1. INTRODUCTION

> *"There* is *a magic in the blood, but what does it consist of? [Emil von] Behring thinks it's a mysterious power in the blood. But if it can be augmented..."*
>
> Edward G. Robinson as Ehrlich in
> *Dr. Ehrlich's Magic Bullet.*
> WARNER BROS., 1940

Anti-peptide antibodies have become essential tools in life science research. They are exploited in a variety of applications and result in the generation of useful information that contributes significantly to the understanding of biological processes.

The use of proteins for antibody production yields polyclonal or monoclonal antibodies with different specificities because multiple epitopes are presented on the surface of the protein. In contrast, it is possible to specifically generate antibodies directed to a single epitope by using synthetic peptides. Anti-peptide antibodies, whether monoclonal or polyclonal, can be used in a variety of biological applications *in vivo* or *in vitro*. These include the identification, characterization and localization of gene products, as well as the monitoring of exon usage, proteolytic processing and post-translational modification of proteins. Anti-peptide antibodies can be immobilized for immunoaffinity purification, or employed in the development of diagnostic reagents and immunoassays. Synthetic peptides and their corresponding antibodies have proved useful in studies of interactions in T-/B-cell receptor MHC peptide complexes and also serve in the development of synthetic vaccines.

The production of anti-peptide antibodies can be a fastidious task since synthetic peptides may be of poor immunogenicity on their own. There are various methods to improve peptide immunogenicity. However, limited data is available on how to efficiently produce anti-peptide antibodies. Covering the most prominent strategies for the production of anti-peptide antibodies with a comparative study is the purpose of this thesis.

1.1. Synthetic peptide antigens

> *"For a considerable period of time after the discovery of serological phenomena and despite an abundance of observations, a method was yet wanting for the systematic investigation, along chemical lines, of specificity in serum reactions. It was indeed clear that serological reactions must somehow be dependent upon the chemical properties of the substances involved (...) but insufficient chemical knowledge concerning the available antigens (proteins) (...) made a closer analysis impossible."*
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> K. Landsteiner. The Specificity of Serological Reactions; Harvard University Press. Cambridge, Massachusetts, 1945, p 156[1].

Interest in the antigenic properties of synthetic peptides stems mainly from the fact that peptides are able to mimic the antigenic sites of proteins. Proteins are the molecules of highest biological interest.

Using peptides to generate antisera has a number of advantages over immunizing with a protein: 1) Antisera of greater specificity are produced because the antibodies are generated only against the peptide of interest. 2) Antisera with higher titers are produced because more antigen is available for immunization. 3) Antibodies are generated against areas of a protein that are antigenic but not immunogenic, or are not surface accessible. 4) T cell tolerance and suppression, which sometimes occur when the protein is too similar to a native protein, are bypassed. 5) Antibodies can be generated against a protein that is toxic, rare, expensive or unavailable.

An unmatched quality of synthetic peptides is their rapid availability. Because of advances in gene cloning and sequencing, the information on protein sequences nowadays is almost completely derived from nucleotide sequence analysis. To a certain extent, automated solid phase peptide synthesis is the logical extension of automated DNA sequencing. Information obtained by DNA sequencing can be instantly translated into a functional molecule. Consequently, current advances in genome sequencing technology has also stimulated improvements in automated peptide synthesis technology. Although historically older than automated DNA-analysis, during the past decade of the "Genomics Era" peptide synthesis technology has been more innovative than ever before. Just a few examples are: non-native amino acid derivatives [1] and chimeric structures such as peptide nucleic acids (PNA) [2]; molecular dissection of protein antigens into multiple peptides simultaneously synthesized on polystyrene pins ("pepscan" [3,4]) or membranes [5]; super-parallel synthesis on beads or in microtiter plates, and robotized synthesis of combinatorial libraries yielding as many as 2 x 10^{14}

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[1] Landsteiner realized that while proteins were immunogenic (capable of inducing an immune response) the lack of structural knowledge of proteins made using them to study the chemical specificity of "serological reactions" impossible. He turned to the use of small molecules which had a defined chemical composition to study antibody specificity. Landsteiner was the first to systematically develop and chart the use of small molecules as probes of antibody recognition.

individual molecules combined with high throughput screening (HTS) for systematically assessing the structural or functional consequences of amino acid sequence variation [6,7].

So, more than 35 years after the innovative work of Merrifield on solid phase peptide synthesis, interest in the virtually unlimited possibilities of this technique has by no means diminished. On the contrary, synthetic peptides are one of the major compounds in combinatorial chemistry strategies. Combinatorial chemistry techniques and their emerging applications are in fact “spin-offs” of the Merrifield technique themselves. Many modern “designer drugs”, such as anti-angiogenesis “anti-tumor” cyclo-peptides (Merck [8]) and HIV-protease inhibitors (Abbott [9]) are synthetic peptides or peptide derivatives.

SPPS is the method of choice when it comes to the synthesis of small peptides, as documented by the successful synthesis of countless peptide hormones, cytokines and other peptide derivatives over the past 36 years. However, the vision of E. Fischer and his contemporaries of an entirely synthetic protein chemistry has remained unattainable. For several reasons, peptide synthesis cannot completely substitute for recombinant protein techniques: defective *in-vitro* folding and the occasional need for post-translational protein modification can thwart a synthetic approach. Moreover, SPPS requires high throughput of environmentally critical organic materials.

On the other hand, cloning techniques are not necessarily the method of choice for the production of larger peptide or proteins. There is no reason *per se* to infer that a recombinant protein or stretch of a protein possesses “native conformation” (i.e. that present in the intact protein *in vivo*). Moreover, a transgenic expression system is no guarantee for proper post-translational protein modification.

In addition to its rapid and straight-forward character, peptide synthesis seldom fails because of solubility or purification problems as do conventional cloning/purification approaches. There are virtually no limitations for physicochemistry or molecular composition, and no lower limit for size. Yet, SPPS is restricted to rather low MW molecules because it is difficult to synthesize polypeptides of much more than 100 amino acids. However, these limitations may vanish with time. New approaches for the generation of synthetic proteins are emerging, such as linking protein precursors “domain-by-domain” (native chemical ligation or modular approaches in SPPS [10-12]). Our understanding of the underlying principles of *in-vitro* protein-folding constantly grows, reviving the notion that the primary protein sequence contains the complete folding information and elucidating the role of protein-folding assistants such as chaperones.

Cloning/purification approaches often have to tackle another problem: contamination. Natural fragments obtained by cleavage of a protein must be separated from each other, and since the level of purity required for immunological studies is extremely high, this is often no mean task. Indeed, if a fragment of a protein devoid of antigenicity is contaminated by a small amount of highly reactive antigenic fragment derived from the same parent molecule, it may happen that the antigenicity is ascribed erroneously to the major, inactive peptide component in the mixture. Such misinterpretations are eliminated when synthetic fragments are used.

1.2. Anti-peptide antibodies and their applications

> *"To use a picture, I will say that enzyme and glucoside must join one another as lock and key in order to be able to exert a chemical effect."*
>
> E. Fischer, 1892 [2]

Antibody molecules, especially of the immmunoglobulin G (IgG) class, are extremely useful reagents for the identification, measurement, purification, and characterization of various molecules [13]. This arises from the fact that antibodies can be induced against most structures of molecular weight greater than 200-300 Da (provided the size is increased by attachment to a carrier in case of small molecules) and these antibodies usually show high avidity and specificity. In addition, IgG molecules are relatively stable and can be labeled and immobilized in various ways without serious damage to their binding abilities.

Antibodies to peptide antigens have become particularly useful tools. While the measurement and subcellular location of peptides *per se* is important, a major use of anti-peptide antibodies has been the characterization of the cognate or parent protein. Increasing understanding of how to use peptides to mimic substructures of proteins has led to a major expansion in the application of anti-peptide antibodies.

One important feature of anti-peptide antibodies is their potential capability to detect cryptotopes, i.e. to recognize protein epitopes which are not detected after immunization with the whole native protein.

Detection and characterization of gene products

Anti-peptide antibodies are important means in the identification of new proteins for which a DNA sequence has been identified, particularly those associated with viruses and oncogenes. The products of such genes are generally of very low abundance within the cell (< 0.1%), making protein isolation and antibody production very difficult. Such tools will become increasingly valuable as the genome sequencing projects progress and as more cDNAs, N-terminal protein sequences and individual spots from 2D gels analysis of cellular proteins become characterized [14-16]. The hunt for "information" (→ DNA-sequence/ORF) is being gradually replaced by a postgenomic hunt for "function" (→ protein). The identification of gene products may ensue *in vivo*/*in situ* or by detecting *in vitro* translation products (Figure 1).

In vitro translation products have been characterized by many different methods. Most of them require the proteins to be labeled during their synthesis by the incorporation of radioactive amino acids. However, if antibodies specific to the

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[2] Paul Ehrlich was influenced by the similarity between enzyme substrate- and antibody antigen-interaction. He introduced Fischer's *lock-and-key* metaphor into immunology, where it has clung tenaciously.

translation products are available, they represent an alternative powerful method for identifying and quantifying these products. The specificity of these antibodies makes it possible to avoid the problems caused by multiple translation that occur when many mRNA species are present. The strategy used for identifying translation products involves either immunoprecipitation experiments or a separation of translated products on SDS-PAGE followed by Western Blot. An early example is provided by Berna *et al.* who used this approach in their characterization of the non-structural proteins of alfalfa mosaic virus (AVM) in a wheat germ or yeast translation system [17,18].

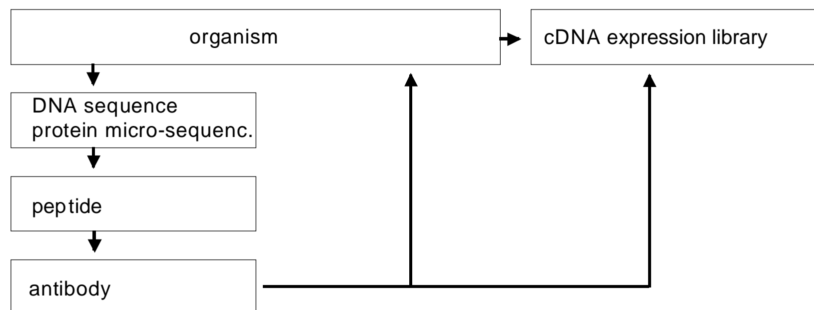

Figure 1: Detection of gene products and *in vitro* translation products using anti-peptide antibodies.

Protein localization

Anti-peptide antibodies can be of great importance for the characterization of the cellular localization and function of proteins [19]. In situ hybridization of mRNA highlights the area(s) within a tissue or cell where a protein is expressed. The use of anti-peptide antibodies complements this technique by showing the site of action of the corresponding protein. The use of anti-peptide antibodies for this technique is exceptionally informative when the site of action of the protein is distant from where the protein is expressed.

Protein purification by immunoaffinity chromatography

Polyclonal anti-peptide antibodies are generated against a specific peptide sequence and have monoclonal-like properties. If this sequence is unique to a particular protein, then the antibodies generated can be specific to that protein. As a result, the antibodies can be used to purify the corresponding protein by a technique known as immunoaffinity chromatography.

This procedure was first used to purify polyoma virus medium T from infected cell extracts [20]. In this example, two different anti-peptide antibodies were used sequentially to provide extensive purification of the medium T.

Assay and vaccine development

Synthetic peptides that elicit antibodies recognizing the parent protein are important for the development of immunodiagnostic reagents or the delineation of critical epitopes in the investigation of potential vaccines [19,21,22]. Many clinical conditions are the result of overexpression or underexpression of physiological proteins, including hormones, enzymes, and acute phase reactants (e.g., C-reactive protein or serum amyloid A). These abnormalities can be used as markers for tissue damage (e.g., liver disease, muscle disease, myocardial infarction), kidney failure, tumors, immune disorders, and infections.

The exploitation of these markers and their corresponding anti-peptide antibodies includes various applications in immunoassay-, immunoblotting-, immunocytochemistry- and immunoprecipitation-techniques, and ranges from a simple classical Western Blot to more advanced automated techniques such as fluorescence-activated cell sorting (FACS), and their utilization as markers and contrast-media in computer-tomography.

Demonstration of protein similarity and dissimilarity

Anti-peptide antibodies can demonstrate protein similarity and dissimilarity. Families of proteins, such as the keratins, are usually composed of conserved regions with similar amino acid sequences and non-conserved areas where the amino acid sequences are unrelated.

Depending on the peptide sequence chosen, different results are obtained. If a sequence within a conserved region is chosen, the anti-peptide antibodies obtained are capable of detecting all of the family members. If a sequence within a non-conserved region is chosen, the antibodies can be used to differentiate one specific family member from another [23].

Other authors successfully employed anti-peptide antibodies to discriminate between enzyme isoforms. Brown and co-workers detected and localized the alpha and beta isoforms of endothelin-converting enzyme-1 (ECE-1) in rat and man [24]. With the help of anti-peptide antibodies, Grazioli *et al.* monitored protein conformational changes of protein tyrosine kinase ZAP-70 induced by T cell receptor (TCR) triggering [25]. Wallukat and colleagues used anti-peptide antibodies for steering of physiological receptor molecules: Antibodies directed against the second extracellular loop of the beta-1-adrenoceptor controlled the beating rate of cultured cardiomyocytes [26].

Determination of protein active sites

Antibodies are also capable of identifying the active site of a given protein. To accomplish this, antibodies are made against synthetic peptides that scan the length of the protein or the proposed active site. The subsequent antibodies are then tested in a functional assay to see which of them inhibit the function of the protein. By looking at the sequence to which the antibody was directed, information can be obtained as to which region of the protein is responsible for its function. Several examples are described in a review by Lerner (1982) [27].

Anti-'wrong' reading frame antibodies

Anti-peptide antibodies can be made against a predicted protein sequence derived from a frameshifted gene sequence. These antibodies can then be used to detect the frameshift gene product. This type of antibody has been prepared in order to study frameshift mutations in different sarcoma virus transforming genes [27]. These antibodies are also useful in the study of wild-type and mutant proteins.

Following protein domains

Anti-peptide antibodies have been useful in following protein domains as a means of studying precursor and post-transitional processing [27-29]. Murakami *et al*. (1992) [30] also described the use of anti-peptide antibodies in defining the protein domains involved in protein-protein interactions within G proteins.

Exon usage

A special application for anti-peptide antibodies involved in following protein domains is the study of exon usage in gene expression. Tamura *et al.* (1991) [31] used anti-peptide antibodies to show the existence of distinct variants of the alpha 6 and alpha 3 subunits of integrin. The differences between the variants were in their cytoplasmic domains and were caused by alternative exon usage, which was cell-type dependent.

Study of proteolytic processing

It is possible to produce antibody reagents specific for different variants resulting from proteolytic processing of proteins. With this approach Hanecak *et al.* [32] and Baron *et al.* [28,33] have demonstrated the existence of a common precursor for the poliovirus replicase and the polio RNA-linked protein, VPg. Differential transcription and differential splicing of the mRNAs coding for proteins can also be detected. Neurath *et al.* showed that the HBV *env* gene has the capacity to code for three related proteins: the S protein (226 aa), the M protein, containing an additional 55 amino acids at the N-terminal end of the S protein (the 'pre-S2 region'), and the L protein, corresponding to the sequence of the M protein with an additional 108 N-terminal amino acids (encoded by the pre-S1 region). Using antibodies stimulated to synthetic peptides corresponding to the pre-S gene, Neurath and co-workers showed that all three *env* encoded proteins were present in HBV particles [34]. In another experiment, site-specific antibodies against neurotensin, as well as against the exposed KLPLVL (K6L) and EKEEVI (E6I) sequences of its precursor, were used to localize the resulting maturation products immunohistochemically from the neurotensin/neuromedin N precursor molecule [35]. Anti-peptide antibodies have also been used to show how proteolytic processing of the ß-amyloid precursor protein is important in the development of amyloid plaques in Alzheimer's disease [36].

Study of posttranslational modifications

Turner and co-workers first adapted peptide derived monoclonal antibodies to the study of posttranslational modifications. In a recent project a panel of antibodies was used to define the distribution of specific acetylated isoforms of core histones in mouse

embryos in different developmental cell stages by laser-scanning confocal microscopy. With the help of these antibodies, which were discriminating between acetylated and non-acetylated lysine residues, information was gained about how replication-dependent acetylation and deacetylation of zygotic chromatin mediates the programming of zygotic transcription [37].

MHC studies

Another area of recent expansion is the use of defined or definable peptide antigens to identify and map T cell receptors. Unlike Ig molecules, T cells principally recognize linear and short amino acid sequences, thus making synthetic peptides (and peptide specific antibodies) a perfect strategy for the exploration of T cell and HLA-/MHC-related topics [38].

1.3. Solid-phase peptide synthesis

"There is a need for a rapid, quantitative, automatic method for the synthesis of long chain peptides."

R. B. Merrifield, laboratory notes, May 26, 1959

The broad range of applications of synthetic peptides observed is the consequence of the development of peptide chemistry over more than ninety years. The synthesis of peptides has been a challenge since the turn of the century. The early endeavors, notably those of Emil Fischer and his colleagues, were stimulated by the emerging theories of protein structure (For a review of the early history of peptide synthesis, see ref. [39]). By the middle of the century, however, the realization that other biologically important molecules had simpler amino acid sequences increased the stimulus and reduced the problem of peptide synthesis to a feasible task.

In 1954 du Vigenaud and his colleagues accomplished the synthesis of a rather small nonapeptide, oxytocin [40]. This lactogenic amide hormone was the first naturally occurring peptide hormone to be prepared in the laboratory. It initiated a new era in both biology and chemistry, and earned du Vigenaud the Nobel Prize for chemistry. In 1963 the first chemical synthesis of human insulin was achieved by Meienhofer *et al.* [41]. These two hormones were synthesized by application of the classical methods of solution-chemistry, which require the purification and characterization of the intermediate peptide at every step, a time-consuming and complicated approach. Other new biologically active peptides were soon isolated, thus demanding new and improved methods for their synthesis. Pharmacological studies required synthesis not only of the often hard-to-isolate natural peptides, but also of numerous analogues, thus permitting investigation of the relationship between chemical structure and biological activity. However, the classical methods of solution peptide synthesis were hard pressed to meet this explosive increase in demand. The total number of steps in synthesis of moderately sized peptides is substantial. Even in skillful hands, yields in peptide bond forming reactions were often only modest, giving low overall yields and contamination with side-products.

This was the background for the search for feasible accelerated procedures in the 1950s, just around the time when Robert Merrifield left the quotation above as a remark in his laboratory journal at Rockefeller University.

The introduction of the concept of solid phase peptide synthesis (SPPS) by Merrifield in 1963 considerably improved the existing state of the art. In this approach, the growing peptide chain is bound covalently through its C-terminus to an insoluble solid support. The procedure was first proposed in 1962 [42], with a full paper published in 1963 [43]. In the latter, Merrifield described the preparation of the tetrapeptide Leu-Ala-Gly-Val by successive addition of benzyloxycarbonylamino acids to a polystyrene resin. To enable cleavage of the benzyloxycarbonyl protecting groups without concomitant detachment of the peptide from the resin support, the latter was nitrated, since the nitrobenzyl ester linking groups are more resistant to acids. The

synthesis was undoubtedly successful, but still not sufficient for extended applications because the coupling and deprotection reaction were not brought to completion and the target tetrapeptide was contaminated with shorter variants. Thus, extension to longer sequences with increased purification problems was unpromising. Within a year, however, the initial approach was improved significantly. In a third publication [44] Merrifield described substantial changes in technique, particularly replacement of benzyloxycarbonyl-protected amino acids by the much more acid-labile *t*-butoxycarbonyl (Boc) derivatives. Hence, the "Boc" method for SPPS was born. Figure 2 presents the standard scheme for SPPS as developed by Merrifield.

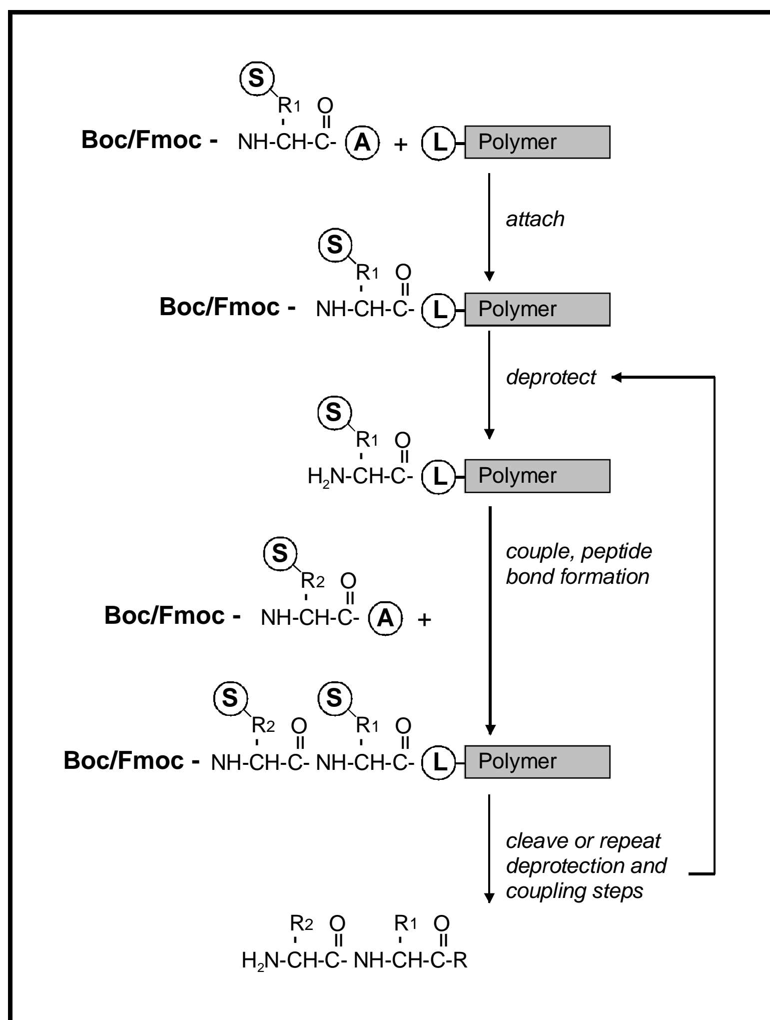

Figure 2: Flow diagram for the solid phase synthesis of peptides. After linking the first N-terminally protected (**Boc/Fmoc**) and C-terminally activated (**A**) amino acid to the resin bound linker **L**, the peptide is elongated by repeated cycles of deprotection and acylation (coupling) steps. Amino acids are introduced as side-chain protected derivatives (**S**). Depending on resin or cleavage chemistry the product can be obtained as a C-terminal acid (R = OH), ester (R = OMe, OEt, etc.), hydrazide (R = $NHNH_2$) or amide (R = NH_2, NHMe, NHEt, etc.).

In essence, the core protocols of the original Merrifield methodology have changed very little from those detailed in the 1963 publication, which attests to the prescience of the instigator of the method. However, there has been a process of

continual refinement in the development of protecting groups for α-amino and side-chain functionalities so as to permit the selective removal of one in the presence of the other, as well as improvement in the methods used to achieve complete deprotection of the final peptide and to effect its efficient and clean removal from the inert solid phase support.

In particular, the introduction of the base-labile 9-fluorenylmethyloxycarbonyl group ("Fmoc", Figure 2) for the protection of amino acid functional groups by Carpino and Han [45], laid the foundation for a genuinely orthogonal solid-phase peptide synthesis strategy in which the removal of the *N*-α-protecting group could be achieved under qualitatively different conditions from those required to effect cleavage of the side-chain protecting groups. This was in direct contrast to the Merrifield method which was based upon quantitative differences in the rates of acidolytic cleavage of Boc-groups and those protecting side chain functionalities. Carpino's findings were subsequently exploited by Meienhofer for the development of a polystyrene based Fmoc strategy [46,47], which has since been perfected (and extended to other applications such as solid-phase nucleotide synthesis) by Sheppard and co-workers [48].

Merrifield's elegant idea to perform chemical reactions on solid phase thus shifting the chemical equilibrium to the product side and, in addition, substantially simplifying product recovery, changed organic chemistry completely. Merrifield was awarded the Nobel Prize in 1984.

1.4. Conjugated peptides

Some authors claim to get satisfactory antibody levels with peptides as short as 6-8 residues [49-52]. However, it is commonly assumed that antigens with a molecular weight smaller than $2\text{-}5\times10^3$ (corresponding to roughly 20 to 50 aa) behave like haptens and are not immunogenic [53,54]. The weak and unpredictable immunogenicity of free synthetic peptides accounts for the widespread practice of immunizing animals with peptides conjugated to suitable carrier molecules and co-administering adjuvants. There are indeed many reports in the literature indicating that immunizing with conjugated peptides leads to antisera of higher titers than immunizing with the corresponding free peptides [55-57].

The widespread use of synthetic peptides as immunogenic components for the induction of defined antibodies specific to the parent protein has been accompanied by steady progress in the enhancement of their immunogenic properties. Besides the established protocols of coupling peptides to larger, foreign proteins, such as BSA or KLH, strategies are used which exclude irrelevant carrier-associated B-cell epitopes in the inoculum. Alternative approaches have been sought to focus the immune system repertoire on structures related to the molecules of interest. This must be done without sacrificing the ability to recruit T-helper cells and stimulate them to release cytokines required for the expansion of relevant B cell clones and switching of the antibody isotype expression. Investigators have tried to enhance immunogenicity by a variety of manipulations [58-60]:

i) by further polymerization to increase antigen mass: assembly of peptides as multimeric sequences branching from a common polylysine core scaffold (multiple antigenic peptide, MAP [61,62]; synthesis of peptides on inert, non-immunogenic polymers such as polyamide or polystyrene beads [63].

ii) by increasing peptide lipophilicity. This can be achieved by attaching fatty acid chains to peptides [64-66]. Attaching peptides to or enclosing them into liposomal bilayers to increase cellular uptake has also been shown to increase immunogenicity.

iii) by enhancing the immunogenicity of the peptide itself. Sequence or structure alteration for the optimization of the paratope-epitope fit have successfully been tested ("epitope enhancement" [67], "constraint peptides" [68]), as well as integrating known T cell epitopes into the synthesized sequence ("synthetic heterotopes"[69]).

Despite their conjugation to carriers and formulation with potent adjuvants, certain peptides may remain poorly immunogenic due to tolerance or non-responsiveness by the host organism (genetic restriction), or shared sequence homology with host proteins [6,70-72].

Protein carrier

More than just increasing the size of the hapten (antigen), the protein carrier serves as a source of Th-cell epitopes. These epitopes induce carrier specific T cell help necessary for efficient B-cell priming.

This method increases the antigenic diversity of the immunizing complex through induction of irrelevant B-cell epitopes, which is an undesired effect. The immune response is directed also against the carrier itself (not only against the antigen), going far beyond the mere utilization of Th-epitopes. An ideal carrier molecule should not elicit a significant antibody response to itself, while providing a strong helper effect for the antibody response to the attached antigen. The carrier must also work well in the context of different major histocompatibility complex (MHC) class II allelic forms. In other words, when processed by the antigen presenting cell (APC) it must produce peptides capable of binding to products of various MHC class II alleles. Universal ("promiscuous") peptides capable of binding to products of almost any MHC class II alleles have indeed been described [73,74].

The conjugation of peptides to proteins can be accomplished using several procedures. Comprehensive information can be found in [54,75,76] . The majority is based on covalent coupling strategies: Most of these methods rely on the presence of free amino (N-terminus, lys), imidazol (his), sulfhydryl (cys), phenolic (tyr), carboxylic (asp, glu) or guanido groups (arg). A wide range of coupling reagents has been reported (Table 1). Conventionally, the conjugation reaction is carried out in solution by first activating the peptide or the carrier with the help of the coupling reagent and then attaching the peptide or carrier respectively. Alternative protocols, such as conjugation to preactivated proteins [77] and direct synthesis of peptides on carrier proteins [78] have been described. Brumeanu *et al.* enzymatically coupled viral peptides to carbohydrate moieties of *self* immunoglobulins, thus taking advantage of the long half life of immunoglobulins and minimizing adverse effects [79,80]. Also,

non-covalent coupling methods have successfully been tested for attaching immunogenic peptides to heat shock proteins [81-83].

Coupling agents	Modified amino acid	
	Primary reaction	Secondary reaction
glutaraldehyde	α-NH_2, ε-NH_2, Cys-SH	Tyr, His
bis imido esters	α-NH_2, ε-NH_2	negligible
BDB	Tyr, Cys-SH, His, ε-NH_2	Trp, Arg
carbodiimides (ECDI, MCDI)	α-NH_2, ε-NH_2, α-COOH, Glu, Asp	Tyr, Cys
MBS	Cys-SH, NH_2 bridges	n/o
SPDP, MCS	Cys-SH, NH_2 bridges	n/o
imido esters (2-iminothiolane)	Cys-SH, NH_2 bridges	n/o
IBCF	-COOH, NH_2 bridges	n/o
toluene diisocyanate	α-NH_2, ε-NH_2	n/o
p-nitrobenzoyl chloride	Tyr, His, Cys-SH, ε-NH_2	Trp, Arg
p-amino phenyl acetic acid	α-NH_2, ε-NH_2	His, Tyr
cystamine dihydrochloride	Cys-SH	n/o
EBIZ	α-NH_2, ε-NH_2, -COOH	n/o

n/o = not observed

Table 1: Principal reagents used for peptide-protein conjugation (Table adapted from [54]).

Carbodiimide and mixed anhydride based procedures yield stable amide crosslinks between carboxyl and amino groups. Bifunctional aldehydes (e.g. glutaraldehyde) yield relatively stable Schiff base crosslinks between primary amino groups. Bifunctional active esters yield stable amide crosslinks between primary amino groups, and heterobifunctional reagents yield crosslinks between amino and thiol groups. As an extension of these methods, the inclusion of non-natural amino acids in the peptides permits an easy quantitation of the conjugation number, i.e. the number of peptide ligands coupled to one carrier molecule [84,85].

Peptides should be coupled without putting the functional integrity of epitopes at risk. Epitopes can be damaged if integral amino acid residues participate in the coupling reaction. The crosslinking function can be situated anywhere on the peptide ligand. For ease of synthesis and spatial accessibility however, a terminal position may be favorable. Hence, the peptide can easily be derivatised even with non-amino acid crosslinkers. Furthermore, “spacer” molecules, such as a poly-alanine stretch, can be introduced to guarantee epitope accessibility. Epitopes corresponding to N-termini of proteins should be coupled through their carboxy-terminal amino acid, whereas peptides representing protein C-termini should be coupled through their amino-terminus. Crosslinkers may lead to carrier polymerization. Especially symmetric i.e. mono functional crosslinkers have to be considered carefully as they allow less geometric control.

Non-protein carrier

Increasing the size of the hapten or antigen can also be accomplished by carrier molecules other than proteins. Non-protein carriers are not capable of providing T cell help but may improve antigen presentation, protect against degradation or exert an adjuvant effect. One prominent example for a carrier structure providing all these features is aluminum hydroxide (alum).

The controlled further polymerization of synthetic peptides has also been studied. Advantages of synthetic polymers are ease of use and the fact that there are almost no size limitations. These strategies try to overcome low valency of conventional linear or branched synthetic peptide preparations (MAPs), and explore ways to integrating large numbers of multiple copies into one macromolecular structure. Another objective is to increase the low heterogeneity of peptide constructs and to combine sets of different antigens for use in vaccines. As an example, polymerization of peptide epitopes derivatised with the acryloyl group (CH_2=CHR-) resulted in synthetic antigen preparations > 500 kDa and allowed the assembly of hundreds of copies of the same or different epitopes into one polymer [86]. This approach admits synthesis of constructs of any complexity with respect to peptide presentation (peptide integration is geometrically random), and to the number or variability of antigenic functions. Jackson *et al.* constructed large polymers containing multiple copies of peptides representing T- and B-cell determinants of influenza virus haemagglutinin. The observed levels of antibody obtained after a single dose of polymeric immunogen were similar or greater than those achieved after repeated doses of the equivalent monomeric peptide control.

Polymerization reactions that are based on radical formation will impair peptide epitopes irreparably. An alternative possibility is the introduction of a polymeric scaffold prior to synthesis: Peptide conjugates have been studied which leave the peptide bound to the resin support, such that the peptide antigens are linked to a macromolecular mesh after synthesis [87]: CD4+ T cell immunity in mice was achieved with a polymeric construct containing two different T cell determinants.

Instead of co-polymerizing peptide antigen with a polymer matrix, engineered polymers have also been used for a better delivery of drugs and antigens. These microspheres consist of biodegradable microparticles of varying composition from which antigen is slowly released over a prolonged period of time. In addition, polymer degradation products have been designed with adjuvant properties. Examples are an L-tyrosine based polyiminocarbonate which greatly increased the immune response of mice against bovine serum albumin [88], and poly DL-lactide-*co*-glycolide microspheres which have also been shown to be immunogenic in a murine model [89].

Lipopeptides and liposomes

The immunogenicity of peptides can be enhanced by coupling them to fatty acid moieties and to liposomes. The potential value of liposomes for eliciting antibodies to peptides is well recognized. They are non-toxic and liposomal components are not immunogenic; the immune response is specifically generated against the peptide antigens. While this is particularly favorable for vaccine development, liposomes are perfectly applicable in research and diagnostics as well. They help to limit non-specific cross-reactions and keep the background low in polyclonal antibody preparations or during the isolation of antigen-specific hybridomas.

For example, a low-molecular weight vaccine (3.4 kDa) against foot-and-mouse-disease virus (FMDV) is composed of a 20 aa synthetic peptide segment coupled to tripalmitoyl-S-glyceryl-cysteine. It induces long-lasting protection after a single administration without any additional adjuvant or carrier [90].

Liposomes were first described as adjuvants for protein antigens, even compatible with human use [91]. Liposomes are non-toxic, biodegradable and do not induce local or systemic reactions after immunization. They are composed of one or more concentric phospholipid bilayers formed in the presence of excess water. Almost any water-soluble compound can be incorporated in appropriate liposomes, as well as lipophilic or particulate compounds (for a comprehensive review refer to [92]).

Liposomes have been used as delivery vehicles for various substances which can be administered by a variety of routes. They also possess considerable configurational fluidity which can be modified according to need. A transdermal drug delivery system has been developed by the Munich-based company *IDEA*: "Transfersomes", chemically modified and, compared to liposomes, more rigid lipoidal vesicles. They showed an intriguing capability of transferring through the skin of vertebrates, realizing non-invasive delivery of insulin and protein vaccines [93,94].

Liposomes may also confer an adjuvant effect. The adjuvant activity of liposomes has been widely demonstrated by using a variety of bacterial, viral, parasitic and tumor antigens [95]. Liposomes interact strongly with macrophages. For immunization, antigens can be entrapped in the aqueous compartment of the vesicles or can be covalently bound to the outer surface of the liposomes. Antibodies against the lipid components of the liposomes are not induced if they contain lecithin [96]. Liposomes as such will not mediate T cell help, as they are *per se* void of T cell epitopes. Hence, T cell epitopes have to be supplied additionally, either as integral components of the peptide antigen or as extra ligands.

Incorporation within liposomes greatly reduces the amount of antigen required to induce an immune response. The immunogenicity of synthetic subunit peptides has been improved upon incorporation into liposomes: Brynestad *et al.* [97] used a synthetic peptide corresponding to aa 1 to 23 of glycoprotein D (gD1-23) of herpes simplex virus type 1 (HSV-1) to immunize mice against HSV-1 infection. The coupling of this peptide to palmitic acid enhanced its immunogenicity. Incorporation of the acyl peptide into liposomes further increased the immunogicity of the peptide.

The alternative approach of covalently coupling peptides to liposomes also enhances immunogenicity. Polymers of the peptide corresponding to aa 135-155 of hepatitis B surface (HBs) antigen were coupled to liposomes and found to be immunogenic in rabbits [98]. Different methods used for anchoring peptides to liposomes have been described [54,99].

Immunostimulating complexes (ISCOMs)

ISCOMs have been described by Morein and co-workers [100]. In ISCOMs antigens are presented in multiple copies and attached by hydrophobic interactions to a matrix built up by the adjuvant component Quil A combined with lipids. Quil A alone has been widely used as an adjuvant for a variety of vaccines in veterinary use. Quil A and the lipid (preferably cholesterol) form a stable cagelike structure which is maintained even after lyophilization. Amphipathic and non-amphipathic substances, as well as small peptides coupled to protein carriers can be incorporated into ISCOMs.

Initially native membrane proteins were used in ISCOMs which, due to their amphipathic characteristics, could be easily integrated. Non-amphipathic proteins can be acid- or heat-treated in order to reveal hydrophobic regions for integration. The efficacy of various ISCOM vaccines against viral envelope antigens has been demonstrated. These include envelope proteins from more than 20 different kinds of viruses. As an example, Takahashi *et al.* were able to prime antigen-specific MHC I-restricted CTL by immunization with exogenous gp160 envelope glycoprotein of HIV-1 using ISCOMs [101].

MAPs, TASPs, tetra-oximes and other synthetic compounds

There are limitations to the augmentation of an immune response. For instance if a carrier is co-administered with the antigen, immunologic capacity is dislocated. KLH for example, is highly immunogenic by itself and the use of KLH has been detrimental to the immune response to coupled peptide antigens, a phenomenon called “antigenic competition” [102,103]. A similar but more complex phenomenon is known from HIV infection: “immunologic exhaustion”. For vaccine carriers, the repeated use of standard carrier molecules for immunization, such as tetanus toxoid, may lead to immunotolerance. This phenomenon, resulting in the inhibition of the antibody response to new epitopes linked to the same “old” protein, is known as “epitope-specific suppression”, or “carrier suppression” [104].

These inherent disadvantages of using a protein carrier can be resolved by renouncing protein carriers and improving the immunogenicity of the peptide itself, either by structural or chemical modifications. Unlike in recombinant protein technology a limitless chemical arsenal is available, ranging from D-amino acids to non-amino acid mimotopes. Immunogenicity might be increased by simply varying one or more amino acid residues (“epitope enhancement” [67]) or by turning the construct into a “retro-inverso” peptide analogue (a metabolism-resistant peptide derivative bearing NH-CO instead of natural CO-NH peptide bonds [1].

In other strategies, known T cell epitopes were included as an additional module to a peptide construct (“synthetic heterotopes” [69]) or structural properties were modified to increase immunogenicity (“constraint peptides” [68,105], helix-stabilization [106,107]).

Another approach that avoids the induction of antibodies to a carrier protein is the multiple antigenic peptide (MAP) system introduced by Tam *et al.* [108]. Tam and his colleagues tried to overcome the usually poor immunogenicity of free peptides by increasing molecular weight and valency of peptide antigens. A MAP consists of a core matrix made up of up to three levels of lysine residues resulting in up to eight amino

terminals for anchoring peptide antigens. This poly-lysine terminus allows a single-run automated synthesis of branched, tree-like peptide multimers. Despite the ease of synthesis, the MAP approach faces two restrictions, however: variability and size. Firstly, the resulting polypeptide is a homo-multimer. It is not possible to link different peptide ligands or effectors, except for arranging them in a linear manner. Secondly, depending on the secondary structure formation propensity of the peptide, spatial restraints increase during the synthesis of the peptide. Eventually, this results in faulty peptide ligands and limits the length of MAPs.

Both, improving structure, as well as combining immunologic functions in a modular manner are realized by the TASP approach (TASP: template assembled synthetic peptides [109]. Here, functional peptides are attached to a synthetic core scaffold in a multiple step modular fashion, yielding large synthetic proteins. The construction of TASP molecules is achieved by standard methods of peptide synthesis. Orthogonal protection techniques in combination with segment-condensation strategies allow for the chemical synthesis of TASPs with a variety of different peptide blocks in a protein-like arrangement. Hence, different epitopes can be grafted on the same carrier. Compared with the multiple antigenic peptide approach, conceived as an immunogenicity amplification system for the grafting of peptides of immunological interest, the TASP approach offers the advantage of better control of the conformational epitopes features. These variable chemoselective ligation techniques also overcome size restrictions of the classical MAPs, where multiple copies of a synthetic peptide are simultaneously synthesized on a branched lysine core.

Implantation

Anti-peptide antibodies have also been stimulated in mice by intraperitoneal implantation of paper disks derivatised with a synthetic peptide [110]. A cysteine variant peptide of 13 amino acids corresponding to an epitope on thymopoietin was coupled by diazo linkages to aminophenyl thioether-derivatised paper disks. Mice were implanted with disks every 3 weeks. After 4 implantations, the mice developed antibodies reactive to native thymopoietin. In contrast, mice immunised conventionally with the peptide alone or with thyroglobulin conjugated peptide failed to produce antibodies. Another successful method was described by Smith and co-workers [111] who implanted subcutaneously into mice pieces of nitrocellulose containing absorbed antigens. Here, only a single implantation was necessary and spleen cells of the inoculated mouse could be used for producing monoclonal antibodies.

1.5. Cytokines as adjuvants

The term “adjuvant” is derived from the Latin term *adjuvare* which means “to help”. Adjuvants have been in use to augment the immune response to antigens for about 70 years since Ramon showed increased antitoxin response to tetanus and diphteria toxoids when they were injected together with other compounds such as agar, tapioca, lecithin, starch, oil, saponin or even breadcrumbs [112,113].

Adjuvants help antigens to elicit a rapid, strong and long-lasting immune response and allow the use of less antigen. Any material increasing the immune response to an antigen is referred to as an adjuvant.

Common adjuvants form a highly heterogeneous group of compounds, both chemically and with regard to their mechanism of action. On the basis of mechanism, adjuvants can be grouped into (1) substances causing depot formation at the site of injection (e.g. minerals, oil-based adjuvants like IFA/CFA [114] or RIBI, biodegradable polymer microspheres of > 10 µm); (2) substances acting as delivery vehicles which help in targeting or presenting antigens to immune competent cells (e.g. liposomes, biodegradable polymer microspheres < 10 µm); (3) substances acting as immunostimulators (e.g. FCA, pertussis toxin (PT), muramyl dipeptide (MDP), lipopolysaccharide (LPS), cytokines).

Several adjuvant preparations fall into multiple categories. For instance, CFA forms a depot, contains oil and supplies a potent inflammatory mycobacterial preparation as an immunostimulator. The liposomes included in this study can be regarded both as carriers and an adjuvants. Another classical adjuvant, alum ($AlK(SO_4)_2 \cdot 12H_2O$ in 10% aqueous solution) [114], is also depot-forming and a "vehicle" providing an efficient (but rather poorly understood) way of antigen presentation.

By using adjuvants, the immune response can be selectively modulated to major histocompatibility complex (MHC) class I or MHC class II, and to Th1 or Th2 type responses, resulting in an antibody (humoral) or cellular response biased immune response [115,116]. Besides being dependent on immuno-stimulators, the route of antigen presentation and existence of MHC I or II binding motifs on the antigen also influence the MHC character of the immune response. An MHC I response is usually associated with intracellular pathogens, such as viruses, leading to CTL (cytotoxic T-lymphocytes) and cell-mediated immunity [71] and is usually not observed with pure protein or peptide antigens. Adjuvants such as immunostimulating complexes (ISCOM) and QS21 can elicit CTL with protein or peptides [101,117-120]. On the other hand, the MHC class II response induces antibody production against protein antigens or inactivated organisms. Most adjuvants elicit MHC class II responses.

Adjuvants can also modulate the immune response to different T-helper subsets (Th1 and Th2) [116,119,121,122]. The Th1 type response is accompanied by IL-2 and IFN_{γ} secretion and is usually observed after intracellular bacterial or parasitic infections and live viruses [123]. Stimulation of the Th1 type response leads to a cell-mediated immune response and production of relatively high levels of antibodies of the IgG2a isotype in mice. In recent years several adjuvant formulations including FCA, MDP and liposomes have been shown to stimulate the Th1 type response in mice [119,124]. The Th2 type response is accompanied by IL-4 and IL-10 secretion and is stimulated after immunization with protein antigens or inactivated organisms. Stimulation of Th2 type response leads to the production of IgG1 and IgE antibodies in mice [116]. Alum and IFA adjuvants are known to favor murine Th2 type response [125,126].

The capacity of a formulation to induce specific T cell immune responses (CD4+, $CD8^+$) to the bound peptide is crucial for immunogenicity. In the present study, Interleukin-4 (IL-4) and granulocyte-macrophage colony-stimulating factor (GM-CSF) were selected for testing as adjuvants.

Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF)

GM-CSF has been extensively studied *in vitro* and *in vivo* as a potentiator for vaccination. It has been shown to have a stimulating systemic effect on primary and secondary responses [116,121,122].

The well-documented effects of GM-CSF on the immune response include (1) activating macrophages [127]; (2) increasing MHC class II antigen expression [128,129]; (3) enhancing dendritic cell maturation and migration as well as immunostimulatory function; (4) inducing localized inflammation at the site of injection [130] and (5) exerting immunomodulatory effects by its systemic impact on the hematopoietic cytokine network [131]. The importance of GM-CSF in the maturation of antigen presenting cells has been confirmed. Larsen *et al.* showed that GM-CSF-cultured dendritic cells (DC) of various backgrounds (spleen and kidney) showed enhanced ability to stimulate both T cell proliferation and cytokine release [132].

GM-CSF may affect titers of antibody to foreign antigens when used as an immunopotentiator as suggested by studies in both animals and humans. Monkeys injected with human IL-3 developed earlier and higher antibody titers to IL-3 if GM-CSF was co-administered. Doses of GM-CSF as low as 0.1 ng per mouse induced serum antibody to BSA 14 days after a single injection of antigen, whereas injecting BSA alone did not [133].

GM-CSF has also successfully been tested as a fusion protein for augmenting an anti-idiotypic immune response in anti-tumor vaccination experiments [134,135]. GM-CSF was fused with an antibody corresponding to a specific idiotype expressed on an immune B-cell lymphoma. Mice immunized with the fusion protein developed antibodies and were protected against the progression of the lymphoma. Here, antibodies did not develop when mice were injected with the idiotypic antibody together with co-administered GM-CSF. *Ex vivo* approaches to the use of GM-CSF have been described as well, where antigen presenting cells (APCs) were stimulated with GM-CSF externally and re-infused. In this approach tumor cells transduced with various retroviruses encoding potential immunomodulators were tested. Tumor cells expressing GM-CSF stimulated potent, long-lasting, and specific anti-tumor immunity [136].

Interleukin 4 (IL-4)

First described as a B-cell stimulating factor, IL-4 is generally considered to stimulate humoral immunity [137-139]. Carter and co-workers showed that IL-4 not only reversed the usual course of *Leishmania major* infection in BALB/c mice but also promoted the generation of protective immunity [140]. Cameron *et al.* showed that IL-4 treatment favors the expansion of regulatory CD4+ Th2 cells in NOD mice, thus stabilizing a protective Th2-mediated (humoral) environment [141]. ElGhazali [142] and colleagues demonstrated that an antibody-dependent Th2 response profile of human individuals to tetanus toxoid was characterized by IL-4 producing cells. In IL-4 deficient mice an overall reduction in antibody production was observed [126].

1.6. Open questions in the generation of anti-peptide antibodies

As described above, several variables can influence the immune response, including the route of administration, the dosage, frequency and timing of immunization, along with the formulation of immunogens and adjuvants and the type of organism to be investigated.

General procedures for raising antibodies have been described by several authors. A comprehensive compilation of available information on the topic is provided by Van Regenmortel *et al.* [54]. However, few systematic studies on the efficient generation of anti-peptide antibodies have been performed [143].

Available work primarily covers methodologic aspects of anti-peptide antibody generation, discussing coupling chemistry [77,144,145], assessment of coupling ratios [146] and the introduction of new carrier structures [81], or is usually restricted to 2 to 3 different carrier structures [70,98,147].

In surveying the literature, KLH and BSA [75], as well as ovalbumin [145] appear to be the most commonly used carriers in the laboratory. Thyroglobulin and the rather expensive tetanus toxoid are also utilized [148]. Tetanus and diphtheria toxoid are the preferred choices as carriers used for vaccines [75]. Some authors claim that certain protein carriers are superior to others, e.g. keyhole limpet hemocyanin with respects to parent protein cross-reactivity [146]. Guidelines on the choice of the appropriate carrier for peptide immunization are restricted to rather vague instructions, like “test more than one” or “the solubility of the final peptide carrier conjugate may determine the choice of the carrier” [54]. Several authors emphasize the importance of “context” on the ability of selected immunogenic epitopes to elicit antibodies [70,148,149]. However, systematic data on this apparent topic are not available.

Some of the open questions are:

- Do synthetic peptides invariably need coupling to carriers in order to yield satisfactory immune responses?
- It is clear that all protein carriers can moderate T cell help to coupled peptide antigens provided that they are sufficiently complex. What about the efficiency of the various carriers in use, though? Are there qualitative differences in cognate protein cross-reactivity of the induced anti-peptide antibodies? Are certain carriers unreliable because of antigenic competition?
- How do synthetic alternatives, such as liposomes, multiple antigenic peptides (MAPs) and template attached synthetic peptides (TASPs) perform compared to protein carriers?

1.7. Aim and scope of this thesis: A comparative study of different common carriers on immunogenicity of synthetic peptides

The overriding objective of the present thesis was to gain comprehensive comparative data on the efficient generation of anti-peptide antibodies by means of different common carriers and immunogen formulations. In other words, to offer a manual for the production of peptide-specific monoclonal and polyclonal antibodies for research and diagnostics to the scientific community.

In order to conceptualize this focus, the project was translated into three work packages or goals:

I. Immunogen synthesis
II. Molecular characterization of immunogens
III. Comparative immunological characterization of immunogens

I. Three distinct synthetic peptide antigens corresponding to epitopes of the human pathogenic bacterium *Neisseria meningitidis* were selected as model antigens. The peptides, two 20- and one 50-mer, were synthesized by solid phase synthesis in their free form and the 20-mers as multiple antigenic peptides (MAPs). Cysteine-variants of these peptides were coupled to liposomes and to protein-carriers. The latter included antigenic peptides conjugated to BSA, ovalbumin, KLH, thyroglobulin and tetanus toxoid. Aminooxyacetyl-modified peptides were further assembled to form tetra-oximes. Additionally, immunogen formulations containing cytokines were prepared to test their feasibility as adjuvants.

II. Synthesis and assembly were paralleled by the molecular characterization of the immunogens by means of mass spectrometry, amino acid analysis and chromatographic methods.

III. The immunogenicity of the various antigenic peptide formulations was analyzed in a BALB/c mouse model. Normalized preparations of the various peptide antigens were tested. The immune response was monitored at the serum level, as well as at a cellular level by ELISPOT.

29 different immunogens were analyzed and their potential to stimulate a peptide specific immune response was assessed. A detailed outline of the work and an introduction to the biological background of the three neisserial synthetic peptide antigens involved, is presented at the beginning of Chapter 3 (RESULTS).