hydrochloride
EDT	1,2-ethanedithiol
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EPT	end point titer
F	fusion protein
FACS	fluorescence-activated cell sorter
FBS-HI	heat inactivated fetal bovine serum
FID	free induction decay
FITC	fluorescein isothiocynate
FMDV	foot and mouth disease virus

Fmoc	9-fluorenylmethoxycarbonyl
FT	Fourier transformation
HCCA	3-Hydroxypicolinic acid
HIV	human immunodeficiency virus
HNE	haemagglutinin noose epitope
HOBt	*N*-hydroxybenzotriazole
HPLC	high performance liquid chromatography
H-protein	haemagglutinin protein
i.m.	intramuscular
i.p.	intraperitoneal
IFA	incomplete Freund adjuvant
IgG	immunoglobin G
IgG-AP	IgG-alkaline phosphatase
LAV	live attenuated virus
LNS	Laboratoire National de Santé
M	matrix protein
mAb	monoclonal antibody
MALDI-TOF	matrix-assisted laser desorption/ionisation - time of flight
MAP	multiple antigen peptides
MES	2-(*N*-morpholino)ethanesulfonic acid
MHC	major histocompatibility factor
MMP	matrix metalloprotease
MMR	Measles Mumps Rubella vaccine
MS	mass spectometry
MV	measles virus
MW	molecular weight
N	nucleoprotein
NDV	Newcastle disease virus
NE	neutralising epitope
NHS	*N*-hydroxy-succinimide
NMP	*N*-methylpyrrolidone
NMR	nuclear magnetic spectroscopy
NOE	nuclear Overhauser effect

NOESY	nuclear Overhauser effect spectroscopy
OD	optical density
PBS	phosphate buffered saline
RPC	reverse phase chromatography
rpm	rounds per minute
RPMI	Roswell Park Memorial Institute
RT	room temperature
s.c.	subcutaneous
SLAM	signaling lymphocyte activation molecule
SPPS	sold phase peptide synthesis
sulfo-NHS	*N*-hydroxysulfosuccinimide
TCE	T cell epitope
TCI	transcutaneous immunisation
TFA	trifluoroacetic acid
TFE	trifluoroethanol
TLR	toll like receptor
TMB	3,3',5,5'-tetramethylbenzidine
TOCSY	total correlation spectroscopy
TT	tetanus toxoid
UV	ultraviolet
WHO	World Health Organisation

List of amino acids

single letter code	amino acid name	abbreviation
A	Alanine	Ala
R	Arginine	Arg
N	Asparagine	Asn
D	Aspartic acid	Asp
C	Cysteine	Cys
E	Glutamic acid	Glu
Q	Glutamine	Gln
G	Glycine	Gly
H	Histidine	His
I	Isoleucine	Ile
L	Leucine	Leu
K	Lysine	Lys
M	Methionine	Met
F	Phenylalanine	Phe
P	Proline	Pro
S	Serine	Ser
T	Threonine	Thr
W	Tryptophan	Trp
Y	Tyrosine	Tyr
V	Valine	Val

L-amino acids are displayed with capital letters using the one-letter-code or the three-letter-code
Small caps one letter codes, small caps three-letter-code, or with “D-“prefix are used for D-amino acids
Non-natural and synthetic amino acids abbreviations are listed in Annexe 1.

Zusammenfassung

Biologisch aktive Peptide sind kurze Polymere von einer Länge von bis zu 100 Aminosäuren. Sie finden Anwendung in vielen Bereichen der Medizin, von Wirkstoffen gegen Krebs, antimikrobiellen Medikamenten bis hin zum Einsatz in der Immuntherapie. Limitierende Faktoren für die gängige Nutzung sind jedoch ihre metabolische Instabilität und strukturelle Flexibilität, sowie die für Impfstoffe erforderliche Immunogenität. In dieser Studie wurden verschiedene, bereits beschriebene, Strategien zur Modifizierung eines Peptids des Haemagglutinin Noose Epitop (HNE) des Masernvirus kombiniert, um dessen Stabilität gegenüber Peptidasen und die Immunogenität zu erhöhen.

Epitop-Peptide mit substituierten Aminosäuren wurden mit Kernspinresonanzspektroskopie untersucht. Die Strukturen einiger Peptide wurden berechnet und die Ergebnisse lieferten Informationen über Anforderungen an die Peptidstruktur zur Aufrechterhaltung der Antigenität mit HNE-spezifischen Antikörpern. Durch Anwendung dieser Methoden wurden die jeweiligen Strategien zur Erhöhung der Biostabilität und Immunogenizität des HNE-Peptid Modells angepasst.

Die Resultate zeigten, dass HNE-Peptide eine C-terminale α-Helix formen und dass Lys387 eine wichtige Rolle in der Antigenität spielt. Ausserdem kann der flexible N-terminale Bereich einiger substituierten Peptide das Binden von Antikörpern behindern, wenn das Peptid unabhängig von den Einschränkungen des Wildtyp-Proteins ist. Der Vergleich unserer Peptidstrukturen mit den kürzlich publizierten Röntgenkristallographiestrukturen vom Haemagglutinin-Protein vertieften unser Verständnis der Antigenität und bestätigten gleichzeitig die strukturelle Ähnlichkeit des Epitops im Protein und der freien HNE-Peptide.
In den Stabilitäts-Untersuchungen wurden veränderte Peptide mit einer erhöhten Stabilität gegenüber Peptidasen in Serum und Intestinalflüssigkeit erfolgreich synthetisiert. Diese Peptide mit einer Vielzahl von verschiedenen ausgetauschten Aminosäuren in der Sequenz oder Änderungen an den Peptid-Enden, reagierten weiterhin mit Epitop-spezifischen Antikörpern. Diese Peptide bewahrten ihre Immunogenität und die induzierten Antikörper konnten mit dem Wiltyp-HNE-Peptid als auch mit dem nativen Haemagglutinin-Protein

kreuzreagieren, allerdings in geringerem Maße. Trotz ihrer erhöhten Stabilität konnten diese Peptide keine bessere Immunantwort hervorrufen.
Neben den Substitutionen und terminalen Änderungen in der Peptidsequenz, wurden ein zyklisiertes und ein gerüstgebundenes HNE-Peptid analysiert. Eine erhöhte Biostabilität und eine aufrechterhaltene Antigenität und Immunogenität wurden bestätigt, jedoch erkannten die induzierten Antikörper das Wildtyp-Peptid und -Protein nicht.

Zusammenfassend zeigten die Ergebnisse dass es möglich ist die Stabilität von HNE-Peptiden deutlich zu erhöhen, aber dass es erhebliche Unterschiede zwischen Antigenität und kreuzreagierender Immunogenität gibt, ein bekanntes Problem von kurzen Peptide. Der Austausch von Aminosäuren muss bedacht gewählt warden, um die korrekte strukturelle Ausrichtung von kurzen Peptiden in Lösung oder in Gerüstkomplexen und somit ihre kreuzreagierende Immunogenität zu gewährleisten.

Abstract

Biologically active peptides, short polymers of less than 100 amino acids, are of great importance in several fields of medicine, from anticancer and antimicrobial drugs to immunotherapy. Their widespread use is however limited by several key problems such as their metabolic instability and their conformational flexibility. The use of peptides in vaccines faces the additional challenging need for immunogenicity. In this study several previously described strategies were combined to modify a peptide of the Haemagglutinin Noose Epitope (HNE) of measles virus, to improve its stability against peptidases and increase its immunogenicity.

Epitope-peptides with amino acid substitutions were analysed by nuclear magnetic resonance spectroscopy. The structures of several of these peptides were resolved and the results provided information on the conformational requirements for antigenicity with antibodies targeting the HNE. These tools have guided the efforts to enhance biostability and immunogenicity of the HNE-peptide model.

The results showed that HNE-peptides form a C-terminal α-helix and that Lys387 plays a crucial role in antigenicity. In addition a flexible N-terminal region in several substituted HNE-peptides can disrupt antibody binding, when the peptide is removed from the constraints of the wild-type protein. Comparisons of these peptide structures with recently published crystallography structures of the wild-type Haemagglutinin protein have further increased the understanding of the antigenicity and confirmed the structural similarities between the epitope in the protein and the shorter free HNE-peptides.
In the stabilisation studies modified peptides with an increased resistance towards peptidases in serum and intestinal fluid were successfully synthesised. These peptides, with a number of substitutions in the epitope sequence and modified flanking regions, retained their reactivity with HNE-specific antibodies. The peptides were also immunogenic and antibodies raised against them were capable of crossreacting with the wild-type HNE-peptide and the native Haemagglutinin protein, but with lower reactivity. Despite their increased stability these peptides did not induce a better immune response.

Additionally to substitutions and additions in the peptide sequence, a scaffold-bound and a backbone cyclised HNE-peptide were analysed and their increased stability and conserved immunogenicity was demonstrated. However, the induced antibodies failed to crossreact with the wild-type peptide and protein.

In conclusion the results showed that the stability of HNE-peptides can be significantly increased, but that there is a discrepancy between antigenicity and crossreactive immunogenicity that is inherent to these small peptides. Residue changes need to be carefully selected to guarantee the correct conformational arrangement of short peptides in solution or in constructs and thus their crossreactive immunogenicity.

Chapter 1: Introduction

1. Peptides as therapeutic agents and their role in vaccination

1.1. Therapeutic peptides

The therapeutic potential of peptides has been known for 40 years (Freidinger 2003). The clinical application of these biologically active peptides, short polymers of less than 50 or 100 amino acids in length, has been hampered by several key problems such as their metabolic instability and their conformational flexibility (Adessi and Soto 2002; Lien and Lowman 2003; Hans et al. 2006; Sato et al. 2006). Extensive peptide-based research has affected many fields of medicine and biology.

Antimicrobial/antibacterial

The current status of antibacterial peptides has been recently reviewed (Lien and Lowman 2003; Marr et al. 2006). Antimicrobial peptides are part of the innate immune system of higher organism, for example magainins, a family of peptides with antibacterial and antifungal properties, have been isolated from the skin of the African clawed frog (*Xenopus laevis*) (Zasloff 1987). More than 600 peptides able to recruit elements of the innate immune system and capable of killing microorganisms including Gram-negative and Gram-positive bacteria, viruses and funghi, have been described. The common features of these peptides are a cationic charge and an induced amphiphilic conformation since half of the residues are hydrophobic (Powers and Hancock 2003). Molecular dynamics simulations have elucidated the mode of action of antimicrobial peptides: they form a pore in the phospholipid membrane, which is permeable to both water and lipids (Leontiadou et al. 2006). The main advantage of these antimicrobial peptides is their activity against methicillin-resistant *Staphylococcus aureus* and multi-drug resistant *Pseudomonas aeruginosa* (Zhang et al. 2005). In addition there is only a remote possibility of development of complete resistance against these peptides due to their mode of action on the membrane and possibly multiple other targets (Marr et al. 2006).

Anti-cancer drugs

Peptides have various potential uses in the treatment of cancers to increase the effectiveness of current anti-cancer treatments and to lower their considerable side-effects. Peptides can act by enhancing cellular uptake and drug targeting, as well as acting as antigens in cancer vaccines (Lien and Lowman 2003). The latter are further described in the Immunotherapy section below.

Matrix metalloproteases (MMP) are capable of degrading the extracellular matrix and are thought to play a role in tumour metastasis as high MMP levels are often linked to poor prognosis (Stetler-Stevenson et al. 1993; Birkedal-Hansen 1995; Murray et al. 1998; Leeman et al. 2002). Koivunen and colleagues have reported the synthesis of cyclic peptides with selective inhibitor activity against MMP-2 and MMP-9, and potent antitumour activity (Koivunen et al. 1999).

The group of Freidinger has developed a peptide-cytotoxic drug specifically targeting prostate tumours. Prostate specific antigen (PSA) is a protease only produced in prostate epithelium and metastasised cells of prostate cancer. Antibody levels induced against circulating PSA are used as prostate cancer marker. The peptide component of the drug is a PSA-specific substrate and the proteolytic cleavage releases the cytotoxic agent in the close environment of prostate cancer cell, thus increasing drug targeting (Freidinger 2003).

1.2. Immunotherapy and the concept of subunit vaccines

Immunotherapy and more specifically subunit vaccines is the most interesting field of peptide-research relating to this work. Traditionally most vaccines are based on whole microorganism or virus antigens, either in killed or attenuated form delivered by injection. With increasing knowledge of the targets of the immune response, there has been a shift away from these classical vaccines towards subunit vaccines. These are reduced to a limited set of microbial or viral proteins or even to the smallest immunogenic part of a protein which can induce a precisely directed immune response against the infectious pathogen (Purcell et al. 2007). These minimal regions are called epitopes and in this work, an epitope of the measles virus is used as a model (Figure 1).

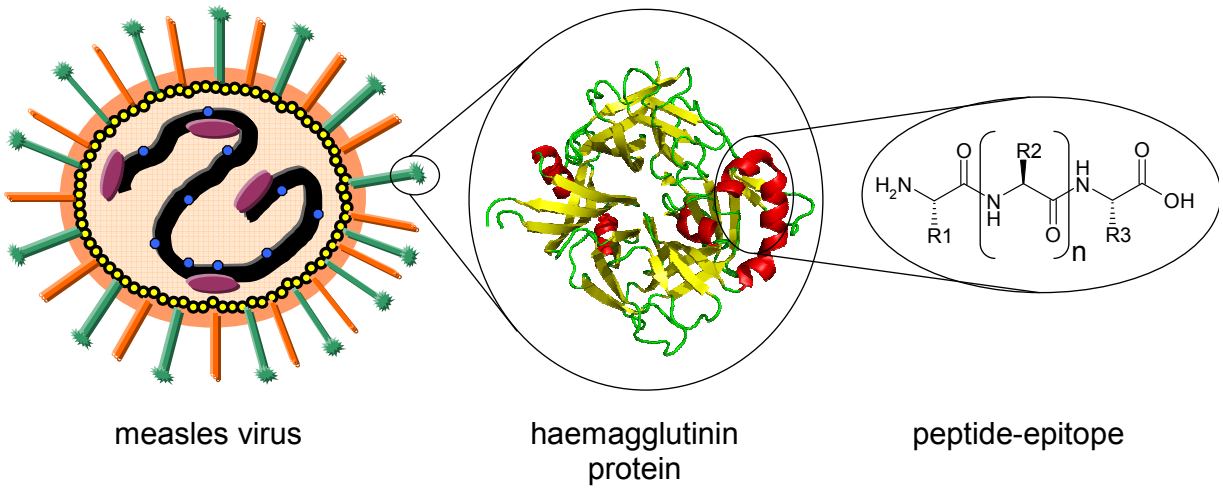


Figure 1: From whole virus to peptide-epitope

Reductionist approach to vaccine design. Starting from whole measles virus, moving to immunogenic surface Haemagglutinin protein to give an immunogenic Haemagglutinin Noose Epitope peptide, capable of inducing a crossreactive immune response.

For these synthetic vaccines to mount an effective immune response they need to incorporate two different antigenic epitopes: the T cell epitope (TCE) needs to be processed and presented by antigen presenting cells (APC) via the major histocompatibility complex (MHCI and MHCII) to activate the cell mediated cytotoxic response and combat intracellular infectious pathogens. The B cell epitope (BCE) binds to preformed cell receptors and with the help of T helper cells (T_H) leads to clonal expansion and maturation of antibody secreting B cells (Purcell et al. 2007). Even when only an antibody response against a BCE is desired, a TCE is still required to induce an effective humoral response (Hans et al. 2006).

The identification of antigenic determinant amino acid sequences are the prerequisite for the successful development of a synthetic vaccine. The methods employed to indentify these sequences are described in detail elsewhere (Zauner et al. 2001; Hans et al. 2006) and in the context of this work, priority is given to the selection of BCEs. Whereas TCEs are purely sequence dependent, the antibody reactive BCEs are related to the structure of the native antigen. These structural recognition patterns are usually located at the solvent exposed sites of the native proteins and can be formed by continuous amino acid residues, i.e. by sequential residues in the primary amino acid sequence, or by discontinuous sequences. These discontinuous BCEs are spatial arrangements of residues which are not closely located in the primary sequence, but brought together by the folding of protein chains into the secondary and tertiary structure, stabilised by disulfide bonds or non covalent interactions (Zauner et al.

2001). These epitopes are much more difficult to recreate synthetically, nevertheless this has been possible in the case of foot and mouth disease virus (FMDV) epitopes (Villen et al. 2004). Continuous BCEs can be indentified by PEPSCAN, a method by which a library of synthetic peptides with overlapping sequences are screened for their reactivity with sera from mice immunised with the infectious pathogen (Hans et al. 2006). This method cannot be applied to discontinuous BCEs and thus the identification of these BCEs is more problematic (Zauner et al. 2001).

1.3. Use of synthetic peptide-based vaccines in immunotherapy

There has been extensive research into peptide-based vaccines, but given the long time required for the development of these therapeutic vaccines and the only recent advances in the field, these synthetic vaccines are only expected to hit the market in the near future.

Cancer therapy

As noted above, peptides can be used in cancer therapy to enhance drug targeting. Another attractive possibility is vaccination against cancers. The strategy of anti-cancer vaccination is based on the observation that cancerous cells express cell surface antigens not present on healthy cells. These tumour associated antigens can be used to develop synthetic peptide-based vaccines that specifically target cancer cells and by upregulating the cytotoxic immune response, can effectively combat tumours (Sahin et al. 1997). Vaccines against a multitude of cancers including cervical, lung, breast, colorectal, leukaemia and melanoma cancer, are currently in development and have been reviewed elsewhere (Hans et al. 2006; Pietersz et al. 2006).

Allergies and other diseases

Larché has reviewed the recent advances in immunotherapy for allergic diseases where peptide therapy showed promising results in preclinical studies against cat allergens, insect venoms and autoimmune diseases and has lead to first clinical studies in humans (Larché 2007). There has also been an increased interest in vaccination against Alzheimer, a disease characterised by the deposition of extracellular β-amyloid (Aβ)-containing plaques. Human

clinical trials with Aβ42 containing peptide-vaccines were effective in reducing plaque formation but had to be prematurely halted because of severe side-effects. New refined peptides vaccines are currently undergoing clinical trials (Hawkes and McLaurin 2007).

Viral infections

The second biggest research area of peptide-based vaccines after anti-cancer vaccines is the vaccination against viruses, further advancing our understanding peptide/immune system interactions. Examples of peptide-vaccines in development include peptide-epitopes of the surface glycoprotein of human respiratory syncytial virus (Beck et al. 2007), influenza, herpes simplex virus (Purcell et al. 2007), human papilloma virus and of course several peptide-epitopes from the surface proteins (gp120 and gp41) of HIV (Hans et al. 2006).
Even though no peptide-based vaccine has yet been licensed for the human market, the feasibility and proof of concept have been confirmed by the successful vaccination of swine against FMDV (Wang et al. 2002). The peptide used in this vaccine is based on an epitope of the VP1 protein (Bittle et al. 1982) and short synthetic peptides of this region were found to induce neutralising antibodies against all seven serotypes of FMDV (Francis et al. 1990).

1.4. Advantages and disadvantages of peptides-based vaccines

Synthetic peptide-based vaccines offer a number of significant advantages over conventional vaccines, which are based on whole organisms, cells or viruses and are heterogeneous mixtures of chemicals and biological materials. These can cause important side-effects and there is concern about the risk of reversion to virulent forms of live attenuated virus vaccines (Johnson 1999; Greensfelder 2000). In addition not all pathogens can be modified by these classical procedures (Zauner et al. 2001; Purcell et al. 2007). The controlled chemical synthesis of peptides eliminates the risk of infectious diseases and side-effects thus increasing safety. The use of several peptides of the same epitope or several epitopes of the same pathogen can potentially bypass genetic restriction arising from mutations in different virus strains (Table 1).

Table 1: Advantages and drawbacks of peptide-based vaccines adapted from Purcell (2007)and Hans (2006)

Advantages	Disadvantages
Absence of infectious material	Low chemical stability of native peptides
Homogeneous and chemically pure	Low conformational stability
Economically large scale synthesis	Poor immunogenicity of simple peptides
Freeze-dried storage (avoids need for cold-chain)	Need for T cell stimulation
No risk of reversion to virulent strain	Limited availability of carriers and adjuvants
(live attenuated viruses)	HLA restrictions for T cell epitopes
No risk of genetic integration	
(DNA-Vaccines)	
Multiple epitopes of one or more pathogens	
Stabilisation by amino acid modification	
Improved quality control	

The development of peptide-based vaccines is hampered by three main interconnected factors: poor immunogenicity, chemical and metabolic instability. While the low immunogenicity of peptides is in fact a favourable and desirable aspect in the context of peptide drugs, it is the pivotal factor in peptide-vaccine design. The poor immunogenicity of peptides is a result of their chemical and conformational instability. Short peptides are rapidly removed from the circulating system by renal clearance and their *in vivo* stability is reduced by proteolytic degradation. Amino- and carboxyexopeptidases cleave single amino acids, dipeptides or tripeptides from the N- and C-termini, respectively, and endopeptidases cleave peptide bonds within the peptide sequence. Proteases do not cut peptides with discrete secondary structure without unwinding and reverting them to random coils that fit into their β-strand preferring active site (Tyndall and Fairlie 1999; Fairlie et al. 2000). Short peptides rarely display a defined secondary structure in solution. It is estimated that the longer peptides are, the more likely they are to fold into complex structures, burying a number of residues and thus increasing the stability towards proteolytic enzymes (Hans et al. 2006). Using this approach conformationally restricted epitopes appear to induce better immune responses than the linear epitopes (Kaumaya et al. 1992; Sundaram et al. 2004; Dakappagari et al. 2005). The relative influence of purely chemical stability and conformational stability becomes blurred as each one influences the other.

In addition to the above listed concrete disadvantages there are doubts that a rational design of peptide-vaccines based purely on epitopes and the corresponding contact residues, is

achievable. This theory focuses on the relationship between peptide antigenicity, i.e. its ability to specifically interact with the antibody paratope, and crossreactive immunogenicity, i.e. the ability of the peptide to induce an immune response capable of recognising the parent protein. It is feasible to modify a peptide-epitope to increase binding to a monoclonal antibody that specifically recognises the epitope's native parent protein. While this reductionist approach down to purely chemical interactions, is possible with respect to antigenicity, the situation with immunogenicity is however different since it relies on complex interactions between various parts of the immune system. A neutralising antibody can trap a flexible peptide-epitope in a conformation corresponding to the structure of the native protein. Such an engineered peptide is unlikely to induce crossreactive antibodies against the parent protein, since its many possible conformations are recognised by a multitude of B cells. The affinity maturation is not limited to a single stable conformation but targets a variety of structures, thus weakening the specific response (Van Regenmortel 1999, 2001b, 2001a; Dormitzer et al. 2008).

Despite these warranted doubts and conceptual difficulties, synthetic peptides mimicking BCEs of FMDV and canine parvovirus have been successfully used for vaccinating the respective animals (Langeveld et al. 1994; Wang et al. 2002). Also peptide-epitopes of measles were shown to induce neutralising and protective antibodies (Obeid et al. 1995; Atabani et al. 1997; Putz et al. 2004).

1.5. Engineering peptide-based vaccines – Stabilisation of peptides

Several strategies have been developed to enhance the metabolic and conformational stability of peptides with the aim of improving immunogenicity (Figure 2). Cocktails of purified proteases generally do not mimic the complex metabolic behaviour of peptides *in vitro* and the use of homogenates, plasma or serum are more appropriate to investigate the stability of peptides (Adessi and Soto 2002). The thought is that the more stable a peptide is, the more persistently it can be presented and processed, thus increasing its immunogenicity (Delamarre et al. 2006). There are numerous strategies to improve the stability, conformation and immunogenicity of peptides-vaccines and only the most relevant will be described here. For a complete and extensive list of modifications the reader is referred to recent reviews (Adessi and Soto 2002; Webb et al. 2003; Werle and Bernkop-Schnurch 2006; Purcell et al. 2007)

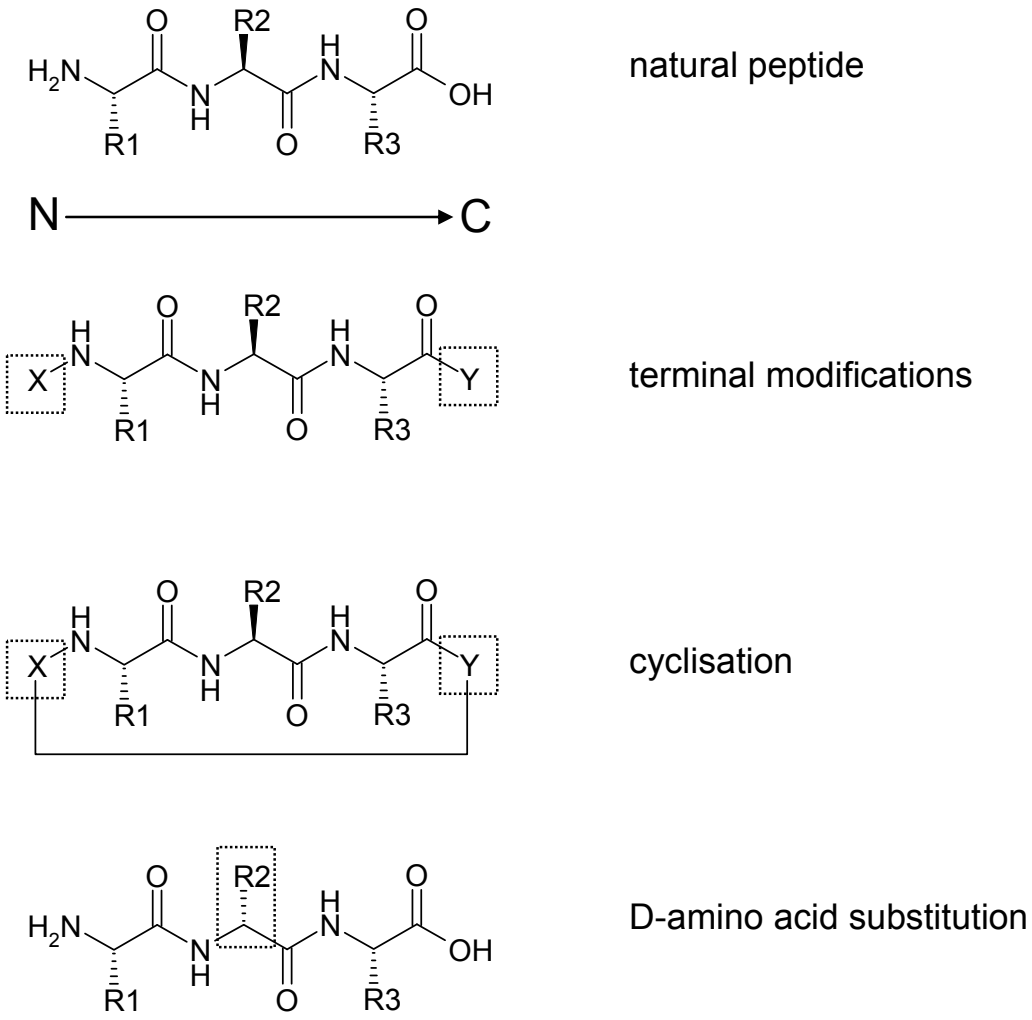


Figure 2: Common peptide modifications to increase proteolytic stability

The location of modifications introduced into linear peptides are illustrated. X and Y represent any chemical group or atom. R1, R2 and R3 denote sidechains of natural amino acids. Figure adapted from (Adessi and Soto 2002)

Chemical modifications

Chemically modified peptides can be divided into three groups, depending on the importance of the changes to the original sequence and chemistry: (i) modified peptides are peptide derivatives with only small modifications which conserve the peptide bonds and the chemical nature of peptides. (ii) Pseudopeptides contain mostly peptide bond modifications and replacements. (iii) Peptide mimetics are organic molecules that mimic the activity of peptides but contain no peptide bonds. In line with the modifications applied in this study the focus

will be mainly on modified peptides. The two other groups are reviewed elsewhere (Adessi and Soto 2002).

N- and C-terminal modifications

Natural L-amino acid peptides with free N- and C- termini are rapidly degraded in serum. The stability of many neuropeptides is known to be dependent on acetylation and amidation, and some hormones and peptides are naturally end-protected. These termini modifications have been widely employed to block exopeptidase activity and to increase peptide drug biostabilities. Mallière and colleagues found that terminal acetylation or amidation of a 13 residue peptide increased its stability compared to non-modified peptide and that the combination of the two modifications further stabilised these peptides (Maillère et al. 1995). A similar improvement was achieved in a pentapeptide (Heavner et al. 1986) and by alkylation of N-terminal amide (Marschütz et al. 2002). Terminal conjugation with polyethylene glycol (PEG) increases the overall size of peptides, decreases renal clearance and also protects peptides against exopeptidase degradation, however the lowered immunogenicity of some conjugated peptides make this modification only attractive for peptide drugs and not for peptide-vaccines (He et al. 1999; Werle and Bernkop-Schnurch 2006).

Cyclisation

Cyclisation of peptides can function as chemical and/or conformational stabilisation. The conformational stability is increased by constraining the peptide in a conformation potentially more closely related to the native protein (Putz et al. 2003b). Cyclised peptides can be formed either by combining the terminal residues of a peptide sequence or by sidechain-sidechain linkage of residues within the sequence. Cyclisation can be achieved by reducing cysteines sidechains, by the formation of ester, ether or thioether bridges, and terminal residues can be backbone cyclised to give an additional peptide bond. The effects of cyclisation on peptides have been reviewed (Adessi and Soto 2002; Werle and Bernkop-Schnurch 2006) and cyclisation is an accepted method for reducing proteolytic cleavage and prolonging biostability (Clark et al. 2005). Tugyi and colleagues compared the biostabilities of thioether, peptide bond and disulfide cyclised peptides compared to their linear analogue and found them to decrease in stability as listed (Tugyi et al. 2005a). It should be noted that cyclisation

does not always lead to more reactive analogues of linear peptides, as has been shown by the cyclisation of a Herpes simplex virus epitope (Schlosser et al. 2003) and anthopleurin A epitope (Gould et al. 1992).

Amino acid substitution

Another strategy to improve the biostability of peptides is the replacement of susceptible amino acid residues. The substitution residue can be another natural amino acid with a chemically similar sidechain or a modified natural amino acid, in order to change the recognition site of proteases yet preserve the activity or antigenicity and immunogenicity of the peptide (Adessi and Soto 2002). For example methylated Lys in histones are known to play an important role in gene regulation and signal transduction (Morgunkova and Barlev 2006; Paik et al. 2007) and have been shown to increase the stability of microbial peptides (Na et al. 2007). Alternatively substitutions with non-natural amino acids such as D-isomers and β-amino acids are also known to increase the biological stability of peptides, though their chirality and backbone elongation can lead to loss of activity or antigenicity. In the context of peptide-based vaccines, the need for conserved antigenicity and structural similarity with the parent protein precludes substitution of critical contact residues with D-amino acids. But substitution of non-conserved or flanking residues is an interesting approach. Powell and colleagues increased the biostability of several peptides by replacing the L-amino acid residues at both termini with their respective D-isomers (Powell et al. 1993). D-amino acids substitutions of terminal residues of antimicrobial peptides have also increased the proteolytic stability in serum and selected proteases without affecting the antimicrobial activity (Hong et al. 1999; Hamamoto et al. 2002). Manea and colleagues added D-amino acids and β-amino acids as non native flanking regions to a plaque specific β-amyloid epitope and observed the same antigenicity than native epitope (Manea et al. 2008). Substitution of flanking residues with D-amino acids in an epitope of mucin glycoprotein (MUC2) improved the stability of peptides in serum and lysosome homogenates, while preserving antigenicity (Tugyi et al. 2005b).

In addition to the above, some more extensive modifications have been employed in the design of pseudopeptides. Amide bond reduction from the natural amide (CO-NH) to a aminomethyl (CH_2-NH) in a glycoprotein TCE of lymphocytic choriomeningitis virus, substantially increases stability of this peptide *in vivo* and maintains MHC binding (Stemmer

et al. 1999). Recently Reuter and colleagues synthesised a series of retro-inverse peptides based on chicken ovalbumin BCEs. Simultaneous reversion of chirality and sequence (Chorev et al. 1979), lead to a peptide that is structurally more related to its L-amino acid parent peptide than the D-analogue. These peptides displayed an increased stability in murine intestinal fluid compared to the natural peptides. Immunisation with these retro-inverse peptides induced similar anti-peptide antibody titres than their normal analogues, but failed to crossreact with the native ovalbumin protein (Reuter 2008).

Conformational stability

Conformational stability can be increased by conjugating the flexible peptide-epitope to a scaffold. Steroids have emerged as an attractive scaffold system due to their rigidity and the possibility of variable functionalisation (Barry et al. 1999; Salunke et al. 2006) and bile acids have attracted a lot of attention for peptide-conjugation (Kramer et al. 1997). Such a steroidal bile acid conjugate incorporating the HNE peptide was synthesised in a collaboration between our laboratory and the University of Ghent by Cathy Bodé (Bodé et al.; Bodé 2007; Bodé et al. 2007).

1.6. Increasing the immunogenicity of peptides

As mentioned before, peptides are only poorly immunogenic due to their size related inherent flexibility and the low biostability. Besides increasing the chemical and conformational stability, the immunogenicity of peptides can be increased by several mechanisms.

Conjugation to carrier proteins

There are suggestions that conjugating labile peptides to poorly digestible carrier proteins can partially protect them from proteolytic cleavage (Delamarre et al. 2006). Coupling of atrial natriuretic peptide to human serum albumin has been shown to increase it's stability (Léger et al. 2003). Reuter and colleagues have increased the stability of ovalbumin derived peptides in muine intestinal fluid by coupling them to cholera toxin B (Reuter 2008). Peptides can be conjugated to a large variety of molecules to increase their immunogenicity, for animal experiments protein carriers such as bovine serum albumin (BSA), keyhole limpet

hemocyanin (KLH), tetanus toxoid (TT) and diphtheria toxoid (DT) are often used. (Zauner et al. 2001; Lien and Lowman 2003). These carrier proteins can also supply TCE, which are essential for a immunological memory (Zauner et al. 2001).

Oligomerisation

Branching several peptides onto a central polylysine core, via α and ε amino groups, form so-called multiple antigen peptides (MAP) which can overcome the ineffectiveness of single linear peptides to induce an immune response (Tam 1996). Alternatively peptides can be assembled into linear chimeric constructs. Branched polyepitope constructs have been shown to induce higher antibody titres than carrier-conjugated peptides, with a significantly reduced response against the carrier molecule (Hudecz 2001). These MAP also allow the coupling of various peptides of the same epitope and peptides of different epitopes of the same or various pathogens, thus potentially overcoming the genetic variability of some pathogens such as HIV (Hewer and Meyer 2005).

Adjuvants

Adjuvants are substances that trigger or enhance an immune response of antigens that would under normal circumstances only be poorly immunogenic. As vaccine development moves away from killed or attenuated whole pathogens towards increasingly pure antigens, with poorer immunogenicity, there is an increasing and vital need for adjuvants to help elicit clinically relevant immune responses. Over the past decades there has been a large expansion in the search of new adjuvant (Table 2), yet despite this, only few adjuvants, including Alum (aluminium salts) and MF59 (a squalene based oil in water formulation) are currently licensed for human use because of stringent regulations. Immunopotentiating reconstituted influenza virosomes (IRIVs) incorporate peptides or proteins on the surface of influenza virus like particles. A hepatitis A vaccine using this adjuvant has been recently licensed for human use (Gluck 1999; Moreno et al. 2001; Westerfeld and Zurbriggen 2005). Alum was first used in 1926 and has dominated the adjuvant market ever since (Glenny et al. 1926). Even though there have been a number of new adjuvants that proved to be as effective as or even more than Alum, they were deemed unsuitable for human use due to local and systemic toxicity. The most notable examples are Freund's adjuvant (Freund et al. 1937) and lipopolysaccharide (LPS) (Johnson et al. 1956). Freund's adjuvant is a water in mineral oil emulsion (incomplete

Freund's adjuvant) that can be complemented with heat-killed mycobacteria (complete Freund's adjuvant) and has been routinely used in veterinary medicine and experimental immunology for decades because of its very strong adjuvanticity and ease of use. Tolerance issues have lead to the development of several substitutes (O'Hagen 2000).

Table 2: Short overview of current adjuvant classes

Adjuvant family	Adjuvant example	References
Mineral salts	Aluminium hydroxide	(Glenny et al. 1926)
	Aluminium phosphate	(Gupta and Siber 1994)
	Calcium phosphate	(He et al. 2000)
Bacterial products	Cholera toxin B subunit	(Holmgren et al. 1993)
	CpG oligonucleotides	(Harandi 2004; Agger et al. 2006)
	E. coli heat-labile enterotoxin non-toxic variants LTK63 LTR72	(Partidos et al. 1996; Giuliani et al. 1998; Tierney et al. 2003)
	Monophosphoryl Lipid A	(Schneerson et al. 1991)
	Muramyl dipeptide (MDP)	(Cohen et al. 1996)
Emulsions	Freund adjuvant	(Freund et al. 1937)
	Montanide	(Aucouturier et al. 2002; Halassy et al. 2006)
	MF59	(Granoff et al. 1997)
Microparticles	virosomes	(Gluck 1999; Moreno et al. 2001)
	ISCOMS	(Sjolander et al. 1997)
Surface-active reagents	Saponins (QUIL-A, QS-21)	(Dalsgaard 1984; Jackson and Opdebeeck 1995; Wong et al. 1999; Boyaka et al. 2001)
Cytokines	Interleukins	(Lynch et al. 2003)
	Interferons	(Odean et al. 1990)

The ideal adjuvant should be non toxic, non immunogenic, generate a depot at the site of inoculation, slowly release the antigen, target antigen presenting cells, etc. (O'Hagen 2000; Zauner et al. 2001). By differently stimulating Th1 and Th2 T-helper cells, adjuvants can modulate the immune response of the host. Despite the increased knowledge of the immune system and the use of adjuvants for decades, their mechanisms of actions are still very poorly

understood. The family of toll like receptors (TLRs) has emerged as a key element of the reactivity of adjuvants (Seya et al. 2006), suggesting that adjuvants activate the innate immune response. Several adjuvants are known to act as TLR activators or ligands such as unmethylated CpGs and LPS, and initiate a Th1 mediated immune response. Though Alum, which stimulates a Th2 response, does not exercise its function through TLRs (Schnare et al. 2001). Until recently it was also thought that Alum exerted its adjuvant function by depot effect , but this has since been disputed (Gupta et al. 1996).

The choice of adjuvants depends on the antigen, the desired immune response and the route of inoculation. With the large variety of adjuvants currently in development or clinical trials, this choice is not simple. A description of the current adjuvants in development goes beyond the scope of this thesis and the reader is referred to recent reviews on adjuvants (Powell and Newman 1995; O'Hagen 2000; Vogel 2000; Zauner et al. 2001; Aguilar and Rodriguez 2007; Del Giudice and Rappuoli 2007). In an effort to further increase their adjuvanticity, many of these adjuvants have been combined and these mixtures of two or more adjuvants are reviewed elsewhere (Fraser et al. 2007).

2. Measles virus epitopes as models for peptide-subunit vaccines

2.1. Family and history

Despite the availability of an effective vaccine, measles, caused by the measles virus (MV), remains one of the most important causes of childhood mortality and morbidity worldwide with around 200,000 deaths and 20 million infected annually particularly in the developing world (WHO 2008). MV is a morbillivirus of the family of paramyxoviridae, which includes peste des petits ruminants, dolphin and porpoise morbillivirus, canine and phocoid distemper virus. The close phylogenetic relation to rinderpest virus suggests an ancestral virus evolving during the early stages of colonisation with close proximity of humans and cattle (Norrby et al. 1992). Measles transmission studies in island populations suggests that the critical community size is 300,000 to 500,000 (Keeling 1997); population sizes of this magnitude were first reached in ancient Egypt and Sumaria around 3000 BC (Griffin 2007). Measles was first precisely described by Abu Becr, an Arab physician of the 9th century also known as Rhazes of Baghdad (Rhazes 1748; Redd et al. 1999) and has caused millions of deaths over the last thousands of years, making it one of the most important human infectious diseases (Griffin 2007).

In the 16th century the disease, together with smallpox, played an important role in the conquest of the Americas, disseminating the native Indian population and facilitating the colonisation (McNeill 1976). Humans are the only natural reservoir of MV (Griffin 2007). The first isolate of MV, the Edmonston strain, was cultured by Enders and Peebles in 1954 from the blood of an infected child, David Edmonston (Enders and Peebles 1954)

2.2. Disease and complications

MV is transmitted from infected persons to susceptible individuals by aerosols or respiratory droplets and enters the organism via the respiratory route, where it infects and replicates in the epithelial cells. 10-14 days after measles infection the first non specific symptoms of measles disease appear: fever, cough, coryza and conjunctivitis. Following the 2-3 day prodrome period, the characteristic maculopapular rash starts on the face and behind the ears and then spreads centrifugally to the trunk and extremities. The rash lasts for 3-4 days and coincides with the immune response, clearance of virus and clinical recovery. Recovery from natural

measles infection is accompanied by a lifelong immunity and hence measles is considered a childhood disease since older members of a community are protected through previous exposure to the virus (Ota et al. 2005; Moss and Griffin 2006; Griffin 2007).

In 60% of cases, patients remit uneventfully from measles, however malnutrition and age-extremes increase the risk of complications caused by the immune suppressive character of measles, which renders patients more susceptible to secondary viral and bacterial infections (Ota et al. 2005). Pneumonia and diarrhoea are the most common complications with the former accounting for 56-86% of measles fatalities (Duke and Mgone 2003).

Rare but serious complications of measles infection can occur in the central nervous system within 2 weeks (Post-measles encephalomyelitis). Measles inclusion body encephalitis (MIBE) and subacute sclerosing panencephalitis (SSPE) can occur months and even 5-15 years, respectively, after acute infection and are caused by persistent MV infection (Moss and Griffin 2006).

2.3. Genome, Proteins and Structure

Measles virus is a negative sense single strand RNA virus of 100-300 nm diameter, whose 16,000 nucleotides contain 6 genes encoding 8 proteins: Nucleoprotein (N), the transmembrane glycoproteins haemagglutinin (H) and fusion (F), matrix protein (M), phosphoprotein (P), large protein (L) as well as 2 non-structural proteins, C and V (Figure 3) (Griffin 2007).

Nucleocapsid protein is first expressed and is the most abundant protein. It self assembles, complexes with L and P, and binds the viral RNA to form the nucleocapsid, the replicase complex of the virus. Nucleocapsid protein is an omnipresent antigen in infected cells and is the first target of the immune response, though antibodies are not neutralising due to it's unavailability at the virus surface (Graves et al. 1984). The viral envelope is formed by matrix protein and cellular lipid bilayer containing haemagglutinin and fusion proteins.

M is a basic protein with several hydrophobic domains and links the nucleocapsid ribonucleoproteins with the envelope proteins during virion assembly. M-protein plays an important role in virion generation and budding (Griffin 2007) but when it is associated with the ribonucleoprotein core, it inhibits transcription (Suryanarayana et al. 1994).

The F-protein is a type I transmembrane glycoprotein and is the most highly conserved protein of MV. It is anchored in the virus membrane via the hydrophobic C-terminal tail and plays a role in cell-cell fusion. Fusion can be inhibited by the presence of anti-F antibodies. A

Cys rich region (F337-381), conserved throughout the paramyxoviridae family, interacts with H-protein facilitating virus-cell fusion (Griffin 2007).

The haemagglutinin protein is a 617 amino acid long type II transmembrane protein expressed on the surface of the virus and infected cells. The N-terminal domain is essential for transport to the cell membrane and acts as a membrane anchor. It appears as disulfide linked homodimers which associate into tetramers on the cell surface (Hu and Norrby 1994). The C-terminal ectodomain of H with its 13 highly conserved cysteine residues, binds to receptors on the cell surface and constitutes the first step in measles infection (Devaux et al. 1996). This event is followed by a functional cooperation with F allowing viral entry into the cell by attaching and fusing viral envelope to host cell membrane (Moss and Griffin 2006; Griffin 2007). Initially CD46, a complement regulatory molecule expressed on all nucleated human cells, was identified as a cellular receptor (Naniche et al. 1993). Tatsuo and colleagues also identified SLAM (lymphocyte activation molecule; CD150) as an additional receptor (Tatsuo et al. 2000). Phylogenetic analysis of MV strains show that wt and vaccine strains both use SLAM but that vaccine strains have evolved to use ubiquitously expressed CD46 as well. There are suggestions that H-protein is not the only receptor binding protein of measles virus and that other MV proteins are also capable of facilitating virus entry (Yanagi et al. 2006). In addition *in-vitro* infection independent of CD46 and SLAM have been reported suggesting the role of not yet identified (inefficient) ubiquitous receptors (Andres et al. 2003; Griffin et al. 2008).

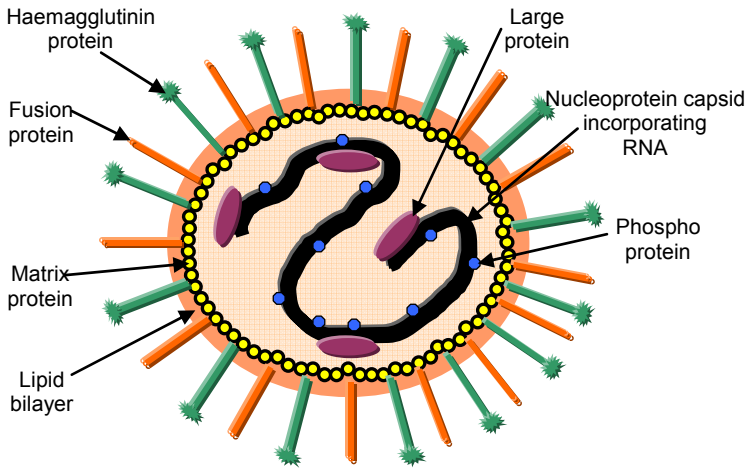


Figure 3: Schematic representation of MV

The core of the measles virus consists of a ribonucleoprotein complex incorporating N-protein encapsulated RNA associated with the polymerase complex of phosphoprotein and the large protein. The ribonucleocapsid is connected via M-protein to an enveloping lipid bilayer incorporating viral transmembrane glycoproteins F and H.

2.4. Immune response to MV infection

The wt virus induces a humoral and cellular response leading to live-long immunity against reinfection (Panum 1938; Black and Rosen 1962; Graves et al. 1984). Passive immunisation with anti-measles antibodies protects against virus infection suggesting that a humoral response is sufficient to avoid infection, as demonstrated by the protection of infants by maternally transferred antibodies (Albrecht et al. 1977). However cellular immunity is crucial for virus clearance after infection (Kaplan et al. 1992). Children with agammaglobulinaemia (a disease state in which B-lymphocytes fail to produce antibodies), are able to recover from MV infection while defects in T-lymphocyte function lead to severe or fatal disease (Moss and Griffin 2006).

Specific antibodies can be detected at the onset of rash, with IgM appearing first and lasting for about 8 weeks. IgG and IgA are detectable a few days after onset and remain for life. IgG antibodies are directed against most viral proteins N, M, F and H, with the majority targetting N, the most abundant protein of MV (Graves et al. 1984). Only anti-H and anti-F antibodies contribute to the neutralisation of the virus (de Swart et al. 2005) and the highest proportion of neutralising antibodies is directed against H–protein (McFarlin et al. 1980; Giraudon and Wild 1985).

The immune response against measles is mediated by CD8+ and CD4+ cells. CD8+ cells are activated during the prodrome. CD4+ Th1 response is essential for virus clearance while a subsequent Th2 response promotes the induction of protective MV-specific antibodies (Griffin 1995). The strong efficient and long lasting immune response against MV is in stark contrast to the ensuing immune suppression that can last for several months (Tamashiro et al. 1987) and increases the susceptibility towards secondary infections. While the exact mechanism of immune suppression remains poorly understood, several parts of the immune system seem to be involved.

2.5. MV-vaccine

The first vaccine against measles was developed at the end of the 1950s using the Edmonston strain. The virus was passaged in primary kidney and amnion cells followed by chick embryo cells and chick fibroblasts (Bellini et al. 1994). This passaged virus induced immunity against measles causing no or only mild clinical symptoms. No neurological sideffects, frequently

associated with measles infection, were observed after intracerebral inoculation of monkeys with the adapted virus (Enders et al. 1960). This first attenuated live vaccine (LAV) (Edmonston B) was patented as a vaccine in 1963. Later on, several attenuated vaccine strains were developed on the basis of wt Edmonston strain. The majority of these strains are still being used as vaccine: the Moraten strain is used primarily in the US whereas Schwarz is used throughout the world. Nucleotide analysis of M, N, H and F sequences showed no more than 0.6% differences between the various vaccine strains derived from Edmonston strain (Redd et al. 1999).

Prior to vaccination an estimated 5-8 million fatalities were attributed annually to measles with 80 million infected. This trend was significantly reduced with the joint efforts of vaccination, the increased nutritional status and the use of antibiotics against secondary bacterial infections. Today the vaccine is routinely injected to 9-15 months old children throughout the world, often together with Mumps and Rubella vaccine (MMR-vaccine). The vaccine has a high seroconversion rate, induces long-term protection against MV infection after a single dose and has an excellent safety record (Redd et al. 1999). As one of the most successful and cost-effective medical intervention it has resulted in the interruption of indigenous measles circulation in a number of developed countries (Finland (Heinonen et al. 1998) and US (CDC 1997)). Several developing countries, especially in Latin America and southern Africa, have made significant progress towards eliminating measles (de Quadros et al. 1998; Biellik et al. 2002).

Vaccination is generally accepted to result in long lasting immunity, accompanied with the standard rate of vaccine failure (Markowitz et al. 1990; Cutts et al. 1999; Redd et al. 1999). Observation in isolated insular population like Palau have shown that after 27 years of no known re-exposure to measles, antibody waning did not increase the risk of infection of vaccinees. But they confirmed that a second administration of vaccine was more protective than single doses (Guris et al. 1996). There have however been reports of measles outbreaks and subclinical measles in highly vaccinated populations (Gustafson et al. 1987; Pedersen et al. 1989; Yeung et al. 2005).

2.6. Drawbacks of the current measles vaccine

The success of the current measles vaccine has lead the WHO to target measles for eradication. Mathematical models estimate that in developed countries herd immunity is likely to be lost and endemic transmission of measles possible when herd immunity drops

below 93-95% (Meissner et al. 2004). Despite the advantages mentioned previously, the current LAV has some important drawbacks that may make it difficult to achieve such levels in the final stages of measles elimination.

1) The common drawback of currently used attenuated measles vaccines is their thermal and chemical instability, requiring a continuous cold-chain and protection from sunlight; factors hindering deployment in third world countries.

2) Since measles vaccine have to be administered subcutaneously or intramuscularly specialised health workers are required and hazardous waste of needles and syringes needs to be properly disposed of (Moss and Griffin 2006).

3) Contrary to the high efficacy in adults and children immunisation with LAV of infants is coupled with a weak immunogenicity (Albrecht et al. 1977; Markowitz et al. 1990; Redd et al. 1999). The reduced efficacy is caused by the presence of interfering maternal anti-MV antibodies (Albrecht et al. 1977; Osterhaus et al. 1998) and by the immaturity of the immune system even in the absence of maternal antibodies (Gans et al. 1998; Gans et al. 2001).
Maternally transferred antibodies via the placenta during gestation protect infants after birth from a variety of infections. The presence of anti-measles maternal antibodies interferes with seroconversion during LAV vaccination by *in vivo* neutralisation prior to vaccine virus replication (Hayden 1979). While early vaccination is complicated by low seroconversion, delayed vaccination to improve seroconversion leaves an important fraction of infants without protection if maternal antibodies wane prematurely, until routine vaccination at 15 months is performed. Waning of maternal antibodies has been reviewed (Caceres et al. 2000; Leuridan and Van Damme 2007). Premature waning of maternal antibodies leads to a susceptibility gap during which infants and children are at an increased risk of measles infection. Studies in Nigerian infants showed that less than half remain protected by maternal antibodies at the age of 4 months (Oyedele et al. 2005) and that there is a considerable difference between maternal antibody waning in Nigerian and German infants, as infants from developed countries remained protected for a longer period (Hartter et al. 2000). To compensate for this observed earlier loss of protection and the danger posed by circulating MV, infants in developing countries are vaccinated at 9 months of age (Fagbule and Orifunmishe 1988). Despite the success of elimination programmes in Latin America a large outbreak in 1997 originating in São Paulo and spreading through other countries showed that under 1 year old and young

adults were most affected (Cutts et al. 1999) A recent study (Martins et al. 2008) suggested that a new group of children may already lose protective immunity by maternal antibodies at the age of 3-5 months. While the authors admit that their study was relatively small, they observed a vaccine efficacy of over 80% in 4.5 month old children. However earlier vaccination combined with the observed antibody waning in vaccinated individuals raises questions about the durability of immunity (Putz 2004; Kremer et al. 2006b). Infants in the developed world are protected by herd immunity and the first vaccine injection is administered at 15 months of age.

4) Several studies have shown that remission from measles results in higher antibody titres than compared to vaccination (Christenson and Bottiger 1994; Bouche et al. 1998; Damien et al. 1998)

5) Antibody titres raised via vaccination wane faster than those from naturally infected persons (Krugman 1983; Christenson and Bottiger 1994; Davidkin and Valle 1998). This phenomenon is likely to be further accentuated by the lack of boosts from subclinical infections (Davidkin and Valle 1998; Bennett et al. 1999) by circulating virus as measles incidence is reduced. Kremer and colleagues suggest that contact with circulating wt virus plays a role in maintaining antibody levels in late convalescent patients (Kremer et al. 2006a). At the same time there are fears that vaccinees with incomplete immunity can transmit measles during the subclinical phase (Mossong et al. 1999; Whittle et al. 1999).

6) The widespread distribution of HIV in the developing world has implications on measles vaccination. A number of studies have reported a lower level of passively transmitted maternal antibodies against MV at birth in infants born to HIV-1 seropositive mothers, thus widening the window of susceptibility. These children were at a 3.8 fold higher risk of acquiring measles before immunisation at 9 months (Embree et al. 1992; Moss et al. 1999; Moss et al. 2007; Scott et al. 2007). HIV-1 infected infants reacted better to vaccine administration at 6 months of age than at 12-15 months presumably because at 6 months, infants were not yet immunocompromised (Rudy et al. 1994; Arpadi et al. 1996). In light of these findings the WHO has recommended vaccination of HIV-1 infected infants at 6 months and a second dose at 9 months, yet the unknown status of HIV-1 infection in infants render this policy difficult to implement (Scott et al. 2007).

Despite initial concerns MMR vaccination had long been recommended for HIV infected children and generally considered safe (Onorato et al. 1989) until in 1994 an HIV-infected 21-year old man with haemophilia A died 10 months after receiving his second dose of measles vaccine. Measles vaccine is no longer recommended for severely immunocompromised HIV-infected patients whose immunological status is defined by age-specific CD4+ T lymphocyte counts (Moss et al. 1999).

2.7. The need for a new vaccine – experimental measles vaccines

The need for high herd immunity will put the current measles vaccine to its ultimate test to achieve worldwide elimination of measles. In the final stages, small flaws of the current vaccine could potentially have wide ranging negative consequences and so, since the mid–eighties, new generations of measles vaccines and/or new administration routes have been investigated (Putz 2004). A recent comprehensive review of those efforts has been published (Putz et al. 2003a). These experimental vaccines have been mostly tested in mice and the rhesus macaque model has been used to assess the risk of atypical measles. The large variety of experimental vaccines is illustrated in Table 3 and Table 4, and includes recombinant viral vectors expressing mostly the H- and F-surface proteins of measles, which lead to neutralising and protective antibodies in rodents and primates. The bacterial recombinant vectors mainly express the N-protein of measles as it is not dependent on the eukaryotic folding machinery. The success of neutralisation and protective activity with these vectors were more limited than for viral vectors. H- and F-proteins incorporated into ISCOMS with the Quil-A adjuvant component, were virus-neutralising and protective in cotton rats and macaques. Synthetic subunit vaccines of H- and F-protein epitopes induced neutralising and protective antibodies in mice and will be described in further detail later.

One novel area of measles vaccine research focuses on aerosol vaccination. The use of nebulised measles vaccine has been trialled in Mexico and South Africa and follow-up studies suggest that vaccination via aerosol is as effective as or more effective than subcutaneous injection (Dilraj et al. 2007; Low et al. 2008). The WHO has recently started the Measles Aerosol Project (WHO 2005) aimed at licensing nebulisers and vaccines for this novel type of delivery which avoids the need for needles and the associated risks (de Swart et al. 2006; Cohen et al. 2008). There has also been recent research into the feasibility of using dry

powder inhalation of measles vaccine to circumvent the need of a cold-chain and the reconstitution of the virus (LiCalsi et al. 2001; de Swart et al. 2007), with the need for further improvements. Administration of these whole virus based vaccines is however still limited by the presence of neutralising maternal antibodies in young infants (Low et al. 2008).

In what could be considered a twist of fate MV itself has become a possible viral vector (de Vries et al. 2008) and live attenuated MV is known to be oncolytic and is thus used in cancer research to specifically target cancer cells (Liniger et al. 2007; Gauvrit et al. 2008).

Table 3: Experimental measles vaccines in rodents

Experimental vaccine	Mode of delivery	Immune response	References
ISCOMs	i.m., s.c.	Neutralising Abs, CTL, protection	(Varsanyi et al. 1987; Stittelaar et al. 2000a; Wyde et al. 2000a; Wyde et al. 2000b)
Recombinant viral vectors			
· Adenovirus vectors	i.n., i.p., p.o.	CTL, protection	(Fooks et al. 1995; Fooks et al. 1998)
· Poxvirus vectors (replication-competent)	i.n., i.p.	Neutralising Abs, CTL, protection	(Drillien et al. 1988; Wild et al. 1992; Galletti et al. 1995; Etchart et al. 1996; Kovarik et al. 2001)
· Poxvirus vectors (replication-deficient)	i.n., i.p.	Neutralising Abs, protection	(Weidinger et al. 2001)
· Avian virus vectors	i.m., i.p.	Neutralising Abs, CTL, protection	(Wyde et al. 2000a; Wyde et al. 2000b; Kovarik et al. 2001)
· Vesicular Stomatitis Virus	i.n., i.p.	Neutralising Abs, protection	(Schlereth et al. 2000b)
· Attenuated parainfluenza virus type 3	i.n.	Neutralising Abs	(Durbin et al. 2000)
Recombinant bacterial vectors			
· BCG	i.p.	IgG, protection	(Fennelly et al. 1995)
· Streptococcus gordonii	s.c.	IgG	(Maggi et al. 2000)
· Salmonella typhimurium	i.p., p.o.	IgG, CTL, protection	(Verjans et al. 1995; Fennelly et al. 1999; Spreng et al. 2000)
· Shigella flexneri	i.n.	IgG, CTL	(Fennelly et al. 1999)
Nucleic Acid vaccines	gene-gun, i.d.,	Neutralising Abs, CTL, protection	(Cardoso et al. 1996; Fooks et al. 1996; Etchart et al. 1997; Yang et al. 1997; Fennelly et al. 1999; Torres et al. 1999; Fooks et al. 2000; Schlereth et al. 2000a; Green et al. 2001)
Plant based vaccines	i.m., i.p.	Neutralising Abs	(Huang et al. 2001; Webster et al. 2002; Bouche et al. 2003; Marquet-Blouin et al. 2003; Bouche et al. 2005)
Epitope based vaccines			
· Synthetic peptides of F protein	i.n., i.p.	Neutralising Abs, protection	(Obeid et al. 1995; Atabani et al. 1997; Partidos et al. 1997; Hathaway et al. 1998)
· Synthetic peptides of H-protein	i.p., i..n.	Neutralising Abs, protection	(Verjans et al. 1995; El Kasmi et al. 1998; El Kasmi et al. 1999b; El Kasmi et al. 2000; Spreng et al. 2000; Putz 2004; Putz et al. 2004; Halassy et al. 2006)
· "Recombinant peptides" of F protein	p.o.	IgG, CTL, protection	(Verjans et al. 1995; Spreng et al. 2000)
· Polyepitopes of H-protein	i.p.	Neutralising Abs	(Bouche et al. 2003; 2005)

A summary of experimental measles vaccines in rodents. i.d.: intradermal; i.m.: intramuscular; i.n.: intranasal; i.p.: intraperitoneal; p.o.: oral; s.c.: subcutaneous. Table and references adapted from Putz (2003a).

Table 4: Experimental measles vaccines in macaques

Experimental vaccine	Mode of delivery	Immune response	References
ISCOMs	i.m.	Neutralising Abs, CTL, protection	(de Vries et al. 1988; Rimmelzwaan and Osterhaus 1995; van Binnendijk et al. 1997; Stittelaar et al. 2000a; Stittelaar et al. 2002c)
Recombinant viral vectors			
· Poxvirus vectors (replication-competent)	i.d., i.m.	Neutralising Abs, CTL, protection	(van Binnendijk et al. 1997; Zhu et al. 2000)
· Poxvirus vectors (replication-deficient)	i.m., i.n.	Neutralising Abs, CTL, protection	(Stittelaar et al. 2000b; Zhu et al. 2000; Stittelaar et al. 2001)
· Attenuated parainfluenza virus type 3	i.n.	Neutralising Abs	(Skiadopoulos et al. 2001)
Recombinant bacterial vectors			
· BCG	i.d., i.n.	IgG, CTL	(Zhu et al. 1997)
Nucleic acid vaccines	gene-gun, i.d.	Neutralising Abs, CTL, protection	(Polack et al. 2000; Stittelaar et al. 2002b)
Mucosal delivery of live attenuated virus			
	aerosol, m.-e.	under investigation	(LiCalsi et al. 1999; LiCalsi et al. 2001; Stittelaar et al. 2002a)

A summary of experimental measles vaccines in macaques. i.d.: intradermal; i.m.: intramuscular; i.n.: intranasal;, m.-e.: micro-encapsulated. Table and references adapted from Putz (2003a).

2.8. Subunit vaccines based on measles specific epitopes

Neutralising epitopes have been described for the fusion (F) and the haemagglutinin (H) surface glycoproteins of MV.

Fusion protein

Since anti-F antibodies have been found to protect mice against measles induced encephalitis, several neutralising epitopes of the F-protein were identified. Atabani and colleagues screened a panel of 15-mer peptide from MV-F with polyclonal sera from African children with acute measles and identified the F388-402 region, which is located in a conserved Cys rich domain of the F protein, as immunodominant. Immunisations of mice with the 15-mer induced MV-crossreactive and neutralising antibodies. Passive transfer of the induced anti-peptide antibodies protected susceptible mice against encephalitis by challenge with a rodent adapted strain of MV (Atabani et al. 1997). Partidos and colleagues tested the F397-420 BCE for its immunogenicity and protective capacity against rodent adapted MV-challenge. The peptide was immunogenic in two mice strains and did not require the addition of a TCE. Analysis of

the fine specificities of induced antibodies showed strain dependent preferences that targeted F408-420 in the case of C57BL/6 mice, whereas BALB/c mice recognised the F407-417 region specifically. Crossreactivity with MV was demonstrated but sera lacked neutralising activity. Despite this, passive transfer (BALB/c) of anti-397-420 peptide antibodies and active immunisation (C57BL/6) with F397-420 peptide protected mice against neuroadapted MV-challenge (Partidos et al. 1997). Several chimeric peptides composed of one F404-414 BCE and one or two copies of the promiscuous TCE (F288) of the F-protein were analysed in several mouse strains and only peptides with two TCE followed by the BCE were found to induce a protective response against MV-challenge (Obeid et al. 1995). Intranasal immunisation of the above TTB chimeric peptide with cholera toxin b subunit adjuvant induced neutralising and protective immunity in mice (Hathaway et al. 1998). A random solid-phase combinatorial peptide library identified an 8-mer mimotope, with structural similarities to F153-161, which induced MV-crossreactive antibodies, demonstrating the potential of mimotopes (Steward et al. 1995).
While the above mentioned epitopes and immunisations with peptides are promising, the fact that they are recognised by human sera, makes it unlikely that they can resist neutralisation by maternal antibodies and are thus not compatible with a PRE-vaccine (Protection by antibody Resistant Epitopes) (El Kasmi and Muller 2001).

Haemagglutinin protein

Two sequential B cell epitopes of the haemagglutinin protein have been identified in our laboratory by screening overlapping peptides with a panel of neutralising and protective monoclonal antibodies: H236-256, termed NE, neutralising epitope (Fournier et al. 1997) and H386-400 named HNE, haemagglutinin noose epitope (Ziegler et al. 1996).

Neutralising Epitope

Several mAb, induced by immunisation with Edmonston strain, recognised the NE domain with different fine specificities, were able neutralise a range of wt-MV strains *in vitro* and protected mice against a challenge with neuroadapted MV. A Lys242Gly mutation occurring in 50% of MV is thought to allow some viruses to escape neutralisation by these mAb. These anti-NE mAbs are functionally similar to anti-F antibodies as they seem to inhibit virus/cell fusion, yet are not able to block virus/receptor binding. Based on the discrepancy between

haemolytic activity and haemagglutination, the location of the NE epitope is suggested to lie at the functional and/or topographical interface between the F- and H-proteins (Fournier et al. 1997). Amino acid Arg243, which is part of the core epitope, is known to play a role in the downregulation of CD-46 receptor, further confirming the importance of the NE domain (Bartz et al. 1996). Using functional fine-mapping with truncation analogues the H236-256 epitope was narrowed down to the core sequence of S_{244}ELSQL_{249}. Circular dichroism in combination with 3D structure homology modelling suggested an α-helical structure for the epitope (Deroo et al. 1998). Chimeric peptides were synthesised incorporating the BCE H236-255 or H236-250 or the 7-mer core epitope H243-250 and various TCEs with varying copy numbers and orientations. Several of these chimera peptides induced crossreactive and neutralising antibodies and the binding motif of NE was defined as E_{245}LXQL_{249} using mAbs. This binding motif was confirmed by anti-peptide antibodies with similar fine-specificities (El Kasmi et al. 1998; El Kasmi et al. 1999b). Passive immunisation with anti-peptide antibodies protected mice against a neuroadapted MV strain and immunisation with the peptide-vaccine was successful in the presence of MV-neutralising antibodies. These experiments demonstrate the potential of using peptide-based vaccines in the presence of maternally transferred antibodies (El Kasmi et al. 1999b). These anti-peptide sera were however only able to neutralise vaccine strain and none of the wt-field isolates. Several explanations were brought forward, such as the influence of low neutralising titres, differences in uptake mechanisms of the used cell lines and the Arg243Gly mutation in some field isolates. A phage display technique lead to several mimotopes of the NE core peptide which were used for immunisation and neutralisation tests. Surprisingly, the mimotopes which bound much more strongly to anti-NE mAb than some BCE peptides (up to 135 times) failed to induce MV-crossreactive antibodies. Even more surprisingly antibodies induced against several BCE peptides and which did not bind to mAb, were able to crossreact with and neutralise MV (El Kasmi et al. 1999a). These finding show the inherent difficulty of a rational design of peptides (El Kasmi and Muller 2001). Recently Oh and colleagues demonstrated that the 10-mer H250-259 was a TCE and induced protective immunity against a challenge with recombinant vaccinia virus expressing H- and F-proteins of the Edmonston strain (Oh et al. 2006).

Haemagglutinin Noose Epitope

The HNE is the second sequential epitope identified by mAb screening. Three of these mAbs (BH216, BH21 and BH6) reacted with MV under non-reducing conditions, neutralised MV *in vitro* and protected mice from a lethal challenge with a neuroadapted MV strain. These three mAbs specifically bound 15-mer peptides forming the H381-400 sequence. This fragment contains three Cys residues (Cys381, Cys386 and Cys394) which are conserved in all morbilliviruses and the 15-mers are not recognised by anti-MV sera of women of child-bearing age (Ziegler et al. 1996). Two peptides of the HNE-epitope (H386-400 and H379-400) containing two and three Cys respectively were combined with one or two copies of promiscuous TCE in different combinations and orientations. Peptides with the shorter BCE induced crossreactive but non-neutralising antibodies. The longer BCE with three Cys residues induced crossreactive sera when combined with two copies of the TCE, and these sera were also MV-neutralising. These peptides were still immunogenic even in the presence of anti-MV antibodies suggesting that maternal antibodies in infants would not interfere with the immune responses of such peptides (El Kasmi et al. 2000). Functional fine-mapping of the HNE H379-410 epitope (E_{379}TCFQQACKGKIQALCEN$_{396}$) was performed by synthesising substitution and truncation analogues. With the help of truncation analogues the core epitope was reduced to Q_{384}ACKGKIQALCEN$_{396}$. Binding of mAb to PEPSCAN peptides, where each amino acid residue of the H379-410 region was replaced with Ala, Glu, Asn, Gln, Arg and Ser, showed that none of these amino acid residues were tolerated in positions Lys387, Gly388, Gln391 and Glu395, with the exception of Arg in position 387. Thus the binding motif of X_7C[KR]GX[AINQ]QX_2CEX_5 with a disulfide bridge was suggested for these protective antibodies BH216, BH21 and BH6. Twenty-one distinct HNE sequences found in 92.9% of all MV-strains, displayed this binding motif and twenty of them reacted with mAb BH216. All, except one, wt-MV isolates, containing the above binding motif, were neutralised *in vitro* by these mAb (Putz et al. 2003b).

Molecular modelling of the HNE-peptide H384-396 by dynamic simulations at 300 K (Kelvin) and 1000 K and superposition of the calculated structures showed an astonishingly rigid structure (Figure 4). The peptide is a flat circular structure and the hydrophilic residues Gln384, Lys387, Lys389, Gln391 and Glu395 are located at what is suspected to be the solvent exposed side. The hydrophobic sidechains of Ala385, Ile390, Ala392 and Lys393 are directed to the opposite side, most likely buried inside the protein (Putz 2004).

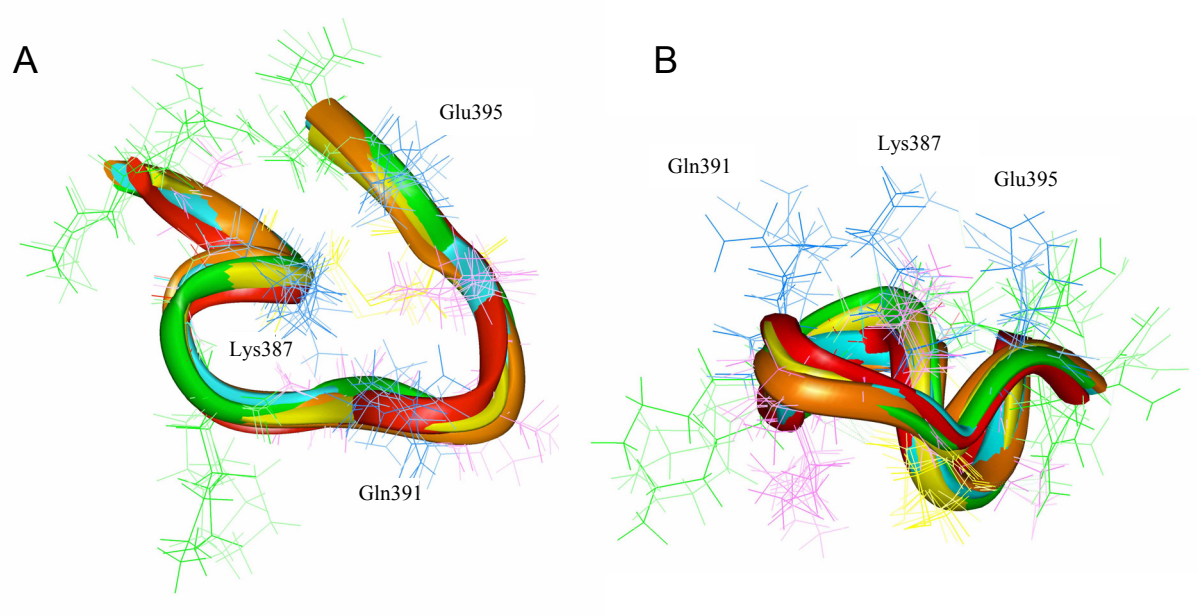


Figure 4: Molecular model of the HNE

Top view (A) and lateral view (B) of 4 representative conformations (peptide backbone as yellow, red, blue and orange ribbon) from simulation runs at 1000 K superposed to a conformation (peptide backbone as green ribbon) from the simulation at 300 K. Sidechains of critical contact residues are shown in blue, hydrophilic/charged sidechains in green, hydrophobic sidechains in pink, disulfide bridge in yellow, peptide backbone as thick ribbon (Putz 2004).

The immunogenicity of HNE-peptides conjugated to DT and TT were tested in the presence of maternally transferred anti-carrier and anti-peptide antibodies and prior vaccination with the carriers. Two injections of HNE-peptide-conjugates in mice previously immunised with the carrier protein induced similar levels than in naïve mice, demonstrating a lack of epitopic suppression. Maternally transferred anti-carrier and anti-peptide antibodies lead to initial suppression of the immune response which could be overcome by additional boosting. In addition, immunisation with the HNE-peptide-conjugates did not exclude the development of an immune response against administered MV as demonstrated by the appearance of anti-F antibodies. These experiments further reinforce the potential of the HNE-peptide-based vaccine in the presence of maternal antibodies and a subsequent immunisation with the current live attenuated MV vaccine (Putz et al. 2004).

The potential of HNE-peptides to elicit crossreactive and neutralising antibodies was demonstrated by a series of high-molecular weight polyepitope constructs produced in

transgenic plants. While the crossreactivity and neutralising activity of paired multiple copies of NE- and HNE-epitopes combined with promiscuous TCEs was highly dependent on the molecular environment in which they were displayed, some constructs correctly displayed the BCEs (Theisen et al. 2000). Immunisation with extracts from transgenic carrots expressing a tandem 8-mer of four copies of the HNE and four copies of a promiscuous TT derived T cell epitope, induced MV crossreactive antibodies in mice. These antibodies were also capable of neutralising a wide range field of isolates of MV, even those with mutations in the HNE binding motif. This may indicate that, in this construct the epitopes may display different conformations and induce a broader range of antibodies which may overcome the genetic variability of some MV strains and ensure neutralisation (Bouche et al. 2003; 2005).

A recent investigation into alternative administration routes of an HNE-peptide subunit vaccine showed that intranasal immunisation with cholera toxin induced antibody at similar levels than via intraperitoneal route. In addition immunisation with DT-conjugated HNE-peptide protected mice against a challenge with neuroadapted MV. Transcutaneous administration was less successful, giving lower anti-peptide titres which were only weakly crossreacting with native H-protein (Putz 2004).

2.9. The rationale of a peptide -based vaccine against measles

The NE- and HNE-epitopes, described above, are potential epitope candidates for a peptide-based vaccine to close the susceptibility gap in newborns and infants. Infants can develop efficient antibody responses against proteins, and antibodies against the surface proteins are sufficient to protect infants from measles (Putz and Muller 2003). As demonstrated earlier, and contrary to most experimental vaccines which are based on whole virus, the immune response against NE and HNE is not suppressed by pre-existing anti-MV antibodies. By using measles unrelated carrier proteins such as TT, or promiscuous TCE, a Th2 priming of measles specific T cells can be avoided. An anomalous Th2 response has been linked to atypical measles (Polack et al. 2002). Children immunised with formalin inactivated measles vaccine, were affected by a severe form of measles after exposure to circulating wt-MV (Fulginiti et al. 1967). The risk of atypical measles hampered the development of new measles vaccines until an animal model for the condition was found (Auwaerter et al. 1999; Polack et al. 1999). There are also suggestions that the current live attenuated vaccination is possible after

vaccination with a peptide-based vaccine. Such a PRE-vaccine (Protection by antibody Resistant Epitopes) could provide transient protection of susceptible infants until routine LAV vaccine can be administered and thus act as a supplement to the current vaccination schedule (El Kasmi and Muller 2001; Putz and Muller 2003).

3. Study Objectives

As described in the previous paragraphs a substantial amount of work has been done in our laboratory on defining BCEs of MV surface proteins and the feasibility of a peptide-based vaccine against measles. Two epitopes of the haemagglutinin protein were described and induced neutralising and protective antibodies in mice.

The current study aims to further characterise the structure and define structurally and chemically important residues of the HNE-peptide with the objective to stabilise the peptide and to increase its immunogenicity.

In a first part, the structure of the wt-HNE-peptide (KGQQACKGKIQALCEN) and several modified peptides will be resolved by Nuclear Magnetic Spectroscopy (NMR) and the influence of substitution will be analysed both by structural comparisons and by antigenicity tests with monoclonal antibodies. These models will shed more light on the structural requirements of the HNE-peptide for antibody binding and complement the current dynamic modelling structure of the HNE-peptide. The resolved structures will then be compared to the X-ray crystallography structures of MV-H-protein that have been published after our structural studies and further explain the differences between free HNE-peptides and the constraint HNE–epitope in the protein.

In a second step we will use the acquired information from both our resolved NMR structures and the previously defined HNE binding motif and rationally apply some of the current strategies to increase the metabolic stability of peptides. The antigenicity of these modified peptides will be assessed by anti-HNE monoclonal antibodies. These modified and stabilised HNE-peptides will then be used *in vivo* to determine their immunogenicity.

In collaboration with Cathy Bodé at the University of Ghent we will analyse the stability, antigenicity and immunogenicity of a bile acid-conjugated scaffold-peptide construct.

Coupling the HNE-peptide via its C- and N-termini to the scaffold will decrease the degradation by exopeptidases. The structural constraints of the scaffold will reduce the flexibility of the HNE-peptide and limit the number of peptide conformations presented to B cells during the immunisation, thus strengthening the immune response.

This study will thus further increase our understanding of the HNE-peptide structure, and the relationship between stability, antigenicity and immunogenicity in the context of epitope-peptide-based vaccines.

Chapter 2: Materials and Methods

Part I: Materials

1. Chemicals and reagents

Commonly used chemicals were purchased from Sigma Aldrich (Bornem, Belgium) and Merck Schuchardt (Hohenbrunn, Germany). All chemicals were used without further purification. All cell culture medium and reagents were purchased Lonza (Basel, Switzerland).Solvents used in mass spectroscopy were from of MS grade from Biosolve (Valkenswaard, The Netherlands)

Compound	Supplier
acetonitrile (ACN)	Biosolve (Valkenswaard, The Netherlands)
Tween 20	Sigma-Aldrich (Bornem, Belgium)
phosphate substrate	Sigma-Aldrich (Bornem, Belgium)
sodium-iodoacetate	Sigma-Aldrich (Bornem, Belgium)
s-NHS/EDC	Pierce (Rockford, IL, USA)
β-mercaptoethanol	Calbiochem (Merck, Darmstadt, Germany)
dithiothreitol (DTT)	Sigma-Aldrich (Bornem, Belgium)
3-Hydroxypicolinic acid (HCCA)	Bruker (Bremen, Germany)
2,5-Dihydroxybenuoic acid (DHB)	Bruker (Bremen, Germany)

2. Peptide synthesis reagents and amino acids

Reagents

Reagents used for automated peptide synthesis were of peptide synthesis grade.

Compound	Supplier
dichloromethane (DMF)	Biosolve (Valkenswaard, The Netherlands)
N-methylpyrrolidone (NMP)	Merck Schuchardt (Hohenbrunn, Germany)
N-hydroxybenzotriazole (HOBt)	Sigma-Aldrich (Bornem, Belgium)
piperidine	Merck Schuchardt (Hohenbrunn, Germany)
N,N'-diisopropylcarbodiimide (DIC)	Fluka/Sigma-Aldrich (Bornem, Belgium)
phenol	Merck Schuchardt (Hohenbrunn, Germany)
ethanedithiol	Merck Schuchardt (Hohenbrunn, Germany)
thioanisole (methyl phenyl sulphide)	Merck Schuchardt (Hohenbrunn, Germany)

Amino acids and resin

All natural L- and D-amino acids were from OrpegenPharma (Heidelberg, Germany).

Compound	Supplier
β-alanine	Novabiochem (Merck, Darmstadt, Germany)
pamba	Fluka/Sigma-Aldrich (Bornem, Belgium)
t-amcha	Fluka/Sigma-Aldrich (Bornem, Belgium)
gaba	Fluka/Sigma-Aldrich (Bornem, Belgium)
achca	Fluka/Sigma-Aldrich (Bornem, Belgium)
aib	Novabiochem (Merck, Darmstadt, Germany)
cha	Novabiochem (Merck, Darmstadt, Germany)
Norleucine (Fmoc-Nle)	Novabiochem (Merck, Darmstadt, Germany)
Norvaline (Fmoc-Nva)	Novabiochem (Merck, Darmstadt, Germany)
diamnio propionic acid (dap)	Bachem (Bubenhof, Switzerland)
trimethylated Lys	Bachem (Bubenhof, Switzerland)
dimethylated Lys	Bachem (Bubenhof, Switzerland)
Fmoc-Rink amide aminomethyl-polystyrene resin	Iris Biotech (Marktredwitz, Germany)

3. Antibodies

Compound	Supplier
anti-MV-H (BH216, BH21, BH6, BH195)	in house produced mAb from hybridomas (Ziegler et al. 1996)
IgG-AP	Southern Biotechnology (Birmingham, AL, USA)
anti-2,4-D (clone E2/G2)	(Franek et al. 1994) for Borstel Research Centre
horseradish-peroxidase-labeled streptavidin (SA-HRP)	Vector, (Burlingame, CA, USA)
FITC-labelled goat anti-mouse IgG (Anti-Mouse IgG (Fc specific) F(ab') fragment-FITC)	Sigma-Aldrich (Bornem, Belgium)

4. Buffers and Solutions

The water used for the buffers and solutions was purified on an Elga Option 4 instrument (Elga Labwater, Ede, The Netherlands)

HPLC

HPLC Solvent A

100%	H_2O
0.1%	TFA

HPLC Solvent B

80%	ACN
20%	H_2O
0.1%	TFA

Oxidation and conjugation

Borate buffer

50 mM	boric acid
150 mM	NaCl
pH 7.4	

Ammonium bicarbonate buffer

0.1 M	NH_4HCO_3
pH 7.8	

MES

0.1 M	MES
0.5 M	NaCl
pH 6	

ELISA and FACS

Carbonate buffer

0.1 M	Na_2CO_3
adjusted to pH 9.6 with	
0.1 M	$NaHCO_3$

Washing buffer

154 mM	NaCl
1 mM	Trisbase
1% (w/v)	Tween 20
pH 8	

Blocking buffer

15 mM	Trizma-Acetate
136 mM	NaCl
2 mM	KCl
1% (w/v)	BSA
pH 7.4	

Dilution buffer

0.1% (w/v)	Tween 20
in blocking buffer	
pH 7.4	

Substrate buffer

1 mM	AMP
0.1 M	$MgCL_2.6H_2O$
pH 10.2	

FACS buffer

0.5% (w/v)	BSA
0.05% (w/v)	NaN_3
in PBS	

Proteolytic assays

PBS (Dulbecco's Phosphate-buffered saline)

2.7 mM	KCl
1.5 mM	KH_2PO_4
136 mM	NaCl
8.1 mM	Na_2HPO_4
pH 7.3	

PBST

0.05% (w/v)	Tween 20
in PBS	

L-PBS ("Lite" PBS)

10 mM	sodium phosphate
10 mM	NaCl
pH 7	

SIFT $CaCl_2$ buffer

1 mM	$CaCl_2$
0.005% (w/v)	Tween 20
in SIF	

SIF-buffer (simulated intestinal fluid)	
4.6 mM	K
111.3 mM	Na
101.5 mM	Cl
in 8 mM	phosphate buffer
pH 7.2	
according to	
(Lockwood and Randall 1949)	

protease inhibitor cocktail	
308 nM	aprotinin
20 µM	leupeptin
400 µM	4-(2-aminoethyl) benzenesulfonyl fluoride
2 mM	EDTA
in L-PBS	

5. Immunisation

Animals

BALB/cOlaHsd mice purchased from Harlan (AD Horst, The Netherlands). They were fed standard food and water *ad libitum*. and kept in cages connected to an air filtering system (Tecniplast, Someren, The Netherlands) under timed 12 h light/dark cycles at 22 ± 2 °C and 40 ± 5% relative humidity. The mice were acclimatised for one week before the start of the immunisation studies. All animal experiments were done in compliance with the rules of the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Adjuvants and anaesthesiacs:

Compound	Supplier
Complete Freund adjuvant	Sigma-Aldrich (Bornem, Belgium)
Incomplete Freund adjuvant	Sigma-Aldrich (Bornem, Belgium)
Montanide ISA50V	Seppic (Paris, France)
Alhydrogel 2% 30mg/mL	Brenntag-Biosector (Frederikssund, Denmark)
Quil-A	Brenntag-Biosector (Frederikssund, Denmark)
Ketaminum hydrochloridum (Imalgene®1000)	Merial, (Lyon, France)
Xylazinum hydrochloridum (Rompun®2%)	Bayer (Brussels, Belgium)

Part II: Methods

1. Peptide synthesis

1.1. Solid-Phase Peptide Synthesis principle

The general principle of Solid Phase Peptide Synthesis (SPPS) is one of addition of protected amino acids residues to a solid insoluble support in repeated deprotection and coupling cycles giving rise to a growing peptide chain. SPPS was pioneered by Bruce Merrifield (Merrifield 1963) and without it modern peptide synthesis would be unthinkable. Indeed its introduction was deemed so revolutionary and essential that Merrifield was awarded the Nobel Prize in Chemistry in 1984. The SPPS method has been described and reviewed in several publication and textbooks (Atherton and Sheppard 1989; Merrifield 1997; Chan and White 2000).

The widespread use of a solid polymer support in peptide synthesis and by extension in organic synthesis can be readily explained by the numerous advantages that the technique offers in contrast to liquid phase synthesis. Removal of reaction mixture, excess reagents, side-products and repeated washing steps can be easily achieved by filtration, leaving only the desired product on the solid support behind with minimal loss of product and hence increased yield. The use of excess reagents allows to push the reaction equilibria towards near completion. The simplicity of dispensing and filtration allows the use of automated systems (synthesising robots) to achieve peptide synthesis with significant savings in times and manpower. Since SPPS does not accommodate for intermediate purification steps, reagents need to be carefully selected to assure fast and near complete reactions as well as to minimise the production of side-products, especially insoluble ones.

In standard SPPS peptides are synthesised starting with their C-terminal amino acid residue, which is either already preloaded to the resin or added in the first step of the synthesis. In order to avoid side-reactions all reactive amino acid residue sidechains are protected and the protecting groups are insensitive to the reagents used throughout the synthesis. To prevent multiple additions of the same amino acid residue on the growing peptide chain, the reactive α-amine group of the backbone is temporarily protected by either of two approaches: an acid labile *t*-BOC protection developed by Merrifield (Merrifield 1964) or an base-labile Fmoc-group (Carpino and Han 1972). Since *t*-BOC-group is cleaved by TFA and Fmoc-group by

piperidine it is obvious that the sidechain protections of BOC-amino acids and Fmoc-amino acids as well as the selection of resins need to be adapted accordingly. Each amino acid residue is added via the unprotected C-terminal carboxy group to the reactive α-amine group of the growing peptide chain (or of the resin if first step) by use of a carbodiimide coupling reagent. The most common method employed consists of diisopopylcarboiimide as coupling reagent and hydrobenzotriaziole (HOBt) to suppress epimerisation (racemisation) Recently the introduction of 1H-benzotriazol-1-yloxy-tris(pyrrolidino)phosphonium hexafluoro-phosphate (PyBOP) has further reduced reaction time, epimerisation and production of toxic side-products. After washing away the excess reagents, the α-Fmoc group of the newly coupled amino acid is removed and the next residue is added. These cycles are repeated until the peptide sequence is completed, after which the peptide is detached from the resin, the conditions of which depend on the α amine protecting group. The peptide can be released with its sidechain protecting groups still present in case of combinatorial synthesis, or more frequently the sidechain protecting groups are removed concurrently with resin cleavage. Peptides are then precipitated from the reaction mixture and ready for further modifications or purification.

1.2. Peptide synthesis: reactor method

Peptides were synthesised by automated solid phase peptide synthesis using standard Fmoc chemistry on a Syro II peptide synthesiser controlled via SyroXP software (Multisyntech, Witten, Germany). Synthesis was carried out either in 400 µL small scale polypropylene tip reactors (Multisyntech, Witten, Germany) on a 96 reactor block or in 2 mL large scale polypropylene reactors (Multisyntech, Witten, Germany) on a 48 reactor block at RT. For large scale 30 mg (small scale: 5 mg) of Fmoc-Rink amide aminomethyl-polystyrene resin with a loading density of 0.5-0.8 mmol/g were used. N-terminal Fmoc-protected amino acids were dissolved overnight at 4 °C at a concentration of 0.45 M in a 0.65 M HOBt solution in NMP with end-over-end rotation. Resins were washed and swollen in DMF and Fmoc group was removed by the addition of 300 µL (small scale: 80 µL) of 40% (v/v) piperidine in DMF twice for 10 min, each step was followed by 5 washes with 500 µL (small scale: 200µL) of DMF. The coupling reactions were started by adding 50 µL of a 3 M DIC solution (DMF:DCM, 1:1, v/v) and 300 µL (small scale: 50 µL) of amino acid solution giving an 8 fold excess of amino acid to resin loading. Reactions were carried out twice for 90 min each,

followed by 5 washing steps each. The coupling step was completed by capping of unreacted NH_2 groups to terminate chain elongation of incomplete coupling reactions, with 300 µL (small scale: 80 µL) of a 10% acetanhydride/5% DIPEA solution (v/v) in DMF for 15 min and washed 5 times with DMF. Capping solution was freshly prepared every 12 h.
After completed synthesis the resin was washed extensively with DMF, followed by three washes with 2 mL (small scale: 300 µL) of methanol and diethyl ether for 1 min each. Peptide sidechain protection removal and peptide cleavage from resin was achieved by adding 2 mL (small scale: 300 µL) of TFA solution with 5% H_2O, 15% scavenger K (6.5% (w/v) phenol, in thianisole:ethanedithiol (2:1)) and rotating end over end for 4 h. Since resin cleavage is faster than sidechain deprotection the reaction mixture was filtered into 50 mL tubes after 2 h reaction time to avoid resin disintegration. Peptides dissolved in TFA were precipitated and washed 3 times with 40 mL (small scale: 8 mL) ice-cold diethyl ether (-80°C). The dried pellet was dissolved in 80% *tert*-butanol, frozen and lyophilised in an Alpha 2-4 lyophilisator (Christ, Osterode am Harz, Germany).
To avoid low yields with longer peptide sequences due to lack of mixing capability of the robot station, coupling reactions were carried out manually after amino acid position 25. Reactors were removed from the rack and the same volumes of DIC and Fmoc-amino acid solutions as described above were added manually and reactions allowed to proceed at RT with end-over-end rotation for 2 h. Deprotection, capping and washing steps were performed via the robot as described above.

1.3. Peptide synthesis: SPOT method

In the context of a collaboration with the Research Center Borstel (Division of Mucosa Immunology) peptide libraries were synthesised by Dr. Steffen Bade on a cellulose membrane support using standard Fmoc-amino acid protection chemistry as described by Frank (Frank 1992). Briefly, peptide SPOTs were defined by automated application of a Boc-Lys(Fmoc) solution on a proline-derivatised cellulose membrane, establishing a cleavable ε-Lys-Pro anchor (Bray et al. 1990). Next, the first label, Fmoc-N-γ-(N-biotinyl-3-(2-(2-(3-aminopropyloxy)-ethoxy)-ethoxy)-propyl)-L-glutamine (Merck Biosciences, Schwalbach, Germany), was applied. The peptide was then assembled in a semiautomated cycle using an ASP 222 peptide synthesiser (Intavis AG, Cologne, Germany): (i) acetyl-capping, (ii) Fmoc-deprotection with 20% (v/v) piperidine in DMF, (iii) bromophenol blue staining for synthesis

control, (iv) drying, (v) triplicate automatic application of 0.2 µL of DIC-activated amino acid solutions (0.2 M Fmoc-protected amino acid in a 0.35 M HOBt in NMP solution). As the last synthesis step, the second label 11-(2-(2,4-dichlorophenoxy)acetylamino)undecanoic acid was attached. This marker had been previously synthesised in the Research Center Borstel (Patents: (Bade et al. 2007a; Bade et al. 2007b); unpublished results Bade et al). For sidechain deprotection and diketopiperazine formation of the ε-lysine-proline anchor the membrane was incubated in 50% TFA, washed and dried. The SPOTs were punched out and transferred individually to polypropylene tubes. Peptides were cleaved from the membrane in 0.1 M triethylammonium acetate, 20% ethanol at 30 °C. Lyophilised peptides were dissolved in 1.5 mL L-PBS with 0.005% (w/v) Tween 20, snap frozen in liquid N_2 and stored at -80 °C. The peptides were then analysed for their biostability.

2. Peptide oxidation

Two methods were used for the oxidation of lyophilised peptides: (i) Purified peptides were dissolved and oxidised at 1 mg/mL in a 20% DMSO solution in borate buffer for 5 h at RT with end-over-end rotation (Tam et al. 1991). The oxidised and reduced isoforms were immediately separated via RP-HPLC and lyophilised. (ii) Crude linear peptides were oxidised using an improved oxidation method adapted from Eichler (Eichler and Houghten 1997): peptides were dissolved in ammonium bicarbonate buffer at a concentration of 0.5 mg/mL and 5 molar equivalents of 0.3% H_2O_2 (stock 30% w/w, Sigma-Aldrich, Bornem, Belgium) were added. Peptides were oxidised at RT for 1 h with end-over-end rotation, after which the reaction mixture was quenched with 10% acetic acid (50% of reaction volume). Solutions were frozen, lyophilised and dissolved in HPLC starting buffer and purified via RP-HPLC.

3. HPLC

3.1. High Performance Liquid Chromatography-principle

In order to analyse any molecules in great detail purification is essential. A useful tool in the purification and characterisation of peptides and proteins is provided by High Performance Liquid Chromatography (HPLC) (Lottspeich and Zorbas 1998). The basis of HPLC is the reversible interaction of the sample in a liquid mobile phase with a solid stationary phase. These interactions can be the result of either polarity, size, charge etc. and the methods used

to separate molecules via these interactions differ accordingly: reverse phase HPLC (RP-HPLC), gel filtration or ion exchange chromatography, respectively. In the context of this work RP-HPLC was used.

Loading of large sample volumes is possible with RP-HPLC as the sample binds to the non-polar phase due to hydrophobic interactions as soon as it comes into contact with the solid phase. Gel chromatography which separates according to size differs in this respect as separation of sample mixture occurs immediately after loading and hence is only suitable for small sample volumes. After the sample mixture is loaded on the column, the sample is eluted with increasing concentrations of organic solvent (often acetonitrile or methanol) and the addition of ion pairing agents (e.g. TFA). The retention times of sample on the column depends on the amount of hydrophobic and hydrophilic groups present on the surface of the molecule and their strong yet reversible interactions with the solid phase. For RP-HPLC the solid phase is usually silica based attached aliphatic chains of variable carbon chain length. In addition structural changes such as those induced by the formation of disulfide bridges, influence retention times.

HPLC in this work was used either in an analytical or semi-prep context:

3.2. Analytical HPLC

To assess peptide purity after peptide synthesis, aliquots of peptides in *tert*-butanol were taken and analytical RP-HPLC runs were performed. These runs were also important in determining elution and scouting parameters for large scale semi-prep HPLC runs. Typically solutions containing 15-50 µg of peptide were diluted to 100uL 10% solvent B in solvent A and injected into a RP-HPLC ÄKTAexplorer 10S system (Amersham Biosciences, Uppsala, Sweden). Elution was performed on a C18 silica gel column [Grom™Sil media, length: 250 mm, diameter: 4 mm, pore diameter: 120 Å, particle size: 5 µm] (Grom, Rottenburg-Hailfingen, Germany) with a linear gradient of 10-100% solvent B in solvent A over 6 column volumes with a flowrate of 1 mL/min. C18 columns were equipped with a 0.2 µm stainless steel filter (Alltech, Lokeren, Belgium) and a pre-column of the same media as the main column to avoid damage to the column from insoluble sample components. Elution was monitored at 215 nm, 230 nm and 280 nm using a UV monitor and peptides were collected via an F950 fraction collector (Amersham Biosciences, Uppsala, Sweden). Peptide mass and oxidative status was confirmed by mass spectroscopy.

3.3. Semi-preparative HPLC

Semi-preparative HPLC was used to purify peptides after peptide synthesis and after oxidation to separate linear and disulfide cyclised isoforms. Lyophilised peptides and peptides in oxidation reaction mixtures containing DMSO were dissolved in 10%-20% solvent B in solvent A at a concentration of 1-2 mg/mL. Peptide solutions were injected into a RP-HPLC ÄKTAexplorer 10S system (Amersham Biosciences, Uppsala, Sweden) and run over either one of two C18 columns [Grom™Sil media, length: 250 mm, diameter: 8 mm, pore diameter: 120 Å, particle size: 5 µm or Grom™Sil media, length: 250 mm, diameter: 20 mm, pore diameter: 120 Å, particle size: 5 µm] (Grom, Rottenburg-Hailfingen, Germany) typically with a linear gradient of 25-65% solvent B in solvent A over 6-8 column volumes with a flowrate of 3 mL/min and 7 mL/min, respectively. C18 columns were equipped with a 0.2 µm stainless steel filter (Alltech, Lokeren, Belgium) and a pre-column of the same media as the main column. Elution was monitored at 215 nm, 230 nm and 280 nm using a UV monitor and peaks were collected via an F950 fraction collector (Amersham Biosciences, Uppsala, Sweden). Peptide solutions were frozen and lyophilised and peptide masses and oxidative status were confirmed by mass spectroscopy.

4. Mass spectroscopy: MALDI-TOF

After synthesis and oxidation reactions, peptide masses and oxidative status were confirmed by mass spectroscopy on an Ultraflex MALDI-TOF/TOF (Bruker, Bremen, Germany). Ground steel massive target mictotitre plates (MTP 384 Bruker, Bremen, Germany) were either coated with a thin layer matrix of HCCA in acetone or positions were individually spotted with 0.2 µL matrix mixture (HCCA, DHB; Bruker, Bremen, Germany) and 0.5 µL peptide solution containing 5 pmoles of peptide were deposed on spots. After drying, samples were recrystallised with 0.5 µL of 80% acetonitrile solution in H_20 with 0.1% TFA to ensure proper crystallisation homogeneticity. External calibration was performed using tryptic digestion of BSA (Bruker, Bremen, Germany) with calibration points covering peptide mass range. Spectra were analysed for mass and isotopic distribution in flexAnalysis 2.4 (Bruker, Bremen, Germany). Furthermore peptide sequence was confirmed using MS-MS (MS2) post source decay fragmentation.

5. Biostability

5.1. Biostability in serum

To evaluate peptide biostability, peptide solutions (1mg/mL in H_2O) were mixed in a 1:1 ratio with mouse serum (Harlan, AD Horst, Netherlands, Species: Mouse female, Stock/Strair: BALB/cOlaHsd, frozen commercial stock or freshly prepared) and incubated at 37 °C. Aliquots of 15 µL were collected and added to 335 µL H_2O 0.1% TFA after 0, 24 and 48 h and frozen until analysis. The aliquots were filtered over previously pre-washed 30 kDa Microcon filters (Millipore, Billerica, MA, USA) and 300 µL of filtrate were injected into an ÄKTAexplorer 10S system (Amersham Biosciences, Uppsala, Sweden) over a Zorbax 300SB-C3 column [length: 150 mm, diameter: 2.1 mm, pore diameter: 300 Å, particle size: 5 µm] (Agilent, Diegem, Belgium) and C18 column [Grom^{TM}Sil, length: 250 mm, diameter: 4 mm, pore diameter: 120Å, particle size: 5µm] (Grom, Rottenburg-Hailfingen, Germany) with a linear gradient of 10-100% solvent B in solvent A over 6 column volumes with a flowrate of 0.5 mL/min and 1mL/min, respectively. Biostability was expressed as percentage of remaining intact peptide determined via automated integration of the area under the curve of the corresponding HPLC peak at wavelengths 215 nm, 230 nm and 280 nm where appropriate.

5.2. Biostability in intestinal fluid

Biostability analysis using murine intestinal fluid was performed by Dr Steffen Bade at the Research Center Borstel (Division of Mucosa Immunology) using a novel patented method (Patent: (Gorris et al. 2007); Publication: (Gorris et al. 2009)). Peptide sequences were flanked C- and N-terminally with specific markers. After incubation with a variety of proteolytic fluids the peptides bind to to specific monoclonal antibody on a plastic support plastic support via the N-terminal marker 11-(2-(2,4-dichlorophenoxy) acetylamino)undecanoic acid. After several washes the peptides are incubated with horse radish peroxidase coupled to streptavidin which binds to the C-terminal biotin of intact peptides and detection is measured using the TMB substrate. By employing this method any cuts in the sequence by proteases will result in the separation of the two markers and no signal will be observed.

Proteolysis reactions were carried out in a 96-well polypropylene microtiter plate. Intestinal fluids were harvested from excised murine intestines (unpublished results Bade et al). The crude enzyme preparation was serially threefold diluted in 80 µL/well of approximately 67 pM peptide in SIFT $CaCl_2$ buffer. The sealed microtiter plate was incubated for 90 min at 37 °C before the enzyme reaction was stopped by addition of 80 µL/well of doubly concentrated protease inhibitor cocktail. The microtiter plate was resealed, stored for 10 min on ice, heated for 10 min to 90 °C, and cooled on ice. From each cavity, 75 µL of the peptide solution was transferred into a corresponding cavity of a high-bind 96-well microtiter plate (Corning, Wiesbaden, Germany) which had been coated with 75 µL/well of 30 ng/mL anti-2,4-D antibody E2/G2 beforehand. The plate was washed three times with 300 µL/well of PBST, blocked with 250 µl/well of 1 % (w/v) casein/PBS for 3-4 h at RT, and again washed four times with PBST. After 2 h 30 min incubation at RT the plate was washed four times with PBST, and incubated for 60 min at RT with 75 µL/well of 1 µg/ml horseradish-peroxidase-labeled streptavidin in 1 % (w/v) casein in PBS. After six washes with PBST, plates were developed with the 3,3',5,5'-tetramethylbenzidine (TMB) substrate system (Frey et al. 2000).
For the data analysis the raw data of the pseudo-first order proteolysis reaction were fitted to an exponential function according to Gorris and colleagues (Patent: (Gorris et al. 2007) Publication: (Gorris et al. 2009)). Hydrolysis half-lives were calculated by nonlinear-regression and differences between hydrolysis half-lives were analysed statistically.

6. Conjugation to carrier – peptide-conjugates

6.1. Principle

Bioconjugation chemistry involves the covalent coupling of two or more molecules to form a novel compound which combines the characteristics of the individual components. The benefits of bioconjugation chemistry have affected nearly every discipline in life sciences. Two interrelated chemical reactions are the basis of bioconjugation: the reactive functional groups on the crosslinking or derivatising reagent and the functional groups available on the target molecules to be conjugated, in this case short peptides and macromolecules. Without the presence and chemical compatibility of these functional groups bioconjugation cannot be achieved. Derivatisation of nonreactive groups to functionally useful groups for one particular conjugation strategy is of great interest to researchers. Knowledge of the basic chemistry

behind coupling reactions between reactive and target functional groups and the careful choice of reagent systems selected on the basis of the available functional groups, form the prerequisite of a successful and intelligent conjugation strategy. It is of particular importance in the context of vaccine and drug research to preserve the *in vitro* and *in vivo* characteristics of the molecules involved in the bioconjugation, thus the chosen reagents and conjugation should not affect functional groups of critical amino acid residues nor impede structure-activity relationship. In the context of peptide-protein carriers the most commonly used functional groups are primary amines, i.e. the N-terminal α-amine or the ε-amine of Lys sidechain; and carboxylate groups, i.e. the C-terminal carboxylate, and β- or γ-carboxylate groups of Asp and Glu acid sidechains respectively. The amine and carboxylate group linkage coupling reaction employs EDC/NHS chemistry. Alternatively coupling reactions using sulfhydryl groups of cysteine sidechains are facilated by SPDP chemistry. Hermanson has published an extensive guidebook on bioconjugation techniques (Hermanson 1996).

6.2. Method

Whole tetanus toxoid (TT) (a generous gift from the Serum Institute of India, Hadaspar, Pune, India) was modified and conjugated to HNE-peptides using an adapted protocol (Prodhomme et al. 2007). First crude whole TT (150 kDa) was purified by buffer exchange using a 10 kDa cut-off Amicon Ultra-4 centrifugal filter (Millipore, Billerica, MA, USA) (3500 g, 20 °C, 15 min) in PBS buffer. Protein concentration was determined by DC Protein Assay (Biorad Laboratories, Hercules, CA, USA) according to manufacturer's recommendation. The purified TT solution was adjusted to 1 mg/mL and reduced by a 200 molar excess of DTT for 20 min at 50 °C. The reduced proteins were immediately alkylated by the addition of a 400 molar excess of sodium-iodoacetate. After 20 min at RT the modified TT was purified by washing with MES buffer [0.1 M MES, 0.5 M NaCl, pH 6] using a 10 kDa cut-off Amicon Ultra-4 centrifugal filter, the solution was adjusted to 10 mg/mL and stored at 4 °C.

In a next step the available carboxyl groups of TT were activated and coupled with the zero-length crosslinking Sulfo-NHS/EDC chemistry (N-hydroxysulfosuccinimide/1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride) to TT in a two step one-pot reaction. First $2x10^3$ molar equivalents of Sulfo-NHS in H_2O and $1x10^3$ molar equivalents of EDC in H_2O were added to the solution of modified TT and solution was adjusted with MES buffer to a final concentration of 1 mg/mL of TT. Reaction was allowed to proceed at RT with end-over-

end rotation for 15 min after which β-mercaptoethanol was added in a $4x10^4$ molar excess. After 10 min a solution of 300 molar excess peptides in PBS-H_2O (1:1) was added and coupling reaction of activated carboxyl groups of TT to free primary N-terminal α-amine and ε-amine of Lys sidechain was carried out overnight at RT in the dark with end-over-end rotation. The conjugates were purified by washing with 3 reaction volumes of 50 mM ammonium bicarbonate buffer (pH 7.8) using 10 kDa cut-off Amicon Ultra-4 centrifugal filters. Protein concentration was measured as above and solutions were stored at 4° C. Coupling of peptides to TT was assessed by ELISA with anti-HNE monoclonal antibodies BH216, BH21 and BH6.

7. ELISA

7.1. Indirect Enzyme Linked Immunosorbent Assay

The principle of Enzyme Linked Immunosorbent Assay (ELISA) is the recognition by enzyme linked antibodies of antigens absorbed onto a specially treated plastic support. Binding to the plate occurs via hydrophobic interaction between chemical groups on the peptide. In order to avoid any other components from the ELISA reaction to bind to the plate and lead to non-specific background noise, all free binding sites are blocked by the addition of a blocking reagent (e.g. BSA, Casein etc). The bound antigen is then exposed to a purified antibody or serum containing antibodies that may (or may not) recognise the coated antigen. After the addition of a secondary antibody covalently linked to an enzyme such as alkaline phosphatase, the samples are incubated with a substrate solution containing a chromophore which is oxidised by the enzyme on the secondary antibody. The colour change of the reaction is measured by a spectrophotometer. This method allows to assess the antigenicity of a given antigen with regard to specific antibodies or to assess the immunogenicity of a peptide by the amount of induced anti-peptide antibodies in sera. The term indirect ELISA is derived from the fact that the antibody bound to antigen is not measured directly but via a secondary antibody.

7.2. Antigenicity of synthetic peptides by indirect ELISA

To assess the antigenicity of synthetic peptides by monoclonal anti-H-protein antibodies, ELISAs were performed in 96-well plates (Maxisorb, Nalge Nunc, Rochester, NY, USA). 50 µL of twofold dilutions of peptides (starting concentration: 3.2 µM) in carbonate buffer adjusted to pH 9.6 were coated overnight at 4 °C. Plates were washed manually with washing buffer and blocked for 2 h with 200 µL blocking buffer at RT. Plates were washed again and incubated with 50 µL of monoclonal antibody BH216, BH21 or BH6 (1:1000 dilution in dilution buffer) for 90 min at RT. After washing, plates were incubated for 90 min at RT with 50 µL of goat anti-mouse IgG-AP antibody (1:750 in dilution buffer). After washing, plates were incubated at 37 °C with 100 µL of a 1.35 mM phosphatase substrate buffer solution. Antibody binding was assessed by measuring the optical density at 405 nm after 60 min on a SPECTRAmax PLUS^{384} microplate reader system (Molecular Devices, Sunnyvale, CA, USA). Wells with no coated peptide, omitted primary or secondary antibody, or nonspecific primary antibody were used as negative controls. wt-HNE-peptide was used as positive controls.

7.3. Anti-peptide reactivity of immunised mice sera by indirect ELISA

Sera from immunised mice were assayed for the presence of peptide-binding antibodies by ELISA. The above protocol was adapted as follows: 50 µL of a peptide solution (1.6 µM) or 125 ng of H-protein in 50 µL were coated in 96-well plates (Maxisorb, Nalge Nunc, Rochester, NY, USA) overnight at 4°C using carbonate buffer adjusted to pH 9.6. Plates were washed manually using washing buffer and blocked for 2 h with 200 µL blocking buffer at RT. Plates were washed again and incubated with 50 µL of a threefold serial dilution of mouse serum in dilution buffer for 90 min at RT. After washing, plates were incubated for 90 min at RT with 50 µL of goat anti-mouse IgG-AP antibody (1:750 in dilution buffer). After washing, plates were incubated at 37 °C with 100 µL of a 1.35 mM phosphatase substrate buffer solution. Optical density was read at 405 nm after 60 min on a SPECTRAmax PLUS^{384} microplate reader system (Molecular Devices, Sunnyvale, CA, USA). Positive controls included monoclonal antibodies BH216, BH21 and BH6. Wells with no peptide coated and sera from mice immunised with adjuvant or adjuvant and carrier protein (tetanus toxoid) were

used as negative controls. Serum end point titres were calculated at five times the response of non-immunised mice using the logit-log plot logarithm.

8. Antigen preparation and *in vivo* animal experiments

8.1. Antigen preparation

Prior to injection into animals the peptide-conjugates were combined with their respective adjuvants. For the *in vivo* animal assays all antigen formulations were prepared just prior to use and were not stored thereafter to avoid degradation.

Freund adjuvant water in oil emulsions were prepared by passing 25 µg of peptide-conjugate in 100 µL PBS mixed with 100 µL of adjuvant via two 2 mL glass syringes connected via a Luer lock constrictor until the solution emulsified. The priming injection formulation was prepared using Complete Freund Adjuvant and booster injections were formulated with Incomplete Freund Adjuvant.

Adjuvant preparation with Montanide was performed according to manufacturer's recommendation: 100 µL of peptide-conjugate solution (containing 25 µg conjugate) was added to an equal volume of Montanide ISA50V (a kind gift from Seppic, Paris, France), vortexed for 30 s and further mixed with five up and down stroked with a syringe. In order to achieve a better emulsion that did not separate within a short time, up to thirty up and down strokes had to be performed.

Absorption of peptide-conjugates onto aluminium hydroxide (Alhydrogel 2% 30 mg/mL) was performed by adding peptide-conjugate solution (25 µg) to 500 µg of Alhydrogel in 100 µL PBS total reaction volume and rotating end-over-end overnight (20 h) at 4 °C. Prior to injection samples were diluted to 200 µL with PBS.

Quil-A, purchased as purified and lyophilised powder, was dissolved to 10 mg/mL solution in PBS. 25 µg of peptide-conjugate were mixed with 25 µg Quil-A and diluted in PBS to a final injection volume of 100 µL.

8.2. Intraperitoneal and subcutaneous route

Groups of 5-6 specific pathogen-free 11-12 week old female BALB/cOlaHsd mice were injected intraperitoneally with 200 µL prepared antigen-formulations (CFA/IFA, Montanide and Aluminium hydroxide) or subcutaneously into the scruff of the neck with 100 µL of prepared antigen-formulations (Quil-A according to manufacturer's recommendation) containing 25 µg of peptide-conjugate. Booster injections were administered on day 14, 28 and 42.

8.3. Blood collection

Mice were put under general anaesthesia by intraperitoneal injection of 200 µL anaesthetics solution containing 2.5 mg Ketaminum hydrochloridum (Imalgene®1000) and 50 µg Xylazinum hydrochloridum (Rompun®2%). Blood (200 µL) was collected via retro orbital bleeding on day 24, 38 and 52. Sera was separated by incubating whole blood samples at 37 °C for 30 min, followed by centrifugation for 30 min at 3000 g at RT.

9. Flow Cytometry

9.1. Principle

Flow Cytometry is an effective method for the identification and characterisation of subpopulations of cells such as lymphocytes or assessing the interaction of fluorescence labelled molecules with cells. Cells are diluted in saline solution and pass through a laser beam in a fluid stream ideally in single file. Flow cytometers that are equipped with a cell counter and separator are called fluorescence-activated cell sorter (FACS). In a first step, cells are incubated with specific antibodies or mixtures containing antibodies such as sera, followed by the addition of a second antibody with a high affinity for the first antibody (similar to ELISA). This second antibody is labelled by a chromophore such as fluorescein isothiocyanate (FITC). As each labelled or unlabelled cell passes through the laser beam, the scattering of light and changes in the wavelength originating from the fluorophore, are measured by a series of detectors located in line with the beam or perpendicular to it (forward and side scatter, respectively). Light scattering can give important information on the size of

the cell and allows setting the calibration to ignore aggregates formed by dead cells. Specific absorption and emission of various fluorophores attached to antibodies provides the possibility to discriminate between subpopulations (e.g. in lymphocytes) in a single experiment (Shapiro 1993).

9.2. Method

Flow cytometry was used to determine the crossreactivity of mouse sera immunised with peptide-conjugates and was performed on a transfected human melanoma cell line (Mel-JuSo-H) expressing the H-protein of MV. Mel-JuSo-H and Mel-JuSo-wt cells were kindly provided by R.L. de Swart (Erasmus University, Rotterdam, The Netherlands (de Swart et al. 1998)). Cell stock was thawed and cultured in BioWhittaker® RPMI-1640 medium supplemented with 10% FBS-HI, 1% penicillin-streptomycin and 1% L-glutamine or ultraglutamine at 5% CO_2. To reduce loss of expression of H-protein in Mel-JuSO-H cells, cells were passaged only once. On the day of the experiment cells were washed and resuspended in FACS buffer and 50 µL added to 1.2 mL tubes at a concentration of $4x10^6$ cells/ml. 50 µL of serum dilution (1:50 or 1:100 dilution in FACS buffer) was added, mixed shortly and incubated on ice for 30 min. Cells were washed twice with 1 mL FACS buffer and stained for 15 min on ice with 50 µL of a 1:200 diluted FITC-labelled goat anti-mouse IgG. Cells were washed again twice with FACS buffer and fixed with 0.4% formol. Five thousand cells were counted and the fluorescence was measured by flow cytometry on an Epics Elite ESP instrument (Coulter Company, Miami, Florida, USA) or BD FACS Canto (BD Biosciences, San Jose, CA, USA) as described previously (Muller et al. 1995). Data are expressed as ratios of arbitrary fluorescence units (AFU) of immunised sera on Mel-Juso-H and Mel-JuSo-wt cells Histogram overlays were produced using WinMDI 2.8. H-protein expression in Mel-JuSo-H cells was confirmed by FITC-labelled H-protein specific BH216 monoclonal antibody and by mouse sera immunised with MV-H-protein. Negative controls included pre-immunisation sera, sera induced against adjuvant alone or against adjuvant carrier (tetanus toxoid) only, irrelevant primary antibody and immunised sera on Mel-JuSo-wt cells.

10. NMR

10.1. History and principles

The description of the intricate workings and details of NMR, couplings and spectroscopy and the physics behind these processes are beyond the context of this work. They are explained in detail in several textbooks (Williams and Fleming 1995; Lottspeich and Zorbas 1998) and with an increased focus on structure determination in Kurt Wüthrichs "NMR of proteins and nucleic acids" (Wüthrich 1986). In this chapter only the basics of NMR will be touched upon to illustrate the usefulness of the technique for the determination of structures of peptides and proteins.

Limitations and comparison to X-ray

NMR and X-ray crystallography (or X-ray diffraction) are the two major methods for the determination of three-dimensional structures of macromolecules and proteins. The two methods can be seen as complementary. X-ray crystallography requires the growing of crystals and is thus based on solid state. The obvious disadvantages are the difficulty in obtaining crystals for a number of molecules and the fact that solid state structure does not necessarily represent the structure of the molecule in a liquid physiological environment. NMR on the other hand is measured mostly in solution which has significant implications on the data obtained. First of all, solution dynamics allow the peptides to adopt a variety of structures and energy transfers from one molecule to the next can easily occur, resulting in signal averaging. The dynamics of solution phase NMR also allow for kinetic analysis of biological processes with real time observation of appearance product specific peaks or the disappearance of starting material specific peaks; a process which is useful in reaction kinetic and protein folding studies. The disadvantages of NMR include the need for larger amounts of sample and the lack of stereospecific resonance assignment.

The phenomenon of NMR was first observed in 1946 and during the sixties it became a routine technique in organic chemistry. After the introduction of the Fourier transformation (FT) in the 1970s the field of NMR has expanded enormously.

Magnetisation field, free induction decay and Fourier Transformation

In order for atoms to be visible in NMR they need to have a nuclear spin. Nuclei with odd mass numbers have non integer spins and can thus orient themselves in a magnetic field. The most important atoms with nuclear spins are ^{1}H and ^{13}C, which have both spins of ½. When exposed to an applied magnetic field (B_0) these atoms can either align themselves with the magnetic field in a low energy state or they can oppose the magnetic field in a high energy state. A broad range of radio frequencies is applied to the system and nuclei can be promoted to the higher energy state if the radio frequency matches the required energy (Bulk magnetisation - M). When nuclei fall back to the low energy state in a process referred to as relaxation, energy is released which is measured. Since the differences in energy levels are low, NMR is a relatively insensitive method requiring much larger amounts of analytes than other spectroscopy methods. The sensitivity and signal to noise ratio can be improved by increasing the number of scans, but a much more effective way is to increase the strength of the applied magnetic field. With higher field strength the differences between ground and excited state are more significant increasing the energy released by relaxing nuclei and hence increasing the signal strength. While NMR machines started out using magnetic fields of 60-100 MHz, most routinely used machines operate in the 400-600 MHz region and high end instruments reach up to 950 MHz.

A radio frequency signal is applied to magnetic field (B_0) at a 90° angle for a short time which results in an oscillating magnetic field (B_1). A receiver coil along one axis records the oscillating signal during the relaxation time. The combined signals of all ^{1}H nuclei in a sample constitute the free induction decay (FID) and are recorded as a series of decaying cosine waves. The Fourier transformation (FT), a mathematical approach to solving the complex mix of waves, converts these into frequencies which are plotted against the signal amplitude and give a 1D NMR spectrum (An analogy of this is an orchestra whose music also consists of large number of sound waves, which are deciphered by our brains into the different musical instruments). In Fourier transform NMR the pulses of radio frequencies are applied in multiple and quick succession cancelling out noise and giving average signal responses.

Chemical shift

The term chemical shift refers to the shift in frequencies at which nuclei are observed with respect to a reference frequency (usually ^{1}H of TMS – Tetramethylsilane). The chemical shift

is defined as the difference between the frequency of the peak of interest and the frequency of a reference peak, divided by the operating frequency of the instrument:

$$\delta = \frac{\upsilon_s\,(Hz) - \upsilon_{ref}\,(Hz)}{operating\ frequency\,(MHz)}$$

The chemical shift has no units and is expressed as ppm. Since it is related to an internal standard it is not influenced by the NMR instrument or operating frequency. The scale of δ is written right to left (0-10 for ^{1}H) with high frequency responses appearing to the left in the downfield region and low frequency signals appearing upfield. The chemical shift is influenced by the environment of the nuclei: each nucleus is surrounded by circulating electrons which produce their own electromagnetic fields opposing the applied field. These nuclei are shielded and a stronger field is required to achieve resonance in such cases. In addition neighbouring atoms and double bonds also effect the shielding of nuclei.

Coupling and Nuclear Overhauser Effect

An important feature of NMR is the interaction of spins from adjacent nuclei. The spin of one nucleus affects the magnetic environment of an adjacent nucleus by transferring some of its energy. This interaction can occur in two ways: scalar coupling (or J-coupling) results from transfer of energy of nuclei via bonds. This effect is limited to nuclei distant by up to 3 chemical bonds. The nuclear Overhauser effect (NOE) on the other hand is the interaction between magnetic fields of nuclei through space via dipolar relaxation and is detectable over short distances of 2-4 Å. This interaction is physically different from scalar coupling. Both of these phenomena are the basis of two dimensional NMR and provide information on the distance of nuclei in complex molecular arrangements like peptides and proteins and hence their structure.

2D-NMR spectra

In order to obtain 2D-NMR spectra an additional experimental step is applied to the traditional 1D-NMR experiment. As previously the magnetisation of the system is changed by bursts of radio frequencies at 90° angle to the established field. Instead of measuring the signals immediately an evolution and mixing time is added during which the spins are allowed to influence each other (either through space or through scalar spin-spin coupling). After the mixing time another series of 90° pulses are applied and the FIDs are acquired. The

setup of the instrument is arranged so that either spin-spin coupled or through space interactions are recorded and displayed diagonally as crosspeaks on a 2D-NMR (Figure 5).

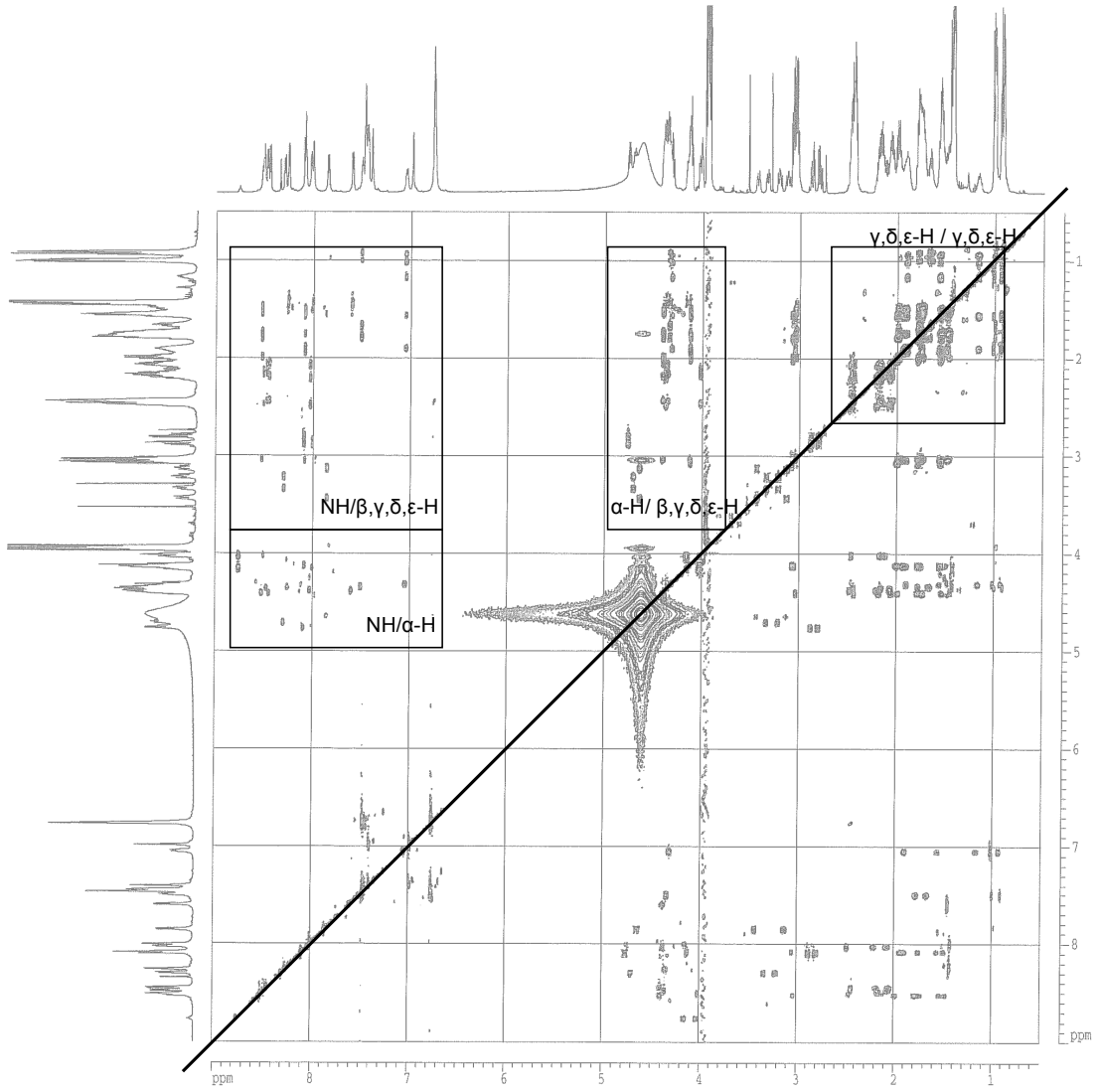


Figure 5: 2D TOSCY spectrum of an HNE-mutant peptide

1D NMR spectra are shown on the axis and intraresidual crosspeaks are shown as 2D spectrum field. The diagonal separates the spectrum into a mirror image. The large peak at the centre is caused by water. The relevant regions containing intraresidual crosspeaks are highlighted and labelled.

Three major 2D-NMR spectra are routinely used:

COSY (COrrelation SpectroscopY) is the 2D representation with crosspeaks originating through single scalar coupling only.

In *TOCSY* (TOtal Correlation SpectroscopY) the magnetisation is successively transferred through the entire spin system via scalar coupling. This allows the differentiation of overlapping signals of nuclei originating from different spin systems and specific assignment of these resonances to their respective spin system, which is essential in structural studies of

peptides and proteins. The advantage of TOCSY is the display of entire spins systems of amino acids resulting in characteristic patterns for each amino acid.

NOESY (Nuclear Overhauser Effect SpectroscopY) is a 2D-NMR experiment which incorporates all proton-proton interactions through space and is essential in the determination of three dimensional structures. The intensities of crosspeaks between protons with a maximal distance of approximately 4 Å are inversely related to the sixth of the power of the distance. The main benefit of NOESY is that protons which are located far apart in the primary structure can induce crosspeaks due to their arrangement in secondary and tertiary structure. Crosspeaks of αH of residue (i) to NH of residue (i+1) are used for sequential assignment. Crosspeaks of αH(i) to NH(i+3) and NH(i+4) as well as NH(i) to NH(i+2) are characteristic of an α-helix. 3_{10} helices show slightly different crosspeaks since they lack αH(i) to NH(i+4) but gain αH(i) to NH(i+2) crosspeaks. β-sheets show none of these crosspeaks and only have NH(i) to NH(i+1) and αH(i) to NH(i+1) sequential crosspeaks, whose intensities however are inverse of helix crosspeaks in the same region (Figure 6).

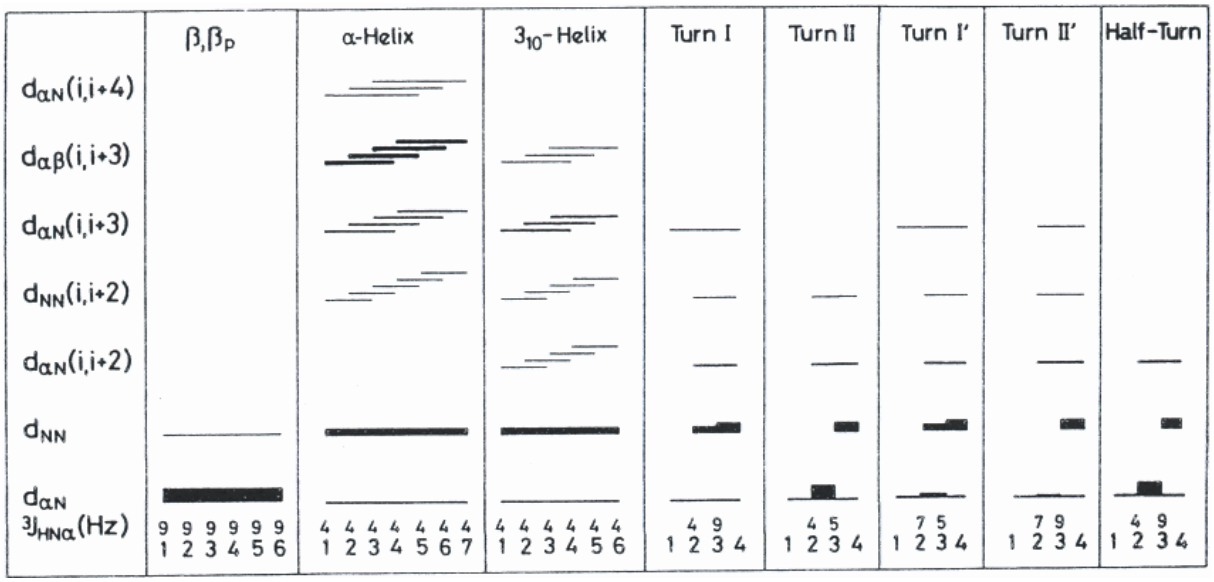


Figure 6: Summary of sequential and medium range 1H -1H NOEs

NOE pattern of sequential and medium range NOE expected in the standard secondary structures of parallel and antiparallel β-sheets, α-helices, 3_{10}-helices, tight turns I, II, I' and II', and half-turns. The numbers at the bottom represent the residue number in the secondary structure elements. Short ^{1}H -^{1}H distances between two residues are shown as bars, the thickness of which represent the crosspeak intensities in NOESY and are proportional to r^{-6}. Figure from (Wüthrich 1986)

10.2. Measurements

4 mg of peptide were dissolved in 350 µL doubly distilled H_2O and 350 µL of TFE-d_2 or D_2O were added to give 700 µL with a ±3 mM concentration. ^{1}H-NMR Spectra were recorded on a Bruker Avance DMX 600 NMR spectrometer (Bruker, Rheinstetten, Germany) at 300 K with mixing times of 110 ms for TOCSY and 250 ms for NOESY. The spectra were referenced to residual water residues at 4.8 ppm or TFE signal at 3.95 ppm.

10.3. NMR spectrum analysis and structure calculations

NMR spectra were processed using XWin NMR software (Bruker, Rheinstetten, Germany). The characteristic crosspeak pattern of each amino acid in 50% TFE-d_2 was determined in amide proton region of 2D-TOCSY ^{1}H NMR spectra (Figure 7). Amide proton to sidechain protons crosspeaks within each amino acid allowed the assignment of NH/αH crosspeak to a specific residue (Wüthrich 1986). This assignment does not give any indication about the residues position in the sequence. Focusing on the fingerprint region of NH/αH in the NOESY spectrum, and superposing it on the corresponding region of the TOCSY, showed additional peaks, which were used for sequential residue assignment (Wüthrich 1986). Briefly, NOE crosspeaks between αH of one residue (i) coupling with HN of the following residue (i+1) in the peptide sequence and allows assignment of each amino acid pattern to the residue number in the sequence (Figure 8). For example Gly2 has a horizontal crosspeak with Gln3 (NH of Gly2 coupling to αH of Gln3). A vertical line down from this crosspeak will lead to αH of Gln3. After assignment of each αH peak, chemical shifts were obtained by using the NOESY spectrum. Experimental α-proton chemical shifts were compared to random coil chemical shifts (Wüthrich 1986), allowing identification of structural elements. According to Hung and Samudrala, ^{1}H α-chemical shifts are higher than for random coil structures (downfield) in extended structures (β-sheet) and lower than average (upfield) in helices (Hung and Samudrala 2003).

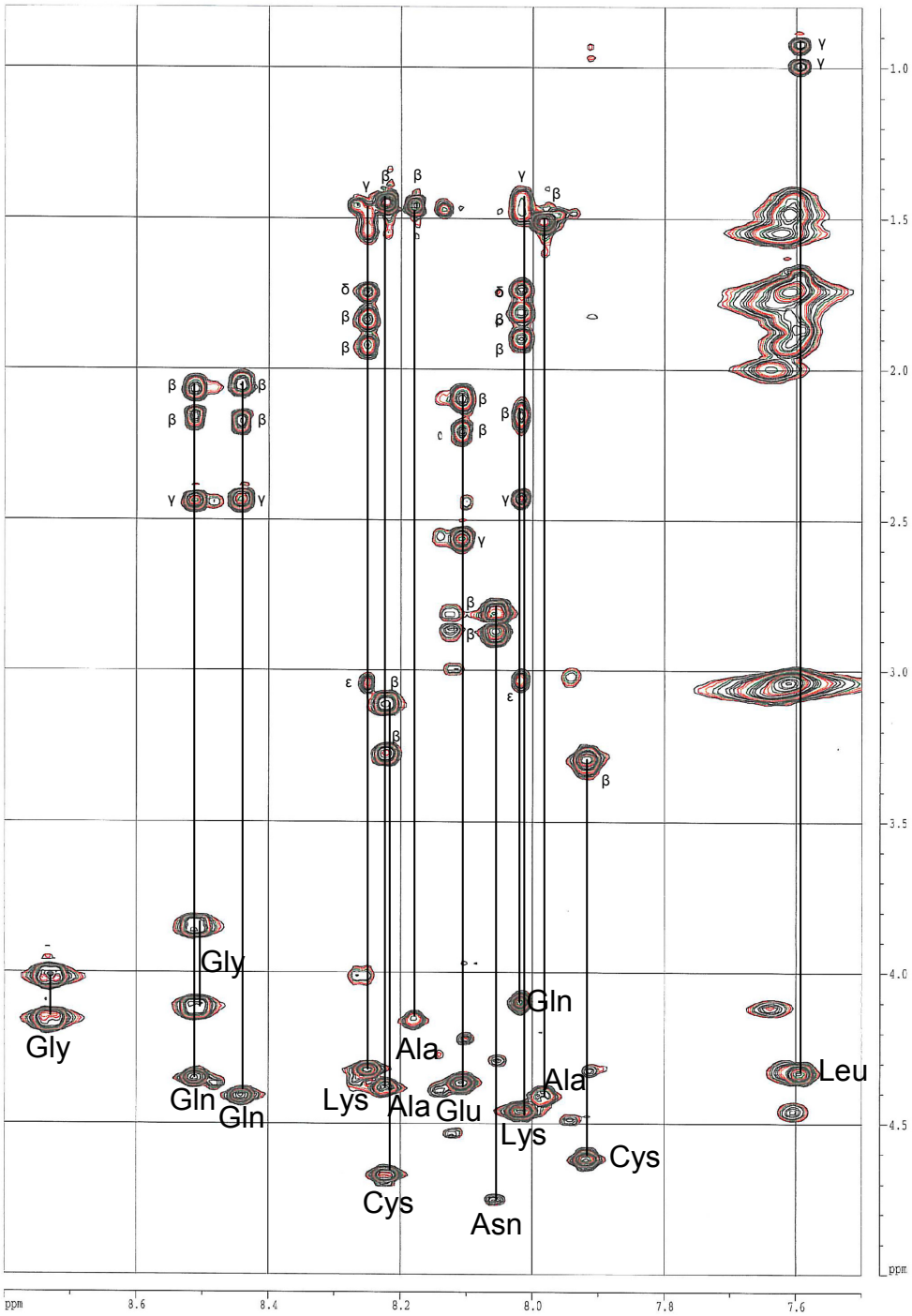


Figure 7: Characteristic crosspeak patterns of amino acids in TOCSY

Cutout of 8.8-7.5 ppm (amide region) and 0.08-5 ppm (sidechain region) of 2D-TOSCY shows amino acid specific crosspeak patterns which allow assignment of amide crosspeaks to specific residues.

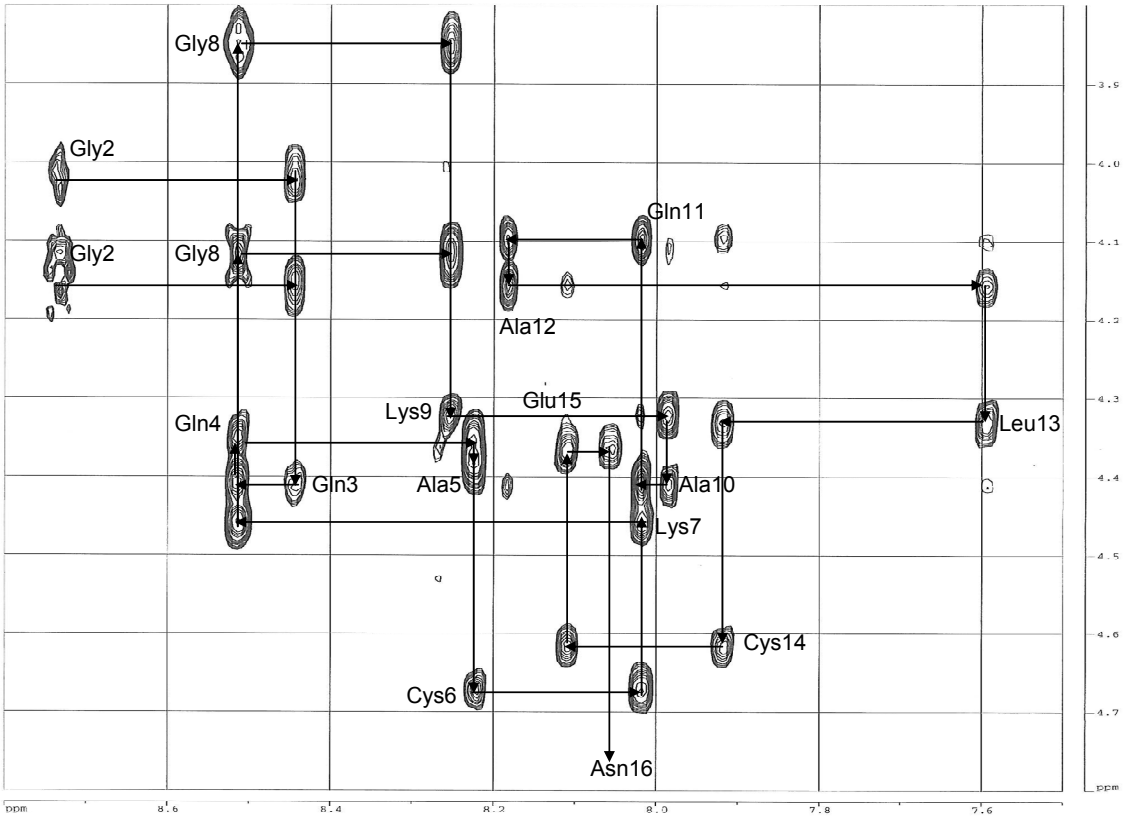


Figure 8: Sequential amino acid assignment in fingerprint NOESY region

Sequential NOE crosspeaks of αH(i) to NH(i+1) in the NOESY fingerprint region (x-axis: 8.8-7.5 ppm and y-axis: 3.8-4.8 ppm) allows sequential assignment of amino acids.

Spectra were analysed using the AURELIA software (Neidig and Kalbitzer 1990) (Bruker, Rheinstetten, Germany) on a Silicon Graphics work station: Threshold level was set arbitrarily, standard peak multiplicity was applied, a peak list was created and additional peaks were added manually to the list when not detected by the software. Each peak was either unambiguously assigned or removed from list. Peak signal integration was performed using side chain NH_2 signals of Gln as calibration for distance calculation. For manually added crosspeaks distances were obtained by comparison (using topographic lines in spectra) to known crosspeaks of similar intensities with calculated distances. Since the interpretation of NOESY lacks stereospecific resonance assignment of sidechain protons, a theoretical proton located centrally with respect to these protons was added via pseudo-atom corrections as described by Wüthrich (Wuthrich et al. 1983). Duplicate signals originating from the peaks located diagonally in the NOESY spectrum were averaged. If the difference between the intensities of identical peaks was greater than 0.5 Å, signals were analysed for splitting or fusion with nearby peaks and corrected accordingly.

Structure calculations were performed using the protocol embedded in the CNSsolve software 1.0 (Brunger et al. 1998) starting with an extended peptide backbone and incorporating the α-proton chemical shifts and NOE data. Initial calculations were computed for 20 structures and NOE with distance violations above 0.5 Å which occurred in 4 or more structures were re-examined carefully in NOESY and distance corrections were applied if necessary. Several rounds of such structure refinement were performed and the cut-off for distance violations was lowered to 0.2 Å. In a final step 100 structures were computed using the refined distance constraints; these were sorted according to lowest number of 0.2 Å distance violations, followed by the lowest E_{NOE} and lowest E_{Total}. The 20 lowest energy structures were further analysed in superposition studies to determine the central structure. Finding an objective and optimal fitting region for the 20 structures is essential to determine the structure with the lowest overall RMSD. Pairwise RMSD were calculated using the consecutive segment method (Blankenfeldt et al. 1996). Short segments of two, three, four and five residues in length were compared pairwise for all 20 lowest energy structures by the INTRMS programme. For instance, in the case of 3 residue segment length, segments Lys1-Gln4, then Gly2-Ala5, Gln3-Cys6 up to Leu13-Asn16 were compared for each structure. The RMSD of the backbone atoms calculated for these fragment were assigned to each residue within the segment. In the three-residue segment calculations, each residue is present in three segments, with the exception of the two C- and N-terminal residues of the sequence. Finally the average of each assigned value was attributed to the residue in question and plotted against the sequence. The resulting plot was used to determine the best fitting regions for allows a section for fitting with an RMSD of 0.2, 0.4 or 0.6 Å (Figure 9). After combining the separate pdb files of the 20 structures to a single pdb file with the MOLEMAN2 module (Uppsala Software Factory, Sweden), LSQMAN calculation (Uppsala Software Factory, Sweden)(Kleywegt 1996) was performed to determine the central structure with the lowest RMSD to a hypothetical mean of all 20 structures within a defined region, selected by the consecutive segment method. The LSQMAN module also calculated RMSD values of each structure to the central structure for the fitted region.

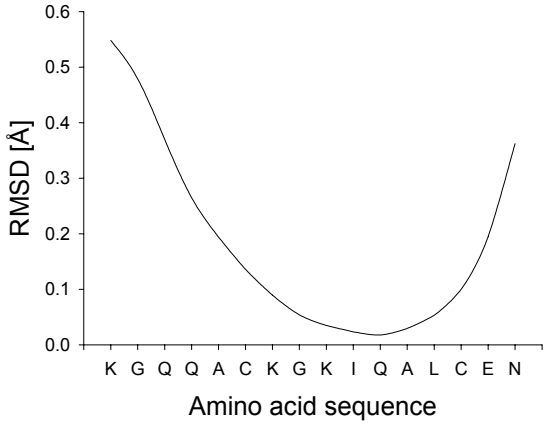


Figure 9: Mean RMSD for backbone atoms

Mean RMSD for backbone atoms in each residue are shown, calculated using consecutive segment method (2-5 amino acid segment length) and plotted against residue number for the 20 lowest energy structures

11. Molecular Modelling

Visualisation and analysis of pdb structure files was performed using a variety of software including BRAGI (Schomburg and Reichelt 1988); Pymol, an open-source projected maintained by DeLano Scientific LLC; ViewerLite 4.2 (Accrelys, San Diego, CA, USA) and Deepview/Swiss-PdbViewer 3.7 (Guex and Peitsch 1997). All the figures of resolved NMR structures in this work have been created with Pymol. Figures of amino acids or chemical compounds were created with MDL ISIS™/Draw 2.5 (MDL, San Ramon, CA, USA).

12. Statistical analysis

Statistical analysis was performed using SigmaStat 3.1 and SigmaPlot 9.0. Serum end point titres from ELISA and AFU ratios from flow cytometry experiments were analysed individually and plotted in boxplot with average values shown as horizontal line and the box area covering 75% data points. Data were analysed by One Way ANOVA logarithm and statistical significance was quantified by Student-Newman-Keuls test unless stated otherwise, with statistical difference being at least $p<0.05$. Half-lives from biostability tests with murine intestinal fluids were analysed using outlier analysis (extreme studentised deviate method), One Way ANOVA and the Bonferroni post hoc test. Statistical and regression analyses were performed on GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA, USA). For all statistical analyses a probability of $p<0.05$ was considered significant.

Chapter 3: Results

Part I: Structures

1. NMR

1.1. Measurements and α-proton chemical shifts

A series of HNE-peptides with substitutions in the epitope core (KGQQACKGKIQALCEN), which was previously defined in our laboratory (Putz et al. 2003b), were synthesised. These peptides were analysed by NMR and observations of α-chemical shifts highlighted structural differences between them and the wt-HNE-peptide, as α-proton chemical shifts are known to be dependent of the secondary structure of peptides and proteins. α-protons located in α-helices experience an upfield shift in four consecutive residues when compared to chemical shifts of protons in random coil conformation (Wüthrich 1986). A downfield shift in three consecutive residues is indicative of β-sheets (Wishart et al. 1992). Figure 10A shows the differences between observed α-proton chemical shifts in 50% TFE-d_2 solutions and random coil values for each amino acid of HNE-peptides in which Gly8 was substituted with a variety of amino acids. The values for the C-terminal region of Gln11-Asn16 of wt and modified HNE-peptides were similar. Based on chemical shift structural predictions the α-helix observed in the wt-peptide seems to be conserved between Gln11 and Glu15 in all mutants. Values for the N-terminal residues (Lys1-Ala5), which have no defined structure, were similarly well conserved and no significant difference in secondary structure was expected within this region. Substituting Gly8 had not only a direct effect on the chemical shift of the amino acid at that position but also lead to significant shifts in adjacent residues, further increasing the flexibility of the random coil loop region (Cys6-Ile10). As expected a Gly to Pro substitution had the largest effect based on the backbone altering character of Pro. The variations in the α-proton chemical shifts observed for the critical contact residues Cys6, Lys7 and Gly8, indicate that the structure of the antibody binding motif of all HNE Gly8 substituted peptides differed from the wt-HNE-peptide, which is likely to affect binding (Figure 10A).

Substitutions of amino acids located within the α-helical region generally show less dramatic effects (Figure 10B). Mirroring the results from Gly8 mutant the values for C- and N-termini of these helix mutants remain largely unchanged with regard to the reference peptide, retaining the N-terminal random coil arrangement and the C-terminal α-helix. One notable exception is the Leu13Asn substituted peptide, whose values changed significantly in the C-terminal region from upfield to downfield shifts, which is likely helix destroying. As demonstrated in the case of Gly8 mutants the region located between Cys6 and Ile10 had no defined structure and HNE peptide mutants Ile10Ala and Ile10Glu exhibiting the largest difference in α-proton chemical shifts in this region.

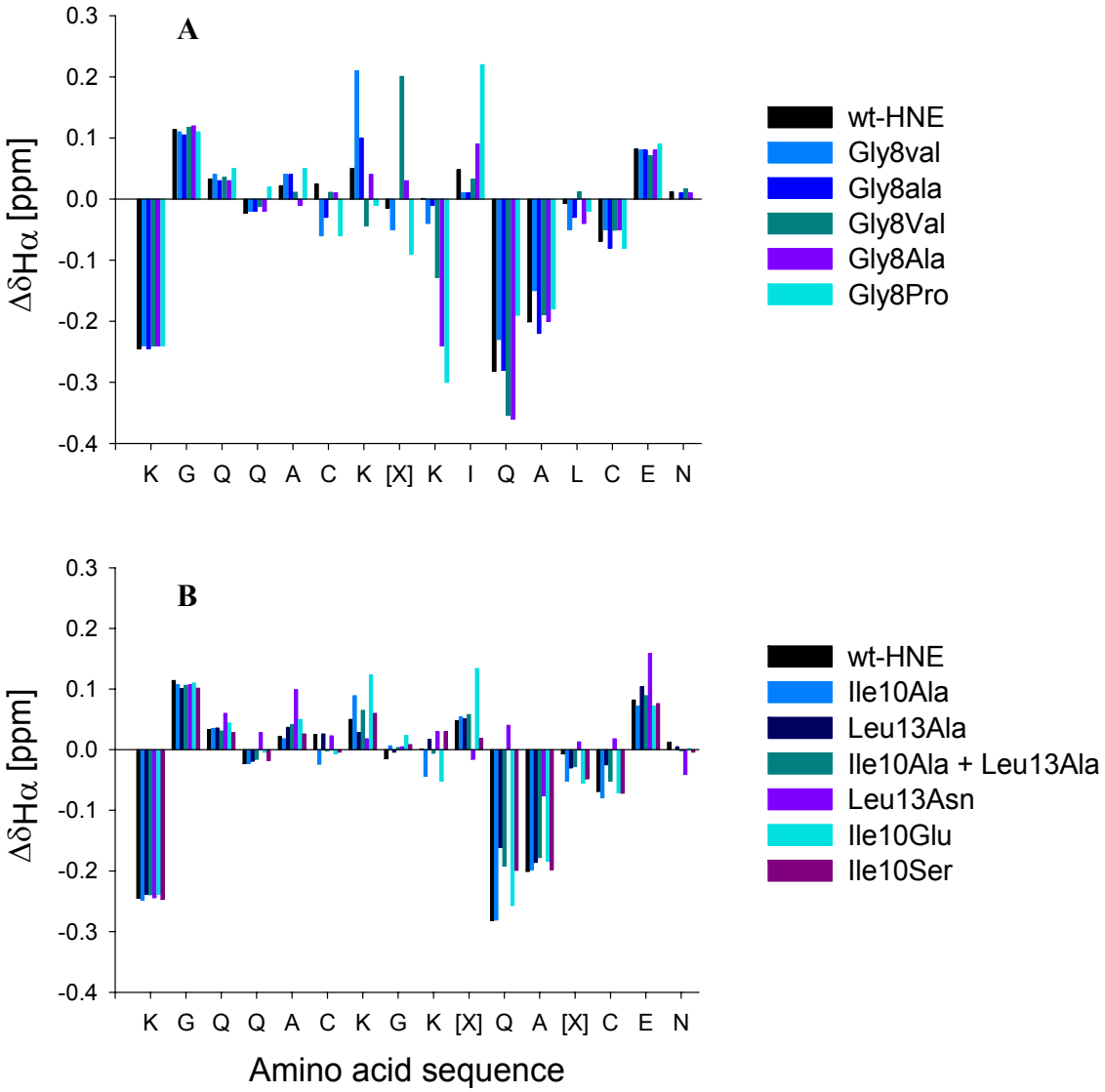


Figure 10: Differences in secondary structure of HNE-peptide based on ^{1}H α-chemical shifts in TFE-d_2
Shown are differences between experimental and theoretical chemical shifts of α-protons of HNE-peptides with Gly8 substitutions (A) and helix substitutions (B) for each amino acid.

Influence of solvent on α-proton chemical shifts and structure

To determine the influence of solvents on α-proton chemical shifts and structure, ^{1}H NMR spectroscopy measurements were performed in H_2O for wt-HNE-peptide and four substituted HNE-peptides (Figure 11A). While α-proton chemical shifts in H_2O of residues located within the α-helical region were less pronounced and thus the helix was better defined in 50% TFE-d_2 solution, the overall secondary structure remained conserved in H_2O in all measured peptides (Figure 11B, C and D).

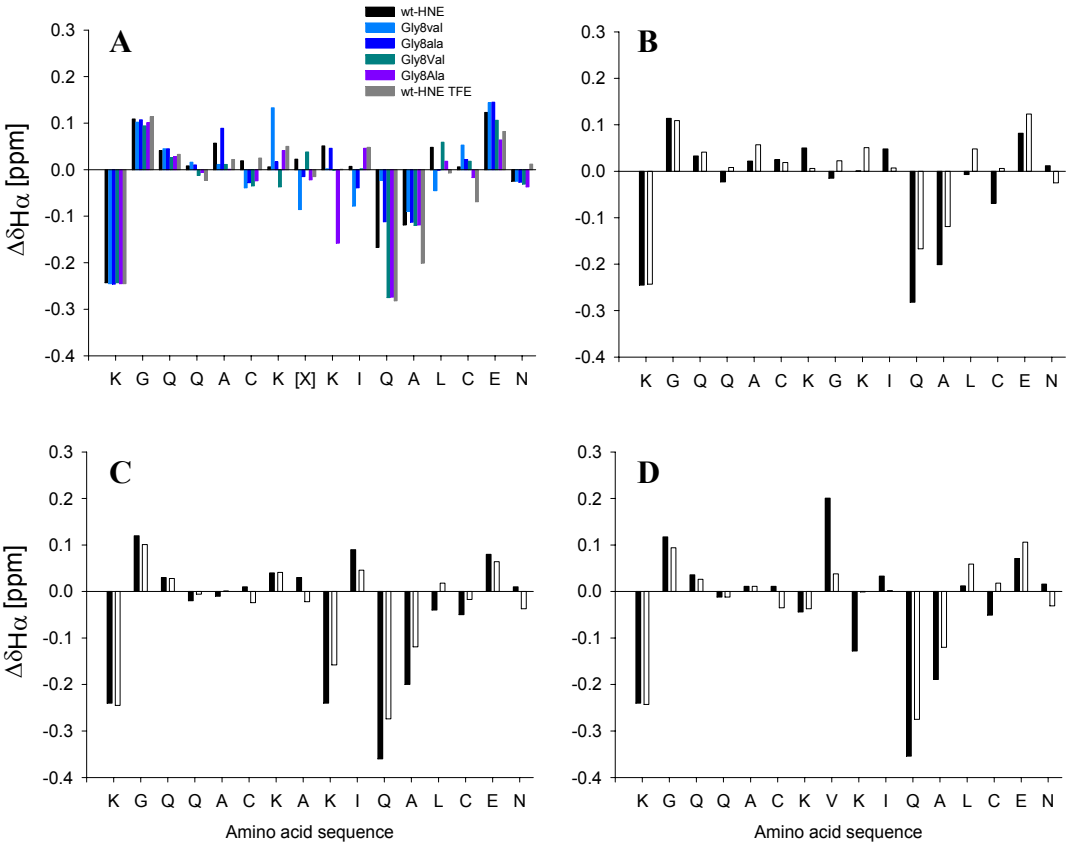


Figure 11: Differences in secondary structure of HNE peptide based on ^{1}H α-chemical shifts in H_2O and 50% TFE-d_2

Shown are differences between experimental and theoretical chemical shifts of α-protons for each amino acid of wt and modified HNE-peptides with Gly8 substitutions in H_2O (A). Direct comparisons between chemical shifts in 50% TFE-d_2 (filled bars) and H_2O (empty bars) of wt-HNE-peptide (B), Gly8Ala mutant (C) and Gly8Val mutant (D) are shown.

Influence of disulfide bond on the structure of HNE-peptide

NMR measurements were performed in 50% TFE-d_2 on both reduced and oxidised Gly8Val substituted HNE-peptides, in order to assess the influence of the disulfide bridge on the structure. Applying the method of using α-proton chemical shifts as easy and reliable criteria for structural domains, the oxidised HNE-peptide formed an α-helix between Lys9 and Cys14 (Figure 12). While no actual structure was determined for either the reduced or oxidised Gly8Val peptide, the reduced isoform showed significant differences in α-proton chemical shifts. These were observed as expected for Cys6 and Cys14, since these residues were no longer constrained by the disulfide bond. This increase in conformational freedom also altered the structure of the loop region between Cys6 and Cys14, with an α-helix now extending N-terminally up to residue Gln3.

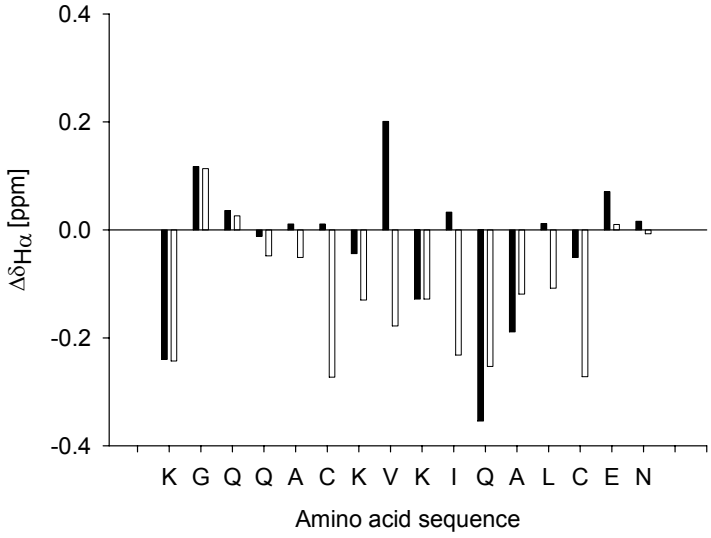


Figure 12: Differences in secondary structure of reduced and oxidised HNE-peptide
Shown are differences between experimental and theoretical chemical shifts of α-protons of reduced (open bars) and oxidised (filled bars) HNE-peptide Gly8Val in 50% TFE-d_2.

1.2. Biological activity of modified HNE-peptides

HNE-peptides with substitutions of critical and non-critical contact residues were tested for their antigenicity (Figure 13). wt-HNE-peptide reacted strongest with HNE-specific monoclonal antibody BH216 while peptides with substitutions of non-critical contact residues Leu13Ala and Ile10Ala showed a 2/3 and 1/3 decreased response, respectively, compared to wt-HNE-peptide. The other single substituted peptides failed to interact with BH216 mAb in

line with previous results obtained for the binding motif (Putz et al. 2003b). A peptide with both Ile10Ala and Leu13Ala substitutions showed no reactivity even though Ile10 and Leu13 are not contact residues and each of these substitutions is allowed separately on their own.

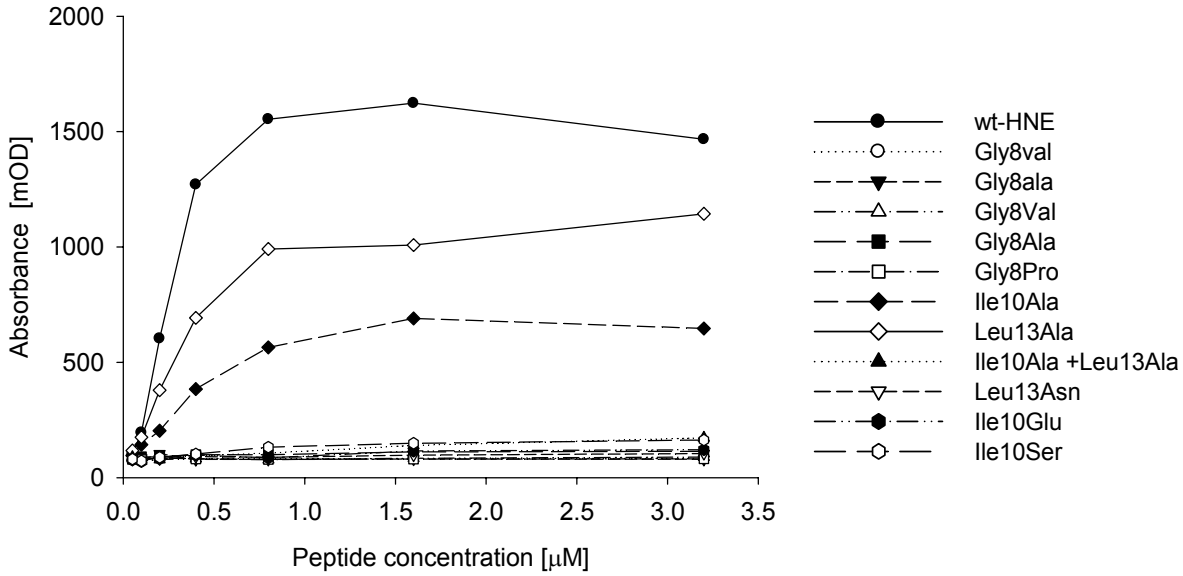


Figure 13: Indirect ELISA of Gly8 and α-helix mutants of HNE-peptides with monoclonal antibody
Wt-HNE-peptide, Gly8 substituted peptides and α-helix substituted peptides were coated and analysed for their ability to bind monoclonal antibodies BH216 in indirect ELISA.

1.3. Structure of wt-HNE-peptide

The structure of the wt-HNE-peptide was determined for our laboratory in collaboration with Dr. Karsten Bruns and Prof. Victor Wray of the Helmholtz Research Centre for Infection Research (Structural Biology, Research Group Biophysical Analysis) in Braunschweig, prior to the start of this work and was essential in developing substitution peptides that were used throughout this work. It was in their laboratory, under their guidance and with their help that I performed all other NMR structure determinations of peptides mentioned throughout this work.

Nuclear Overhauser effect (NOE) crosspeaks for KGQQACKGKIQALCEN in 50% TFE-d_2 were assigned as described before. The qualitative information obtained from comparison of observed α-proton chemical shifts with those from random coil structures, indicated that starting from Gln11, the C-terminus has a helical structure. The N-terminus appears largely unstructured (Figure 10A). The analysis of short and medium range NOE, showed that the C-terminus contained significantly more interresidual interactions than the N-terminus (Figure

14). A number of these medium range interactions (αH(i) to NH(i+3) and NH(i+4)) are characteristic of an α-helical structure. The quantitative NOE data was used as distance constraints in molecular dynamics and energy minimisation calculations. A total of 197 NOE crosspeaks were assigned unambiguously, composed of 59 intraresidual, 73 sequential, 64 medium range (up to i-i+4) and 1 long range (over i-i+4) interactions (Figure 15). These NOE were used to generate 100 conformations of which the 19 lowest energy conformations were used for fitting analysis. The consecutive segment method was applied to these structures, as described before, and amino acid sequence of Gln3 to Asn16 showed a backbone RMSD below 0.4 Å (Figure 16). This region was selected as fitting region and for central structure determination. Fitting of the lowest energy structures highlights regions with well conserved structural features and less defined domains (Figure 17), as suggested by qualitative α-proton chemical shift data and quantitative NOE. The peptide consists of a well conserved α-helix between Ile10 and Asn16 and an N-terminal linkerarm Lys1-Ala5, with pronounced flexibility. The critical HNE-motif residues Cys6, Lys7 and Gly8 as determined by Putz (2003b) are arranged in an exposed loop held in place by the disulfide bond between Cys6 and Cys14. The presence of the disulfide bridge was confirmed by NOE of β-H of Cys6 to amide proton of Cys14.

The critical hydrophilic residues of the antibody binding motif (Cys, Lys, Gly, Gln and Glu) are located on top of the α-helix (Figure 18). The bottom of the helix is formed by essentially hydrophobic uncharged residues (Ile, Ala and Leu), which have no role in binding. Ile10 can only be substituted by Ala, Glu and Gln, and plays most likely a structural role, as no direct contact with the antibody is expected. The linkerarm and Asn16, none of which are part of the binding motif, are directed away from the motif. A number of low energy structures are characterised by a backbone torsion around Gly8 causing the linkerarm to point into the antibody binding domain (Figure 19). Several Gly8 substituted HNE-peptides were synthesised to investigate this backbone flip and the structure of Gly8Ala has been resolved.

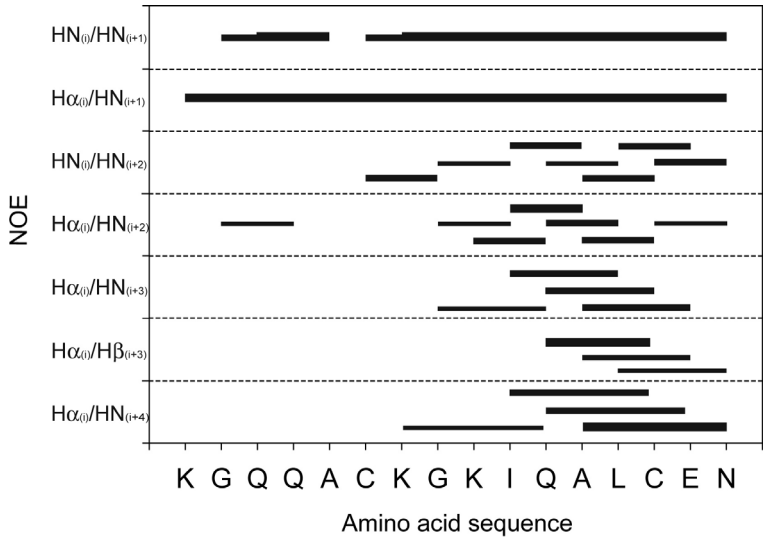


Figure 14: Structurally important short and medium range NOEs in wt-HNE-peptide

Summary of observed structurally important sequential and non sequential short and medium range NOEs in the wt-HNE-peptide.

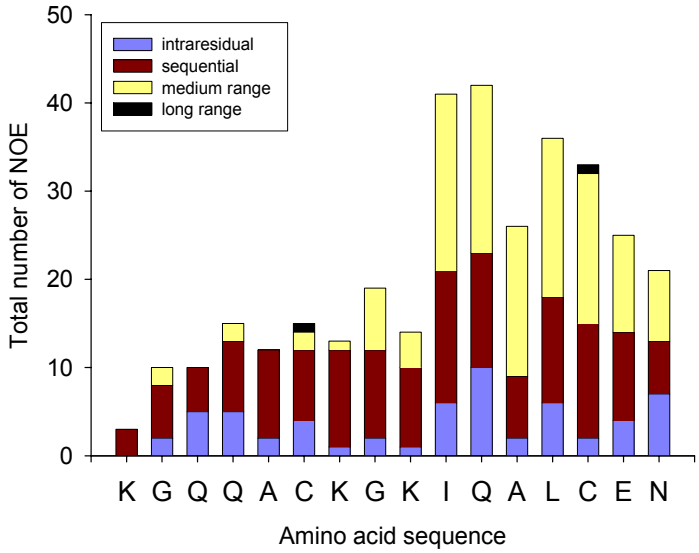


Figure 15: Total quantitative NOE crosspeaks observed and assigned for wt-HNE-peptide

Distribution of quantitative NOE observed and assigned to each residue for wt-HNE-peptide and used for molecular dynamic calculations. Apart from intraresidual NOE, each interresidual NOE appears twice as both residues to which they are assigned are shown.

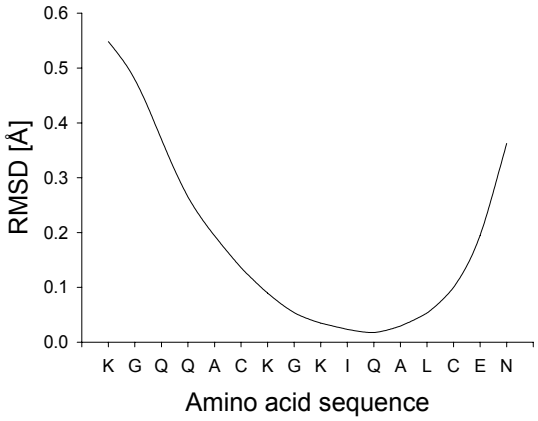


Figure 16: Mean RMSD for backbone atoms of wt-HNE-peptide

Mean RMSD for backbone atoms in each residue are shown, calculated using consecutive segment method (2-5 amino acid segment length) and plotted against residue number for the 19 lowest energy structures.

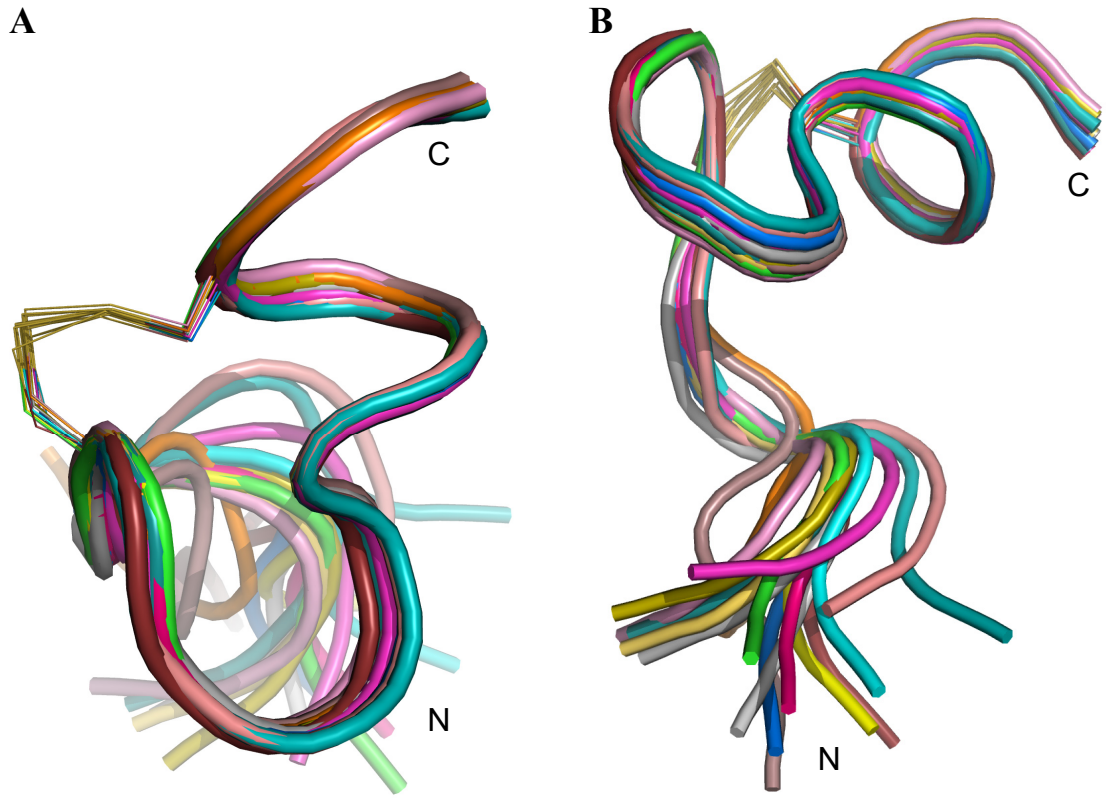


Figure 17: Structure of the 19 lowest energy conformations of wt-HNE-peptide

Superposition of the 19 lowest energy structures calculated for wt-HNE-peptide after alignment of the backbone atoms of Gln3 to Asn16. Peptide backbones are shown as tubes. Central structure is shown in green, disulfide bridges as yellow lines. (A) Top view, (B) lateral view.

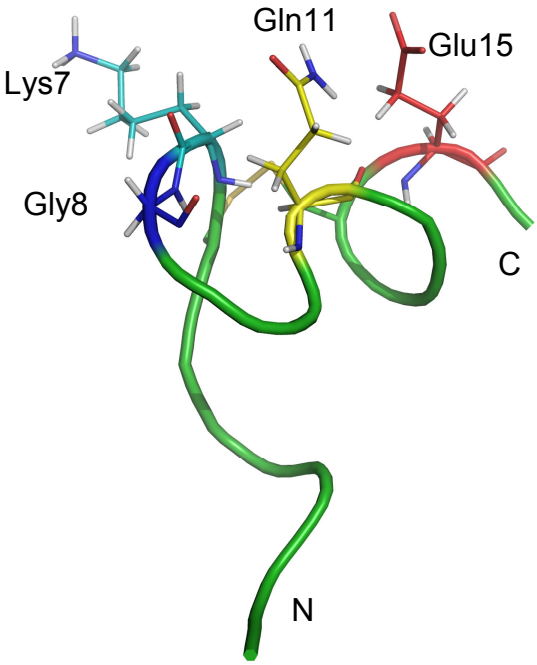


Figure 18: Central structure of wt-HNE-peptide

The critical contact residues of the antibody binding domain are displayed as coloured sticks and labelled. The peptide backbone is shown as tube.

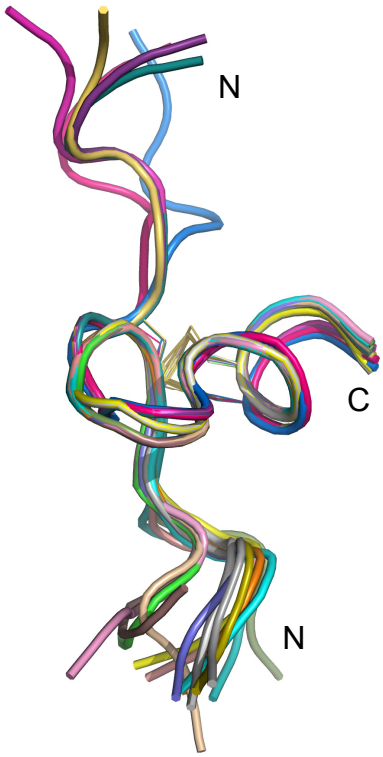


Figure 19: Backbone torsion around Gly8 of wt-HNE-peptide

Alignment showing flip of N-terminal linkerarm for 6 structures of the 20-40 lowest energy conformations of wt-HNE peptide. Peptide backbones are shown as tubes, disulfide bridge as yellow lines.

1.4. Structure of Gly8Ala-HNE-peptide

The Gly8Ala substituted peptide (KGQQACKAKIQALCEN) was one of several synthesised to further determine the characteristics of the HNE-loop and the observed flip in the wt-structure. NOE crosspeaks in 50% TFE-d_2 were assigned as described before. α-proton chemical shift data (Figure 10A) and short and medium range couplings (Figure 20) indicate the presence of a C-terminal helical structure starting at Gln11 and a relatively unstructured N-terminus. A total of 211 NOE unambiguously assigned crosspeaks were composed of 104 intraresidual, 59 sequential, 45 medium range (up to i-i+4) and 3 long range (over i-i+4) interactions (Figure 21) and used as distance constraints in structure modelling.
After repeated and improved calculation steps, the 20 lowest energy structures of the last calculation were selected for structural analysis. Mean backbone RMSD values, obtained by the consecutive segment method, showed large backbone variations in the N-terminal domain. A mean RMSD below 0.4 Å was observed between residues Ala8 and Glu15 (Figure 22). This interval highlights a relatively stable region with a conserved secondary structure in the 20 lowest energy conformations. The structure with the lowest RMSD to all other conformations was selected as the central structure and was used as a template for the alignment of the remaining 19 structures between Ala8 and Glu15 (Figure 23). The superposition confirmed indications of α-proton chemical shifts and qualitative NOE (Figure 20) that the C-terminus of the peptide forms an α-helix between residue Gln11 and Glu15. Thus the Gly8Ala substitution did not affect the α-helix to any large extent. Disulfide bonds between Cys6 and Cys14 were confirmed by long range NOE interactions between Cys6 (βH) and Cys14 (NH) as well as Ala5 (βH) - Cys14 (NH) and Ala8 (βH) - Leu13 (γH). The Lys1-Ala8 region exhibits substantial flexibility as observed for the wt-HNE-peptide structure. The structure of the peptide differs significantly from the wt-HNE-peptide with regard to the Gly loop and the orientation of the linkerarm. The Gly8Ala structure is more closely related to the observed flipped structure of wt-HNE-peptide. This random coil structure shows a preference to point upwards, into the antibody binding domain in 19 of the lowest energy conformations, with the central structure adopting a conformation in the plane of the α-helix. It is important to note that the central structure was determined as such for the fitting region Ala8-Glu15 of the backbone atoms, independently of orientation and structure of the Lys1-Lys7 sequence. Fitting clearly shows a preference for perpendicular arrangement of the sidearm with regard to the α-helix for majority of conformations.

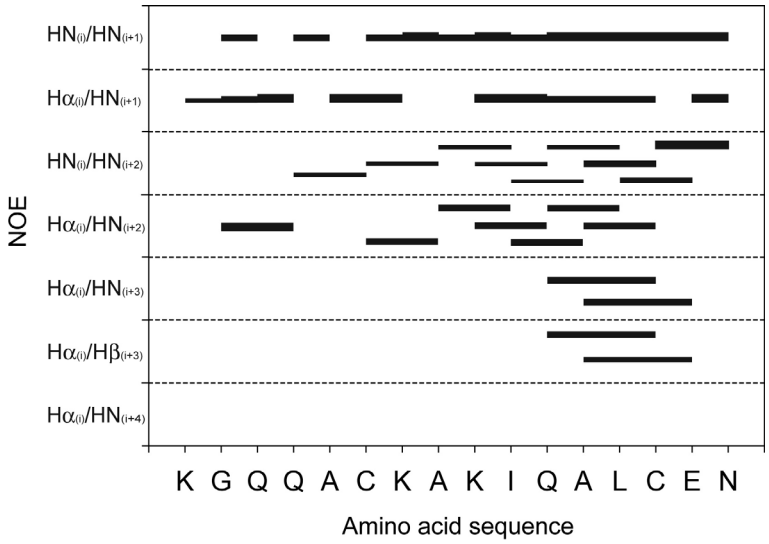


Figure 20: Qualitative summary of observed short and medium range NOE in Gly8Ala peptide

Summary of observed structurally important sequential and non sequential short and medium range NOEs in the Gly8Ala substituted HNE-peptide.

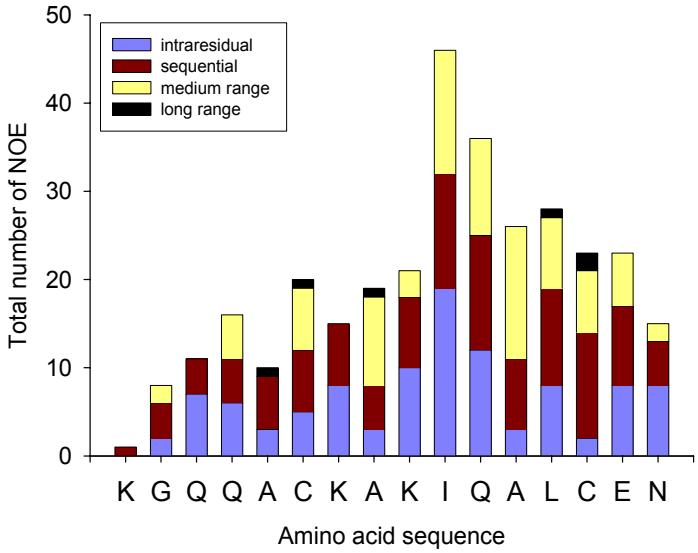


Figure 21: Total quantitative NOE crosspeaks observed and assigned for Gly8Ala mutant

Distribution of quantitative NOE observed and assigned to each residue for Gly8Ala mutant and used for molecular dynamics calculation. Apart from intraresidual NOE, each interresidual NOE appears twice as both residues to which they are assigned are shown.

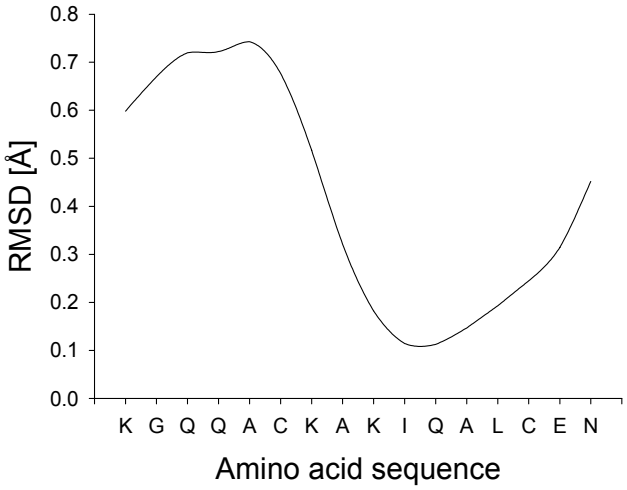


Figure 22: Mean RMSD for backbone atoms of Gly8Ala mutant peptide

Mean RMSD for backbone atoms in each residue are shown, calculated using consecutive segment method (2-5 amino acid segment length) and plotted against residue number for the 20 lowest energy structures.

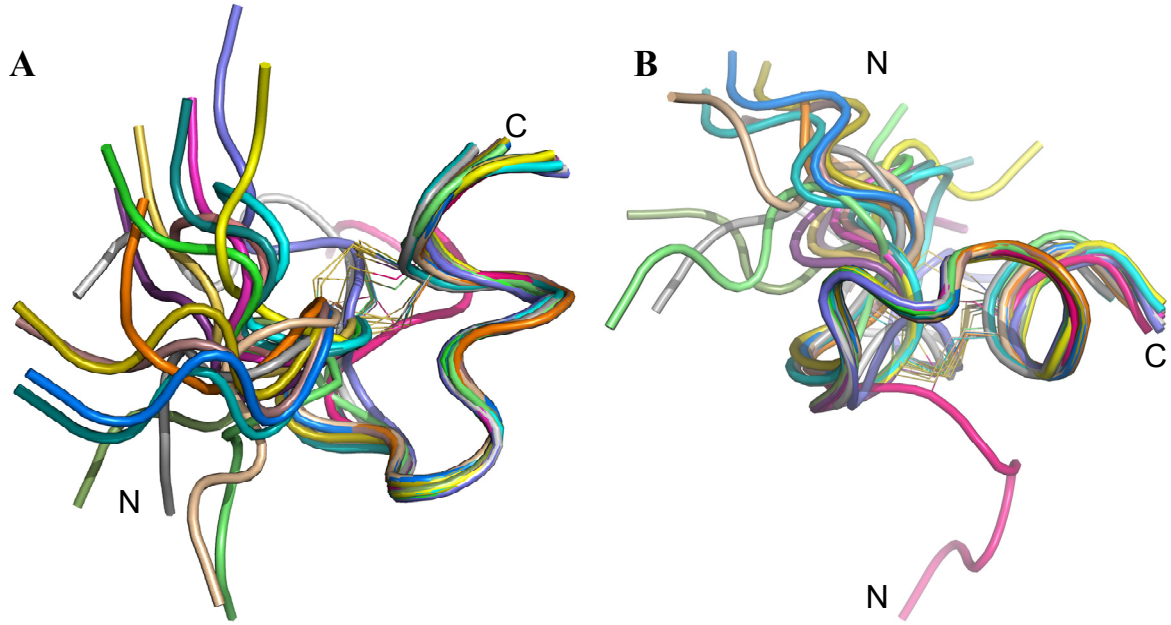


Figure 23: Structure of the 20 lowest energy conformations of Gly8Ala mutant

Superposition of the 20 lowest energy conformations calculated for Gly8Ala mutant after alignment of the backbone atoms residues 8 to 15. Peptide backbones are shown as tubes, disulfide bridge as yellow lines. Central structure is shown in pink. (A) Top view, (B) lateral view.

1.5. Structure of Ile10Ala-HNE-peptide

The Ile10Ala substituted HNE-peptide is part of a series of peptides with potential α-helical stabilising substitutions. α-proton chemical shifts (Figure 10A) and quantitative observations of assigned NOE crosspeaks (Figure 24) indicated a C-terminal helical domain and an unstructured N-terminus. A total of 154 NOE crosspeaks were assigned unambiguously composed of 74 intraresidual, 55 sequential, 26 medium range (up to i-i+4) and 1 long range (over i-i+4) interactions (Figure 25).

After gradual improvements of the NOE data, 100 structures were calculated and the 20 lowest energy structures were selected for further structural analysis. The consecutive segment method was applied to these structures and amino acid sequence of Lys9 to Cys14 with a backbone RMSD below 0.4 Å was selected as fitting region and central structure determination (Figure 26). After pairwise fitting of the 20 lowest structures over the region of Lys9-Cys14, the central structure was determined. Alignment of the remaining 19 structures to the central structure showed that the α-helical region is well conserved as expected (Figure 27). The peptide backbone around C-terminal Glu15 and Asn16 residues is considerably more flexible and these residues are not part of the α-helix as predicted by qualitative NOE and α-proton chemical shifts. The α-helix from Ile10 to Glu15 is preceded by a flexible N-terminus correlating with the observations from the structures of the wild-type and Gly8Ala substituted HNE-peptides. The disulfide bond between Cys6 and Cys14 is confirmed by long range NOE interaction of Cys6 (βH) and Gln11 (αH).

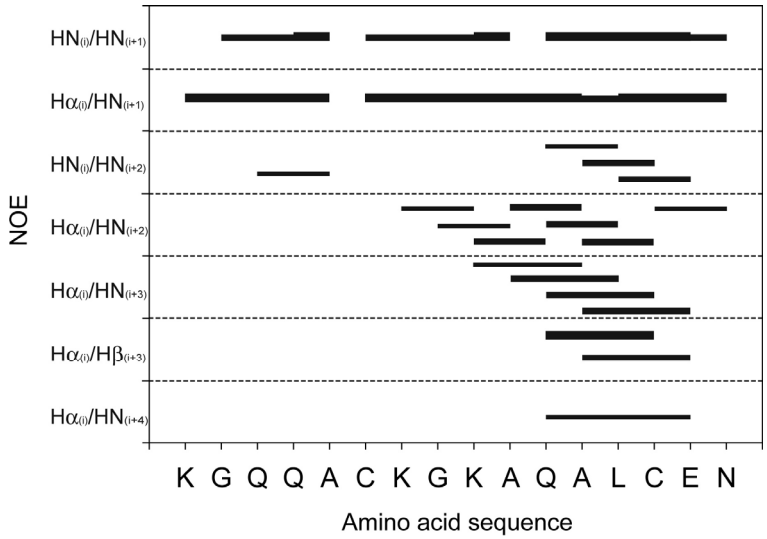


Figure 24: Qualitative summary of observed short and medium range NOE in Ile10Ala mutant peptide
Summary of observed structurally important sequential and non sequential short and medium range NOEs in the Ile10Ala substituted HNE-peptide.

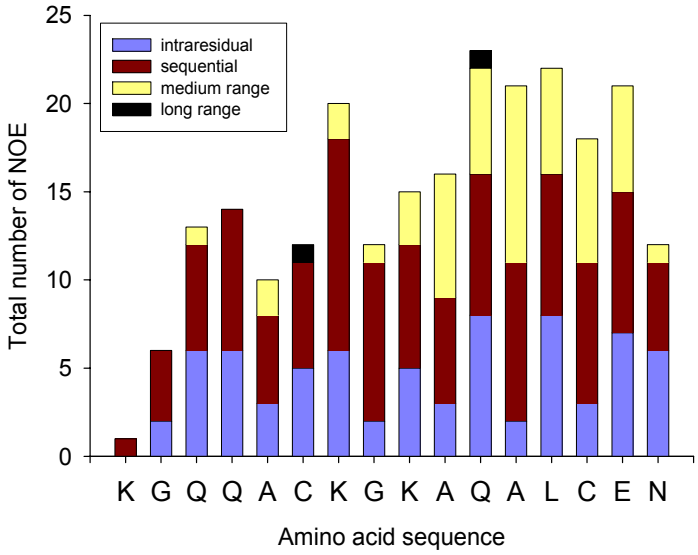


Figure 25: Total quantitative NOE crosspeaks observed and assigned for Ile10Ala mutant peptide

Distribution of quantitative NOE observed and assigned to each residue for Ile10Ala mutant peptide and used for molecular dynamic calculations. Apart from intraresidual NOE, each interresidual NOE appears twice as both residues to which they are assigned are shown.

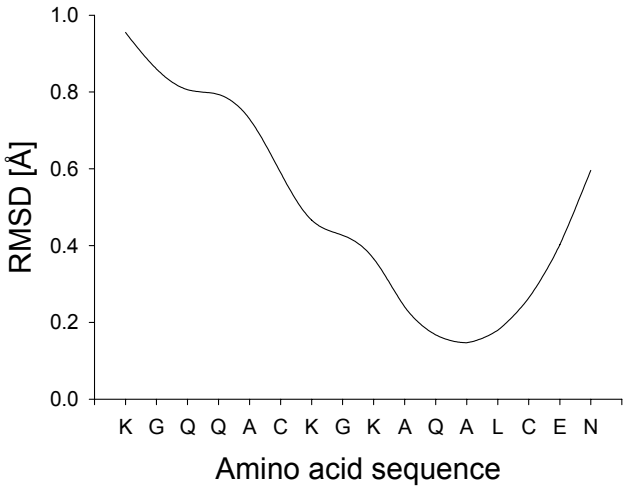


Figure 26: Mean RMSD for backbone atoms of Ile10Ala mutant peptide

Mean RMSD for backbone atoms in each residue are shown, calculated using consecutive segment method (2-5 amino acid segment length) and plotted against residue number for the 20 lowest energy structures.

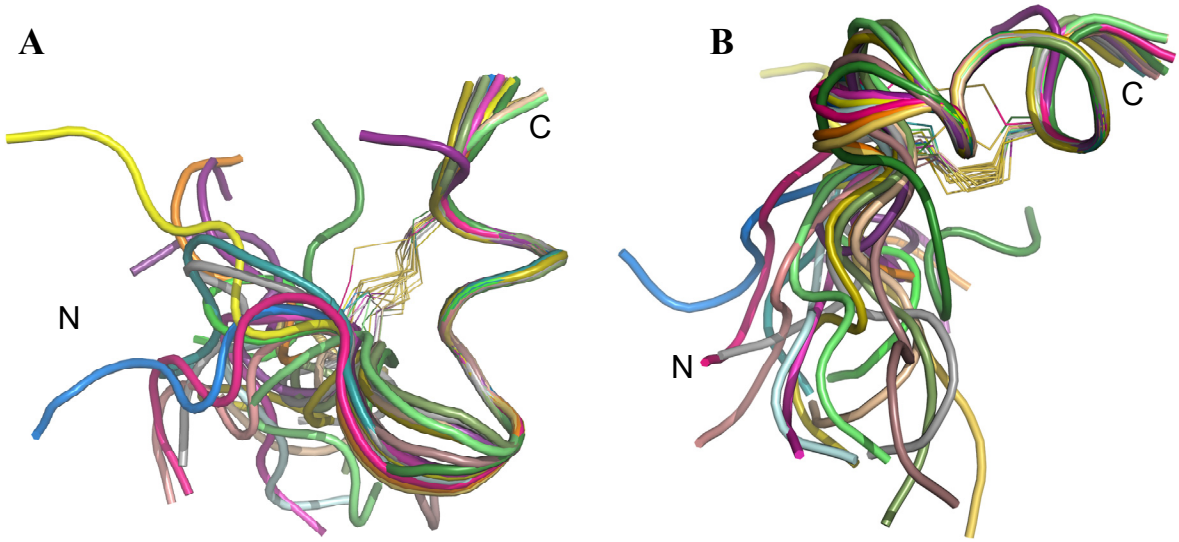


Figure 27: Structure of the 20 lowest energy conformations of Ile10Ala mutant peptide

Superposition of the 20 lowest energy structures calculated for Ile10Ala mutant peptide after alignment of the backbone atoms residues 9 to 14. Peptide backbones are shown as tubes, disulfide bridges as yellow lines. Central structure is shown in blue. (A) Top view, (B) lateral view.

1.6. Structure of Ile10Ser-HNE-peptide

The structure of KGQQACKGKSQALCEN was resolved to assess the influence of a Ser substitution in the α-helix domain of the wt-HNE-peptide. α-proton chemical shifts (Figure 10A) and quantitative NOE data (Figure 28) suggest that the α-helix was in fact retained but the lower number of crosspeaks in the C-terminal domain could be a sign of a weakened helix. Structure calculations were performed using a total of 222 unambiguously assigned NOE crosspeaks composed of 112 intraresidual, 71 sequential, 35 medium range (up to i-i+4) and 4 long range (over i-i+4) interactions (Figure 29).

The 20 lowest energy structures were selected after structure calculations and the consecutive segment method was applied, as described above. Amino acids Lys7 to Cys14 with a backbone RMSD below 0.4 Å were selected as fitting region and central structure determination (Figure 30). The 19 lowest energy structures were aligned with the central structure for the fitting region of Lys7 to Cys14 (Figure 31). Generally the region between disulfide bound Cys exposed a well conserved structure in all 20 lowest energy conformations. Long range NOEs of β-protons of Cys6 with α and amide protons of Gln11 and Cys14 respectively, and β-proton of Cys14 with the amide proton of Cys6 confirm the presence of a disulfide bond between the two Cys. The C-terminus is characterised by an α-helix between Gln11 and Glu15, correlating with α-proton chemical shift data and results

obtained from the structures of wt-HNE-peptide and other substituted HNE-peptides. It is interesting to note that the C-terminus for this structure is considerably more flexible. For position Asn16, 11 structures align with the central structure corresponding to the structures of wt-HNE-peptide, Gly8Ala and Ile10Ala substituted peptides. However 8 conformations exhibit a backbone twist at Glu15 and as a result Asn16 of these structures is moved outside the α-helix. More importantly the side chain of Glu15 is directed away from the antibody binding site and hidden inside the α-helix. The random coil structure of the N-terminal linkerarm displays the expected flexibility and is located in the same plane as the α-helix.

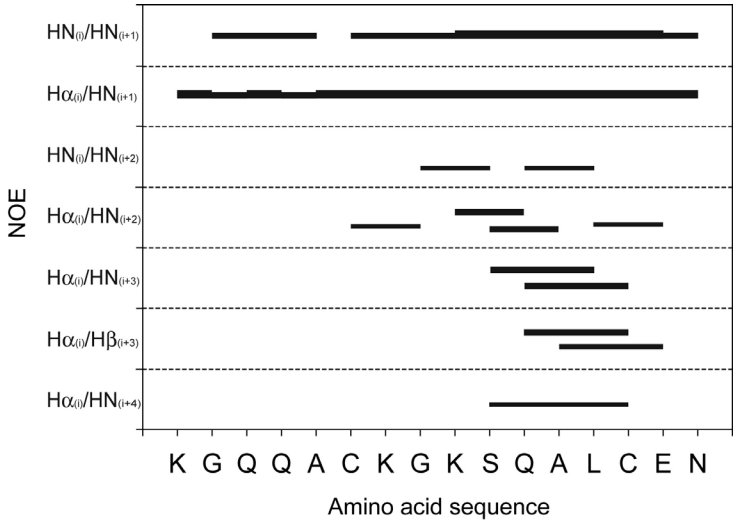


Figure 28: Qualitative summary of observed short and medium range NOE for Ile10Ser mutant peptide
Summary of observed structurally important sequential and non sequential short and medium range NOEs in the Ile10Ser substituted HNE-peptide.

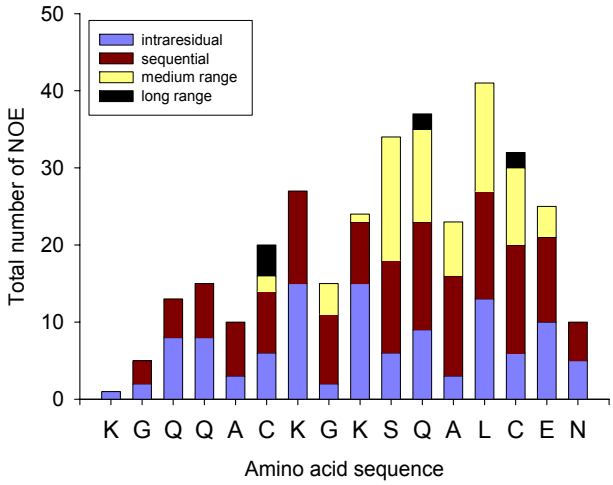


Figure 29: Total quantitative NOE crosspeaks observed and assigned for Ile10Ser mutant peptide

Distribution of quantitative NOE observed and assigned to each residue for Ile10Ser mutant peptide and used for molecular dynamic calculations. Apart from intraresidual NOE, each interresidual NOE appears twice as both residues to which they are assigned are shown.

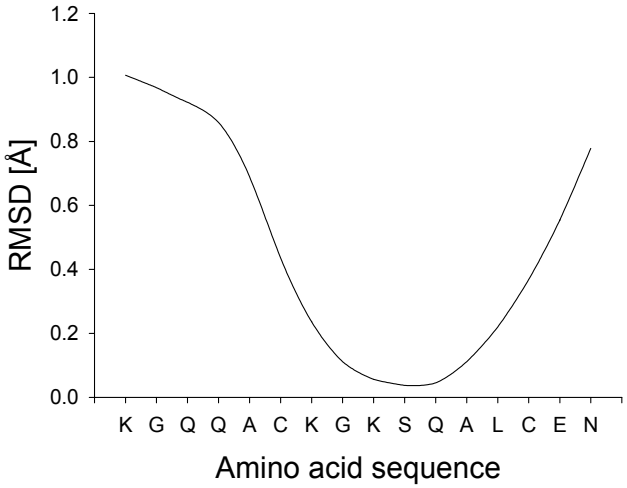


Figure 30: Mean RMSD for backbone atoms of Ile10Ser mutant peptide

Mean RMSD for backbone atoms in each residue are shown, calculated using consecutive segment method (2-5 amino acid segment length) and plotted against residue number for the 20 lowest energy structures.

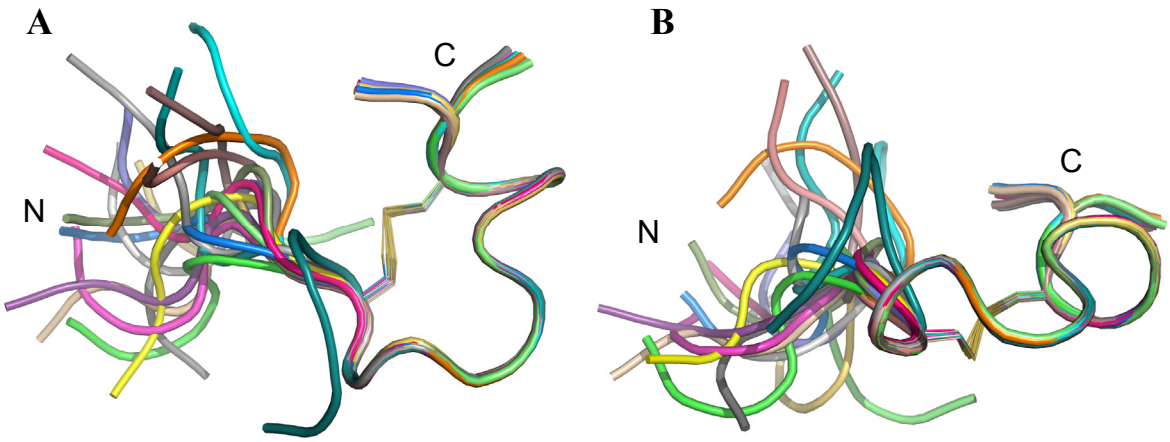


Figure 31: Structure of the 20 lowest energy conformations of Ile10Ser mutant peptide

Superposition of the 20 lowest energy structures calculated for Ile10Ser mutant after alignment of the backbone atoms residues 7 to 14. Peptide backbones are shown as tubes, disulfide bridges as yellow lines. Central structure is shown in yellow. (A) Top view, (B) lateral view.

2. Functional importance and location of disulfide bond in HNE-peptides

The amino acid sequence of the HNE loop of measles virus H-protein contains three Cys that can potentially form disulfide bonds. To assess the location and importance of the disulfide bonds and the structural role of each Cys, Cys of full length HNE peptides were monosubstituted with amino butyric acid (Cys381B, Cys386B or Cys394B) and a full length HNE-peptide sequence with all three substitutions was synthesised as control. Binding of these peptides to anti-HNE specific monoclonal antibodies (mAbs) BH216, BH21 and BH6 was assessed. Monosubstituted Cys386B, Cys394B and trisubstituted (Cys381B, Cys386B, Cys394B) peptides failed to bind anti-HNE specific antibodies (Figure 32). Only the oxidised peptide Cys381B with a disulfide bond between Cys386 and Cys394 reacted within this peptide series. BH216 (Figure 32A) recognises C381B peptide much stronger than the oxidised shorter HNE peptide sequence ($KGQ_{383}QACKGKIQALCEN_{396}$). Binding profiles of BH21 with peptides were very similar (data not shown). BH6 shows a different binding specificity than BH216 with the same peptides: the importance of the Cys386-Cys394 disulfide bridge is confirmed and no other substituted peptide reacts with BH6 (Figure 32B). This mAb does however exhibit stronger binding to the shortened oxidised HNE-peptide. These results, indicating a disulfide bridge between Cys386-Cys394, corroborate observations made by Putz (2003b) and were later confirmed by the publications of crystal structures of the MV H-protein (Colf et al. 2007; Hashiguchi et al. 2007).

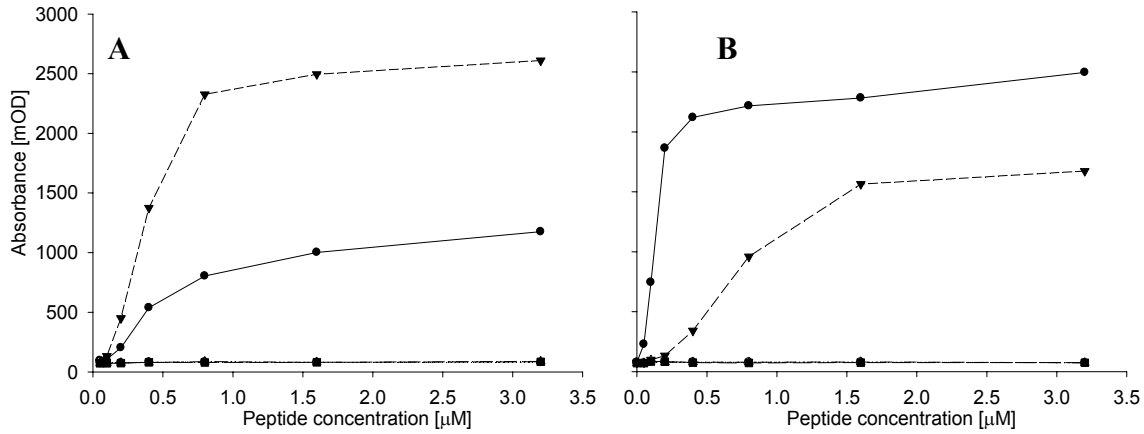


Figure 32: Indirect ELISA of mono and trisubstituted full length HNE peptides with monoclonal antibodies.

Each Cys in the full length HNE sequence $E_{379}TCFQQACKGKIQALCENPEWA_{400}$ was substituted with amino butyric acid (Cys381B ▼, Cys386B △ or Cys394B ■, or trisubstituted ○) and peptides were coated and analysed for their ability to bind mAbs BH216 (A) and BH6 (B) in indirect ELISA. Oxidised shortened wt-HNE-peptide ● ($KGQ_{383}QACKGKIQALCEN_{396}$) was included as control.

Part II: Stability and antigenicity

1. wt-HNE-peptide non-recognition by anti-MV sera

The binding specificities of sera and anti-H-protein mAb to HNE peptide and H-protein were assessed in ELISA. Results showed that the shortened wt-HNE-peptide $KGQ_{383}QACKGKIQALCEN_{396}$ was only recognised by BH216 antibody (Figure 33) and related mAb BH21 and BH6 (data not shown), which specifically bind the HNE-loop. Sera from naturally immunised (late convalescent) and vaccinated individuals showed no reactivity with the HNE-peptide. mAb BH195 is known to bind only to reduced full length HNE peptides and selectively to the H397-400 region of the denatured H-protein and is routinely used as a negative control in ELISA with shortened HNE-peptides. As expected BH195 did not bind the HNE-peptide, however some reactivity with H-protein was observed possibly due to the presence of some denatured H-protein which exposed the H397-H400 region. The H-protein was strongly recognised by mAb BH216 and sera from late convalescent and vaccinated individuals. MV negative sera did not bind the HNE peptide or the MV H-protein. These binding specificities, in particular the binding of the MV neutralising and protective BH216 and the absence of binding of human sera to peptide, confirmed the choice of the HNE-peptide as a lead peptide for peptide-based vaccine against MV in the presence of anti-MV antibodies.

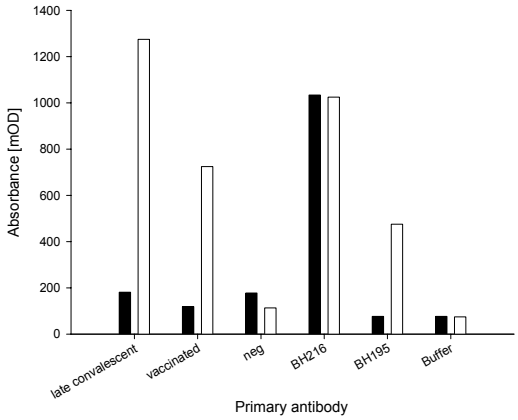


Figure 33: Differential recognition of H-protein and shortened HNE-peptide

ELISA responses of human sera from naturally infected (late convalescent), immunised and vaccinated individuals and monoclonal anti-HNE antibodies binding to coated H-protein (empty bars) or shortened wt-HNE-peptide ($KGQ_{383}QACKGKIQALCEN_{396}$) (filled bars).

2. Fine specificities of monoclonal antibodies

The anti-HNE specific monoclonal antibodies BH216, BH21, BH6 and BH195 used throughout this work have different binding specificities to HNE-peptides. Confirming earlier results, BH195 binding was negative for both reduced and oxidised forms of the wt-HNE-peptide (Figure 34A). Even the other anti-HNE mabs showed considerable differences in reactivity with the wt-HNE-peptide: BH216 only binds HNE-peptide with cyclised Cys residues. The reduced form was only poorly recognised. BH6 on the other hand, while predominantly binding the oxidised isoform, had a much higher tolerance for the lack of a disulfide bond in the wt-HNE-peptide, with approximately 50% of antibodies also binding the reduced isoform.

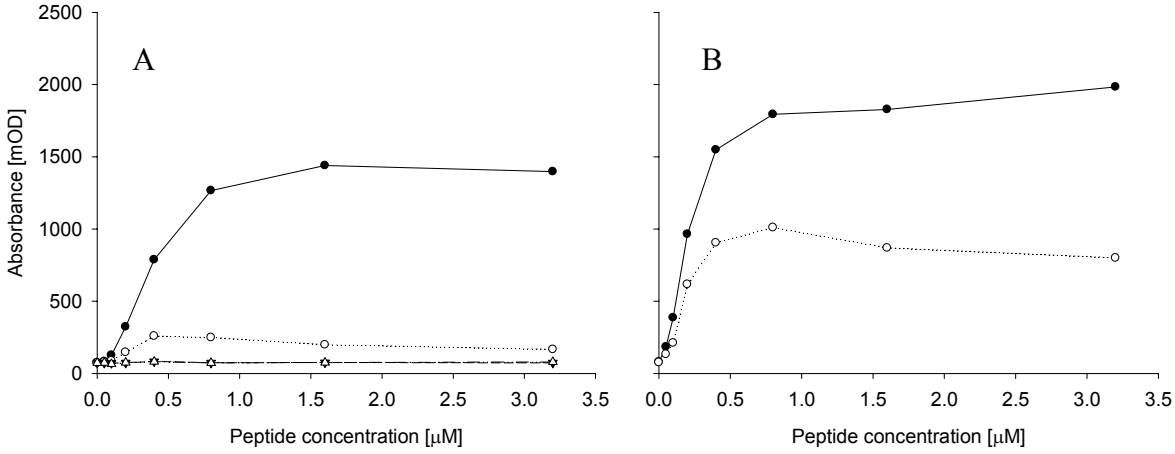


Figure 34: Fine specificities of anti-HNE monoclonal antibodies to shortened HNE-peptide

HNE-peptide ($KGQ_{383}QACKGKIQALCEN_{396}$) was coated either in reduced (empty symbols) or oxidised (filled symbols) form and binding specificities of monoclonal antibody BH216 (circles) and BH195 (triangles) (A), and BH6 (B) were assessed in ELISA.

3. Stability of wt-HNE-peptide in serum

Initially the ELISA technique was assessed for its capacity to analyse peptide stability in serum. The method allows monitoring peptide fragments from degradation process for their interaction anti-HNE mAb. Peptides were coated on ELISA plates and incubated with serum at different time points and degraded peptides were analysed for mAb binding. In an alternative approach peptide was incubated with serum and the reaction mixtures were coated and standard ELISA protocol was followed thereafter. Both these methods were assessed and found to be inadequate. The major drawbacks of these techniques were background noise due to serum binding to plastic support and the apparent inability of serum proteases to degrade peptide that is coated to the plastic support (data not shown). The ELISA approach to peptide stability was abandoned and HPLC based stability tests were developed.

The HPLC based biostability test of the reference peptide KGQQACKGKIQALCEN in mouse serum from the frozen stock revealed a half life of 11.8 hours (Figure 35A). In fresh serum the half life was even shorter (8.9 hours, Figure 35B). Frozen commercial mouse serum stock was chosen for further biostability tests because of the homogenicity of pooled sera compared to freshly prepared serum and thus higher reproducibility of results.

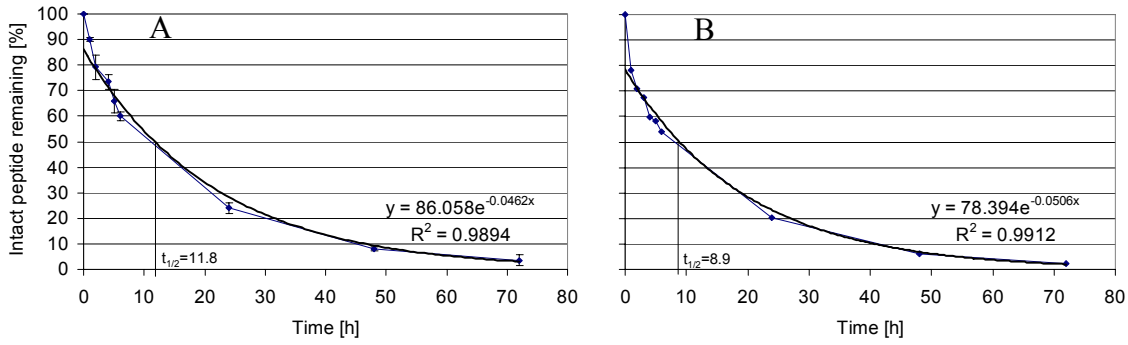


Figure 35: Degradation of wt-HNE peptide in mouse serum over a 72 h time span

Biostability of wt-HNE-peptide was assessed in serum from frozen commercial stock (A) or from freshly prepared (B) mouse serum (n=1-2). Trendlines (black) were used to determine half-lives.

4. Enhancing the stability of wild-type HNE-peptide

4.1. Preliminary peptide synthesis

In a first preliminary peptide synthesis two sets of peptides were produced to assess different functional aspects and to establish interesting modifications for further syntheses.

1) In a first set of peptides, the N-terminus of the peptide chain featured a Fmoc protecting group on the Lys backbone amine. Prior to immunisation, peptides are generally coupled to carrier protein which is likely to block degradation of the peptide N-terminus. The presence of the Fmoc protecting group was intended to simulate the carrier protein and at the same time facilitate the monitoring of peptide degradation. But the Fmoc group was unable to protect the N-terminus against exopeptidase mediated proteolytic degradation, because of spontaneous cleavage of the protecting moiety and accelerated peptide degradation (data not shown).

2) Our structures of the wt-HNE-peptide and substitution HNE-peptides have shown that amino acids following the C-terminal residue Glu15 are directed away from the antibody binding site and are unlikely to affect binding to the critical HNE contact residues.
The second set of synthesised peptides were part of a library with different C-terminal extensions by non-natural amino acids (see Annexe 1), to identify modifications that enhanced peptide biostability without affecting its antigenicity. The various extension residues included large and modified amino acids and D-amino acids whose sidechains did not include functional groups. In contrast to expectations most single additions of non-natural amino acids such as pamba (4-(aminomethyl)benzoic acid) and gaba (γ-aminobutyric acid) or the additions of one or two D-amino acids had little effect on the stability towards serum proteases after 4 hours of incubation or reduced peptide antigenicity (data not shown). In contrast the analysis suggested that *t*-amcha (trans-4-(aminomethyl)cyclohexanecarboxylic acid), achca (1-(amino)cyclohexanecarboxylic acid), aib (α-amnioisobutyric acid), D-Phe, D-Ala, and D-Val amongst others (data not shown), were potentially interesting.
The combination of different N- and C-termini within the same peptides complicated the conclusions drawn from this exploratory peptide synthesis.

4.2. C-terminally extended peptides

A new synthesis of peptides with the above mentioned C-terminal extensions or the C-terminal addition of natural (PEWA) or modified (PEWa and PEWAa) HNE-sequence residues, were produced (see fold out page Annexe 2). The N-terminal Lys of all of these peptides, including the wt-HNE-peptide, was blocked via acetylation for the stability testing, as such a modification is known to stabilise peptides against exopeptidases(Maillère et al. 1995) and allowed focus on the C-terminus only. The antigenicity measured binding of anti-HNE mAb, and the biostability, expressed in terms of % of intact peptide remaining after 48 h incubation in serum, were plotted against each other. This plot shows peptides with interesting mutations in the top right corner (Figure 36). Those modified peptides exhibit increased stability and retained antigenicity, while peptides located at the bottom left of the plot contain the least favourable modifications. Only a few C-terminal additions such as D-Ala, PEWAa and PEWa retained antigenicity and were as stable as the N-acetylated wt-HNE-peptide (Figure 36). Unexpectedly, the large majority of modified peptides showed decreased biostability compared to the wt-HNE-peptide, although some of these reacted better with anti-HNE mAbs. The addition of two D-amino acids to the C-terminus inhibited antibody binding, presumably because of steric interactions.

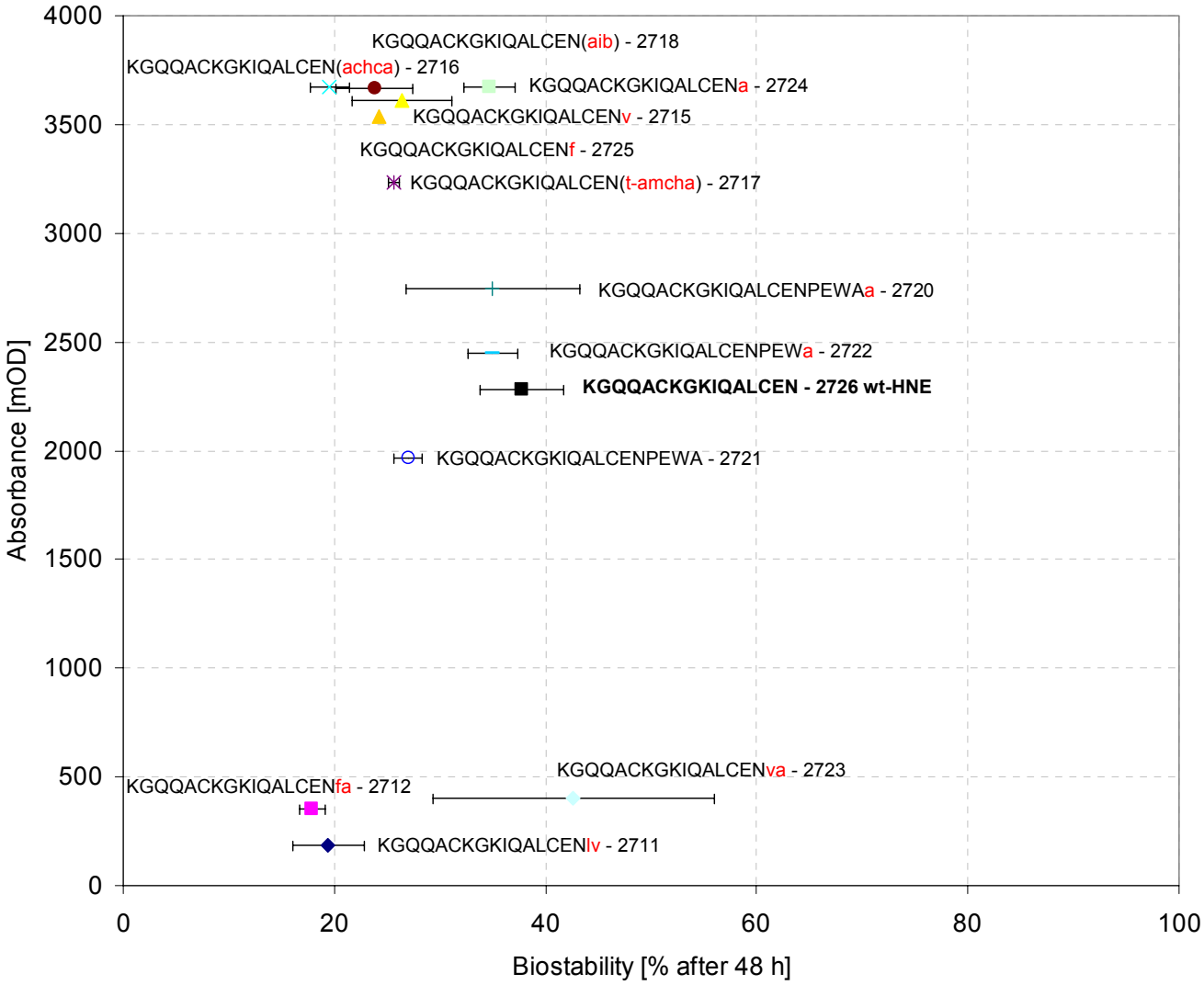


Figure 36: Biostability-antigenicity correlation plot of C-terminally extended peptides

The biostability (x-axis) and antigenicity (y-axis) of modified HNE-peptides are plotted against one another. Biostability is calculated as % intact peptide and mean values (±SD) of 2 independent experiments are shown. Antigenicity is expressed as absorbance in ELISA with BH216 mAb binding to 0.8 µM coated peptide.wt-HNE-peptide is shown in bold.

4.3. Internally and N-terminally modified peptides

Since C-terminal extensions did not increase the biostability of the HNE-peptide, a number of peptides with internal modification were synthesised. Substitution studies in the HNE binding motif, which were previously done in our laboratories (Putz et al. 2003b), guided the selection of acceptable substitution sites. The critical antibody binding residues were left unaltered; instead the noncritical Lys9 which is also expected to be a major endopeptidase target was substituted with a variety of amino acids. Substitution of Lys9 with Ala, His, Ser and Gln produced peptides, which were more stable than the wt-HNE-peptide or as stable as in the case of Lys9Asn (Figure 37). However, with the exception of Lys9Ala substitution, their antigenicity was reduced or totally lost.

The N-terminal Lys of the HNE-peptide is important for optimal coupling to the carrier protein and was added as a linker to the HNE-sequence during earlier work in our laboratory (Putz 2004). Since Lys is a known target of peptidases, modifications of the N-terminus such as branching extensions (G_4K_2KGQQ… and G_2KGQQ...) were produced which could increase the stability of the peptides.The additional residues were added to the backbone and sidechain amine of N-terminal Lys. The increased number of N-terminal amine groups could also reduce the risk of coupling via sidechain amines of Lys residues located inside the antibody binding domain (Lys7 and Lys9). Coupling via the latter Lys was suggested to destroy the critical epitope. While improving the biostability to 48% (from 37%), a reduction in reactivity with mAb BH216 was observed for these peptides (Figure 37). The substitution of the N-terminal Lys with its D-isomer did not increase the stability, suggesting that the responsible aminoexopeptidase is not stereospecific. Thus only the Lys9Ala (peptide 2734) mutant showed both improved stability and antigenicity and was selected as the lead substitution for further rounds of synthesis.

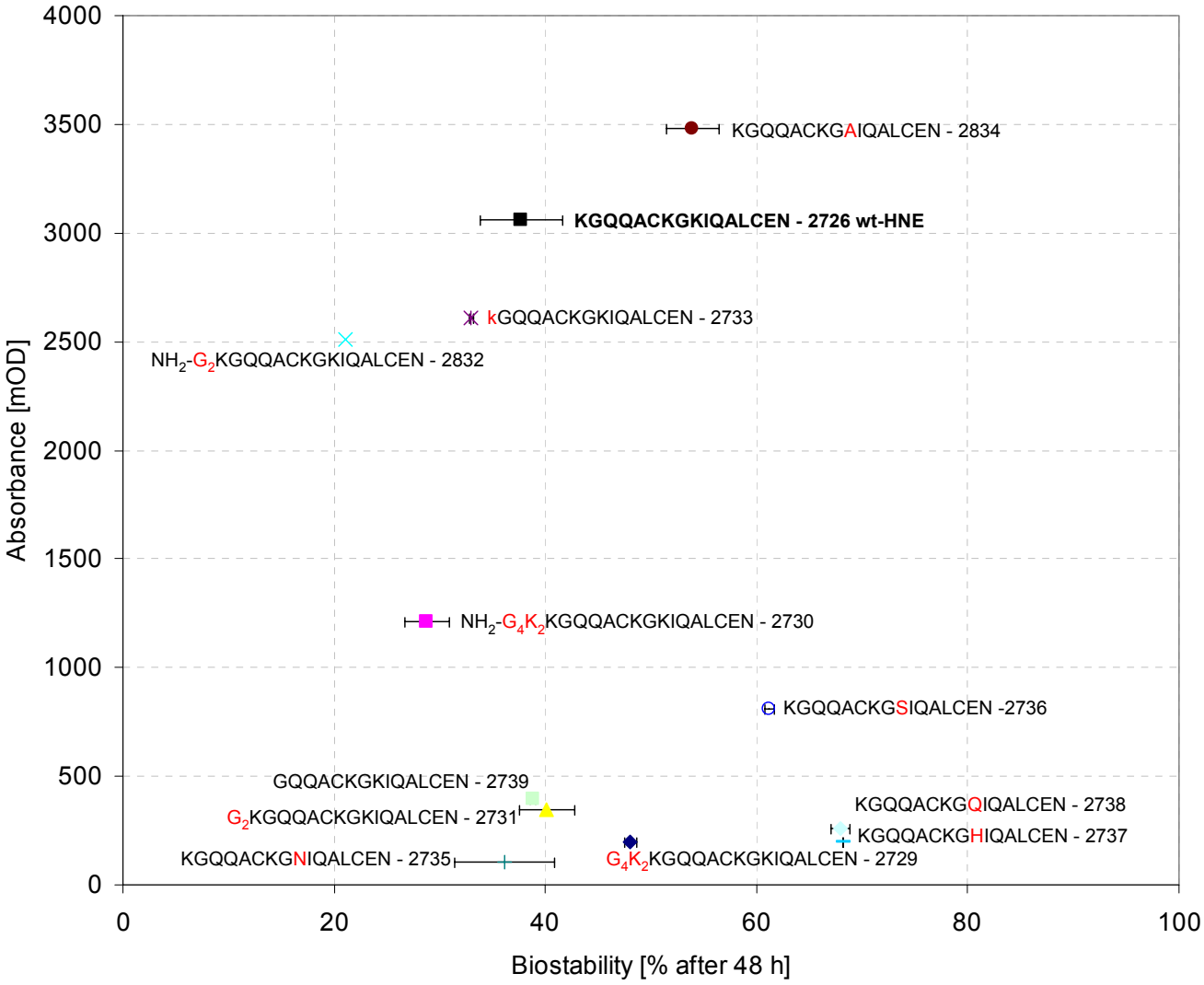


Figure 37: Biostability-antigenicity correlation plot of internally and N-terminally modified peptides

The biostability (x-axis) and antigenicity (y-axis) of modified HNE-peptides are plotted against one another. Biostability is calculated as % intact peptide and mean values (±SD) of 2 independent experiments are shown. Antigenicity is expressed as absorbance in ELISA with BH216 mAb binding to 0.8 µM coated peptide. Branched peptides: G_4 and G_2 = Gly coupled to preceding Lys via backbone and sidechain NH_2, K_2 = Lys coupled to preceding Lys via backbone and sidechain NH_2,. wt-HNE-peptide is shown in bold

4.4. Internally and C-terminally modified peptides

Based on the results observed for the C-terminal modifications and internal substitutions, a synthesis combining these individual changes, was performed. This resulted in peptides KGQQACKGAIQALCENPEWa (2741), KGQQACKGAIQALCENa (2744), KGQQACKGAIQALCENv (2745) and KGQQACKGAIQALCEN (2751), with better stability in mouse serum compared to wt-HNE-peptide KGQQACKGKIQALCEN (2752): stabilities increased from 37% to 47%, 54%, 50%, and 53%, respectively (Figure 38). These peptides also retained the reactivity with respect to mAb BH216.

The substitution of Lys7 to D-Lys (peptide 2749) increased stability from 37% to 59% compared to wt-HNE-peptide. The addition of a Lys9Ala substitution to this peptide (peptide 2750) lead to an additional increase in stability to 71%. However peptides whose Lys7 was replaced with its D-isomer failed to bind mAb. Substituting Lys with Arg had a destabilising effect suggesting that Arg is even more readily cleaved by proteases than Lys.

It is interesting to note that the consecutive C-terminal extension of D-Val and D-Ala, on a peptide with a Lys9Ala substitution, abolished the reactivity of this peptide with BH216 mAb, confirming earlier results of loss of antigenicity due to the addition two D-amino acid additions. HoweverInstead, the addition of two consecutive D-Ala residues to the C-terminus conserved the peptide's antigenicity. Presumably the steric hindrance with respect to the antibody binding domain by two small D-Ala residues is much less pronounced than with larger D-amino acids.

The four peptides with an enhanced biological stability listed above, were singled out for immunological studies.

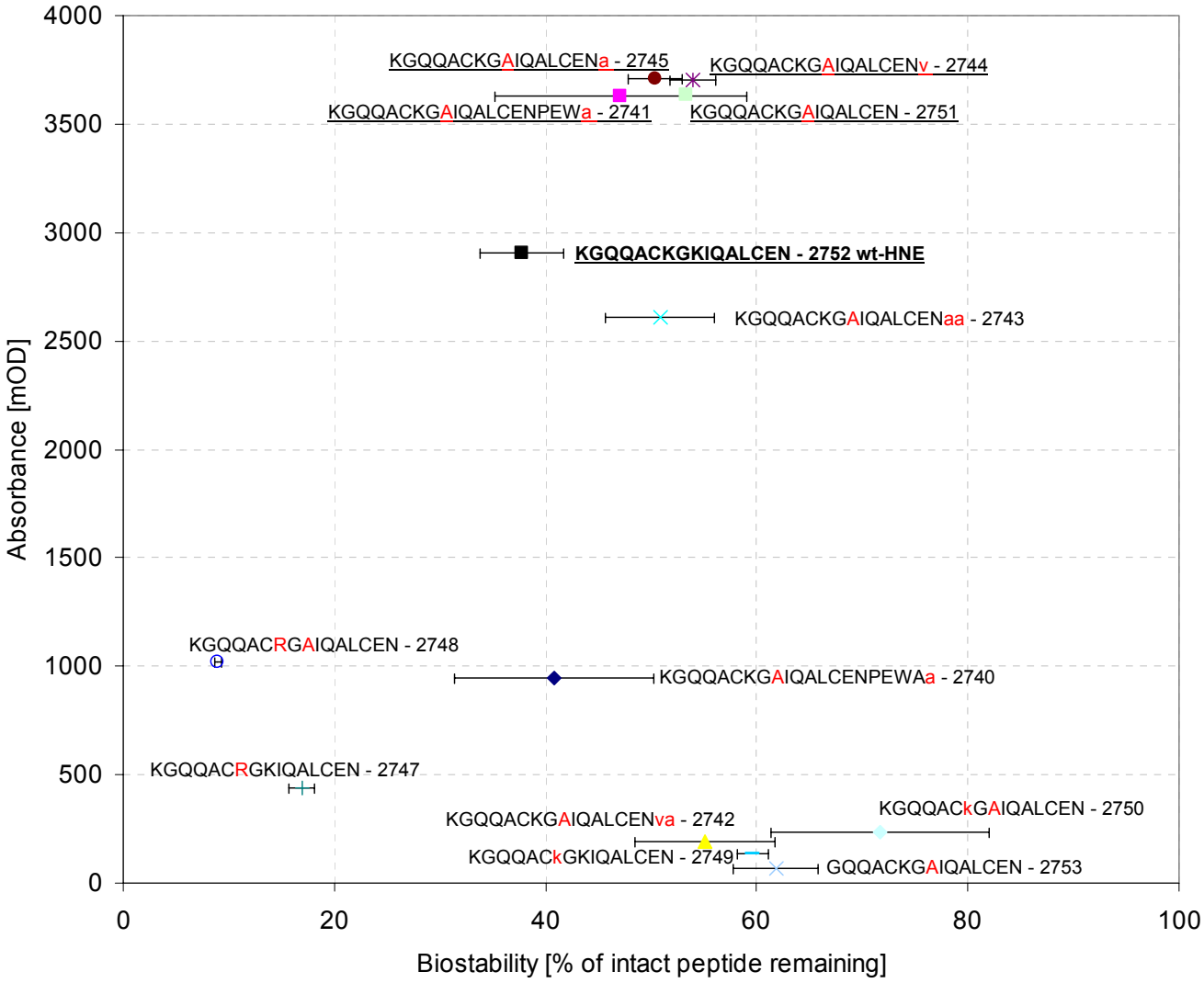


Figure 38: Biostability-antigenicity correlation plot of internally and C-terminally modified peptides

The biostability (x-axis) and antigenicity (y-axis) of modified HNE-peptides are plotted against one another. Biostability is calculated as % intact peptide and mean values (±SD) of 2 independent experiments are shown. Antigenicity is expressed as absorbance in ELISA with BH216 mAb binding to 0.8 µM coated peptide. Underlined peptide sequences were selected for immunisation studies. wt-HNE-peptide is shown in bold.

4.5. Further improved internally and C-terminally modified peptides

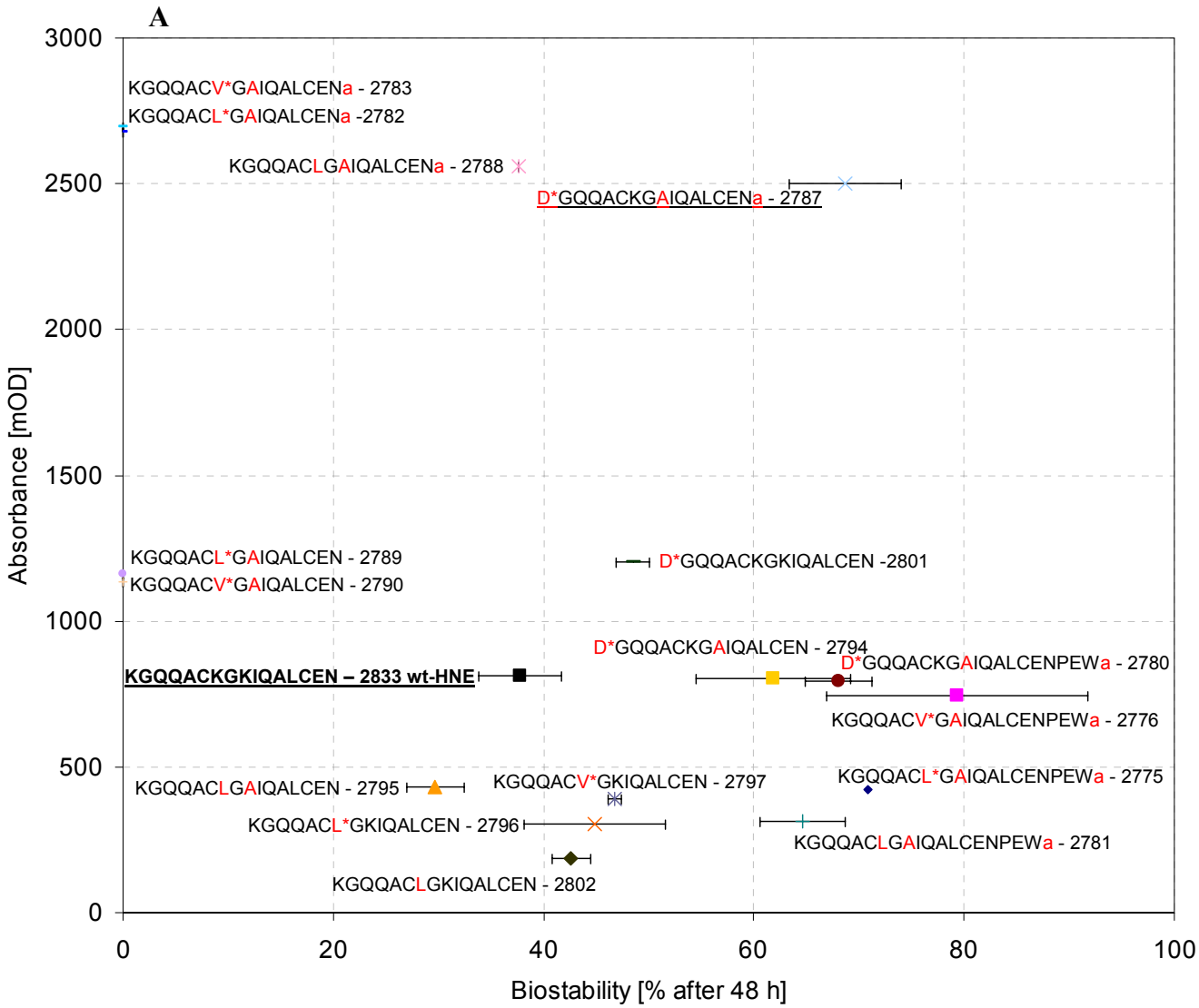
Following the immunisation with peptides 2741, 2744, 2745 and 2751 with the Lys9Ala mutation and various C-terminal modifications, an additional set of peptides were synthesised in an attempt to further stabilise the HNE-peptide. The immunisation results obtained with the above peptides directly influenced the selection of the new modifications that were included in the new synthesis. In this synthesis the D-Val C-terminal extension was eliminated to exclude solubility problems.

The results obtained for these new peptides with additional modifications in the antibody binding motif, shed more light on the structure required for antibody binding, especially with respect to Lys7. Substitution with diaminopropionic acid (dap) with a shorter basic sidechain, in this position completely abolished antibody binding, whereas norvaline (Nva) and norleucine (Nle) preserved antibody binding, despite their lack of a basic sidechain (Figure 39A). For most peptides with Nle and Nva substitutions no biostability measurements were possible: The Nle and Nva substitution peptides bind more strongly to the used cellulose filter since the substitution of basic residues by aliphatic ones increased hydrophobicity of the peptides. These peptides are thus only plotted on the y-axis showing the antigenicity. Only peptides containing at least two Lys (2796 and 2797) gave a reliably increased biostability, but their antigenicity was reduced or lost. No peptide stability assays were performed on Lys7dap substituted peptides, because they were not recognised by the mAbs.

Dap was also investigated as a possible replacement of N-terminal Lys. Lys1dap substitution in the wt-HNE-peptide increased the stability from 37% to 47% and conserved the antigenicity (Figure 39A). When this substitution was combined with additional modifications such as Lys9Ala substitution and C-terminal extensions, the stability was further increased without compromising the antigenicity compared to wt-HNE-peptide. Peptide 2787 with Lys1Dap, Lys9Ala and C-terminal D-Ala modifications was substantially stabilised (to 68%) and was one of the most stable peptides that still reacted with BH216 and BH21 mAbs.

Several Lys7 substitutions lead to peptides with both increased stability and conserved antigenicity (Figure 39B). These modified peptides included substitutions of Lys7 with sidechain ε-di- or tri-methylated Lys, but their stability was very dependent on additional modifications. A simple replacement of Lys7 with di-methylated sidechain Lys (peptide 2798) did not improve the stability compared to wt-HNE-peptide. However when this substitution was combined with Lys9Ala (peptide 2791), or Lys9Ala and C-terminal D-Ala addition (peptide 2784), very stable and antigenic peptides were obtained.

A similar observation was made when Lys7 was substituted with tri-methylated sidechain Lys: this change alone increased the antigenicity of the peptide (peptide 2811) but the stability remained unchanged. An additional replacement such as Lys9Ala (peptide 2809) or Lys9Ala combined with either PEWa (peptide 2805) or a C-terminal extension with D-Ala (peptide 2807), lead to some of the most stable and reactive peptides in this synthesis (Figure 39B).
These further improvements in stability meant that nearly twice as much intact peptide was detected after 48 h incubation in serum compared to wt-HNE-peptide. Since the antigenicity of these peptides was also further improved, they were selected for immunisation (peptides 2784, 2787, 2791, 2805, 2807 and 2809).



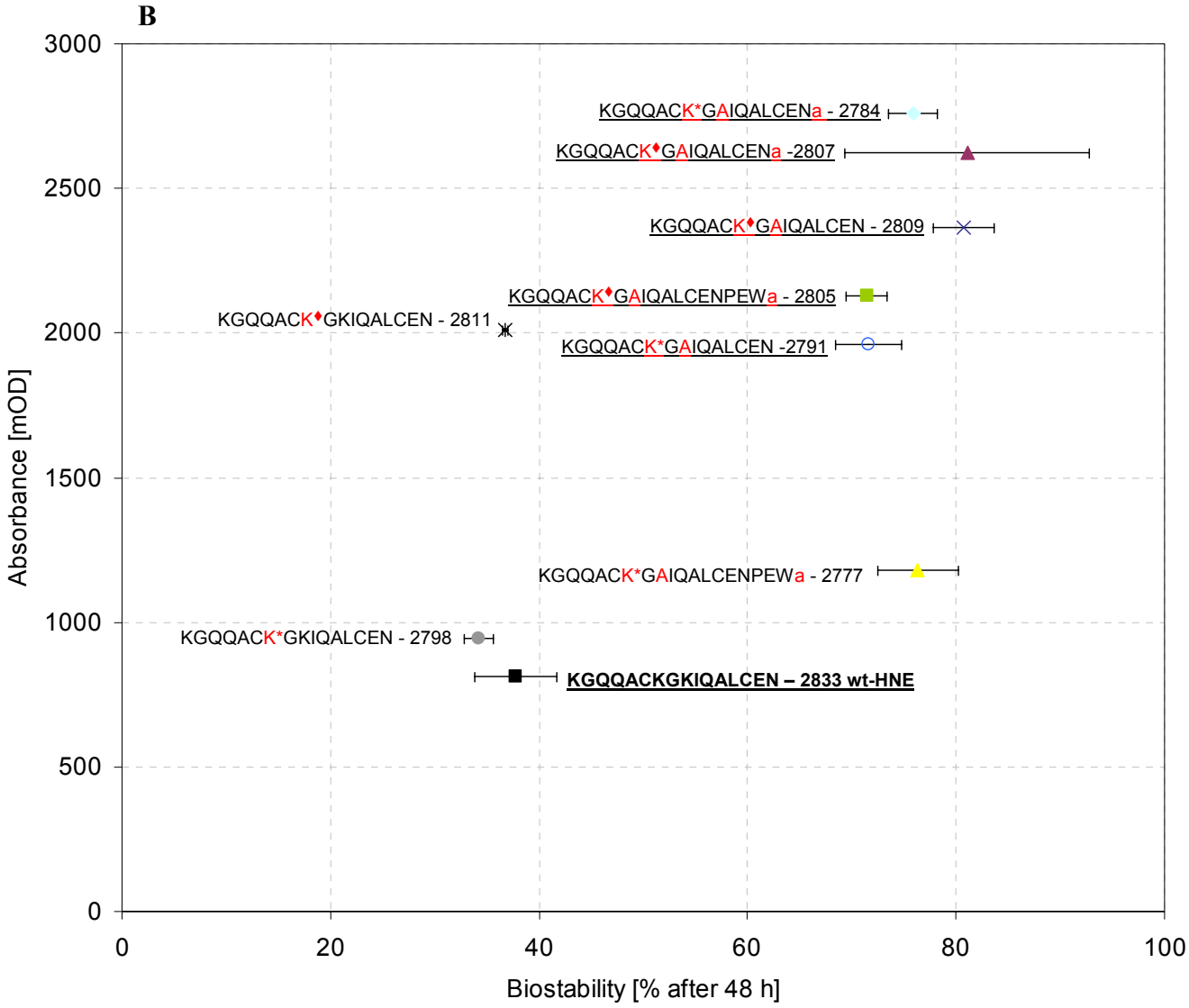


Figure 39: Biostability-antigenicity correlation plot of internally and C-terminally modified peptides improvements following results of immunisations

The biostability (x-axis) and antigenicity (y-axis) of modified HNE-peptides are plotted against one another. Biostability is calculated as % intact peptide and mean values (±SD) of 2 independent experiments are shown. Antigenicity is expressed as absorbance in ELISA with BH216 mAb binding to 0.4 µM coated peptide. (A) Lys7 substitutions with L* = Nle, V* = Nva, D* = dap. (B) Lys7 substitutions with K* = di-methylated Lys, K♦ = tri-methylated Lys. Underlined peptide sequences were selected for immunisation studies. wt-HNE-peptide is shown in bold.

5. Stability of HNE-peptides in intestinal juice

A number of peptides selected on the basis of their stability, were further analysed for their biostability in intestinal juice, in a collaboration with Dr. Steffen Bade at the Research Center Borstel (Research group: Mucosa Immunology). The method is performed on peptides that are flanked by 11-(2-(2,4-dichlorophenoxy)acetylamino)undecanoic acid and biotin on the N- and C-terminus, respectively. Peptides can be incubated with a variety of different physiological fluids (serum, intestinal wash, peritoneal wash etc). These peptides are then fixed N-terminally to an ELISA plate coated with the antibody specific for the N-terminal residue. The detection step is performed with a horse radish peroxidise coupled to streptavidin. This enzyme only detects intact peptides using the biotin/streptavidin interaction on the C-terminus. The half-lives of peptides were calculated via a logarithm based on serial dilutions of murine intestinal juice from intestinal washes. Wt-HNE-peptide $KGQ_{383}QACKGKIQALCEN_{396}$ had a half-life of 0.8 s and all tested modified peptides were more stable (Figure 40). The half-lives of peptides 2741, 2744, 2745 and 2751, which were tested in the first round of immunisation, correlate well with the stabilities observed in serum; for example peptide 2751 with only a Lys9Ala mutation, showed a 1.4 fold increase in stability in both serum and intestinal fluid. The peptides for the second immunisation (2807, 2784, 2787, 2809 and 2791) were as stable as or more stable than the peptides used in the first immunisation. Peptide 2809 with a tri-methylated Lys and Ala substitution of Lys7 and Lys9 respectively, showed a two fold increase in stability compared to wt-HNE-peptide. Only peptide 2805 showed a slight decrease in stability compared to the above peptides, but was still 1.3 times more stable than wt-HNE-peptide. Peptides of the second immunisation were overall more stable than those used for the first round of immunisation, but the increase in stability in intestinal fluid was less pronounced than in serum. An interesting outlier in the tested peptides was (dap)GQQACKGAIQALCEN (peptide 2787) whose half-life increased by a factor of 8. Peptide 2745, which lacks the dap substitution but is otherwise identical, is not stabilised to such an extent. In serum peptide 2787 was nearly twice as stable as wt-HNE-peptide. These differences between intestinal fluid and serum suggest that exopeptidases with different specificities and affinities are present in both media.

This novel method for the determination of biostability also allowed the analysis of peptides with Lys387Nva and Lys387Nle substitutions, which could not be tested in serum using the standard HPLC analysis method because of their absorption on the filter. These peptides (2776, 2775, 2783, 2782, 2790, 2789, 2797 and 2796) with an additional Lys9Ala and or C-terminal extensions, were not more stable than the other modified peptides.

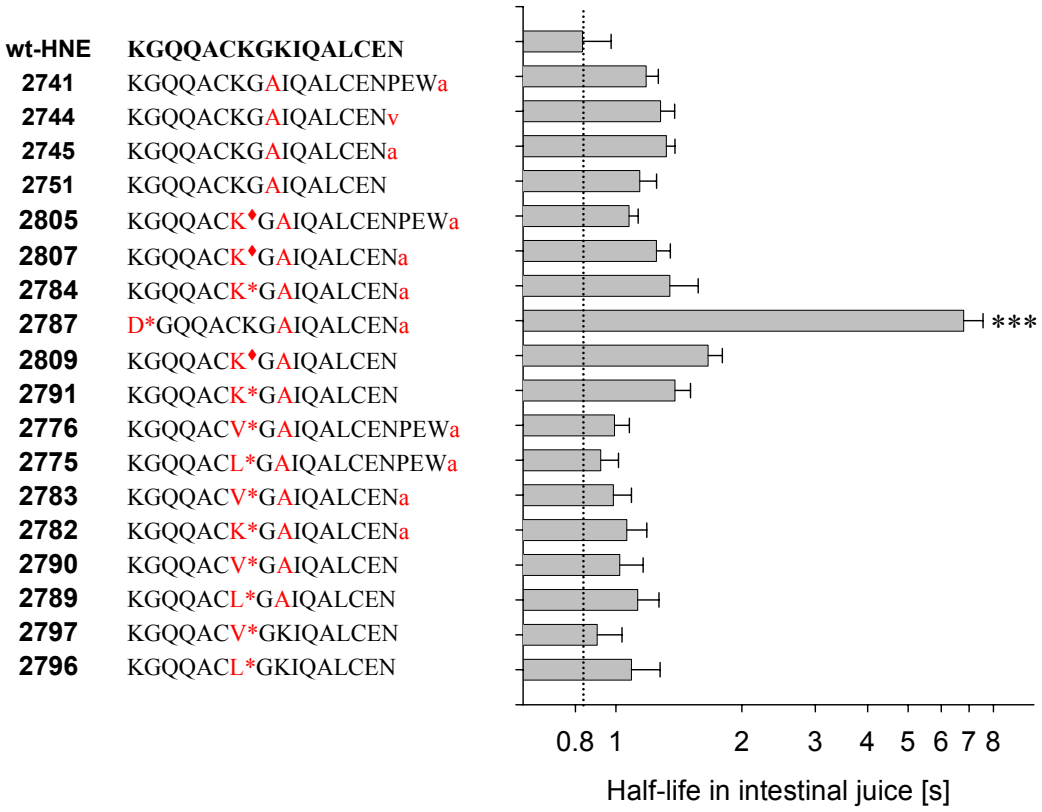


Figure 40: Half-lives of selected peptides in murine intestinal juice

Biostability was tested using a patented procedure (Gorris et al. 2007). Peptides were incubated with murine intestinal juice and intact peptide was measured by C-terminal marker specific revelation step. Half-life is expressed in s and shown as bars (mean +-SEM; n=6). One Way Anova was applied and significance levels are indicated as *** $p<0.001$ as determined by the Benferroni post hoc test.

6. Efficiency of peptide-conjugation to carrier protein

Peptides with increased stability and conserved antigenicity were coupled to tetanus toxoid (TT) using zero-length crosslinking sulfo-NHS/EDC chemistry. Before using these peptide-conjugates for immunisation, the efficiency of peptide conjugation to TT was confirmed by testing the conjugates with mAb BH216 (Figure 41A) and BH6 (Figure 41B) in ELISA. BH21 (not shown) and BH216 binding profiles were very similar. Binding of the mAbs to the unconjugated carrier TT served as negative control. Constructs segregated into two groups according to their binding to BH216: TT-2807, TT-2784 and TT-2787 reacted three times better than the other constructs. The same pattern of reactivity was observed with BH21 mAb (data not shown). In contrast BH6 bound equally well to all constructs with the exception of TT-2805. The differences in binding to BH216 mAb may have suggested lower coupling efficiencies for some peptides, however in light of the BH6 results, peptide coupling seems similar between the different conjugates. A second batch of conjugates gave the same reactivities with BH216, BH21 and BH6 mAb as observed here (data not shown).

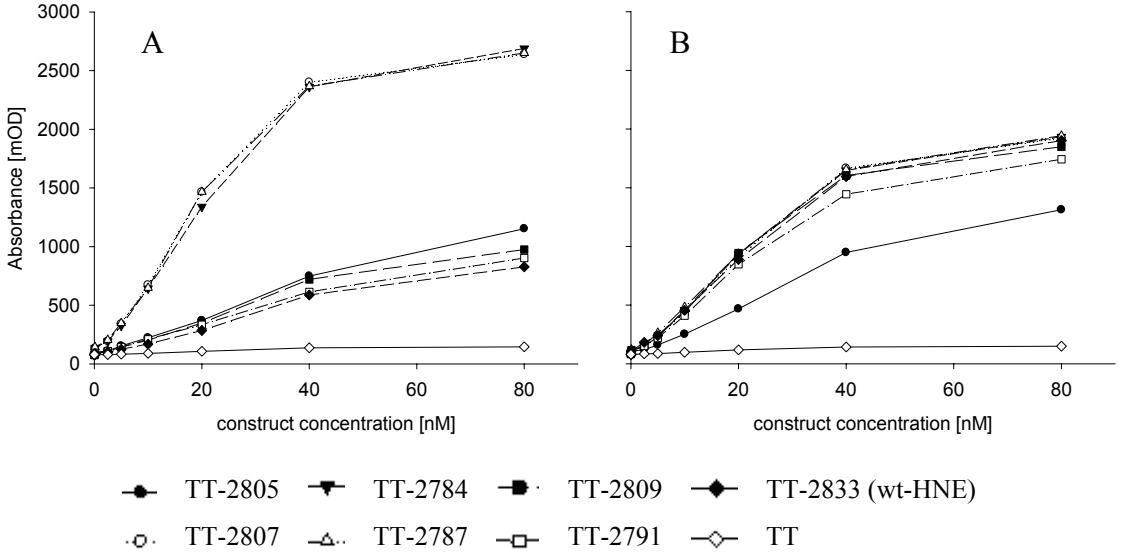


Figure 41: Antigenicity of TT-peptide-conjugates with BH216 and BH6 mAb

Prior to immunisation TT-peptide-conjugates were assessed for their coupling efficiency during conjugation reaction. TT-conjugates were coated in indirect ELISA and analysed for their ability to bind monoclonal antibodies BH216 (A) and BH6 (B).

Part III: Immunogenicity

1. Reactivity of mouse sera against homologous peptides

Table 5: List of peptides used for immunisation

peptide code	sequence[1)]
2752 / 2833	KGQQACKGKIQALCEN - **wt-HNE**
2741	KGQQACKGAIQALCENPEWa
2744	KGQQACKGAIQALCENv
2745	KGQQACKGAIQALCENa
2751	KGQQACKGAIQALCEN
2784	KGQQACK*GAIQALCENa
2787	D*GQQACKGAIQALCENa
2791	KGQQACK*GAIQALCEN
2805	KGQQACK♦GAIQALCENPEWa
2807	KGQQACK♦GAIQALCENa
2809	KGQQACK♦GAIQALCEN

[1)] D* = diamoni propionic acid (dap) , K* = di-methylated Lys, K♦ = tri-methylated Lys

Antigenic peptides with enhanced stability were selected from the previous experiments. They were coupled to TT and the TT-peptide-conjugates were assessed by immunisation. Peptide specific antibodies were measured by ELISA after 4 injections with TT-peptide–conjugates (Table 5, peptides 2741, 2744, 2745, 2751 and 2752). Median end point titres (EPT) for each group showed that similar levels of antibody were induced against TT-2741 and TT-2752 (wt-HNE- peptide) (Figure 42A). TT-2744 gave significantly lower EPT values compared to TT-2741 and TT-2752 ($p<0.05$) despite its increased stability. We assume that the lower solubility of peptide 2744 has impaired coupling resulting in reduced peptide binding to the carrier. In conclusion, increases in the stability of peptides 2741, 2744, 2745 and 2751 did not induce higher levels of antibodies than the wt-HNE-peptide.

Based on the results obtained from these immunogenicity tests, further stabilising substitutions were performed. Selected peptides (Table 3, peptides 2784, 2787, 2791, 2805, 2807, 2809 and 2833) were conjugated to TT and evaluated for their immunogenicity (Figure 42C). Peptide-conjugates TT-2787 and TT-2833 (wt-HNE-peptide) induced similar levels of antibodies and these levels were higher than those of the other peptide-conjugates. There was

little variation in the levels of antibodies raised against TT-2805, TT-2807, TT-2784, TT-2809 and TT-2791.

It is interesting to note that the clustering observed during TT-peptide-conjugate quality control with BH216 did not lead to the same groups when assessing the levels of antibodies induced against these conjugates. BH216 reacted better with peptide-conjugates TT-2807 and TT-2784 than with TT-2809 and TT-2791, yet levels of antibodies raised against these peptide-conjugates were similar, further suggesting that the difference in reactivity with the antibody is related to fine-specificities of anti-HNE mAb binding rather than the quantity of peptides conjugated to the carrier.

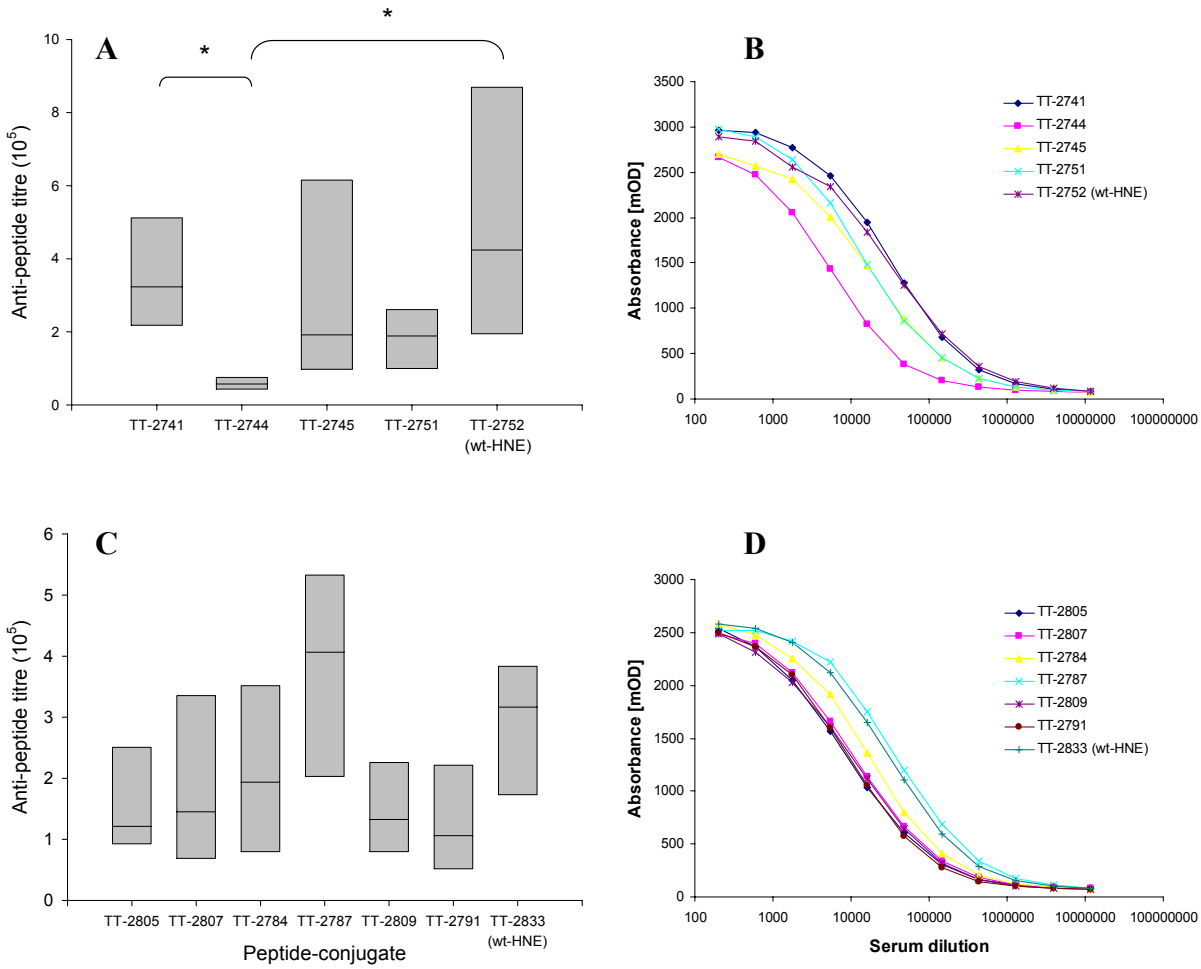


Figure 42: Assessment of anti-peptide specific response after immunisation with peptide-conjugates

Peptide specific serum end point titres (EPT) after 4 injections of TT-peptide-conjugates were determined in ELISA at 5 times OD response of negative sera. The bottom line of the box plot indicates the 25 percentile, the top line the 75 percentile. The median is shown as horizontal bar. Sera (n=4-6) were titrated against coated homologous peptide alone (not coupled to TT) revealing peptide specific antibodies only. (A) First round of immunisation; (C) further modified and stabilised peptides. One Way Anova was applied and significant difference is indicated as * $p<0.05$ as determined by the Student-Newman-Keuls method. (B) and (D) show median serum dilution curves of groups of mice (n=4-6) against the corresponding homologous peptide.

2. Reactivity of mouse sera against heterologuous wt-sequence

2.1. wt-HNE-peptide

All sera from immunised mice were analysed for their crossreactivity with wt-HNE-peptide (KGQQACKGKIQALCEN) (Figure 43 top panel). Mice immunised with TT-2741 gave a significantly higher antibody titres against wt-HNE-peptide compared to those induced by TT-2744 ($p<0.05$). The low level of crossreacting antibodies induced against TT-2744 could be explained by the lower solubility of this peptide. No significant differences in antibody titres were observed between TT-2741, TT-2745 and TT-2751, similar to their reactivity with the homologous peptide (Figure 42A).

Evaluation of anti-wt-HNE-peptide antibody levels induced against peptide-conjugates with additional modifications, showed that these conjugates form two distinct groups with respect to their ability to induce crossreactive antibodies (Figure 43B). Sera from mice immunised with constructs TT-2787, TT-2809 and TT-2791 recognised wt-HNE-peptide better than those immunised with TT-2805, TT-2807 and TT-2784, with a statistical significance between TT-2805 and TT-2787 and TT-2809, respectively ($p<0.05$). There was little difference between the various peptide-conjugates within the two groups. The clustering of groups against wt-HNE-peptide differed from the results observed against homologous peptide where TT-2787 induced higher levels of antibodies than the other modified HNE-peptide-conjugates. It was estimated that a higher proportion of the anti-peptide antibodies induced against TT-2809 and TT-2791 crossreacted with wt-HNE-peptide than those induced against the other peptide-conjugates. Based on these results, peptide-conjugates TT-2787 and TT-2809 were selected to analyse the influence of a variety of adjuvants.

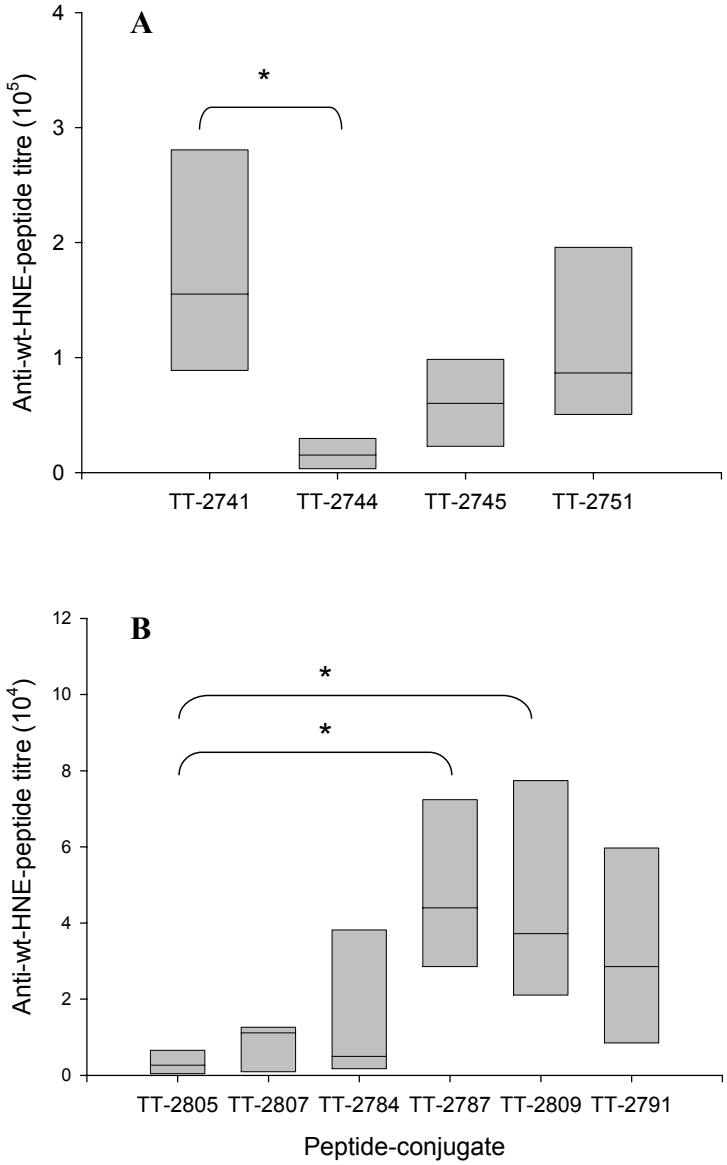


Figure 43: Assessment of crossreactivity with wt-HNE-peptide induced against different TT-peptide-conjugates

wt-HNE-peptide specific serum end point titres after 4 injections of TT-peptide-conjugates were determined in ELISA at 5 times OD response of negative sera. The bottom line of the box plot indicates the 25 percentile, the top line the 75 percentile. The median is shown as horizontal bar. Sera (n=4-6) were titrated against coated heterologous wt-HNE-peptide (KGQQACKGKIQALCEN) alone (not coupled to TT) revealing wt-HNE-peptide specific antibodies only. (A) First round of immunisation; (B) further modified and stabilised peptides. One Way Anova was applied and significant difference is indicated as * $p<0.05$ as determined by the Student-Newman-Keuls method.

2.2. recombinant MV-H-protein

In a further effort to demonstrate the HNE-crossreactivity of induced antibodies, sera from mice immunised with the first set of stabilised peptides, were titrated against recombinant H-protein in ELISA (Figure 44) and showed only weak levels of crossreacting antibodies. Immunisation with wt-HNE-peptide-conjugate induced the highest level of antibodies. There was no significant difference in the levels of antibodies raised by peptide-conjugates TT-2741, TT-2745 and TT-2751. Sera from mice immunised with TT-2744 showed the lowest crossreactivity with recombinant H-protein compared to wt-HNE-peptide ($p<0.05$) confirming the results obtained throughout the study.

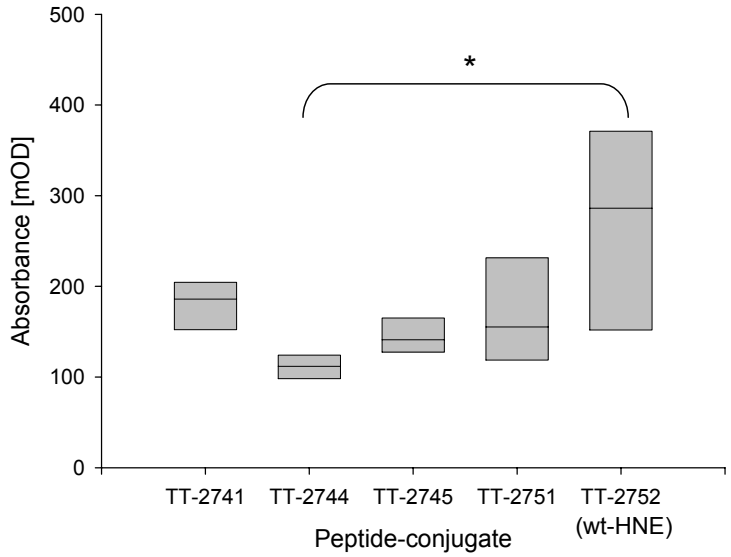


Figure 44: Crossreactivity with recombinant MV-H-protein of sera from mice immunised with HNE-peptide-conjugates.

H-protein specific crossreactivity after 4 injections of TT-peptide-conjugates were determined in ELISA using mouse sera (1:200 dilution) titrated against 125ng of coated recombinant H-protein and are expressed as absorbance in mOD. The bottom lines of the box plots indicate the 25 percentile, the top line the 75 percentile. The median is shown as horizontal bar. . One Way Anova was applied and significant difference is indicated as * $p<0.05$ as determined by the Student-Newman-Keuls method.

3. Effect of adjuvants

3.1. Reactivity of mouse sera against homologous peptides

Irrespective of adjuvant used, serum EPT values of mice immunised with peptide-conjugates TT-2787 and TT-wt-HNE-peptide (TT-2833) showed no significant difference between each other when titrated against coated homologous peptide (Figure 45). In contrast TT-2809 induced lower anti-peptide antibodies ($p<0.001$). Immunisation of TT-wt-HNE-peptide (2833) with Montanide lead to significantly lower anti-peptide antibody titres compared to Alum, Quil-A and Freund adjuvant ($p<0.05$). Similarly, the combination of TT-2787 with Montanide was less potent than the other adjuvants, especially Freund adjuvant ($p<0.05$). When all peptide-conjugates are considered, Montanide induced significantly less antibodies than Alum ($p<0.01$), Freund's adjuvant ($p<0.01$) and Quil-A ($p<0.05$).

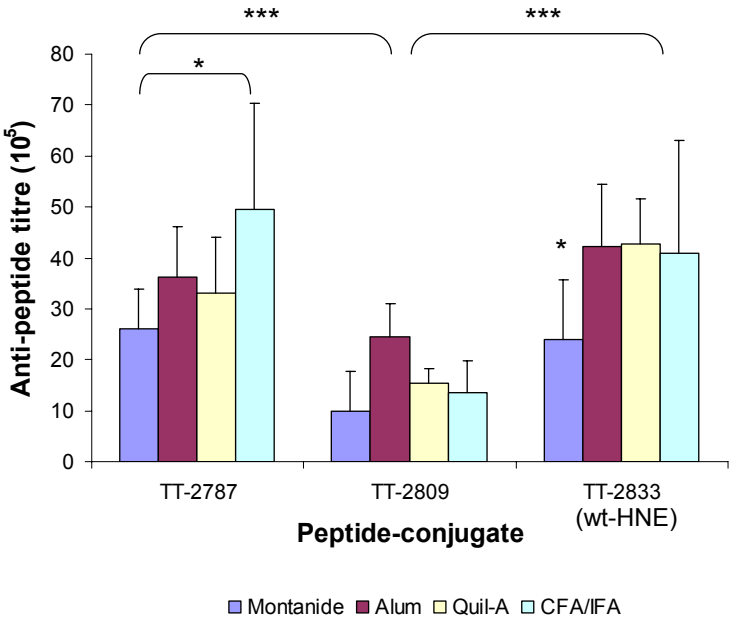


Figure 45: Assessment of anti-peptide specific response after immunisation with peptide-conjugates using different adjuvants

Anti-peptide specific end point titres (mean ±SD) after 4 injections of TT-peptide-conjugates determined in ELISA at 5 times OD response of negative sera. Sera (n = 4-6) were titrated against coated homologous peptide alone (not coupled to TT) revealing peptide specific antibodies only. Two Way Anova was applied and significant difference is indicated as * $p<0.05$ and *** $p<0.001$ as determined by the Student-Newman-Keuls method.

3.2. Reactivity of mouse sera against heterologuous wt-HNE-peptide

Irrespective of the adjuvant used, peptide-conjugate TT-2787 induced higher levels of wt-HNE-peptide crossreactive antibodies than TT-2809 ($p<0.01$) (Figure 46). TT-2787 peptide-conjugate worked significantly better than TT-2809 when injected in combination with Quil-A ($p<0.01$). The TT-2787 peptide-conjugate showed no difference in antibody levels irrespective of the adjuvants used. These data showed that there were significant differences between the adjuvants depending on which peptide-conjugate was used. In general Alum was the most appropriate universal adjuvant.

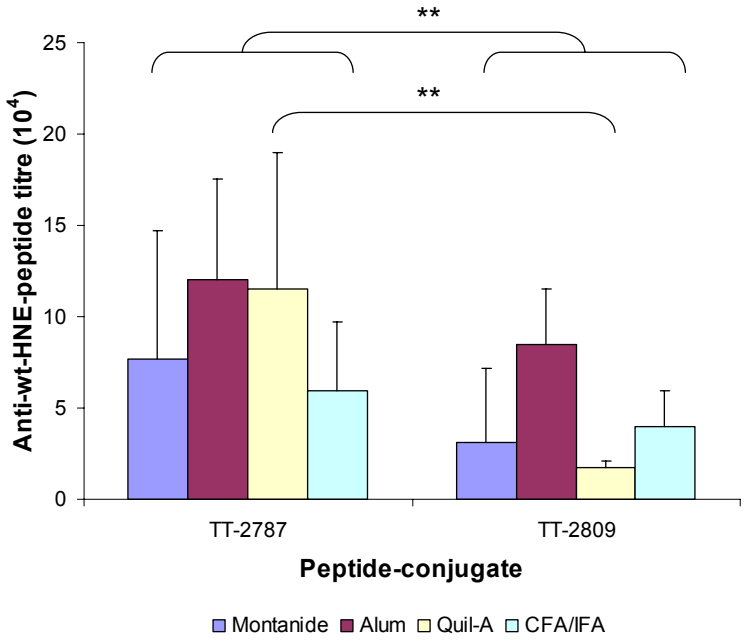


Figure 46: Assessment of crossreactivity to wt-HNE-peptide induced against different TT-peptide-conjugates

wt-HNE-peptide specific crossreactive end point titres (mean ±SD) after 4 injections of TT-2787 and TT-2809 peptide-conjugates determined in ELISA at 5 times OD response of negative sera. Sera (n = 4-6) were titrated against coated heterologous wt-HNE-peptide (KGQQACKGKIQALCEN). Two Way Anova was applied and significant difference is indicated as ** $p<0.01$ as determined by the Student-Newman-Keuls method.

4. Flow Cytometry – Facs

Flow cytometry with Mel-JuSo transfected cell lines expressing the MV-H-protein was performed to test the crossreactivity of antibodies with native MV-H-protein after immunisation with TT-peptide-conjugates. Sera, immunised with different peptide-conjugates recognised native MV-H-protein with varying affinities: Similar to the results with recombinant H-protein in ELISA, wt-HNE-peptide-conjugate induced always higher levels of crossreactive antibodies compared to TT-2741 ($p<0.05$), TT-2744 ($p<0.001$), 2745 ($p<0.05$) and 2751 ($p<0.05$) (Figure 47A). Peptide-conjugate TT-2741 immunised mice reacted better than those injected with TT-2745 and TT-2751. When compared to the antibody binding to recombinant H-protein in ELISA, the crossreactivity of TT-2741 to native H-protein is increased relative to TT-2745 and TT-2751. TT-2744 immunised mice essentially lacked crossreactivity similar to our observations in H-protein ELISA.

Of the second series of peptide-conjugates, TT-2787 (1 out of 6 sera) and TT-wt-HNE-peptide (3 out of 6) induced crossreactive antibodies with H-protein. None of the stabilised HNE-peptides, containing di- and tri-methylated Lys induced antibodies crossreacting with the H-protein when coinjected with Freund adjuvant (data not shown).

To assess whether adjuvants could affect the crossreactivity of the antibodies, Montanide, Quil-A or Alum were coinjected with TT-2787, TT-2809 or TT-2833 (wt-HNE-peptide) conjugate. When TT-2787 and TT-2809 were administered with Montanide, 2 out of 6 or 1 out of 6 H-protein crossreactive sera were obtained. No crossreactivity was observed when TT-2787 and TT-2809 were injected with Quil-A or Alum (data not shown). H-protein binding of sera immunised with TT-wt-HNE-peptide-conjugate was observed irrespective of the used adjuvants (Figure 47B). The efficacy of adjuvants to induce crossreactive antibodies varied with Montanide and Quil-A inducing 4 out of 6 and 5 out of 6 crossreactive sera, respectively, whereas in the Alum and Freund adjuvant groups only 2 out of 5 and 3 out of 6 mice sera were positive, respectively.

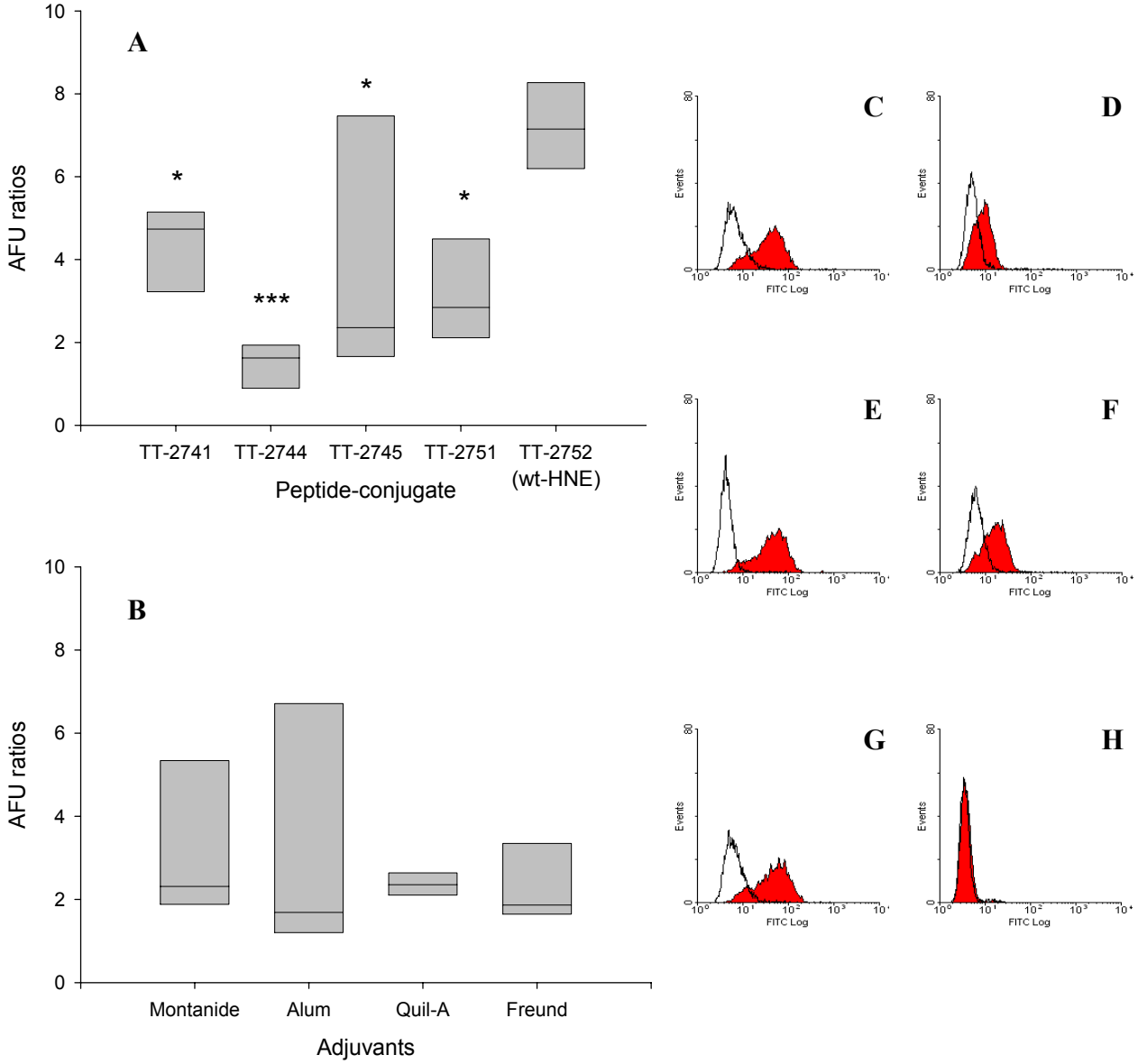


Figure 47: Crossreactivity with native MV-H-protein of anti-HNE-peptide sera

Crossreactivity with MV-H-protein of mouse sera after immunisation with TT-peptide-conjugates was measured by flow cytometry on Mel-JuSo-H transfected and Mel-JuSo-wt cells. Boxplot bars represent median values of AFU ratios (AFU H / AFU wt). The bottom lines of the box plots indicate the 25 percentile, the top line the 75 percentile. Ratios above 2 are considered positive. (A) 1/50 serum dilution of mice immunised with various TT-peptide-conjugates (n=4-5); (B) 1/100 serum dilutions of mice immunised with TT-wt-HNE-peptide-conjugate (n=5-6) in combination with 4 different adjuvants. One Way Anova was applied and significant difference is indicated as * $p<0.05$ and *** $p<0.001$ as determined by the Student-Newman-Keuls method. (C-H) Typical flow cytometry histograms of crossreactivity with H-protein on Mel-JuSo-H cells (red histogram) and Mel-JuSo-wt cells (open histogram) TT-2741 (C), TT-2744 (D), TT-2745 (E), TT-2751 (F), TT-2752 (wt-HNE) (G) and adjuvant only (H)

Part: IV Bile-acid peptide-scaffold analysis

1. Synthetic pathway

1.1. Synthesis of scaffold-peptide construct

In collaboration with Catherine Bodé at the Department of Organic Chemistry at the University of Ghent, we have evaluated an HNE-peptide-scaffold construct. We investigated the feasibility to restrict the conformation of a the HNE-peptide using a bile-acid based scaffold. The detailed description of the chemistry of the scaffold-peptide construct is beyond the scope of this thesis (Bodé et al.; Bodé 2007; Bodé et al. 2007) . In the following paragraphs only a short summary of the chemical synthesis pathway of the scaffold-peptide construct will be described, followed by a more in depth analysis of the biological data observed.

An orthogonally protected bile-acid based steroidal scaffold was coupled to a functionalised Tentagel resin for SPPS, via a photolabile linker, which allowed easy deprotection and chemical analysis of reaction intermediates. Two approaches for the synthesis of the peptide on the scaffold were investigated: The two-strand approach is based on convergent solid phase peptide synthesis. One part of the peptide was synthesised on the C-12 amine of the scaffold linked to the resin. The second part of the peptide sequence was assembled separately on another resin. After resin cleavage the sidechain-protected peptide was coupled via the N-terminus to the C-3 amine of the scaffold via compatible linkers (succinic acid). After successful coupling, the two strands were connected via simple backbone cyclisation to yield the final product. This two strand approach with convergent SPPS has a clear advantage in the synthesis of difficult and/or large peptide sequences. The second peptide sequence can be purified prior to coupling to the scaffold, thus reducing the number of by-products. This two strand approach was however complicated by several factors including difficulties of coupling the second strand to the scaffold.

The single strand approach is based on the stepwise synthesis of the peptide strand starting from Fmoc-protected Gly linker residue coupled to the scaffold. After completion of the peptide sequence, the peptide was oxidised to form the disulfide bridge. The peptide strand

was coupled to the C-3 amine of the scaffold via the succinic acid linker thus closing the peptide loop. The orthogonal sidechain protection groups were removed by TFA and the scaffold-peptide construct was cleaved from the resin by UV-irradiation. After purification of the scaffold-peptide construct we analysed it in a collaboration for its stability, antigenicity and immunogenicity (Figure 48).

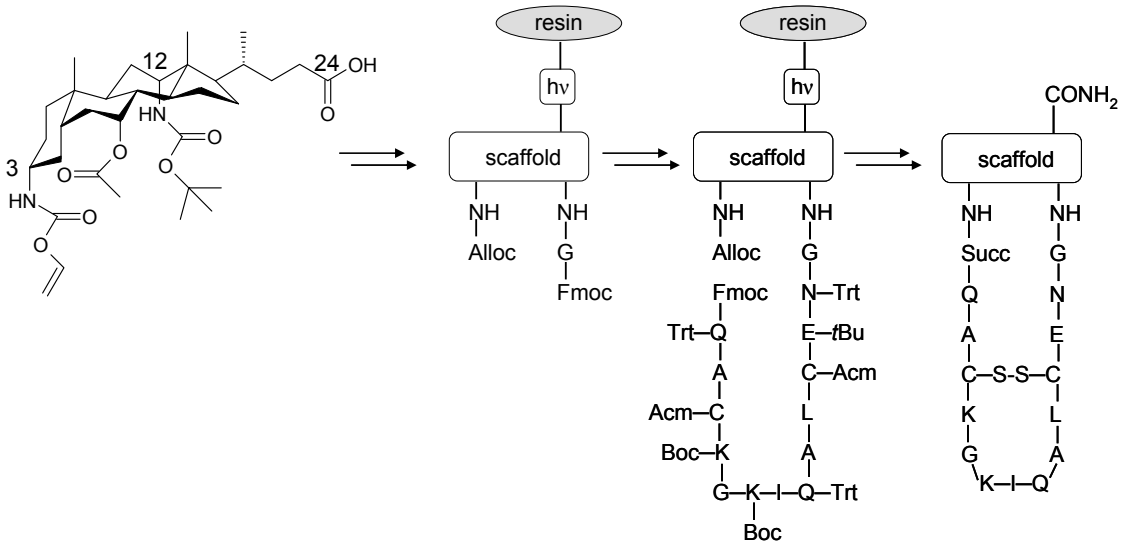


Figure 48: Synthetic pathway for scaffold-peptide construct

The synthetic pathway starting with steroidal scaffold, linkage to resin and functionalisation of scaffold, one strand peptide synthesis on scaffold, followed by cystine formation, backbone cyclisation, sidechain protection group removal and cleavage from resin to yield final scaffold-peptide construct.

1.2. Synthesis of cyclic control peptide

To assess the influence of the scaffold of the scaffold-peptide construct on biostability, antigenicity and immunogenicity, a peptidic control sequence was synthesised. This control peptide contains the same peptide sequence as the scaffold-peptide construct including the disulfide bridge and merely lacks the scaffold itself. The synthetic pathway started with a Fmoc-Glu_{395}-OAll residue which was covalently coupled via its sidechain to chlorotrityl resin and the peptide chain was elongated via SPPS until completion of the peptide sequence. The intermediate peptide "Fmoc-N-G-Q-A-C-K-G-K-I-Q-A-C-E-(resin)" was oxidised to form the disulfide bridge, followed by backbone cyclisation between amine group of N-terminal Asn residue and the carboxy group of Glu, to form the cyclised control peptide with a Gly spacer between "C-terminal" Glu and "N-terminal" Asn. Cyclisation of the peptide sequence prior to cystine formation caused dimerisation of the peptide chains presumably because of the high

loading of the resin. In this context it is important to differentiate between cyclic and oxidised peptides. Here cyclisation refers to the backbone connection of N- and C-terminal amino acids thus forming a cyclic peptide. Peptides such as the HNE-peptide with a disulfide bridge in the sequence are referred to as linear oxidised peptides.

2. Biological evaluation

2.1. Biostability

Preliminary tests of the biostability of the scaffold-peptide construct and the cyclised control peptide indicate that after 24 h incubation in mouse serum, over 75% of the peptides remain intact (data not shown). This was a significant improvement in comparison with the natural wt-HNE-peptide which was degraded to 25% within the same time. Despite these improvements neither the scaffold nor the cyclisation increased the peptide stability to the same extent as our internal and C-terminal modifications.

2.2. Antigenicity

The cyclic peptide and scaffold-peptide construct were tested for their reactivity with anti-HNE mAb BH216, BH21 and BH6. Binding patterns of BH216 and BH21 were similar, showing that binding to scaffold-peptide construct was stronger (Figure 49A) than binding to linear oxidised wt-HNE-peptide (KGQQACKGKIQALCEN). The cyclic control peptide gave a much weaker binding. Binding patterns to BH6 were predictably different: the linear oxidised control peptide gave the highest binding followed by the scaffold-peptide construct and cyclic peptide. It is interesting to note that the cyclic peptide gave much better binding with BH6 than it did with BH216 (Figure 49B). No binding of peptides to negative control BH195 were observed, which specifically recognises the PEWA region of denatured H-protein.

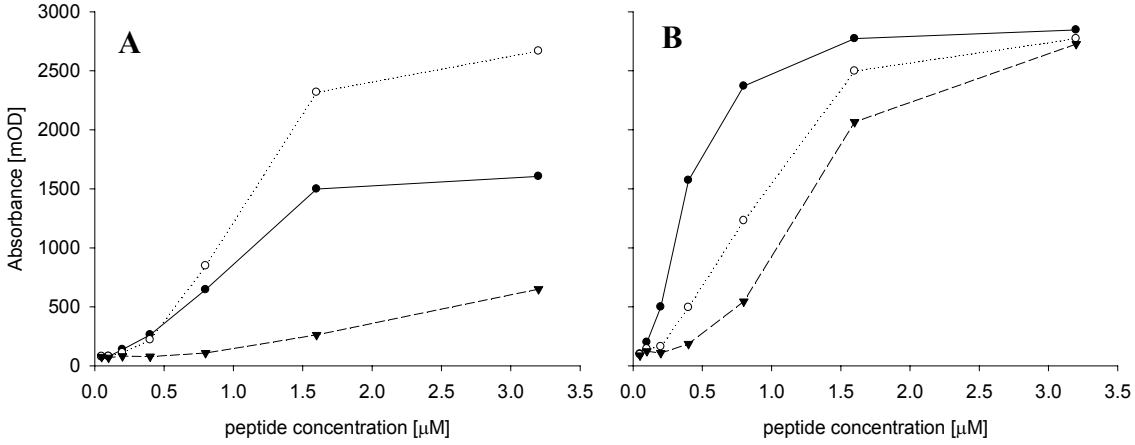


Figure 49: Antigenicity of scaffold-peptide construct and cyclic control peptide tested with anti-HNE monoclonal antibodies.

Scaffold-peptide construct (open circle) and cyclic control peptide (triangle) were analysed for their ability to bind monoclonal antibodies BH216 (A) and BH6 (B) in indirect ELISA in relation to wt-HNE-peptide (KGQQACKGKIQALCEN) (closed circle).

2.3. Immunogenicity

The chemistry of the scaffold-peptide construct and of the cyclic peptides did not allow covalent coupling to tetanus toxoid. The only available reactive amine and carboxy groups were located on sidechains and coupling would likely destroy the epitope. Thus peptides were merely mixed with TT in immunisation tests. Following the same immunisation schedule with Freund adjuvant described previously we injected 20 µg of each peptide either alone or mixed with 20 µg TT. After 3 injections sera of immunised mice were analysed for the presence of anti-peptide antibodies (Figure 50). The scaffold-peptide construct and the cyclic peptide induced high and comparable titres when coinjected with TT. In the absence of TT, the scaffold-peptide construct induced a much lower antibody titre and cyclic peptide was essentially non-immunogenic, demonstrating the benefit of additional T cell epitopesof TT.

In order to establish the crossreactivity of the induced antibodies, sera of mice immunised against scaffold-peptide construct were titrated against coated cyclic control peptide in ELISA (data not shown). Poor binding was observed for scaffold-peptide construct mixed with TT and scaffold-peptide construct injected alone showed no crossreactivity. This poor binding can be explained either by the fact that most antibodies raised against the scaffold-peptide construct targeted the scaffold only, or more likely, that the structure of the cyclic peptide

differs substantially from the one adopted by the same peptide sequence constrained by the scaffold.

Neither the scaffold-peptide construct nor the cyclic peptide induced sera crossreacting with recombinant MV-H-protein or with the native MV-H-protein expressed by Mel-JuSo-H cells assessed by ELISA and flow cytometry, respectively (data not shown)

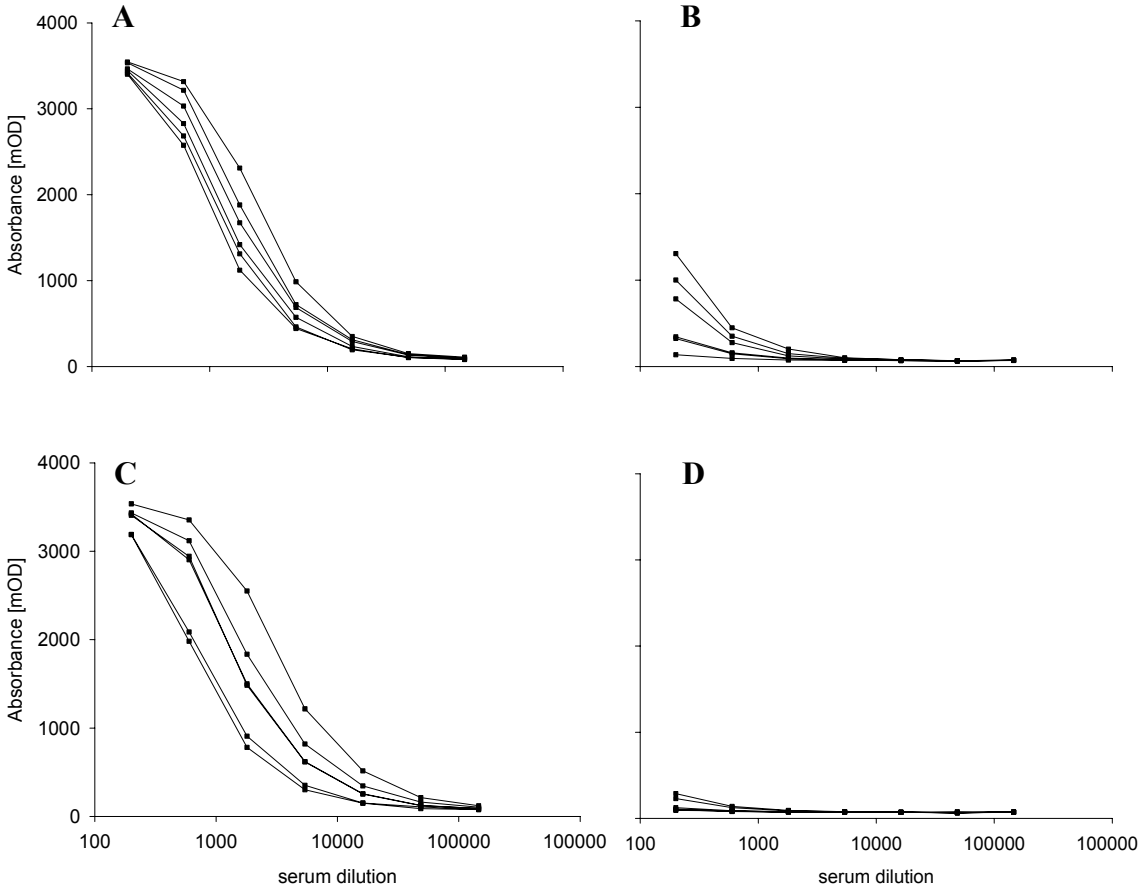


Figure 50: Assessment of anti-peptide specific response after immunisation with scaffold-peptide construct and cyclic peptide with or without TT

Serum dilution curves of mice (n=6) after three injections of scaffold-peptide construct mixed with TT (A), scaffold-peptide construct alone (B), cyclic peptide mixed with TT (C) and cyclic peptide alone (D) in ELISA against coated scaffold-peptide construct (A,B) and coated cyclic peptide (C,D).

Chapter 4: Discussion

1. Role of Cysteine bridges and of the HNE

The recent publication of two X-ray crystallography structures of the measles H-protein (Colf et al. 2007; Hashiguchi et al. 2007) has allowed us to put our structure of the wt- HNE-peptide into the context of the native H-protein. So far only few models were available: (1) a 3D structure obtained by homology modelling on the basis of the crystal structure of influenza virus neuramidase (Langedijk et al. 1997). After the publication of the crystal structure of the haemagglutinin-neuraminidase (HN) protein of Newcastle disease virus (NDV) (Crennell et al. 2000) the model of MV-H-protein was refined: (2) A new model of the H-protein based on the HN-protein of NDV was proposed (Vongpunsawad et al. 2004). (3) A second model with NDV as a template using sequence homology, secondary structure prediction and protein folding was created (Masse et al. 2004). (4) A fourth structure was predicted by concerted use of homology modelling, fold recognition and de novo folding techniques also based on the crystal structure of the HN-protein of NDV (Damien et al unpublished).

While these models gave a good indication on the location of protruding accessible loops of the H-protein, the structure of these loops was not well defined and the proposed Cys bridges remained a matter of debate for some time. The importance of the Cys in the structure and function of the H-protein has been recognised early on (Hu and Norrby 1994) and this is reflected in the conservation in number and position of the Cys in all MV isolates. The Cys pattern is not limited to MV but Cys are also conserved in the H-protein of rinderpest virus (Tsukiyama et al. 1987), canine (Curran et al. 1991), phocine (Kovamees et al. 1991) distemper viruses and all other morbilliviruses (Ziegler et al. 1996). However there has been conflicting data on which Cys bridges are important in the structure of the HNE. Hu and Norrby suggested that Cys381, Cys394 and Cys494 are essential for the function of the H-protein and that Cys386 is most likely unpaired (Hu and Norrby 1994). Cys381 was suggested to form a disulfide bond with Cys494. The model by Langedijk on the other hand predicts the disulfide bridges Cys381-Cys386 and Cys394-Cys494. Earlier work within our laboratory by Ziegler could only show a functional importance for Cys394 and El Kasmi found that the presence of all three HNE Cys in peptides were necessary for the induction of MV-neutralising antibodies (Ziegler et al. 1996; El Kasmi et al. 2000). More recent work by Putz

demonstrated that peptides containing only the Cys386-Cys394 bridge induced MV-neutralising sera (Putz et al. 2003b). In this study we have also confirmed by Cys substitution of full length HNE peptides, the recognition of peptides with a Cys386-Cys394 bridge by anti-HNE mAbs. The importance of the Cys386-Cys394 disulfide bridge was also confirmed by the two crystal structures of the H-protein; both of them predict Cys bridges between Cys386-Cys394 and Cys381-Cys494. The structure of the loop and Cys positions preclude the formation of both Cys381-Cys386 and Cys381-Cys394 bridges, since Cys381 is locked in an α-helix pointing in the opposite direction of Cys 386 and Cys394 (Figure 51).

While it seems that the crystal structures and our results contradict the data by Hu and colleagues, these data could represent different states of the H-protein: The disulfide bridges in a native protein are the end result of a process of oxidation, reduction and isomerisation of disulfide bonds by enzymes such as protein disulfide isomerase (Creighton et al. 1995), suggesting that different disulfide bridge arrangements may exist in the H-protein at different stages of its life cycle. It has also been reported that reduction and rearrangement of disulfide bridges in native proteins act as switches for biological functions (Hogg 2003). For example cleavage of various disulfide bonds within the bound complex of CD4 immune cell receptor and gp120 surface protein of HIV-1 has been reported to be essential for virus entry and cell-cell fusion (Fenouillet et al. 2001; Gallina et al. 2002; Matthias et al. 2002). Even though such an effect has never been directly suggested in the case of MV-H it cannot be excluded that disulfide bridges undergo functional rearrangements.

Disulfide bridges are important for stabilising the conformation of proteins by lowering the entropy of the unfolded state by restricting the degrees of freedom (Sternberg 1997). They also ensure the structural integrity of proteins and can protect them against oxidants and proteolytic cleavage (Arolas et al. 2006). In the HNE the disulfide bond between Cys386 and Cys394, located in two distinct α-helices, stabilises a turn between these two α-helices. The α-proton chemical shift data obtained for the reduced Gly8Val substituted peptide suggests an elongation of the α-helix in the absence of disulfide link induced loop. This structural change may be responsible for the lack of reactivity of anti-HNE mAb with reduced HNE-peptides. It is however unknown whether a lack of disulfide bonds in the HNE would lead to the same structural change in the H-protein. This seems unlikely since the HNE is more constrained within the H-protein compared to the flexible free peptide. The disulfide bridge in the HNE-peptides is essential for maintaining a structure able to mimic the HNE arrangement of the H-protein.

The actual role of the HNE and its conserved Cys residues remains unclear. Since the HNE-antibody binding motif is conserved in most known MV isolates (Putz et al. 2003b) and the Cys located within the HNE are conserved in all known morbilliviruses it very likely that this region has a very important but unknown function.

It has been known for some time that the H-protein of wt-MV preferentially binds SLAM (CD150) and not CD46 (Yanagi et al. 2006). Mutational studies have shown that Pro486 and Ile487 are critical for H-protein binding to CD46 (Vongpunsawad et al. 2004) and substitutions in the vaccine strains (Asn390Ile, Asn481Tyr) further enable CD-46 binding (Bartz et al. 1996; Tahara et al. 2007). Residue 390 is located in the HNE, and the other amino acids are located in the opposite loop, which is kept in close proximity to the HNE by the Cys381-Cys494 disulfide bridge. It is thus possible that the HNE, with its proposed Cys386-Cys394 and Cys381-Cys494 disulfide bonds may stabilise the secondary structure required for CD46 binding and retain the function of regions located further away or possibly support conformational changes after binding which play a role in cell-cell fusion.

Hashiguchi showed that although N-linked sugars hide several H-protein domains, the receptor binding sites and the HNE-loop remain exposed. A role in receptor binding would explain the relative conservation of the HNE-sequence (Hashiguchi et al. 2007). This would also explain the neutralising capacity of our HNE-targeting mAbs (BH216, BH21 and BH6).

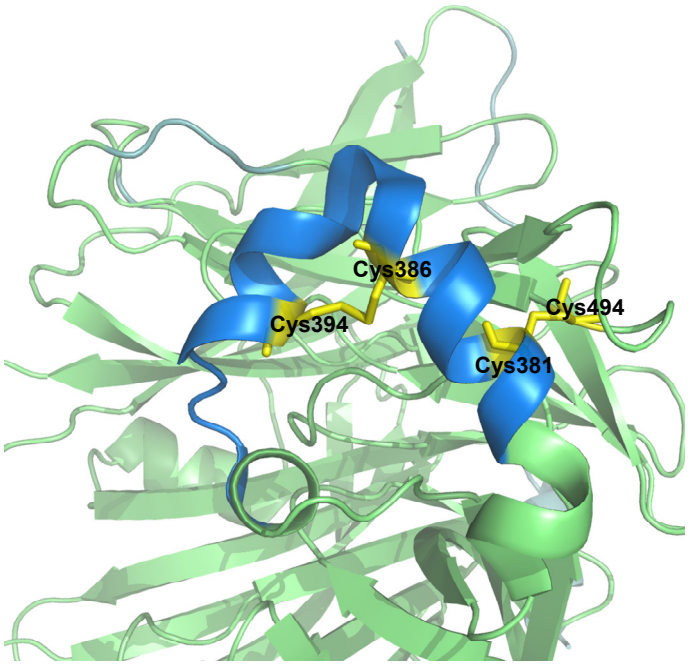


Figure 51: HNE cysteine residues

The crystal structure of H-protein (Hashiguchi et al. 2007) is shown as cartoon type, HNE region (E_{379}TCFQQACKGKIQALCENPEWA$_{400}$) is coloured in blue, HNE Cys residues are labelled and disulfide bridges between Cys381-Cys494 and Cys386-Cys394 are shown in yellow.

2. HNE-peptide structures

Comparisons of observed α-proton chemical shifts with literature values of random coil structures provided qualitative information on the position and nature of secondary structure elements in the modified HNE-peptides, without the need to resolve complete structures. It was thus shown that the majority of substitutions of Gly8, Ile10 and Leu13 of the wt-HNE-peptide (KGQQACKGKIQALCEN) did not lead to an apparent loss of α-helical structure of the C-terminus. Notable exceptions were Gly8Pro, which due to the backbone twist affected the loop around Gly8, and Leu13Asn. Asn is known to be strongly helix-destructive and the α-proton chemical shifts confirm that the helix formation was inhibited and the whole peptide adopted a random coil structure (Pace and Scholtz 1998). N-terminal Asn may also account for the break in the α-helix observed in both our HNE-peptides and the HNE of the H-protein. We observed that some modified and wt-HNE-peptide retained most of their secondary structure in water even if the helix was weakened. This is surprising since short peptides usually do not adopt a secondary structure in water (Shepherd et al. 2005). We suspect that the disulfide bridge between Cys6 and Cys14 can account for this high stability. At the same time we showed that a Gly8Val substitution and a break in the disulfide bond increased the α-helical content of the modified HNE-peptide, further confirming the tendency of this region to adopt such a secondary structure.

2.1. wt-HNE-peptide

Peptides can adopt a multitude of structures when they are removed from the constraints of the protein of which they are part. This effect was also obvious in our NMR structures. Comparisons of the crystal structure of the HNE region (E_{379}TCFQQACKGKIQALCENPEWA$_{400}$) of the H-protein (Hashiguchi et al. 2007) and our NMR structure of the wt-HNE-peptide (K_1GQQACKGKIQALCEN$_{16}$) clearly show that the structure of the flexible N-terminal linkerarm region observed in the HNE-peptide does not correlate to the structure of the N-terminal HNE in the H-protein (Figure 52). In the H-protein the amino acids preceding the loop around Gly8 form an α-helix keeping the critical contact residue Lys 7 in a more constrained conformation compared to the free peptide. In addition the N-terminal linkerarm in the free HNE-peptide adopts an orientation that would not be allowed in the protein because of steric hindrance from other structural domains. The helical region with the critical contact residues for antibody binding (BH216) on the other hand is

conserved in the free HNE-peptide, indicating that the structural informations for the α-helix are contained in the Ile10 to Asn16 sequence, independent of protein environment. The N-terminal region Lys1-Lys7 requires other structural features of the H-protein to keep it in a helical conformation. It is also possible that the Lys1-Gly2 extension prevents helix formation. Gly has a greater conformational freedom because of its lack of sidechain (Sternberg 1997) and breaks the α-helix as shown by Gly8 in the HNE-peptide and Gly388 in the H-protein.

Our resolved NMR structure of the wt-HNE-peptide was considerably more accurate than the molecular model of the HNE-peptide (QACKGKIQALCEN) previously generated by dynamic simulations (Putz 2004). The critical and non-critical residues were correctly located in opposite planes however that model did not predict the α-helix that we observed in the NMR structure. The relative positions of the critical residues were significantly different between the model and the NMR structure. This indicates that a purely dynamic modelling, based on force-fields, cannot give a correct representation of the structure of small peptides.

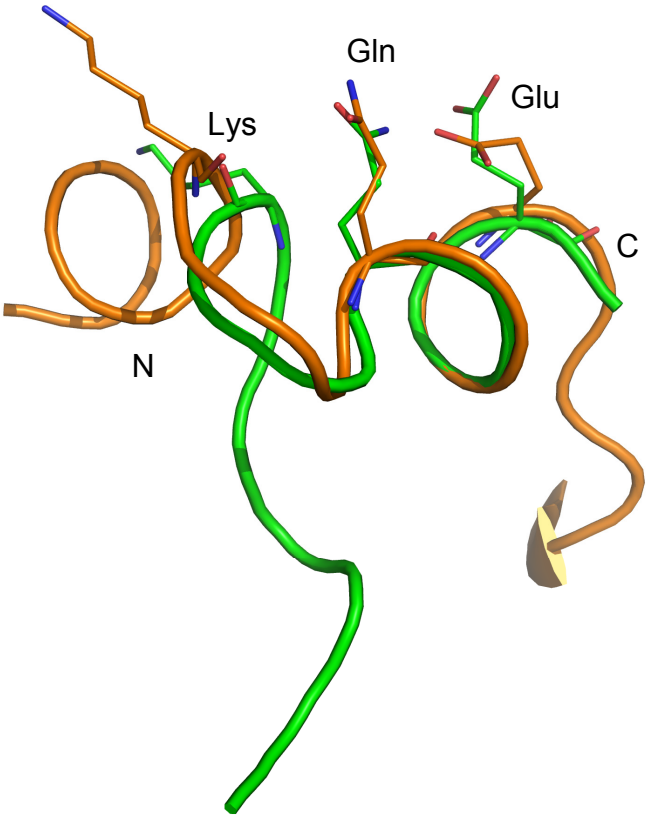


Figure 52: Superposition of H-protein and wt-HNE-peptide structures

Crystal structure of H-protein (orange) and central structure wt-HNE-peptide (green) superposed by alignment of residues 390 to 395/10 to 15. Peptide backbones are shown as tubes and critical contact residues Lys387/7, Gln391/11 and Glu395/15 are shown as sticks.

2.2. Structure of Gly8Ala

The superposition of the structures calculated for HNE-peptide mutants gave important clues about the effects of certain amino acid substitutions on the peptide structure, effects that are difficult to predict. Together with the results of the ELISA with mAb BH216 of HNE-peptides these changes could shed light on the biological results and especially on the structural requirements for sustained antigenicity of the HNE-peptide.

The Gly8Ala substituted peptide was part of a series of Gly8 mutants synthesised to investigate the importance and structure of the Gly loop formed by disulfide bond in the HNE-peptide structure and to further investigate the orientation of the N-terminal linkerarm observed in some of the wt-HNE-peptide conformations. Alignment of Gly8Ala to wt-HNE-peptide was somewhat complicated by the peculiar conformation of the central structure. As seen in Figure 23, the orientation of the linkerarm of the central conformation did not align with those of the other 19 low energy conformations, while the the structural features of the loop between Cys6 and Cys14 are conserved in all these conformations. Comparisons between wt-HNE-peptide and Gly8Ala peptide showed that the position and orientation of the critical contact residues Gln11 and Glu15 sidechains within the helix are well conserved (Figure 53). The loop distortion caused by the substitution of Gly to Ala has severe effects on the position of Lys7 which is moved out of the plane formed by the contact residues in the wt-HNE-peptide. This shift together with the tendency of the linkerarm to point into the binding domain explains the absence of interaction of mAb BH216 with this peptide. These results are confirmed by comparisons with the structure of the H-protein. Superposition of the central structure of Gly8Ala (Figure 54) and the twenty lowest energy conformations of Gly8Ala mutant (Figure 55) with H-protein confirmed the loop distortion between Cys6 and Cys14 and linkerarm orientation into the antibody binding domain. The lack of binding of mAb BH216 to Gly8Ala peptide could be explained by comparison to both the HNE-peptide NMR structure as well as the H-protein crystal structure. The linkerarm orientation observed after Gly8Ala substitution may be caused by steric hindrance of the Ala sidechain with the backbone of the neighbouring residues, which causes a distortion of the Gly loop and a rearrangement of the peptide into an energetically preferred conformation. These results explain why no substitution of Gly8 is allowed without abolishing antigenicity.

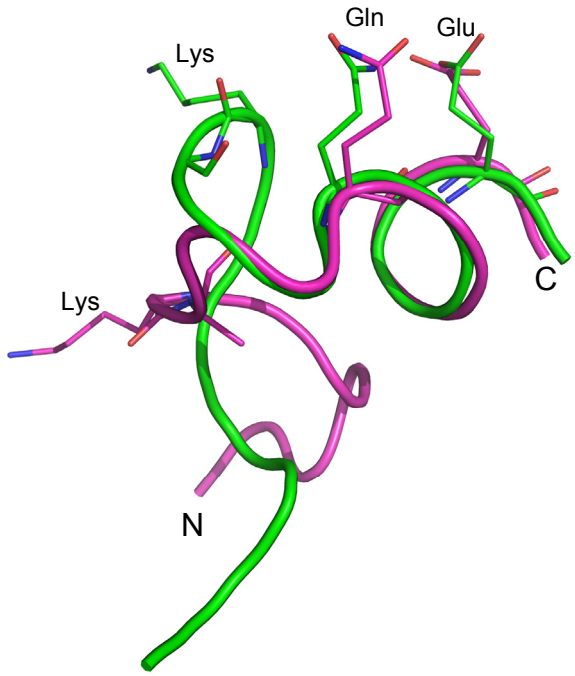


Figure 53: Superposition of wt-HNE-peptide and Gly8Ala mutant peptide structures

Central structures of wt-HNE-peptide (green) and Gly8Ala mutant peptide (pink) superposed by alignment of residues 10 to 15. Peptide backbones are shown as tubes and critical contact residues Lys7, Gln11 and Glu15 are shown as sticks.

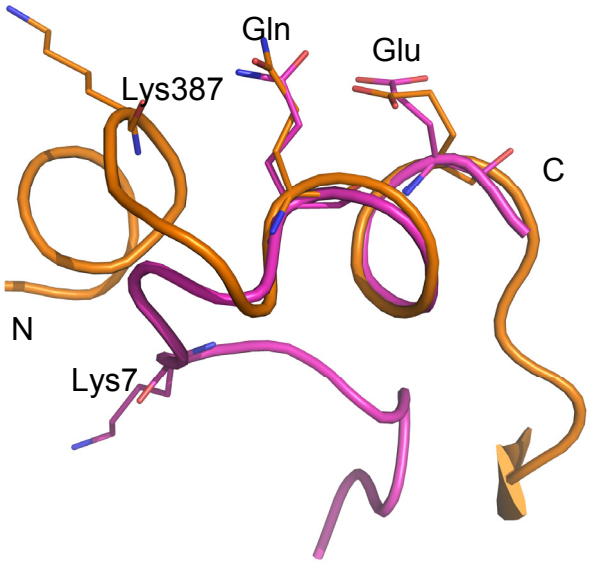


Figure 54: Superposition of H-protein and Gly8Ala mutant peptide structures

Crystal structure of H-protein (orange) and central NMR structure of Gly8Ala mutant peptide (pink) superposed by alignment of residues 390 to 395/10 to 15. Peptide backbones are shown as tubes and critical contact residues Lys387/7, Gln391/11 and Glu395/15 are shown as sticks.

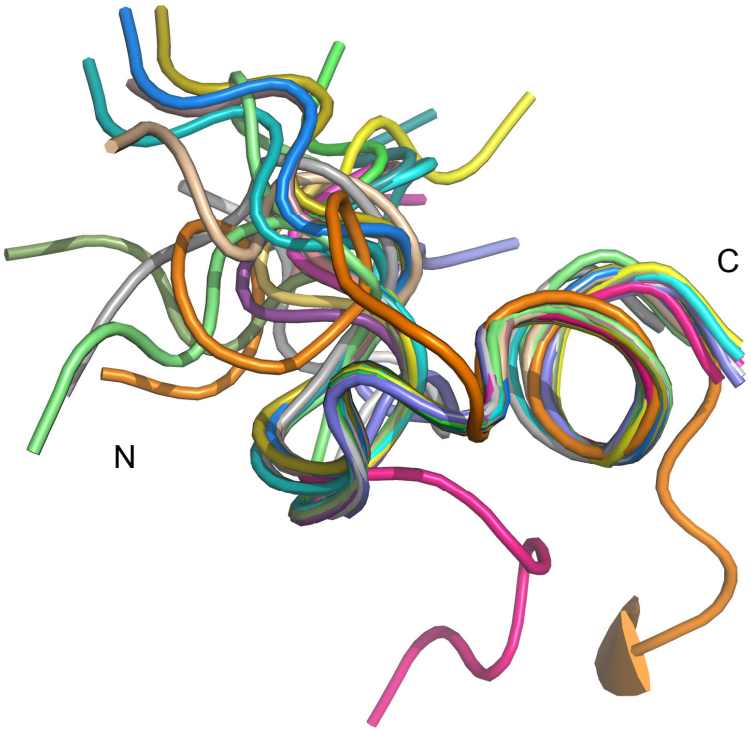


Figure 55: Superposition of H-protein and 20 lowest energy conformations of Gly8Ala mutant peptide
Crystal structure of H-protein (orange) and 20 lowest NMR energy conformations of Gly8Ala mutant peptide (coloured) superposed by alignment of residues 390 to 395/10 to 15. Peptide backbones are shown as tubes, central structure of Gly8Ala is in pink.

2.3. Structure of Ile10Ala

To increase the reactivity of the wt-HNE-peptide with mAbs we tried to reduce the flexibility of the α-helix in the peptide (Gurunath et al. 1995). While Ile10Ser lost antibody binding, the Ile10Ala substitution preserved some of the reactivity with mAb BH216 (Putz et al. 2003b). Since Ala is at the top of the propensity scale and thus the most preferred amino acid in α-helices (Chou and Fasman 1974), the helix is well conserved in comparison to the wt-HNE-peptide (Figure 56). Despite its distant location, the Ile10Ala substitution still influenced the linkerarm orientation with Lys7 pointing much further towards the antibody binding region. This would explain the partial loss in reactivity with mAb in the ELISA. The comparison of the Ile10Ala peptide with the H-protein shows that both align very well (Figure 57). There is a much better superposition of Lys7 of the Ile10Ala substituted peptide with Lys387 of H-protein than there is of wt-HNE-peptide Lys7 and the H-protein Lys387 residue. Thus, in principle Ile10Ala should react better with BH216 than the wt-HNE-peptide. However, the

reduced reactivity with BH216 is probably again linked to the orientation of the linkerarm of the peptide. The linkerarm in the wt-HNE-peptide points in the opposite direction of the binding domain and is thus less likely to sterically interfere with an approaching antibody than the linkerarm of the Ile10Ala mutant which is located in the same plane as the antibody binding domain. The small position shift of Lys7 observed for the wt-HNE-peptide may be compensated by induced fit, thanks to the inherent flexibility of the peptide, by a slight distortion of the loop between Cys6 and Cys14, which would superpose the two Lys and allow antibody binding.

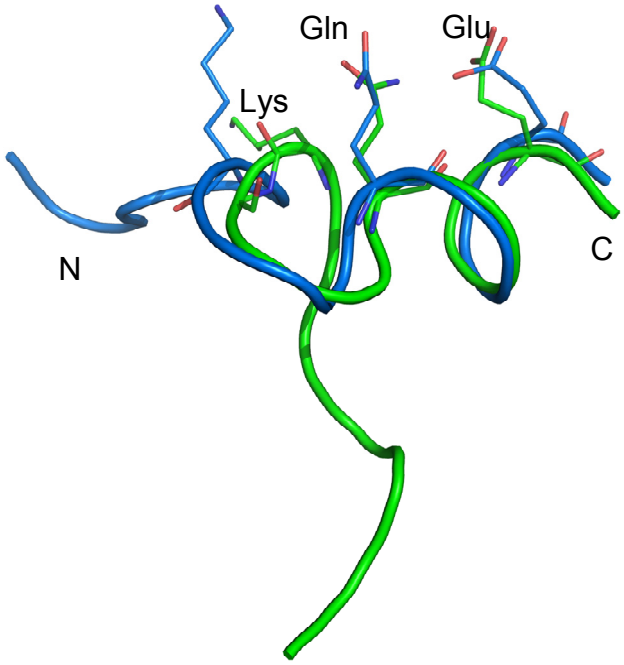


Figure 56: Superposition of wt-HNE-peptide and Ile10Ala mutant peptide structures

Central structures of wt-HNE-peptide (green) and Ile10Ala mutant peptide (blue) superposed by alignment of residues 10 to 15. Peptide backbones are shown as tubes and critical contact residues Lys7, Gln11 and Glu15 are shown as sticks.

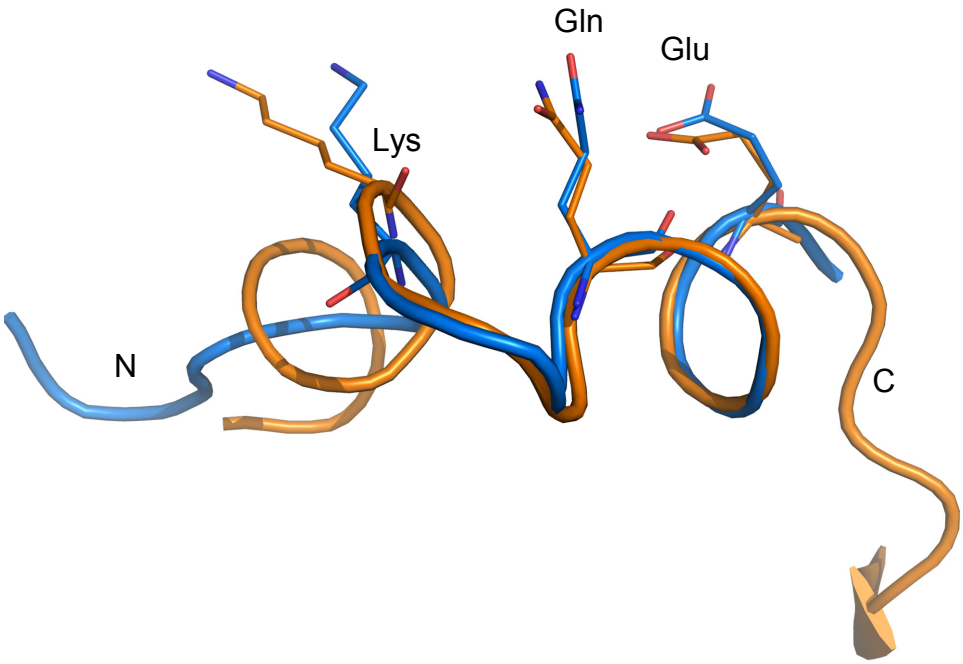


Figure 57: Superposition of H-protein and Ile10Ala mutant peptide structures
Crystal structure of H-protein (orange) and central structure of Ile10Ala mutant peptide (blue) were superposed by alignment of residues 390 to 395/10 to 15. Peptide backbones are shown as tubes and critical contact residues Lys387/7, Gln391/11 and Glu395/15 are shown as sticks.

2.4. Structure of Ile10Ser

As demonstrated by Putz (2003b), an HNE-peptide with an Ile10Ser mutation lost the ability to react with anti-HNE mAb. This may seem surprising since Ile10 is not a contact residue but most likely plays a structural role. Negative effects on the α-helix of the HNE-peptide and consequently antibody binding were not expected since Ile and Ser are situated midfield on the propensity scale with 0.41 and 0.5 kcal/mol, respectively. The scale ranges from 0 kcal/mol for Ala which is the amino acid most preferred in α-helices, to 1 kcal/mol for Gly, the least preferred residue (Pace and Scholtz 1998). Comparisons between the Ile10Ser mutant and wt-HNE-peptide and the HNE-epitope in the H-protein confirm that the α-helix together with the position and orientation of the critical contact residue Gln11 remain fairly well conserved (Figure 58A, Figure 59). As previously shown, the C-terminal region of the helix is more flexible compared to the rest of the HNE-peptide structures and the twists seen for Glu15 are expected to account for some of the reduced reactivity with antibodies. Ser also has a major influence on the structure of the loop between the two Cys residues. The loop appears more elongated compared to the wt structures (HNE-peptide and H-protein) resulting

in a significant shift of Lys7 (Figure 58B). In addition the flexible linkerarm tends to lie in the same plane as the Lys7 / Lys387 sidechain of the HNE and is likely to block access to the antibody (Figure 58A, Figure 59).

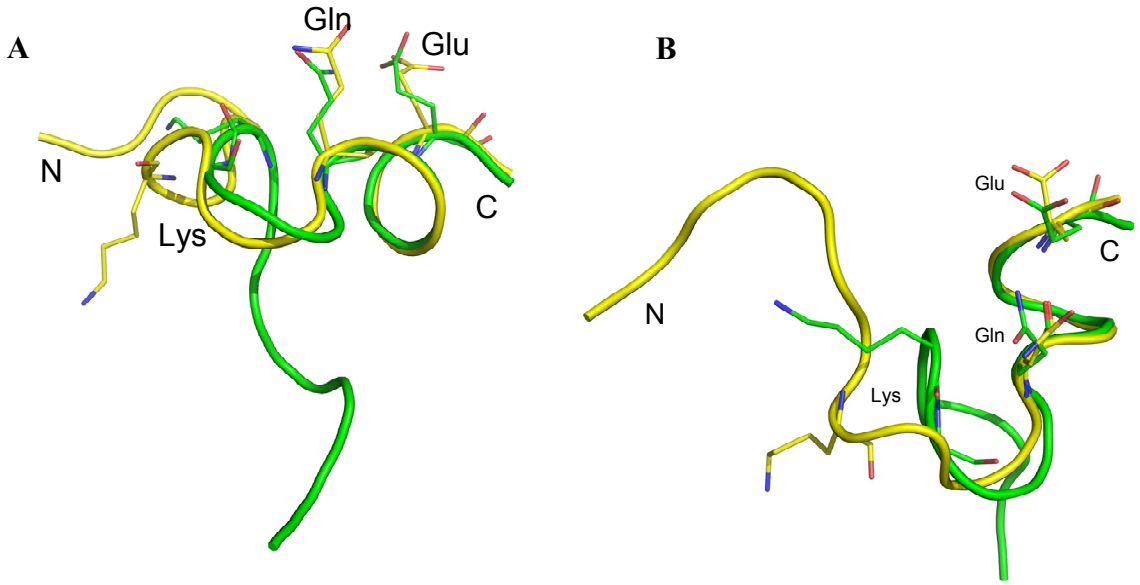


Figure 58: Superposition of wt-HNE-peptide and Ile10Ser mutant peptide structures

Central structures of wt-HNE-peptide (green) and Ile10Sser mutant peptide (yellow) superposed by alignment of residues 10 to 15. Peptide backbones are shown as tubes and critical contact residues Lys7, Gln11 and Glu15 are shown as sticks. Lateral view (A), top view (B).

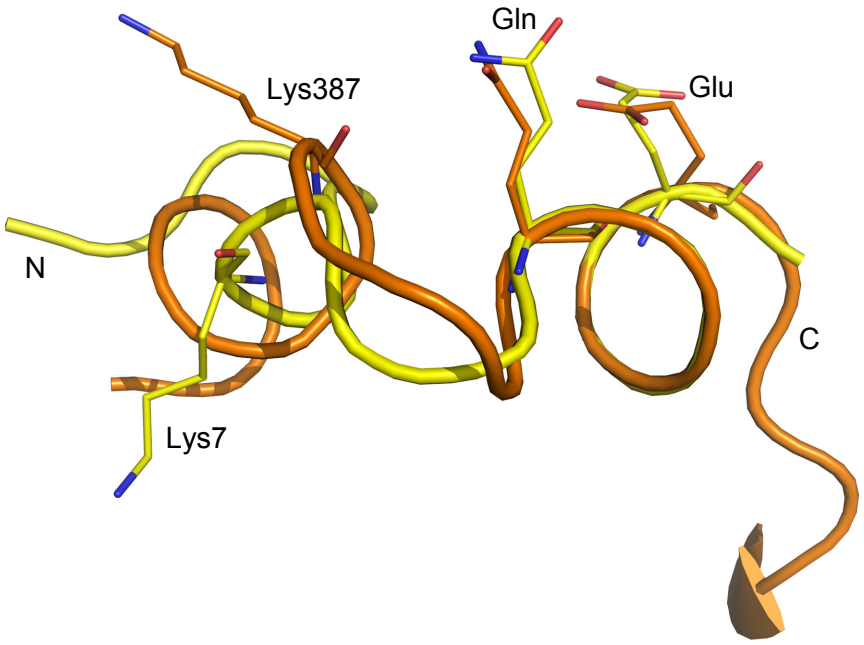


Figure 59: Superposition of H-protein and Ile10Ser mutant peptide structures

Crystal structure of H-protein (orange) and central structure of Ile10Sser mutant peptide (yellow) were superposed by alignment of residues 390 to 395/10 to 15. Peptide backbones are shown as tubes and critical contact residues Lys387/7, Gln391/11 and Glu395/15 are shown as sticks.

3. Stability and antigenicity

The half-life of wt-HNE-peptide was the benchmark to which peptides modified for enhanced stability would be compared to. The stability of the peptides was tested in mouse serum, which is commercially available and known to represent a high protease activity, even if immunisations are not done by intravenous route but by intraperitoneal injection. Intraperitoneal fluid is rarely available and collection cannot be easily standardised. The use of serum is therefore generally accepted as a useful fluid to assess the stability of peptides to be used in a clinical setting (Brinckerhoff et al. 1999). For instance, degradation experiments comparing peptide stabilities in human serum and synovial fluid found no difference between the two (Powell et al. 1992). Thus as a model, serum was an appropriate choice for stability testing of peptides and given the context of the experimental settings, serum from mice was chosen.

Analysis of the wt-HNE-peptides revealed a number of factors known to stabilise peptides: (i) the C-terminal amide stabilises peptides against exopeptidase degradation (Maillère et al. 1995; Brinckerhoff et al. 1999; Marschütz et al. 2002) and has been used throughout our study. (ii) Disulfide bridges stabilise peptide conformations and to some extent also block peptidase activity (Tugyi et al. 2005a). (iii) Prior to immunisation HNE-peptide was coupled to tetanus toxoid, which acted as carrier and provided important T cell epitopes to induce an immune response. The conjugation, via the N-terminal amine groups, blocked the HNE-peptide N-terminus from aminoexopeptidases specifically.

Our NMR studies have helped our understanding of the structural effects of amino acid substitutions on peptide antigenicity. Initial modifications of the HNE-peptide to increase the stability without compromising its reactivity with the antibodies, focused on the extension of the C-terminus, because even substitutions of non-critical residues of the α-helix had negative effects on antigenicity. Previous work in our laboratory (Putz 2004) established that residues $N_{396}PEWA_{400}$ could be replaced with any number of residues without interfering with antibody binding. Our NMR structure of the wt-HNE-peptide has shown that the C-terminus following Glu15 points away from the antibody binding site and therefore C-terminally extended amino acids were not expected to severely influence antigenicity. The orientation of the backbone was also confirmed in later publications of the X-ray crystal structures of the native H-protein (Colf et al. 2007; Hashiguchi et al. 2007).

To simulate the carrier protein and hopefully block degradation via aminoexopeptidases, the N-terminal Fmoc protection group was assessed. Blocking the N-terminus would have allowed us to focus only on carboxypeptidases and endopeptidases which are the only peptidases that can degrade N-terminally conjugated HNE-peptides. However, our studies have shown that due to premature cleavage in serum, the Fmoc group was not suitable and acetylation was more appropriate. N-terminal acetylation has previously been shown to protect synthetic peptides against N-terminal degradation (Maillère et al. 1995; Brinckerhoff et al. 1999). In fact, in eukaryotes many proteins are protected against N-terminal degradation by acetylation (Bell and Strauss 1981; Bradshaw et al. 1998).

To protect the C-terminus against degradation, candidate residues for C-terminal modification were selected from the literature as well as based on structural considerations (see Annexe 1). D-amino acids and modified natural amino acids, amongst others, were considered for this purpose. A limitation was the commercial availability of Fmoc-protected amino acids required for peptide synthesis. Candidate amino acids should not add additional functional groups which would potentially influence the peptide structure and affect the interaction with the antibody. At the same time they must be structurally different enough from natural amino acids to reduce peptide susceptibility towards peptidases.

Surprisingly the variety of non-natural amino acids failed to significantly improve the biostability of peptides. β-amino acids have been reported to increase the stability of peptides (Steer et al. 2002; Hook et al. 2004) without loss of biological activity (Frackenpohl et al. 2001; Sagan et al. 2003). A methyl insertion into the peptide backbone increases peptide flexibility (DeGrado et al. 1999; Beke et al. 2006). In our hands this did not adversely affect antibody binding since β-Ala was C-terminal and outside the binding domain, however the increase in stability was only minimal (data not shown).

Despite their often unusual structures, nonnatural amino acids such as trans-4-(aminomethyl)cyclohexane carboxylic acid (*t*-amcha), 1-amino-cyclohexane carboxylic acid (achca) and 4-(aminomethyl)benzoic acid (pamba; data not shown) failed to increase the stability of peptides, although antigenicity was conserved as expected.

D-amino acid residues added C-terminally included the small D-Ala and D-Val and the larger D-Phe and D-Leu. As observed with the other non-natural residues, no stabilisation was achieved by single amino acid extensions. Only the peptide with a C-terminal D-Ala was as stable as wt-HNE-peptide in serum. While several studies have reported increased stabilities using one or more D-amino acids at the C-terminus of core epitopes, these were either in combination with other N-terminal modifications (Brinckerhoff et al. 1999) or have consisted

of D-isomer substitutions of L-amino acids already present in the core peptide sequence (Hong et al. 1999). Here additions and not substitutions of residues to the peptide sequence were analysed and results are comparable to those obtained by Tugyi and colleagues who substituted three C-terminal L-amino acids and observed lower peptide stability (Tugyi et al. 2005b). More surprisingly the addition of two D-amino acids without other modifications, failed to stabilise peptides and all permutations of the above four D-amino acids, with the exception of 2 D-Ala residues, reduced or even abrogated antibody binding (amongst others peptides: 2711, 2712, 2723, 2742, and 2743; other data not shown). It is thought that the second amino acid, regardless of its nature, is located too close to the antibody binding domain and therefore reduces antigenicity.

Extending the peptide by the natural sequence in the H-protein ($P_{397}EWA_{400}$) generated peptides with a stability that was similar to that of wt-HNE-peptide only when combined with a D-Ala addition (PEWAa and PEWa). Presumably the increase in stability by D-Ala is negatively compensated by the presence of additional cleavage sites.

The failure to design more stable peptides solely by introducing C-terminal modifications suggested that carboxypeptidases play a less important role in degradation than aminopeptidases (Hong et al. 1999; Galati et al. 2003). Nevertheless this cannot explain the failure to improve stability and even the decrease in stability for certain C-terminal additions. While these terminal modifications can protect the peptide against exopeptidases, they have no protective effect against endopeptidases. In fact, similar to our findings, Marchütz and colleagues have also observed an increased susceptibility of terminally modified peptides towards endopeptidases. It was suggested that terminal modifications alter the peptide structure, thus exposing endopeptidase susceptible amide bonds (Marschütz et al. 2002). Thus C-terminal modifications may gain from combinations with amino acid substitutions within the core epitope (Webb et al. 2003).

To investigate this synergic effect and to increase the biostability of modified peptides, several internal substitutions were performed. Based on the fine-mapping work of the epitope by Putz (2003b) Lys9 was an interesting substitution site since it could be replaced by a variety of amino acids without loss of antibody binding. As a basic residue it is also a preferred cleavage site of peptidases. While the stability of the HNE-peptides with Lys9 substituted by several amino acids (Lys9Ala, Lys9Asn, Lys9Ser, Lys9His, Lys9Gln) were markedly increased compared to the wt-HNE-peptide, only the Lys9Ala substitution did not compromise antibody binding. Thus this substitution was further used in subsequent stability studies. The reduced reactivity with mAb BH216 of the other Lys9 substituted peptides does

not necessarily question the validity of the established binding motif, but may indicate that the shorter HNE-peptide would be more sensitive to a change in the proximity of the binding motif than the full length HNE-peptides, which were used in the fine-mapping studies.

In an effort to further increase the stability and immunogenicity of modified peptides, the number of Lys in the peptide sequence was reduced. The hypothesis is that endopeptidases would be deprived of their cleavage sites. At the same time coupling to the carrier protein via ε-amine groups of Lys in the antibody binding domain would be reduced, thus increasing the number of N-terminally coupled and biologically active molecules in peptide-protein-conjugates. Substitutions of Lys7 without loss of antibody binding were expected to be difficult since Lys7 is a critical part of the binding motif. D-Lys substitution of Lys7 showed a 2 fold increased stability, but the loss of antibody binding suggested that the paratope of BH216 is stereospecific. Similarly Hong and Na observed a loss of activity of an antimicrobial peptide after D-Lysine substitution in the core epitope (Hong et al. 1999; Na et al. 2007). This observation is in line with the structural NMR data of the HNE-peptide and the H-protein crystal X-ray crystal structure. The sidechain of D-Lys is likely to point further into the antibody binding domain and the loop around Gly8 is expected to be distorted to accommodate the new sidechain orientation, thus reducing antibody binding.

Methylation of basic amino acid sidechains has been known for some time to be an important post-transcriptional modification in a large number of proteins and can occur as mono-, di- or tri-methylation. The most well known Lys methylation is that reported in histones, which is important for gene regulation (Bannister et al. 2002; Paik et al. 2007). Methylation of Lys residues in the heparin-binding haemagglutinin adhesion protein in mycobacteria was reported to render the protein resistant to proteolysis (Pethe et al. 2002). Tri-methylation of Lys115 in calmodulin has been shown to inhibit ubiquitin mediated proteolysis (Gregori et al. 1987). Na and colleagues increased the stability of an antimicrobial peptide using mono-methylated Lys (Na et al. 2007). In our study substitution of Lys7 of the HNE-peptide showed that generally tri-methylated Lys are slightly more stable and more antigenic than di-methylated Lys substituted peptides. Most surprisingly substitutions with di- and tri-methylated Lys only increased the stability of HNE-peptides compared to wt-HNE-peptide when used in combination with further modifications (such as Lys9Ala substitutions), suggesting a synergic effect with respect to the additional modification. It is suspected that the

methylation of Lys7 on its own actually increases susceptibility of Lys9 towards endopeptidases via steric changes in the peptide structure.
Norvaline- and norleucine-substitution in position 7 conserved or lowered antigenicity with mAb BH216 depending on the other substitutions in the same peptide. These data show that the binding pocket of the antibody can accommodate a variety of residues at position 7, and is relatively independent of functional sidechains since substitution by methylated Lys, norleucine and norvaline gave the best results in that order. The binding pocket does, however, show selectivity with respect to sidechain orientation (as demonstrated by D-Lys) as well as size: diamino propionic acid (dap) with its shorter sidechain loses all reactivity with the antibody while the simple addition of one methyl as in norvaline reverses that effect.
These data showed that an increase in stability is possible but the conservation of antigenicity is much more delicate. Overall, our results show that substitutions of Lys7 with methylated Lys are interesting since they manage to tread the thin line between not fitting into the peptidase active site and yet preserving good interaction with the antibody paratope.

An additional point of interest was the N-terminal linker Lys-Gly, which increased the probability of a correct N-terminal coupling to the carrier by adding an additional N-terminal amine group and thus reducing peptide coupling via Lys (7 and 9) in the binding motif. This additional Lys, however, increased the susceptibility towards exopeptidases *in vitro* and possibly endopeptidases *in vivo* after coupling to the carrier. Branched peptides in which Lys sidechain amine groups were used to couple additional amino acids, were more stable than the wt-HNE-peptide but exhibited reduced antigenicity. We hypothesise that the substantial increase in size of the linkerarm can sterically hinder the accessibility of the antibody or influence the coating efficacy during ELISA. Similar problems were observed with other N-terminal substitutions or deletions. A possible approach in further tests could be to use biotin for N-terminal coating of the peptide in the streptavidin-ELISA system. In the case of branched peptides this may in fact strengthen binding compared to wt-HNE-peptide as a result of the increased number of N-terminal amine groups. Despite these problems with the current experimental setup, dap proved useful as N-terminal Lys replacement since it conserved antigenicity and increased peptide stability.

Biostability analysis of a number of stabilised peptides in murine intestinal juice gave results that were comparable to those in murine serum. All peptides tested were more stable than the wt-HNE-peptide in both serum and intestinal juice. The increased stability of peptides used in

the second immunisation following further modification, was also confirmed by the latter. Surprisingly peptide 2787 with a dap substitution of N-terminal Lys was significantly more stable in intestinal juice than in serum. The reasons for this observation are still under investigation. This may be explained by differences in exopeptidase activity in serum and intestinal juice. The observation that the stability of peptide 2787 was increased by 8 fold in intestinal juice yet did not lead to a similar increase in anti-peptide titres would support this hypothesis. Even after only 2 injections with the TT-2787 conjugate there was no significant increase in anti-peptide antibody titres compared to other less stable peptides (data not shown). Further testing *in vitro* and *in vivo* of wt-HNE-peptide with a dap substitution, would provide more information on the exact effect of this substitution.

4. Immunogenicity

During the course of this work we have iteratively combined various peptide modifications that increase the stability against the peptide without losing the antigenicity of the parent wt-HNE-peptide. The antigenicity, based on the interaction with the anti-HNE mAbs, specifically BH216, is an interesting tool to determine the effects of substitutions and modifications on peptide structure. Antigenicity, the capacity of a molecule to be recognised by antibodies, is however only a first step on the road to immunogenic peptide-based vaccines. Immunogenicity, the capacity of an antigen to induce an immune response, and specifically crossreactive immunogenicity, i.e. the reactivity with the protein from which the peptide is derived, are further critical properties. Here we investigated whether modified peptides induce antibodies that crossreact with the parent wt-HNE-peptide and with the native H-protein.

Most peptides tested *in vivo* induced antibodies levels against the homologous peptide that were comparable with the wt-HNE-peptide, showing that in most cases the conserved antigenicity correlates with immunogenicity. Only peptides with di- and tri-methylated Lys in position 7, gave slightly lower titres compared to the two peptides with the natural Lys. We suspect that while these peptides bound anti-HNE antibodies to a high degree by induced fit, their largely modified sidechains may slightly reduce immunogenicity. The considerable increases in stability that were achieved with our modifications did, however, not induce an increase in antibody titres compared to the wt-HNE-peptide. Peptide 2787 was as stable in serum as other peptides, but in murine intestinal juice the peptide showed a significant

increase in stability. It was the only modified peptide with such a discrepancy between serum and intestinal juice. This peptide showed a slight increase in anti-peptide antibody titres compared to wt-HNE-peptide, but to a lesser extent than the increase in stability in intestinal fluid (8 fold).

While the levels of anti-peptide antibodies are similar between the different peptide-conjugates, the extent of crossreactivity is much more variable. In the first immunisation experiments KGQQACKGAIQALCENPEWa (2743) conjugated to TT induced higher levels of antibodies crossreacting with the wt-HNE-peptide than the other peptide-conjugates. Crossreactivity with the recombinant H-protein or H-protein expressed on the cell surface of Mel-JuSo-H cells was on the other hand more similar to the other peptide-conjugates.

Putz found that antibodies induced against full length HNE-peptides predominantly recognised the C-terminal PEWA residues (Putz 2004). We have shown that conjugates with peptides KGQQAC(KMe_3)GAIQALCENPEWa (2805) and KGQQAC(KMe_3)GAIQALCEN (2809), which differ only by their C-terminal extension, induce similar levels of anti-peptide antibodies, but KGQQAC(KMe_3)GAIQALCEN (2809) has a significantly higher crossreactivity with wt-HNE-peptide. This finding confirms that the majority of antibodies induced against the former peptide targeted the C-terminus and little or none targeted the core epitope. Based on additional C-terminal extensions we further suggest that any addition to the C-terminus after $C_{14}E_{15}N_{16}$ is more immunogenic than the epitope core (i.e. the antibody binding motif). Peptides KGQQAC(KMe_3)GAIQALCENa (2807) and KGQQAC(KMe_2)GAIQALCENa (2784) induced similar levels of anti-peptide antibodies than their homologues without the C-terminal D-Ala residue KGQQAC(KMe_3)GAIQALCEN (2809) and KGQQAC(KMe_2)GAIQALCEN (2791). Crossreactivity with wt-HNE-peptide on the other hand shows that the peptides with an unmodified C-terminus induced more antibodies against the epitope core of the wt-HNE-peptide than the C-terminally modified peptides. There is an apparent contradiction with peptide 2809 with an unmodified C-terminus and 2787 with a D-Ala C-terminus: Immunisation with 2809 induced antibodies that crossreacted to a lesser degree with wt-HNE-peptide when combined with several adjuvants, than those induced against 2787. However we speculate that the increased complexity of the epitope core of 2809 with a tri-methylated Lys at position 7 and a Lys9Ala substitution leads to a structure that differs too much from that of the the wt-HNE-peptide and peptide 2787, which has only a Lys9Ala substitution in the antibody binding domain. Hence peptide 2787 could induce higher levels of crossreactive antibodies despite the immunodominant C-terminus.

A rapidly advancing field of vaccinology is the development of adjuvants since currently only few adjuvants (e.g. aluminium hydroxide) are licensed for human use. Here we have assessed the immunopotentiating efficacy of three different adjuvants: Montanide, Alum and Quil-A compared to Freund adjuvant. Due to its toxicity and side-effects Freund adjuvant is not allowed for human use and the use in animals is under scrutiny. After intraperitoneal injection with Freund adjuvants (complete and incomplete), mice showed signs of abdominal discomfort such as arched back and fluffy fur. No such side-effects were observed with any other tested adjuvant. Montanide induced lower levels of anti-peptide antibody titres, but levels of crossreactive antibody titres were similar to those induced by Freund adjuvant. Thus we can only partially confirm the results obtained by other groups which found Montanide equally effective as Freund adjuvant both for peptides and proteins (Martinez et al. 1996; Halassy et al. 2006). Nevertheless thanks to the absence of major side-effects Montanide remains an interesting alternative to Freund adjuvant, if an oil-based adjuvant with Th1 dominated Th response is desired.

In our tests Alum adjuvant was the most promising adjuvant. In addition to the absence of visual side-effects and discomfort on mice, Alum was the only adjuvant to induce wt-HNE-peptide crossreactive antibody levels when formulated with TT-2809, and these were comparable to those of TT-2787. The use of Alum adjuvant is however not straightforward: The reproducibility of antigen absorption onto aluminium hydroxide gel and desorption later on, remain issues that can significantly affect *in vivo* results (O'Hagen 2000). Alum adjuvants can be injected subcutaneously (s.c.), intraperitoneally (i.p.) and intramuscularly (i.m.) and predominantly induce a Th2 biased Th response, which makes them particularly useful in peptide-vaccines where only an antibody response is required.

Quil-A, a saponin based adjuvant, is also commonly used in ISCOMS (immunostimmulating complexes) inducing mainly Th1 immune responses. In our hands Quil-A reacted equally well as Alum and Freund's adjuvant. The low titre of crossreactive antibody observed when used in combination with TT-2809 is more likely related to the general tendency of TT-2809 to give lower responses and not due to the efficacy of Quil-A, as demonstrated by the levels induced by Freund and Montanide. Compared to the other adjuvants, Quil-A is easy to use: a solution of Quil-A is simply mixed to the antigen and injected. There are however limitations with regard to injection sites and methods. Quil-A is recommended for s.c. injections since i.p injections induce chemical peritonitis and fibrous adherences between various organs in the body cavity. (Dr. Erik B. Lindblad; personal communication), although use of Quil-A in i.p. (Lee et al. 2007) and i.m. (Jackson and Opdebeeck 1995) has been reported. We have

observed only slight skin irritation after s.c. injection of 25 µg of Quil-A into the scruff of the neck. These side-effects may be even lower when purified varieties of saponin derived adjuvants such as QS-21 are used (Powell and Newman 1995; O'Hagen 2000).

5. Fine-specificities of binding of HNE mAbs

Throughout this work we have shown that anti-HNE monoclonal antibodies BH216, BH21 and BH6, produced by distinct hybridomas (Ziegler et al. 1996), exhibit different fine-specificities towards modified HNE-peptides. Our and previous experiments have confirmed that the three mAb bind to the HNE-epitope only when C386 and C394 form a disulfide bridge. BH6 is less stringent with respect to the oxidation of the disulfide bond and shows some reactivity with the reduced peptide while BH216 and BH21 do not. The Cys substituted peptides also showed that BH6 preferentially binds shortened HNE-peptide (KGQQACKGKIQALCEN) rather than full length HNE-peptide (ETBFQQACKGKIQA-LCENPEWA).

Analysis of the peptide coupling efficiency to tetanus toxoid carrier lead to the clustering of peptides-conjugates into two distinct groups, when these conjugates were tested with BH216: Peptide-conjugates TT-2807, TT-2784 and TT-2787, with a C-terminal D-Ala reacted three times stronger than peptides with the "PEWa" extension or no extension. In contrast BH6 bound equally well to all peptide-conjugates irrespective of the different C-terminal modifications. It is therefore unlikely that the differences in binding observed for BH216 are caused by differences in the coupling efficiency to the carrier protein. The preference of BH216 for peptides with a C-terminus extended by a single amino acid was also observed for unconjugated modified HNE-peptides during the antigenicity tests, irrespective of the additional amino acid.

We conclude that (i) BH6 is less stringent with respect to oxidation of the peptide, (ii) preferentially binds to shorter HNE-peptides and (iii) is essentially insensitive to C-terminal extensions. BH216 and BH21 on the other hand have a preference for the oxidised HNE-peptides. The amino acid sequence data by Dr Fred Fack (unpublished results) further substantiate these findings. Sequence alignments of the complementarity determining regions (CDR) of the heavy chains of BH216, BH21 and BH6 to a germ-line sequence showed that BH216 and BH21 cluster differently than BH6 (Figure 60A). BH6 shows several mutations compared to BH216 and BH21 in CDR H2 and H3 as well as frameworks regions 2 and 3.

The alignment of the amino acid sequence of the kappa light chains with a germ-line sequence indicated that BH216 and BH6 clustered together (Figure 60B). But most of the substitutions in the kappa light chains were conservative replacements, where amino acids were substituted with residues of similar structure or property (polarity, basicity, acidity). A structure of the BH216 heavy and kappa light chains based on homology modelling with an anti-influenza H-protein antibody shows that some of these mutations are solvent exposed and may play a role in epitope binding (data not shown). It is unclear what influence the framework region mutations may have on this binding.

In the context of a collaboration with Prof Yanagi (Department of Virology, Faculty of Medicine, Kyushu University, Japan), work is currently underway to crystallise and structurally resolve the BH216 antibody/H-protein complex. This will shed more light on the antibody paratope and the residues interacting with the HNE-epitope of H-protein. The X-ray structure will also be important in determining the effects of the mutations observed in the heavy chains of BH6 and the consequences these may have on the antibody binding fine specificity.

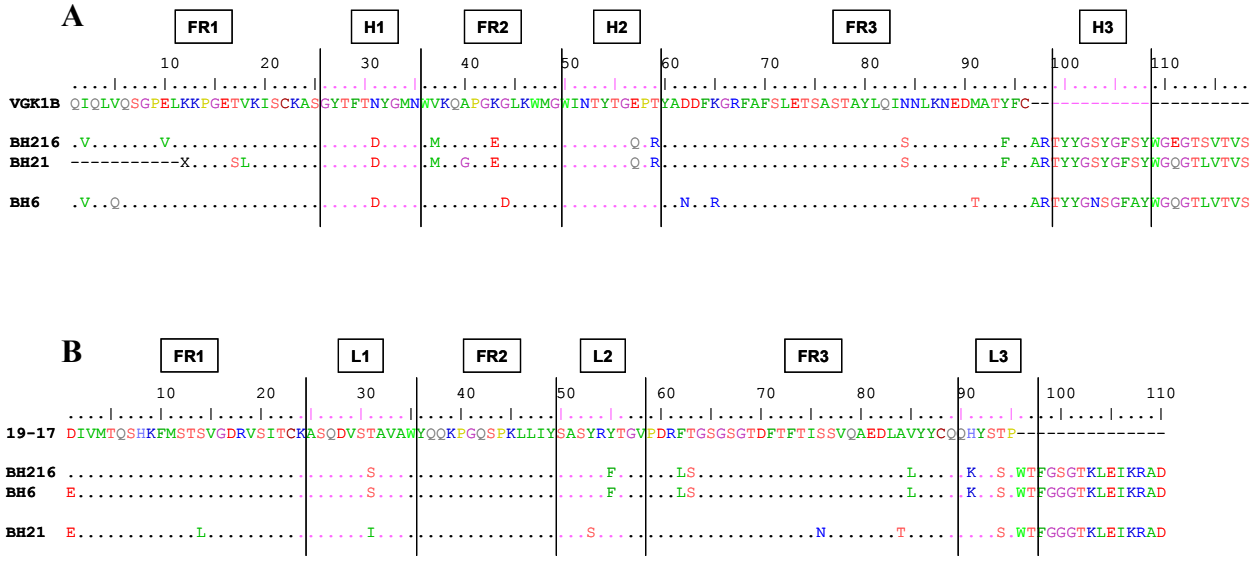


Figure 60: Amino acid sequence alignment of heavy and light chains of three anti-HNE antibodies with germ-line sequences

Amino acid sequences of heavy (A) and kappa light chains (B) of BH216, BH21 and BH6 aligned with germ-line sequences. Conserved residues are marked with a dot, deletions with a hyphen. Framework regions are labelled as FR, heavy and light region CDRs are marked as H and L. (Figure adapted from and with kind authorisation by Dr. Fred Fack)

6. Bile-acid peptide scaffold

In fulfilment of her PhD thesis Cathy Bodé (University of Ghent) synthesised a backbone cyclised peptide and a scaffold-peptide construct, incorporating the HNE sequence QACKGKIQALCEN with a C-terminal Gly spacer. Biological testing of these constructs confirmed their antigenicity with respect to the three anti-HNE mAbs. We could again show the differences in fine-specificities between BH6 on one hand and BH216 and BH21 on the other hand, with BH6 being less selective. The antibody binding profiles showed that the scaffold-peptide construct mimics the structure of the HNE more closely than the same peptide after end to tail cyclisation.

The biostability data of the cyclic and scaffold-peptide construct suggest that both scaffold and the backbone cyclisation increased the peptide stability *in vitro* compared to the oxidised wt-HNE-peptide (KGQQACKGKIQALCEN). This is in line with the results from various groups showing that cyclised peptides are more resistant to proteolytic degradation than linear peptides (Clark et al. 2005; Tugyi et al. 2005a). The stability of these constructs however was not higher than the stability observed using our internal and terminal substitution strategies. This also confirms our previous results that blocking exopeptidases alone does not prevent peptide degradation to the same extent as by preventing endopeptidase attack by substitution of susceptible residues in the peptide core.

In immunisation assays these peptides induced high levels of anti-peptide antibodies. Antibodies raised against the scaffold-peptide construct reacted only weakly with the cyclic peptide. It is not yet clear if the majority of anti-scaffold-peptide construct antibodies targeted mainly the scaffold-peptide interface instead of the core epitope, or whether the structural differences between the two peptides is too important. Antibodies induced against both cyclic control peptide and scaffold-peptide construct did not crossreact with the H-protein. We hypothesise that both peptides adopt conformations that can still bind the monoclonal antibodies by induced fit, but that are too distant from the natural conformation to induce crossreactive antibodies.

It may be possible to improve the structure of these peptides by varying the length of the scaffold-bound peptide, or by reducing the disulfide bridge, giving the peptide more conformational freedom to adopt an energetically more favourable structure (Bodé 2007).

7. Concluding remarks and perspectives

In this study we analysed the relationship between stability, antigenicity and immunogenicity of modified HNE-peptides and provided new information on the structural requirements of the HNE-epitope for antibody binding. The data obtained from our NMR structures guided our strategy to further improve peptide stability and to understand antibody binding in the antigenicity studies. We show that Lys387 plays a crucial role in this regard.

Our study is one of only few that analysed the substitution by D-amino acids in flanking regions of epitope cores (Tugyi et al. 2005b; Manea et al. 2008). Our efforts to stabilise peptides based on a rational iterative approach and the NMR structures of HNE-peptides, have lead to the synthesis of several peptides with significantly increased stability without loss of antigenicity. These peptides induced strong antibody titres, showing that they also retained immunogenicity. The failure of these antibodies to strongly crossreact with the natural MV-H-protein confirms that antigenicity does not guarantee crossreactive immunogenicity. It is important to recognise that there may be large discrepancies between antigenicity and immunogenicity. Increased stability against degradation by peptidases was achieved by modifying amino acid sidechains. Alternatively a steroidal scaffold-system blocked degradation by exopeptidases. By restricting peptide flexibility, the scaffold limited the number of possible conformations that are presented during the immunisation process to the cell surface immunoglobulins of B cells. The preferred peptide conformations resulting from these modifications were still antigenic and bound the antibody via induced fit. They may however not be the conformations that are closest to the native protein structure, thus reducing crossreactive immunogenicity. Earlier studies in our laboratory demonstrated that some antigenic peptides failed to induce crossreactive antibodies, whereas some peptides which lacked antigenicity even induced neutralising antibodies (El Kasmi et al. 1999a). But the successful protection against FMDV and canine parvovirus of swine and dogs, respectively, using peptide-based vaccines demonstrated the feasibility of vaccination with peptides (Langeveld et al. 1994; Wang et al. 2002).

An important question throughout this study was to what extent the wt-HNE-peptide could be further stabilised by chemical and conformational approaches, before crossreactive immunogenicity started to decrease. Especially a shorter peptide is less likely to accommodate amino acid substitutions without a change in its structure. In principle, most increases in biostability were penalised by changes in peptide structure and thus a reduction of crossreactive immunogenicity. We conclude that stability against degradation may not be the

most important factor in peptide-based vaccines and that the correct conformation of a peptide compensates to some extent the lower stability.

The knowledge obtained from the peptide modifications and the NMR structures provide a blueprint for a more focused approach to modifying and coupling of the peptide to the steroidal scaffold. It would be possible that moderate internal modifications such as a Lys9Ala substitution can increase the biological stability of the scaffold-bound HNE-peptide. Changing the spacer amino acid residues to better conserve the loop structure of the peptide would be another step to improve the structure within the scaffold-system. A combination of the two approaches could create a construct with a more stable and yet structurally conserved peptide, able to induce a stronger crossreactive immune response.

References

1. Adessi C,Soto C (2002). "Converting a peptide into a drug: strategies to improve stability and bioavailability." Curr Med Chem **9**(9): 963-78.
2. Agger EM, Rosenkrands I, Olsen AW, Hatch G, Williams A, Kritsch C, Lingnau K, von Gabain A, Andersen CS, Korsholm KS,Andersen P (2006). "Protective immunity to tuberculosis with Ag85B-ESAT-6 in a synthetic cationic adjuvant system IC31." Vaccine **24**(26): 5452-60.
3. Aguilar JC,Rodriguez EG (2007). "Vaccine adjuvants revisited." Vaccine **25**(19): 3752-62.
4. Albrecht P, Ennis FA, Saltzman EJ,Krugman S (1977). "Persistence of maternal antibody in infants beyond 12 months: mechanism of measles vaccine failure." J Pediatr **91**(5): 715-8.
5. Andres O, Obojes K, Kim KS, ter Meulen V,Schneider-Schaulies J (2003). "CD46- and CD150-independent endothelial cell infection with wild-type measles viruses." J Gen Virol **84**(Pt 5): 1189-97.
6. Arolas JL, Aviles FX, Chang JY,Ventura S (2006). "Folding of small disulfide-rich proteins: clarifying the puzzle." Trends Biochem Sci **31**(5): 292-301.
7. Arpadi SM, Markowitz LE, Baughman AL, Shah K, Adam H, Wiznia A, Lambert G, Dobroszycki J, Heath JL,Bellini WJ (1996). "Measles antibody in vaccinated human immunodeficiency virus type 1-infected children." Pediatrics **97**(5): 653-7.
8. Atabani SF, Obeid OE, Chargelegue D, Aaby P, Whittle H,Steward MW (1997). "Identification of an immunodominant neutralizing and protective epitope from measles virus fusion protein by using human sera from acute infection." J Virol **71**(10): 7240-5.
9. Atherton E,Sheppard RC (1989). Solid Phase Peptide Synthesis: A Practical Approach. Oxford, Oxford University Press.
10. Aucouturier J, Dupuis L, Deville S, Ascarateil S,Ganne V (2002). "Montanide ISA 720 and 51: a new generation of water in oil emulsions as adjuvants for human vaccines." Expert Rev Vaccines **1**(1): 111-8.
11. Auwaerter PG, Rota PA, Elkins WR, Adams RJ, DeLozier T, Shi Y, Bellini WJ, Murphy BR,Griffin DE (1999). "Measles virus infection in rhesus macaques: altered immune responses and comparison of the virulence of six different virus strains." J Infect Dis **180**(4): 950-8.
12. Bade S, Röckendorf N, Franek M, Gorris H-H,Frey A (2007a). Kits for highly sensitive detection assays. **PCT-patent application, Publication number WO 2007/051603 A2**.
13. Bade S, Röckendorf N, Gorris H-H,Frey A (2007b). Novel 2,4-dichlorophenoxyacetic acid derivatives and use thereof in diagnostic and analytic methods. **PCT-patent application, Publication number WO 2007/051604 A2**.
14. Bannister AJ, Schneider R,Kouzarides T (2002). "Histone Methylation: Dynamic or Static?" Cell **109**(7): 801.
15. Barry JF, Davis AP, Nieves Pérez-Payan M, Elsegood MRJ, Jackson RFW, Gennari C, Piarulli U,Gude M (1999). "A trifunctional steroid-based scaffold for combinatorial chemistry." Tetrahedron Lett **40**(14): 2849.

16. Bartz R, Brinckmann U, Dunster LM, Rima B, Ter Meulen V,Schneider-Schaulies J (1996). "Mapping amino acids of the measles virus hemagglutinin responsible for receptor (CD46) downregulation." Virology **224**(1): 334-7.
17. Beck A, Klinguer-Hamour C, Bussat MC, Champion T, Haeuw JF, Goetsch L, Wurch T, Sugawara M, Milon A, Van Dorsselaer A, Nguyen T,Corvaia N (2007). "Peptides as tools and drugs for immunotherapies." J Pept Sci **13**(9): 588-602.
18. Beke T, Somlai C,Perczel A (2006). "Toward a rational design of beta-peptide structures." J Comput Chem **27**(1): 20-38.
19. Bell JR,Strauss JH (1981). "In vivo NH2-terminal acetylation of Sindbis virus proteins." J Biol Chem **256**(15): 8006-11.
20. Bellini WJ, Rota JS,Rota PA (1994). "Virology of measles virus." J Infect Dis **170 Suppl 1**: S15-23.
21. Bennett J, Whittle H, Samb B, Cisse B, Simondon F,Aaby P (1999). "Seroconversions in unvaccinated infants: further evidence for subclinical measles from vaccine trials in Niakhar, Senegal." Int J Epidemiol **28**(1): 147-51.
22. Biellik R, Madema S, Taole A, Kutsulukuta A, Allies E, Eggers R, Ngcobo N, Nxumalo M, Shearley A, Mabuzane E, Kufa E,Okwo-Bele JM (2002). "First 5 years of measles elimination in southern Africa: 1996-2000." Lancet **359**(9317): 1564-8.
23. Birkedal-Hansen H (1995). "Proteolytic remodeling of extracellular matrix." Curr Opin Cell Biol **7**(5): 728-35.
24. Bittle JL, Houghten RA, Alexander H, Shinnick TM, Sutcliffe JG, Lerner RA, Rowlands DJ,Brown F (1982). "Protection against foot-and-mouth disease by immunization with a chemically synthesized peptide predicted from the viral nucleotide sequence." Nature **298**(5869): 30-3.
25. Black FL,Rosen L (1962). "Patterns of measles antibodies in residents of Tahiti and their stability in the absence of re-exposure." J Immunol **88**: 725-31.
26. Blankenfeldt W, Nokihara K, Naruse S, Lessel U, Schomburg D,Wray V (1996). "NMR spectroscopic evidence that helodermin, unlike other members of the secretin/VIP family of peptides, is substantially structured in water." Biochemistry **35**(19): 5955-62.
27. Bodé CA, Bechet T, Prodhomme EJ, Gheysen K, Gregoir P, Martins JC, Muller CP,Madder A "Design, Synthesis and Biological Evaluation of a Cyclic Bile Acid-Peptide Conjugate: Towards the Conformational Mimicry of the Measles Virus HNE Loop." submitted to Org Biomol Chem.
28. Bodé CA (2007). Evaluation of a Steroidal Scaffold for the Conformational Restriction of Peptides in the Development of Peptide Vaccines. Department of Organic Chemistry. Ghent, University of Ghent. **PhD**.
29. Bodé CA, Muller CP,Madder A (2007). "Validation of a solid-phase-bound steroid scaffold for the synthesis of novel cyclic peptidosteroids." J Pept Sci **13**(11): 702-8.
30. Bouche F, Ammerlaan W, Fournier P, Schneider F,Muller CP (1998). "A simplified immunoassay based on measles virus recombinant hemagglutinin protein for testing the immune status of vaccinees." J Virol Methods **74**(1): 77-87.
31. Bouche FB, Marquet-Blouin E, Yanagi Y, Steinmetz A,Muller CP (2003). "Neutralising immunogenicity of a polyepitope antigen expressed in a transgenic food plant: a novel antigen to protect against measles." Vaccine **21**(17-18): 2065-72.
32. Bouche FB, Steinmetz A, Yanagi Y,Muller CP (2005). "Induction of broadly neutralizing antibodies against measles virus mutants using a polyepitope vaccine strategy." Vaccine **23**(17-18): 2074-7.

33. Boyaka PN, Marinaro M, Jackson RJ, van Ginkel FW, Cormet-Boyaka E, Kirk KL, Kensil CR,McGhee JR (2001). "Oral QS-21 requires early IL-4 help for induction of mucosal and systemic immunity." J Immunol **166**(4): 2283-90.
34. Bradshaw RA, Brickey WW,Walker KW (1998). "N-terminal processing: the methionine aminopeptidase and N alpha-acetyl transferase families." Trends Biochem Sci **23**(7): 263-7.
35. Bray AM, Joe Maeji N,Mario Geysen H (1990). "The simultaneous multiple production of solution phase peptides; assessment of the geysen method of simultaneous peptide synthesis." Tetrahedron Letters **31**(40): 5811.
36. Brinckerhoff LH, Kalashnikov VV, Thompson LW, Yamshchikov GV, Pierce RA, Galavotti HS, Engelhard VH,Slingluff CL, Jr. (1999). "Terminal modifications inhibit proteolytic degradation of an immunogenic MART-1(27-35) peptide: implications for peptide vaccines." Int J Cancer **83**(3): 326-34.
37. Brunger AT, Adams PD, Clore GM, DeLano WL, Gros P, Grosse-Kunstleve RW, Jiang JS, Kuszewski J, Nilges M, Pannu NS, Read RJ, Rice LM, Simonson T,Warren GL (1998). "Crystallography & NMR system: A new software suite for macromolecular structure determination." Acta Crystallogr D Biol Crystallogr **54**(Pt 5): 905-21.
38. Caceres VM, Strebel PM,Sutter RW (2000). "Factors determining prevalence of maternal antibody to measles virus throughout infancy: a review." Clin Infect Dis **31**(1): 110-9.
39. Cardoso AI, Blixenkrone-Moller M, Fayolle J, Liu M, Buckland R,Wild TF (1996). "Immunization with plasmid DNA encoding for the measles virus hemagglutinin and nucleoprotein leads to humoral and cell-mediated immunity." Virology **225**(2): 293-9.
40. Carpino LA,Han GY (1972). "9-Fluorenylmethoxycarbonyl amino-protecting group." J. Org. Chem. **37**(22): 3404-3409.
41. CDC (1997). "Measles--United States, 1996, and the interruption of indigenous transmission." MMWR Morb Mortal Wkly Rep **46**(11): 242-6.
42. Chan WC,White PD (2000). Fmoc Solid Phase Peptide Synthesis: A Practical Approach. Oxford, Oxford University Press Inc.
43. Chorev M, Shavitz R, Goodman M, Minick S,Guillemin R (1979). "Partially modified retro-inverso-enkephalinamides: topochemical long-acting analogs in vitro and in vivo." Science **204**(4398): 1210-2.
44. Chou PY,Fasman GD (1974). "Conformational parameters for amino acids in helical, beta-sheet, and random coil regions calculated from proteins." Biochemistry **13**(2): 211-22.
45. Christenson B,Bottiger M (1994). "Measles antibody: comparison of long-term vaccination titres, early vaccination titres and naturally acquired immunity to and booster effects on the measles virus." Vaccine **12**(2): 129-33.
46. Clark RJ, Fischer H, Dempster L, Daly NL, Rosengren KJ, Nevin ST, Meunier FA, Adams DJ,Craik DJ (2005). "Engineering stable peptide toxins by means of backbone cyclization: stabilization of the alpha-conotoxin MII." Proc Natl Acad Sci U S A **102**(39): 13767-72.
47. Cohen BJ, Parry RP, Andrews N, Bennett AM,Dennis JH (2008). "Laboratory methods for assessing vaccine potency retained in aerosol outputs from nebulizers: application to World Health Organization measles aerosol project." Vaccine **26**(27-28): 3534-9.
48. Cohen LY, Bahr GM, Darcissac EC,Parant MA (1996). "Modulation of expression of class II MHC and CD40 molecules in murine B cells by various muramyl dipeptides." Cell Immunol **169**(1): 75-84.

49. Colf LA, Juo ZS,Garcia KC (2007). "Structure of the measles virus hemagglutinin." Nat Struct Mol Biol **14**(12): 1227-8.
50. Creighton TE, Zapun A,Darby NJ (1995). "Mechanisms and catalysts of disulfide bond formation in proteins." Trends Biotechnol **13**(1): 18-23.
51. Crennell S, Takimoto T, Portner A,Taylor G (2000). "Crystal structure of the multifunctional paramyxovirus hemagglutinin-neuraminidase." Nat Struct Biol **7**(11): 1068-74.
52. Curran MD, Clarke DK,Rima BK (1991). "The nucleotide sequence of the gene encoding the attachment protein H of canine distemper virus." J Gen Virol **72 (Pt 2)**: 443-7.
53. Cutts FT, Henao-Restrepo A,Olive JM (1999). "Measles elimination: progress and challenges." Vaccine **17 Suppl 3**: S47-52.
54. Dakappagari NK, Lute KD, Rawale S, Steele JT, Allen SD, Phillips G, Reilly RT,Kaumaya PT (2005). "Conformational HER-2/neu B-cell epitope peptide vaccine designed to incorporate two native disulfide bonds enhances tumor cell binding and antitumor activities." J Biol Chem **280**(1): 54-63.
55. Dalsgaard K (1984). "Assessment of the dose of the immunological adjuvant Quil A in mice and guinea pigs, using sheep red blood cells as model antigen." Zentralbl Veterinarmed B **31**(9): 718-20.
56. Damien B, Huiss S, Schneider F,Muller CP (1998). "Estimated susceptibility to asymptomatic secondary immune response against measles in late convalescent and vaccinated persons." J Med Virol **56**(1): 85-90.
57. Davidkin I,Valle M (1998). "Vaccine-induced measles virus antibodies after two doses of combined measles, mumps and rubella vaccine: a 12-year follow-up in two cohorts." Vaccine **16**(20): 2052-7.
58. de Quadros CA, Hersh BS, Nogueira AC, Carrasco PA,da Silveira CM (1998). "Measles eradication: experience in the Americas." Bull World Health Organ **76 Suppl 2**: 47-52.
59. de Swart RL, Vos HW, UytdeHaag FGCM, Osterhaus ADME,van Binnendijk RS (1998). "Measles virus fusion protein- and hemagglutinin-transfected cell lines are a sensitive tool for the detection of specific antibodies by a FACS-measured immunofluorescence assay." Journal of Virological Methods **71**(1): 35.
60. de Swart RL, Yuksel S,Osterhaus AD (2005). "Relative contributions of measles virus hemagglutinin- and fusion protein-specific serum antibodies to virus neutralization." J Virol **79**(17): 11547-51.
61. de Swart RL, Kuiken T, Fernandez-de Castro J, Papania MJ, Bennett JV, Valdespino JL, Minor P, Witham CL, Yuksel S, Vos H, van Amerongen G,Osterhaus AD (2006). "Aerosol measles vaccination in macaques: preclinical studies of immune responses and safety." Vaccine **24**(40-41): 6424-36.
62. de Swart RL, LiCalsi C, Quirk AV, van Amerongen G, Nodelman V, Alcock R, Yuksel S, Ward GH, Hardy JG, Vos H, Witham CL, Grainger CI, Kuiken T, Greenspan BJ, Gard TG,Osterhaus AD (2007). "Measles vaccination of macaques by dry powder inhalation." Vaccine **25**(7): 1183-90.
63. de Vries P, van Binnendijk RS, van der Marel P, van Wezel AL, Voorma HO, Sundquist B, Uytdehaag FG,Osterhaus AD (1988). "Measles virus fusion protein presented in an immune-stimulating complex (iscom) induces haemolysis-inhibiting and fusion-inhibiting antibodies, virus-specific T cells and protection in mice." J Gen Virol **69 (Pt 3)**: 549-59.
64. de Vries RD, Stittelaar KJ, Osterhaus AD,de Swart RL (2008). "Measles vaccination: new strategies and formulations." Expert Rev Vaccines **7**(8): 1215-23.

65. DeGrado WF, Schneider JP,Hamuro Y (1999). "The twists and turns of beta-peptides." J Pept Res **54**(3): 206-17.
66. Del Giudice G,Rappuoli R (2007). Adjuvants and subunit vaccines In: Microbiology and Microbial Infections. 10th. S. H. E. Kaufmann and M. W. Steward, Topley & Wilson's. **Immunology:** p925-946.
67. Delamarre L, Couture R, Mellman I,Trombetta ES (2006). "Enhancing immunogenicity by limiting susceptibility to lysosomal proteolysis." J Exp Med **203**(9): 2049-55.
68. Deroo S, El Kasmi KC, Fournier P, Theisen D, Brons NH, Herrmann M, Desmet J,Muller CP (1998). "Enhanced antigenicity of a four-contact-residue epitope of the measles virus hemagglutinin protein by phage display libraries: evidence of a helical structure in the putative active site." Mol Immunol **35**(8): 435-43.
69. Devaux P, Loveland B, Christiansen D, Milland J,Gerlier D (1996). "Interactions between the ectodomains of haemagglutinin and CD46 as a primary step in measles virus entry." J Gen Virol **77 (Pt 7)**: 1477-81.
70. Dilraj A, Sukhoo R, Cutts FT,Bennett JV (2007). "Aerosol and subcutaneous measles vaccine: measles antibody responses 6 years after re-vaccination." Vaccine **25**(21): 4170-4.
71. Dormitzer PR, Ulmer JB,Rappuoli R (2008). "Structure-based antigen design: a strategy for next generation vaccines." Trends Biotechnol **26**(12): 659-67.
72. Drillien R, Spehner D, Kirn A, Giraudon P, Buckland R, Wild F,Lecocq JP (1988). "Protection of mice from fatal measles encephalitis by vaccination with vaccinia virus recombinants encoding either the hemagglutinin or the fusion protein." Proc Natl Acad Sci U S A **85**(4): 1252-6.
73. Duke T,Mgone CS (2003). "Measles: not just another viral exanthem." Lancet **361**(9359): 763-73.
74. Durbin AP, Skiadopoulos MH, McAuliffe JM, Riggs JM, Surman SR, Collins PL,Murphy BR (2000). "Human parainfluenza virus type 3 (PIV3) expressing the hemagglutinin protein of measles virus provides a potential method for immunization against measles virus and PIV3 in early infancy." J Virol **74**(15): 6821-31.
75. Eichler J,Houghten RA (1997). "Synthesis of Cyclic Disulfide Peptides: Comparison of Oxidation Methods." Protein and Peptide Letters **4**(3): 157-164.
76. El Kasmi KC, Theisen D, Brons NH,Muller CP (1998). "The molecular basis of virus crossreactivity and neutralisation after immunisation with optimised chimeric peptides mimicking a putative helical epitope of the measles virus hemagglutinin protein." Mol Immunol **35**(14-15): 905-18.
77. El Kasmi KC, Deroo S, Theisen DM, Brons NH,Muller CP (1999a). "Crossreactivity of mimotopes and peptide homologues of a sequential epitope with a monoclonal antibody does not predict crossreactive immunogenicity." Vaccine **18**(3-4): 284-90.
78. El Kasmi KC, Theisen D, Brons NH, Ammerlaan W, Klingele M, Truong AT,Muller CP (1999b). "A hemagglutinin-derived peptide-vaccine ignored by virus-neutralizing passive antibodies, protects against murine measles encephalitis." Vaccine **17**(19): 2436-45.
79. El Kasmi KC, Fillon S, Theisen DM, Hartter H, Brons NH,Muller CP (2000). "Neutralization of measles virus wild-type isolates after immunization with a synthetic peptide vaccine which is not recognized by neutralizing passive antibodies." J Gen Virol **81**(Pt 3): 729-35.
80. El Kasmi KC,Muller CP (2001). "New strategies for closing the gap of measles susceptibility in infants: towards vaccines compatible with current vaccination schedules." Vaccine **19**(17-19): 2238-44.

81. Embree JE, Datta P, Stackiw W, Sekla L, Braddick M, Kreiss JK, Pamba H, Wamola I, Ndinya-Achola JO, Law BJ,et al. (1992). "Increased risk of early measles in infants of human immunodeficiency virus type 1-seropositive mothers." J Infect Dis **165**(2): 262-7.
82. Enders JF,Peebles TC (1954). "Propagation in tissue cultures of cytopathogenic agents from patients with measles." Proc Soc Exp Biol Med **86**(2): 277-86.
83. Enders JF, Katz SL, Milovanovic MV,Holloway A (1960). "Studies on an attenuated measles-virus vaccine. I. Development and preparations of the vaccine: technics for assay of effects of vaccination." N Engl J Med **263**: 153-9.
84. Etchart N, Wild F,Kaiserlian D (1996). "Mucosal and systemic immune responses to measles virus haemagglutinin in mice immunized with a recombinant vaccinia virus." J Gen Virol **77 (Pt 10)**: 2471-8.
85. Etchart N, Buckland R, Liu MA, Wild TF,Kaiserlian D (1997). "Class I-restricted CTL induction by mucosal immunization with naked DNA encoding measles virus haemagglutinin." J Gen Virol **78 (Pt 7)**: 1577-80.
86. Fagbule D,Orifunmishe F (1988). "Measles and childhood mortality in semi-urban Nigeria." Afr J Med Med Sci **17**(3): 181-5.
87. Fairlie DP, Tyndall JD, Reid RC, Wong AK, Abbenante G, Scanlon MJ, March DR, Bergman DA, Chai CL,Burkett BA (2000). "Conformational selection of inhibitors and substrates by proteolytic enzymes: implications for drug design and polypeptide processing." J Med Chem **43**(7): 1271-81.
88. Fennelly GJ, Flynn JL, ter Meulen V, Liebert UG,Bloom BR (1995). "Recombinant bacille Calmette-Guerin priming against measles." J Infect Dis **172**(3): 698-705.
89. Fennelly GJ, Khan SA, Abadi MA, Wild TF,Bloom BR (1999). "Mucosal DNA vaccine immunization against measles with a highly attenuated Shigella flexneri vector." J Immunol **162**(3): 1603-10.
90. Fenouillet E, Barbouche R, Courageot J,Miquelis R (2001). "The catalytic activity of protein disulfide isomerase is involved in human immunodeficiency virus envelope-mediated membrane fusion after CD4 cell binding." J Infect Dis **183**(5): 744-52.
91. Fooks AR, Schadeck E, Liebert UG, Dowsett AB, Rima BK, Steward M, Stephenson JR,Wilkinson GW (1995). "High-level expression of the measles virus nucleocapsid protein by using a replication-deficient adenovirus vector: induction of an MHC-1-restricted CTL response and protection in a murine model." Virology **210**(2): 456-65.
92. Fooks AR, Jeevarajah D, Warnes A, Wilkinson GW,Clegg JC (1996). "Immunization of mice with plasmid DNA expressing the measles virus nucleoprotein gene." Viral Immunol **9**(2): 65-71.
93. Fooks AR, Jeevarajah D, Lee J, Warnes A, Niewiesk S, ter Meulen V, Stephenson JR,Clegg JC (1998). "Oral or parenteral administration of replication-deficient adenoviruses expressing the measles virus haemagglutinin and fusion proteins: protective immune responses in rodents." J Gen Virol **79 (Pt 5)**: 1027-31.
94. Fooks AR, Sharpe SA, Shallcross JA, Clegg JC,Cranage MP (2000). "Induction of immunity using oral DNA vaccines expressing the measles virus nucleocapsid protein." Dev Biol (Basel) **104**: 65-71.
95. Fournier P, Brons NH, Berbers GA, Wiesmuller KH, Fleckenstein BT, Schneider F, Jung G,Muller CP (1997). "Antibodies to a new linear site at the topographical or functional interface between the haemagglutinin and fusion proteins protect against measles encephalitis." J Gen Virol **78 (Pt 6)**: 1295-302.
96. Frackenpohl J, Arvidsson PI, Schreiber JV,Seebach D (2001). "The outstanding biological stability of beta- and gamma-peptides toward proteolytic enzymes: an in vitro investigation with fifteen peptidases." Chembiochem **2**(6): 445-55.

97. Francis MJ, Hastings GZ, Clarke BE, Brown AL, Beddell CR, Rowlands DJ,Brown F (1990). "Neutralizing antibodies to all seven serotypes of foot-and-mouth disease virus elicited by synthetic peptides." Immunology **69**(2): 171-6.
98. Franek M, Kolar V, Granatova M,Nevorankova Z (1994). "Monoclonal Elisa for 2,4-Dichlorophenoxyacetic Acid: Characterization of Antibodies and Assay Optimization." Journal of Agricultural and Food Chemistry **42**(6): 1369-1374.
99. Frank R (1992). "Spot-synthesis: an easy technique for the positionally addressable, parallel chemical synthesis on a membrane support." Tetrahedron **48**(42): 9217.
100. Fraser CK, Diener KR, Brown MP,Hayball JD (2007). "Improving vaccines by incorporating immunological coadjuvants." Expert Rev Vaccines **6**(4): 559-78.
101. Freidinger RM (2003). "Design and synthesis of novel bioactive peptides and peptidomimetics." J Med Chem **46**(26): 5553-66.
102. Freund J, Casals J,Hosmer EP (1937). "Sensitization and antibody formation after injection of tubercle bacilli and paraffin oil." Proc. Exp. Biol. Med. **37**: 50-513.
103. Frey A, Meckelein B, Externest D,Schmidt MA (2000). "A stable and highly sensitive 3,3',5,5'-tetramethylbenzidine-based substrate reagent for enzyme-linked immunosorbent assays." J Immunol Methods **233**(1-2): 47-56.
104. Fulginiti VA, Eller JJ, Downie AW,Kempe CH (1967). "Altered reactivity to measles virus. Atypical measles in children previously immunized with inactivated measles virus vaccines." Jama **202**(12): 1075-80.
105. Galati R, Verdina A, Falasca G,Chersi A (2003). "Increased resistance of peptides to serum proteases by modification of their amino groups." Z Naturforsch [C] **58**(7-8): 558-61.
106. Galletti R, Beauverger P,Wild TF (1995). "Passively administered antibody suppresses the induction of measles virus antibodies by vaccinia-measles recombinant viruses." Vaccine **13**(2): 197-201.
107. Gallina A, Hanley TM, Mandel R, Trahey M, Broder CC, Viglianti GA,Ryser HJ (2002). "Inhibitors of protein-disulfide isomerase prevent cleavage of disulfide bonds in receptor-bound glycoprotein 120 and prevent HIV-1 entry." J Biol Chem **277**(52): 50579-88.
108. Gans H, Yasukawa L, Rinki M, DeHovitz R, Forghani B, Beeler J, Audet S, Maldonado Y,Arvin AM (2001). "Immune responses to measles and mumps vaccination of infants at 6, 9, and 12 months." J Infect Dis **184**(7): 817-26.
109. Gans HA, Arvin AM, Galinus J, Logan L, DeHovitz R,Maldonado Y (1998). "Deficiency of the humoral immune response to measles vaccine in infants immunized at age 6 months." Jama **280**(6): 527-32.
110. Gauvrit A, Brandler S, Sapede-Peroz C, Boisgerault N, Tangy F,Gregoire M (2008). "Measles virus induces oncolysis of mesothelioma cells and allows dendritic cells to cross-prime tumor-specific CD8 response." Cancer Res **68**(12): 4882-92.
111. Giraudon P,Wild TF (1985). "Correlation between epitopes on hemagglutinin of measles virus and biological activities: passive protection by monoclonal antibodies is related to their hemagglutination inhibiting activity." Virology **144**(1): 46-58.
112. Giuliani MM, Del Giudice G, Giannelli V, Dougan G, Douce G, Rappuoli R,Pizza M (1998). "Mucosal adjuvanticity and immunogenicity of LTR72, a novel mutant of Escherichia coli heat-labile enterotoxin with partial knockout of ADP-ribosyltransferase activity." J Exp Med **187**(7): 1123-32.
113. Glenny AT, Pope CC, Waddington H,Wallace V (1926). "The antigenic value of toxoid precipitated by potassium alum." J. Pathol. Bacteriol. **29**: 38-45.
114. Gluck R (1999). "Adjuvant activity of immunopotentiating reconstituted influenza virosomes (IRIVs)." Vaccine **17**(13-14): 1782-7.

115. Gorris H-H, Bade S, Röckendorf N,Frey A (2007). Methods for determining the cleavability of substrates. **PCT-patent application, Publication number WO 2007/051605 A2**.
116. Gorris HH, Bade S, Rockendorf N, Albers E, Schmidt MA, Franek M,Frey A (2009). "Rapid Profiling of Peptide Stability in Proteolytic Environments." Anal Chem.
117. Gould AR, Mabbutt BC, Llewellyn LE, Goss NH,Norton RS (1992). "Linear and cyclic peptide analogues of the polypeptide cardiac stimulant, anthopleurin-A. 1H-NMR and biological activity studies." Eur J Biochem **206**(3): 641-51.
118. Granoff DM, McHugh YE, Raff HV, Mokatrin AS,Van Nest GA (1997). "MF59 adjuvant enhances antibody responses of infant baboons immunized with Haemophilus influenzae type b and Neisseria meningitidis group C oligosaccharide-CRM197 conjugate vaccine." Infect Immun **65**(5): 1710-5.
119. Graves M, Griffin DE, Johnson RT, Hirsch RL, de Soriano IL, Roedenbeck S,Vaisberg A (1984). "Development of antibody to measles virus polypeptides during complicated and uncomplicated measles virus infections." J Virol **49**(2): 409-12.
120. Green TD, Newton BR, Rota PA, Xu Y, Robinson HL,Ross TM (2001). "C3d enhancement of neutralizing antibodies to measles hemagglutinin." Vaccine **20**(1-2): 242-8.
121. Greensfelder L (2000). "INFECTIOUS DISEASES: Polio Outbreak Raises Questions About Vaccine." Science **290**(5498): 1867b-1869b.
122. Gregori L, Marriott D, Putkey JA, Means AR,Chau V (1987). "Bacterially synthesized vertebrate calmodulin is a specific substrate for ubiquitination." J Biol Chem **262**(6): 2562-7.
123. Griffin DE (1995). "Immune responses during measles virus infection." Curr Top Microbiol Immunol **191**: 117-34.
124. Griffin DE (2007). Measles Virus In: Fields Virology. 5th. D. M. Knipe and P. M. Howley (eds). Philadelphia, Lippincott Williams & Wilkins. **2:** p1551-1586.
125. Griffin DE, Pan CH,Moss WJ (2008). "Measles vaccines." Front Biosci **13**: 1352-70.
126. Guex N,Peitsch MC (1997). "SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling." Electrophoresis **18**(15): 2714-23.
127. Gupta RK,Siber GR (1994). "Comparison of adjuvant activities of aluminium phosphate, calcium phosphate and stearyl tyrosine for tetanus toxoid." Biologicals **22**(1): 53-63.
128. Gupta RK, Chang AC, Griffin P, Rivera R,Siber GR (1996). "In vivo distribution of radioactivity in mice after injection of biodegradable polymer microspheres containing 14C-labeled tetanus toxoid." Vaccine **14**(15): 1412-6.
129. Guris D, McCready J, Watson JC, Atkinson WL, Heath JL, Bellini WJ,Polloi A (1996). "Measles vaccine effectiveness and duration of vaccine-induced immunity in the absence of boosting from exposure to measles virus." Pediatr Infect Dis J **15**(12): 1082-6.
130. Gurunath R, Beena TK, Adiga PR,Balaram P (1995). "Enhancing peptide antigenicity by helix stabilization." FEBS Lett **361**(2-3): 176-8.
131. Gustafson TL, Lievens AW, Brunell PA, Moellenberg RG, Buttery CM,Sehulster LM (1987). "Measles outbreak in a fully immunized secondary-school population." N Engl J Med **316**(13): 771-4.
132. Halassy B, Mateljak S, Bouche FB, Putz MM, Muller CP, Frkanec R, Habjanec L,Tomasic J (2006). "Immunogenicity of peptides of measles virus origin and influence of adjuvants." Vaccine **24**(2): 185-94.

133. Hamamoto K, Kida Y, Zhang Y, Shimizu T,Kuwano K (2002). "Antimicrobial activity and stability to proteolysis of small linear cationic peptides with D-amino acid substitutions." Microbiol Immunol **46**(11): 741-9.
134. Hans D, Young PR,Fairlie DP (2006). "Current status of short synthetic peptides as vaccines." Med Chem **2**(6): 627-46.
135. Harandi AM (2004). "The potential of immunostimulatory CpG DNA for inducing immunity against genital herpes: opportunities and challenges." J Clin Virol **30**(3): 207-10.
136. Hartter HK, Oyedele OI, Dietz K, Kreis S, Hoffman JP,Muller CP (2000). "Placental transfer and decay of maternally acquired antimeasles antibodies in Nigerian children." Pediatr Infect Dis J **19**(7): 635-41.
137. Hashiguchi T, Kajikawa M, Maita N, Takeda M, Kuroki K, Sasaki K, Kohda D, Yanagi Y,Maenaka K (2007). "Crystal structure of measles virus hemagglutinin provides insight into effective vaccines." Proc Natl Acad Sci U S A **104**(49): 19535-40.
138. Hathaway LJ, Obeid OE,Steward MW (1998). "Protection against measles virus-induced encephalitis by antibodies from mice immunized intranasally with a synthetic peptide immunogen." Vaccine **16**(2-3): 135-41.
139. Hawkes CA,McLaurin J (2007). "Immunotherapy as treatment for Alzheimer's disease." Expert Rev Neurother **7**(11): 1535-48.
140. Hayden GF (1979). "Measles vaccine failure. A survey of causes and means of prevention." Clin Pediatr (Phila) **18**(3): 155-6, 161-3, 167.
141. He Q, Mitchell AR, Johnson SL, Wagner-Bartak C, Morcol T,Bell SJ (2000). "Calcium phosphate nanoparticle adjuvant." Clin Diagn Lab Immunol **7**(6): 899-903.
142. He XH, Shaw PC,Tam SC (1999). "Reducing the immunogenicity and improving the in vivo activity of trichosanthin by site-directed pegylation." Life Sci **65**(4): 355-68.
143. Heavner GA, Kroon DJ, Audhya T,Goldstein G (1986). "Biologically active analogs of thymopentin with enhanced enzymatic stability." Peptides **7**(6): 1015-9.
144. Heinonen OP, Paunio M,Peltola H (1998). "Total elimination of measles in Finland." Ann Med **30**(2): 131-3.
145. Hermanson GT (1996). Bioconjugate Techniques. London, Academic Press Ltd.
146. Hewer R,Meyer D (2005). "Peptide immunogens designed to enhance immune responses against human immunodeficiency virus (HIV) mutant strains: a plausible means of preventing viral persistence." J Theor Biol **233**(1): 85-90.
147. Hogg PJ (2003). "Disulfide bonds as switches for protein function." Trends Biochem Sci **28**(4): 210-4.
148. Holmgren J, Lycke N,Czerkinsky C (1993). "Cholera toxin and cholera B subunit as oral-mucosal adjuvant and antigen vector systems." Vaccine **11**(12): 1179-84.
149. Hong SY, Oh JE,Lee KH (1999). "Effect of D-amino acid substitution on the stability, the secondary structure, and the activity of membrane-active peptide." Biochem Pharmacol **58**(11): 1775-80.
150. Hook DF, Gessier F, Noti C, Kast P,Seebach D (2004). "Probing the proteolytic stability of beta-peptides containing alpha-fluoro- and alpha-hydroxy-beta-amino acids." Chembiochem **5**(5): 691-706.
151. Hu A,Norrby E (1994). "Role of individual cysteine residues in the processing and antigenicity of the measles virus haemagglutinin protein." J Gen Virol **75 (Pt 9)**: 2173-81.
152. Huang Z, Dry I, Webster D, Strugnell R,Wesselingh S (2001). "Plant-derived measles virus hemagglutinin protein induces neutralizing antibodies in mice." Vaccine **19**(15-16): 2163-71.

153. Hudecz F (2001). "Manipulation of epitope function by modification of peptide structure: a minireview." Biologicals **29**(3-4): 197-207.
154. Hung L-H,Samudrala R (2003). "Accurate and automated classification of protein secondary structure with PsiCSI." Protein Sci **12**(2): 288-295.
155. Jackson LA,Opdebeeck JP (1995). "The effect of various adjuvants on the humoral immune response of sheep and cattle to soluble and membrane midgut antigens of Boophilus microplus." Vet Parasitol **58**(1-2): 129-41.
156. Johnson AG, Gaines S,Landy M (1956). "Studies on the O antigen of Salmonella typhosa. V. Enhancement of antibody response to protein antigens by the purified lipopolysaccharide." J Exp Med **103**(2): 225-46.
157. Johnson RP (1999). "Live attenuated AIDS vaccines: hazards and hopes." Nat Med **5**(2): 154-5.
158. Kaplan LJ, Daum RS, Smaron M,McCarthy CA (1992). "Severe measles in immunocompromised patients." Jama **267**(9): 1237-41.
159. Kaumaya PT, VanBuskirk AM, Goldberg E,Pierce SK (1992). "Design and immunological properties of topographic immunogenic determinants of a protein antigen (LDH-C4) as vaccines." J Biol Chem **267**(9): 6338-46.
160. Keeling MJ (1997). "Modelling the persistence of measles." Trends Microbiol **5**(12): 513-8.
161. Kleywegt GJ (1996). "Use of non-crystallographic symmetry in protein structure refinement." Acta Crystallogr D Biol Crystallogr **52**(Pt 4): 842-57.
162. Koivunen E, Arap W, Valtanen H, Rainisalo A, Medina OP, Heikkila P, Kantor C, Gahmberg CG, Salo T, Konttinen YT, Sorsa T, Ruoslahti E,Pasqualini R (1999). "Tumor targeting with a selective gelatinase inhibitor." Nat Biotechnol **17**(8): 768-74.
163. Kovamees J, Blixenkrone-Moller M, Sharma B, Orvell C,Norrby E (1991). "The nucleotide sequence and deduced amino acid composition of the haemagglutinin and fusion proteins of the morbillivirus phocid distemper virus." J Gen Virol **72 (Pt 12)**: 2959-66.
164. Kovarik J, Gaillard M, Martinez X, Bozzotti P, Lambert PH, Wild TF,Siegrist CA (2001). "Induction of adult-like antibody, Th1, and CTL responses to measles hemagglutinin by early life murine immunization with an attenuated vaccinia-derived NYVAC(K1L) viral vector." Virology **285**(1): 12-20.
165. Kramer W, Wess G, Enhsen A, Falk E, Hoffmann A, Neckermann G, Schubert G,Urmann M (1997). "Modified bile acids as carriers for peptides and drugs." J. Control. Release **46**(1-2): 17.
166. Kremer JR, Bouche FB, Schneider F,Muller CP (2006a). "Re-exposure to wild-type virus stabilizes measles-specific antibody levels in late convalescent patients." J Clin Virol **35**(1): 95-8.
167. Kremer JR, Schneider F,Muller CP (2006b). "Waning antibodies in measles and rubella vaccinees--a longitudinal study." Vaccine **24**(14): 2594-601.
168. Krugman S (1983). "Further-attenuated measles vaccine: characteristics and use." Rev Infect Dis **5**(3): 477-81.
169. Langedijk JP, Daus FJ,van Oirschot JT (1997). "Sequence and structure alignment of Paramyxoviridae attachment proteins and discovery of enzymatic activity for a morbillivirus hemagglutinin." J Virol **71**(8): 6155-67.
170. Langeveld JP, Casal JI, Osterhaus AD, Cortes E, de Swart R, Vela C, Dalsgaard K, Puijk WC, Schaaper WM,Meloen RH (1994). "First peptide vaccine providing protection against viral infection in the target animal: studies of canine parvovirus in dogs." J Virol **68**(7): 4506-13.

171. Larché M (2007). "Peptide immunotherapy for allergic diseases." Allergy **62**(3): 325-31.
172. Lee YH, Shin DW, Lee JH, Nam HW,Ahn MH (2007). "Vaccination against murine toxoplasmosis using recombinant Toxoplasma gondii SAG3 antigen alone or in combination with Quil A." Yonsei Med J **48**(3): 396-404.
173. Leeman MF, McKay JA,Murray GI (2002). "Matrix metalloproteinase 13 activity is associated with poor prognosis in colorectal cancer." J Clin Pathol **55**(10): 758-62.
174. Léger R, Robitaille M, Quraishi O, Denholm E, Benquet C, Carette J, van Wyk P, Pellerin I, Bousquet-Gagnon N, Castaigne JP,Bridon D (2003). "Synthesis and in vitro analysis of atrial natriuretic peptide-albumin conjugates." Bioorg Med Chem Lett **13**(20): 3571-5.
175. Leontiadou H, Mark AE,Marrink SJ (2006). "Antimicrobial peptides in action." J Am Chem Soc **128**(37): 12156-61.
176. Leuridan E,Van Damme P (2007). "Passive transmission and persistence of naturally acquired or vaccine-induced maternal antibodies against measles in newborns." Vaccine **25**(34): 6296-304.
177. LiCalsi C, Christensen T, Bennett JV, Phillips E,Witham C (1999). "Dry powder inhalation as a potential delivery method for vaccines." Vaccine **17**(13-14): 1796-803.
178. LiCalsi C, Maniaci MJ, Christensen T, Phillips E, Ward GH,Witham C (2001). "A powder formulation of measles vaccine for aerosol delivery." Vaccine **19**(17-19): 2629-36.
179. Lien S,Lowman HB (2003). "Therapeutic peptides." Trends Biotechnol **21**(12): 556-62.
180. Liniger M, Zuniga A,Naim HY (2007). "Use of viral vectors for the development of vaccines." Expert Rev Vaccines **6**(2): 255-66.
181. Lockwood JS,Randall HT (1949). "The place of electrolyte studies in surgical patients." Bull N Y Acad Med **25**(4): 228-43.
182. Lottspeich F,Zorbas H (1998). Bioanalytik. Heidelberg, Spektrum Akademischer Verlag.
183. Low N, Kraemer S, Schneider M,Restrepo AM (2008). "Immunogenicity and safety of aerosolized measles vaccine: systematic review and meta-analysis." Vaccine **26**(3): 383-98.
184. Lynch JM, Briles DE,Metzger DW (2003). "Increased protection against pneumococcal disease by mucosal administration of conjugate vaccine plus interleukin-12." Infect Immun **71**(8): 4780-8.
185. Maggi T, Oggioni MR, Medaglini D, Bianchi Bandinelli ML, Soldateschi D, Wiesmuller KH, Muller CP, Valensin PE,Pozzi G (2000). "Expression of measles virus antigens in Streptococcus gordonii." New Microbiol **23**(2): 119-28.
186. Maillère B, Mourier G, Hervé M,Ménez A (1995). "Fine chemical modifications at N- and C-termini enhance peptide presentation to T cells by increasing the lifespan of both free and MHC-complexed peptides." Mol Immunol **32**(17-18): 1377-85.
187. Manea M, Kalaszi A, Mezo G, Horvati K, Bodor A, Horvath A, Farkas O, Perczel A, Przybylski M,Hudecz F (2008). "Antibody recognition and conformational flexibility of a plaque-specific beta-amyloid epitope modulated by non-native peptide flanking regions." J Med Chem **51**(5): 1150-61.
188. Markowitz LE, Sepulveda J, Diaz-Ortega JL, Valdespino JL, Albrecht P, Zell ER, Stewart J, Zarate ML,Bernier RH (1990). "Immunization of six-month-old infants with different doses of Edmonston-Zagreb and Schwarz measles vaccines." N Engl J Med **322**(9): 580-7.

189. Marquet-Blouin E, Bouche FB, Steinmetz A,Muller CP (2003). "Neutralizing immunogenicity of transgenic carrot (Daucus carota L.)-derived measles virus hemagglutinin." Plant Mol Biol **51**(4): 459-69.
190. Marr AK, Gooderham WJ,Hancock RE (2006). "Antibacterial peptides for therapeutic use: obstacles and realistic outlook." Curr Opin Pharmacol **6**(5): 468-72.
191. Marschütz MK, Zauner W, Mattner F, Otava A, Buschle M,Bernkop-Schnurch A (2002). "Improvement of the enzymatic stability of a cytotoxic T-lymphocyte-epitope model peptide for its oral administration." Peptides **23**(10): 1727-33.
192. Martinez D, Perez JM, Sheikboudou C, Debus A,Bensaid A (1996). "Comparative efficacy of Freund's and Montanide ISA50 adjuvants for the immunisation of goats against heartwater with inactivated Cowdria ruminantium." Vet Parasitol **67**(3-4): 175-84.
193. Martins CL, Garly ML, Bale C, Rodrigues A, Ravn H, Whittle HC, Lisse IM,Aaby P (2008). "Protective efficacy of standard Edmonston-Zagreb measles vaccination in infants aged 4.5 months: interim analysis of a randomised clinical trial." Bmj **337**: a661.
194. Masse N, Ainouze M, Neel B, Wild TF, Buckland R,Langedijk JP (2004). "Measles virus (MV) hemagglutinin: evidence that attachment sites for MV receptors SLAM and CD46 overlap on the globular head." J Virol **78**(17): 9051-63.
195. Matthias LJ, Yam PT, Jiang XM, Vandegraaff N, Li P, Poumbourios P, Donoghue N,Hogg PJ (2002). "Disulfide exchange in domain 2 of CD4 is required for entry of HIV-1." Nat Immunol **3**(8): 727-32.
196. McFarlin DE, Bellini WJ, Mingioli ES, Behar TN,Trudgett A (1980). "Monospecific antibody to the haemagglutinin of measles virus." J Gen Virol **48**(Pt 2): 425-9.
197. McNeill WH (1976). Plagues and Peoples. Garden City, New York, Anchor Press/Doubleday.
198. Meissner HC, Strebel PM,Orenstein WA (2004). "Measles Vaccines and the Potential for Worldwide Eradication of Measles." Pediatrics **114**(4): 1065-1069.
199. Merrifield B (1997). "Concept and early development of solid-phase peptide synthesis." Methods Enzymol **289**: 3-13.
200. Merrifield RB (1963). "Solid Phase Peptide Synthesis. I. The Synthesis of a Tetrapeptide." J. Am. Chem. Soc. **85**(14): 2149-2154.
201. Merrifield RB (1964). "Solid Phase Peptide Synthesis. II. The Synthesis of Bradykinin." J. Am. Chem. Soc. **86**(2): 304-305.
202. Moreno R, Jiang L, Moehle K, Zurbriggen R, Gluck R, Robinson JA,Pluschke G (2001). "Exploiting conformationally constrained peptidomimetics and an efficient human-compatible delivery system in synthetic vaccine design." Chembiochem **2**(11): 838-43.
203. Morgunkova A,Barlev NA (2006). "Lysine methylation goes global." Cell Cycle **5**(12): 1308-12.
204. Moss WJ, Cutts F,Griffin DE (1999). "Implications of the human immunodeficiency virus epidemic for control and eradication of measles." Clin Infect Dis **29**(1): 106-12.
205. Moss WJ,Griffin DE (2006). "Global measles elimination." Nat Rev Microbiol **4**(12): 900-8.
206. Moss WJ, Scott S, Mugala N, Ndhlovu Z, Beeler JA, Audet SA, Ngala M, Mwangala S, Nkonga-Mwangilwa C, Ryon JJ, Monze M, Kasolo F, Quinn TC, Cousens S, Griffin DE,Cutts FT (2007). "Immunogenicity of standard-titer measles vaccine in HIV-1-infected and uninfected Zambian children: an observational study." J Infect Dis **196**(3): 347-55.

207. Mossong J, Nokes DJ, Edmunds WJ, Cox MJ, Ratnam S,Muller CP (1999). "Modeling the impact of subclinical measles transmission in vaccinated populations with waning immunity." Am J Epidemiol **150**(11): 1238-49.
208. Muller CP, Beauverger P, Schneider F, Jung G,Brons NHC (1995). "Cholera toxin B stimulates systemic neutralizing antibodies after intranasal co-immunization with measles virus." J Gen Virol **76**(6): 1371-1380.
209. Murray GI, Duncan ME, O'Neil P, McKay JA, Melvin WT,Fothergill JE (1998). "Matrix metalloproteinase-1 is associated with poor prognosis in oesophageal cancer." J Pathol **185**(3): 256-61.
210. Na DH, Faraj J, Capan Y, Leung KP,DeLuca PP (2007). "Stability of antimicrobial decapeptide (KSL) and its analogues for delivery in the oral cavity." Pharm Res **24**(8): 1544-50.
211. Naniche D, Varior-Krishnan G, Cervoni F, Wild TF, Rossi B, Rabourdin-Combe C,Gerlier D (1993). "Human membrane cofactor protein (CD46) acts as a cellular receptor for measles virus." J Virol **67**(10): 6025-32.
212. Neidig K-P,Kalbitzer HR (1990). "Improved representation of two-dimensional NMR spectra by local rescaling." Journal of Magnetic Resonance (1969) **88**(1): 155.
213. Norrby E, Kovamees J, Blixenkrone-Moller M, Sharma B,Orvell C (1992). "Humanized animal viruses with special reference to the primate adaptation of morbillivirus." Vet Microbiol **33**(1-4): 275-86.
214. O'Hagen D (2000). Vaccine Adjuvants - Preparation methods and research protocols. New Jersey, Humana Press.
215. Obeid OE, Partidos CD, Howard CR,Steward MW (1995). "Protection against morbillivirus-induced encephalitis by immunization with a rationally designed synthetic peptide vaccine containing B- and T-cell epitopes from the fusion protein of measles virus." J Virol **69**(3): 1420-8.
216. Odean MJ, Frane CM, Van derVieren M, Tomai MA,Johnson AG (1990). "Involvement of gamma interferon in antibody enhancement by adjuvants." Infect Immun **58**(2): 427-32.
217. Oh S, Stegman B, Pendleton CD, Ota MO, Pan CH, Griffin DE, Burke DS,Berzofsky JA (2006). "Protective immunity provided by HLA-A2 epitopes for fusion and hemagglutinin proteins of measles virus." Virology **352**(2): 390-9.
218. Onorato IM, Orenstein WA, Hinman AR, Rogers MF,Koplan JP (1989). "Immunization of asymptomatic HIV-infected children with measles vaccine: assessment of risks and benefits." Med Decis Making **9**(2): 76-83.
219. Osterhaus A, van Amerongen G,van Binnendijk R (1998). "Vaccine strategies to overcome maternal antibody mediated inhibition of measles vaccine." Vaccine **16**(14-15): 1479-81.
220. Ota MO, Moss WJ,Griffin DE (2005). "Emerging diseases: measles." J Neurovirol **11**(5): 447-54.
221. Oyedele OO, Odemuyiwa SO, Ammerlaan W, Muller CP,Adu FD (2005). "Passive immunity to measles in the breastmilk and cord blood of some nigerian subjects." J Trop Pediatr **51**(1): 45-8.
222. Pace CN,Scholtz JM (1998). "A helix propensity scale based on experimental studies of peptides and proteins." Biophys J **75**(1): 422-7.
223. Paik WK, Paik DC,Kim S (2007). "Historical review: the field of protein methylation." Trends in Biochemical Sciences **32**(3): 146.
224. Panum P (1938). "Observations made during the epidemic of measles on the Faroe Islands in the year 1846." Medical Classics **3**: 829-886.

225. Partidos CD, Pizza M, Rappuoli R,Steward MW (1996). "The adjuvant effect of a non-toxic mutant of heat-labile enterotoxin of Escherichia coli for the induction of measles virus-specific CTL responses after intranasal co-immunization with a synthetic peptide." Immunology **89**(4): 483-7.
226. Partidos CD, Ripley J, Delmas A, Obeid OE, Denbury A,Steward MW (1997). "Fine specificity of the antibody response to a synthetic peptide from the fusion protein and protection against measles virus-induced encephalitis in a mouse model." J Gen Virol **78 (Pt 12)**: 3227-32.
227. Pedersen IR, Mordhorst CH, Glikmann G,von Magnus H (1989). "Subclinical measles infection in vaccinated seropositive individuals in arctic Greenland." Vaccine **7**(4): 345-8.
228. Pethe K, Bifani P, Drobecq H, Sergheraert C, Debrie AS, Locht C,Menozzi FD (2002). "Mycobacterial heparin-binding hemagglutinin and laminin-binding protein share antigenic methyllysines that confer resistance to proteolysis." Proc Natl Acad Sci U S A **99**(16): 10759-64.
229. Pietersz GA, Pouniotis DS,Apostolopoulos V (2006). "Design of peptide-based vaccines for cancer." Curr Med Chem **13**(14): 1591-607.
230. Polack FP, Auwaerter PG, Lee SH, Nousari HC, Valsamakis A, Leiferman KM, Diwan A, Adams RJ,Griffin DE (1999). "Production of atypical measles in rhesus macaques: evidence for disease mediated by immune complex formation and eosinophils in the presence of fusion-inhibiting antibody." Nat Med **5**(6): 629-34.
231. Polack FP, Lee SH, Permar S, Manyara E, Nousari HG, Jeng Y, Mustafa F, Valsamakis A, Adams RJ, Robinson HL,Griffin DE (2000). "Successful DNA immunization against measles: neutralizing antibody against either the hemagglutinin or fusion glycoprotein protects rhesus macaques without evidence of atypical measles." Nat Med **6**(7): 776-81.
232. Polack FP, Hoffman SJ, Moss WJ,Griffin DE (2002). "Altered synthesis of interleukin-12 and type 1 and type 2 cytokinesin rhesus macaques during measles and atypical measles." J Infect Dis **185**(1): 13-9.
233. Powell MF, Grey H, Gaeta F, Sette A,Colon S (1992). "Peptide stability in drug development: a comparison of peptide reactivity in different biological media." J Pharm Sci **81**(8): 731-5.
234. Powell MF, Stewart T, Otvos L, Jr., Urge L, Gaeta FC, Sette A, Arrhenius T, Thomson D, Soda K,Colon SM (1993). "Peptide stability in drug development. II. Effect of single amino acid substitution and glycosylation on peptide reactivity in human serum." Pharm Res **10**(9): 1268-73.
235. Powell MF,Newman MJ (1995). Vaccine Design - The subunit and adjuvant approach. New York, Plenum Press.
236. Powers JP,Hancock RE (2003). "The relationship between peptide structure and antibacterial activity." Peptides **24**(11): 1681-91.
237. Prodhomme EJ, Ensch C, Bouche FB, Kaminski T, Deroo S, Seck P, Kirsch G,Muller CP (2007). "Synthesis of 4-[2-aminoethyl(nitrosamino)]-1-pyridin-3-yl-butan-1-one, a new NNK hapten for the induction of N-nitrosamine-specific antibodies." Bioconjug Chem **18**(6): 2045-53.
238. Purcell AW, McCluskey J,Rossjohn J (2007). "More than one reason to rethink the use of peptides in vaccine design." Nat Rev Drug Discov **6**(5): 404-14.
239. Putz MM, Bouche FB, de Swart RL,Muller CP (2003a). "Experimental vaccines against measles in a world of changing epidemiology." Int J Parasitol **33**(5-6): 525-45.

240. Putz MM, Hoebeke J, Ammerlaan W, Schneider S,Muller CP (2003b). "Functional fine-mapping and molecular modeling of a conserved loop epitope of the measles virus hemagglutinin protein." Eur J Biochem **270**(7): 1515-27.
241. Putz MM,Muller CP (2003). "The rationale of a peptide-conjugate vaccine against measles." Vaccine **21**(7-8): 663-6.
242. Putz MM (2004). Ein Peptidkonjugatvakzin gegen Masern - A peptide-conjugate vaccine against measles. Fakultät für Chemie und Pharmazie. Tübingen, Eberhard-Karls-Universität Tübingen. **PhD**.
243. Putz MM, Ammerlaan W, Schneider F, Jung G,Muller CP (2004). "Humoral immune responses to a protective peptide-conjugate against measles after different prime-boost regimens." Vaccine **22**(31-32): 4173-82.
244. Redd SC, Markowitz LE,Katz SL (1999). Measles Vaccine In: Vaccines. 3rd. S. A. Lotkin and W. A. Orenstein, Saunders**:** p222-266.
245. Reuter F (2008). Verdauungsstabilität von Antigenen im Dünndarm - Bedeutung für orale Immunisierungen. Mathematisch-Naturwissenschaftlichen Fakultät. Kiel, Christian-Albrechts-Universität. **PhD**.
246. Rhazes A-B (1748). Treatise on the Smallpox and Measles. London, J Brindley.
247. Rimmelzwaan GF,Osterhaus AD (1995). "A novel generation of viral vaccines based on the ISCOM matrix." Pharm Biotechnol **6**: 543-58.
248. Rudy BJ, Rutstein RM,Pinto-Martin J (1994). "Responses to measles immunization in children infected with human immunodeficiency virus." J Pediatr **125**(1): 72-4.
249. Sagan S, Milcent T, Ponsinet R, Convert O, Tasseau O, Chassaing G, Lavielle S,Lequin O (2003). "Structural and biological effects of a beta2- or beta3-amino acid insertion in a peptide." Eur J Biochem **270**(5): 939-49.
250. Sahin U, Tureci O,Pfreundschuh M (1997). "Serological identification of human tumor antigens." Curr Opin Immunol **9**(5): 709-16.
251. Salunke DB, Hazra BG,Pore VS (2006). "Steroidal conjugates and their pharmacological applications." Curr Med Chem **13**(7): 813-47.
252. Sato AK, Viswanathan M, Kent RB,Wood CR (2006). "Therapeutic peptides: technological advances driving peptides into development." Curr Opin Biotechnol **17**(6): 638-42.
253. Schlereth B, Germann PG, ter Meulen V,Niewiesk S (2000a). "DNA vaccination with both the haemagglutinin and fusion proteins but not the nucleocapsid protein protects against experimental measles virus infection." J Gen Virol **81**(Pt 5): 1321-5.
254. Schlereth B, Rose JK, Buonocore L, ter Meulen V,Niewiesk S (2000b). "Successful vaccine-induced seroconversion by single-dose immunization in the presence of measles virus-specific maternal antibodies." J Virol **74**(10): 4652-7.
255. Schlosser G, Mezo G, Kiss R, Vass E, Majer Z, Feijlbrief M, Perczel A, Bosze S, Welling-Wester S,Hudecz F (2003). "Synthesis, solution structure analysis and antibody binding of cyclic epitope peptides from glycoprotein D of Herpes simplex virus type I." Biophys Chem **106**(2): 155-71.
256. Schnare M, Barton GM, Holt AC, Takeda K, Akira S,Medzhitov R (2001). "Toll-like receptors control activation of adaptive immune responses." Nat Immunol **2**(10): 947-50.
257. Schneerson R, Fattom A, Szu SC, Bryla D, Ulrich JT, Rudbach JA, Schiffman G,Robbins JB (1991). "Evaluation of monophosphoryl lipid A (MPL) as an adjuvant. Enhancement of the serum antibody response in mice to polysaccharide-protein conjugates by concurrent injection with MPL." J Immunol **147**(7): 2136-40.
258. Schomburg D,Reichelt J (1988). "BRAGI: A comprehensive protein modeling program system." Journal of Molecular Graphics **6**(3): 161.

259. Scott S, Moss WJ, Cousens S, Beeler JA, Audet SA, Mugala N, Quinn TC, Griffin DE,Cutts FT (2007). "The influence of HIV-1 exposure and infection on levels of passively acquired antibodies to measles virus in Zambian infants." Clin Infect Dis **45**(11): 1417-24.
260. Seya T, Akazawa T, Tsujita T,Matsumoto M (2006). "Role of Toll-like receptors in adjuvant-augmented immune therapies." Evid Based Complement Alternat Med **3**(1): 31-8; discussion 133-7.
261. Shapiro HM (1993). Practical Flow Cytometry. New York, Wiley-Liss.
262. Shepherd NE, Hoang HN, Abbenante G,Fairlie DP (2005). "Single turn peptide alpha helices with exceptional stability in water." J Am Chem Soc **127**(9): 2974-83.
263. Sjolander A, van't Land B,Lovgren Bengtsson K (1997). "Iscoms containing purified Quillaja saponins upregulate both Th1-like and Th2-like immune responses." Cell Immunol **177**(1): 69-76.
264. Skiadopoulos MH, Surman SR, Riggs JM, Collins PL,Murphy BR (2001). "A chimeric human-bovine parainfluenza virus type 3 expressing measles virus hemagglutinin is attenuated for replication but is still immunogenic in rhesus monkeys." J Virol **75**(21): 10498-504.
265. Spreng S, Gentschev I, Goebel W, Weidinger G, ter Meulen V,Niewiesk S (2000). "Salmonella vaccines secreting measles virus epitopes induce protective immune responses against measles virus encephalitis." Microbes Infect **2**(14): 1687-92.
266. Steer D, Lew R, Perlmutter P, Smith AI,Aguilar MI (2002). "Inhibitors of metalloendopeptidase EC 3.4.24.15 and EC 3.4.24.16 stabilized against proteolysis by the incorporation of beta-amino acids." Biochemistry **41**(35): 10819-26.
267. Stemmer C, Quesnel A, Prevost-Blondel A, Zimmermann C, Muller S, Briand JP,Pircher H (1999). "Protection against lymphocytic choriomeningitis virus infection induced by a reduced peptide bond analogue of the H-2Db-restricted CD8(+) T cell epitope GP33." J Biol Chem **274**(9): 5550-6.
268. Sternberg M (1997). Protein Structure Prediction: A Practical Approach. Oxford, Oxford University Press, Inc.
269. Stetler-Stevenson WG, Aznavoorian S,Liotta LA (1993). "Tumor cell interactions with the extracellular matrix during invasion and metastasis." Annu Rev Cell Biol **9**: 541-73.
270. Steward MW, Stanley CM,Obeid OE (1995). "A mimotope from a solid-phase peptide library induces a measles virus-neutralizing and protective antibody response." J Virol **69**(12): 7668-73.
271. Stittelaar KJ, Boes J, Kersten GF, Spiekstra A, Mulder PG, de Vries P, Roholl PJ, Dalsgaard K, van den Dobbelsteen G, van Alphen L,Osterhaus AD (2000a). "In vivo antibody response and in vitro CTL activation induced by selected measles vaccine candidates, prepared with purified Quil A components." Vaccine **18**(23): 2482-93.
272. Stittelaar KJ, Wyatt LS, de Swart RL, Vos HW, Groen J, van Amerongen G, van Binnendijk RS, Rozenblatt S, Moss B,Osterhaus AD (2000b). "Protective immunity in macaques vaccinated with a modified vaccinia virus Ankara-based measles virus vaccine in the presence of passively acquired antibodies." J Virol **74**(9): 4236-43.
273. Stittelaar KJ, Kuiken T, de Swart RL, van Amerongen G, Vos HW, Niesters HG, van Schalkwijk P, van der Kwast T, Wyatt LS, Moss B,Osterhaus AD (2001). "Safety of modified vaccinia virus Ankara (MVA) in immune-suppressed macaques." Vaccine **19**(27): 3700-9.
274. Stittelaar KJ, de Swart RL, Vos HW, van Amerongen G, Agafonov AP, Nechaeva EA,Osterhaus AD (2002a). "Enteric administration of a live attenuated measles

vaccine does not induce protective immunity in a macaque model." Vaccine **20**(23-24): 2906-12.
275. Stittelaar KJ, de Swart RL, Vos HW, van Amerongen G, Sixt N, Wild TF,Osterhaus AD (2002b). "Priming of measles virus-specific humoral- and cellular-immune responses in macaques by DNA vaccination." Vaccine **20**(16): 2022-6.
276. Stittelaar KJ, Vos HW, van Amerongen G, Kersten GF, Osterhaus AD,de Swart RL (2002c). "Longevity of neutralizing antibody levels in macaques vaccinated with Quil A-adjuvanted measles vaccine candidates." Vaccine **21**(3-4): 155-7.
277. Sundaram R, Lynch MP, Rawale SV, Sun Y, Kazanji M,Kaumaya PT (2004). "De novo design of peptide immunogens that mimic the coiled coil region of human T-cell leukemia virus type-1 glycoprotein 21 transmembrane subunit for induction of native protein reactive neutralizing antibodies." J Biol Chem **279**(23): 24141-51.
278. Suryanarayana K, Baczko K, ter Meulen V,Wagner RR (1994). "Transcription inhibition and other properties of matrix proteins expressed by M genes cloned from measles viruses and diseased human brain tissue." J Virol **68**(3): 1532-43.
279. Tahara M, Takeda M, Seki F, Hashiguchi T,Yanagi Y (2007). "Multiple amino acid substitutions in hemagglutinin are necessary for wild-type measles virus to acquire the ability to use receptor CD46 efficiently." J Virol **81**(6): 2564-72.
280. Tam JP, Wu C-R, Liu W,Zhang J-W (1991). "Disulfide bond formation in peptides by dimethyl sulfoxide. scope and applications." J Am Chem Soc **113**: 6657-6662.
281. Tam JP (1996). "Recent advances in multiple antigen peptides." J Immunol Methods **196**(1): 17-32.
282. Tamashiro VG, Perez HH,Griffin DE (1987). "Prospective study of the magnitude and duration of changes in tuberculin reactivity during uncomplicated and complicated measles." Pediatr Infect Dis J **6**(5): 451-4.
283. Tatsuo H, Ono N, Tanaka K,Yanagi Y (2000). "SLAM (CDw150) is a cellular receptor for measles virus." Nature **406**(6798): 893-7.
284. Theisen DM, Bouche FB, El Kasmi KC, von der Ahe I, Ammerlaan W, Demotz S,Muller CP (2000). "Differential antigenicity of recombinant polyepitope-antigens based on loop- and helix-forming B and T cell epitopes." J Immunol Methods **242**(1-2): 145-57.
285. Tierney R, Beignon AS, Rappuoli R, Muller S, Sesardic D,Partidos CD (2003). "Transcutaneous immunization with tetanus toxoid and mutants of Escherichia coli heat-labile enterotoxin as adjuvants elicits strong protective antibody responses." J Infect Dis **188**(5): 753-8.
286. Torres CA, Yang K, Mustafa F,Robinson HL (1999). "DNA immunization: effect of secretion of DNA-expressed hemagglutinins on antibody responses." Vaccine **18**(9-10): 805-14.
287. Tsukiyama K, Sugiyama M, Yoshikawa Y,Yamanouchi K (1987). "Molecular cloning and sequence analysis of the rinderpest virus mRNA encoding the hemagglutinin protein." Virology **160**(1): 48-54.
288. Tugyi R, Mezo G, Fellinger E, Andreu D,Hudecz F (2005a). "The effect of cyclization on the enzymatic degradation of herpes simplex virus glycoprotein D derived epitope peptide." J Pept Sci **11**(10): 642-9.
289. Tugyi R, Uray K, Ivan D, Fellinger E, Perkins A,Hudecz F (2005b). "Partial D-amino acid substitution: Improved enzymatic stability and preserved Ab recognition of a MUC2 epitope peptide." Proc Natl Acad Sci U S A **102**(2): 413-8.
290. Tyndall JD,Fairlie DP (1999). "Conformational homogeneity in molecular recognition by proteolytic enzymes." J Mol Recognit **12**(6): 363-70.

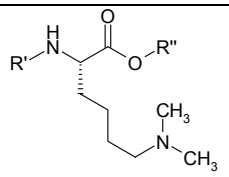
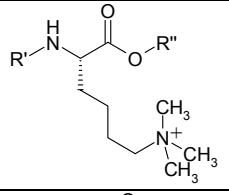
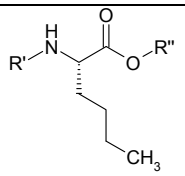
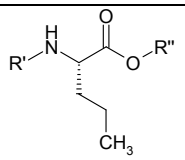
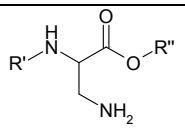
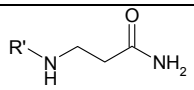
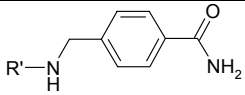
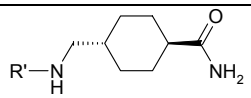
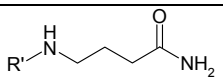
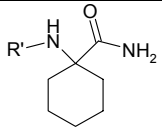
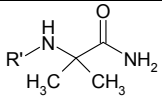
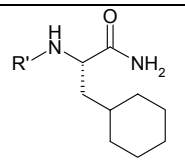
291. van Binnendijk RS, Poelen MC, van Amerongen G, de Vries P,Osterhaus AD (1997). "Protective immunity in macaques vaccinated with live attenuated, recombinant, and subunit measles vaccines in the presence of passively acquired antibodies." J Infect Dis **175**(3): 524-32.
292. Van Regenmortel MH (1999). "Molecular design versus empirical discovery in peptide-based vaccines. Coming to terms with fuzzy recognition sites and ill-defined structure-function relationships in immunology." Vaccine **18**(3-4): 216-21.
293. Van Regenmortel MH (2001a). "Antigenicity and immunogenicity of synthetic peptides." Biologicals **29**(3-4): 209-13.
294. Van Regenmortel MH (2001b). "Pitfalls of reductionism in the design of peptide-based vaccines." Vaccine **19**(17-19): 2369-74.
295. Varsanyi TM, Morein B, Love A,Norrby E (1987). "Protection against lethal measles virus infection in mice by immune-stimulating complexes containing the hemagglutinin or fusion protein." J Virol **61**(12): 3896-901.
296. Verjans GM, Janssen R, UytdeHaag FG, van Doornik CE,Tommassen J (1995). "Intracellular processing and presentation of T cell epitopes, expressed by recombinant Escherichia coli and Salmonella typhimurium, to human T cells." Eur J Immunol **25**(2): 405-10.
297. Villen J, de Oliveira E, Nunez JI, Molina N, Sobrino F,Andreu D (2004). "Towards a multi-site synthetic vaccine to foot-and-mouth disease: addition of discontinuous site peptide mimic increases the neutralization response in immunized animals." Vaccine **22**(27-28): 3523-9.
298. Vogel FR (2000). "Improving vaccine performance with adjuvants." Clin Infect Dis **30 Suppl 3**: S266-70.
299. Vongpunsawad S, Oezgun N, Braun W,Cattaneo R (2004). "Selectively receptor-blind measles viruses: Identification of residues necessary for SLAM- or CD46-induced fusion and their localization on a new hemagglutinin structural model." J Virol **78**(1): 302-13.
300. Wang CY, Chang TY, Walfield AM, Ye J, Shen M, Chen SP, Li MC, Lin YL, Jong MH, Yang PC, Chyr N, Kramer E,Brown F (2002). "Effective synthetic peptide vaccine for foot-and-mouth disease in swine." Vaccine **20**(19-20): 2603-10.
301. Webb A, Aguilar M-i,Purcell A (2003). "Optimisation of peptide-based cytotoxic T-cell determinants using." Int J Pept Res Ther **10**(5): 561.
302. Webster DE, Cooney ML, Huang Z, Drew DR, Ramshaw IA, Dry IB, Strugnell RA, Martin JL,Wesselingh SL (2002). "Successful boosting of a DNA measles immunization with an oral plant-derived measles virus vaccine." J Virol **76**(15): 7910-2.
303. Weidinger G, Ohlmann M, Schlereth B, Sutter G,Niewiesk S (2001). "Vaccination with recombinant modified vaccinia virus Ankara protects against measles virus infection in the mouse and cotton rat model." Vaccine **19**(20-22): 2764-8.
304. Werle M,Bernkop-Schnurch A (2006). "Strategies to improve plasma half life time of peptide and protein drugs." Amino Acids **30**(4): 351-67.
305. Westerfeld N,Zurbriggen R (2005). "Peptides delivered by immunostimulating reconstituted influenza virosomes." J Pept Sci **11**(11): 707-12.
306. Whittle HC, Aaby P, Samb B, Jensen H, Bennett J,Simondon F (1999). "Effect of subclinical infection on maintaining immunity against measles in vaccinated children in West Africa." Lancet **353**(9147): 98-102.
307. WHO. (2005). "Measles Aerosol Project." last accessed January 3, 2009, from http://www.who.int/immunization_delivery/new_vaccines/technologies_aerosol/en/index.html.

308. WHO. (2008). "Measles fact sheet." last accessed January 3, 2009, from http://www.who.int/mediacentre/factsheets/fs286/en/index.html.
309. Wild TF, Bernard A, Spehner D,Drillien R (1992). "Construction of vaccinia virus recombinants expressing several measles virus proteins and analysis of their efficacy in vaccination of mice." J Gen Virol **73 (Pt 2)**: 359-67.
310. Williams DH,Fleming I (1995). Spectroscopic Methods in Organic Chemistry. Berkshire, McGraw-Hill.
311. Wishart DS, Sykes BD,Richards FM (1992). "The chemical shift index: a fast and simple method for the assignment of protein secondary structure through NMR spectroscopy." Biochemistry **31**(6): 1647-51.
312. Wong CP, Okada CY,Levy R (1999). "TCR vaccines against T cell lymphoma: QS-21 and IL-12 adjuvants induce a protective CD8+ T cell response." J Immunol **162**(4): 2251-8.
313. Wuthrich K, Billeter M,Braun W (1983). "Pseudo-structures for the 20 common amino acids for use in studies of protein conformations by measurements of intramolecular proton-proton distance constraints with nuclear magnetic resonance." J Mol Biol **169**(4): 949-61.
314. Wüthrich K (1986). NMR of Proteins and Nucleic Acids. New York, John Wiley & Sons.
315. Wyde PR, Moore-Poveda DK, De Clercq E, Neyts J, Matsuda A, Minakawa N, Guzman E,Gilbert BE (2000a). "Use of cotton rats to evaluate the efficacy of antivirals in treatment of measles virus infections." Antimicrob Agents Chemother **44**(5): 1146-52.
316. Wyde PR, Stittelaar KJ, Osterhaus AD, Guzman E,Gilbert BE (2000b). "Use of cotton rats for preclinical evaluation of measles vaccines." Vaccine **19**(1): 42-53.
317. Yanagi Y, Takeda M, Ohno S,Seki F (2006). "Measles virus receptors and tropism." Jpn J Infect Dis **59**(1): 1-5.
318. Yang K, Mustafa F, Valsamakis A, Santoro JC, Griffin DE,Robinson HL (1997). "Early studies on DNA-based immunizations for measles virus." Vaccine **15**(8): 888-91.
319. Yeung LF, Lurie P, Dayan G, Eduardo E, Britz PH, Redd SB, Papania MJ,Seward JF (2005). "A limited measles outbreak in a highly vaccinated US boarding school." Pediatrics **116**(6): 1287-91.
320. Zasloff M (1987). "Magainins, a class of antimicrobial peptides from Xenopus skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor." Proc Natl Acad Sci U S A **84**(15): 5449-53.
321. Zauner W, Lingnau K, Mattner F, von Gabain A,Buschle M (2001). "Defined synthetic vaccines." Biol Chem **382**(4): 581-95.
322. Zhang L, Parente J, Harris SM, Woods DE, Hancock RE,Falla TJ (2005). "Antimicrobial peptide therapeutics for cystic fibrosis." Antimicrob Agents Chemother **49**(7): 2921-7.
323. Zhu Y, Rota P, Wyatt L, Tamin A, Rozenblatt S, Lerche N, Moss B, Bellini W,McChesney M (2000). "Evaluation of recombinant vaccinia virus--measles vaccines in infant rhesus macaques with preexisting measles antibody." Virology **276**(1): 202-13.
324. Zhu YD, Fennelly G, Miller C, Tarara R, Saxe I, Bloom B,McChesney M (1997). "Recombinant bacille Calmette-Guerin expressing the measles virus nucleoprotein protects infant rhesus macaques from measles virus pneumonia." J Infect Dis **176**(6): 1445-53.

325. Ziegler D, Fournier P, Berbers GA, Steuer H, Wiesmuller KH, Fleckenstein B, Schneider F, Jung G, King CC,Muller CP (1996). "Protection against measles virus encephalitis by monoclonal antibodies binding to a cystine loop domain of the H protein mimicked by peptides which are not recognized by maternal antibodies." J Gen Virol **77 (Pt 10)**: 2479-89.

Annexes

1. Table of non-natural amino acids

single letter code	common abbreviation	full name	structure
K*	$K(Me)_2$	di-methylated lysine	
K♦	$K(Me)_3$	tri-methylated lysine	
L*	Nle	norleucine	
V*	Nva	norvaline	
D*	dap	diamino propionic acid	
	β-Ala	β-alanine	
	pamba	4-(aminomethyl)benzoic acid	
	t-amcha	trans-4-(aminomethyl)cyclohexane carboxylic acid	
	gaba	γ-aminobutyric acid	
	achca	1-amino-cyclohexane carboxylic acid	
B	aib	α-aminoisobutyric acid	
	cha	β-cyclohexyl-L-alanine	

R' = N-terminal peptide sequence extension
R'' = C-terminal peptide sequence extension

2. Table of synthesised peptides

code	*C-terminally extended peptides*		
2711	KGQQACKGKIQALCENlv	**2721**	KGQQACKGKIQALCENPEWA
2712	KGQQACKGKIQALCENfa	**2722**	KGQQACKGKIQALCENPEWa
2715	KGQQACKGKIQALCENv	**2723**	KGQQACKGKIQALCENva
2716	KGQQACKGKIQALCEN(achca)	**2724**	KGQQACKGKIQALCENa
2717	KGQQACKGKIQALCEN(t-amcha)	**2725**	KGQQACKGKIQALCENf
2718	KGQQACKGKIQALCEN(aib)	**2726**	KGQQACKGKIQALCEN **wt-HNE**
2720	KGQQACKGKIQALCENPEWAa		

code	*internally and N-terminally modified peptides*		
2729	G_4K_2KGQQACKGKIQALCEN	**2735**	KGQQACKGNIQALCEN
2730	NH_2-G_4K_2KGQQACKGKIQALCEN	**2736**	KGQQACKGSIQALCEN
2731	G_2KGQQACKGKIQALCEN	**2737**	KGQQACKGHIQALCEN
2732	NH_2-G_2KGQQACKGKIQALCEN	**2738**	KGQQACKGQIQALCEN
2733	kGQQACKGKIQALCEN	**2739**	GQQACKGKIQALCEN
2734	KGQQACKGAIQALCEN	**2726**	KGQQACKGKIQALCEN **wt-HNE**

code	*internally and C-terminally modified peptides*		
2740	KGQQACKGAIQALCENPEWAa	**2748**	KGQQACRGAIQALCEN
2741	KGQQACKGAIQALCENPEWa	**2749**	KGQQACkGKIQALCEN
2742	KGQQACKGAIQALCENva	**2750**	KGQQACkGAIQALCEN
2743	KGQQACKGAIQALCENaa	**2751**	KGQQACKGAIQALCEN
2744	KGQQACKGAIQALCENv	**2752**	KGQQACKGKIQALCEN **wt-HNE**
2745	KGQQACKGAIQALCENa	**2753**	GQQACKGAIQALCEN
2747	KGQQACRGKIQALCEN		

code	*Further improved internally and C-terminally modified peptides*		
2775	KGQQACL*GAIQALCENPEWa	**2794**	D*GQQACKGAIQALCEN
2776	KGQQACV*GAIQALCENPEWa	**2795**	KGQQACLGAIQALCEN
2777	KGQQACK*GAIQALCENPEWa	**2796**	KGQQACL*GKIQALCEN
2780	D*GQQACKGAIQALCENPEWa	**2797**	KGQQACV*GKIQALCEN
2781	KGQQACLGAIQALCENPEWa	**2798**	KGQQACK*GKIQALCEN
2782	KGQQACL*GAIQALCENa	**2801**	D*GQQACKGKIQALCEN
2783	KGQQACV*GAIQALCENa	**2802**	KGQQACLGKIQALCEN
2784	KGQQACK*GAIQALCENa	**2805**	KGQQACK♦GAIQALCENPEWa
2787	D*GQQACKGAIQALCENa	**2807**	KGQQACK♦GAIQALCENa
2788	KGQQACLGAIQALCENa	**2809**	KGQQACK♦GAIQALCEN
2789	KGQQACL*GAIQALCEN	**2811**	KGQQACK♦GKIQALCEN
2790	KGQQACV*GAIQALCEN	**2833**	KGQQACKGKIQALCEN **wt-HNE**
2791	KGQQACK*GAIQALCEN		

L* = Nle, V* = Nva, D* = dap, K* = di-methylated Lys, K♦ = tri-methylated Lys.
Changes compared to wt-HNE-peptide sequence are marked red. Peptides used for immunisations are highlighted yellow.

Publications and conference participations

Publications:

Bechet, T., Prodhomme, E.J., Bruns, K., Wray, V., Muller C.P., Structure of HNE-peptide mutants. *In preparation*

Bechet, T., Prodhomme, E.J., Bade, S., Muller C.P., Stability, antigenicity and immunogenicity of HNE-peptide mutants. *In preparation*

Schote, A., Bechet, T., Pirrotte, P., Turner J.D., Muller, C.P., Characterisation of a new DNA binding domain deficient Chicken Ovalbumin Upstream Promoter- Transcription Factor II Δisoform in the human brain. *In preparation*

Bodé, C.A., Bechet, T., Muller, C.P., Madder, A., Design, Synthesis and Biological Evaluation of a Cyclic Bile Acid-Peptide Conjugate: Towards the Conformational Mimicry of the Measles Virus HNE Loop. *submitted to Organic and Biomolecular Chemistry*

Conference participations - Talks:

SarLorLux Meeting, Nancy, France, September 13, 2006, "Structure and stability of a pre-vaccine peptide based on the haemagglutinin noose epitope of measles virus"

ArbeitsKreis (AK) Vakzine Meeting, Borstel, Germany, June 12, 2008, "Structure, stability and immunogenicity of a pre-vaccine peptide based on the hemagglutinin noose epitope of measles virus"

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