Ein Peptidkonjugatvakzin gegen Masern

A Peptide-Conjugate Vaccine against Measles

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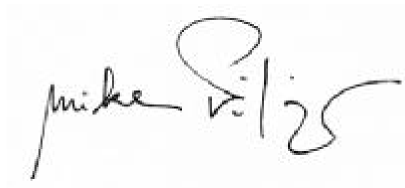

List of Abbreviations

Abu	amino butyric acid (also ‘B’)
AFU	arbitrary fluorescence units
Alum	aluminium hydroxide gel (also ‘alhydrogel’)
APC	antigen presenting cell
ATCC	American type culture collection
B	amino butyric acid (also ‘Abu’)
BCE	B-cell epitope
BCG	Bacille Calmette-Guerin
BSA	bovine serum albumin
Boc	*tert*-butoxycarbonyl
BTC	bis-(trichloromethyl)carbonate (also ‘triphosgene’)
CDC	Centers for Disease Control and Prevention
CDV	canine distemper virus
CFA	complete Freund’s adjuvant
CFF	consistent forcefield
95% CI	95% confidence intervals
CPV	canine parvovirus
CRM197	‘crossreactive material’, nontoxic mutant of diphtheria toxin
ct	cholera toxin
ctb	nontoxic cholera toxin B subunit
CTL	cytotoxic T-lymphocyte
CVFF	consistent valence forcefield
DCM	dichloromethane
DIC	1,3-diisopropylcarbodiimide
DIPEA	*N,N*-diisopropylethylamine
DMEM	Dulbecco’s modified Eagle’s medium

DMF	*N,N*-dimethylformamide
DMSO	dimethyl sulfoxide
DNA	desoxyribonucleic acid
dt	diphtheria toxoid
DT	HNE-peptide-diphtheria-toxoid conjugate
DTP	diphtheria-tetanus-pertussis vaccine
DTT	1,4-dithiothreitol
EBV	Epstein-Barr virus
EDC	*N*-ethyl-*N*'-[(3-dimethyl-amino)propyl] carbodiimide hydrochlorid
EDT	1,2-ethanedithiol
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
EPI	Expanded Programme of Immunization
EPT	end point titer
ESI	electron spray ionization
F	fusion protein
FACS	fluorescence-activated cell sorter
FBS-HI	heat-inactivated fetal bovine serum
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
Fmoc	9-fluorenylmethoxycarbonyl
FMDV	foot and mouth disease virus
H	hemagglutinin protein
HBSS	HEPES buffered salt solution
HIV	human immunodeficiency virus
HEPES	*N*-(2-hydroxyethyl) piperazine-*N*'-(2-ethanesulfonic acid)
HNE	hemagglutinin noose epitope
HOBt	1-hydroxybenzotriazole
HPLC	high performance liquid chromatography
IC50%	50%-inhibiting concentration
IFA	incomplete Freund's adjuvant
IFN	interferon
IgG	immunoglobulin G
IgG-AP	IgG-alkaline phosphatase

IL	interleukin
IgM	immunoglobulin M
i.d.	intradermal
i.p.	intraperitoneal
i.m.	intramuscular
i.n.	intranasal
ISCOM	immune stimulating complex
L	large protein
LAV	live-attenuated vaccine
LNS	Laboratoire National de Santé
lt	heat-labile enterotoxin
ltb	nontoxic heat-labile enterotoxin B subunit
M	matrix protein
mAb	monoclonal antibody
MES	2-(*N*-morpholino) ethanesulfonic acid
MHC	major histocompatibility complex
mRNA	messenger RNA
MS	mass spectrometry
MV	measles virus
MVA	modified vaccinia virus Ankara
MW	molecular weight
N	nucleoprotein
NALT	nasal-associated lymphoid tissue
NE	neutralizing epitope
NHS	*N*-hydroxy-succinimide
NK	natural killer cell
NMP	*N*-methylpyrrolidone
OD	optical density
ova	ovalbumin
OVA	HNE-peptide-ovalbumin conjugate
P	phosphoprotein
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PSG	penicillin-streptomycin-L-glutamin

PRN	plaque reduction neutralization
RNA	ribonucleic acid
RP-HPLC	reverse phase high performance liquid chromatography
rpm	rounds per minute
RPMI	Roswell Park Memorial Institute
RU	resonance units
RSV	respiratory syncytial virus
RT	room temperature
SD	standard deviation
SLAM	signaling lymphocytic activation molecule (also ‘CDw150’)
SPDP	*N*-succinimidyl 3-(2-pyridyldithio) propionate
SPPS	solid phase peptide synthesis
SPR	surface plasmon resonance
SSPE	subacute sclerosing panencephalitis
TCE	T-cell epitope
TCI	transcutaneous immunization
$TCID_{50}$	tissue culture infective doses
TCR	T-cell receptor
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TNF	tumor necrosis factor
tt	tetanus toxoid
TT	HNE-peptide-tetanus-toxoid conjugate
UNICEF	United Nations Children's Fund
UV	ultraviolet
WHO	World Health Organisation

L-amino acids are displayed with capital letters
using the one-letter-code and the three-letter-code

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Abstract

Today there is a large consensus that the development of new measles vaccines cannot wait until in the final stage of an eventual WHO measles elimination program when its need will become urgent. Strategies for vaccination during early infancy and revaccination of adults include peptide based conjugate and recombinant polyepitope vaccines for potentially non-invasive routes of administration such as the nose or the skin. The inclusion of peptide-based antigens in an experimental subunit vaccine implies the careful evaluation of its structural characteristics and the biological significance thereof. Although peptides are conformationally very unstable, studies of antigenicity and immunogenicity paired with molecular modeling iteratively shape the understanding of peptide-antibody interactions. Here we describe such an approach for a peptide epitopes of the measles virus hemagglutinin protein.

Neutralizing and protective monoclonal antibodies (mAbs) were used to fine-map the highly conserved hemagglutinin noose epitope (H379-410, HNE) of the measles virus. Short peptides mimicking this epitope were previously shown to induce virus-neutralizing antibodies. The epitope contains three cysteine residues, two of which (Cys386 and Cys394) form a disulfide bridge critical for antibody binding. Substitution and truncation analogues revealed four residues critical for binding (Lys387, Gly388, Gln391 and Glu395) and suggested the binding motif X7C[KR]GX[AINQ]QX2CEX5 for three distinct protective mAbs. This motif was found in more than 90% of the circulating wild-type measles viruses and its presence in wild-type strains correlates with their neutralization by anti-HNE mAbs. An independent molecular model of the core epitope predicted an amphiphilic loop

displaying a remarkably stable and rigid loop conformation. The three hydrophilic contact residues Lys387, Gln391 and Glu395 pointed on the virus towards the solvent-exposed side of the planar loop and the permissive hydrophobic residues Ile390, Ala392 and Leu393 towards the solvent-hidden side of the loop, precluding antibody binding. The high affinity (Kd = 7.60 nM) of the mAb BH216 for the peptide suggests a high structural resemblance of the peptide with the natural epitope and indicates that most interactions with the protein are also contributed by the peptide. Improved peptides designed on the basis of these findings induced sera that crossreacted with the native measles virus hemagglutinin protein and protected against an intracranial challenge with a rodent-neuroadapted MV strain. Interestingly, and targeted the same critical residues as defined in the HNE binding motif, although with less stringency than the mAbs. Stable backbone conformation and side chain orientation of critical contact residues is essential for reliable crossreactive immunogenicity. Indeed, the orientation of its critical side chains could not be altered even in a chemically modified backbone that was N-methylated at positions A392 or L393 and conjugated peptides continued to induce hemagglutinin crossreactive sera.

For successful inclusion of a peptide-conjugate in vaccine formulations and schedules designed for early infancy, its peptide-specific immunogenicity has to be evaluated carefully in the presence of prior immunity or passively acquired antibodies against the carrier protein as both factors are known to interfere often with subsequent infant immunization, although with changing degrees of clinical incidence. In addition, its compatibility with a succeeding administration with the live attenuated virus has to be assessed.

Mice pre-immunized with the free carrier proteins diphtheria toxoid or tetanus toxoid developed after two succeeding injections with the peptide-conjugate similar protective antibody levels against the peptide than animals without prior immunization with the carrier protein. As expected, no epitopic suppression of the anti-peptide response occurred, presumably because of efficient activation of carrier-specific T cells. However, passive transfer of anti-carrier or anti-peptide conjugate antibodies decreased peptide-specific antibody titers significantly and the antibody response against the homologous carrier protein was also reduced. Nonetheless, the suppressive effect of passive antibodies can be overcome by additional boosting.

These results confirm previous studies pointing out that the response to a hapten is enhanced after active priming with the carrier protein and that the hapten-specific antibody response is mainly suppressed by passively acquired antibodies. These findings stress the importance of the memory T-cell immunity for the immunogenicity of peptide-conjugate vaccines, as carrier-specific CD4+ helper cells govern the activation and clonal expansion of peptide- and carrier-specific B cells. Moreover, prior antibodies against the HNE epitope does not preclude an efficient active antibody response against other epitopes of the MV, underlining thus that a immunization with a peptide-conjugate is compatible with a subsequent administration of the live attenuated measles vaccine. The possibility to prime or boost using a chimeric peptide containing a promiscuous T-cell epitope collinearly synthesized to the HNE peptide B-cell epitope was also investigated, but none of the studied schedules lead to an improved anti-peptide response and the titers were even lowered in comparison to the immunization with the conjugate.

Peptide-conjugates induce also high titers of protective antibodies after intranasal administration. Moreover, peptide-conjugates efficiently induced peptide-specific antibodies after application on shaved, intact skin. Needle-free delivery of a peptide-conjugate vaccine against measles via non-invasive routes such as the nose or the bare skin would largely increase its safety and compliancy for early infancy use. An intranasal spray or a transcutaneous administration via a patch would be compatible with self-administration and would thus represent a major advantage for revaccination of adults with waning immunity to measles.

Zusammenfassung

Es besteht heute ein breiter Konsens, dass die Entwicklung eines neuen Impfstoffs gegen Masern nicht bis zur Endphase eines potentiellen Eliminierungsprogramms der WHO aufgeschoben werden kann, wenn sein Einsatz unmittelbar dringend wird. Impfstrategien für die frühe Kindheit und das Revakzinieren von Erwachsenen sehen den Gebrauch von Peptidkonjugat- und rekombinanten Polyepitopvakzinen vor, welche unter anderem auch für nicht-invasive Verabreichungswege wie die Nase oder die Haut ausgelegt sind. Die Formulierung eines Peptidantigens in einem experimentellen Impfstoff setzt eine detailgetreue Beschreibung seiner strukturellen Chrakteristika und deren biologischer Signifikanz voraus. Obwohl Peptide aus konformationeller Sicht sehr unstabile Gebilde sind, können Antigenizitäts- und Immunogenizitätsstudien, unterstützt durch molekulare Modellisierungsalgorithmen, viel zum Verständnis von Peptid-Antikörper-Wechselwirkungen beitragen. Diese Arbeit beschreibt eine solche Studie über ein Peptidepitop des Masernvirus Hemagglutininproteins.

Neutralisierende und protektive monoklonale Antikörper wurden benutzt für die Feinkartierung des konservierten *Hemagglutinin Noose Epitop* (H379-410, HNE) des Masernvirus. Es konnte in früheren Arbeiten gezeigt werden, dass kurze Peptidmimetika dieses Epitops neutralisierende Antikörper induzieren. Das Epitop enthält drei Cysteinreste, von denen zwei (Cys386 und Cys394) eine Disulfid-Brücke bilden, welche ausschlaggebend für die Antikörperbindung ist. Mit Hilfe einer Bibliothek, bestehend aus substituierten und verkürzten Epitopanaloga, konnten 4 Aminosäurereste (Lys387, Gly388, Gln391 und Glu395) eingegrenzt werden, welche kritisch für die Wechselwirkung sind und im Bindungsmotif $X_7C[KR]GX[AINQ]QX_2CEX_5$ für 3 unterschiedliche protektive Antikörper definiert werden. Dieses Motif kommt in mehr als 90% der zirkulierenden Widtypviren vor und dessen Präsenz im Wildtypstamm korreliert mit Virusneutralisation durch monoklonale HNE-spezifische Antikörper. Unabhängig davon wurde die Konformation der Epitopkernsequenz mit Hilfe von Modellisierungsalgorithmen

berechnet und eine amphiphile Schleife mit aussergewöhnlich stabiler und verwindungssteifer Struktur wurde gefunden. Die 3 hydrophilen Kontaktreste Lys387, Gln391 und Glu395 befinden sich an der exponierten Oberfläche der kompakten, platten Epitopschleife auf dem Virusprotein im Gegensatz zu den ersetzbaren, hydrophoben Resten Ile390, Ala392 und Leu393, welche auf der wasserunzugänglichen Seite zu finden und für die Antikörperwechselwirkungen nicht zu erreichen sind. Die hohe Affinität (K_d = 7.60 nM) des monoklonalen Antikörpers BH216 für das Peptid weist auf eine hohe Übereinstimmung mit der Originalstruktur des Epitops auf dem H-Protein hin und bedeutet, dass die meisten Antikörperwechselwirkungen im Proteinepitop auch im Peptidepitop stattfinden. Optimisierte Peptidepitopsequenzen konnten in ein Peptidkonjugat implementiert werden und induzierten Antikörper welche mit dem nativen Masern-H-Protein kreuzreagierten und in einem Mausmodell gegen eine lethale Infektion mit einem neuroadaptierten Masernvirus schützten. Interessanterweise wiesen diese induzierten polyklonalen Seren ein ähnliches - obwohl weniger genaues - Bindungsmotif auf als die monoklonalen Antikörper. Stabile Konformation des Peptidrückgrats und Orientierung der kritischen Seitenketten sind unabdingbare Voraussetzungen für eine zuverlässige kreuzreaktive Immunogenizität bei einem Peptidimpfstoff. Die Orientierung der so wichtigen Reste konnte auch in einem Peptid mit chemisch modifiertem, *i.e.* N-methyliertem, Backbone nicht verändert werden und konjugierte Peptide induzierten weiterhin H-Protein-kreuzreagierende Antikörper.

Eine erfolgreiche Entwicklung eines Peptidimpfstoffes und dessen dazugehörigen Immunisierungszeitplan für das frühe Kindesalter setzt eine sorgfältige Auswertung der peptidspezifischen Immunogenizität bei bestehender Immunität oder passiv erworbener Antikörper gegen das Trägerprotein im Konjugat voraus. Es ist bekannt, dass beide Faktoren eine humorale Antwort entschieden beeinflussen, obgleich in variierendem Masse an klinischer Signifikanz. Darüber hinaus sollte gewährleistet werden, dass mehrere Immunisierungen mit einem Peptidkonjugat kompatibel sind mit einer späteren Vakzinierung mit dem Lebendmasernimpfstoff.

Nach zwei Injektionen mit einem Peptidkonjugat, basierend auf Diphtheria- oder Tetanus-Toxoid als Trägerprotein, entwickelten Mäuse gleiche peptid- und trägerspezifische Antikörpertiter, unabhängig von einer vorherigen aktiven

Immunisierung mit dem Trägerprotein. Wie erwartet, findet keine sogenannte *epitopische Suppression* statt; vermutlich wegen einer vorherigen effizienten Aktivierung einer trägerspezifischen T-Zell Antwort. Die Päsenz passiver peptid- oder trägerspezifischer Antikörper hemmte jedoch eine humorale Antwort sowohl gegen das Peptid wie auch gegen das Trägerprotein in unspezifischer Weise, aber in einem Umfang abhängig von der Dosis der passiven Antikörper. Nichtsdestotrotz kann dieser suppressive Effekt durch zusätzliche *Booster*-Injektionen überwunden werden. Diese Resulte stimmen mit vorherigen Studien überein, welche einerseits die stimulierende Rolle einer vorherigen aktiver Trägerimmunisierung hervorheben und andererseits eine Inhibition einer Antikörper Antwort hauptsächlich auf die Präsenz passiver Antikörper zurückführen. Die Resultate aus unserer Studie unterstreichen die Wichtigkeit von vorhandenen *Memory*-T-Zellen für die Immunogenizität in Peptidkonjugatimpfstoffen, da trägerspezifische $CD4^+$ Helferzellen die Aktivierung und die klonale Expansion von peptid- und trägerspezifischen B-Zellen steuern. Darüber hinaus konnte gezeigt werden, dass peptidspezifische Antikörper die Induktion von Antikörper gegen andere Masernepitope nicht inhibiert, was soviel bedeutet wie, dass eine vorherige Immunisierung mit Peptidkonjugat tatsächlich kompatibel ist mit einer darauffolgenden Injektion mit Lebendimpfstoff. Auch wurden Möglichkeiten untersucht, die peptidspezifische humorale Immunantwort durch vorherige oder spätere Injektionen mit schimärischen Peptiden, welche T- und B-Zell-Epitope enthalten, zu verbesseren. Bei keiner der angewandten Strategien konnten jedoch die erreichten Antikörpertiter mit einer Immunisierung mit dem Peptidkonjugat alleine übertroffen werden.

Nicht nur in einer intraperitonealen, sondern auch in einer intranasalen Verabreichung induzierten diese Peptidkonjugate hohe und protektive peptidspezifische Antikörpertiter. Überdies waren diese Konjugate in der Lage nach einfacher Applikation auf intakte, rasierte Haut gute peptidspezifische Antikörpertiter zu induzieren. Eine spritzenfreie Verabreichung auf nicht-invasiven Wegen - wie in diesem Fall die Nase oder die nackte Haut - würde die Sicherheit und die Akzeptanz von Peptidimpfstoffen für die frühe Kindheit entscheidend vergrössern. Ein nasaler Spray oder die transkutaneale Aufbringung durch ein Pflaster wären für eine Selbstverabreichung gut geeignet und würden auch eine Möglichkeit bieten zur Revakzinierung von Erwachsen mit schwindender Masernimmunität.

Chapter 1: Introduction

Vaccination with the current live attenuated measles vaccine is one of the most successful and cost-effective medical interventions. Global measles incidence and mortality decreased dramatically after the introduction of routine vaccination. A single dose of the live-attenuated vaccine administered at 9-15 months of age provides long-lasting protection. However, as a result of persisting maternal antibodies and immaturity of the infant immune system, this vaccine is poorly immunogenic in children < 9 months old. Immunity against the live vaccine is less robust than natural immunity and protection less durable. There may also be some concern about (vaccine) virus spread during the final stage of an eventual measles eradication program. Opinions may differ with respect to the potential threat that some of these concerns may be to the WHO goal of measles elimination, but there is a consensus that the development of new measles vaccines cannot wait. Indeed, to interrupt virus transmission, 95% of a population must be immune. Candidate vaccines are based on viral or bacterial vectors expressing recombinant viral proteins, naked DNA, ISCOMs or synthetic peptides mimicking neutralizing epitopes. Selected peptides corresponding to sequential, subdominant B-cell epitopes of measles virus glycoproteins have been shown to induce neutralizing and protective antibodies even in the presence of whole virus antibodies. Similar to polysaccharide-conjugate vaccines, which are highly effective in infants a peptide-conjugate vaccine against measles is proposed. Such a vaccine induces carrier-specific T cells, avoiding measles-specific Th2 cells associated with the risk of atypical measles. This work is dedicated to discuss the rationale of such a strategy and its future potential.

1. The measles virus

Measles is a highly infectious childhood disease. Today, still an estimated 40 million annual cases contract the disease with an outcome of about 800.000 fatal complications (CDC, 2003). The virus is spreading through airborne transmission and initially affects the respiratory tract from where it disseminates to the draining lymphnodes and into the blood stream.

There is no natural animal reservoir for measles, although the virus can be transmitted to laboratory macaque monkeys (Goldberger et Anderson, 1911). Measles virus (MV) is a member of the *Morbillivirus* genus belonging to the *Paramyxoviridae* family. Other members of the *Morbillivirus* genus include zoonotic viruses like rinderpest, peste-des-petits-ruminants, canine distemper, phocine distemper, dolphin distemper and porpoise distemper virus and whale morbillivirus (Figure 1.1). It is commonly believed that MV evolved in a habitat in which humans and cattle lived closely together (Norrby et al., 1992).

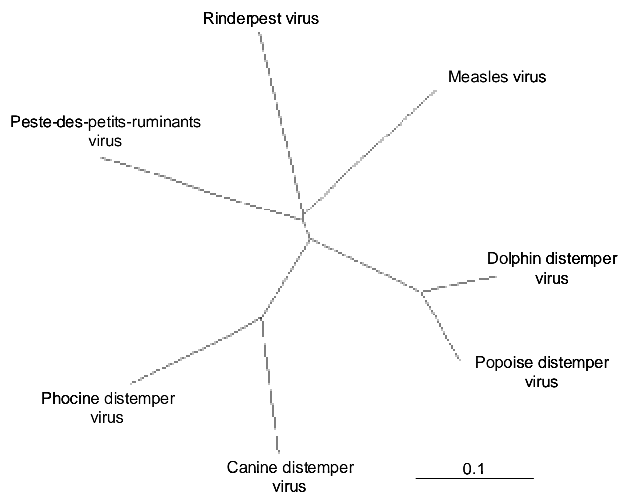

Figure 1.1: The *morbillivirus* genus

Genetic relationships between morbilliviruses are based on the comparison of the nucleotide sequences of their N genes (phylogenetic tree was performed courtesy of J. Kremer)

2. Genes and Proteins

MV is a monotypic virus with a genome made of a linear, single stranded, non-segmented, negative-sense RNA molecule of approximately 15900 ribonucleotides, containing six ORFs flanked by a 3'-leader and a 5'-trailer sequence. Its genes encoding 6 structural proteins, the nucleocapsid (N), the phospho protein (P), the large protein (L), the matrix protein (M), the fusion protein (F) and the hemagglutinin

protein (H) are shared by all morbilliviruses (Figure 1.2). Additionally, the P gene encodes for another two non-structural components, the C and the V protein.

The N protein is the first and most abundantly expressed among the MV proteins. It is synthesized on free ribosomes and folded in the cytoplasm, where it self-assembles into the nucleocapsid packaging the viral RNA molecule. This structure is the template for replication and transcription and complexes with the L and P proteins. Because of its omnipresence in infected cells, the N protein is a major antigen in the immune response against MV, accounting for the most abundant and most rapidly induced antibodies (Graves et al., 1984). Absence of N-specific antibodies is a reliable indicator of seronegativity. However, due to its cytoplasmic synthesis and its unavailability on the MV surface, no neutralizing antibodies are generated against the N protein. Indeed, its intra-cytoplasmic function and origin induce MHC class I responses and numerous CTL-epitopes of the N protein have been described (Nanan et al., 1995).

As most RNA viruses, MV uses its own RNA-dependant RNA polymerase lacking proof-reading activity. The viral polymerase derives from the L protein, which clusters together with the P protein on the ribonucleoprotein complex formed by the N protein with the RNA molecule. It is present in low amounts in the infected cells and its sequence is highly homologous with the polymerase sequences of other negative stranded viruses (Blumberg et al., 1988). Hitherto, only little is known about the its immunological role, but it is probably minimal.

The P protein is a phosphorylated protein also participating in the replication complex. It is very sensitive to proteolysis and is only found in small quantities in the packaged virus in comparison to its overabundance in infected cells. As stated above, the P gene codes for the C and V proteins, which regulate the shift from mRNA transcription to viral replication (Escoffier et al., 1999). The C and V proteins have also been proposed to function as virulence factors (Patterson et al., 2000).

The viral envelope is constituted of three components: the M protein and two transmembrane glycoproteins, namely F and H. The M protein is associated with the nucleocapsid and with the inner layer of the cytoplasmic membrane in infected cells. It exhibits several conserved hydrophobic regions interacting with the transmembrane regions of the F and H proteins, apparently stabilizing the membrane environment and thus coordinating virion maturation and budding (Tyrell and Ehrnst, 1979). However, when the M protein is associated to the viral ribonucleoprotein complex, it inhibits

transcription (Suryanarayana et al., 1994). The immunological role of the M protein is low and very little antibodies are elicited against this protein during normal MV infection, except in atypical measles (Graves et al., 1984, Machamer et al., 1980), a severe, aggravated form of the disease (see section 6.3.).

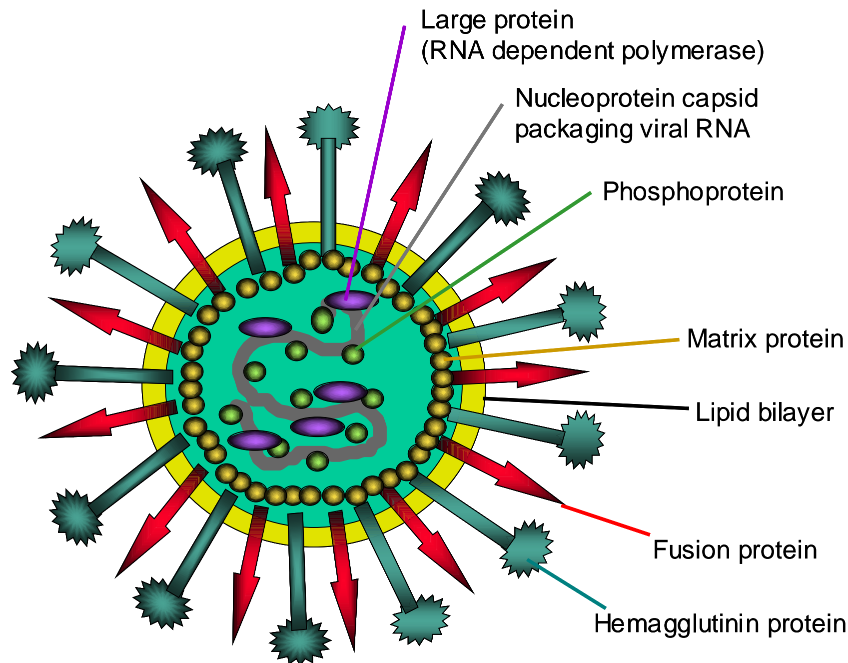

Figure 1.2: Schematic representation of measles virus.

The ribonucleoprotein complex consists of a nucleocapsid (formed by the nucleoprotein) packaging the viral RNA molecule and clustering with the Large protein (RNA dependent polymerase) and the Phosphoprotein. The envelope components comprise the Matrix protein, a lipid bilayer and two transmembrane surface glycoproteins, the Fusion and the Hemagglutinin proteins.

The F protein, one of the two surface glycoproteins, is the most conserved MV protein. It is a type I membrane protein anchored in the virus membrane via a C-terminal hydrophobic domain. The F protein is synthesized as a biologically inactive precursor protein, subsequently glycosylated and tetramerized. The glycosylation step is required for further processing and translocation towards the membrane (Sato et al., 1988). During its transport to the surface, disulfide-linked F_1 and F_2 subunits are generated in the *trans*-Golgi compartment after cleavage by a protease of the host cell (Hardwick and Bussel, 1978). A highly conserved hydrophobic region at the N-terminus of the F_1 is suggested to mediate virus fusion with the cell, prior to virus

entry. This process can be precluded by F-specific antibodies. Virus-cell fusion occurs preferentially in presence of the H protein, interacting also with the F_1 subunit (Schmid et al., 1992; Taylor et al., 1991; Wild et al., 1991). The F protein is easily accessible to humoral immunity and induces neutralizing antibodies, although to a much lesser extent than the H protein. In addition, a large amount of $CD4^+$ T-cell epitopes indicating an MHC class II restricted response were detected on the F protein (Partidos and Stewart, 1990, 1992; Partidos et al., 1991; Obeid and Stewart 1994; Muller et al., 1995b, 1996a; Steward et al., 1995; Obeid et al., 1995; Atabani et al., 1997).

The H protein is a type II transmembrane glycoprotein and consists of 617 amino acids. Its N-terminal hydrophobic domain acts as well as a signalling sequence targeting the protein to the endoplasmic reticulum, where it is folded and dimerized (Bellini et al., 1983; Malvoisin et Wild, 1993, 1994), and as a transmembrane anchor (Hummel and Bellini, 1995). Similar to the F protein, its glycosylation plays an important role in the folding, dimerization and transport to the cell surface, where the H protein occurs as disulfide-bridged homodimers, its biologically active form (Alkhatib and Briedis, 1986; Hu and Norrby, 1994). These dimers have been shown to associate into tetramers (Plemper et al., 2000; Schneider-Schaulies et ter Meulen, 2002). Its primary function is the initiation of the infection by enabling the virus to bind to the receptor on the target cell (Varsanyi et al., 1984). CD46 was initially identified as the receptor for H-mediated cell binding (Naniche et al., 1993), but this only accounts for Edmonston derived MV strains (Minagawa et al., 2001). Recently, SLAM has been found to serve as a second receptor for entry and MV wild-type strains use this latter one (Tatsuo et al., 2000a; Minagawa et al., 2001). Subsequently to receptor binding, an H protein tetramer interacts with a F protein trimer to govern the fusion of the virus with the host cell membrane (Schmid et al., 1992; Wild et al., 1991; Schneider-Schaulies et ter Meulen, 2002). In the extracellular domain of the H protein, 13 highly conserved cysteines form intra- and intermolecular disulfide bonds, crucial for maintaining the conformational structure of the protein and thus preserving its antigenic characteristics (Hu and Norrby, 1994). The H protein is highly immunogenic and induces the major part of MV-neutralizing and protective antibodies (McFairlin et al., 1980; Giraudon et Wild, 1985). Numerous B-cell epitopes have been detected and investigated (Makela et al., 1989a, 1989b; El Kasmi et al., 1998, 1999, 2000; Beauverger et al., 1994).

3. Pathogenesis and Pathology

Measles is a typical childhood disease and recovery provides life-long protection to reinfection. MV spreads via aerosol droplets and enters via the respiratory route. The initial infection settles in the mucosa of the respiratory tract from where it extends to the local lymphatic tissues. Virus amplification in the regional lymph nodes results in dissemination via the blood to the spleen, the lung, the thymus and the skin. After an incubation period of 10-14 days, the first clinical symptoms including fever, coryza, cough and conjunctivitis appear. This short prodrome is followed by the onset of a characteristic buccal enanthem, the Koplik spots, before the typical maculopapular rash finally appears. The rash initiates in the face from where it spreads to the trunk and the extremities and lasts about 4 days (Griffin et Bellini, 1996).

Recovery from measles is the rule however severe complications affecting the respiratory and the intestinal tract and the nervous system are frequently. Despite an efficient and long-lasting immune response generated during acute measles, paradoxically, a generalized immune suppression to secondary infections occurs, promoting bacterial or viral superinfections. Symptoms regularly accompanying measles include pneumonia, otitis, diarrhoea, encephalitis and childhood blindness (Griffin et Bellini, 1996; Schneider-Schaulies et al., 2002; Schneider-Schaulies et ter Meulen, 2002). Indeed, pneumonia accounts for 60% of the measles related deaths in infants (Barkin, 1975). Central nervous system complications can manifest days (postinfectious encephalomyelitis, PIE) or months (measles body encephalitis, MIBE) to even years (subacute sclerosing panencephalitis, SSPE) after the acute measles infection. Atypical measles, a severe form of measles, was observed in the late 1960s in recipients of formalin-inactivated measles vaccine who were subsequently exposed to wild-type MV (see section 6.3).

4. Molecular Epidemiology

There is only one serotype known for MV and although the maximum diversity between MV field isolates is relatively low (97.6% overall genomic sequence identity), genetic variations among circulating wild-type strains exists. The viral RNA polymerase lacks proof-reading activity thus allowing mutations to accumulate with every round of replication. *In vitro* the virus' mutation rate has been estimated to about $9 \cdot 10^{-5}$ per base per replication resulting in a genomic mutation rate of 1.43

bases per replication round (Schrag et al., 1999). *In vivo* estimates of mutation rates of $4 - 5 \cdot 10^{-4}$ per site per year were based on the genetic variability of two genotypes C2 and D3 (Rima et al., 1997). The majority of mutations occur at the third base of a codon and remain silent. However, non-synonymous mutations may influence virus ability to escape *in vitro* neutralization by monoclonal antibodies (Fayolle et al., 1999; Truong et al., 1999) or even serum of vaccines (Klingele et al., 2000).

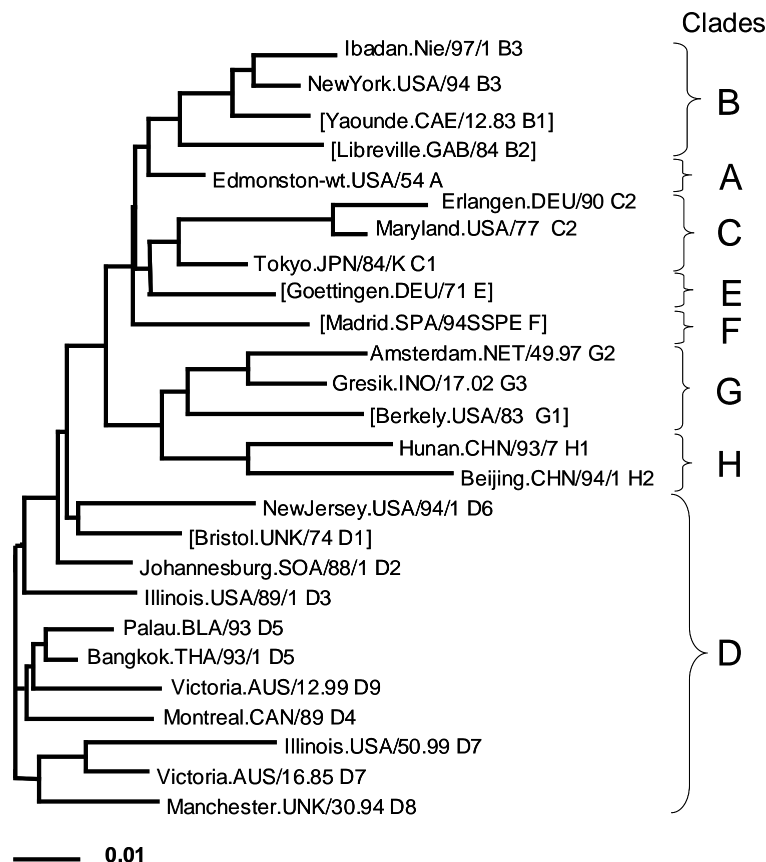

Figure 1.3: Phylogenetic tree of measles virus reference strains.

The phylogenetic tree is based on an alignment of the hypervariable region of the N gene (450 C-terminal nucleotides) of the measles reference viruses used for genotype classification of the currently known 22 genotypes. Corresponding Clades are indicated (A-H). ClustalX has been used to calculate the tree. References between brackets are genotypes of MV strains no longer circulating. (adapted from Kremer et Muller, 2003)

Among the MV protein genes, the genes coding for the N and H proteins are the less conserved and display a sequence identity of 97.4% and 97.1% respectively. Across the hypervariable region located in the C-terminal part of the N gene, the sequence identity drops to 95.4%. Therefore nucleotide sequence analysis is carried out on the

carboxy-terminal region of the N gene or on the complete H gene for genotyping (Mori et al., 1993). Phylogenetic analysis generates evolutionary trees from numerous aligned sequences. Molecular epidemiology has grown in importance during the last years and constitutes an important tool in revealing the origin of imported viruses, identifying transmission routes and monitoring the extent of virus circulation (Rota et al., 1996; Bellini et Rota, 1998; Muller et Mulders, 2002).

Recently, the WHO defined minimal protocols for sequence analysis and adopted a unique and unifying nomenclature for genotyping MV strains (Anonymous, 1998; WHO, 2001a, 2001b). MV wild-type strains are currently assigned to 8 clades, designated by letters A to H, containing 22 genotypes (Figure 1.3). Most genotypes show a restricted geographic distribution, others have been found throughout the world (Bellini et Rota, 1998; Muller et Mulders, 2002; Kremer et Muller, 2003). Some genotypes are not circulating anymore and are considered inactive (genotypes B1, B2, D1, E, F, G1).

5. Immune responses to MV

Both the humoral and the cellular immune responses are important for efficient clearance of MV and recovery from infection. Recovery results in life-long immunity. However, it remains unknown how measles antibodies persist for decades after acute infection.

IgM antibodies can be first detected when the rash appears. IgG1 and IgG4 can be monitored later, shortly after recovery. Antibodies are induced against most viral proteins, namely N, F, H and M. N-specific antibodies are the most abundant (Graves et al., 1984), while the H protein generates the biggest proportion of neutralizing antibodies (McFairlin et al., 1980; Giraudon et Wild, 1985). Antibodies are sufficient for protection because infants are protected by passively transferred maternal antibodies (Albrecht et al., 1977). Maternally derived antibodies can last for up to 9 months, but in general the protective effect fades within 4-5 months after birth (De Serres et al., 1997). Moreover, antibodies transmitted from vaccinated mothers are lost even more rapidly than those from mothers who went through natural infection (De Serres et al., 1997). This subject is further discussed in section 6.2.2. Passive transfer of immune serum also partially protects children against measles, but can interfere with measles vaccination (Siber et al., 1993).

An efficient immune response includes adequate $CD4^+$ and $CD8^+$ T-cell responses. This is especially important for vaccination against measles: immunization with a killed vaccine can introduce an detrimental Th2 bias in the response priming for atypical measles (see section 6.3.). However, while MV effectively activates MV-specific $CD4^+$ and $CD8^+$ T cells during the prodromal phase, it suppresses in parallel pre-existing T-cell reactivity against non-measles antigens, leading to an increased susceptibility to secondary infections.

6. Vaccination against measles

6.1. The Live Attenuated Measles Vaccine

Soon after the first isolation and adaptation of measles virus (MV) to propagation *in vitro* in 1954 (Enders and Peebles, 1954), the first experimental infections in macaques led to the development of a live attenuated measles vaccine (Peebles et al., 1957; Enders et al., 1960). Passaging of the MV Edmonston-wild type strain *in vivo* in chicken embryos and *in vitro* through chicken embryo fibroblasts generated a virus that induced MV-specific immunity, but only mild or no clinical symptoms. Intracerebral inoculation of monkeys with live attenuated vaccine did not cause neurological complications commonly associated with measles in humans (Enders et al., 1960). Today, this inexpensive vaccine is routinely given to 9 to 24 months old children, in probably every single country in the world. The vaccine has a high seroconversion rate and provides long-term protection after a single injection (Redd et al., 1999). It also has an excellent safety record (Table 1.1). In particular a number of expert review panels including a recent Medical Research Council panel were unanimous in their conclusion that a causal link between MMR vaccine and 'autistic enterocolitis' and autism spectrum disorders was not proven and that current epidemiological evidence did not support such a link (Anonymous, 2001; Madsen et al., 2002).

Today, vaccination with the current measles vaccine is one of the most successful and cost-effective medical interventions (Table 1.1). Introduction of routine vaccination has dramatically reduced measles prevalence and mortality worldwide. In some developed countries (e.g. US, Finland) circulation of indigenous virus has been interrupted (CDC, 1997; Heinonen et al., 1998). Developing countries, in particular in Latin America (de Quadros et al., 1998) and southern Africa (Biellik et al., 2002)

have also achieved major progress towards measles elimination. The high uptake of the vaccine and its worldwide success have encouraged the WHO to target measles for global elimination. This highly desirable but ambitious goal will put the live vaccine to its ultimate test. Two-dose schedules have been implemented in many countries to reach the high level of population immunity (>>90%) required for effective measles control. Despite excellent features, the live vaccine also has some drawbacks, which will be discussed in next section. Considering the high level of immunity required, relatively minor flaws of the vaccine could have important consequences for eventual eradication. Therefore, since the second half of the 1980's efforts have been made to develop new generation MV vaccines tested in rodents and monkeys and are discussed in section 7.

Table 1.1: Assets and drawbacks of the live attenuated measles vaccine

Assets	Drawbacks
Inexpensive to produce	Technical aspects: • low thermal stability • reconstitution prior to injection • safety of injections • safe disposal of injection devices
High seroconversion rates	
Long-term protection after single injection	
Excellent safety record	Vaccination of infants: • low immunogenicity • lack of resistance to neutralising maternal antibodies
High cost-effectivity	
Balanced Th1/Th2 immune response	Potential risks of the live vaccine: • pathogenicity in severely immunosuppressed individuals • reintroduction of circulating vaccine strains
Effective in combination with rubella and mumps vaccine	
High uptake and acceptance worldwide	Protection related: • second dose for sufficient population immunity • lower titres and accelerated waning of antibodies in vaccinees in comparison to natural immunity • virus transmission by asymptomatic individuals with weak or waning immunity

6.2. Drawbacks of current measles vaccines

6.2.1. Technical aspects

Although some progress has been made to improve the thermal stability of lyophilised vaccines, the need for maintaining an uninterrupted cold chain remains a major technical, logistical and financial burden. Improper handling of vaccine stocks and of multidose vials at the point of care is a continuing challenge to measles control in

low-income developing countries and in remote rural areas. New generation vaccines which are stable at ambient temperatures of the tropics would greatly advance the measles control.

Another technical issue that requires increasing attention is the safety of injection devices and the safe disposal of needles and syringes. According to WHO estimations, up to one third of all injections administered in developing countries are unsafe (WHO, 1996a, 1996b). Autodestruct syringes, which are irreversibly blocked after injection, should be available for all mass campaigns (WHO, 1998). However, alternative routes of immunization, which are non-invasive, safe, cost effective and which would greatly reduce the need for training of health personnel, would be particularly suitable for vaccination of large cohorts during mass campaigns. Vaccines delivered by the mucosal route are also likely to be more resistant to maternal serum antibodies (Cutts et al., 1997).

6.2.2. Vaccination of infants

Although highly effective in children and adults, the live attenuated measles vaccine is weakly immunogenic in infants less than 6 to 9 months of age (Albrecht at al., 1977; Markowitz et al., 1990a; Johnson et al., 1994; Redd et al., 1999). Limited efficacy in infants is in part due to interference by persisting measles-specific maternal antibodies during the first months of life (Albrecht et al., 1977; Osterhaus et al., 1998). In addition, the immaturity of the immune system is responsible for an impaired antibody response to the vaccine even in the absence of maternal antibodies (Gans et al., 1998, 2001).

Antibody levels of mothers were identified as the prime determinant of antibody titres in infants as well as of the duration of protection (Caceres et al., 2000). During the last trimester, the mother actively transfers antibodies transplacentally onto the child (Landor, 1995; Malek et al., 1996). Maternal antibodies protect newborns and infants during their early lives against infections including measles. Waning of maternal antibodies is highly variable in infants and depends on a number of factors reviewed by Caceres et al. (2000). Vaccination of infants is complicated by neutralisation of the vaccine virus by persisting maternal antibodies, severely reducing vaccine efficacy (Albrecht et al., 1977; Taylor et al., 1988c). Thus, early vaccination reduces seroconversion rates in those children that are still protected by persisting maternal antibodies and leaves many unprotected once passive antibodies have waned. In

contrast, delayed vaccination improves seroconversion, but a considerable proportion of children with low levels and premature waning of maternal antibodies are at risk of disease until they receive routine vaccination at the age of 15 months. Numerous studies report the early loss of maternal antibodies in children from low-income developing countries (Black, 1989).

Early waning of antibodies and delayed vaccination leave children with a susceptibility gap exposing them to a considerable risk of contracting measles. Vaccination at 9 months in developing countries (unlike at 15 months in most industrialised countries) is a compromise between the risk of disease sustained by infants unprotected by maternal antibodies and sustained by older children who failed to seroconvert because of maternal antibodies. In the developed countries infants are largely protected by community immunity. In many developing countries, where community immunity is incomplete and contacts with susceptibles are intense, the incidence of measles is high in this age group (McCormick et al., 1977; Fagbule and Orifunmishe, 1988; Ibia and Asindi, 1990). Even successful vaccination programs leave infants under 1 year of age at risk. During the resurgence of measles in Latin America in 1997, the age-specific attack-rates were highest in infants under 1 year of age (and in adults) (Cutts et al., 1999). A considerable proportion of the 800.000 annual fatal measles cases occur before the age of vaccination. In the future the susceptibility gap is likely to increase even further, although increasing community immunity might reduce the chance of actually becoming infected during this period.

In most of the above studies children were born to mothers that were protected by wild-type measles infection. In general, vaccinees develop considerably lower antibody titres and waning of their antibodies is faster than in measles late convalescents (Bouche et al., 1998a, 1998b; Damien et al., 1998; Van den Hof et al., 1999). Therefore vaccinated mothers have lower levels of specific antibodies than mothers with a history of measles (Brugha et al., 1996; Johnson et al., 1994). The rate of transplacental transfer was not influenced by the mothers' vaccination status (Brugha et al., 1996; De Serres et al., 1997). As a result, children born to vaccinated mothers acquire less antibodies than those with natural immunity (Krugman et al., 1977; Yeager et al., 1977; Lennon and Black 1986; Maldonado et al., 1995; Brugha et al., 1996; Markowitz et al., 1996) and are less long protected by maternal antibodies (Papania et al., 1999). Although studies showing similar correlations in developing countries are rare, it is reasonable to assume that protection by maternal antibodies

will become shorter in children when vaccinated birth cohorts reach childbearing age. Many countries with poor measles control also suffer from an increasing prevalence of HIV-infections. Children born to HIV-positive mothers have an increased risk of developing measles during early childhood. Accelerated waning of maternal antibodies in HIV infected infants left >90% of them unprotected at 6 months of age (Embree et al., 1992).

For these and other reasons, we anticipate that passive protection by maternal antibodies will further erode. Although higher vaccination coverage increases the age at which the children acquire measles, during outbreaks, a considerable proportion of children in developing countries will be unprotected for most of the first 9 or 12 months of their lives until they can be vaccinated (Muller, 2001). As a consequence, measles-associated fatalities sustained by infants may further justify attempts to develop a measles vaccine, which could already be protective early after birth.

6.2.3. Specific problems of the live vaccine

HIV infected individuals. The implications of the HIV-epidemic on measles vaccination and eradication have been reviewed by Moss et al. (1999). Measles-mumps-rubella (MMR) vaccination has long been recommended for HIV-infected children, although the serological response appears to be weaker the older the child is at vaccination (Arpadi et al., 1996). This is presumably due to advancing immunosuppression. Despite initial concerns, immunization with the standard live vaccine has been considered safe (Onorato et al., 1989) until the death of a HIV-infected young man 15 months after measles vaccination (CDC, 1996). As a consequence, the risk/benefit of measles vaccination should be evaluated individually in severely immunosuppressed HIV patients (CDC, 1998; Anonymous, 1999).

What about clade A field viruses? Vaccine strains as well as clade A field isolates are genetically almost identical, at least in the genes of the MV haemagglutinin (H) protein and the C-terminal region of the nucleoprotein (N), which are considered to be the most variable regions of the genome (reviewed by Muller and Mulders, 2002). Clade A viruses occur sporadically in the field and are not geographically confined; they show virtually no diversity over time and space. Most other MV genotypes have geographic prevalence patterns and display a considerable genomic diversity. While most wild-type viruses do not grow on Vero cells, clade A viruses grow spontaneously on this cell line. These and other observations would be compatible

with an occasional reintroduction of clade A viruses from a unique source. Although the development of a vigorous immune response would be expected to be associated with viremia, early studies conducted in the 1960s failed to demonstrate replicating virus in the blood of normal vaccinees (Katz et al., 1960, 1962). Only rare reports limited to the canine cell derived vaccine virus (McCrumb et al., 1961b; Hornick et al., 1962) or to monkeys (van Binnendijk et al., 1997) have recovered vaccine virus from the blood or from other tissues (Mitus et al., 1959; Morfin et al., 2002). Also early contact studies failed to demonstrate transmissible virus (Katz et al., 1960, 1962; McCrumb et al., 1961a; Hornick et al., 1962). However, studies with more sensitive techniques, including immune suppressed individuals, may be required to detect a rare but potentially significant spread of vaccine strain as discussed by us and others (Rima et al., 1995; Muller and Mulders, 2002). At present, both the likelihood of such a scenario and its consequences are difficult to evaluate. However, in the final stages of an eventual measles elimination, the reintroduction of a circulating vaccine strain which has (partially) lost attenuation could be a serious threat.
These examples show that there may be a need for a non-replicating vaccine for susceptible individuals and during the final stages of an eventual measles elimination program, in particular if vaccination cannot be discontinued (Stittelaar et al., 2002d).

6.2.4. Protection by live attenuated measles vaccine

It has been suggested that under optimal conditions protection by this vaccine may be lifelong with occasional vaccine failures (Markowitz et al., 1990b; Redd et al., 1999; Cutts et al., 1999). Long-term protection has been observed in remote Micronesia. After 27 years of no known exposure to measles, time since vaccination was not a significant risk factor for developing measles (Guris et al., 1996). Although such studies are rare, they suggest that a single dose of the live vaccine provides long-term immunity in most vaccinees.
However, while it is generally accepted that natural immunity protects life-long (Panum, 1939; Black and Rosen, 1962), overwhelming evidence shows that vaccine-induced immunity is less robust than natural immunity.

(i) Vaccinees develop lower antibody levels than naturally infected individuals (Bouche et al., 1998b; Damien et al., 1998; van den Hof et al., 1999).
(ii) Antibody titres decline more rapidly in vaccinees than in naturally infected individuals and may fall below detection levels (Krugman, 1983; Dai et al.,

1991; Christenson and Bottiger, 1994; Boulianne et al., 1995; Davidkin and Valle, 1998, van den Hof et al., 1999). Seroconverted individuals with initially low antibody levels developed measles within 10 years after vaccination (Mathias et al., 1989). In the absence of boosting by circulating wild-type virus waning of antibodies may be accelerated (Dai et al., 1991; Davidkin and Valle, 1998; Bennett et al., 1999; Whittle et al., 1999). By some accounts antibody half-lives have been estimated at 25 years (Mossong et al., 1999) or less (Mossong et al., 2000).

(iii) A distinct group of individuals with weak immunity may be protected against disease but not necessarily against infection, as suggested by an asymptomatic boost of their IgG response (Muller et al., 1996; Ozanne and d'Halewyn, 1992; Bennett et al., 1999; Whittle et al., 1999). The role of these individuals in virus transmission is not known, although such a role has been suggested in some cases (de Swart et al., 2000). In the absence of measles this was taken as an indication that the virus could circulate in a population of individuals who were otherwise protected against disease (Pedersen et al., 1989). It was estimated that their proportion is up to 10 times higher among vaccinees than among measles late convalescents (Damien et al., 1998).

(iv) As mentioned above, maternal antibodies and the immaturity of the infant immune system reduce vaccine efficacy at an early age of vaccination (Albrecht et al., 1977; Gans et al., 1998, 2001). Since in developing countries children are vaccinated already at nine months, the durability of protective immunity may be less than in most developed countries. It is an open but important question until what age these children will be protected, even if vaccinated under appropriate conditions, and how their immunity will evolve in the absence of boosting by field virus, even when a second-dose of vaccine is given.

If the immune response is thought to be less durable in vaccinees, this is mainly based on observations of eroding specific antibodies from seroprevalence studies. Although antibodies are sufficient for full protection even in the absence of a T-cell response, the role of the T-cell response for protection is less clear. In particular, the durability and the role of a residual T-cell response after waning of antibodies in vaccinees is an open question. A more persistent T-cell response could explain observations that vaccinees with low or undetectable antibody titres are still protected. Independently of

eroding antibodies, no decline in protection was observed in Micronesia (Guris et al., 1996). However, in light of the excessively high levels of protection that are required it is unlikely that vaccine-induced immunity is robust enough to maintain the high levels of protection (>>90%) necessary to avoid outbreaks.

It must be emphasised that the impact of each one of these issues, let alone their combined effect, is at present difficult to assess. If today some of the issues may appear theoretical, they should be considered as worst case scenarios which may later complicate measles eradication. Their potential impact is likely to increase along with the proportion of individuals protected by vaccine-induced immunity (rather than natural immunity) and when (or if) adult vaccinees start loosing their immunity.

Vaccination of adult vaccinees who have lost protection may become an issue in a more or less distant future. It will be difficult to determine the best strategy for revaccination of vaccinees, but a vaccine that would escape recognition by pre-existing antibodies would be an advantage (Osterhaus et al., 1998; Bouche et al., 2003; Muller et al., 2003).

6.3. Atypical measles

The development of new measles vaccines has been complicated by observations in the 1960's and 1970's of immunopathology in children vaccinated with the early formalin-inactivated vaccine. This vaccine provided insufficient protection and sometimes predisposed infants for enhanced, atypical measles after natural infection (Fulginiti et al., 1967). The risk of atypical measles precluded clinical studies with inactivated MV vaccines, especially since elucidation of the underlying mechanism took until the late 1990's. Interestingly, a similar vaccination approach in the 1960's for another human respiratory pathogen of the family of Paramyxoviridae, the respiratory syncytial virus, also predisposed infants for enhanced disease upon natural infection (Kapikian et al., 1969). Studies in rodent models of respiratory syncytial virus suggested that the disease was caused by formalin-inactivated respiratory syncytial virus-specific $CD4^+$ T cells associated with a type 2 cytokine profile (Graham, 1995; Openshaw et al., 2001). A similar hypothesis was proposed for atypical measles (Griffin et al., 1994), which was finally tested when a macaque model became available. Initially atypical measles was attributed to a dearth of fusion glycoprotein-specific antibodies (Norrby et al., 1975). Today there is increasing

evidence that this severe disease is caused by priming of non-protective T helper 2 cells. Th2-skewing is associated with a reduced IL-12 and an augmented IL-4 production further enhancing immunosuppression during atypical measles (Polack et al., 2002a). In addition, complement-fixing virus-specific antibodies of low avidity were induced and immune complex formation was shown, which apparently also played a role in the pathogenesis of the enhanced disease (Polack et al., 1999, 2002b, 2003). The possibility to assess the risk of atypical measles in rhesus macaques (Polack et al., 1999, 2002a) removed a major obstacle to the development of new candidate (subunit) MV vaccines.

7. Experimental vaccines against measles

Several animal models have been developed for the evaluation of new candidate measles vaccines. Originally, experiments in small laboratory animals like mice, rats, ferrets and hamsters were mainly carried out using Edmonston-derived rodent-adapted MV strains (Johnson, 1981; Thormar et al., 1985; Brinckmann et al., 1991; Niewiesk et al., 1993). These animal species are not susceptible to infection with wild-type MV strains, unless xenografted with human cells (Auwaerter et al., 1996; Valsamakis et al., 1999). More recently, MV vaccination and challenge models were developed in cotton rats which proved to be susceptible to wild-type MV infection (Niewiesk, 2001; Wyde et al., 1992, 1999, 2000a, 2000b).
Non-human primates have been used in measles research since the beginning of the previous century. Inoculation of filtered respiratory tract secretions of measles patients was found to induce measles-like symptoms in macaques, thereby providing evidence of a viral aetiology (Josias, 1898; Goldberger and Anderson, 1911). Since then, several monkey species have been shown to be susceptible to MV infection (Van Binnendijk et al., 1995). Although not infected with MV in their natural environment, several MV outbreaks have been described in captive monkeys, some of them with substantial mortality. MV isolated in Vero cells or other continuous cell lines proved to have limited pathogenicity in monkeys, but the introduction of the lymphoblastoid B-cell line B95-8 of non-human primate origin for the isolation of wild-type MV strains (Kobune et al., 1990, 1996) provided the tools for development of vaccination and challenge models in macaques.

Studies of experimental vaccines against measles carried out in rodents and in non-human primates have been summarized in Tables 1.2 and 1.3, respectively.

7.1. Immune stimulating complexes (ISCOMs) and Quil A-based vaccines

Preparations of haemagglutinin (H) and fusion (F) proteins obtained from F-, or H-depleted MV-lysates were incorporated into ISCOMs (Varsanyi et al., 1987). Quil A was identified as a strong adjuvant which, either alone or as a component of ISCOMs (Morein et al., 1984), mediated a strong neutralizing antibody responses as well as MHC class I- and class II-restricted T-cell responses (Rimmelzwaan and Osterhaus, 1995). In preliminary immunization studies, only ISCOMs containing both F and H protein induced neutralizing antibodies (De Vries et al., 1988). Mice were inoculated subcutaneously with either H or F-ISCOMs. In all cases, mice produced haemolysis-inhibiting antibodies. Sera from animals immunized with ISCOMs containing the H protein also developed haemagglutination-inhibiting antibodies. Despite this difference both ISCOM preparations fully protected mice against a lethal MV challenge (Varsanyi et al., 1987). In infants well-defined, non-toxic, purified Quil A components are required. Recently, MV-ISCOM preparations based on different purified Quil A components were tested in intramuscular immunizations in mice (Stittelaar et al., 2000a). Differences in the relative amounts of the MV-F to H ratio, Quil A-components and lipids did not influence the MV-specific antibody response. All candidates gave rise to virus neutralizing antibodies in all cases. However, these variations had a profound effect on their capacity to generate $CD8^+$ MV-F specific human cytotoxic T cell (CTL) cell clones *in vitro* (Stittelaar et al., 2000a). In mice, MV-ISCOMs containing both solubilised F and H induced predominantly F-specific IgG1 antibodies, whereas antibodies induced with MV-ISCOM-matrix, containing β-propiolactone-inactivated virus that is not deliberately incorporated into ISCOMs (therefore called matrix), were mainly of the IgG2a subclass. ISCOMs containing both MV proteins were also highly immunogenic in juvenile macaques both in the absence or presence of passively transferred MV-specific neutralizing antibodies (Van Binnendijk et al., 1997). These animals proved to be largely protected against an intratracheal challenge with the wild-type MV Bilthoven (Bil) strain 1 year after vaccination. Although this vaccine looked very promising, problems associated with

toxicity of certain Quil A components slowed down further studies including clinical testing of this adjuvant.

Table 1.2: Experimental measles vaccines in rodents

Experimental vaccine	Mode of delivery	Immune response	References
ISCOMs	i.m., s.c.	Neutralizing Abs, CTL, protection	Varsanyi et al, 1987; Stittelaar et al., 2000a; Wyde et al., 2000a, 2000b
Recombinant viral vectors			
· Adenovirus vectors	i.n., i.p., p.o.	CTL, protection	Fooks et al., 1995, 1998
· Poxvirus vectors (replication-competent)	i.n., i.p.	Neutralizing 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.	Neutralizing Abs, protection	Weidinger et al., 2001
· Avian virus vectors	i.m., i.p.	Neutralizing Abs, CTL, protection	Wyde et al., 2000a, b; Kovarik et al., 2001
· Vesicular Stomatitis Virus	i.n., i.p.	Neutralizing Abs, protection	Schlereth et al., 2000b
· Attenuated parainfluenza virus type 3	i.n.	Neutralizing 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., i.m., i.n., i.p.	Neutralizing Abs, CTL, protection	Cardoso et al., 1996; Fooks et al., 1996, 2000; Etchart et al., 1997; Yang et al., 1997; Fennelly et al., 1999; Torres et al., 1999; Schlereth et al., 2000a; Green et al., 2001
Plant based vaccines	i.m., i.p.	Neutralizing Abs	Huang et al., 2001; Webster et al., 2002; Marquet-Blouin et al., 2003; Bouche et al., 2003
Epitope based vaccines			
· Synthetic peptides of F protein	i.n., i.p.	Neutralizing 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.	Neutralizing Abs, protection	El Kasmi et al., 1998, 1999, 2000
· "Recombinant peptides" of F protein	p.o.	IgG, CTL, protection	Verjans et al., 1995; Spreng et al., 2000
· Polyepitopes of H protein	i.p.	Neutralizing Abs	Bouche et al., 2003

i.d.: intradermal; i.m.: intramuscular; i.n.: intranasal; i.p.: intraperitoneal; p.o.: oral; s.c.: subcutaneous

However, in recent years purified Quil A components without undesired side effects have been confirmed as potent adjuvants (Stittelaar et al., 2000a). β-propiolactone-inactivated MV antigen in association with one of these purified components, either in the form of ISCOMs, ISCOM-matrix or uncomplexed, induced strong and long-lasting neutralizing antibody responses in macaques (Stittelaar et al., 2002c).

7.2. Vaccines based on recombinant MV proteins

7.2.1. Recombinant Viral Vectors

A number of replicating or replication-defective recombinant live viruses expressing one or more of the immunodominant proteins of MV (F, H, N) have been developed. These studies have considerably improved our understanding of the role of these viral proteins in protection and the type of response required for protection. Some of these vectors also proved to circumvent problems associated with current vaccine.

Adenovirus vectors

Alkhatib and Briedis (1988) showed that a recombinant live adenovirus vector produced levels of H protein in eukaryotic cells comparable to MV infection. Proper surface expression of the MV-H did not require the co-expression of other MV proteins. Similarly, F protein was glycosylated, cleaved and transported to the cell surface without loss of activity (Alkhatib and Briedis, 1988; Alkhatib et al., 1990; Fooks et al., 1998). Neither intraperitoneal nor oral immunization with the H- or the F-recombinant adenovirus vectors resulted in detectable neutralizing antibodies in mice. Intraperitoneal or oral immunization with H-expressing adenovirus protected 85% or 50% of mice against fatal encephalitis, while the F-expressing vector showed no protection. (Fooks et al., 1998). The absence of neutralizing antibodies suggested that protection was mediated by a cellular rather than a humoral response. After intraperitoneal immunization of mice with an adenovirus expressing N protein (Warnes et al., 1994), a significant MHC class I-restricted CTL response was induced against the target protein but not against the recombinant vector (Fooks et al., 1995). Primed spleen cells lysed target cells infected with vaccinia virus expressing N but not MV-infected target cells. This cellular response was as protective in mice as immunization with N-expressing vaccinia virus (Fooks et al., 1995). Intraperitoneal immunization of cotton rats with H- or F-expressing adenovirus vectors significantly reduced MV titres in the lungs after intranasal challenge with the CAM strain (Fooks et al., 1998). However, after intranasal immunization no protection was observed in this animal model.

Poxvirus vectors

Recombinant replication-competent poxviruses - H and F protein genes were introduced into a recombinant vaccinia virus (Drillien et al., 1988; Wild et al., 1992). After parenteral immunization of mice, the recombinant vaccinia virus-H induced higher neutralizing antibody titres than the vaccinia virus-F (Drillien et al., 1988). In addition, parenteral immunization of mice with vaccinia virus-H induced a strong class I-restricted CTL response and protection against MV encephalitis (Drillien et al., 1988; Wild et al., 1992; Galletti et al., 1995; Etchart et al., 1996). However, in the presence of passively transferred anti-MV antibodies, induction of the H-specific humoral response was inhibited whereas the specific CTL response was not affected (Galletti et al., 1995). Similarly, recombinant vaccinia virus expressing the F and H protein were shown to be very effective in inducing MV-specific antibody and T-cell responses in juvenile macaques, although responses were slightly lower than in animals which received an MV ISCOM preparation in the same study (Van Binnendijk et al., 1997). Again, passively transferred MV-specific neutralizing antibodies substantially reduced the immunogenicity of this vaccine. Surprisingly, after challenge with wild-type MV strain Bil one year after a second vaccination the animals proved to be largely protected, supposedly by specific T-cell responses. Immunization of infant macaques with a similar vaccine also induced good MV-specific antibody responses in four out of five naïve animals. However, antibody responses were poor in nine out of nine animals vaccinated in the presence of passively transferred MV-specific antibodies or in eight out of eight animals with maternally derived MV-specific antibodies (Zhu et al., 2000). MV-specific CTL responses were also reduced in eight out of 17 animals vaccinated in the presence of MV-specific antibodies. Half of these animals proved to be largely protected against a wild-type MV challenge 4 months after a second vaccination. Intranasal inoculation of mice with vaccinia virus-H induced both a mucosal IgA response as well as a systemic H-specific CTL response (Etchart et al., 1996). The CTL response was similar to the one obtained after parenteral immunization. The intranasal co-administration of cholera toxin with vaccinia virus-H further improved the response.

The attenuated vaccinia virus vector NYVAC(K1L) replicates in human and rodent cells. NYVAC(K1L) containing the H gene was used to immunized mice during early life (Perkus et al., 1989; Kovarik et al., 2001). A single dose induced a vigorous CTL response in suckling mice which was similar in adult mice. Also, interferon-γ levels

were similar in both age groups, while IL-5 secretion was not detectable in either group. The NYVAC(K1L)-H seemed to induce adult-like antibody-, Th1- and CTL-responses even in Th2-prone 1-week-old mice (Kovarik et al., 2001). Also anti-H IgG antibody titres were high and similar in both groups (Kovarik et al., 2001). However, in the presence of maternal antibodies, specific antibody induction was depressed even after prime/booster, whereas CTL responses as well as interferon-γ secretion were similar to the response obtained after a single immunization, irrespective of passively transferred H-specific IgG antibodies.

Replication-deficient poxviruses – The use of replication-competent vaccinia viruses in humans is dangerous due to the relatively high risk of side-effects including generalised poxvirus infections in immunocompromised individuals. Recombinant viruses based on Modified Vaccinia virus Ankara (Sutter and Moss, 1992), a virus which is replication deficient in mammalian cells, have been proven safe and effective as vaccine vector for respiratory infections (Sutter et al., 1994; Wyatt et al., 1996, 1999), especially for the elderly and young children. They can be administered in much higher doses than the replication-competent recombinant vaccinia virus vaccine and they can also be safely administered to immunocompromised individuals (Sutter and Moss, 1992). This vector expressing the H protein was tested in mice and cotton rats (Weidinger et al., 2001) and in monkeys (Zhu et al., 2000; Stittelaar et al., 2000b, 2001). Modified vaccinia virus Ankara expressing H induced mainly IgG2a antibodies in mice suggesting a Th1 phenotype. $CD4^+$ T-cell-depleted mice were protected against an intracranial challenge with a neurotropic MV strain (Weidinger et al., 2001). The same authors showed that both intraperitoneal and intranasal administration of this recombinant vector protected cotton rats against an intranasal challenge with MV. In cotton rats the induction of neutralizing antibodies in the presence of passively transferred maternal antibodies was reduced in comparison to mice or monkeys (Siegrist et al., 1998a, 1998b, 1998c; Stittelaar et al., 2000b; Zhu et al., 2000; Weidinger et al., 2001).

This vector, recombinant for measles F and H genes, proved to be highly effective in inducing MV-specific neutralizing antibodies as well as $CD8^+$ T-cell responses in juvenile macaques, both in the absence or presence of passively transferred MV-specific antibodies (Stittelaar et al., 2000b). All animals proved to be largely protected against a wild-type MV (Bil) challenge 1 year after the second vaccination. A similar

recombinant modified vaccinia virus Ankara vector also induced a protective immune response in naïve infant macaques, but was less effective in infant macaques with passively transferred antibodies (Zhu et al., 2000). Four out of four animals vaccinated in the absence and two out of four animals vaccinated in the presence of passively transferred antibodies proved to be protected against a wild-type MV challenge 4 months after the second vaccination. It remains unclear whether the difference between the latter studies were due to the age of the animals or differences in the levels of passively transferred antibodies.

Avian virus vectors

Fowlpox virus is a member of *Avipoxvirus* genus that has been developed more than 10 years ago as a live virus vector for the delivery of antigens to mammalian hosts (Taylor et al., 1988a,b). The F protein was cloned and expressed in primary embryo fibroblast using the fowlpox virus expression system (Taylor et al., 1990; Spehner et al., 1990), but no reports of successful immunization data were published using this recombinant vector.

Canarypox virus**,** another member of the *Avipoxvirus* genus has also been engineered to express heterologous proteins (Boyle et al., 1987; Taylor et al., 1988a,b). Recombinant canarypox virus-H and -F expressed authentic proteins on the surface of avian and non-avian cells (Taylor et al., 1992). No productive viral replication was observed in non-avian cells, which makes this vector, as modified vaccinia virus Ankara and fowlpox virus, an attractive vector for use in humans. Using the canarypox vector ALVAC co-expressing F and H protein, Wyde et al. (2000a, 2000b) induced neutralizing antibodies after a single intramuscular administration in cotton rats and all sero-converted animals were protected. Kovarik et al. (2001) showed that the ALVAC vector expressing the H gene induced a specific CTL cell response in adult mice but not in suckling mice. Unlike in adult mice the vaccine was unable to induce specific interferon-γ secreting CD8 cells in the young mice (Kovarik et al., 2001).

Vesicular stomatitis virus

Vesicular stomatitis virus, as the MV, is a member of the *Mononegavirales*. A chimeric vesicular stomatitis virus carrying both H and F was created recently (Tatsuo et al., 2000b). Using this chimeric virus, the authors showed that the difference in cell

tropism between the wild-type virus and vaccine-derived strain was largely determined by virus entry, in which the H protein plays a decisive role. A chimeric vesicular stomatitis virus expressing only the H protein has been reported to induce high titres of neutralizing antibodies after intranasal but not after intraperitoneal immunization of cotton rats in the presence of increasing levels of passively transferred MV-antibodies (Schlereth et al., 2000b). Irrespective of the amount of anti-MV antibodies transferred, most animals were protected after intranasal immunization against an intranasal challenge with MV (Schlereth et al., 2000b). The successful immunization of cotton rat in the presence of maternal antibodies seemed to depend on the replication of the vesicular stomatitis virus vector.

Attenuated parainfluenza virus type 3

Parainfluenza virus type 3 is a member of the *Respirovirus* genus of the *Paramyxoviridae* family in the order *Mononegavirales*. A live attenuated cold-passaged parainfluenza virus vaccine, *cp45*, represents a promising candidate for vaccination of infants and children (Karron et al., 1995), even in the presence of passively acquired antibodies (Durbin et al., 1999). The recombinant parainfluenza type 3 virus was used as a vector to express the MV-H protein (Durbin et al., 2000). After intranasal inoculation, very high titers of MV neutralizing antibodies were obtained in Syrian hamsters (Durbin et al., 2000). Immunization of macaques with a chimeric human-bovine parainfluenza virus type 3 mediating the expression of the H protein resulted in significant MV-specific neutralizing antibody responses (Skiadopoulos et al., 2001). Although these animals were not challenged with wild-type MV, this candidate vaccine may be suitable for intranasal vaccination against both parainfluenza virus type 3 and MV.

Table 1.3: Experimental measles vaccines in primates

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

i.d.: intradermal; i.m.: intramuscular; i.n.: intranasal; m.-e.: micro-encapsulated

7.2.2. Recombinant Bacterial Vectors

Different bacteria have been used as vectors to develop recombinant live vaccines. In contrast to recombinant viral vaccines expressing measles glycoproteins, bacterial systems were mainly used to express either the N protein, which does not require the eucaryotic folding machinery for its conformation, or linear B- and T-cell epitopes (Stover et al., 1991; Fennelly et al., 1995; Zhu et al., 1997; Maggi et al., 2000).

The most widely used vaccine in the world, the ***Bacille Calmette-Guerin*** (BCG) was developed into a recombinant live vaccine since it can be administrated at birth without the need for boosting (Stover et al., 1991). MV N protein was expressed in BCG. After i.p. immunization of mice, high anti-MV antibodies were detected only 5 weeks after a second boost (Fennelly et al., 1995). Only 30% of mice survived an intracranial challenge with a neuro-adapted measles strain. This recombinant vector was further tested for immunogenicity and efficacy in newborn macaques (Zhu et al., 1997). The rationale for this study was that BCG can be administered at birth. By choosing the N protein as immunogen the authors hoped to immunise effectively in the presence of maternal antibodies and prime for a MV-specific T-cell response. Vaccination indeed induced low-level N-specific lymphoproliferative and CTL

responses. However, vaccinated animals were not protected when challenged 5 months later with a wild-type MV strain, although a reduction of lung inflammation and of levels of virus shedding (in some animals) was observed.

Streptococcus gordonii is a Gram-positive commensal bacterium colonising the upper respiratory tract of healthy humans. It was genetically engineered to express either H or F proteins as chimeric proteins with the M6 surface protein. Subcutaneous inoculation of mice with transformed *Streptococcus gordonii* expressing H or F protein induced MV-reactive IgG, which however did not neutralize virus *in vitro* (Maggi et al., 2000).

Attenuated recombinant ***Salmonella typhimurium*** was used as a vector to express the MV-N protein as described in Section 3.3 (Fennelly et al., 1999) or selected MV epitopes as part of a chimeric protein as described in Section 3.5.2 (Verjans et al., 1995; Spreng et al., 2000).

A genetically attenuated strain of ***Shigella flexneri*** was used as a delivery system for recombinant plasmid DNA coding for the N, H and F genes of MV as described in Section 3.3 (Fennelly et al., 1999).

7.3. Nucleic acid vaccines

Direct targeting of dendritic cells by DNA vaccination is an attractive way to produce viral proteins *in vivo* and present them most efficiently to the immune system (Koprowski and Weiner, 1998). Plasmids expressing the N, F or H proteins were used for DNA immunization of mice, cotton rats and rabbits (Cardoso et al., 1996; Fooks et al., 1996, 2000; Etchart et al., 1997; Yang et al., 1997; Fennelly et al., 1999; Torres et al., 1999; Schlereth et al., 2000a; Green et al., 2001) and monkeys. The H protein was expressed as a membrane protein or a soluble protein after genetic modification of its N-terminal sequence (Yang et al., 1997; Torres et al., 1999). Gene-gun immunizations with the membrane H protein or the soluble H protein plasmid induced long-lasting neutralizing antibodies in mice, but in rabbits antibodies were short-lived. Highest H-specific titres were obtained in mice when the protein was expressed under its native form (Yang et al., 1997). It was shown *in vitro* that the membrane H protein plasmid produced more proteins than the plasmid coding for the soluble H protein (Torres et al., 1999). Gene-gun immunization of mice with a plasmid containing the soluble H protein fused to three tandem copies of the complement component C3d induced

higher anti-H antibody titres and higher neutralizing antibodies than the plasmid coding for the soluble H protein alone (Green et al., 2001). Intramuscular immunization with the membrane form of H protein generated predominantly IgG2a antibodies associated with a Th1 response, the soluble form induced predominantly IgG1 antibodies (Cardoso et al., 1996, 1998; Martinez et al., 1997; Torres et al., 1999). This was also the dominant subclass after gene-gun inoculation of the H plasmid (Cardoso et al., 1998). Similar as for the H plasmid, the N plasmid induced IgG2a antibodies, which were switched to IgG1 when co-administrated with the H plasmid (Cardoso et al., 1998). In mice, as well as in cotton rats, F plasmid immunization induced lower neutralizing titres than H plasmid (Yang et al., 1997; Schlereth et al., 2000a).

After intramuscular immunization of mice with H plasmid, a strong class I-restricted CTL response was elicited (Cardoso et al., 1996, 1998; Etchart et al., 1997; Martinez et al., 1997). The response after mucosal immunization was less strong, but after intragastric delivery it was considerably improved by co-administration of cholera toxin or cationic lipids (Etchart et al., 1997). A specific CTL response was also obtained in mice with N plasmid when attenuated bacteria such as *Shigella flexneri* or *Salmonella typhimurium* were used for intranasal or intraperitoneal DNA delivery, respectively. CTL responses were similar for both delivery systems (Fennelly et al., 1999).

Intradermal DNA immunization of cotton rats (*Sigmodon hispidus*) with plasmid expressing the N protein induced the same levels of MV-specific antibodies as the H plasmid, albeit without neutralizing activity (Schlereth et al., 2000a). A robust protective immunity against an intranasal challenge was obtained by intradermal immunization with plasmids expressing H or F or both genes, but only in the absence of passive MV-specific antibodies (Schlereth et al., 2000a). Based on these data, the resolution of lung infection in cotton rats seems to require neutralizing antibodies. This observation is in contrast with earlier experiments in which mice and rats were protected against CNS infection with viral vectors expressing N protein without the requirement of neutralizing antibodies (Bankamp et al., 1991; Finke and Liebert, 1994; Fennelly et al., 1995; Fooks et al., 1995).

Intradermal and gene gun vaccination of juvenile macaques with plasmids encoding the F or H or both genes resulted in some animals in significant MV-specific neutralizing antibody titres, and partial protection from wild-type MV challenge 7

months after the second vaccination (Polack et al., 2000). In another study, gene gun vaccination of juvenile macaques with plasmids encoding either H, H and F, or H, F and N genes did not result in detectable neutralizing antibody titres. N-specific antibodies were produced only in animals receiving all three plasmids (Stittelaar et al., 2002a). One year after the second vaccination animals showed no significant protection, but the accelerated kinetics of the MV-specific antibody response indicated that DNA vaccination had primed the animals for a secondary immune response.

7.4. Plant based vaccines

The MV-H protein was expressed in tobacco (Huang et al., 2001; Webster et al., 2002) as well as in transgenic carrots which are part of the diet of infants as well as adults and can be eaten either raw or boiled (Marquet-Blouin et al., 2003). While in carrots expression of the unmodified H protein was efficient, expression in tobacco required the addition of a retention signal for the endoplasmic reticulum. After intraperitoneal immunization of mice with the H protein extract of carrots or tobacco plants virus crossreactive and neutralizing antibodies were induced (Huang et al., 2001; Marquet-Blouin et al., 2003). The transgenic carrots induced both IgG1 and IgG2a, indicative of a balanced Th1/Th2 response (Marquet-Blouin et al., 2003). After gavaging with tobacco extract mixed with relatively high doses of Vibrio cholerae enterotoxin B, mice developed only low levels of anti-H antibodies (Huang et al., 2001). However, the potential role of an edible vaccine was shown in a prime boost strategy. After intramuscular immunization with a DNA plasmid coding for a soluble H protein containing a secretion signal peptide of the CD5 gene, mice were boosted by gavaging with tobacco extract co-administered with a mixture cholera toxin and cholera toxin B (Webster et al., 2002). MV-specific and neutralizing titres were significantly higher after oral boosting.

7.5. Vaccines based on selected epitopes

Neutralizing and protective B-cell epitopes are generally conformational antigenic sites, which are difficult to mimic by synthetic peptides (Van Regenmortel and Muller, 1999). However, a number of protective sequential epitopes have been identified and effective vaccination with a peptide vaccine against canine parvovirus

and foot and mouth disease has been demonstrated in the natural animal (Langeveld et al., 1994a, 1994b; Wang et al., 2002). In the case of measles, neutralizing antibodies are directed against the H and F protein, and sequential epitopes have been identified in their surface glycoproteins. A number of studies have shown that peptides corresponding to these epitopes can induce neutralizing and protective antibodies in small animal models (Obeid et al., 1995; Atabani et al., 1997; El Kasmi et al., 1999, 2000).

7.5.1. Synthetic peptides of the fusion protein

Steward and colleagues described two adjacent sequential epitopes within a short sequence (F388-420) of the F protein. Sera from African children with acute measles consistently reacted with peptide F388-402 in a pepscan ELISA. This peptide was immunogenic in mice and induced MV-crossreactive and neutralizing antibodies. Passive transfer of anti-peptide serum conferred significant protection in the mouse model against a neuroadapted strain of MV (Atabani et al., 1997). Peptide F397-420, partially overlapping with the latter peptide, was co-linearly synthesized with one or two copies of a promiscuous T-cell epitope. Although some of these chimeric peptides induced antibodies with no detectable neutralizing activity *in vitro*, they were reported to protect in two different strains of mice against MV-induced encephalitis (Obeid et al., 1995). Analysis of the fine specificity revealed that these antibodies targeted residues 407-417 (Partidos et al., 1997). Intranasal co-immunization of a chimeric synthetic peptide containing two copies of a T-cell epitope and one copie of a B-cell epitope together with cholera toxin B adjuvant induced MV neutralizing antibodies and protected mice against an intracranial challenge with a neuroadapted MV strain (Hathaway et al., 1998).

7.5.2. Recombinant peptides of the fusion protein

Attenuated bacteria represent a potentially safe alternative to express and secrete recombinant proteins bearing T- and B-cell epitopes for vaccination purposes. Three copies of the F protein B-cell epitope F404-414 (Obeid et al., 1995) or two copies of a T-cell epitope of the N protein (N79-99) were fused to the C-terminus of the secretion signal of *E. coli* haemolysin and expressed by the attenuated *Salmonella typhimurium aroA* strain (Verjans et al., 1995; Spreng et al., 2000). After oral immunization, *S. typhimurium* secreting these antigens induced a specific MV-related humoral and

cellular response. A combination of both recombinant proteins partially protected susceptible mice against a lethal challenge with a rodent adapted MV strain.

7.5.3. Polyepitopes of the haemagglutinin protein

On the basis of the above results, a series of chimeric high-molecular weight polyepitope constructs (24500-45500) based on the permutational recombination of multiple paired copies of the Haemagglutinin Noose Epitope and the Neutralizing Epitope sequences with a promiscuous T-cell epitope of the tetanus toxoid (tt830) were developed (Theisen et al., 2000). Although the crossreactive and neutralizing immunogenicity of the B-cell epitopes was highly sensitive to the molecular environment in which they were displayed, some of these constructs displayed the natural conformation of the epitope. A number of these constructs (including the $[L_4T_4]_2$, a tandem 8-mer of four copies of the Haemagglutinin Noose Epitope (HNE) and four copies of a promiscuous T-cell epitope, tt830, derived from tetanus toxoid) were also shown to efficiently stimulate a tt830-844 specific human T-cell line, demonstrating efficient processing and presentation of the T-cell epitope by antigen presenting cells. At least one polyepitope construct, the $[L_4T_4]_2$, was shown to induce high levels of neutralizing antibodies in mice (Bouche et al., 2003). These sera neutralized a wide range of field isolates from developing countries (Bouche et al., 2003).

7.5.4. Synthetic peptides of the haemagglutinin protein

Two sequential B-cell epitopes of the H protein have been identified in our laboratory by screening a panel of neutralizing and protective monoclonal antibodies (mAbs) against a complete set of overlapping peptides (Ziegler et al., 1996; Fournier et al., 1997). Several mAbs reacted with peptides defining the sequential epitope H236-256 (Neutralizing Epitope domain, NE). Evidence from electron spin resonance, amino acid substitution scans, prediction algorithms, homology modelling and mimotopes indicated that the epitope had a helical structure (Deroo et al., 1998; El Kasmi et al., 1998). Interestingly, these mAbs blocked haemolysis of monkey erythrocytes without inhibiting haemagglutination, suggesting that they acted at the functional interface of the H and F protein and did not inhibit virus binding to host cells (Fournier et al., 1997). When the core sequence of this epitope (H243-250) was combined in different orientations and copy numbers with various T-cell epitopes, some of these peptides

induced MV-crossreacting and neutralizing antibodies. Binding studies with the mAbs identified the contact residues $E_{244}LXQL_{249}$. The neutralizing anti-peptide sera exhibited a similar fine-specificity (El Kasmi et al., 1998). After passive transfer, these sera were shown to protect young mice against a lethal challenge with a neuroadapted MV strain. Peptides derived from this Neutralizing Epitope domain were not recognised by mouse sera generated by immunization with whole virus (El Kasmi et al., 1999). It was further shown that anti-MV antibodies did not suppress the antibody response against a peptide, which induced protective antibodies. Thus, even in the presence of protective levels of passively transferred MV-specific antibodies, the peptide was able to generate a neutralizing response.

Another set of mAbs identified a second sequential epitope of the H protein spanning residues H381-400 (haemagglutinin noose epitope, HNE). This fragment contains three Cys residues (in positions H381, H386 and H394) which are conserved in all morbilliviruses (Ziegler et al., 1996). By iterative optimisation, peptides were designed that induced MV-crossreactive (El Kasmi et al., 2000). Peptides corresponding to the HNE domain were not recognised by sera of women of child-bearing age, suggesting that passively acquired maternal antibodies in infants would not interfere with the immune response to these peptides. This was demonstrated in the mouse model where passively transferred protective levels of anti-whole virus serum did not suppress the immunogenicity of such a peptide. These peptides are now being conjugated to immunogenic carrier proteins licensed for use in infants such as diphtheria and tetanus toxoids to develop candidate vaccines compatible with current vaccination schedules.

8. A peptide-conjugate vaccine against measles

8.1. Rationale

The HNE and NE peptide candidate antigens, presented above, are designed for immunization early after birth in order to close the window of susceptibility in newborns and infants. The design of the recombinant and the synthetic (conjugate) vaccine candidates was guided by the following considerations:

(1) Newborns and infants develop efficient antibody responses against protein and conjugate vaccines (Fedson et al., 1999; Ward et Zangwill, 1999). At this age antibodies are the main mechanisms of vaccine-induced protection.

(2) In measles, antibodies against the two glycoproteins provide full protection even in the absence of a T-cell response (Giraudon and Wild, 1985; Albrecht et al., 1977). In particular, newborns are solely protected by passive antibodies.

(3) Most vaccines based on recombinant proteins are susceptible to neutralization by maternal antibodies. In contrast, the immune response to the Haemagglutinin Noose Epitope and the Neutralizing Epitope peptides is not suppressed by pre-existing anti-whole virus antibodies (El Kasmi et al., 1999, 2000). Women in child-bearing age do not have antibodies reacting with these peptides (Ziegler et al., 1996).

(4) The Haemagglutinin Noose Epitope sequence is highly conserved in field isolates. In particular, the prevalent viruses in Africa and Asia (genotypes B3, D2, D4, D8, H2, and G2) which are responsible for the highest measles mortality in children show few mutations in this epitope.

(5) The risk of atypical measles is a major obstacle for the development of new measles vaccines. It appears that this enhanced disease is due to an aberrant Th2-biased immune response (Polack et al., 1999, 2002a, 2003). However, this is both the preferred response in infants (Barrios et al., 1996; Siegrist, 2000, 2001) and the one required for generating protective antibodies. To avoid a (unfavorable) Th2-priming of measles specific T cells, measles specific sequences were kept to a minimum and measles-unrelated carrier proteins or promiscuous T-cell epitopes (tt830-844; Demotz et al., 1993) were used instead.

(6) The antibody response against measles would benefit from the Th2 primed T cells induced by early vaccination against tetanus and/or diphtheria.

(7) Furthermore, some evidence suggests that vaccination with the whole virus (e.g. live attenuated vaccine) after vaccination with a peptide vaccine may still be possible. Thus an epitope vaccine combines a number of features that are compatible with pre-existing maternal antibodies and possibly with recommended routine vaccination schedules. Although peptide immunogens have a number of limitations, these may be less relevant or even assets in the case of a PRE-vaccine (Protection by antibody Resistant Epitopes) that would provide protection of limited duration at least against severe disease, until the live vaccine can be administered.

8.2. The Hemagglutinin Noose Epitope (HNE)

In previous studies, it was shown that the MV-neutralizing and protective monoclonal antibodies (mAbs) BH216, BH21 and BH6 bind to synthetic peptides corresponding to amino acid residues 361-410 of the H protein (Ziegler et al., 1996). This domain contains three cysteine residues (C381, C386, C394), highly conserved among field isolates. Short peptides mimicking the immunogenicity of this Hemagglutinin Noose Epitope (HNE) induced high levels of antibodies crossreacting with the H protein (El Kasmi et al., 2000). Most of the different peptides were efficiently recognized by the mAbs, demonstrating that they assumed conformations that are congruent to the antibody binding site. A compendious structural analysis of the HNE domain and related peptides thereof is difficult, as no crystallographic structure of the H protein has been obtained to date. In *in vivo* immunization experiments, the HNE peptides presented multiple conformations to the B-cell receptors only a few of which induced crossreactive antibodies depending on the flanking sequences of the B-cell epitope. Despite these conceptual and practical difficulties to predict the outcome of the immune response (van Regenmortel et Muller, 1999; van Regenmortel, 2001a, 2001b), peptides mimicking BCEs of a number of pathogens have been reported, which induced strong virus neutralizing and protective humoral responses (Bittle et al., 1982; Emini et al., 1983; Francis et al., 1990; Langeveld et al., 1994a, 1994b; Obeid et al., 1995; El Kasmi et al., 1998). Some of these studies also showed that much can be learned from antibody-peptide binding studies to improve virus-crossreactive immunogenicity of the peptides. This work is dedicated in the functional implementation of the HNE epitope in a peptide-conjugate suited for early infancy use.

8.3. Immunogencity of a peptide-conjugate in relation to prior immunity

An antibody response against candidate peptides requires a potent T-cell help. Since the peptides are relatively short (<20 amino acids), they cannot be expected to contain T-cell epitopes recognized by (the MHC II of) a sizeable proportion of a human population. Similarly, polysaccharides representing neutralizing and protective B-cell epitopes of bacterial pathogens do not contain (efficient) T-cell epitopes. In order to induce a sustainable immune response polysaccharides are normally coupled to proteins providing potent T-cell epitopes. Antigenic proteins derived from vaccines licensed in humans are attractive candidates as carrier proteins. These include diphtheria toxoid, tetanus toxoid, purified protein derivative (PPD), recombinant hepatitis B surface protein, cholera toxin and others. Conjugate vaccines based on diphtheria toxoid or tetanus toxoid as carrier proteins have been successfully developed against bacterial infections such as *Haemophilus influenzae* type b (Hib) (Ward et Zangwill, 1999) and *Streptococcus pneumoniae* (Fedson et al., 1999). They are effective both in adults and in infants, although immaturity of the immune system of infants also affects antibody responses to conjugate vaccines. For instance, an age-dependent stepwise increase in IgG antibody response has been observed after a single dose of Hib conjugate given at 2 to 17 months of age (Einhorn et al., 1986). In many studies tetanus toxoid has been found to be more immunogenic than diphtheria toxoid. Passive transfer of anti-tetanus antibodies did not seem to inhibit the immune response against the peptide (Siegrist et al., 1998b). In light of widespread vaccination of mothers against neonatal tetanus in developing countries a careful evaluation of both carriers is warranted. Conjugate vaccines can be administered with DTP in a single injection (CDC, 1993). With a more than 80% global coverage by two doses of DTP (WHO, 1999), future conjugate vaccines free-loading on this vaccine have great potential to reach many of the world's children early after birth.

Prior immunity against the carrier has been shown to modulate the subsequent antibody response to an antigen conjugated to the same carrier. The reports have often been contradictory and the biological and clinical significance of these observations seems difficult to predict. T lymphocytes that were primed against the carrier protein have been initially shown to enhance the humoral response against a small molecular hapten (DNP) in adoptive recipients after subsequent injection with the hapten-

carrier-conjugates (Mitchison, 1971). Herzenberg and colleagues (1980) described the hapten-specific suppression, i.e. a reduced antibody response against the hapten occurring in mice following prior injection with the carrier protein. Similar observations were made for antibody responses for peptide-conjugates in mice (Schutze et al., 1985) and in humans (Di John et al., 1989). Oppositely, prior immunity to the carrier was also shown to enhance the antibody response to a conjugated protein moiety in adults (Shah et al., 1999). Similarly, active priming with the carrier protein resulted in an enhanced polysaccharide-specific antibody response in children (Barington et al., 1994). However, the presence of passively acquired carrier-specific antibodies suppressed the antibody response against the polysaccharide in a dose-dependent manner (Barington et al., 1994). Alike observations were made in rodents for the specific antibody responses against polysaccharides, which was increased after active priming with the carrier (Schneerson et al., 1980; Anderson, 1983).

9. Aims of the study

The study had three major issues:

1) Defining the optimal HNE peptide sequence and conformation to be implemented in a peptide-conjugate in order to induce antibodies crossreacting with the native MV H protein and protecting in an in vivo challenge/protection model
2) Estimate the impact of different pre-immune statuses (prior immunity against the carrier, passively acquired antibodies against the carrier or the peptide) and prime-boost strategies on the peptide- and the carrier-specific antibody responses
3) Evaluate the immunogenicity of the peptide-conjugate using non-invasive immunization routes such as the nose or the bare skin

Chapter 2: Materials & Methods

This chapter is assigned to the description of the methods and the materials used in this study. Chemical protocols including solid phase peptide synthesis (SPPS) and bio-conjugation techniques were used for the synthesis of the various peptide and conjugate antigens that were subsequently analyzed and purified by appropriate HPLC separation techniques (reverse phase, gel filtration, protein G). Immunological methods like ELISA, surface plasmon resonance and flow cytometry defined qualitatively and quantitatively HNE-peptide-antibody interactions and fine-mapped the antigenicity and the crossreactive immunogenicity of the HNE peptide. In silico molecular modeling tools provided additional evidence for the structural description of the epitope and the biological relevance of these data were assessed using in vitro neutralization assays with different wild-type MV strains. Animal experiments in vivo demonstrated the crossreactive and protective immunogenicity of the HNE peptide-conjugates and thoroughly characterized the peptide-specific immunogenicity in relation to existing immunity statuses. Non-invasive routes of immunization (intranasal and transcutaneous) were also tested as needle-free delivery systems of the peptide-conjugates.

1. Synthetic peptides

1.1. The solid phase principle

Solid phase peptide synthesis (SPPS) is based on sequential addition to an insoluble polymeric support of amino acid residues whose α-amino and side chain functions are protected by acid- or base-labile groups and was initially described by Merrifield (1963). A number of excellent descriptions of SPPS have been published, e.g. Atherton et Sheppard (1989), Chan et White (2000).

The peptide chain assembly on an solid support has obvious advantages: separation of the intermediate peptides from soluble reagents and solvents can be carried out simply by filtration and thorough washing providing the procedure its speed, simplicity and efficiency over the corresponding operations in solution synthesis; reagents can be used in excess helping to drive reactions to almost completion; physical losses of formed product are minimized as the peptide chain stays attached to the solid support during the whole synthesis; most operations are compatible with automation with consequent savings in time and effort.

The peptide chain synthesis starts with the C-terminal residue and elongation proceeds from its amino-end by successive carbodiimide couplings. Thus, the protection of the α-amino group is temporary and is removed prior to every coupling with the carboxy group of the next residue. Two approaches have been developed for the α-amino protection, i.e. using the acid-labile Boc-group (Merrifield, 1964) or the base-labile Fmoc-group (Carpino, 1972). Cleavage of the Boc-group or Fmoc-group is achieved by TFA or piperidine, respectively. Any functional groups in amino acid side chains must be masked with permanent protecting groups that are not affected by the reaction conditions occurring throughout the synthesis. After deprotection of the peptide α-amino group of the peptide chain on the support, the next (protected) residue is added in excess and coupled to the peptide chain through its carbodiimide-activated carboxy group. After every deprotection or coupling step, reagents in excess are removed by extensive washing and filtration. This procedure is repeated until the desired peptide sequence is assembled. Finally, the peptide is released from its solid support and the side-chain protecting groups are removed. In Boc chemistry, this is achieved under harsh acidic conditions, i.e. using HF or TFMSA, whereas Fmoc chemistry uses the

less strong TFA. Following the cleavage reaction, the peptide is usually precipitated by addition of ice-cold diethyl ether, washed, shortly dried, dissolved in 80% *tert*-butanol and lyophilized.

1.2. Peptide Synthesis

Peptides were assembled by automated solid-phase peptide synthesis using standard Fmoc chemistry (Wiesmüller et al., 1992) on a SYRO peptide synthesizer (Multisyntech, Witten, Germany). The synthesis was carried out at RT on a 48-reactor block using 2 ml polypropylene reactors (Multisyntech, Witten, Germany) or on a 96-reactor block using 200 µl Eppendorf-tip-reactors (Eppendorf AG, Hamburg, Germany). 30 mg of Fmoc-amide-(aminomethyl)-resin (*Rink*) (PepChem, Tübingen, Germany) displaying a loading density of 0.58 mmol/g were used for the 2 ml reactors.

Prior to the initial deprotection, the resin was extensively washed and swollen with DMF. Fmoc deprotection was carried out after incubating the resin three times in 300 µl 40% piperidine for 10 min, each time followed by 6 washing steps with 500 µl DMF. Coupling reactions were carried out by adding 50 µl of a 3.0 M DIC solution in DMF:DCM (1:1) and 200 µl of a 0.45 M Fmoc amino acid (OrpegenPharma, Heidelberg, Germany) solution in NMP, containing 0.65 M HOBt, to the deprotected resin or peptide chain in the reactors. The coupling reaction was allowed to proceed for 90 min, carried out twice for every step and followed by 6 washing steps with 500 µl DMF. After the coupling steps, a capping reaction using 300 µl of a 10 % acetanhydrid/5% DIPEA solution in DMF was carried out for 15 min to terminate permanently chain elongation in uncompleted coupling reactions and followed by 6 washing steps with 500 µl DMF.

Once the synthesis completed, the resin was again washed and swollen using DMF and subsequently washed with methanol and ether. Side-chain protected peptides were deprotected and released form the resin using 2 ml of TFA containing 5% ddH_2O and 10% Scavenger K [6.5% phenol in thioanisole:ethanedithiol (2:1)] for 5 h. The peptide dissolved in TFA was removed form the reactors, precipitated and washed using ice-cold diethyl ether (-20°C). The precipitate was let dry and dissolved in 80% *tert*-butanol and lyophilized using a Alpha 2-4 lyophilisator (Christ, Osterode am Harz, Germany).

Peptides with altered backbone containing N-methylated peptide bonds were synthesized until the coupling of the N-α-Fmoc-N-methyl amino acid residue (Novabiochem, Läufelfingen, Switzerland) using automated SPPS. After subsequent deprotection and capping reaction, reactors were removed from the block and synthesis was carried out manually. Coupling of the next residue to the N-methylated α-amino group was performed via *in situ* generation of Fmoc-amino acid chlorides using bis-(trichloromethyl)carbonate (BTC, 'triphosgene') with the procedure of Falb et al. (1999). The resin containing the assembled peptide chain was washed with tetrahydrofuran (THF) and heated to 50°C. The protected Fmoc amino acid residue to be coupled was dissolved (0.45 M, 6x excess) together with BTC (0.20 M, 2x excess) in THF to which 2,4,6-collidine (14x excess) was added to yield a white suspension. After 1 min the suspension was added to the resin and the coupling reaction was allowed to proceed for 1 h at 50°C. The resin was subsequently washed with THF and an identical coupling reaction was carried out for a second time. After extensive washing steps using THF and DMF, the reactors were put again on the reactor block and the remaining peptide sequence was synthesized using automated SPPS.
The lyophilized peptides were analyzed and purified by reverse phase (RP) high-performance liquid chromatography (HPLC) (see section 3.3).

1.3. The HNE peptide and pepscan libraries

The HNE peptide corresponds to residues 379-400 [E_{379}TCFQQACKGKIQALCENPEWA_{400}] of the H protein of the MV Edmonston strain. Substitution analogues were prepared by replacing each amino acid by Ala, Arg, Asn, Gln, Glu or Ser residues. Peptides with defined cystines were obtained by replacing the Cys residues also by amino butyric acid (Abu) to mimic the hydrophobicity of the thiol group by a methyl group.
The shortened HNE peptide [KGQ_{383}QACKGKIQALCEN_{396}], elongated at the N-terminus by a Gly and a Lys spacer residue, was used as the main immunogen (see also section XX) in the peptide-conjugates. Prime-boost immunization experiments were also performed using a chimeric construct containing the HNE peptide co-linearly synthesized with a TCE [QYIKANSKFIGITELGQQACKGKIQALCEN]. The promiscuous T-cell epitope is derived from tetanus toxoid (tt830-844; Q_{830}YIKANSKFIGITEL_{844}).

```
ETCFQQACKGKIQALCENPEWA
X---------------------
-X--------------------
--X-------------------
---X------------------
----X-----------------
-----X----------------
------X---------------
-------X--------------
--------X-------------
---------X------------
----------X-----------
-----------X----------
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--------------------X-
---------------------X
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Table 2.1: HNE peptide substitution analogues.

Substitution analogues were prepared by replacing each position in the HNE sequence by Ala, Arg, Asn, Gln, Glu or Ser residues.

1.4. Peptide Oxidation and Reduction

For oxidation, RP-HPLC purified HNE peptides were dissolved in DMSO. The DMSO and the peptide concentrations were subsequently set to 20% and 0.8-1.5 mg/ml respectively using borate buffer [50 mM boric acid, 150 mM NaCl, pH 7.4] (Tam et al., 1991). The reaction was carried out at RT during 5 h in 50 ml Falcon tubes under end-over-top rotation. Finally, the oxidized and the linear isoforms were immediately separated via RP-HPLC and lyophilized.

For reduction, RP-HPLC purified HNE peptides were dissolved in ammonium carbonate buffer [0.1 M NH_4HCO_3, pH 8.0] at a final peptide concentration of 0.8-2.0 mg/ml (Chan et White, 2000). 50-100 equivalents of DTT were added using 1M stock solution aliquots of DTT stored at –20°C. The reaction was carried out at RT during 30-60 min in 50 ml Falcon tubes. Subsequently, the oxidized and the linear isoforms were immediately separated via RP-HPLC and lyophilized.

2. Bioconjugation chemistry and peptide-conjugates

Bioconjugation chemistry involves the covalent linking of two molecules to form a novel conjugate displaying the combined characteristics of its individual components. Synthetic and natural entities with distinct properties can thus be chemically combined to create a unique substance possessing carefully engineered *in vitro* or *in vivo* activities. It is not surprising that the bioconjugation technology has affected nearly every discipline in the life science. Modification and conjugation techniques rely on two interrelated chemical reactions: the reactive functional groups present on the various derivativatizing reagents, also termed ‘crosslinkers’, and the functional groups available on the target molecules, i.e. low molecular weigth moieties, short peptides or protein macromolecules, to be modified. Without the presence of both types of functional groups and chemical compatibility thereof, the process of conjugation would not be possible. The compendious knowledge of the mechanisms underlying the coupling reactions of the reactive with the target functional groups and the careful choice of the adequat reagent systems able to react with the chemical groups available on target molecules form the basis for an intelligent and successful conjugation strategy. Preserving the distinctive *in vitro* and *in vivo* activities of the involved molecules has to be addressed with vigilance during the bioconjugation reactions. The chosen reagents and conjugation strategy should by no means affect any functional groups of critical residues or alter structure-activity relationships. The use of a selected bifunctional crosslinker thus enables the chemical linking of two biomolecules using varying functional groups. The functional groups involved in peptide-protein crosslinking reactions are commonly primary amine groups, i.e. the N-terminal α-amine or the ε-amine of lysine side chains; carboxylate groups, i.e. the C-terminal α-carboxylate, the β-carboxylate or the γ-carboxylate of aspartic or glutamic acid side chains respectively; sulfhydryl groups of cysteine side chains. An excellent bioconjugation guidebook has been published by Hermanson (1996).

2.1. EDC/NHS chemistry

Available carboxylate functions of the carrier proteins diphtheria toxoid (dt, MW 62000), tetanus toxoid (tt, MW 150000) and ovalbumin (ova, MW 43000) were activated using *N*-ethyl-*N*’-[(3-dimethyl-amino)propyl] carbodiimide hydrochloride /

N-hydroxy-succinimide (EDC/NHS) chemistry (Pierce, Rockford, IL, USA; www.piercenet.com). Prior to the chemical activation, the carrier proteins were extensively dialysed in MES buffer [0.1 M MES, 0.5 M NaCl, pH 6.0] using Slide-A-Lyzer® cassettes (3 ml, 10,000 MWCO, Pierce, Rockford, IL, USA). 500 µl aliquots of 8-12 mg/ml dialyzed carrier protein stock solutions were added to priorly weigthed 100 molar equivalents of EDC (MW 191.7) and 200 equivalents of Sulfo-NHS (MW 217.1). After 15 min incubation at RT the 500 µl sample containing the activated carrier protein was injected on a HPLC Gel Filtration column [Superdex™ 200 HR10/30, Amersham Biosciences, Uppsala, Sweden] in order to remove the excess of reactive EDC and sulfo-NHS molecules (see section 3.2). Elution was monitored using a UV detector (230 and 280 nm), the activated carrier was collected and immediately added to lyophilized, purified, oxidized short HNE peptide [$KGQ_{383}QACKGKIQALCEN_{396}$]. The coupling to the activated carboxylate group on the carrier was carried out via the free N-terminal α-amine or ε-amine of the lysine side chain. The reaction was allowed to proceed at RT during 24 h. Finally, the HNE-peptide-conjugates DT, TT or OVA were purified, quantified and eluted in borate buffer [50 mM boric acid, 150 mM NaCl, pH 7.4] using HPLC Gel Filtration (see section 3.2) and stored at 4°C. dt and tt were kindly provided by the Serum Institute of India Ltd., Hadapsar, Pune, India. Throughout the text and the figures, lower case letters designate the carrier proteins (dt, tt, ova) solely, and upper case letters the HNE-peptide-conjugates of the respective carrier (DT, TT, OVA).

2.2. SPDP chemistry

Available primary amine functions of the carrier proteins diphtheria toxoid (dt, MW 62000) and tetanus toxoid (tt, MW 150000) were activated using the heterobifunctional crosslinker *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) (Pierce, Rockford, IL, USA; www.piercenet.com). Prior to the chemical activation, the carrier proteins were extensively dialysed in borate buffer [50 mM boric acid, 150 mM NaCl, pH 7.4] using Slide-A-Lyzer® cassettes (3 ml, 10,000 MWCO, Pierce, Rockford, IL, USA). 500 µl aliquots of 8-12 mg/ml dialyzed carrier protein stock solutions were added to priorly weigthed 100 molar equivalents of SPDP (MW 312.4) dissolved in DMSO. After 30 min incubation at RT the 500 µl sample containing the activated carrier protein was injected on a HPLC Gel Filtration column [Superdex™

200 HR10/30, Amersham Biosciences, Uppsala, Sweden] in order to remove the excess of reactive SPDP crosslinker (see section 3.2). Elution was monitored using a UV detector (230 and 280 nm), the activated carrier was collected and immediately added to lyophilized, purified, oxidized or reduced full length HNE peptide [E_{379}TCFQQACKGKIQALCENPEWA$_{400}$]. The coupling to the activated primary amine function on the carrier was carried out via a free thiol function of an available cysteine side chain of Cys381, Cys386 or Cys394. The reaction was allowed to proceed at RT during 24 h. Finally, the HNE-peptide-conjugates DT or TT were purified, quantified and eluted in borate buffer [50 mM boric acid, 150 mM NaCl, pH 7.4] using HPLC Gel Filtration (see section 3.2) and stored at 4°C.

3. Analytical and semi-preparative HPLC

Analysis and purification of peptides and proteins is a key step in every scientific investigation about biological structure and function. The careful dissection of specific interactions or mechanisms and the reproducibility of highly sensitive assays can only be granted after prior purification and isolation of the reactive species.
High-performance liquid chromatography (HPLC) is an essential tool for the characterization and purification of peptides and proteins (Unger, 1989; Lottspeich et Zorbas, 1998). The separation techniques are funded on sample partitioning due to reversible interactions between a mobile (solvent) and a stationary phase (solid matrix). The choice of the chromatographic method and the type of equipment are determined by the molecular nature of the components to be studied and the aim of the investigation. HPLC analysis and purification of the molecules involved in this study were performed using an ÄKTAexplorer 10S system (Amersham Biosciences, Uppsala, Sweden). This device represents a versatile and efficient working platform for all chromatographic techniques allowing the use of wide ranges of sample load, working pressure and flow rate.

3.1. Reversed Phase-HPLC: Peptide Analysis and Purification

RP-HPLC separates proteins and peptides with differing hydrophobicity based on their reversible interaction with the hydrophobic surface of a chromatographic medium. Samples bind to the solid matrix as soon they are loaded onto the column.

Due to the nature of the matrices (commonly silica-based media) used in RP-HPLC, the binding is usually very strong and requires the use of organic solvents (commonly acetonitrile or aceton) and other additives (ion pairing agents) for elution. Elution is carried out by increasing the concentration of the organic solvent.

Analytical RP-HPLC was used to assess purity of crude peptide samples immediately after peptide synthesis, scout and optimize elution parameters prior to peptide purification and monitor peptide oxidation and reduction reactions. Peptide analysis was typically performed by injecting 50-200 μg samples of lyophilized peptide dissolved in 1-2 ml of 10% of solvent B in solvent A [solvent A: water, 0.1% TFA, pH 2.1; solvent B: water, 60% acetonitrile, 0.1% TFA] on a C18 silica gel column [NUCLEOSIL® C18, length: 250 mm, diameter: 4mm, pore diameter: 120 Å, particle size: 5 μm] (Machery-Nagel, Hoerdt, France) or on a polystyrene/divinyl benzene column [SOURCE 5RPC, length: 150 mm, diameter: 4.6mm, particle size: 5 μm] (Amersham Biosciences, Uppsala, Sweden) using an ÄKTAexplorer 10S system (Amersham Biosciences, Uppsala, Sweden). Peptide elution was typically performed with 4-10 column volumes of a linear gradient of 20%-80% of solvent B in solvent A using a flow rate of 0.8-1.5 ml/min. Elution was monitored using an UV detector at three different wavelengths (205 nm, 230 nm, 280 nm).

Semi-preparative RP-HPLC was used for purification of crude peptides after synthesis or isolating disulfide-bridged and linear isofoms of the HNE peptide after oxidation or reduction reactions. Peptide purification was typically performed by injecting 12-25 mg samples of lyophilized peptide dissolved in 12-30 ml of 10% of solvent B in solvent A [solvent A: water, 0.1% TFA; solvent B: water, 60% acetonitrile, 0.1% TFA] on a C18 silica gel column [GROMSIL ODS-4 HE, length: 250 mm, diameter: 8mm, pore diameter: 120 Å, particle size: 5 μm] (Grom, Herenberg-Kayn, Germany) using an ÄKTAexplorer 10S system (Amersham Biosciences, Uppsala, Sweden). Peptide elution was typically performed with 3-8 column volumes of a linear gradient of 20%-60% of solvent B in solvent A using a flow rate of 2.0-3.0 ml/min. Elution was monitored using an UV detector at three different wavelengths (205 nm, 230 nm, 280 nm). Peaks were fractionated in 0.5-1.0 ml fractions using a F950 fraction collector (Amersham Biosciences, Uppsala, Sweden). Relevant peak fractions were

pooled and immediately lyophilized. Peptide mass and disulfide bond formation was confirmed by electron spray ionization (ESI) technique in positive mode on a LCQDuo instrument (ThermoFinnigan, San Jose, CA, USA) courtesy to our in house facility (Dr. Isabelle Kolber and Dr. Serge Schneider, Division de Toxicology, Laboratoire National de Santé, Luxembourg).

3.2. Gel Filtration: Bioconjugation and peptide-conjugate purification

Gel filtration separates proteins solely on the basis of differences in molecular size. Separation of the different molecules contained in a sample is achieved using a porous matrix to which the given species' have different degrees of access, i.e. smaller molecules can access to a much larger volume as they can migrate inside the porous beads, being thus retained in the column, whereas larger molecules cannot and move rapidly through the column, thus emerging first. Samples are eluted isocratically and separation occurs immediately during loading onto the column. The technique is best suited for small sample volumes.

The HNE peptide was conjugated to the carrier proteins diphtheria toxoid (dt), tetanus toxoid (tt) and ovalbumin (ova) using NHS/EDC chemistry or a heterobifunctional linker SPDP (Pierce, Rockford, IL, USA). Gel filtration was used to separate the activated carrier protein from the crosslinking reagents used in large excess, avoiding thus to alter the peptide side-chains. Separation was achieved using a Superdex™ 200 HR10/30 column [matrix of crosslinked agarose and dextran, length: 300 mm, diameter: 10 mm, bed volume 24 ml] (Amersham Biosciences, Uppsala, Sweden) with a flow rate of 0.6-0.8 ml/min on a ÄKTA Explorer system (Amersham Biosciences, Uppsala, Sweden). Relevant peaks were fractionated in 1.0 ml fractions using a F950 fraction collector (Amersham Biosciences, Uppsala, Sweden), pooled and added immediately to the lyophilized HNE peptide to be conjugated. Elution was monitored using an UV detector at two different wavelengths (230 nm, 280 nm). The elution volumes were regularly calibrated using a Molecular weight marker kit (Sigma MW-GF-200, Sigma-Aldrich, Bornem, Belgium). Diphtheria toxoid, tetanus toxoid and ovalbumin eluted after 15.0 ± 0.1 ml, 12.8 ± 0.1 ml and 15.8 ± 0.1 ml, respectively, corresponding to their respective masses of 62 kDa, 150 kDa and 43 kDa.

The coupling reaction was allowed to proceed for several hours or even overnight. The peptide-conjugate was then separated from the free, unconjugated peptide added in excess using a similar protocol. Elution was carried out in borate buffer [50 mM boric acid, 150 mM NaCl, pH 7.4] using a Superdex™ 200 HR10/30 column (Amersham Biosciences, Uppsala, Sweden) with a flow rate of 0.4-0.8 ml/min on a ÄKTA Explorer system (Amersham Biosciences, Uppsala, Sweden). Relevant peaks were fractionated in 1.0 ml fractions using a F950 fraction collector (Amersham Biosciences, Uppsala, Sweden) and pooled.

3.3. Protein G affinity chromatography: monoclonal antibody purification

Affinity chromatography separates proteins on the basis of a reversible interaction between a protein and a specific ligand attached to a chromatographic matrix. The separation is highly selective as the protein of interest is specifically retained on the column through the ligand. The sample is loaded under conditions optimal for protein-ligand interaction. Unbound material is removed from the column through extensive washing. The target protein is released form the column by changing buffer conditions precluding binding and promoting desorption.

Protein G is a protein derived from *Streptoccocus aureus* binding specifically to the Fc domain of IgG-type antibodies. The recombinant Protein G (MW 22000) contained in the HiTrap Protein G HP columns (Amersham Biosciences, Uppsala, Sweden) has been genetically engineered in order to lack the albumin-binding region and resist to proteolytic degradation.

After the harvest of monoclonal antibodies (BH216, BH21, BH6) from hybridoma supernatant produced in a Cell-Line system (Integra, Wallisellen, Switzerland) (see section 8), they were purified using a HiTrap Protein G HP column (Amersham Biosciences, Uppsala, Sweden) on a ÄKTA Explorer system (Amersham Biosciences, Uppsala, Sweden). Samples were loaded, unbound material was washed away using borate buffer [50 mM boric acid, 150 mM NaCl, pH 7.4] and the target mAb was eluted using glycine buffer [100 mM glycine, pH 2.7]. 1.0 ml fractions were collected using a F950 fraction collector (Amersham Biosciences, Uppsala, Sweden), pooled and dialyzed in borate buffer [50 mM boric acid, 150 mM NaCl, pH 7.4]. The concentration was adjusted to 1.55 mg/ml (=10 µM) for storage. Elution was monitored using an UV detector at two different wavelengths (230 nm, 280 nm).

4. Enzyme-linked immunosorbent assay (ELISA)

A common feature to all ELISA systems is the use of antibodies. In ELISAs, solid-phase reactants are generated by adsorbing an antigen (a peptide or a protein) or an antibody onto plastic support, commonly 96-well plastic microtiter plates. Adsorption occurs via non-covalent, hydrophobic interactions between the antigen (or the antibody) and the specially activated surface of the plates. To avoid adsorption of the reagents of subsequent steps of the protocol which would lead to non-specific background signals, free binding sites in the wells are blocked through adsorption of bovine serum albumin (BSA). The coated reagents are then incubated subsequently to a second or a third reactant (an antibody) covalently coupled to an enzyme, commonly horseradish peroxidase or alkaline phosphatase. After every incubation step, the unbound reagents are washed away. Finally, a chromogenic or fluorogenic substrate is added and as it is hydrolyzed by the antibody-bound enzyme, a colored or fluorescent product is generated which can be monitored visually or by a microtiter plate reader (Crowther, 2001).

4.1. Monitoring antigenicity by indirect ELISA and inhibition ELISA

Binding of mAbs BH216, BH21 and BH6 to HNE peptide analogues was assessed using a classic indirect ELISA protocol. Ninety-six well plates (Maxisorp, Nalge Nunc, Rochester, NY, USA) were coated overnight at 4°C with 50 µl of twofold or threefold dilutions of peptide in carbonate-bicarbonate buffer (pH 9.6). Plates were washed with washing buffer [154 mM NaCl, 1 mM TrisBase, 1.0% Tween 20, pH 8.0] and blocked for 120 min at room temperature (RT) with 200 µl of blocking buffer [136 mM NaCl, 2 mM KCl, 15 mM Tris-Acetate, 1.0% BSA, pH 7.4]. Plates were washed again and incubated for 90 min at RT with 50 µl of 1 nM mAb BH216, BH21 or BH6 in dilution buffer [blocking buffer, 0.1% Tween 20]. After washing, plates were incubated with 50 µl of goat anti-mouse IgG-AP (1:1000 dilution; Southern Biotechnology, Birmingham, AL, USA) in dilution buffer for 60 min at RT. After washing, 100 µl of a 1.35 mM phosphatase substrate (SIGMA 104®, Sigma-Aldrich, Bornem, Belgium) solution was added and the optical density was measured after 30 and 60 min at 405 nm on a SPECTRAmax PLUS^{384} microplate reader system (Molecular Devices, Sunnyvale, CA, USA). Peptide end point titers (EPT) were

considered as the concentration of coated peptide where its optical density equaled the average value of the negative controls plus three standard deviations (SD). Negative controls included wells with no coated peptide and wells where the 1st-step-mAb (BH216, BH21 or BH6) was omitted.

Similarly, binding of mAbs BH216, BH21 and BH6 to HNE peptide analogues was also monitored with inhibition ELISA. This procedures enables to study the binding of mAbs to soluble antigens. The antigens in solution are competing with the coated antigens for mAb binding, thus reducing the binding to the coated reactants. For the inhibition ELISA the above protocol was modified as follows. Microtiter plates were coated coated overnight at 4°C with 1 µM of HNE reporter peptide in carbonate-bicarbonate buffer (pH 9.6). Plates were washed with washing buffer [154 mM NaCl, 1 mM TrisBase, 1.0% Tween 20, pH 8.0] and blocked for 120 min at room temperature (RT) with 200 µl of blocking buffer [136 mM NaCl, 2 mM KCl, 15 mM Tris-Acetate, 1.0% BSA, pH 7.4]. After washing, 400 pM of BH216, BH21 and BH6 in 50 µl dilution buffer [blocking buffer, 0.1% Tween 20], preincubated for 2 h at RT with two-fold dilutions of the inhibiting peptide of interest, were added to the wells. After washing, plates were incubated with 50 µl of goat anti-mouse IgG-AP (1:1000 dilution; Southern Biotechnology, Birmingham, AL, USA) in dilution buffer for 60 min at RT. After washing, 100 µl of a 1.35 mM phosphatase substrate (SIGMA 104®, Sigma-Aldrich, Bornem, Belgium) solution was added and the optical density was measured after 30 and 60 min at 405 nm on a SPECTRAmax PLUS^{384} microplate reader system (Molecular Devices, Sunnyvale, CA, USA). For each inhibiting peptide the concentration, which reduced antibody binding to the reporter peptide by 50% (50%-inhibiting concentration, IC50%), was determined. Negative controls included wells with no coated peptide and wells where the 1st-step-mAb (BH216, BH21 or BH6) was omitted. Positive controls (no inhibition) included wells without soluble competitor peptide.

4.2. Anti-peptide reactivity of mouse immune sera by indirect ELISA

After immunization with the peptide-conjugates, the immune sera were tested for peptide binding using ELISA. For this ELISA the above protocol was modified as follows. Plates were coated overnight at 4°C with 0.4 µM reporter HNE peptide in

carbonate buffer (pH 9.6). Plates were washed with washing buffer [154 mM NaCl, 1 mM TrisBase, 1.0% Tween 20, pH 8.0] and blocked for 120 min at room temperature (RT) with 200 µl of blocking buffer [136 mM NaCl, 2 mM KCl, 15 mM Tris-Acetate, 1.0% BSA, pH 7.4]. After washing, 50 µl of threefold serial dilutions of mouse serum in dilution buffer [blocking buffer, 0.1% Tween 20] were added for 90 min at RT. After washing, plates were incubated with 50 µl of goat anti-mouse IgG-AP (1:1000 dilution; Southern Biotechnology, Birmingham, AL, USA) in dilution buffer for 60 min at RT. After washing, 100 µl of a 1.35 mM phosphatase substrate (SIGMA 104®, Sigma-Aldrich, Bornem, Belgium) solution was added and the optical density was measured after 30 and 60 min at 405 nm on a SPECTRAmax PLUS^{384} microplate reader system (Molecular Devices, Sunnyvale, CA, USA). Serum end point titers (EPT) were considered as the serum dilution where its optical density equaled the average value of the negative controls plus three standard deviations (SD). Negative controls included wells with no coated peptide and wells without serum. Mouse immune sera were all tested individually as pooled sera were found to reflect an observed experimental situation only with little accuracy. Detected EPT values were subsequently evaluated by statistical analysis (see section 11).

5. Surface Plasmon Resonance measurements (BIACORE)

5.1. The BIACORE technology

The BIACORE biosensor technology (www.biacore.com) is based on the optical phenomenon of surface plasmon resonance (SPR) (Jönsson et al., 1991; Jönsson et Malmqvist, 1992). It allows the sensitive detection of molecular interactions, thus to measure specific binding in real time, without the use of labels. The interactions are monitored by measuring the mass concentration of molecules close to a surface. Changes in mass concentrations cause changes in the refractive index and an SPR signal is generated.

SPR relies on the reflection of light under certain conditions from a conducting metallic film at the interface between two media having different refractive indexes. In BIACORE instruments the sample and the glass of the sensor chip are the two

media and the film is a thin layer of gold, about 50 nm thick, on the chip surface. In addition, the gold film is covered with a layer of carboxymethylated dextran presenting a matrix for covalent attachment of molecules. Moreover, this hydrogel matrix supplies a hydrophilic environment optimal for preserving a non-denatured structure of the so-called ligand (attached on the chip surface) and studying interactions between the ligand and the analyte (contained in the sample and passed through a flow cell). Thus, prior to every BIACORE experiment, the sensor chip is derivatized by immobilizing the ligand on its surface activated using a relevant bioconjugation chemistry.

The experiment itself is performed under continuous flow conditions using microfluidics to deliver the sample to be studied to the sensor surface. High precision valves guarantee an accurate control over the time-point and the duration of sample delivery. A thin-layer flow cell ensures a laminar flow providing a fast and reproducible transfer of the molecules from the sample to the sensor surface. When the analyte binds to the immobilized ligand, its local concentration increases, the refractive index of the liquid medium increases, altering the angle of incidence of the reflected light beam and an SPR response is measured.

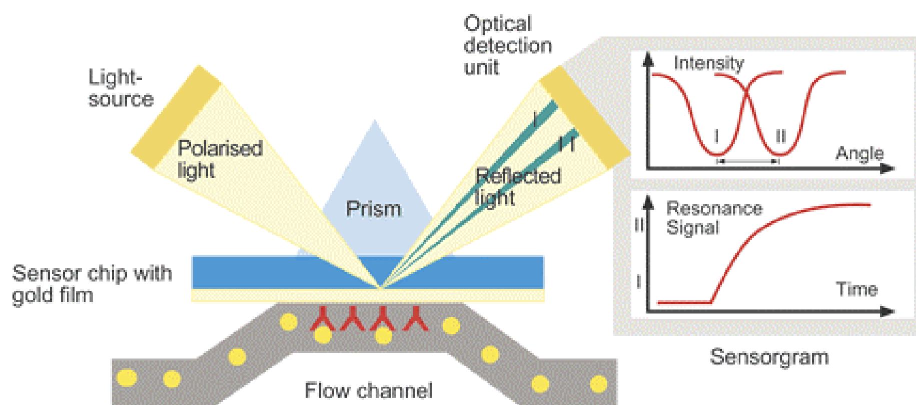


Figure 2.1. Illustration of BIACORE detector with sensor chip.

After ligand immobilization on the surface of the sensor chip, analyte arrives through the flow cell to the sensor surface, where interaction between binding partners occurs. Binding of analyte causes changes in mass concentration at the surface, changes in the refractive index and changes in the intensity of the reflection angle of light beam and generates an SPR signal (Illustration adapted from www.biacore.com).

SPR response values are expressed in resonance units (RU). 1 RU represents a change of 0.0001° in the angle of the reflection intensity. For most proteins, this is equivalent to a change in concentration of about 1 pg/mm^2 on the sensor surface. The response is directly proportional to the mass of analyte molecules binding to the ligand on the surface. However, the exact conversion factor between measured RU and the analyte surface concentration depends on intrinsic properties of the sensor surface and the precise nature of the analyte. Interestingly, the light comes not in contact and is not absorbed by the molecules in the sample: instead the light energy is dissipated through SPR in the thin gold film, i.e. the angle of minimum reflected light intensity is actually measured without the light used to assess the analyte-ligand interactions penetrating the sample contained in the flow cell. Using the BIACORE technology, molecular interactions can thus be studied without the requirement of any labels or reporter moieties, making this technique straight forward in use and eliminating the risk that labels may interfere with the interaction being studied.

When molecules in the sample bind to the sensor surface, the detected SPR response is plotted against time during the course of the interaction and provides a quantitative measure of the progress of the interaction. This plot is called a sensorgram. After an interaction cycle, the chip surface can be regenerated using appropriate buffer conditions that remove bound analyte without affecting the activity of the immobilized ligand. In such a way, a derivatized surface can be used up to 400 times providing reproducible results, stable baseline, high chemical stability and low non-specific binding.

The quantitative characterization of the interactions between binding partners using SPR allows the elucidation of many facets of binding events (Nagata et Handa, 2000): the ***specificity*** of an interaction can be assessed; the biosensor can be used to measure the concentration, or better the exact ***active concentration*** of an analyte in a sample; binding ***kinetics*** can be defined by measuring the association and the dissociation rates, enabling to evaluate the binding ***affinity***. However, for each purpose, appropriate settings for a number of parameters have to be chosen, such as for the ligand density on the chip surface, analyte concentration and flow rate. The binding rate, and thus the SPR signal, are mainly dependent of 2 factors: interaction kinetics and mass-transport limitations. For kinetic measurements, the lowest possible level of

immobilized ligand should be used in order to ensure that the binding rate is dictated by the interaction kinetics and not limited by mass transport processes in the sample solution. The mass transfer of analyte from the bulk flow to the surface with the ligand is much faster in that case and the measured binding reflects exclusively binding kinetics. For active analyte concentration measurements, a high ligand loading should be used for the dextran matrix. Mass transport is then binding rate limiting, obtained sensorgrams represent the mass transport which directly reflects the analyte concentration (Richalet-Sécordel et al., 1997; Christensen et al., 1997).

5.2. Preparation of sensor surfaces

Reporter HNE peptide was coupled to the sensor surface as advised by the supplier (BIAapplications Handbook, Biacore, Uppsala, Sweden). A 100 µM solution of oxidized HNE peptide in 10 mM formic acid (pH 4.3) was injected onto a CM5 senor chip and the peptide was conjugated to the carboxylated dextran matrix either by thiol activation of available sulfhydryl groups in the HNE sequence or by N-hydroxy-succinimide/N-ethyl-N'-[(3-dimethyl-amino)propyl] carbodiimide hydrochloride (NHS/EDC) coupling via an ε-amino group of an additional N-terminal lysine, added to the HNE sequence. A control surface was prepared by immobilizing an irrelevant peptide (GIIDLIEKRKFNQNSNSTYCV) in the second flow cell of the CM5 sensor chip. 290 resonance units (RU) of oxidized peptide (MW: 2495.625) were immobilized on the sensor surface corresponding to about 250-300 $pg \cdot mm^{-2}$ of peptide on the chip. We calculated a theoretical R_{max} of $(290/2496.8) \cdot 150000 = 17422$ RU, if every immobilized peptide molecule would bind one antibody molecule. With 15 nM of mAb BH216, R_{eq} values of 144 RU were observed, which would mean that only 0.83 % of the immobilized peptide were recognized as epitopes. Because of the low density of functional peptide on the sensor surface, the binding rate was assumed to be predominantly determined by interaction kinetics and to a lesser extent limited by mass transport processes and therefore suitable for kinetic measurements.

5.3. Interaction kinetics

Kinetic measurements of the oxidized reporter peptide were performed on a BIACORE3000 instrument (Biacore Inc., Uppsala, Sweden) using increasing concentrations of active mAb BH216 (0.625 - 10 nM). The concentration of active mAb was determined by varying flow rates under conditions of partial mass transport limitation, using the method described by Richalet-Secordel et al. (1997). Thus, the exact concentration of active mAb could be determined without the need of a calibration curve. The active mAb concentration $[BH216]_{act}$ of 14.54 nM corresponds to about 50% of a mAb concentration of 30 nM determined by HPLC and Bradford assay. Binding of mAb BH216 to the immobilized reporter peptide was recorded by sensorgrams allowing an association time of 300 s and a dissociation of 180 s under a constant flow rate of 20 µl/min at 25°C. The sensorgram profile of each run was subtracted by the signal of the irrelevant peptide on the control surface. Data were analyzed according to a '1:1 Langmuir Binding' model (χ^2 = 0.769), a 'Two-State Reaction' model assuming a conformational change (χ^2 = 0.548) and a 'Bivalent Analyte' (χ^2 = 0.539) model using the BIA EVALUATION 3.01 software. It was not possible to generate kinetic data for the linear and most of the substituted HNE peptides because of their very low affinity for mAb BH216.

5.4. SPR solution competition assays

Interaction analysis was carried out at 25°C in HEPES buffered salt solution (HBSS) [10 mM Hepes, 150 mM NaCl, 3.4 mM EDTA, 0.005% surfactant P20, pH 7.4]. RU values were measured in the presence of soluble inhibitor peptide. 10 µM of reduced or oxidized competitor peptide was equilibrated for 2 h with 20 nM of active mAb. Sensorgrams measured binding of uncompeted mAb BH216 in solution to the immobilized reporter peptide during an association time of 180 s and a dissociation time of 120 s at a constant flow rate of 20 µl/min at 25°C (Figure 2.2). The sensorgram profile of each inhibiting peptide analyte was subtracted by the signal to the control canal with the irrelevant peptide. Binding of BH216 to soluble peptide was measured by estimating RU_{eq} using the BIA EVALUATION 3.01 software. The relative RU values (RU_{rel}) were obtained by normalizing the calculated RU_{eq} values with the average RU_{eq} value measured with mAb BH216 alone.

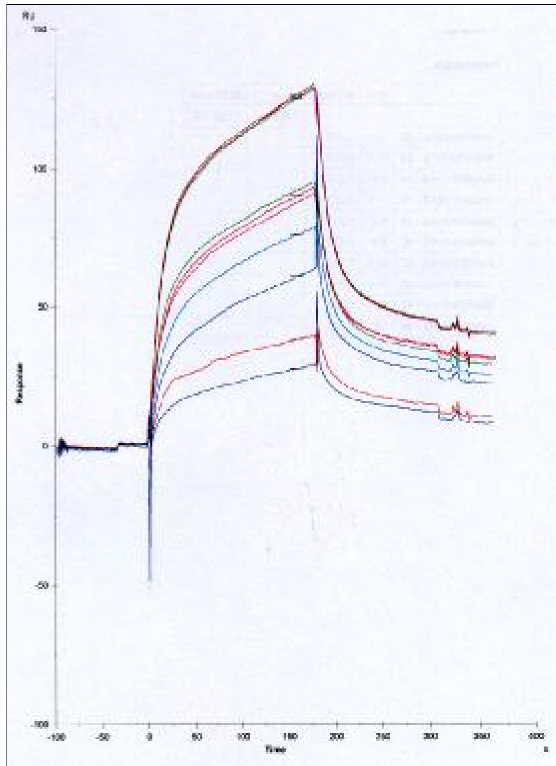


Figure 2.2. Sensorgram profiles after SPR solution competition assays.

The sensorgrams measured association to and dissociation from the immobilized HNE peptide ligand by mAb BH216 uncompeted or not by free HNE analogues in solution. High RU signals in the association rates reflect no binding to mAb BH216 by free HNE analogues (and thus no inhibition) and low signals reflect binding of free analogues and thus inhibition of binding to immobilized reporter peptide. The relative RU values (RU_{rel}) (Figure 3.7) were obtained by normalizing the calculated RU_{eq} values with the average RU_{eq} value measured with mAb BH216 alone.

The chips were washed and regenerated with 50 mM HCl. Full biological activity of the ligand surface was confirmed after every ten runs, by performing a kinetic run with mAb BH216 without competitor peptide. 148 runs were performed with the same sensor surface. No degradation or memory effect was observed.

6. Flow Cytometry

The flow cytometry technique is a powerful tool for defining and counting lymphocytes or characterizing the binding characteristics of antibodies targeting proteins or receptors expressed on the surface of living cells. By detecting and counting individual, labeled cells passing in a stream through a laser beam, a flow cytometer provides qualitative and quantitative information about single cells or molecules incubated with cells. A flow cytometry instrument equipped to separate and count the identified cells is called a fluorescence-activated cell sorter (FACS). These highly sophisticated devices enable the compendious characterization of the properties of cell subsets which are identified using mAbs to distinctive cell-surface proteins. Individual cells within a polyclonal, mixed population are marked after a first incubation with specific antibodies followed by a staining during a second incubation

with anti-immunoglobulin antibodies labeled with a fluorescent low molecular weight chromophore, commonly fluorescein isothiocyanate (FITC). The (labeled and unlabeled) cells, accommodated in a large volume of saline, are then forced through a nozzle, thus creating a fine stream of liquid containing single cells separated by regular intervals. As each cell passes through a laser beam it scatters the laser light and the dye molecules carried by the antibodies bound to the cell will be excited and will fluoresce at a characteristic wavelength. The FITC fluorochrome is for example excited at a wavelength of 495 nm and emits then a light at a wavelength of 519 nm. The use of separate mAbs stained with a unique dye permits thus the study of multiple properties of a cell surface. Sensitive photomultiplier tubes detect on the one hand the scattered light, giving information about the size and the granularity of the cell, and on the other hand the specific fluorescence emissions, providing qualitative information on the binding of distinctive, labeled mAbs and hence on the expression of cell-surface proteins by each cell (Shapiro, 1993).

In our study, flow cytometry was used to monitor the binding capacity of mouse immune sera induced with HNE-peptide-conjugates to cells expressing the MV H-protein in its native conformation, and thus characterize the crossreactive immunogenicity of peptide-conjugate immunogens. The crossreactivity of immune sera (1:100) was tested on a transfected human melanoma cell line (Mel-JuSo) expressing the native H protein. Mel-JuSo-H and -wt cell lines were kind gifts of R.L. de Swart, Institute of Virology, Erasmus University, Rotterdam, The Netherlands (de Swart et al., 1998). Briefly, the Mel-JuSo-H and Mel-JuSo-wt cells were thawed, cultured for three days at 37°C in RPMI 1640 medium (Cambrex Bio Science, Verviers, Belgium) supplemented with 5% heat-inactivated fetal bovine serum (FBS-HI) and 1% Penicillin-Streptomycin-L-Glutamin (PSG) (Invitrogen Corporation, Merelbeke, Belgium), harvested, washed in FACS medium [PBS, 0.5% BSA, 0.05% NaN_3] and plated in 96-well U-bottom plates at a concentration of $4 \cdot 10^6$ cells/ml. Cells were incubated for 60 min on ice in serum samples (1:100) diluted in FACS medium, washed and stained for 30 min on ice with a 1:200 diluted FITC labeled goat-anti-mouse IgG (Sigma-Aldrich, Bornem, Belgium). The fluorescence was measured by flow cytometry on an Epics Elite ESP instrument (Coulter company, Miami, FL, USA) as described previously (Muller et al., 1995a). FITC-conjugate alone, pre-immunization sera on Mel-JuSo-H, anti-DT sera on Mel-JuSo-H and anti-

peptide-DT conjugate immune sera on Mel-JuSo-wt cells were used as negative controls. Data were expressed as arbitrary fluorescence units (AFU). Mouse immune sera were all tested individually as pooled sera were found to reflect an observed experimental situation only with little accuracy. Detected AFU values were evaluated by statistical analysis (see section 11).

7. *In silico* molecular modeling

3D structures generated by crystallographic or NMR studies have remarkably enhanced our understanding of (inter)action of biological macromolecules and structure/activity relationships. Structural data of this kind is however laborious and moreover very difficult to generate. Particularly, in the case of the MV H protein no crystallographic structure is available to date. Computer assisted molecular design enables the simulation of the structure, behavior and interactions of biomolecules using powerful computers. Dynamic changes in structures can readily be visualized and the interaction of complex chemicals can be predicted accurately over time. In addition, this graphical simulation allows a rapid trial *in silico* of a vast array of ideas and focus precisely on the most promising candidate structures. Using adequate molecular modeling software, the rational design of structures becomes reality and can thus suggest compounds for synthesis eventually. Structure determination of macromolecules can be performed through homology modeling techniques based on X-ray crystallography or NMR structural data of homologous molecules. An integrated approach facilitates rapid verification and corroboration of simulated data against experimental data or vice versa. Modeling tools also enables to construct *de novo* on the computer molecular structures that can subsequently be further simulated and analyzed. Such structures are built iteratively by sketch and aggregation of existing fragments of a structural library, applying symmetry operators or importing whole fragments from other structural systems (www.accelrys.com).

The generation of 3D structural models using molecular modeling tools relies on the application of molecular mechanics/dynamics and force field technology. However, atomistic molecular simulations have often been limited by the lack of adequate force field parameters for the biological systems of interest and the accuracy of the force field was often found to be unsatisfactory, especially when applied to unusual types of

molecules, structures or environments. Earlier generations of developed force fields were only based on scarce data of largely differing accuracies and types of measurements. Regardless, most of the nowadays used force field parameters have been created by computing the properties of over 1000 different molecules resulting in at least a million quantum mechanically computed energies and energy derivatives. Sophisticated molecular modeling software allows to select from a variety of force fields, such as Amber, CVFF, CFF, ESFF, CHARMm, Engh and Huber or XPLORh when it comes to perform mechanics or dynamics simulation runs (Dauber-Osguthorpe et al., 1988; Hwang et al., 1998).

The force field used in this study was the Consistent Valence Force Field (CVFF), a quadratic diagonal force field (class I force field) (Dauber-Osguthorpe et al., 1988):

$$V = \sum^{n\text{bonds}} V_{bond} + \sum^{n\text{angles}} V_{angles} + \sum^{n\text{torsions}} V_{torsion} + \sum^{n\text{pairs}} V_{non\text{-}bond}$$

The conformational space of molecules is explored using molecular mechanics. Simple minimization runs delineate potential strains and stresses in conformationally flexible molecules, while dynamics allow the exploration of the entire conformational space available to a molecular system in varying simulated conditions. Even concerted molecular motions can be assessed in more complex simulation experiments and include modeled cascades of minimizations, molecular dynamics for any length or number of steps, which may be further combined with heating, equilibration and cooling stages. The modeling software usually allow to select among several types of simulation environments: *in vacuo*; implicit solvation models with either a distance dependent dielectric constant or a generalized Born approximation thereof; models having recourse to explicit water molecules with either cubic or spherical boundary conditions. Hydrogen bonds can be added automatically and the simulated molecules can be soaked in water or other solvents.

Advanced software tools offer numerous visualization options to suit for a specific purpose or study. Meaningful conclusions from large amounts of simulated data, otherwise difficult to interpret, can readily be drawn using the accurate analytical software tool.

The INSIGHT II software (Accelrys, San Diego, CA, USA; www.accelrys.com) was used for molecular modeling on a Silicon Graphics workstation. The core residues 384-396 (QACKGKIQALCEN) of the HNE peptide were modeled with the BIOPOLYMER module. The peptide was cyclized with a disulfide bond between C386 and C394 and the molecule was protonated at pH = 7.4. The model was then energy-minimized using the DISCOVER module of the INSIGHT II package. Energy minimization was based on CVFF potentials and carried out in 1000 cycles of steepest descent, followed by 2000 cycles of conjugate gradient minimization. Energy minimization was discontinued when the final derivatives were less than 0.001 $kcal \cdot mol^{-1} \cdot A^{-1}$. In order to assess the stability of this loop conformation, dynamic energy sampling runs were performed in a periodic box of explicit water molecules at simulation temperatures of 300 K and 1000 K using the method described by Bartels et al. (1998). At lower temperatures (e.g. 300 K), free energy barriers between distinct conformations can trap the system in a local, higher minimum energy and prevent the system to explore the entire space of possible conformers. The peptide was centered in a cubic cell (30.0 Å) and explicit water molecules were added using the SOLVATATION module of the INSIGHT II software. Unallowed steric overlaps were automatically excluded by the SOAK module. The resulting system contained 2649 atoms; 201 peptide atoms and 816 water molecules. The dynamic simulation runs were performed during 50 ps using integrator steps of 1 fs. The conformers corresponding to distinct local energy minima were subsequently energy minimized as described above and superimposed in order to compare the peptide backbone conformation and the side chain orientation.

8. Monoclonal antibodies (mAbs)

The technique to produce monoclonal antibodies (mAbs) has initially been developed by Köhler et Milstein (1975) for which they were awarded the Nobel Prize in 1984.
The mAbs BH216, BH21, BH6 and BH195 used in this study have previously been generated against native MV (BH216, BH21, BH6) and denatured MV (BH195) and found to crossreact with peptides mimicking the HNE domain of the H protein (Ziegler et al., 1996). 8-10-week-old BALB/c mice were immunized by i.p. or multifocal subcutaneous injections with purified native or denatured (5 min boiling in 1 mg/ml SDS under non-reducing conditions) MV emulsified in incomplete Freund's

adjuvant (Sigma-Aldrich, Bornem, Belgium). The animals were boosted with the same antigen preparation on day 21 and 38 (denatured MV) or day 49 (native MV). On day 41 or 52, spleen cells were explanted and fused with Sp2/0 myeloma cells. Hybridomas specific for MV were isolated by ELISA or by Western blot and were cloned by limiting dilution in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 mM hypoxanthine, 1 mM glutamine, 1 mM sodium pyruvate, 100 IU/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum (FBS) (all from Gibco, Brussels, Belgium). Monoclonal antibodies BH216, BH 21, BH6, BH195 (Ziegler et al., 1996) were harvested from ascites or from hybridoma supernatant produced in a Cell-Line system (Integra, Wallisellen, Switzerland), purified by affinity chromatography using a HiTrap Protein G HP column (Amersham Biosciences, Uppsala, Sweden) on a ÄKTA Explorer system (Amersham Biosciences, Uppsala, Sweden) (see section 3.3), and dialyzed in a 50 mM borate, 150 mM NaCl buffer (pH 7.5). The concentration was adjusted to 1.55 mg/ml (=10 μM). Ig subclasses and isotypes were determined with a mouse mAb isotyping kit (Mouse Typer® Sub-Isotyping Kit, BIO-RAD Laboratories, Nazareth Eke, Belgium) .

9. *In vitro* neutralization assays

Neutralization assays represent an *in vitro* format for assessing the neutralizing capacity of mAbs or polyclonal immune serum samples which have been generated against a specific pathogen, protein toxin or antigenic determinant. For a virus neutralization assay, cells susceptible to the virus are commonly grown on flat bottomed 96-well plates. A previously defined and constant amount of virus, priory incubated with serial dilutions of a specific mAb or serum, is then added to the cells. With increasing mAb or serum dilution less virus particles can potentially be neutralized. Consequently cells are infected and a typical cytopathic effect (CPE) of syncytia formation associated with virus replication can be observed. For the reliable comparison of the neutralization of different virus strains by a given mAb or serum sample, identical tissue-culture-infective-doses ($TCID_{50}$) for each specific virus have to be used. The read-out of this neutralization assay is only qualitative (a quantitative *in vitro* format is the so-called Plaque Reduction Neutralization (PRN) assay, not used in this study). The neutralization titer of the neutralizing mAb or serum is the dilution

at which the cytopathic effect is prevented and can be precisely calculated using an appropriate algorithm.

The $TCID_{50}$ of different virus stocks was determined by titration of the MV virus infectivity on Vero SLAM cells as described earlier (Norrby et Gollmar, 1972; Muller et al., 1995a). An early passage of MV Edmonston strain (American Type Culture Collection (ATCC VR-24), USA) was used as a reference strain. MV wild-type isolates/reference strains for the different clades and genotypes were kind gifts of P.A. Rota, Centers for Disease Control and Prevention, Atlanta, GA, USA, and are gratefully acknowledged. For each MV isolate, 75 µl of a 1:100 dilution in Dulbecco's Modified Eagle's Medium (DMEM) (Cambrex Bio Science, Verviers, Belgium) of virus stock aliquots stored at –80°C was disposed on a sterile, flat bottomed 96-well plate (CELLSTAR, greiner bio-one, Frickenhausen, Germany) kept on ice and diluted DMEM using 2-fold serial dilution steps. Subsequently, $7.5 \cdot 10^3$ Vero SLAM cells in 75 µl of 'Triple medium' [DMEM (Cambrex Bio Science, Verviers, Belgium) supplemented with 3% Ultroser-G (CIPHERGEN, BioSepra s.a., Cergy-Saint-Christophe, France) and 3% Penicillin-Streptomycin-L-Glutamin (PSG) (Invitrogen Corporation, Merelbeke, Belgium)] and 75 µl of DMEM were added to each well. Cells were incubated for 5 days in a 37°C, 5% CO_2, humidified incubator before the read-out was carried out. $TCID_{50}$ determination was performed in triplicates or quadruplicates. The $TCID_{50}$ was defined as the final virus dilution where a cytopathic effect was observed in 50% of the wells of identical virus dilution and calculated using the computer program ID-50 version 5.0 (National Center for Biotechnology, Rockville, MD, USA).

MV neutralization was measured in the presence of serial dilutions of mAbs BH216, BH21 and BH6 on Vero SLAM cells. An early passage of MV Edmonston strain (American Type Culture Collection (ATCC VR-24), USA) was used as a reference strain. For each mAb, 75 µl of a 1:100 dilution in DMEM (Cambrex Bio Science, Verviers, Belgium) of mAb ascites stock aliquots stored at –80°C was disposed on a sterile, flat bottomed 96-well plate (CELLSTAR, greiner bio-one, Frickenhausen, Germany) kept on ice and diluted in 4°C DMEM using 2-fold serial dilution steps. Subsequently, 75 µl containing 100 $TCID_{50}$ of each MV isolate (Table 2.2) in 4°C

DMEM were added to each well and incubated for 3 h at 4°C. Finally, $7.5 \cdot 10^3$ Vero SLAM cells in 75 µl of ‘Triple medium’ [DMEM (Cambrex Bio Science, Verviers, Belgium) supplemented with 3% Ultroser-G (Invitrogen Corporation, Merelbeke, Belgium) and 3% Penicillin-Streptomycin-L-Glutamin (PSG) (Invitrogen Corporation, Merelbeke, Belgium)] were added to each well. Cells were incubated for 5 days in a 37°C, 5% CO_2, humidified incubator before the read-out was carried out. Neutralization assays for each mAb and each MV isolate (Table 2.2) were performed in triplicates. The neutralization titer was defined as the final mAb dilution where a cytopathic effect was prevented in 50% of the wells of identical mAb dilution and calculated using the computer program ID-50 version 5.0 (National Center for Biotechnology, Rockville, MD, USA). Titers below 1:100 were considered negative.

Table 2.2: Wild-type MV isolates tested in neutralization assays

Clade	Genbank	Protbank	MV isolate[1]	Location	HNE domain sequence
A	U03669	AAA56659.1	Edmonston wt	USA	ETCFQQACKGKIQALCENPEWA
A	U08417	AAA50551	Philadelphia 26	Philadelphia USA	---------R------------
D3	L46763	AAA74535	TX-92	Texas USA	-----------N----------
D3	AF009581	AAB63783	CA:7/28/96	California USA	-----------N----K-----
D3	M81896	AAA46426	Chicago-2	Illinios USA	----------R-----------
D5	L46757	AAA74532	PAL-93	Palau	--------Q-------------
G1	AF079553	AAC29441	Berkeley	California USA	--------------I--D----
H1	AF045196	AAC03028	China93-2	China	------------------L---
H2	AF045203	AAC03035	China94-1	China	-----------------D----

[1] MV wild-type isolates/reference strains for the different clades and genotypes were kind gifts of P.A. Rota, Centers for Disease Control and Prevention, Atlanta, GA, USA.

10. *In vivo* animal experiments

10.1. Intraperitoneal immunizations (i.p.)

Immunization of mice in order to induce a humoral immune response involves the repeated injection of the immunogen in presence of an adjuvant. Despite the extensive use of Complete Freund's Adjuvant (CFA) in intraperitoneal (i.p.) immunization protocols, the ethics of administration thereof is at present disputed, because of its severe side-effects including granulomas, abscesses at the site of infection and carcinogenicity (Powell et Newman, 1995). Furthermore, its strong inflammatory action promotes a Th1 response with a predominance of IgG2a and IgG2b antibody isotypes (Chuang et al., 1997), moreover directed mostly against cryptic epitopes (Gomez et al., 2003). The induced immune response with CFA (and boosting with Incomplete Freund's Adjuvant (IFA)) may therefore be biased and not representative or compared to a potential humoral response elicited with Aluminium hydroxide gel (Alum, $Al(OH)_3$), the only adjuvant presently approved by the FDA for use in humans and stimulating predominantly a Th2 response, consequently promoting the production of mainly IgG1 antibodies (Gupta et Siber, 1995; Rowe et al., 2000). In our study, the usage of Alum was thus the preferred adjuvant in i.p. injections using the peptide-conjugates DT and TT.

In the crossreactive immunogenicity studies (Chapter 2, Results Part I), groups of five 10-14 week-old specific-pathogen-free BALB/c mice ($H2^d$) were immunized i.p. with 200 µl containing 50 µg of peptide-conjugate DT (corresponding to 5.0 µg of coupled peptide) or free dt in borate buffer [50 mM boric acid, 150 mM NaCl, pH 7.4], adsorbed on 500 µg aluminium hydroxide gel (Alhydrogel, Superfos Biosector, Frederikssund, Denmark). Mice were boosted on day 21 and serum was obtained on day 29. Prior to the bleeding procedure via the retroorbital vein, mice were anesthetized i.p. with 200 µl containing 2.5 mg Ketaminum hydrochloridum [Imalgène®1000, 100 mg/ml, MERIAL, Lyon, France], 50 µg Xylazinum hydrochloridum [Rompun®2%, 20 mg/ml, BAYER, Brussels, Belgium] and 50 µg Atropinum sulfuricum [Atropinum sulfuricum solutum 1%, 10 mg/ml, eurovet WDT, Garbsen, Germany].

In the immunogenicity studies relating to differential immunity statuses (Chapter 2, Results Part II), groups of five 9 week-old specific-pathogen-free BALB/c mice ($H2^d$) (B&K Universal Ltd, Hull, UK) were immunized i.p. with 200 µl containing either 10 µg of peptide-conjugate DT or TT (corresponding to 1.0 µg of coupled peptide) or free carrier protein dt or tt in borate buffer [50 mM boric acid, 150 mM NaCl, pH 7.4], adsorbed on 500 µg aluminium hydroxide gel (Superfos Biosector, Frederikssund, Denmark), or containing 50 µg of TB peptide dissolved in borate buffer (50 mM boric acid, 150 mM NaCl, pH 7.4) and emulsified (1:1) in CFA (Sigma-Aldrich, Bornem, Belgium). For the active priming, mice received a so-called priming immunization on day 0, and were then immunized on day 21 and 42. For the passive priming, mice received i.p. 150 µl of anti-carrier ([dt], [tt]) or 50 µl of anti-peptide-conjugate serum ([DT], [TT]) 3 days prior to the injection on day 0 and were then boosted on day 21 and 42. Mice were 'prebled' on day –1 and immune serum was obtained on days 29 and 50.

For assessing the compatibility of HNE-peptide-conjugate and subsequent measles virus immunizations (Chapter 2, Results Part II), groups of five 9 week-old BALB/c mice were immunized 3 times at 3 week intervals i.p. with 200 µl containing either 50 µg of DT or 50 µg of purified MV-H protein adsorbed on 500 µg aluminium hydroxide gel (Superfos Biosector, Frederikssund, Denmark). On day 52, they received i.p. $2.0 \cdot 10^5$ PFU of MV Edmonston strain in 200 µl of PBS and CFA (1:1). Mice were 'prebled' on day –1 and serum was obtained on days 50 and 60.

10.2. Intranasal immunizations (i.n.)

The use of non-invasive routes for vaccine delivery such as intranasal (i.n.) immunization remains a promising and compliant way for the generation of an effective immune response, both systemically and locally, as there is a large amount of associated lymphoid tissue and antigen presenting cells available (Bergquist et al., 1995). Mucosal surfaces are specifically structured for the development of humoral and cellular responses against pathogens or immunogenic proteins invading the host via the mucosal route. After initial contact sensitization, antigen-specific B and T cells migrate from the nasal-associated lymphoid tissue (NALT) to the regional lymph

nodes. A subsequent immune cell distribution called 'common mucosal immune system' warrants that all mucosal surfaces become replete with antigen-specific immunocompetent cells for protection. Experimental measles vaccines could be successfully delivered in young children after administration as an aerosol via the intranasal route (Bennett et al., 2002; Sepulveda-Amor et al., 2002). The choice of an appropriate adjuvant for i.n. immunization is often the key for an efficient induction of neutralizing and protective immune responses since many antigens introduced via the mucosal route are poorly immunogenic and may even induce a state of tolerance in the absence of a potent adjuvant. Bacterial toxins like cholera toxin (87 kDa) from *Vibrio cholerae* and the heat-labile enterotoxin (86 kDa) of *Escherichia coli* have been used successfully as mucosal (oral and nasal) adjuvants in many experimental studies to enhance the immune response to vaccines when co-administered (Snider, 1995). They induce predominantly Th2 responses by inhibiting the production of IL-12.

In the studies assessing the crossreactive and protective immunogenicity of the peptide-conjugates DT, TT and OVA in i.n. immunizations a modified protocol of Shen et al. (2001a, 2001b) was used. Groups of five 10-14 week-old specific-pathogen-free BALB/c mice ($H2^d$) were immunized i.n. with 50 µg of peptide-conjugate DT, TT or OVA (corresponding to 5 µg of coupled peptide) or 100 µg of chimeric TB peptide in borate buffer [50 mM boric acid, 150 mM NaCl, pH 7.4], mixed to 1.5 µg cholera toxin (ct) (Sigma-Aldrich, Bornem, Belgium). Each dose was divided and given on two consecutive days with a micropipettor in a volume of 20-30 µl. Prior to the i.n. administration of the immunizing solution, mice were anesthetized i.p. with 100 µl containing 1.25 mg Ketaminum hydrochloridum [Imalgène®1000, 100 mg/ml, MERIAL, Lyon, France], 25 µg Xylazinum hydrochloridum [Rompun®2%, 20 mg/ml, BAYER, Brussels, Belgium] and 25 µg Atropinum sulfuricum [Atropinum sulfuricum solutum 1%, 10 mg/ml, eurovet WDT, Garbsen, Germany]. Mice were boosted on days 21 and 42 and serum was obtained on days 29 and 50. Negative control groups were immunized with 1.5 µg ct in PBS.

10.3. Transcutaneous immunizations (TCI)

Transcutaneous immunization (TCI) is a new method of vaccine delivery using a topical application of an antigen mixed to an adjuvant on intact, wetted skin without physical penetration by needles (Glenn et al., 1998, 2000). It should be stressed that TCI differentiates form intradermal or subcutaneous inoculations, which are invasive routes of immunization. In TCI no needles are used, the skin is not penetrated or disrupted and the immunizing solution is simply applied on the hydrated skin left intact. Needle-free delivery has reached a global importance because of the risk of diseases associated with re-use and improper disposal of needles (WHO, 1996a, 1996b). Vaccine administration via a patch has other obvious practical merits. Vaccination through the skin may be particularly beneficial, as the epidermis extensively provides competent antigen presenting cells, called Langerhans cells. Langerhans cells, lying closely to the most superficial layer of the skin, the stratum corneum, are contained in a network of immune cells underlying 25% of the skin's total surface area. In fact, the principal barrier to skin penetration is constituted by the stratum corneum, mostly composed of dead cells and lipids, and can be readily permeabilized by hydratation using a patch applied on the naked skin, possibly allowing antigens to diffuse into the epidermis. After antigen uptake, Langerhans cells migrate to the draining lymph nodes where systemic immune responses are induced (Kripke et al., 1990).

As for most immunization protocols, TCI relies on the adequate use of potent adjuvants. Similar to the i.n. delivery, bacterial toxins like cholera toxin from *Vibrio cholerae* and the heat-labile enterotoxin of *Escherichia coli* are co-administered, or more precisely co-applied on the skin, in order to act both as adjuvants and antigens.

In the studies assessing the crossreactive immunogenicity of the peptide-conjugates DT, TT and OVA in TCI immunizations a modified protocol of Glenn et al. (1998) and Scharton-Kersten et al. (2000) was used. Groups of five 10-14 week-old specific-pathogen-free BALB/c mice ($H2^d$) were shaved on the dorsum using a clipper and rested for 48 h. Prior to immunization, mice were anesthetized i.p. with 250 µl containing 3.12 mg Ketaminum hydrochloridum [Imalgène®1000, 100 mg/ml, MERIAL, Lyon, France], 62.5 µg Xylazinum hydrochloridum [Rompun®2%, 20

mg/ml, BAYER, Brussels, Belgium] and 62.5 µg Atropinum sulfuricum [Atropinum sulfuricum solutum 1%, 10 mg/ml, eurovet WDT, Garbsen, Germany] during the immunization procedure to prevent grooming. The naked, shaved skin was hydrated by wetting with PBS-drenched cotton wool for 10 min and slightly blotted with dry cotton wool prior to immunization. TCI was performed by placing using a micropipettor 100-120 µl immunizing solution containing 50 µg of peptide-conjugate DT, TT or OVA (corresponding to 5 µg of coupled peptide) or 100 µg of chimeric TB peptide in borate buffer [50 mM boric acid, 150 mM NaCl, pH 7.4] mixed to 15.0 µg cholera toxin (ct) (Sigma-Aldrich, Bornem, Belgium) on the naked skin area for 60 min. The mice were then extensively washed, tails down, under running tap water at RT. Mice were boosted on days 21 and 42 and serum was obtained on days 29 and 50. Negative control groups were immunized with 15.0 µg ct in PBS.

10.4. Challenge/Protection experiments

The animal model used in this study is suited to assess *in vivo* the protective capacity of antibodies, which can be either mAbs or polyclonal immune serum, against measles infection. Mice are normally not susceptible to MV infection. However, after intracranial infection, a rodent-adapted MV (CAM/RB) causes encephalitis in mice (in particular with $H2^k$-restricted MHC haplotype) which is characterized by typical brain lesions. The strain CAM/RB has been expanded in mice and the TCID50 titer has been determined *in vitro* using Vero cells. The MV CAM/RB strain was a kind gift from Dr. U.G. Liebert, University of Würzburg, Germany (Liebert et ter Meulen, 1987) and is gratefully acknowledged.

Groups of six 3 week-old specific-pathogen-free CBA mice ($H2^k$) (B&K Universal Ltd, Hull, UK) were immunized intraperitoneally with 100-200 µl of pooled anti-peptide-conjugate serum generated in the different immunization schedules described above and were challenged simultaneously by intracerebral injection of a lethal dose (12500 $TCID_{50}$) of a CBA-adapted MV (derived from the CAM/RB strain). Control mice received either solely anti-carrier protein serum (dt_dt_dt, tt_tt_tt) or PBS or an injection with a protective mAb BH81. Survival was monitored for 30 days.

11. Statistical analysis

For monitoring the End Point Titers (EPT) and the crossreactivity (AFU values), mouse immune sera were all tested individually in ELISA or in flow cytometry. Pooled sera were found to reflect an observed experimental situation only with little accuracy. Detected EPT and AFU values were subsequently evaluated by statistical analysis (Bland, 1996).

The ELISA and FACS data obtained with mouse sera are presented in the figures as the geometric means of the values obtained with five individual animals. The error bars represent 95% confidence intervals, where $n = 5$. Comparisons between antibody levels for given groups were performed by using an unpaired, two-tailed Student's *t* test. p values of <0.05 were considered significant and p values of <0.01 and <0.001 are displayed when detected.

In the animal challenge/protection experiments, the significance of the differences in survival rate following intracerebral challenge with the rodent-adapted CAM/RB virus between the different groups of mice ($n = 6$) was analyzed using the Fisher's exact test (Bland, 1996).

Chapter 3:

Results. Part I.

In order to understand the native conformation of the HNE epitope in the virus, the structural features of peptides corresponding to the HNE domain were investigated using protective anti-MV mAbs. This should generate evidence as to the specific interactions provided by the viral protein required for optimal design of an immunogenic peptide which would be able to induce antibodies crossreacting with the native epitope with a similar fine-specificity. Both, solvent- as well as matrix-molecules play a major role in stabilizing the conformation of a peptide in binding assays and peptide-antibody complexes (Goldbaum et al., 1996; Ochoa et al., 2000). Peptides can adopt different conformations, whether they are adsorbed, conjugated or free in solution, possibly leading to different results in different immuno-assays (van Regenmortel et Muller, 1999). Therefore, binding studies were performed using solution and solid phase formats including surface plasmon resonance. Binding data were corroborated by a molecular model of the epitope and by immunization experiments with peptide-conjugates. Key residues for antigenic binding and crossreactive immunogenicity were delineated and their presence in wild-type MV strains correlated with neutralization thereof.

1. Importance of a disulfide bond

It was reported that mAb BH216 recognizes MV only under non-reducing conditions, suggesting that a disulfide bond in the epitope is required for binding (Ziegler et al., 1996). Kinetic antibody binding studies were performed using surface plasmon resonance on the immobilized oxidized HNE reporter peptide. When the binding data were analyzed using a '1:1 Langmuir Binding' model (χ^2 = 0.769), an apparent association rate constant k_a = 2.49 x 10^5 M^{-1} s^{-1} and a dissociation constant k_d = 1.89 x 10^{-3} s^{-1} were determined corresponding to a high affinity constant of 7.60 nM. In the 'Two State Reaction' model assuming a two step association or an 'induced fit' binding, a χ^2 = 0.548 was obtained. Although suggestive of a two step association, the difference between the above χ^2 values was not considered significant enough to retain this hypothesis. It was not possible to generate kinetic data for the linear HNE peptide because of its low affinity for mAb BH216.

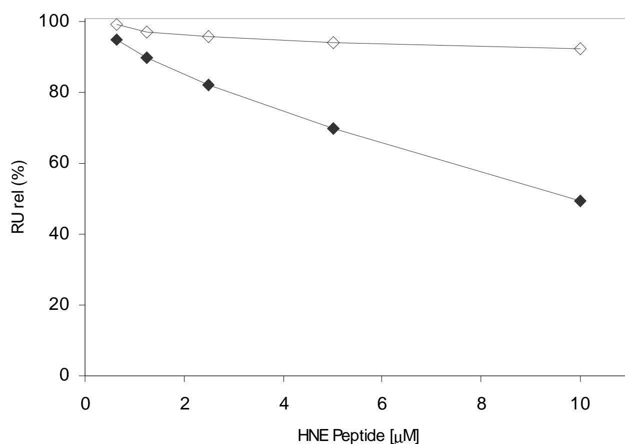


Figure 3.1: BIACORE binding competition of mAb BH216 to immobilized HNE reporter peptide.

Binding of mAb (20 nM) was monitored in the presence of increasing concentrations of oxidized (closed symbols) and reduced (open symbols) competitor HNE peptide. Relative resonance units (RUrel) were measured by surface plasmon resonance (SPR).

Using a SPR solution competition assay, RU_{rel} were measured in the presence of soluble reduced and oxidized competitor HNE peptide. The oxidized species reduced binding to 49.4% RU_{rel}. In contrast, even at concentrations of 10 µM, the reduced peptide inhibited antibody binding to the reporter peptide only by less than 7.3% (Figure 3.1). All other SPR solution competition assays were carried out at this concentration.

2. Oxidized peptide isoforms

The HNE peptide bears three cysteine residues in its primary sequence. Hence, a peptide molecule containing three cysteines can exist as three possible disulfide-linked forms (Browning et al., 1986). Stabilizing one of these isoforms is a difficult outcome as disulfide scrambling promotes an equilibrium where all three possible isomers coexist. Especially under alkaline conditions disulfide interchange of the free sulfhydryl with the existing disulfide bond readily occurs in oxidized fractions (Creighton, 1984). Linear HNE peptide elutes at 57.0% solvent B in RP-HPLC (Figure 3.2A). The mass of this peak (2497.867 Da) measured by MS corresponded to the calculated mass of the reduced peptide (2497.877 Da). Different substitution analogues of the HNE peptide were oxidized with DMSO and analyzed by RP-HPLC. The different oxidized peptides eluted as individual peaks corresponding to the reduced and oxidized isoforms. By monosubstituting each Cys with an amino butyric acid residue, the peaks eluting at 50.2%, 50.6% and 55.1% were assigned to defined isoforms, corresponding to the C381-C394 (Figure 3.2B), C381-C386 (Figure 3.2C) and C386-C394 (Figure 3.2D) bridged species, respectively.

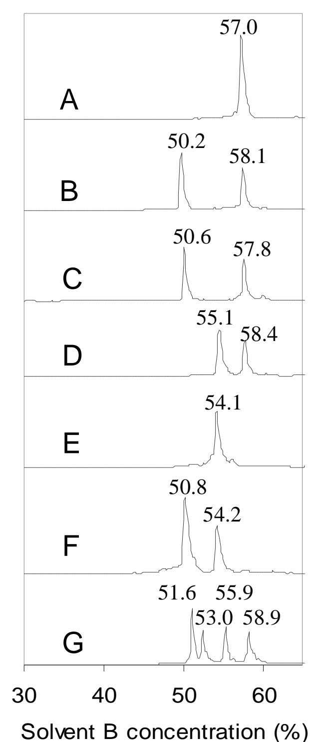


Figure 3.2: HPLC chromatograms of oxidized and reduced HNE peptides.

Reduced HNE peptide (A); monosubstituted C386B (B), C394B (C), C381B (D) and Q384A (G) after 4 h oxidation in 20% DMSO; purified C386-C394 bridged HNE (E); disulfide scrambling of purified C386-C394 bridged HNE after 6 h in ddH2O (F). Chromatograms were performed with 100 mg of peptide, except for G (200 mg) and F (300 mg) and monitored at 230 nm.

Some oxidized Ala-substitution analogues (e.g. A382F, A384Q, A393L, A395E) eluted as three distinct oxidized peaks (Figure 3.2G) whereas others eluted as two oxidized peaks. For instance, the original, unsubstituted HNE peptide oxidized in position C386-C394 eluted at 54.1% (Figure 3.2E), whereas its C381-C394 and C381-C386 bridged derivatives co-eluted as a single peak at 50.8% (Figure 3.2F) and could not be separated by HPLC. The lower mass of the oxidized species was confirmed (2495.625 Da) by MS and the peaks were sensitive to reduction by dithiothreitol treatment. The yield of the oxidation reaction for most substitution analogues was between 65-75% and the three different isoforms were found in similar amounts. When different isomers were purified by preparative HPLC (Figure 3.2E), lyophilized and dissolved in ddH_2O, disulfide scrambling occurred and a new equilibrium was rapidly reached, where all isomers coexist (Figure 3.2F). In cases where the C381-C394 and C381-C386 isoforms co-eluted, this peak represented about two third of the total peptide material. Preferential binding of mAb BH216 to the oxidized HNE peptide was confirmed by classical indirect ELISA. As expected the disulfide bonds were more stable under acid conditions than under basic conditions (Creighton, 1984). Although the stability decreased, the coating efficiency in microtiter plates increased at high pH (Figure 3.3). Under the basic conditions optimal for coating, the HNE peptide was at least partially oxidized and the signal of the reduced species increased as a result of oxidation, as mAb BH216 exclusively binds to oxidized HNE.

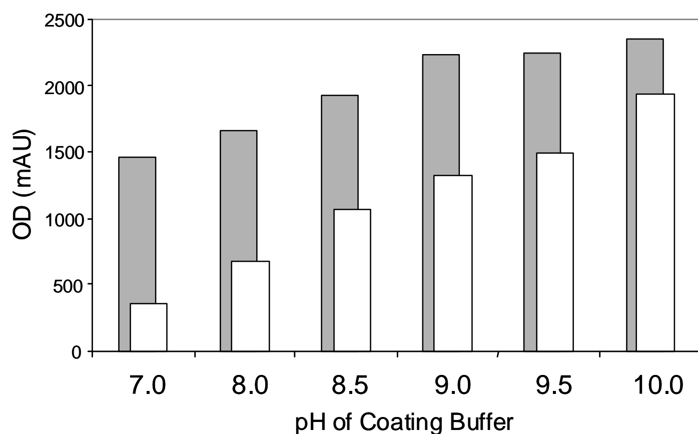


Figure 3.3: pH dependent binding response of mAb BH216 in ELISA.

MAb Binding to oxidized (closed bars) and non-oxidized (open bars) HNE reporter peptide (125ng/well) coated in PBS buffer with increasing pH. OD was measured 60 min after adding the substrate.

3. Identification of the active isoform

Because of disulfide scrambling HPLC-purified isoforms of the oxidized HNE peptide rapidly re-equilibrate, so that binding to the individual isomers cannot be assessed directly. In order to identify the active isomer, the cysteine residues of the HNE peptide were monosubstituted by amino butyric acid (C381B, C386B, C394B) or Ala (C381A, C386A, C394A). In indirect ELISA (Figure 3.4A) and in inhibition ELISA (Figure 3.4B), replacing C386 or C394 precluded antibody binding and mAb BH216 recognized only the oxidized peptides C381B and C381A, where C381 of the HNE peptide was substituted.

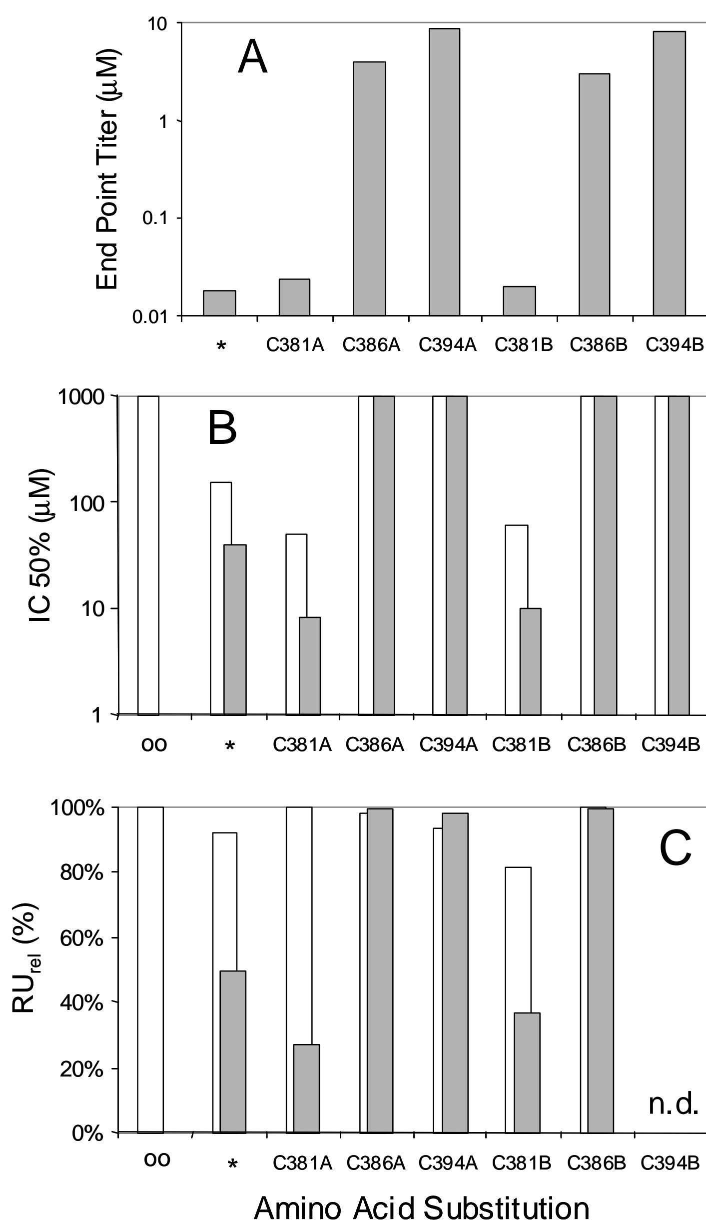


Figure 3.4: Indirect ELISA (A), Inhibition ELISA (B) and SPR solution competition binding assay (C) with monosubstituted full length HNE peptides.

Each Cys was replaced by an Ala (C381A, C386A, C394A) or an amino butyric acid residue (C381B, C386B, C394B). Oxidized (closed symbols) and reduced (open symbols) substituted peptides were tested for their capacity to bind, when coated, directly (A) to mab BH216 or to inhibit binding, when free in solution, of mAb BH216 to coated (B) or immobilized (C) HNE peptide. No inhibition of binding was observed when BH216 was used in the absence of competitor peptide (°°). Unsubstituted HNE peptide (*). Values not determined (n.d.)

Interestingly, the latter two peptides inhibited more strongly (IC50% = 8 μM) than the unsubstituted HNE peptide (IC50% = 40 μM), probably as a result of disulfide scrambling in the unsubstituted peptide. Similar results were obtained by SPR solution competition binding assay, where RU_{rel} of 49.6% were measured for the unsubstituted HNE peptide, compared to 26.9% for C381A, or 36.7% for C381B (Figure 3.4C). Species substituted at either positions C386 or C394 by alanine or amino butyric acid also abrogated the peptides' ability to inhibit antibody binding to the immobilized reporter peptide. As expected, inhibition with the reduced substitution analogues was very weak. Both ELISA and BIACORE results demonstrate that among the three oxidized isoforms, only the one with a cystine bridge linking C386 and C394 is recognized by mAb BH216.

	E 395	N 396	P 397	E 398	W 399	A 400
E 379	>10	0.04	0.03	0.08	0.02	0.02
T 380	>10	0.12	0.10	0.20	0.02	0.08
C 381	>10	0.18	0.04	0.20	0.10	0.06
F 382	>10	0.10	0.03	0.12	0.04	0.04
Q 383	>10	2	0.80	0.80	0.10	0.12
Q 384	>10	4	0.25	0.80	0.10	0.10

Figure 3.5: Reactivity of BH216 with C- and N-terminally truncated HNE peptide analogues in indirect ELISA.

Letters designate the last C-terminal amino-acid (columns) or the first N-terminal amino acid (rows) of the peptide ETCFQQACKGKIQALCENPEWA. Rows corresponds to peptides with the same N-terminus and truncated from the C-terminal end. Data are expressed as End Point Titers (EPT) (mM). Antibody binding to truncated HNE peptide (EPT < 1.0 mM) is shown as open fields. No binding is shown as filled fields (EPT > 1.0 mM).

4. Epitope localization with truncation analogues

In order to determine the minimal core sequence of the HNE peptide necessary for binding to mAb BH216, the peptide was gradually truncated from the N- and the C-termini and the shortened analogues were assessed for binding of mAb BH216 in

standard indirect ELISA (Figure 3.5). The five first amino acids of the N-terminus were omitted without any loss of binding activity. Similarly, the C-terminus could be shortened by the four last amino acid positions. Thus, the core of the HNE epitope is Q_{384}ACKGKIQALCEN$_{396}$, including C386 and C394. The disulfide bridge between these two Cys residues reflects the structural constraint required for binding to the key residues of the epitope.

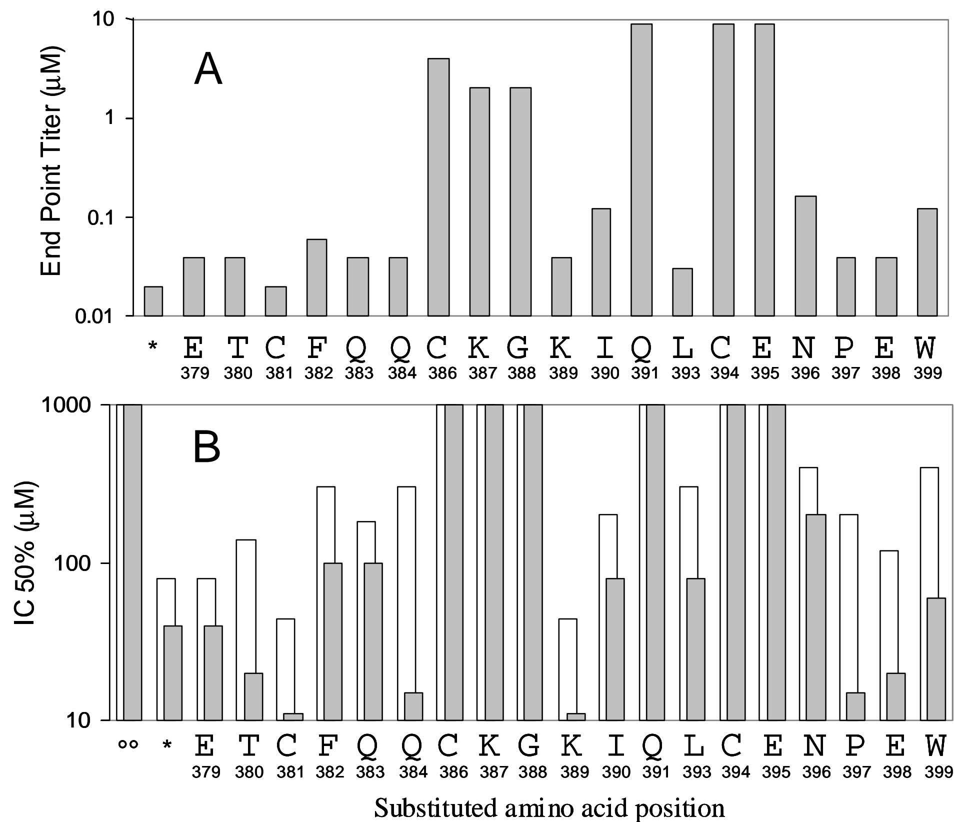


Figure 3.6: ELISA binding assays with Ala-substituted HNE peptides.

(A) End Point Titers of coated, substituted, oxidized HNE peptide [E_{379}TCFQQACKGKIQALCENPEWA$_{400}$], in which each position was substituted by Ala, after assessing of direct binding to mAb BH216 using indirect ELISA. (B) 50% Inhibition Concentration of substituted oxidized (grey bars) and reduced (white bars) competitor peptide after assessing of binding inhibition of mAb BH216 to coated, oxidized, unsubstituted HNE peptide (1.0 µM). No inhibition of binding was observed when BH216 was used alone in the absence of peptide (°°). Unsubstituted HNE peptide (*).

5. Critical contact residues and the HNE binding motif

Individual residues critical for binding of the HNE peptide to the protective mAb BH216 were determined by substitutional analysis in which each position of the HNE peptide was replaced by an Ala residue. Binding was abrogated in indirect ELISA (Figure 3.6A), in inhibition ELISA (Figure 3.6B) and in SPR solution competition binding assays (Figure 3.7A) when K387, G388, Q391 and E395 were substituted. A less pronounced inhibition was observed in peptides substituted in position I390, L393 and N396 (Figure 3.7A).

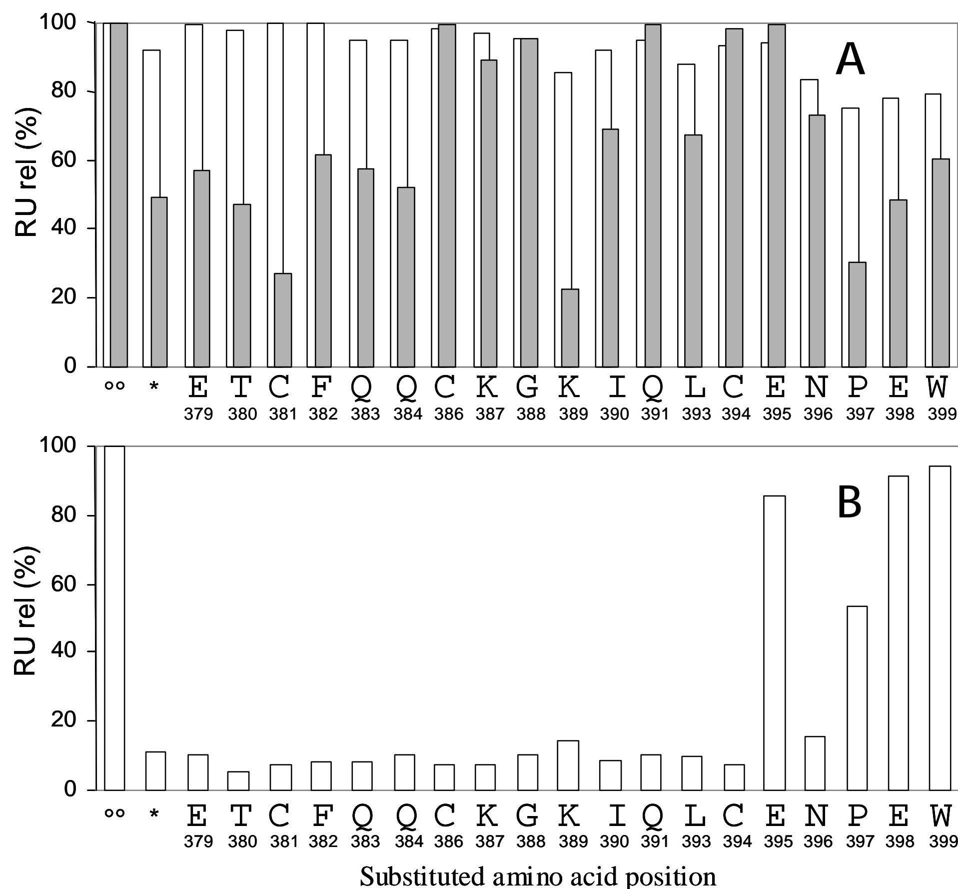


Figure 3.7: SPR solution competition assay with Ala-substituted HNE peptides.

Binding inhibition of (A) mAb BH216 (20 nM) and of (B) mAb BH195 (20 nM) was assessed by measuring RU_{rel} in the presence of 10 µM of monosubstituted oxidized (black bars) and reduced (white bars) competitor peptide [E_{379}TCFQQACKGKIQALCENPEWA_{400}] in which each position was substituted by Ala. Positions with original Ala are not represented. No inhibition of binding was observed when BH216 or BH195 were used alone in the absence of peptide (°°). Unsubstituted HNE peptide (*).

The other residues were substituted without significant loss of binding. As expected, a very weak inhibition of binding was observed with reduced substitution analogues (Figure 3.7A).Interestingly, Ala substitutions of positions C381, K389 and P397 increased dramatically the binding of the oxidized HNE competitor peptide. The C381A substitution precluded the formation of inactive oxidized isoforms as a result of disulfide scrambling (Figure 3.7A). Peptides monosubstituted with an Asn, Arg, Gln, Glu or a Ser residue were tested for binding in classical indirect ELISA to three distinct protective mAbs BH216, BH21 and BH6. In Figure 3.8, the results for BH216 are shown. Irrespective of the mAb, most amino acid positions could be replaced without any significant influence on antibody binding. However, none of the above amino acids was tolerated in positions of the key residues K387, G388, Q391 and E395, with the exception of K387, which tolerated also Arg. I390 can be replaced by Ala, Asn and Gln, but not by Glu, Arg or Ser. Thus, these positional scans suggest the binding motif $X_7C[KR]GX[AINQ]QX_2CEX_5$ of protective antibodies.

Substituted amino acid position

*	E	T	C	F	Q	Q	A	C	K	G	K	I	Q	A	L	C	E	N	P	E	W	A
	379	380	381	382	383	384	385	386	387	388	389	390	391	392	393	394	395	396	397	398	399	400
A	0.02	0.04	0.04	0.02	0.06	0.04	0.04	0.03	4	2	2	0.04	0.12	>10	0.02	0.03	>10	>10	0.16	0.04	0.04	0.12	0.02
E	0.03	0.03	0.04	0.10	0.12	0.04	0.04	0.08	>10	>10	>10	0.50	>10	>10	0.01	0.25	>10	0.03	0.30	0.04	0.02	4	0.08
N	0.03	0.08	0.14	0.02	0.18	0.18	0.08	0.02	4	>10	>10	0.16	0.03	>10	0.08	>10	>10	>10	0.03	0.04	0.04	0.80	0.08
Q	0.03	0.08	0.50	0.08	0.18	0.03	0.03	0.06	8	3	>10	0.40	0.25	0.02	0.20	0.08	>10	>10	0.40	0.04	0.03	0.50	0.40
R	0.03	0.04	0.16	0.03	0.40	0.16	0.03	0.04	4	0.10	>10	0.12	1.5	>10	0.40	0.12	>10	>10	0.40	0.04	0.08	0.06	>10
S	0.03	0.04	0.03	0.04	0.14	0.04	0.04	0.03	8	>10	>10	0.40	>10	>10	0.03	0.12	>10	>10	0.08	0.08	0.03	0.12	0.03
	X	X	X	X	X	X	X	C	K	G	X	AI NQ	Q	X	X	C	E	X	X	X	X	X

Binding motif

Figure 3.8: HNE Binding motif of a protective immune response by substitutional analysis.

Each position of the HNE reporter peptide (E379TCFQQACKGKIQALCENPEWA400) was substituted by an Ala, Glu, Asn, Gln, Arg and Ser residue. End-point titers of BH216 were measured in indirect ELISA. Binding to substituted HNE peptide (EPT < 1.0 mM) is shown in open boxes; no binding is shown as closed boxes (EPT > 1.0 mM). EPT observed with mAbs BH21 and BH6 were very similar (data not shown).

Similarly, the critical binding residues of mAb BH195 were defined by substitutional analysis in SPR solution competition binding assays (Figure 3.7B). In contrast to the above mAbs, BH195 was induced with denatured MV and although it binds to HNE peptides it does not recognize native virus (Ziegler et al., 1996). Thus, mAb BH195

displays neither crossreactive binding to native H protein in flow cytometry, nor MV neutralization *in vitro* (Ziegler et al., 1996). Interestingly, this mAb exhibits a radically different binding pattern: it binds to the HNE peptide irrespective of any cystine bridge and targets essentially the C-terminal residues E395, P397, E398 and W399 (Figure 3.7B).

6. High conservation of the HNE sequence

Table 3.1: Frequency of mutant HNE sequences in public databases

HNE sequence	EPT (µM)[1]	Binding Motif[2]	n[3]	%
ETCFQQACKGKIQALCENPEWA	**0,06**[4]	**Yes**	227	76,69
-----------N----------	**0,25**	**Yes**	20	6,76
--------Q-------------	2	No	8	2,70
------------------L---	**0,06**	**Yes**	4	1,35
-----------------D----	**0,14**	**Yes**	3	1,01
---------S------------	>10	No	3	1,01
--------R-------------	**0,08**	**Yes**	2	0,68
---------------------T	**0,08**	**Yes**	2	0,68
-----------N-V--------	**0,10**	**Yes**	2	0,68
-----H----------------	**0,12**	**Yes**	2	0,68
-----------D----------	1,5	No	2	0,68
----------------D-----	>10	No	2	0,68
------E---------------	**0,08**	**Yes**	1	0,34
---------------------G	**0,08**	**Yes**	1	0,34
-------------------D--	**0,10**	**Yes**	1	0,34
---L------------------	**0,10**	**Yes**	1	0,34
--------------I--D----	**0,12**	**Yes**	1	0,34
----------R-----------	**0,12**	**Yes**	1	0,34
--------------H-------	**0,18**	**Yes**	1	0,34
---L--------------L---	**0,20**	**Yes**	1	0,34
----R---------P-------	**0,60**	**Yes**	1	0,34
--------------------CV	**0,60**	**Yes**	1	0,34
-----------N--F-------	**0,60**	**Yes**	1	0,34
----------Q-----------	**0,60**	**Yes**	1	0,34
-------F--------------	**0,80**[5]	No	1	0,34
---------S--------L---	1,5	No	1	0,34
--------R-EV-----D----	3	**Yes**	1	0,34
---------D------------	>10	No	1	0,34
--------E-------------	>10	No	1	0,34
-----------N----K-----	>10	No	1	0,34
---------R------------	>10	No	1	0,34

[1] End point titers (EPT) to mAb BH216 were assessed by indirect ELISA.
[2] Presence or absence of binding motif $X_7C[KR]GX[AINQ]QX_2CEX_5$ in HNE sequence.
[3] number of corresponding wild-type MV sequences found in non-redundant databases GenBank, EMBL, Swissprot and DDBJ. Vaccine strain and incomplete sequences were not considered.
[4] binding to mutant peptide is shown in bold EPT (EPT < 1.0 µM).
[5] A very weak maximal binding was observed for this peptide. Despite, an EPT < 1.0 µM, this peptide was considered negative for binding.

An interesting and important feature of the HNE is its high degree of conservation among field isolates. The non-redundant GenBank, EMBL, DDBJ and SwissProt databases listed 31 different HNE sequences in 324 MV field isolates, of which 13 vaccine strain sequences and 15 incomplete sequences were rejected (status 07.11.03) (Table 3.1). The 22-amino acids of the HNE region are totally conserved in 227 wild-type viruses. Only one virus showed a single mutation in one of the Cys residues, which are otherwise conserved in all known morbilliviruses. 59 viruses contain a single HNE- mutation and only one viral sequence has more than two mutations. 21 distinct HNE sequences found in 92.9% of all MV strains, were found to display the above binding motif $X_7C[KR]GX[AINQ]QX_2CEX_5$ and 20 peptides corresponding to these sequences were recognized by mAb BH216 (Table 3.1). Furthermore, the 10 HNE sequences, which did not match the binding motif, were also not recognized by mAb BH216.

7. Neutralization of wild-type MV isolates

Here we tested whether the presence of the binding motif $X_7C[KR]GX[AINQ]QX_2CEX_5$ in the HNE domain of wild-type MV isolates can also be considered as a necessary condition for neutralization by protective mAbs BH216, BH21 and BH6.

Indeed, when these mAbs were used in *in vitro* neutralization assays, they neutralized every wild-type MV isolate whose HNE sequence contained the binding motif, except for one, which was resistant despite the presence of the binding motif (Table 3.2). Thus, by substitutional analysis we defined a binding constraint with an inherent biological significance for three protective mAbs. The binding motif describes the recognition of mutated peptides as well as the neutralization of wild-type viruses.

Table 3.2: Neutralization of wild-type MV isolates

Clade	Genbank	Isolate	HNE sequence	Binding Motif	BH216	BH21	BH6
A	U03669	Edmonston wt	ETCFQQACKGKIQALCENPEWA	Yes	1:500	1:400	1:1200
A	U08417	Philadelphia 26	---------R------------	No	No	No	No
D3	L46763	TX-92	-----------N----------	Yes	1:1800	1:1600	1:5000
D3	AF009581	CA:7/28/96	-----------N----K-----	No	No	No	No
D3	M81896	Chicago-2	----------R-----------	Yes	1:7300	1:1900	1:15000
D5	L46757	PAL-93	--------Q-------------	No	No	No	No
G1	AF079553	Berkeley	--------------I--D----	Yes	1:2800	1:3200	1:4400
H1	AF045196	China93-2	------------------L---	Yes	No	No	No
H2	AF045203	China94-1	-----------------D----	Yes	1:2000	1:2300	1:5000

Neutralization Titers of protective mAbs BH216, BH21 and BH6 for wild-type MV isolates from different clades and geographic origins. For every MV isolate its respective Clade, Genbank reference, HNE sequence, presence or absence of binding motif X_7C[KR]GX[AINQ]QX_2CEX_5 in HNE sequence, and if yes, neutralization titer for the respective mAb are indicated.

8. Molecular modeling of the HNE peptide

The HNE peptide 384-396 [QACKGKIQALCEN], which corresponds essentially to the minimal epitope in the truncation studies, was modeled by dynamic simulations at 300 K and at 1000 K. Simulation at high temperatures (1000 K) lowers the effect of the free energy barriers and enables the system to move across higher local energy minima and explore more possible peptide conformations. In order to analyze the rigidity and/or flexibility of the peptide and assess the conformational stability of its backbone, conformers corresponding to distinct local energy minima were sampled during the dynamic simulation runs at 1000 and superimposed to a minimum energy conformer resulting from the 300 K simulations. Remarkably, all conformers displayed quasi-identical backbone structures and side-chain orientations (Figure 3.9A, B). The circular peptide appears as a fairly flat structure with an amphiphilic character. The hydrophilic amino acids (Q384, K387, K389, Q391 and E395) cluster on the ‘upper face’ of the loop and can be expected to be solvent-exposed in the virus (Figure 3.10A). Similarly, the hydrophobic residues (A385, I390, A392 and L393) can be found on the ‘lower side’ of the loop, surrounding the hydrophobic sulfur atoms of the disulfide bridge (Figure 3.10B). The model clearly shows that the sequential discontinuity corresponds to a conformational clustering of interacting and non-interacting residues, resulting from the C386-C394 bridge.

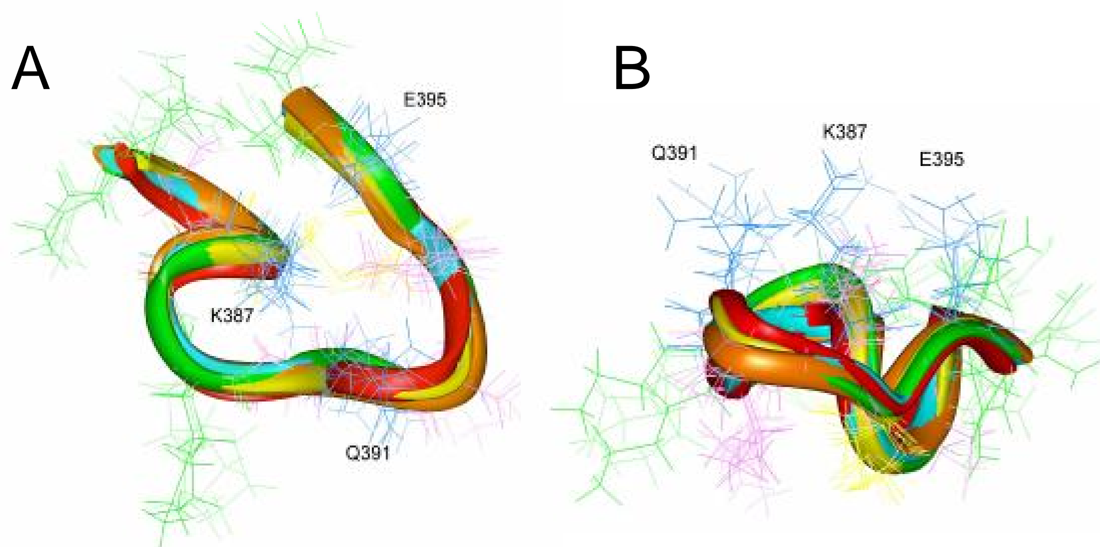


Figure 3.9: Molecular modeling of HNE peptide (1).

Top view (A) and lateral view (B) of 4 representative conformations (peptide backbone as yellow, red, blue and orange ribbon) from simulation runs with explicit water molecules at 1000 K superimposed to a conformation (peptide backbone as green ribbon) from the simulation at 300 K. Side chains of critical contact residues are shown in blue, hydrophilic/charged side chains in green, hydrophobic side chains in pink, disulfide bridge in yellow, peptide backbone as thick ribbon.

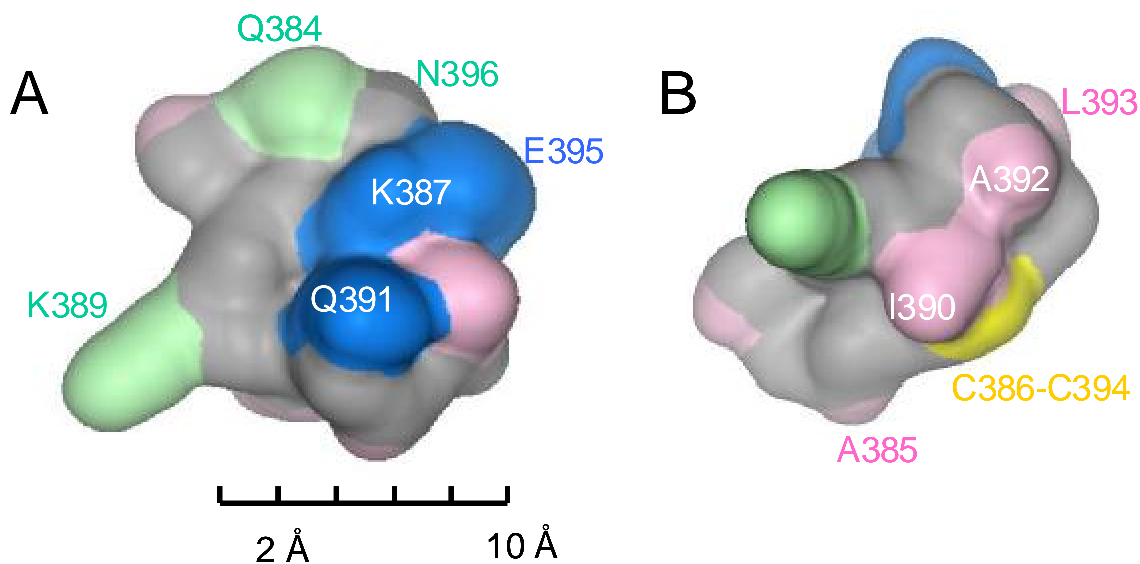


Figure 3.10: Molecular modeling of HNE peptide (2).

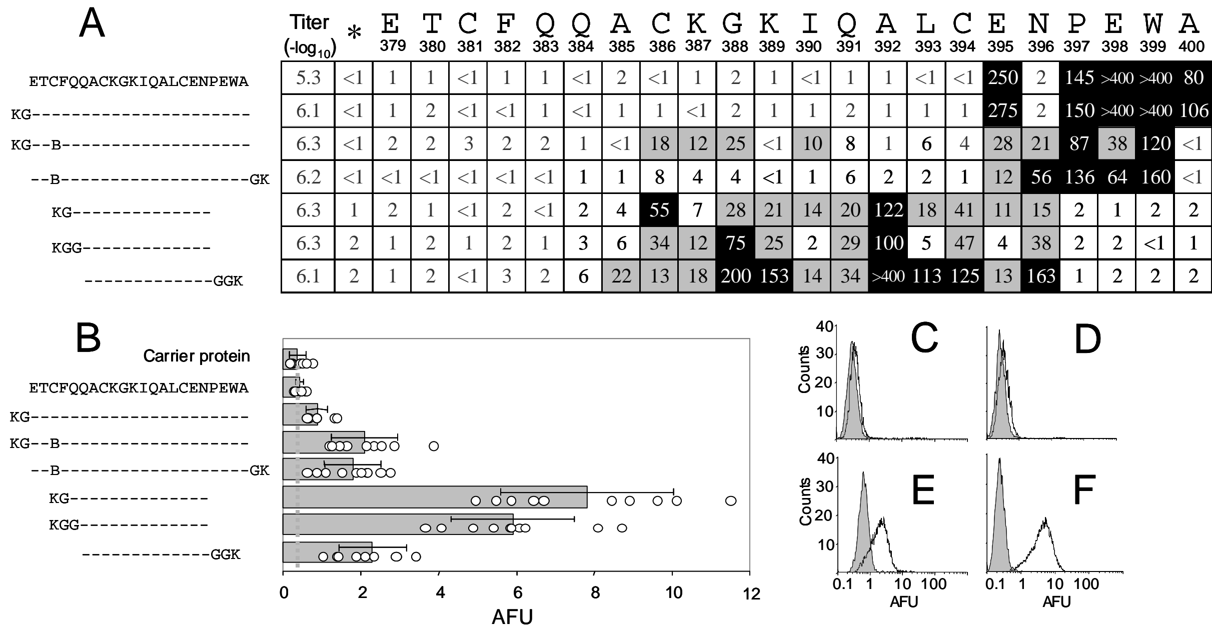
Top view (A) of the HNE epitope: clustering of hydrophilic/charged residues on solvent accessible surface of the epitope. Bottom view (B) of the HNE epitope: Clustering of hydrophobic residues on the lower side of the epitope. Side chains of critical contact residues are shown in blue, hydrophilic/charged side chains in green, hydrophobic side chains in pink, disulfide bridge in yellow.

When this structure was compared to the binding data, the critical contact residues K387, Q391 and E395 (and G388) seem to cluster on top of the planar loop structure formed by the peptide backbone (Figures 3.9A,B; 3.10A). The high structural similarity between the simulated conformers suggests that the peptide folds into a rather rigid conformation stabilized by the cystine bridge. In some of the conformers a hydrogen bond was predicted between the carbonyl atom of the Cys386 residue and the main chain nitrogen atom of residue Ile390. The total contact surface of the epitope can be estimated to 300-400 $Å^2$.

9. Peptide immunogenicity

When the full length, oxidized HNE peptide, containing all three cysteine residues, was conjugated to diphtheria toxoid (dt) either via the free available sulfhydryl function using a heterofunctional crosslinker SPDP [ETCFQQACKGKIQALCENPEWA] or via an additional Lys residue at the N-terminus using EDC/NHS chemistry [KGETCFQQACKGKIQALCENPEWA], it induced anti-peptide immune sera with high anti-peptide titers ($1{:}10^{5.3\text{-}6.1}$) (Figure 3.11A), but failing to crossreact in flow cytometry with the H protein expressed in its native conformation on the surface of Mel-JuSo cells (Figure 3.11B, D). The binding specificity of these sera, revealed by substitutional analysis, was found to target exclusively the C-terminal residues E395, P397, E398, W399 and A400 (Figure 3.11A). Interestingly, these sera showed the same binding specificity as mAb BH195 (Figure 3.7B), generated with denatured MV and unable to crossreact with the native H protein (Ziegler et al., 1996). The Cys381 was then substituted with an amino butyric acid residue in order to prevent disulfide scrambling and a N- or C-terminal Lys was added to conjugate the full length, oxidized HNE peptide to the carrier protein [KGETBFQQACKGKIQALCENPEWA or ETBFQQACKGKIQALCENPEWAGK]. With these peptides, some reactivity with the core of the epitope emerged (Figure 3.11A), and a significant crossreactivity with the native H protein was obtained (Figure 3.11B, E). While with the latter peptides most anti-peptide antibodies were directed towards the C-terminal residues $E_{395}NPEW_{399}$, truncated peptides containing mainly the core residues and the critical Cys386-Cys394 bridge [KGQQACKGKIQALCEN or KGQACKGKIQALCEN or QACKGKIQALCENGK] induced an additional four-fold increase in crossreactivity with

the intact protein (Figure 3.11B, F). For the binding of the sera to the HNE peptide, the importance of the Cys residues was relatively low, in comparison to the binding with the mAbs BH216, BH21 and BH6, suggesting that antibodies may have been partially induced against the linear isoform of the HNE peptide.



A	Titer (-log10)	*	E 379	T 380	C 381	F 382	Q 383	Q 384	A 385	C 386	K 387	G 388	K 389	I 390	Q 391	A 392	L 393	C 394	E 395	N 396	P 397	E 398	W 399	A 400
ETCFQQACKGKIQALCENPEWA	5.3	<1	1	1	<1	1	1	<1	2	<1	1	2	1	<1	1	1	<1	<1	250	2	145	>400	>400	80
KG----------------------	6.1	<1	1	2	<1	<1	1	<1	1	1	<1	2	1	1	2	1	1	1	275	2	150	>400	>400	106
KG--B-------------------	6.3	<1	2	2	3	2	2	1	<1	18	12	25	<1	10	8	1	6	4	28	21	87	38	120	<1
--B-------------------GK	6.2	<1	<1	<1	<1	<1	<1	1	1	8	4	4	<1	1	6	2	2	1	12	56	136	64	160	<1
KG--------------	6.3	1	2	1	<1	2	<1	2	4	55	7	28	21	14	20	122	18	41	11	15	2	1	2	2
KGG-------------	6.3	2	1	2	1	2	1	3	6	34	12	75	25	2	29	100	5	47	4	38	2	2	<1	1
-------------GGK	6.1	2	1	2	<1	3	2	6	22	13	18	200	153	14	34	>400	113	125	13	163	1	2	2	2

Figure 3.11: Fine-specificity and crossreactivity with MV-H protein of anti-HNE-peptide sera.

(A) Mean anti-HNE-peptide titers (-log10) and mean serum reactivity (EPT) of five sera after immunization with HNE-DT conjugates made with HNE peptides of different length. Mean serum reactivity was measured in indirect ELISA against a dilution range of Ala-monosubstituted HNE peptides, or Arg-, Glu- (not shown) and Ser-substituted (not shown) peptides in case of original Ala residues (A385, A392, A400). Reduced binding is shown in grey (EPT > 10 nM) and in black boxes (EPT > 50 nM). SD of anti-peptide reactivity 5-15%. Unsubstituted HNE reporter peptide (*). (B) Crossreactivity of ten mouse sera with H protein after immunization with HNE-DT conjugates measured by flow cytometry. Each open circle reflects the AFU value of an individual mouse, horizontal bars represent the mean crossreactivity ± SD, anti-diphtheria toxoid sera was used as negative control and corresponds the net background AFU value (dotted line). (C, D, E, F) Typical flow cytometry histograms of crossreactivity with H protein on Mel-JuSo-H cells (open histogram) and Mel-JuSo-wt cells (grey histogram) of mouse sera induced against diphtheria toxoid (C), against conjugates with oxidized, full length HNE peptide with three Cys residues [ETCFQQACKGKIQALCENPEWA] (D), or only Cys386 and Cys394 [KGETBFQQACKGKIQALCENPEWA] (E) or the shortened HNE peptide [KGQACKGKIQALCEN] (F).

Although the binding pattern may be somewhat blurred by these antibodies and by the polyclonal nature of the sera, the importance of residues C386, G388, Q391 and C394 as contact residues seems to be confirmed. It is noteworthy, that all HNE-peptide-conjugates induced high anti-peptide titers (Figure 3.11A). Peptide amidation and N- or C-terminal conjugation had little effect on anti-peptide titers (Figure 3.11A) and on the specific binding domain of the immune sera, suggesting that differences in peptide degradation *in vivo* were not a critical issue.

10. Crossreactive immunogenicity of altered peptides

It was shown above that antibody binding is governed by specific contact residues on the peptide chain (Figure 3.8). In addition to binding, these critical residues also play a crucial role in wild-type virus neutralization (Table 3.2). The implication of single amino acid mutations on crossreactive immunogenicity was tested using substitution analogues of the HNE peptide, where K387 and/or K389 were altered (Figure 3.12).

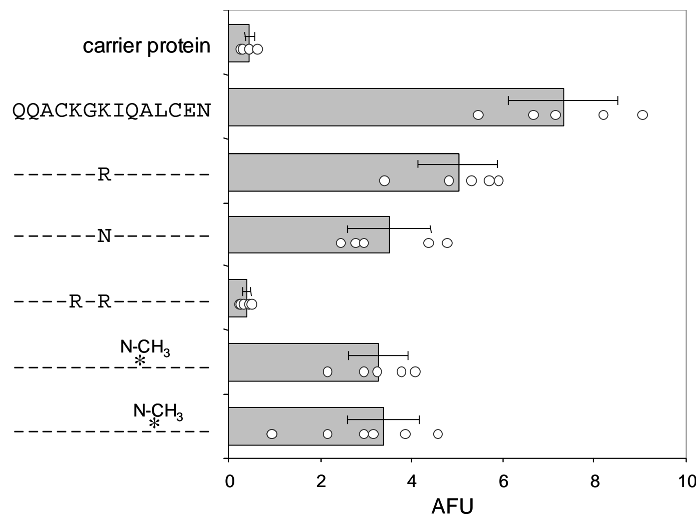


Figure 3.12: Crossreactivity with MV-H protein of anti-HNE-peptide sera.

Conjugated HNE peptide analogues were substituted as indicated on positions K387 and/or K389 or contained an N-methylated peptide bond in their backbone at positions A392 or L393. Crossreactivity of five mouse sera with H protein after immunization with HNE-DT conjugates was measured in flow cytometry. Each open circle reflects the AFU value of an individual mouse, horizontal bars represent the mean crossreactivity ± SD, anti-diphtheria toxoid sera was used as negative control.

Whereas K387 is contained in the HNE binding motif and its substitution, except by arginine, impeded peptide antigenicity with protective mAbs, the mutation of K389 had no effects on antibody binding (Figure 3.8). Conjugated HNE peptides where the non-critical K389 was substituted by an arginine or asparagine residue still induced antibodies crossreacting with the native H protein, though with a lesser extend (Figure 3.12). However, the mutation of the critical K387 in the immunogen thwarted the crossreactive immunogenicity of the HNE peptide. Although an arginine substitution of K397 is tolerated for crossreactive antigenicity (Figure 3.8), it is not for crossreactive immunogenicity (Figure 3.12).
Similarly, the importance of backbone conformation for peptide immunogenicity was tested by introducing at selected positions N-methylated amino acid residues during synthesis, leading to HNE peptides with altered peptide backbones containing N-methylated peptide bonds at positions A392 and I393. When conjugated to diphtheria toxoid, these HNE analogues still induced H-protein crossreactive immune serum, although with lowered AFU values compared to the conjugate with the unsubstituted HNE peptide (Figure 3.12). Apparently, the disulfide-bond-mediated loop conformation has an inherent rigidity which is only minimally affected by peptide backbone modifications.

11. Protective immune response

The protective capacity of the immune sera from the group of mice which yielded the highest crossreactivity in the above immunogenicity study (Figure 3.11A, B, F) was assessed after passive transfer in a rodent challenge/protection model (Figure 3.13). Mice were injected with 100 µl immune serum obtained after 3 injections with the DT peptide-conjugate containing the shortened and modeled HNE peptide [QQACKGKIQALCEN] or with the diphtheria toxoid carrier alone and were then immediately challenged via intracranial injection with a neuroadapted MV strain. The group of mice transferred with anti-peptide conjugate serum displayed significantly higher survival rate than the group which obtained anti-carrier serum ($p=0.0238$) (Figure 3.13).

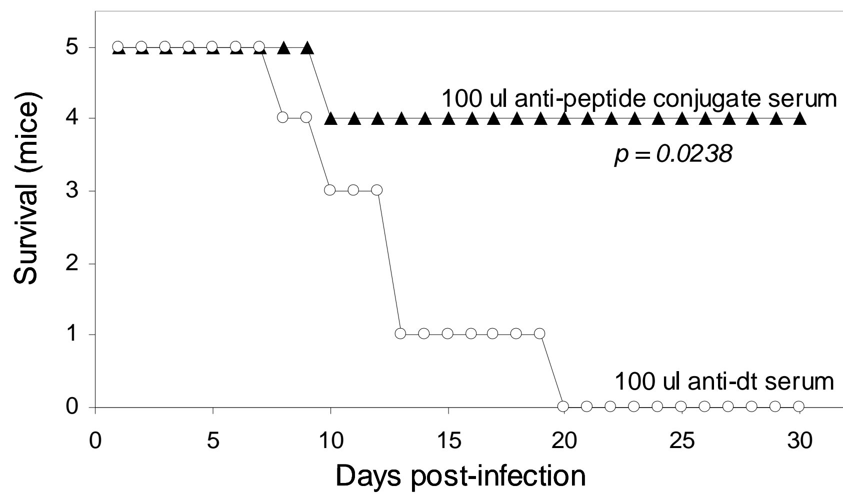


Figure 3.13: Challenge/Protection experiment using anti-peptide-conjugate immune serum.

Two groups of mice (n=5), injected with 100 µl anti-peptide-conjugate (containing the shortened HNE peptide [QQACKGKIQALCEN]) or with anti-carrier pooled immune serum were immediately challenged and monitored for survival during 30 days.

Results. Part II.

The immunogenicity of a peptide-conjugate designed for early infancy use has to be evaluated carefully in the presence of prior immunity or passively acquired antibodies against the carrier protein as both factors are known to interfere often with subsequent infant immunization, although with changing degrees of clinical incidence (Siegrist et al., 1998; Siegrist, 2001). In addition, its compatibility with a succeeding administration with the live attenuated virus has to be assessed. Unlike polysaccharides which are mainly T-independent type 2 antigens inducing B-cell responses characterized by low-affinity antibodies and a lack of immunologic memory, peptide-specific B-cell activation relies on T-cell help. The immunogenicity of peptide-conjugates in mice using diphtheria toxoid (dt) and tetanus toxoid (tt) as carrier proteins was investigated after active priming with the carrier or after passive transfer of anti-carrier antibodies. Additionally, prime-boost strategies using chimeric T-cell-epitope-B-cell-epitope peptide-constructs were also evaluated. The effective immunogenicity was correlated with challenge-protection experiments and finally the compatibility of a peptide-conjugate immunization with a succeeding injection of live attenuated measles vaccine was established.

Note: Throughout the text and the figures, lower case letters designate the carrier proteins diphtheria toxoid (dt) and tetanus toxoid (tt) solely, and upper case letters the HNE-peptide-conjugates of the respective carrier (DT, TT) containing the shortened HNE peptide [`QQACKGKIQALCEN`]. Passive priming is designated using brackets (anti-carrier immune serum: [dt], [tt] or anti-peptide-conjugate serum: [DT], [TT]). The chimeric TCE-BCE peptide is abbreviated TB.

1. Antibody responses to peptide-conjugates

HNE peptide specific antibodies were measured by ELISA after one or two injections with the peptide-conjugates DT or TT (containing the shortened HNE peptide [`QQACKGKIQALCEN`]), based either on the same or on different carriers (diphtheria toxoid, dt, or tetanus toxoid, tt) (Figure 3.14). As expected, a single injection with either peptide-conjugate induced only a weak anti-peptide response (DT_tt [GMT=$4.50 \cdot 10^{-4}$] and TT_dt [GMT=$2.15 \cdot 10^{-4}$] in Figure 3.14 A,B or C,D), which was boosted up to a 100-fold by a second immunization with the same conjugate (DT_DT [GMT=$2.24 \cdot 10^{-6}$] and TT_TT [GMT=$1.32 \cdot 10^{-6}$]), compared to the background obtained with the carrier proteins alone (dt_dt or tt_tt).

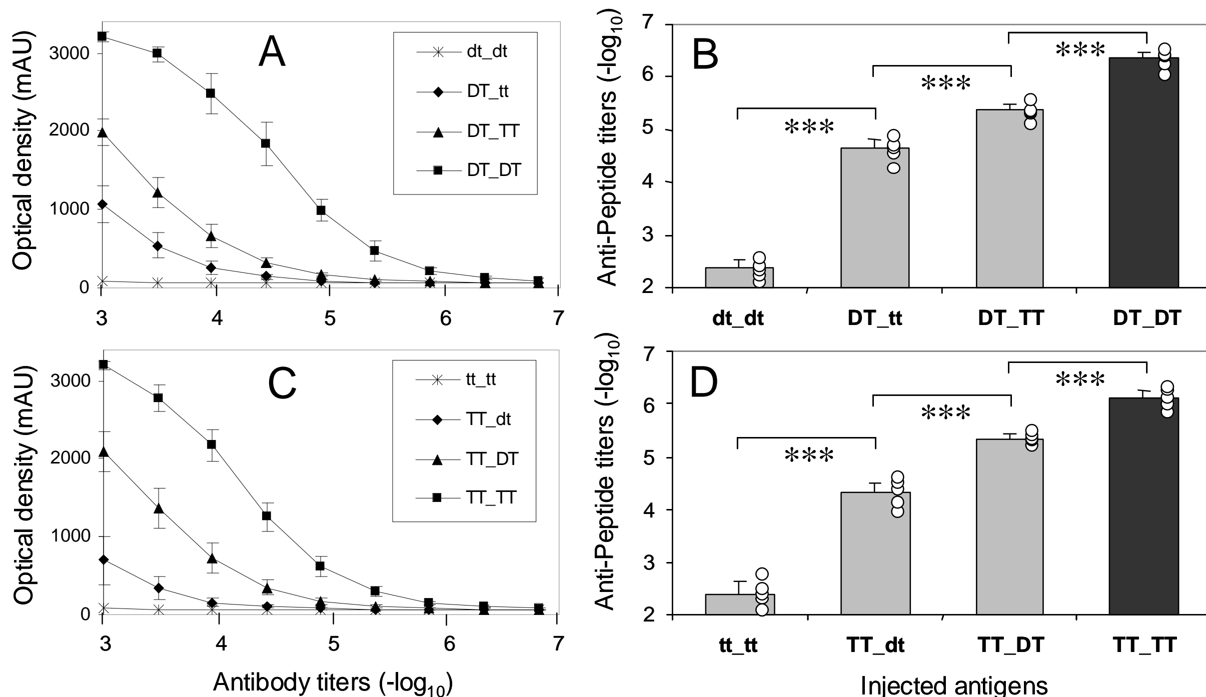


Figure 3.14: Assessment of anti-peptide responses using varying immunization schedules by ELISA.

Average sera dilution curves of groups of mice (*n=5*) after two injections with DT peptide-conjugate or dt carrier protein (A), or with TT peptide-conjugate or tt carrier protein (C). Corresponding geometric mean peptide-specific EPT (B) and (D). Sera were collected 8 days after the last immunization. Each open circle represents the titer of an individual animal. 95% confidence intervals (*n=5*) and significance levels of differences between groups are indicated, where *** means $p<0.001$. Lower case letters designate the carrier proteins (dt, tt) solely, and upper case letters the peptide-conjugates of the respective carrier (DT, TT) (containing the shortened HNE peptide [`QQACKGKIQALCEN`]). EPT after DT_DT and TT_TT (black bars) will be used as reference titer in the figures below.

The booster effect was much weaker (about 3-fold) when the heterologous conjugate was used for the second injection (DT_TT [GMT=$2.33\cdot10^{-5}$] and TT_DT [GMT=$2.30\cdot10^{-5}$] in Figure 3.14). We assume that anti-peptide specific B cells activated after the first peptide-conjugate injection could successfully recruit T-cell help from a new T-cell population specific for the second carrier protein. There are 3 possibilities for this: crossreacting T cells, polyclonal mitigated activation of T cells or peptide-specific T cells.

Similar carrier-specific antibody responses were obtained whether mice were injected with the free or conjugated carrier (dt_DT, DT_DT or dt_dt: GMT = $2.18\cdot10^{-6}$-$3.13\cdot10^{-6}$; tt_TT, TT_TT, tt_tt GMT = $2.46\cdot10^{-6}$-$3.48\cdot10^{-6}$) (Figure 3.15).

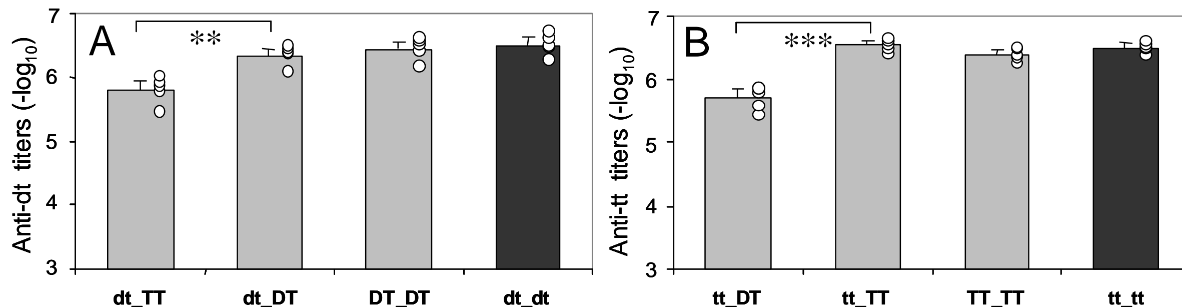


Figure 3.15: Anti-carrier protein responses measured in ELISA.

(A) Geometric mean dt-specific EPT (*n=5*) after two injections with DT peptide-conjugate or dt carrier protein. (B) Geometric mean tt-specific EPT after two injections with TT peptide-conjugate or tt carrier protein. 95% confidence intervals and significance levels of differences between groups are indicated (**: $p<0.01$, ***: $p<0.001$). Injections using carrier proteins (dt, tt) solely and peptide-conjugates (DT, TT) are indicated. EPT after dt_dt and tt_tt (black bars) will be used as reference titer in the figures below.

2. Experimental setup and schedules used

Peptide- and carrier-specific immunogenicity of two injections with the above mentioned peptide-conjugates (DT_DT and TT_TT) was investigated after various ways of priming (Table 3.3). Mice were either actively primed using the carrier proteins (dt or tt) or the peptide-conjugates (DT or TT). Other groups of mice were passively primed by an injection using anti-carrier ([dt] ot [tt]) or anti-conjugate ([DT] or [TT]) immune serum. In every case an homologous and an heterologous priming,

regarding the carrier protein used, was investigated for the following injections. In addition to this a chimeric peptide containing a promiscuous T-cell epitope derived from tetanus toxoid and the HNE B-cell epitope was used for active priming and for the subsequent injections. Thus, peptide- and carrier-specific immunogenicity could be assessed in mice with different priming situations, i.e. antibody (anti-peptide and anti-carrier) or cellular mediated immunity (Table 3.2).

Table 3.3: Immunity after priming injection in relation to subsequent injections

Schedule [1]	Anti-peptide antibodies	Anti-carrier antibodies	T-cells
dt_DT_DT		+	+
dt_TT_TT			
tt_TT_TT		+	+
tt_DT_DT			
DT_DT_DT	+	+	+
DT_TT_TT	+		
TT_TT_TT	+	+	+
TT_DT_DT	+		
[dt]_DT_DT		+	
[dt]_TT_TT			
[tt]_TT_TT		+	
[tt]_DT_DT			
[DT]_DT_DT	+	+	
[DT]_TT_TT	+		
[TT]_TT_TT	+	+	
[TT]_DT_DT	+		
DT_TB_TB	+		
TT_TB_TB	+		
TB_DT_DT	+		
TB_TT_TT	+		+

[1] Lower case letters designate the carrier proteins diphtheria toxoid (dt) and tetanus toxoid (tt), and upper case letters the HNE-peptide-conjugates of the respective carrier (DT, TT) containing the shortened HNE peptide [QQACKGKIQALCEN]. Passive priming is designated using brackets (anti-carrier immune serum: [dt], [tt] or anti-peptide-conjugate serum: [DT], [TT]). The chimeric TCE-BCE peptide is abbreviated TB.

3. Effect of active priming with the free or conjugated carrier protein

Mice pre-immunized with the free carrier proteins dt or tt developed after two succeeding injections with the peptide-conjugate similar antibody levels against the peptide (dt_DT_DT, tt_DT_DT, tt_TT_TT, dt_TT_TT) than animals without prior immunization (DT_DT, TT_TT) (Figure 3.16A, B). As expected, no epitopic suppression of the anti-peptide response occurred, presumably because of efficient activation of carrier-specific T cells. Also carrier-specific antibody titers were unaffected by previous dt or tt injections (Figure 3.17A, B).
After two injections with the same peptide-conjugates (DT_DT, TT_TT) a 'response ceiling' was reached that was not boosted by a third injection (DT_DT_DT, TT_TT_TT) (Figure 3.16A, B). This 'response ceiling' could no longer be reached after a first immunization with the heterologous conjugate (TT_DT_DT, DT_TT_TT): After two immunizations antibody levels were 5-6 times lower ($p<0.001$) and similar to a single injection without heterologous pretreatment (Figure 3.16A, B). This was not due to ineffective priming but to a blocking effect caused by the heterologous immunization. Presumably anti-peptide antibodies generated by the heterologous priming injection inhibited in absence of a specific T-cell priming the induction of an effective immune response. Carrier-specific antibody responses were also affected, although to a lesser extend (2-3 times lower, $p<0.001$) (Figure 3.17).

4. Effect of passive priming with anti-carrier antibodies

The immunogenicity of the peptide-conjugate was further tested in presence of passive anti-toxin antibodies (150 µl of serum containing about 5 IU antitoxin). Three days later mice were immunized with the homologous or heterologous peptide-conjugate. At this time mice displayed average serum levels of 1.54 IU/ml diphtheria antitoxin, which is well above the 1.0 IU/ml associated with long-term protection (Efstratiou et Maple, 1994). After a second injection with the peptide-conjugate ([dt]_DT_DT, [tt]_TT_TT), only weak anti-peptide antibody levels were obtained in comparison to mice with passive antibodies against the heterologous carrier ([tt]_DT_DT, [dt]_TT_TT) (Figure 3.16A, B). Anti-peptide antibody levels of the latter animals were equivalent to the control groups (DT_DT, TT_TT). Passive

transfer of anti-carrier antibodies decreased peptide-specific GMTs by more than a 100-fold (p<0.001) ([dt]_DT_DT, [tt]_TT_TT) (Figure 3.16A, B); the antibody response against the homologous carrier protein was reduced >180-fold and >220-fold in the case of dt and tt respectively (p<0.001) (Figure 3.17A, B). Active antibodies are even more suppressed than apparent from the mean titers in Figures 3.16 and 3.17, since passively transferred homologous antibodies are also monitored by the ELISA.

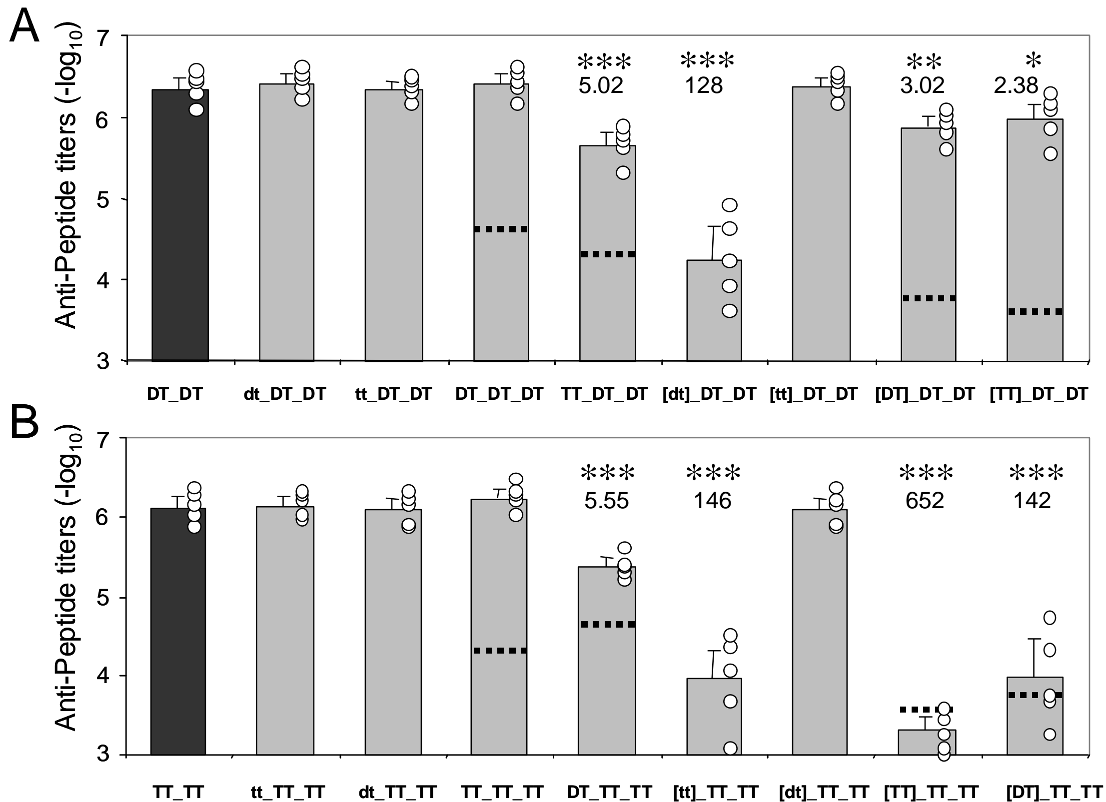


Figure 3.16: Peptide-specific responses following different immunization schedules.

Geometric mean peptide-specific EPT (*n=5*) after previous active or passive priming and two injections with DT peptide-conjugate (A) or with TT peptide-conjugate (B). Sera were collected 8 days after the last immunization. Each open circle represents the titer of an individual animal and 95% confidence intervals are shown. Lower case letters designate injections with carrier proteins (dt, tt) solely, and upper case letters with peptide-conjugates of the respective carrier (DT, TT). For the passive priming, mice received i.p. 150 µl of anti-carrier ([dt], [tt]) or 50 µl of anti-peptide-conjugate serum ([DT], [TT]) 3 days prior to the first injection. Mean EPT after DT_DT and TT_TT (black bars) are used as reference titers and every group of mice is compared to the respective reference titer. Significance levels of differences (where *: *p<0.05*,**: *p<0.01*, ***: *p<0.001*), given ratio with the reference titer and estimated contribution to measured titer due to priming injection (dotted line, cf Figures 3.14C, 3.14D, 3.18B) are indicated.

The proportion of passive antibodies can only be estimated on the basis of the waning curves in Figure 3.18 and are displayed in Figures 3.16 and 3.17. In some cases, the expected passive titers lie well above the actually measured values, proving that passive antibodies are used up more rapidly due to immunocomplex formation after the injections with the peptide-conjugate and that the induction of active antibodies is almost completely suppressed.

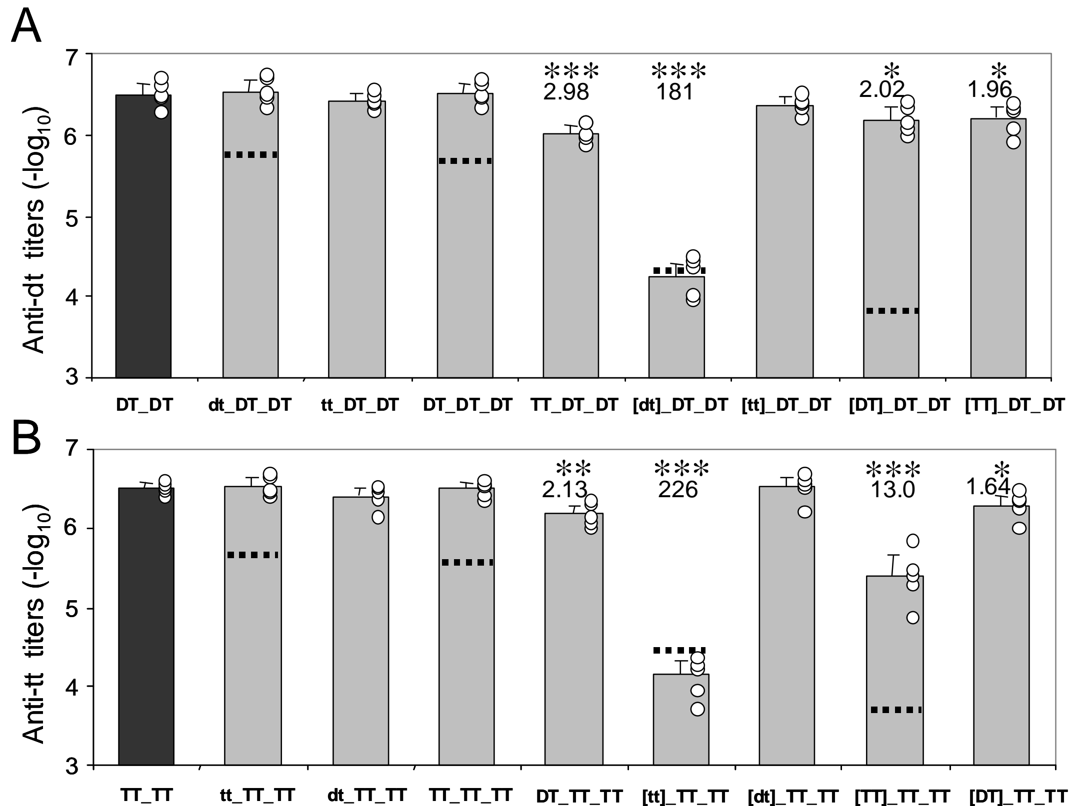


Figure 3.17: Carrier-specific responses following different immunization schedules.

Geometric mean dt-specific (A) or tt-specific (B) EPT (*n=5*) after previous active or passive priming and two injections with DT peptide-conjugate (A) or with TT peptide-conjugate (B). Titers of individual animals (open circles) and 95% confidence intervals are shown. Injections with free carrier proteins (dt, tt) and with peptide-conjugates (DT, TT) are indicated. For the passive priming, mice received i.p. 150 µl of anti-carrier ([dt], [tt]) or 50 µl of anti-peptide-conjugate serum ([DT], [TT]) 3 days prior to the first injection. Mean EPT after DT_DT and TT_TT (black bars) are used as reference titers and every group of mice is compared to the respective reference titer. Significance levels of differences (where *: $p<0.05$,**: $p<0.01$, ***: $p<0.001$), given ratio with the reference titer and estimated contribution to measured titer due to priming injection (dotted line, cf Figure 3.18B) are indicated.

Nevertheless, a third injection with the peptide-conjugate ([dt]_DT_DT_DT, [tt]_TT_TT_TT) boosted antibodies against the HNE peptide to 'ceiling' levels comparable to those of control groups (DT_DT, TT_TT) (Figure 3.19). Apparently, the carrier-specific antibody levels were low enough at the timepoint of the third injection not to interfere with a subsequent humoral response. Waning after passive transfer was assessed (Figure 3.18) and titers decreased with a half-life of 10.7 days for dt- and 12.6 for tt-specific antibodies. When the mice received their second and third injection, titers had approximately decreased by 2- or 4-fold respectively. It is also possible that carrier-specific antibodies are used up and decreased more rapidly when peptide-conjugates are injected.

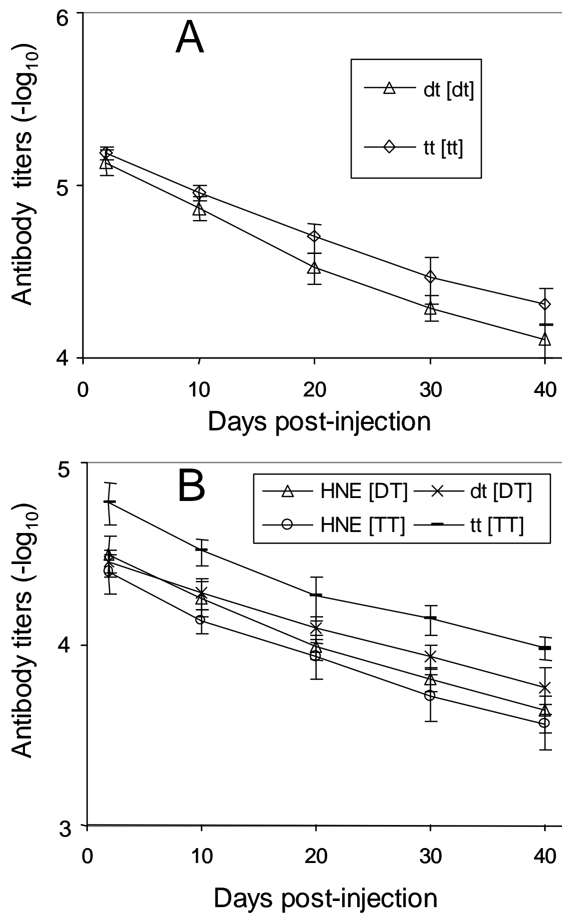


Figure 3.18: Antibody waning after passive transfer.

(A) Geometric mean carrier-specific (dt or tt) EPT (*n=5*) after passive priming where mice received i.p. 150 µl of anti-carrier ([dt], [tt]) at given timepoints after the transfer. 95% confidence intervals are shown.

(B) Geometric mean peptide-specific (HNE) or carrier-specific (dt or tt) EPT (*n=5*) after passive priming where mice received i.p. 50 µl of anti-peptide-conjugate serum ([DT], [TT]). 95% confidence intervals are shown.

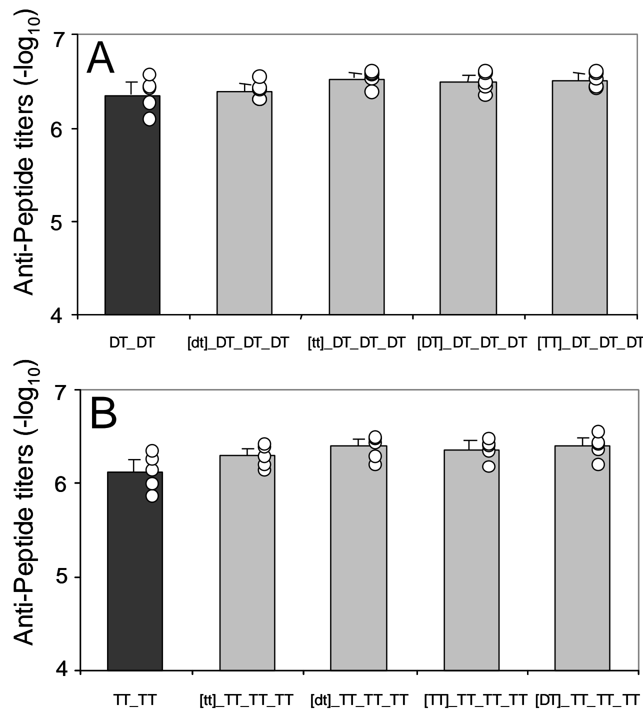


Figure 3.19: Peptide-specific responses after additional boosting.

Geometric mean peptide-specific EPT (*n=5*) after passive priming and two injections with DT peptide-conjugate (A) or with TT peptide-conjugate (B). Titers of individual animals (open circles) and 95% confidence intervals are shown. For the passive priming, mice received i.p. 150 µl of anti-carrier ([dt], [tt]) or 50 µl of anti-peptide-conjugate serum ([DT], [TT]) 3 days prior to the first injection. Mean peptide-specific EPT after DT_DT or TT_TT (black bars) are used as reference titers.

5. Effect of passive priming with anti-conjugate antibodies

Similar to the previous experiment, the effect of passively administered antibodies against peptide-conjugate was tested using equivalent levels of anti-peptide antibodies for the two conjugates, although the priming dose was lower than in the previous experiment (50 µl serum). Half-lives of these antibodies (Figure 3.18B) were similar to those of Figure 3.18A described above: peptide-specific titers decreased with half-lifes of 11.9 or 11.8 days and carrier-specific titers with half-lifes of 16.2 or 11.6 days after passive [DT] or [TT] transfer, respectively.

Passive antibodies against DT and TT significantly inhibited anti-peptide antibodies after even two immunizations with heterologous conjugate ([TT]_DT_DT in Figure 3.16A or [DT]_TT_TT in Figure 3.16B). Interestingly the suppressive effect was of similar extent whether mice were passively primed with the homologous or the heterologous conjugate. In both cases, peptide antibodies were, however, much more suppressed when TT was used as a conjugate ([TT]_TT_TT, [DT]_TT_TT) (Figure

3.16B). In particular in the case of TT, anti-peptide antibodies were much more suppressed than anti-carrier antibodies (Figure 3.16B and 3.17B).
A third active immunization with DT or TT ([DT]_DT_DT_DT, [TT]_DT_DT_DT, [TT]_TT_TT_TT, [DT]_TT_TT_TT) brought antibody to 'ceiling' levels of the control groups (Figure 3.19).
It is interesting that anti-carrier antibodies are more suppressed by passive homologous carrier antibodies ([dt]_DT_DT, [tt]_TT_TT) than after passive (homologous) conjugate antibodies ([DT]_DT_DT, [TT]_TT_TT) (Figure 3.17). In the case of anti-peptide antibodies the same observation was only made for immunizations with DT, but not with TT (Figure 3.16).

6. Effect of mixed schedules using peptide-conjugates and a chimeric TCE-BCE-peptide

The previous results showed that antibodies tend to suppress a humoral response only in absence of a specific T-cell response. Therefore the effect of T-cell priming with a major epitope of the tetanus toxin (which does not induce tt-reacting antibodies, data not shown), was investigated using a chimeric peptide 'TB' containing the BCE sequence collinearly synthesized with the promiscuous TCE from tt.
Prevaccination with TB inhibits subsequent immunization with DT or TT (Figure 3.20A, B) in a similar way than heterologous inhibition caused by active DT (DT_TT_TT) or TT (TT_DT_DT) (Figure 3.16). In the case of TB_DT_DT this can be explained by inhibiting antibodies and by the absence of a shared TCE. In the case of TB_TT_TT, one might expect that at least one TCE is shared. However, whereas T-cell priming is totally absent in the former case, in the latter it is probably very scarce. Although the tt-derived TCE sequence [QYIKANSKFIGITEL] is promiscuous for MHC class II molecules, the collinearly synthesized BCE-TCE peptide contains probably numerous neo-TCE-sequences, which could be efficiently used, in addition to the QYIKANSKFIGITEL sequence to supply T-cell help for the humoral response to the HNE peptide without T-cell priming for subsequent immunization using the TT (or DT) peptide-conjugate. As an example, the presence of potentially generated H2-Ek restricted neo-TCEs was confirmed for the TB peptide using a MHC class II TCE prediction algorithm (Table 3.4).

As expected, two subsequent immunizations with the chimeric TB peptide (TB_TB) generated lower peptide-specific antibody titers than with the peptide-conjugates DT or TT (Figure 3.20A,B). Surprisingly, a priming injection with the peptide-conjugates DT or TT induced increased peptide-specific antibody levels (Figure 3.20A,B), although to a small extend. Apparently, anti-peptide antibodies have no suppressive effect on the humoral response after TB immunization, despite the lack of efficient T-cell priming.

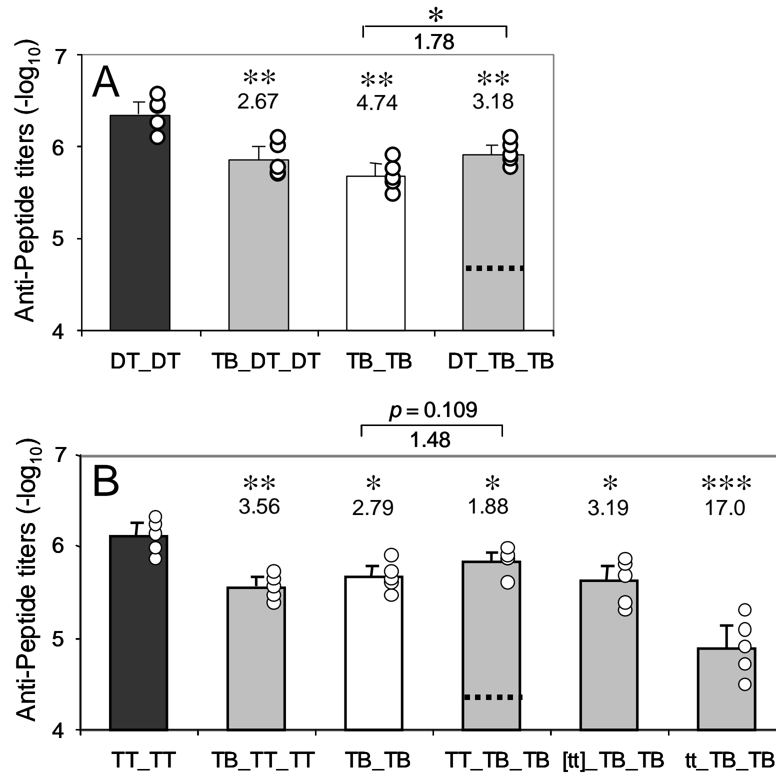


Figure 3.20: Peptide-specific responses in mixed immunization schedules using TB.

Geometric mean peptide-specific EPT (*n=5*) after two injections with the peptide-conjugates or the TB peptide (chimeric TCE-BCE peptide) and previous priming injections using the TB peptide, the peptide-conjugate, passive anti-carrier antibodies or the carrier protein, respectively. Mixed schedules using DT (A) or TT (B) peptide-conjugate. Mice were immunized at three-week intervals and serum was collected 8 days after the last injection. Titers of individual animals (open circles) and 95% confidence intervals are displayed. Mean EPT after DT_DT and TT_TT (black bars) are used as reference titers and every group of mice is compared to the respective reference titer. Significance levels of differences (where *: $p<0.05$,**: $p<0.01$, ***: $p<0.001$), given ratio with the reference titer and estimated contribution to measured titer due to priming injection (dotted line, cf Figures 3.14C, 3.14D, 3.18B) are indicated.

In addition, TB is not neutralized by preexisting anti-carrier antibodies ([tt]) (Figure 3.20B), unlike TT (Figure 3.16B). Active priming with the carrier protein however suppressed the peptide-specific antibody response elicited by the TB peptide (tt_TB_TB) (Figure 3.20B).

Table 3.4: H2-Ek restricted neo-TCEs generated in the TB peptide [1]

Location [2]	1	2	3	4	5	6	7	8	9	0	1	2	3	4	5	Score [3]
9	**F**	I	G	**I**	T	**E**	L	G	**Q**	Q	A	C	K	G	K	20
12	**I**	T	E	**L**	G	**Q**	Q	A	**C**	K	G	K	I	Q	A	18
6	**N**	S	K	**F**	I	**G**	I	T	**E**	L	G	Q	Q	A	C	16
10	**I**	G	I	**T**	E	**L**	G	Q	**Q**	A	C	K	G	K	I	12
3	**I**	K	A	**N**	S	**K**	F	I	**G**	I	T	E	L	G	Q	10
7	**S**	K	F	**I**	G	**I**	T	E	**L**	G	Q	Q	A	C	K	10
1	**Q**	Y	I	**K**	A	**N**	S	K	**F**	I	G	I	T	E	L	6
4	**K**	A	N	**S**	K	**F**	I	G	**I**	T	E	L	G	Q	Q	6
8	**K**	F	I	**G**	I	**T**	E	L	**G**	Q	Q	A	C	K	G	4
11	**G**	I	T	**E**	L	**G**	Q	Q	**A**	C	K	G	K	I	Q	4
14	**E**	L	G	**Q**	Q	**A**	C	K	**G**	K	I	Q	A	L	C	4

[1] Potential 15mers TCE sequences restricted for MHC II H2-Ek molecules contained in the 30-amino-acid TB peptide [QYIKANSKFIGITELGQQACKGKIQALCEN] calculated using the SYFPEITHI prediction algorithm freely available via the internet at the site http://syfpeithi.bmi-heidelberg.com/Scripts/MHCServer.dll/EpPredict.htm courtesy to Rammensee and colleagues (1999). For a 15mer TCE, positions P1, P4, P6 and P9, buried in the MHC II binding pockets, are indicated in bold.

[2] Location of the indicated P1 position in the 30-mer peptide.

[3] Score yielded using the SYFPEITHI prediction algorithm.

7. In vivo protection experiments

The protective potential of the above immune sera was assessed in a challenge/protection model in CBA mice with a neuroadapted MV strain. Passive immunization with anti-peptide serum obtained by the schedules DT_DT_DT, dt_DT_DT and tt_DT_DT showed significantly higher survival rates (p=0.03) than the control group receiving anti-carrier serum (dt_dt_dt) (Figure 3.21A).

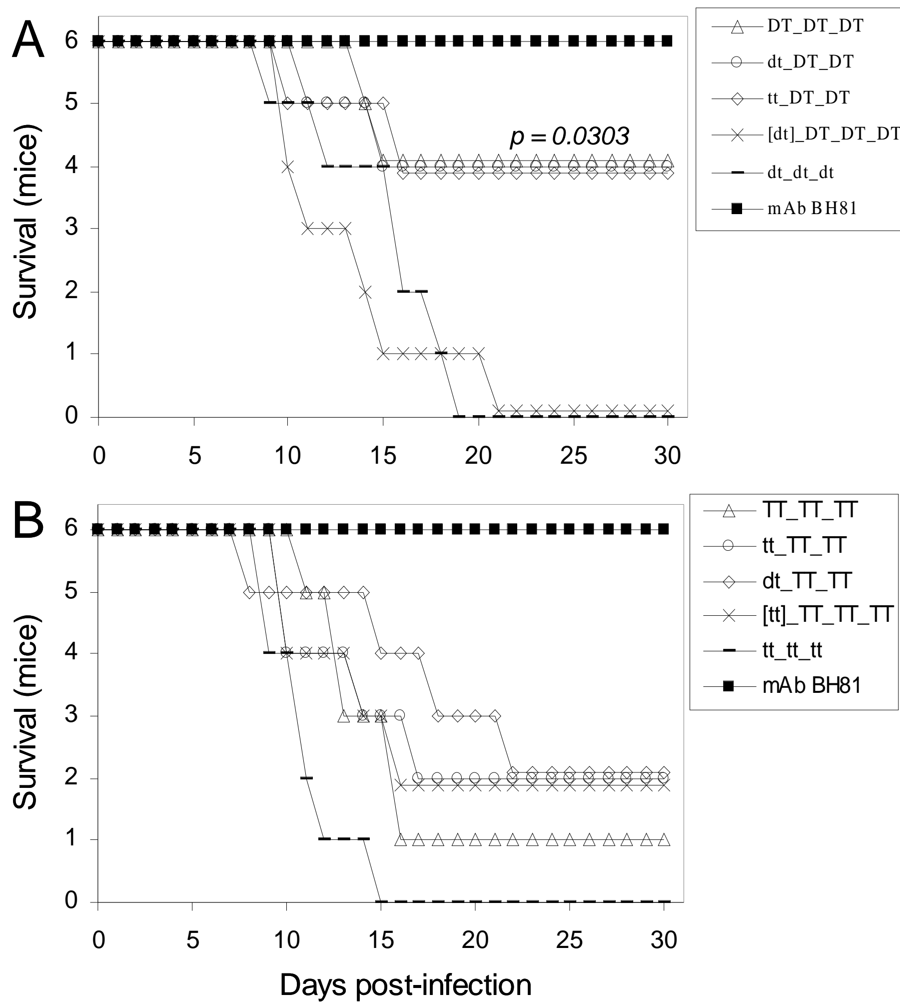


Figure 3.21: Challenge/Protection experiments using anti-peptide-conjugate immune serum.

Groups of mice (n=6) were passively injected with 200 µl of pooled immune serum induced against peptide-conjugate DT (A) or peptide-conjugate TT (B) from the above mentioned immunization experiments, immediately challenged and monitored for survival during 30 days. Injections using anti-carrier protein sera were used as negative controls (dt_dt_dt and tt_tt_tt) and protective mAb BH81 as positive control. A significant survival rate is indicated using the Fisher's Exact Test (*p=0.0303*).

The serum from mice passively primed by carrier-specific antibodies displayed no protective effect. Although a similar tendency was observed with the TT conjugates, survival differences with the control group where not significant ($p>0.05$) (Figure 3.21B).

8. Compatibility of peptide-conjugate immunizations with a subsequent immunization using the live-attenuated MV

We showed previously that the DT peptide-conjugate efficiently induces antibodies which crossreact with the hemagglutinin protein of measles virus. In order to evaluate compatibility of vaccination with the live-attenuated MV with a prior immunization with the protective DT peptide-conjugate, mice sequentially immunized with three doses of DT and $2.0 \cdot 10^5$ PFU of MV. Immune sera were analyzed by flow cytometry with MV-H and F expressing cells for crossreactivity. As expected, DT immunization induced crossreactive antibodies against the H (Figure 3.22), but not against the F protein (Figure 3.23).

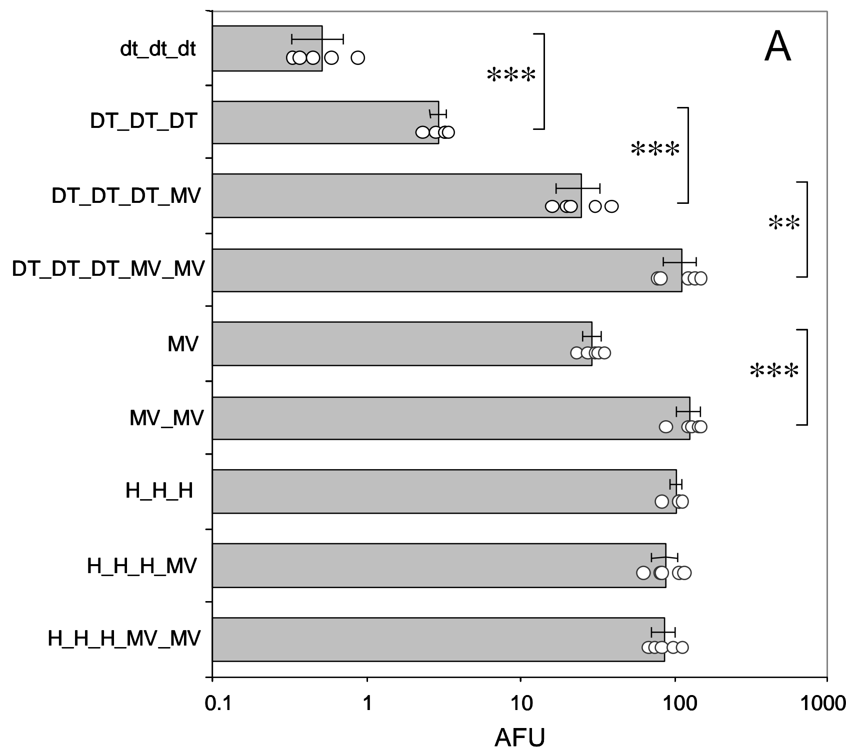


Figure 3.22: MV-H protein crossreactivity of immune sera measured in flow cytometry.

Geometric mean H-specific crossreactivity after three injections with the DT peptide-conjugate and 'on top' immunization with MV detected on MelJuSo cells expressing native H protein. Sera (*n=5*) were collected 8 days after the last immunization. Each open circle represents the signal of an individual animal and 95% confidence intervals (*n=5*) are shown. Significance levels of differences (where **: $p<0.01$, ***: $p<0.001$) are indicated. Immune sera raised against the free carrier were used as negative control (dt_dt_dt).

After a single injection with MV antibody titers against both H (Figure 3.22) and F (Figure 3.23) were similar to those obtained after an immunization with MV alone. Apparently, prior antibodies against the HNE epitope do not preclude an efficient active antibody response against other epitopes of the MV.

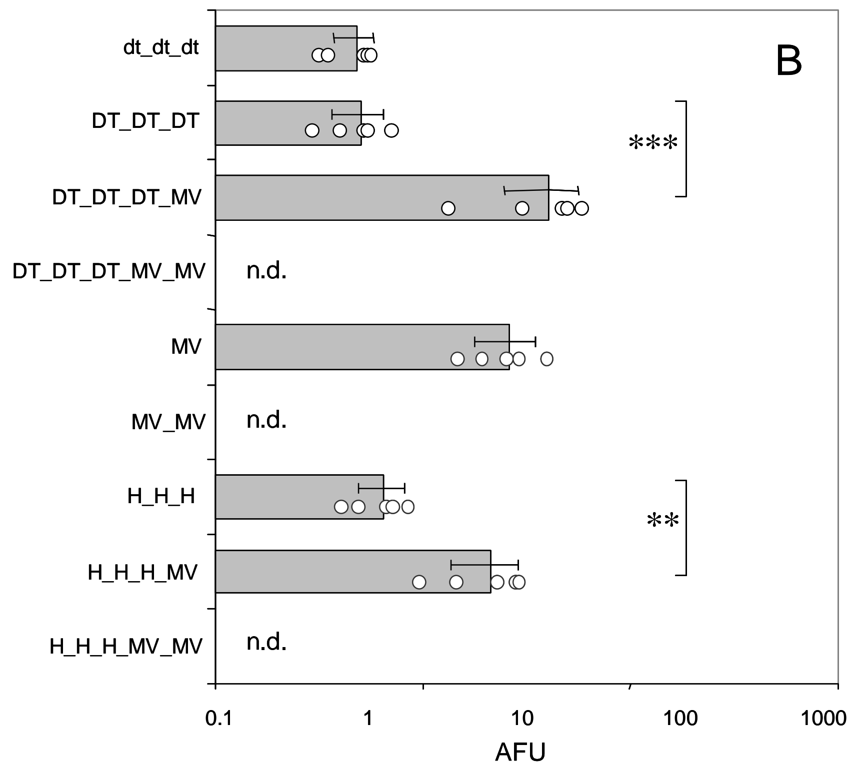


Figure 3.23: MV F-protein crossreactivity of immune sera measured in flow cytometry.

Geometric mean F-specific crossreactivity after three injections with the DT peptide-conjugate and 'on top' immunization with MV detected on MelJuSo cells expressing native F protein. Sera (*n=5*) were collected 8 days after the last immunization. Each open circle represents the signal of an individual animal and 95% confidence intervals (*n=5*) are shown. Significance levels of differences (where **: $p<0.01$, ***: $p<0.001$) are indicated. Immune sera raised against the free carrier were used as negative control (dt_dt_dt).

Results. Part III.

Needle-free delivery of a peptide-conjugate vaccine against measles via non-invasive routes such as the nose or the bare skin would largely increase its safety and compliance for use during early infancy. In addition, an intranasal spray or a transcutaneous administration via a patch would be compatible with self-administration and would thus represent a major advantage for revaccination of adults with waning immunity to measles (Muller et al., 2003). Protein components such as diphtheria or tetanus toxoid were proven to be immunogenic when mixed with cholera toxin and applied on shaved, intact skin (Glen et al., 1998, Scharton-Kersten et al., 2000). Moreover, transcutaneous immunization (TCI) is well suited to boost preexisting immunity, relying on T-cell responses previously primed via the intramuscular route (Hammond et al., 2001). MV-derived peptides were previously shown to induce protective antibodies after intranasal administration (Hathaway et al., 1997) and very recently, antibodies protecting against RSV were successfully induced after transcutaneous immunization using a 101 amino acid recombinant polyepitope (Godefroy et al., 2003). The immunogenicity of the protective HNE peptide was investigated when conjugated to three different carrier proteins, mixed to cholera toxoid and administered intranasally and transcutaneously. The biological relevance of the humoral responses generated was assessed an in vivo protection model.

1. Immunogenicity of peptide-conjugates after intranasal administration

When HNE peptide-conjugates, based on various carrier proteins, were co-administered intranasally together with cholera toxin (ct), high peptide-specific antibodies could be induced (Figure 3.24). The DT peptide-conjugate was significantly more immunogenic than the conjugates TT or OVA. Intriguingly, no anti-peptide antibodies were found after immunization with a chimeric TCE-BCE peptide TB.

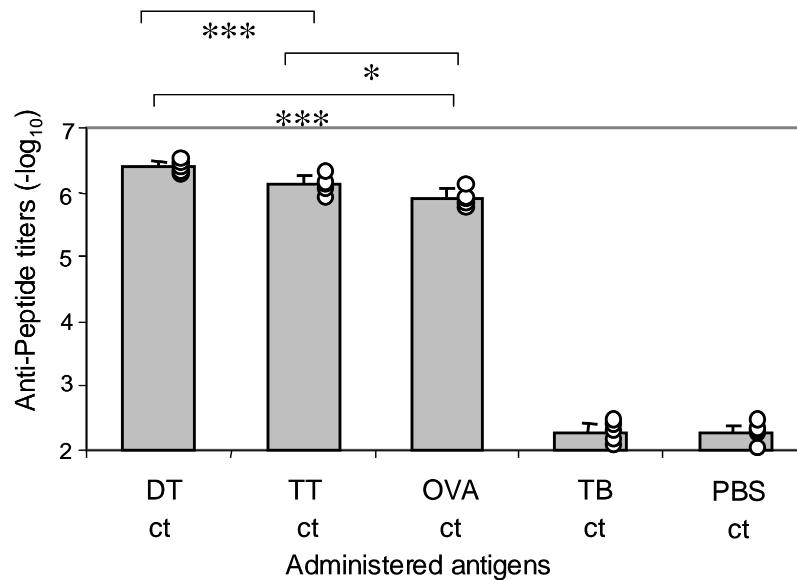


Figure 3.24: Anti-peptide responses after intranasal administration.

Geometric mean peptide-specific titers after two doses with the DT, TT or OVA peptide-conjugate or a chimeric TB peptide together with cholera toxin (ct). Sera (*n=5*) were collected 8 days after the last immunization. Each open circle represents the titer of an individual animal and 95% confidence intervals (*n=5*) are shown. Significance levels of differences (where *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$) are indicated. Immunization with PBS/ct was used as negative control.

A third dose with TT and OVA, administered i.n., could significantly increase the peptide-specific response (Figure 3.25A). For DT however, a maximal response was already observed after the second administration (Figure 3.25A). Similarly, only a minor increment in carrier-specific antibodies was found after a third administration with the peptide-conjugates (Figure 3.25B). Interestingly, TT induced highest carrier-specific antibody titers (Figure 3.25B).

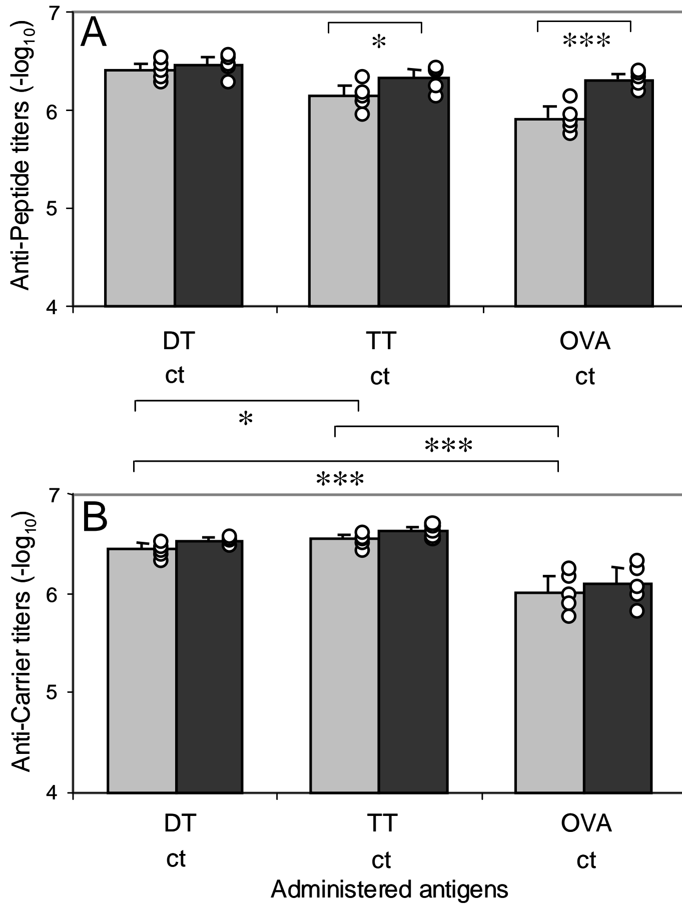


Figure 3.25: Antibody responses after two or three intranasal administrations.

Geometric mean peptide-specific (A) or carrier-specific (B) titers after two (grey bars) or three (black bars) doses of the DT, TT or OVA peptide-conjugate co-administered i.n. with cholera toxin (ct). Sera (*n=5*) were collected 8 days after the last immunization. Each open circle represents the titer of an individual animal and 95% confidence intervals (*n=5*) are shown. Significance levels of differences (where *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$) are indicated.

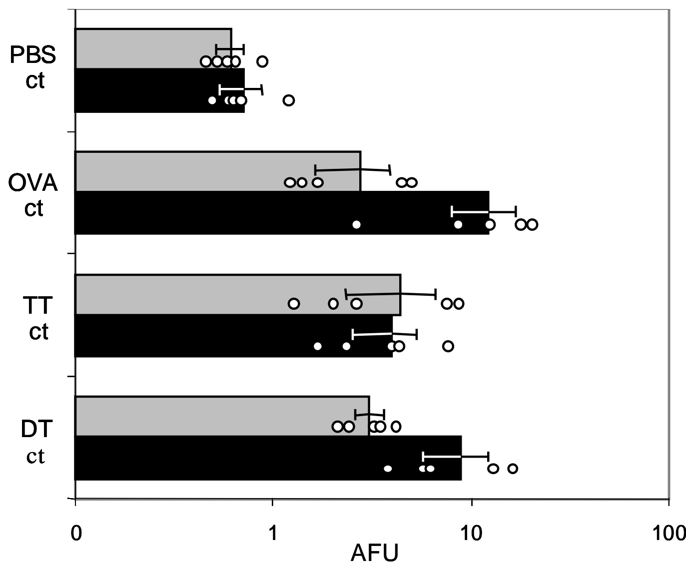


Figure 3.26: Crossreactive responses after i.n. administrations measured.

Geometric mean crossreactivity signal with native H-protein after two (grey bars) or three (black bars) immunizations with the DT, TT or OVA peptide-conjugate co-administered with cholera toxin (ct). Each open circle represents the signal of an individual animal and 95% confidence intervals (*n=5*) are displayed. Sera against PBS/ct were used as negative controls.

The crossreactivity of the i.n. induced immune sera with native MV H protein was assessed by flow cytometry and all three peptide-conjugates were shown to induce anti-peptide antibodies crossreacting with the native MV H-protein (Figure 3.26). No

significant differences in crossreactivity were found between the peptide-conjugates used.

2. Protective effect of i.n. induced anti-peptide response

The immune sera induced i.n. against the peptide-conjugates DT, TT and OVA were assessed for their protective potential in challenge/protection experiments. Passive immunization with anti-peptide serum obtained after i.n. administration with DT showed significantly higher survival rates (p=0.0390) than the control group receiving serum induced against cholera toxin alone (Figure 3.27). However, no protective effect could be demonstrated for the anti-TT or anti-OVA sera.

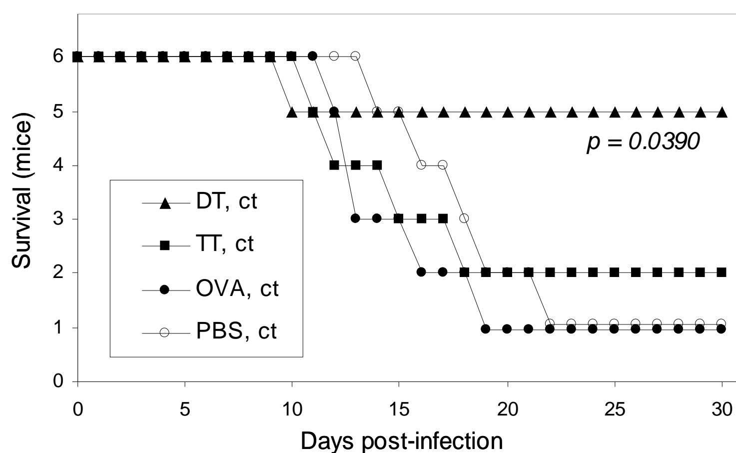


Figure 3.27: Challenge/Protection experiments with anti-peptide-conjugate immune sera.

Groups of mice (n=6) were passively injected with 200 µl of pooled immune serum induced i.n. against peptide-conjugate DT, TT and OVA from the above mentioned immunization experiments, immediately challenged and monitored for survival during 30 days. Injections using anti-ct sera were used as negative control. A significant survival rate is indicated using the Fisher's Exact Test (*p=0.0390*).

3. Immunogenicity of HNE-peptide-conjugates after transcutaneous immunization (TCI)

The same peptide-conjugates were used to determine their immunogenicity in transcutaneous immunization (Figure 3.28). When these conjugates were mixed in PBS to ct and applied for 1 h on shaved, wetted, intact skin, peptide-specific antibodies were efficiently induced (Figure 3.29). The DT peptide-conjugate revealed to be by far the most immunogenic conjugate and induced high antibody titers against the HNE peptide. In addition, high carrier-specific titers were observed (Figure 3.30).

Figure 3.28: Transcutaneous immunization experiments with peptide-conjugates.

Groups of mice (n=5) were shaved and rested for 48h. Upon anesthetization, their skin was extensively wetted for 10 min and 50 µg of DT, TT and OVA peptide-conjugates or TB peptide, mixed together with 10 µg of ct, in 100 µl PBS was topically applied for 1 h on the intact skin.

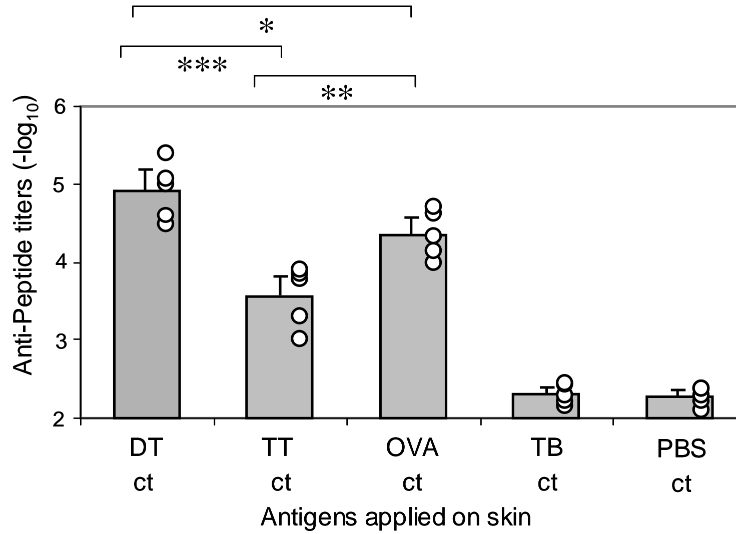


Figure 3.29: Anti-peptide responses after transcutaneous immunization.

Geometric mean peptide-specific titers after three administrations with the DT, TT or OVA peptide-conjugate or a chimeric TB peptide together with cholera toxin (ct). Sera (*n=5*) were collected 8 days after the last immunization. Each open circle represents the titer of an individual animal and 95% confidence intervals (*n=5*) are shown. Significance levels of differences (where *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$) are indicated. Immunization with PBS/ct was used as negative control.

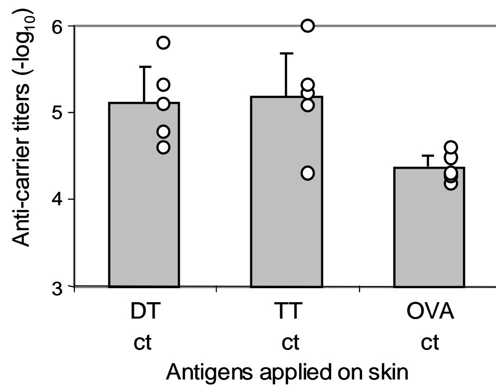


Figure 3.30: Anti-carrier responses after transcutaneous immunization.

Geometric mean carrier-specific titers after three administrations with the DT, TT or OVA peptide-conjugate, mixed with ct. Each open circle represents the titer of an individual animal and 95% confidence intervals (*n=5*) are shown.

Chapter 4: Discussion

In the previous section most of our findings have been shortly discussed in order to create logical links between the presented data and thus enhance the understanding while reading. In this section the results are put in broader context and discussed extensively with respect to the literature. A short discussion about the basic concepts in peptide-antibody interactions and the development of new vaccines against measles opens and closes, respectively this chapter.

1. A sensible amount of rationality in synthetic vaccine design

During the past years, numerous X-ray crystallographic and NMR studies of antigen-antibody and peptide-MHC-TCR complexes increased considerably our understanding of the molecular basis of immunological interaction and recognition. These data also shaped a structural perception of concepts like 'specificity', 'antigenicity', 'crossreactivity' and the 'rational design' of molecules has become realistic.

'Specificity' is an operationally defined notion, widely used in biology and more particularly in immunology, and a characteristic property of biomolecules (Van Regenmortel, 1998; Marchalonis et al., 2001). 'Absolute specificity' has been considered by Medawar and Medawar (1978) as: 'Specificity is the exact complementary relationship between an agent and something acting on, or between *instruction* and *performance* fulfilled'. However, the binding specificity of antibodies is not absolute and Landsteiner (1962) fine-tuned already the notion of 'exquisite specificity', as an ability of antibodies allowing them to discriminate among extremely fine structural changes in small organic moieties, called haptens. Antigen-antibody interactions have been termed fuzzy (Van Regenmortel, 1998, 1999, 2000), as there is no quantitative scale to measure the difference between two antigenic determinants enabling to define clear-cut boundaries between two different epitopes or predict their recognition by an antibody. The 'fuzzy' nature of these interactions is also reflected in the polyspecific or polyreactive binding of monoclonal antibodies. Indeed, monoclonal antibodies, deriving de facto from a single antigenic determinant, can bind promiscuously to several distinct epitopes, sharing no homologies in sequence or backbone conformation (Kramer et al., 1997). Indeed, sequentially and conformationally different peptides can efficiently bind to a paratope surface with varying affinities. In every peptide a unique key residue pattern governs the binding to a different set of critical antibody residues (Kramer et al., 1997). The notion 'molecular mimicry' has thus to be employed with caution, as peptide epitopes with a common 'antigenicity' do not always mimic each other with respect to sequence, conformation and binding mode.

'Antigenicity' relates to the ability of an antigenic moiety to interact with the functional binding site of an antibody, the paratope. Studying the 'antigenicity' of a peptide epitope relies on the detailed description of the chemical interactions between peptide and paratope atoms in order to define the structural basis of the specificity of these interactions. In this immunochemical analysis, the epitope-paratope interactions are reduced to a purely chemical system where binding events are studied on the basis of physicochemical properties of their components, abstraction made of the biological system whereof they are deriving (Van Regenmortel, 2001a, 2001b). Not every residue of a peptide epitope necessarily participates in the interaction with the antibody. These non-critical residues can be substituted by any other amino acid without impairing the binding capacity of the peptide (Geysen et al., 1988; Kramer et al., 1997). Critical contact residues however can hardly be replaced, if only by amino acids with similar side-chains. A detailed substitutional and positional analysis defines a binding motif for a given peptide-antibody interaction. This so-called supertope delineates the structural requirements for binding at each position of the relevant peptide (Kramer et al., 1997). But, as already mentioned, one paratope surface may accept the binding of more than one supertopic entity and involve each time a different fraction of peptide contacting residues. In addition, because of the high flexibility of short peptide epitopes, it is often necessary to increase the rigidity of the conformation in order to improve the structural resemblance between the peptide epitope and the corresponding domain in the wild-type protein, and enhancing thus the 'antigenicity' of a peptide. For this purpose, peptides are often cyclized via disulfide bonds, side-chain anchoring or head-to-tail cyclization (Valero et al., 1999; 2000) or undergo chemical backbone modifications to stabilize the peptide in a particular conformation.

'Crossreactivity' refers to events where critical interactions with homologous entities are identical to those found in the wild-type antigen (Keitel et al., 1997), i.e. on one hand, a mAb isolated after induction with a viral protein can crossreact with a short peptide, a so-called sequential epitope, derived from this protein and, on the other hand, polyclonal antiserum induced with a short, synthetic peptide may crossreact with the cognate, wild-type protein. In the latter case, the term 'crossreactive immunogenicity' is used to relate to the capacity of a synthetic immunogen to elicit anti-peptide antibodies that bind to the respective epitope on the intact protein. Such

'crossreactive' antibodies are the goal of immunogenicity studies during the development of an experimental, synthetic vaccine. High structural and sequential homology of the peptide antigen with the corresponding region on the parent protein is generally believed to be crucial for the induction of crossreactive antibodies. However, the outcome of immunization experiments is usually difficult to predict. So-called peptide mimotopes without any sequential homology to the native epitope have been shown to induce MV-crossreactive and neutralizing antibodies (Steward et al., 1995). One should to be aware that there is no causal relationship between 'antigenicity' and 'immunogenicity' of a peptide epitope (Van Regenmortel, 2001a, 2001b). Indeed, whereas highly antigenic peptide mimotopes were not able to elicit crossreactive antibodies, non-antigenic mimotopes induced MV-crossreactive and even neutralizing antibodies (El Kasmi et al, 2000). However, a careful analysis of the antigenic and immunogenic peptide binding specificities, i.e. the binding pattern of the mAbs used in the antigenicity studies and the induced, crossreactive antibodies, provide useful evidence about the binding mode of the respective antibodies and the structural resemblance of the peptide epitope with the original antigenic determinant in the cognate protein. These studies also delineate the crucial residues for antigenic binding and crossreactive immunogenicity. If these critical residues are conserved among circulating MVs, the immunization with one single, conserved, synthetic epitope can induce antibodies targeting and thus neutralizing a variety of wild-type MV strains.

The development of experimental synthetic vaccines primarily aims at the development of a certain 'activity' or 'function', rather than at the development of a certain 'structure'. But chemical synthesis generates in a first instance a structure, which is then subject of a functional characterization. The study of 'structure-activity' or the 'structure-function' relationships supposedly delineates the critical parameters of a synthetic molecule necessary to fulfil an expected activity. Synthetic vaccine design focus on questions like 'What type of antibodies are neutralizing and protective?' and 'How can neutralizing and protective antibodies be induced?' Whereas the first question targets the characterization (binding specificity, structure) of antibodies fulfilling a wanted activity, the second question relates to the prerequisites (structure, binding mode) of a vaccine to induce a functional antibody population. Responding to the first question might not immediately generate an

answer to the second question. There is no simple causal link between a structure and a function, than a complex relational nexus where many parameters simultaneously influence the two entities (Van Regenmortel, 2000, 2001b). However, an integrated study of the structure-activity space can generate correlations between critical components of the system of interactions. This can be achieved using an iterative approach involving independent and complementary immuno-assays and formats. *In vitro* and *in vivo* experiments as well as *in silico* studies can explore these interactions and a set of correlates can thus be operationally defined referring explicitly to the experimental conditions used for its detection. However, no study may ever hope to capture completely all information relating to the interactions analysed, and even if such a complete model would exist, it would be too complicated to use in any practical way. The understanding of structure-activity relationships is thus built from relevant empirical data on the basis of trial-and-error, complemented with some heuristics or intuition.

'Rational vaccine design' refers to the sensible usage of different assays, methods and synthesis protocols in the course of the investigation and careful and precise analysis of the data resulting thereof. The way how you see things impresses upon the way how you do things. A genuine understanding of the concepts like 'specificity', 'antigenicity', 'crossreactivity' or 'function' affects the layout of experiments and analytical methods used.

2. Functional epitope mapping and molecular modeling

Continuous epitopes of a protein antigen can be considered as surface-accessible loop structures, more or less constrained by the scaffold formed by flanking sequences. Interactions with the microenvironment of the protein further reduce the plasticity /flexibility of such an epitope. In contrast, the flexibility and folding of a synthetic peptide corresponding to the sequential epitope are unconstrained by these interactions. Preformed antibodies directed against a sequential epitope can partially substitute the protein environment and generate the cognate structure of the peptide by induced fit. In the absence of these constraints multiple peptide conformations are free to interact with and induce a repertoire of antibodies, many of which may not

crossreact with the cognate protein. The natural structure of the epitope can provide important guidelines for stabilizing the peptide and improving its crossreactive immunogenicity. However, in the case of the HNE domain no structural information is available and data about the role of the cysteines are conflicting. Hu and Norrby (1994) suggested that C381 and C494 participate in unspecified intramolecular disulfide bridges and that C386 and C394 are normally unpaired or participate in intermolecular disulfide bridges. The model of Langedijk *et al.* (1997) based on homology with the influenza virus predicts cystine bridges between C381-C386 and C394-C494. Ziegler *et al.* (1996) could only show an important role of C394 for peptide binding to neutralizing antibodies and El Kasmi *et al.* (2000) demonstrated that the induction of MV-neutralizing serum required peptides containing the three cysteines C381, C386 and C394. However, our binding studies paired with MS measurements demonstrate that only intramolecular C386-C394 bridged peptides are recognized by MV-neutralizing mAbs. Similarly, only C386-C394 bridged peptides precluding intramolecular cysteine scrambling induced sera crossreacting with MV H protein (Figure 3.11). Whether the above data are in conflict or represent different functional states of the H protein remains an intriguing question. Alternatively, the C386-C394 bridge may best mimic the constraints imposed by the protein scaffold.

Disulfide bonds have been shown in several systems to critically stabilize natural epitopes or peptides mimicking their conformation. Examples include both conformational as well as sequential epitopes with intermolecular and intramolecular Cys-bonds. Specific cystine bonds stabilized two epitopes associated with the receptor-binding site of the bovine thyrotropin beta-subunit (Fairlie et al., 1996). A cystine knot-like motif containing three disulfide bonds stabilizes a conformational epitope of the apical membrane antigen-1 of *P. falciparum* (Hodder et al., 1996). Although the sequential epitope of VP1 of foot and mouth disease does not contain intramolecular cystine bonds, it has an inherent compact cyclic structure (Logan et al., 1993). This very flexible and disordered structure was optimally mimicked by a peptide constrained by cyclization with an internal cystine bond (Valero et al., 2000).
The minimal epitope revealed with truncated HNE peptides extended from C386 to N396. While it was difficult to model the structure of the unconstrained full-length HNE peptide, the introduction of the cystine bond into a shorter peptide containing the core epitope predicted an amphiphilic loop matching the binding data. Simulation

runs at high temperature (1000 K) revealed a rather rigid conformation of this loop. According to the model, the three residues K387, Q391 and E395 critical for antibody interaction pointed towards the ‘upper side’ of the planar loop. We expect that their side chains account for most of the epitope-paratope contacts. The permissive hydrophobic residues I390, A392 and L393, were directed towards the ‘lower side’ of the loop, precluding antibody binding. Although these residues were indifferent to substitutions they may still contribute to antibody binding by backbone interactions with the paratope as described for other epitopes (Nair et al., 2000, 2002). The importance of the G388 may be due to the inherent flexibility of this amino acid facilitating binding by induced-fit. Gly was shown to support loop formations in many systems including sequential epitopes (Verdaguer et al., 1995) and CDR loops of antibodies (Dokurno et al., 1998). Even a small side chain in position 388 would result in steric hindrance with the main-chain nitrogen atom of K389, damaging the shape of the loop. The above spatial arrangement explains that the HNE epitope does not form a continuous stretch of contact residues. According to this model, the loop conformation is further stabilized by an H-bond between the main-chain carbonyl oxygen atom of the C386 residue and possibly the nitrogen atom of residue I390. Intrapeptide H-bonds typically stabilize flexible turns and loops of sequential peptide epitopes into conformations congruent with the antibody paratope (Rini et al., 1993; Kanyo et al., 1999).

The model agrees with observations that most of the binding energy is normally contributed by a few contact residues defining the ‘energetic’ or ‘functional’ epitope (Jin et Wells, 1994; Benjamin et Perdue, 1996; Dall’Acqua et al., 1996) and antibody binding to peptides is usually more or less tolerant to replacement of the other residues by a variety of amino acids (Geysen et al., 1988; Kramer et al., 1997). However, these non-interacting amino acids flanking the critical contact residues are known to contribute to the binding affinity (Ferrieres et al., 2000). This is probably why peptides are normally poor images of natural epitopes with affinity constants of only 10^{-6}-10^{-7} M for anti-protein antibodies. The affinity of the anti-H mAb BH216 for the peptide is unusually high (K_d = 7.60 nM), compared to the maximal values of 10^{-10} M that have been suggested for antibody affinity (Foote et Eisen, 1995). This suggests a high structural resemblance of the peptide with the natural epitope and indicates that most interactions with the protein are also contributed by the peptide. For instance,

K387, which can only be substituted by Arg, is likely to be involved in a salt bridge or a cation-π interaction with a paratope residue.

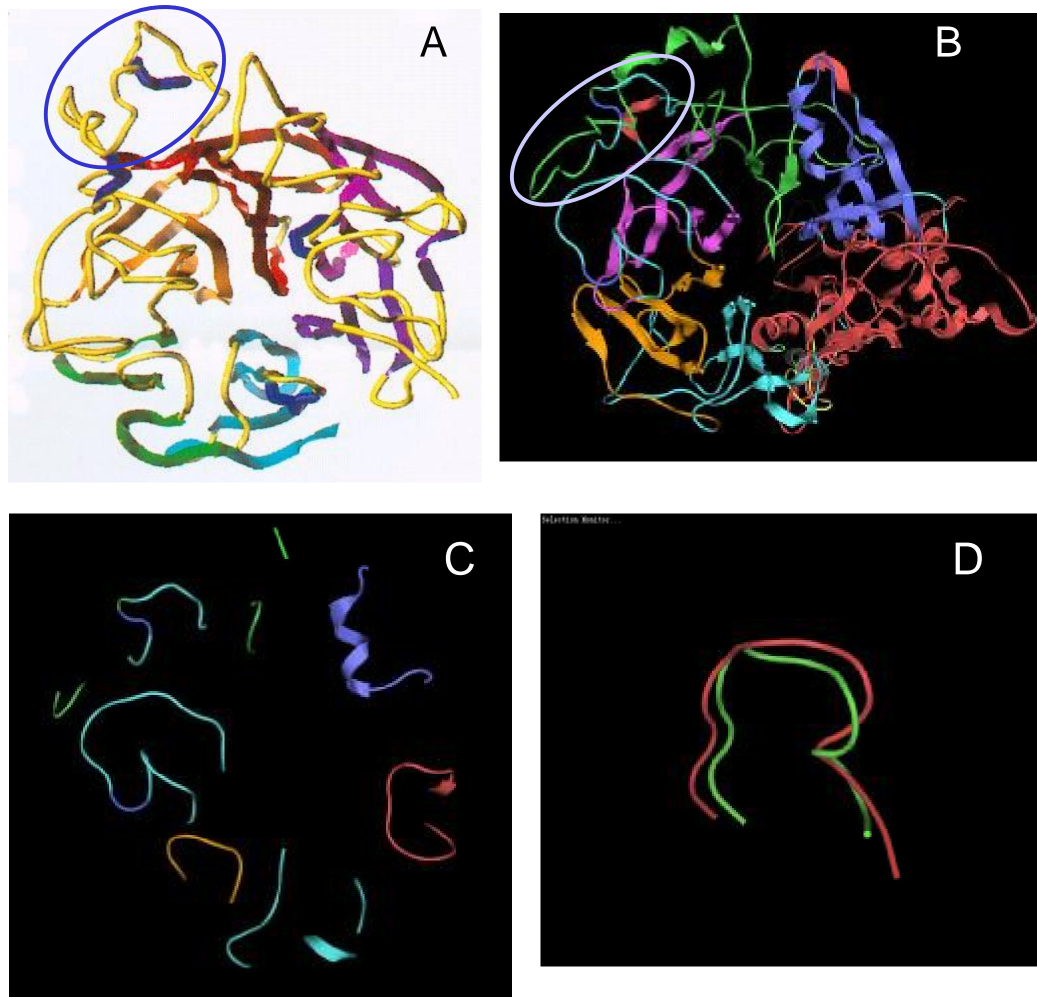


Figure 4.1: Molecular models of the MV H-protein.

(A) Hypothetical 3D structure by homology modeling on the basis of the crystal structure of influenza virus neuraminidase (Langedijk et al., 1997). HNE domain is highlighted by blue circle, predicted disulfide bridges are displayed by bold dark blue ribbons. (B) Very recent model of MV-H protein predicted by the concerted usage of *homology modeling*, *fold recognition* and *de novo folding* techniques based on the crystal structure of the H-protein of Newcastle disease virus (Damien et al., unpublished results). HNE domain is highlighted by white circle. (C) Predicted structure of the protruding, accessible loops on the surface of the H-protein model (Damien et al., unpublished results). (D) Comparison of predicted HNE structure in this study (green backbone) and the one by Damien et al. (red backbone).

Electrostatic interactions between contact residues have also been reported recently to occur within peptide epitopes (Möller et al., 2002). Similarly, the ε-amino group of K387 could form a salt bridge with the close carboxyl group of E395 and further stabilize the peptide structure within the complex with the antibody. In the virus, the amphiphilic loop may be lying flat on top of the protein, possibly stabilized by solvent-hidden hydrophobic residues pointing towards the protein core. In any case, the hydrophilic side chains K387, Q391 and E395 which are intolerant to amino acid substitutions are being solvent-exposed on the protein surface and represent a minimal target for neutralizing antibodies.

At present date, there is no crystal structure of the MV H-protein available. Despite a hypothetical 3D structure calculated by Langedijk and colleagues (1997) (Figure 4.1A) by homology modeling on the basis of the crystal structure of influenza virus neuraminidase, the structure of the flexible loops protruding on the surface of the H-protein is still not well understood and especially the proposed cystine bridges are debated. Indeed, in a very recent model of MV-H protein predicted by the concerted usage of *homology*, *fold recognition* and *de novo folding* modeling techniques (Damien et al., unpublished results) (Figure 4.1B) based on the crystal structure of the H-protein of Newcastle disease virus, the conformation of some loops, including the HNE loop, differed substantially from the model found by Langedijk et al. (1997), whereas the structure for most of the loops was conserved (Figure 4.1C).

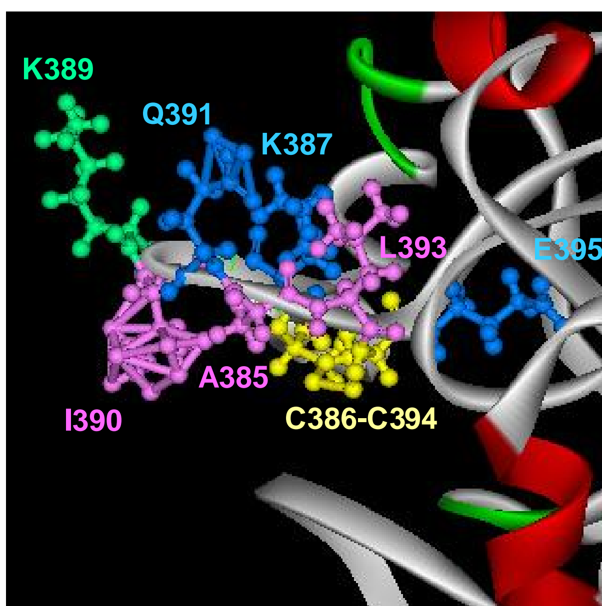


Figure 4.2: Lateral view of the HNE domain on the modeled MV H-protein

Clustering of hydrophilic/charged residues on solvent accessible surface of the epitope. Side chains of critical contact residues are shown in blue (K387, Q391, E395), hydrophilic/charged side chains (K389) in green, hydrophobic side chains in pink (A385, I390, L393), disulfide bridge in yellow (C386-C394) (Damien et al., unpublished results).

In particular, the disulfide bridge and the conformation predicted for the HNE domain correlated surprisingly well with those determined in our binding and *in silico* modeling studies (Figure 4.1D). In addition the clustering and orientation of the critical side chains is very similar in the modeled cognate HNE (Figure 4.2) to the one determined by us (Figures 3.9, 3.10).

Neutralization of wild-type MVs *in vitro* correlated with the presence of the binding motif in their wild-type HNE sequence (Table 3.2). Furthermore, neutralization escape mutants with mutations in positions of the contact residues (G388D, G388S and E395K; Hu et al., 1993; Liebert et al., 1994) further underline the consistency between protein and peptide model. The permissive K389 is predicted to extend within the plane of the loop allowing ionic interactions with both the protein core and/or the solvent. In the peptide, this amino acid can be favorably replaced by Ala. We speculate that in the absence of the ionic interaction in the peptide a small uncharged amino acid such as Ala may reduce steric hindrance. In this context, it is interesting that the introduction of a negative charge in position 392 also improves binding of the A392E substituted peptide. Our substitution studies further show that the critical residues of mAb BH195 are E395, P397, E398 and W399 flanking the C-terminal end of the HNE epitope (Figure 3.7B). This non-neutralizing antibody only reacts with the denatured virus suggesting that in the native protein, the residues extending the C-terminus – although highly immunogenic per se – are probably buried in the protein core. In this case, the improved binding to mAb BH216 of the P397A peptide could be explained by the replacement of a bulky residue (which does not contribute to the binding) by a smaller peptide causing less steric hindrance. Immunizing with the HNE peptide showed that the highly immunogenic region $E_{395}NPEWA_{400}$ which is supposedly not solvent exposed in the virus and does not contribute to virus crossreactivity competed with less immunogenic domains.

The above model allowed us to understand the critical features of the HNE epitope and to suggest a specific binding motif $X_7C[KR]GX[AINQ]QX_2CEX_5$ for neutralizing antibodies. Despite a high level of conservation of the HNE domain among wild-type MV isolates and of the three Cys residues among all other morbilliviruses (Langedijk et al., 1997), some field isolates with mutations in the HNE sequence have been reported. Binding studies with peptides corresponding to all

known virus mutants confirmed (with a single exception) the reactivity of all peptides exhibiting the binding motif. More than 90% of the MV-HNE sequences found in the databases were recognized by the neutralizing antibodies. In the absence of studies with the mutant viruses this is a strong indication that the binding motif is preserved in the vast majority of field isolates.

This study shows that in the presence of antibodies Cys386-Cys394-cyclized peptides of the HNE domain fold into conformations, which seem to be largely independent of the natural flanking sequences. These interactions seem to be similar to those found in the natural epitope of most field viruses. The redesigned peptides induce virus-crossreactive antibodies and as lead-structures they provide the blue-print for the development of antigens with similar structures but enhanced conformational stability also in the absence of antibodies. While this epitope has proven its potential as fully synthetic peptide-vaccine (El Kasmi et Muller, 2001) as well as a recombinant polyepitope vaccine (Bouche et al., 2003), a more robust chemistry, at least for loop cyclization, may be required for the efficient induction of neutralizing antibodies by a conjugate vaccine.

3. Immunogenicity in relation to immunity

It was shown previously that the HNE peptide was not recognized by maternal antibodies and induced equal peptide-specific titers in presence or absence of passively acquired MV antibodies (El Kasmi et al., 2000). Therefore this peptide is an interesting candidate to be included in a peptide-conjugate vaccine for use during early infancy. After optimizing the peptide length, this peptide, once combined to dt, induced antibodies crossreacting with the MV H protein. In this study, we demonstrated that the DT peptide-conjugate induces antibodies protecting against a lethal MV challenge *in vivo*. In addition to this, an immunization with the peptide-conjugate is compatible with active immunity to the carrier protein and with a subsequent MV administration, underlining its great potential as a subunit immunogen designed for infant vaccination.

Conjugate vaccines respond differentially to prior immunity against the carrier protein and the outcome of epitopic suppression experiments is conditioned upon active or passive priming to the carrier and in both situations in a dose-dependent way.

On the one hand, active immunity to the carrier was reported as well to enhance (Rajewski et al., 1969, Mitchison, 1971; Schneerson et al., 1980; Anderson, 1983; Peeters et al., 1991, 1992; Barington et al., 1994; Granoff et al., 1994; Shah et al., 1999) as to suppress (Herzenberg et al., 1980; Schutze et al., 1985; Lise et al., 1987; Di John et al., 1989; Peeters at al., 1991, 1992) antibody responses to conjugated haptens. On the other hand, passive immunity to the carrier protein was also shown to impair (Sarvas et al., 1992; Barington et al., 1994; Karlsson et al., 2001) the subsequent immune response to the coupled epitope moiety, whereas in other cases no interference was found (Kurikka et al., 1996; Siegrist et al., 1998a, 1998b). Most of these studies were carried out for conjugates with polysaccharides or low molecular weight haptens and it is difficult to estimate the biological relevance of these observations for peptide-conjugates. From the present study, we conclude that the peptide- and carrier-specific immunogenicity of two different peptide-conjugates (DT and TT) is not impaired after prior immunization with carrier protein alone. However, when mice were passively primed via transfer of a biologically relevant quantity of anti-carrier antibodies, anti-peptide and anti-carrier responses were almost completely suppressed after two injections with the same peptide-conjugate ([dt]_DT_DT or [tt]_TT_TT). Mice passively primed with the irrelevant carrier developed 'normal' antibody titers ([tt]_DT_DT or [dt]_TT_TT). Similarly, a suppressive effect was also observed for the conjugates after active heterologous priming (TT_DT_DT, DT_TT_TT) and after passive homologous ([DT]_DT_DT, [TT]_TT_TT) and heterologous ([TT]_DT_DT, [DT]_TT_TT) priming priming with anti-conjugate antibodies. These results stress the importance of the T-cell immunity for the immunogenicity of peptide-conjugate vaccines, as carrier-specific $CD4^+$ helper cells govern the activation and clonal expansion of peptide- and carrier-specific B cells and carrier-specific T cells.

These observations confirm early studies in mice and rabbits pointing out that the response to a hapten is enhanced after previous active priming with the carrier protein (Rajewski et al., 1969; Mitchison, 1971; Schneerson et al., 1980; Anderson, 1983).

Similarly, prior active immunity to the carrier led to enhanced humoral responses for an human chorionic gonadotropin based birth-control conjugate vaccine in female adults (Shah et al., 1999). Most importantly, young infants immunized shortly after birth with DTP developed enhaced Hib-specific titers when immunized with a Hib-conjugate based on CRM197 or tt (Granoff et al., 1994). On the other hand, passively acquired maternal antibodies were shown to interfere with immunizations with the routine tetanus vaccine (Sarvas et al., 1992) and a tt-based Hib-conjugate vaccine (Barington et al., 1994) in young infants. Furthermore, our findings discussed above are in accordance with a previously described phenomenon, termed 'non-epitope-specific suppression', as an immunization with one component impaired the subsequent response to both components of the vaccine (Barington et al., 1993).

Nonetheless, our findings are in conflict with observations made in infant mice where high levels of maternal anti-tt antibodies only affected the induction of antibodies against the carrier and did not alter the specific response against an MV derived peptide (Siegrist et al., 1998b). Clinical studies with a Hib-tt conjugate came to the same conclusions showing that maternally inherited tt-specific antibodies did not alter the response against the Hib polysaccharide (Kurikka et al., 1996). Immune responses induced in infants and adults might relate differentially to passively acquired antibodies (Barrios et al., 1996). Maternally acquired antibodies may inhibit completely the carrier-specific antibody response without interfering with the respective T-cell responses (Siegrist et al., 1998b). Additionally, they can interfere more strongly with the first dose of tt immunizations in infants than with the second dose (Sarvas et al., 1992) and in most cases three dose vaccination schedules prevail over the effects of maternal antibodies. The humoral response induced in infants is predominantly Th2 polarized (Barrios et al., 1996; Rowe et al., 2000; Siegrist et al., 2001) and the development of adult-like Th1/Th2 responses to conjugate vaccine antigens occurs earlier than the induction of adult-like antibody responses (Siegrist et al., 2001). The inherent Th1/Th2 balance of the used antigen or carrier protein is critical. Optimal activation of Th1 cells requires antigen presentation by dendritic cells, whereas the B cells and macrophages preferentially support the differentiation of Th2 cells (Gajewski et al., 1991; De Becker et al., 1994). Epitopic suppression strongly affects the IgG2a, but only weakly the IgG1 response and there is evidence that the lack of anti-hapten Th1-like response is due to antigen presentation by cells

incompetent to govern the development of cells through the Th1 pathway (Renjifo et al., 1998). Thus, antigen presentation by dendritic cells instead of B cells can prevent epitopic suppression (Renjifo et al., 1998). The different serum samples in our study were also monitored for their peptide-specific IgG1, IgG2a and IgG2b isotypes. IgG1 represented more than 90% of the total IgG measured and no significant differences were determined in the IgG2a/IgG1 or IgG2b/IgG1 ratios using the different schedules (data not shown).

Some theoretical mechanisms have been advanced to explain epitopic suppression, including the presence of anti-carrier antibodies neutralizing the conjugate by preventing the binding of the conjugate vaccine to hapten-specific B cells and accelerating Fc-R mediated removal (Di John et al., 1989; Karlsson et al, 2001); the competition for antigen capture and presentation between carrier-specific and hapten-specific B cells (Schutze et al., 1987, 1989); the clonal dominance of carrier-specific B cells after previous priming (Schutze et al., 1987, 1989); the induction and activation of suppressor T cells (Tada et Takemori, 1974; Herzenberg et Tokuhisa, 1982); the biased antigen presentation by carrier-specific B lymphocytes in preimmunized animals rather than by dendritic cells in naïve recipients (Renjifo et al., 1998).

Despite the critical role of efficient T-cell priming, epitopic suppression occurs in many aspects in a dose-dependent way. The priming dose of actively injected carrier and passively transferred anti-carrier antibodies both affect the subsequent hapten-specific antibody response as does the dose of conjugate-vaccine used for immunization. The concept of epitopic suppression was initially defined by Herzenberg and colleagues (1980) for antibody responses against dinitrophenyl, a low molecular weight hapten, after priming with high amounts of carrier protein (100 μg KLH). Peptide-specific responses were hampered using similar priming doses of carrier protein (Schutze et al., 1985; Lise et al., 1987) and a dose-dependent role for carrier priming in epitopic suppression was eventually described for polysaccharides (Peeters at al., 1991, 1992). In comparison, also passively acquired antibodies inhibited polysaccharide-specific responses in a dose-dependent manner (Barington et al., 1994) and there was a significant dose-dependent negative correlation in tetanus-

vaccinated adult recipients of low doses of conjugate vaccine and the subsequent lowered antibody titers developed against a *Plasmodium falciparum* peptide linked to tt (Di John et al., 1989).

Our results are in accordance with these observations. Active priming with a reasonable dose (10 µg) of carrier protein did not impede the anti-peptide responses after peptide-conjugate immunization and in the case of passive priming, the degree of epitopic suppression was largely depended on the amount of passive anti-carrier or anti-peptide antibodies present at the time of immunization. High doses of anti-carrier antibodies (GMT = $1.5 \cdot 10^{-5}$) almost completely inhibited a subsequent antibody response ([dt]_DT_DT or [tt]_TT_TT), whereas as 6-fold lower levels (GMT = $2.5 \cdot 10^{-4}$) suppressed the response to a lesser extent. The fact that a similar effect was observed as well for the homologous ([DT]_DT_DT) as for the heterologous passive priming ([TT]_DT_DT) confirms the fact that passive antibodies against one component of the conjugate are able to suppress non-specifically humoral responses against the carrier and the hapten. However, the intensity of the suppressive effect may be different from conjugate to conjugate. Despite the passive transfer of similar amounts of peptide-specific antibodies in both situations, the response to TT was massively suppressed: an almost 150-fold reduced peptide-specific antibody response was observed after TT immunization ([DT]_TT_TT), whereas the suppressive effect for the DT conjugate was less than 3-fold ([TT]_DT_DT). It is subsequently difficult to estimate what proportion of antibodies was eventually induced at the second or the third injection and what proportion was eliminated in the formation of immunocomplexes with the injected conjugates.

The suppressive effect of passive antibodies can be overcome by additional boosting. On the one hand, T-cell activation, expansion and dissemination takes time, and carrier-specific T cells supposedly 'catch up' at last and supply peptide-specific B cells with the imperative help. But most probably, passively acquired antibodies wane rapidly to a titer irrelevant for epitopic suppression.

To circumvent carrier-specific epitopic suppression after active priming, carrier-derived non-B T-helper epitopes conjugated to the protein antigen have been

recommended (Sad et al., 1992; Kaliyaperumal et al., 1995; Mandokhot et al., 2000). However, in our hands a chimeric peptide combining a major T-cell epitope of tt with the HNE peptide BCE suppressed peptide-specific antibody responses to DT, similarly to the schedules TT_DT_DT and [TT]_DT_DT, due to the absence of primed $CD4^+$ helper cells specific for the tt derived class II peptide. Although the TCE is a major $H2^d$-restricited epitope similar observations were made for the schedules using TT. This suggests that even the use of a very strong immunodominant T-cell epitope cannot ensure a sufficient overlap of TCE usage with TT to avoid epitopic immune suppression. Surprisingly, no epitopic suppression was observed after priming with DT and TT, prior to immunizations with TB. The high amount of peptide (50 µg) used for immunization most probably could escape the suppressive effect of the anti-peptide antibodies. In addition, we cannot exclude that the use of a very potent adjuvant (CFA) for the peptide immunizations (in comparison to alum for the peptide-conjugates) also helped to overcome epitopic suppression.

Two injections with the peptide-conjugate vaccine (DT_DT or TT_TT) induced antibody responses significantly higher than those induced using the ‘TB’ peptide and which could not be boosted after reinjection of the same conjugate (DT_DT_DT or TT_TT_TT). Similar ‘response ceiling’ have been reported to occur also in adult humans immunized with *Staphylococcus aureus* or *Streptococcus pneumoniae* derived polysaccharide-conjugate vaccines (Fattom et al., 1990, 1993). A maximum allowable antibody response might be internally regulated by immune mechanisms or preexisting antibodies may form, in a dose-dependent way, immunocomplexes with the injected conjugate containing a further boosting of the antibody response. Antibodies can either completely suppress or enhance the subsequent antibody responses to their specific antigens (Stoner et Terres, 1963; Stoner et al., 1975; Brüggemann et Rajewsky, 1982; Karlsson et al., 1999; Heyman, 2000) and their impact was shown to differ whether studied *in vitro* or *in vivo* (Karlsson et al., 2001). Antibody/antigen ratio in the complexes was proven to be a crucial factor in enhancing antigen presentation (Manca et al., 1988, 1991). Complexes with a moderate antibody excess resulted in optimal T-cell activation in opposition to complexes with extremely high antibody excess failing to activate T cells efficiently (Manca et al., 1991). Antibody modulated uptake via the Fc receptor and enhanced

activation of T cells was also proposed for short MV nucleoprotein derived class II peptides (Fournier et al., 1996) and antibody-complexed vaccine formulations are currently commercially available against infectious bursal disease virus, an avian birnavirus (Haddad et al., 1997). We speculate a similar mechanism to be responsible for the compatibility of a MV immunization with previous peptide-conjugate immunizations. Indeed, 'normal' amounts of MV-H and MV-F crossreacting antibodies were induced by an MV immunization even after three previous injections with DT. Similarly, whereas maternal anti-tt antibodies largely suppressed the induction of antibodies against the carrier in pubs, the response against an MV-derived peptide conjugated to tt was unaffected (Siegrist et al., 1998b). Intriguingly, antibody levels generated after one injection with the peptide-conjugate (DT_tt or TT_dt) could be boosted by an injection with the heterologous conjugate (DT_TT or TT_DT). But a second immunization with the heterologous conjugate (TT_DT_DT or DT_TT_TT) showed that the priming injection suppressed the overall peptide- and carrier-specific antibody responses in comparison to the unprimed (DT_DT or TT_TT) or homologously primed animals (DT_DT_DT or TT_TT_TT). Most probably, peptide-specific B cells induced after the first injection were supported by 'new' T-helper cells induced after the second injection and specific for the other carrier. Antibody/antigen ratio in the complexes at the site of injection might be the critical switch for antibody generation or suppression during immune responses.

The simultaneous administration of several conjugate vaccines sharing the same carrier together with the carrier itself can also result in lower antibody responses against the coupled epitope moieties. Co-injection of free carrier and conjugate-vaccines in carrier-primed mice resulted in a competition for the recruitment of the primed T cells, i.e. implying that the epitopic load rather than the antigenic load at the site of injection generated the lowered hapten-specific immunogenicity (Fattom et al., 1999). Thus, the quantity of carrier proteins and conjugate vaccines involved when investigating the immune responses to conjugate vaccines or evaluating prime-boost strategies in various immunity statuses in a given animal or a clinical model is a critical parameter. Serotype-specific immunogenicities have been shown for multivalent pneumococcal conjugate vaccines using dt or tt as carrier proteins and a mixed carrier vaccine has been proposed (Sigurdardottir et al., 2002). Chedid and co-workers observed an enhanced peptide-specific antibody response for a tt-conjugated

Plasmodium falciparum derived peptide after preimmuniztion with tt, albeit a similar conjugate containing a Plasmodium knowlesi peptide lead to reduced antibody responses (Lise et al., 1987). Carrier-specific differences in the polysaccharide-specific responses to active priming with diphtheria-tetanus vaccine have also been reported for three commercially available meningococcal conjugate vaccines using tt or CRM197 as carrier proteins (Burrage et al., 2002). The CRM197 based vaccine displayed enhanced polysaccharide-specific responses after priming, taking profit from the crossreactive T-cell priming with dt and from the reduced dt-specific antibody crossreactivity.

These data generated evidence showing that the HNE peptide-conjugate elicits a protective immune response against measles which is compatible with a subsequent immunization with the live-attenuated MV. The anti-peptide response is not affected by active immunity to dt or tt, when potentially administered after an immunization with the diphtheria-tetanus-pertussis vaccine. Despite a possible suppression of a peptide-specific antibody response by passively acquired maternal anti-dt or anti-tt antibodies, the implementation of the peptide-conjugate in a 3-dose schedule similar to Hib-conjugate vaccines would induce a protective level of peptide-specific antibodies. These results further promote the HNE epitope as an ideal candidate to be included in a subunit vaccine against measles designed for use in early infancy.

4. Peptide-conjugates and non-invasive immunization strategies

Immunogens admixed with bacterial toxin adjuvants like whole cholera toxin (ct), the nontoxic cholera toxin B subunit (ctb) or heat-labile enterotoxin (lt) induce strong humoral and cellular immune responses, both systemically and locally, after intranasal (i.n.) administration (Bergquist et al., 1995). In our hands, the HNE peptide, once conjugated to different carriers (dt, tt, ova) and mixed with ct, induced high peptide-specific titers after i.n. immunization. Highest peptide-specific serum IgG antibody titers were elicited using the DT conjugate, whereas the TT conjugate induced highest carrier-specific titers. It was shown in previous studies that a *Haemophilus influenzae* type B (Hib) derived polysaccharide, when first conjugated to tt and then coupled to ctb induced high polysaccharide-specific serum IgG antibodies when administered i.n. or subcutaneously (Bergquist et al., 1998). The polysaccharide coupled to tt and

mixed to ctb or directly linked to ctb induced lower antibody titers (Bergquist et al., 1998). Similarly, type III capsular polysaccharide derived from *Streptococcus agalactiae* group B induced systemically antibodies after i.n. delivery either directly conjugated to tt and ctb, or indirectly to ctb via tt (Shen et al., 2001a, 2001b). Potent serum IgG and mucosal IgA responses could also been elicited with conjugate vaccines based on *Neisseria meningitidis* group C and Hib derived polysaccharides using CRM197, the non-toxic mutant of dt, as carrier (Ugozzoli et al., 2002). Even chimeric synthetic peptides containing two copies of a promiscuous TCE and one copy of a BCE, both derived from MV F-protein, induced neutralizing and protective antibodies when mixed with ctb (Hathaway et al., 1997). In our in vivo experiments however, no peptide-specific antibodies were induced using the chimeric TB peptide mixed to ct. Most probably T-cell help was to scarce due to the presence of only one copy of TCE. The dt and tt carrier-proteins provided however strong T-cell help to the conjugated HNE moiety and high titers of anti-HNE antibodies which crossreacted with the MV H-protein and protected against an intracranial challenge with adapted MV, similar to those obtained via the i.p. route using Alum, could be induced.

Whole ct and lt are exceptionally powerful mucosa-binding adjuvants, but they are not suited for human use because of their toxic effects, even at low doses. Their respective subunits, i.e. ctb or ltb, are non-toxic and display still a potent adjuvant activity similar to that obtained with the whole toxins ct or lt (Wu et Russell, 1993; Bergquist et al., 1998; Rask et al., 2000). They have been successfully used in humans without negative side effects (Bergquist et al., 1997a; Rudin et al., 1999). Novel adjuvants suited for i.n. and transcutaneous delivery are currently investigated and 2 mutants of the lt, i.e. LTK63 and LTR72, are promising candidates, because of their combined potency and safety (Pizza et al., 2001; Ugozzoli et al., 2002; Tierney et al., 2003).

Epitopic suppression was reported to occur in mice with preexisting immunity to ctb (Bergquist et al., 1997b). Both the specific mucosal and serum antibody response was inhibited to a ctb-conjugated dextran epitope after i.n. immunization of mice previously immunized with the ctb carrier. This suppressive effect could however be overcome by using higher doses of conjugate for i.n. immunization. On the other hand, passively transferred anti-ctb antibodies suppressed both the serum and the local

antibody response against ctb, while the dextran-specific specific response stayed unaffected (Bergquist et al., 1997b).

We also investigated the immunogenicity of peptide-conjugates DT, TT and OVA by transcutaneous immunization (TCI), a novel delivery strategy where the immunogens are mixed with mucosal adjuvants and applied onto shaved, intact skin. This non-invasive route of immunization was developed recently by Glenn and colleagues (Glenn et al., 1998; Glenn et al., 2000) and they could show that, similar to i.n. administration, bacterial toxins, i.e. ct and lt, strongly enhance immune responses induced against admixed antigens when coapplied on wet skin (Scharton-Kersten et al., 2000; Yu et al., 2002). Using this technique, we could elicit commendable peptide-specific titers after TCI with the peptide-conjugates DT, TT and OVA mixed with ct. Nonetheless, anti-peptide titers were about 30-fold lower than those obtained after i.n. or i.p. delivery and the immune sera displayed only a very weak crossreactivity against the H-protein when measured by flow cytometry. It is difficult to estimate the relevance of the titers we detected as no comparable study with short peptide immunogens was performed yet. But the titers are consistent with, although much higher than those obtained very recently after TCI using a recombinant, 101-amino-acid polypeptide containing G fragment (G2Na) derived form RSV (Godefroy et al., 2003). The polypeptide-specific antibodies were dominated by the IgG1 isotype, an indication for a Th2 type response, and could significantly decrease the RSV titers in lung tissues and the nasal tract, respectively, after i.n. challenge of mice (Godefroy et al., 2003). The amounts of antigens used in this study, i.e. 150 μg G2Na mixed with 100 μg of ct, were however much higher than the quantities used in TCI, i.e. 50 μg DT, TT or OVA, corresponding to 5 μg of HNE peptide, mixed with 10 μg of ct.

Effective peptide- and virus-specific T-cell priming was performed by TCI using a short 13-mer peptide derived from influenza virus hemagglutinin, as measured in vitro by an IL-2 secretion assay (Beignon et al., 2001). TCI induces typically systemic antigen specific T-cell responses with a mixed Th1/Th2 phenotype, but with a Th2 bias. Interestingly, ct and ctb differ in their qualitative adjuvanticity when used in TCI as opposed to a mucosal route where their effect is very similar (Wu et Russell, 1993).

It was shown that ctb, when admixed with antigens, potentiated a Th-1 driven response after TCI, whereas whole ct induced both Th1 and Th2 cytokine production (Anjuère et al, 2003).

TCI can boost existing immunity in an extent dependent of the antibody titer present at the timepoint of TCI boosting (Hammond et al., 2001). Mice, primed intramuscularly using very small amounts of tt, with resulting low tt-specific antibody levels could be boosted to more than a 100-fold increased anti-tt titers by TCI (Hammond et al., 2001). This shows that TCI follows immunological principles that govern other routes of vaccine delivery as discussed in the previous section. Dual immunization regimes involving oral inoculation and transcutaneous boosting or vice versa are currently also under investigation (John et al., 2002).

The development of novel subunit vaccines compatible with non-invasive, needle-free routes of administration has become an important outcome as a large proportion of vaccines delivered via the parenteral route is considered unsafe due to needle-borne diseases or faulty disposal of used injection devices (WHO, 1996a, 1996b). Intranasal delivery or TCI present promising routes suited for mass vaccination, compliant and safe immunization during early infancy or revaccination of adults via uncomplicated and efficacious self-administration. These routes are particularly well adapted for the administration of peptide-conjugates, biologically safe and stable antigens.

5. The need of a new measles vaccine in a world of changing epidemiology

Candidate vaccines based on a highly potent adjuvant (Quil A), a variety of replicating (parainfluenza virus type 3, vaccinia virus,) or replication deficient viral vectors (adenovirus, canarypox virus, fowlpox virus, modified vaccinia virus Ankara, vesicular stomatitis virus), bacterial vectors (BCG), as well as naked nucleic acids utilise either one or more of the immunodominant MV proteins (H, F, N) as antigens. Most of these vaccines are meant to vaccinate infants early after birth. Several have been demonstrated to be immunogenic in infant macaques even in the presence of passively transferred or maternal anti-MV antibodies. Among the vaccines evaluated in monkeys, the Quil A- and recombinant modified vaccinia virus Ankara-based

vaccines might well be the most promising candidates. Quil A-based vaccines induced high level and long-lasting neutralizing antibody responses after a single vaccination and proved to be immunogenic even in the presence of passively transferred MV antibodies. If production of highly purified Quil A components with low toxicity is feasible without loss of adjuvanticity, this vaccine should be evaluated in clinical trials. The modified vaccinia virus Ankara (and the Quil A-based) vaccine are also safe in immunosuppressed monkeys. The modified Ankara vector is also a very promising candidate, especially in view of recent bioterrorist threats. Vaccination with this vector would not only protect against measles but most probably also against smallpox (Stittelaar and Osterhaus, 2001; Stittelaar et al., 2002c). The chimeric vector based on attenuated parainfluenza virus type 3 would potentially protect against both MV and parainfluenza type 3 virus.

Most of these vectors, in particular DNA and peptide vaccines, are less heat-sensitive than the live vaccine, reducing the need of a cold chain and the risk of loss of activity in the tropics.

Although the experience with recombinant vector antigens is limited in humans, the duration of protection by non-replicating vectors can be expected to be much shorter than that of replicating vectors. Except for some vaccinia derived vectors, protection would in most cases be much shorter than for the live vaccine. Vaccines with shorter protection require regular booster injections which would be a considerable disadvantage in comparison with the live vaccine. Alternatively, the development of vaccines that would be compatible with current EPI vaccination recommendations has been proposed (El Kasmi and Muller, 2001). The strategy is based on conjugate peptide antigens or a recombinant string of protective, sequential B-cell epitopes as minimal vaccines to close the window of susceptibilty in infants until the live vaccine can be given. Although candidate peptides have been shown to induce a neutralizing and protective immune response in small animals, even in the presence of maternal antibodies, the compatibility with a later immunization with the live whole virus remains to be determined.

While some experimental vaccines may have some potential also for non-invasive mucosal delivery (ISCOMs, parainfluenza virus type 3, synthetic peptides, plants),

this will have to be confirmed in humans. For non-invasive, needle-free and safe delivery the aerosolised live vaccine has already proven itself in field trials. This approach bears great potential for mass campaigns in children (Cutts et al., 1999). Aerosol vaccine also seems to be more resistant to maternal antibodies (Cutts et al., 1997). Similarly, adults with waning immunity could be boosted by aerosolised live vaccine, should the need arise in the future. Oral, plant based vaccines could also facilitate vaccination of children or adult in mass campaign.

Effective subunit vaccines would be suitable to replace the live vaccine during the final stage of an eventual eradication program, provided that protection is robust and long-lasting; immunogenicity in the presence of maternal antibodies would be an advantage.

The most successful among these candidate vaccines await clinical trials. However, before immunization of seronegative infants, the vaccine must be tested in infant rhesus macaques to ensure that it does not predispose for atypical measles (Wild et al., 1999). It was not until recently that the risk of atypical measles after vaccination could be experimentally assessed and that a major obstacle to clinical evaluation of new measles vaccines was removed (Polack et al., 1999, 2002a).

However, any new vaccine has to prove itself against the benefits of the live vaccine. This vaccine is used world-wide as an integral part of vaccination schedules, fine-tuned over several decades. The optimal age for vaccination has been determined by most countries to account for epidemiological and sociological differences. Many countries use the live vaccine in combination with rubella and mumps (MMR). Furthermore, a number of clinical features of the live vaccine are difficult to match by new vaccines. Any new measles vaccine that would be introduced would be more expensive than the current live vaccine, which proliferates in the vaccinated host (reducing production) and relies on depreciated technology and production facilities (reducing investment).

It would require a major effort to change vaccination schedules to replace the live vaccine by another vaccine. Such an effort may be warranted when measles elimination targets cannot be met or during the final stages of eradication. However,

compatibility with the current live vaccine and recommended vaccination schedules may turn out to be critical for the successful introduction and uptake of a new measles vaccine. Early vaccination with subunit vaccines containing MV proteins would severely prejudice the subsequent use of live vaccine. Some of the drawbacks of this vaccine are more of a practical nature or are already reflected in current vaccine recommendations. Other issues may seem more theoretical and their consequences may not be felt until the later stages of measles elimination (or never), but they need to be addressed in a timely manner. Considering the differences in the robustness and durability of vaccine strain- and wild-type virus-induced immunity, measles eradication may be possible as long as a considerable proportion of mankind is protected by natural immunity. As the proportion of vaccinees increases every year by about 1-2%, we may very well miss the chance to eradicate measles if we do not reach the goal within the next ten years or so. Time may become a crucial factor (Muller, 2001). Opinions may differ with regard to the feasibility of global eradication of measles with the current vaccine and whether true saturation immunization can be reached. However, there seems to be a consensus that the development of new experimental vaccines cannot wait until we find out.

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1. Pütz, M.M., Muller, C.P., 2003. The rationale of a peptide-conjugate vaccine against measles. *Vaccine* 21: 663-666.

2. Pütz, M.M., Hoebeke, J., Ammerlaan, W., Schneider, S., Muller, C.P., 2003. Functional fine-mapping and molecular modeling of a conserved loop epitope of the measles virus hemagglutinin protein. *Eur. J. Biochem.* 270: 1515-1527.

3. Pütz, M.M., Bouche, F.B., de Swart, R.L., Muller, C.P., 2003. Experimental vaccines against measles in a world of changing epidemiology. *Int. J. Parasitol.* 33: 525-545.

4. Pütz, M.M., Jung, G., Muller, C.P., Synthetic and recombinant vaccines based on peptides, mimotopes and permutational polyepitopes. *In press.*

5. Pütz, M.M., Ammerlaan, W., Schneider, F., Jung, G., Muller, C.P. Immune responses to a protective peptide-conjugate against measles after T- and B-cell priming. *Submitted.*

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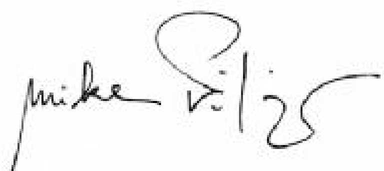

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