SYNTHETIC PEPTIDES FOR ANTIBODY PRODUCTION

Cover:
Spatial representations of the amino acid sequences (amino acids 335-350) from α_1-antitrypsin variants. Top: the α-helical peptide from the Z variant; Bottom: the α-helical peptide from the M variant. The glutamic acid (M variant) and lysine (Z variant), which form the only amino acid difference in the protein, are accented in magenta. (Images made with the kind assistance of Dr. Jeanine van Rooyen and Dr. Jan Raap, RUL)

SYNTHETIC PEPTIDES FOR ANTIBODY PRODUCTION

SYNTHETISCHE PEPTIDEN VOOR ANTILICHAAM PRODUKTIE

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Erasmus Universiteit van Rotterdam
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prof. dr. P.W.C. Akkermans M.A.
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Neeltje Dina Zegers

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Wie alsmaar let op de wind, komt aan zaaien niet toe en wie naar de wolken blijft kijken, komt niet tot oogsten.
Begin in de morgen te zaaien en gun je hand tot de avond geen rust. Je weet immers niet of het de ene keer lukt of de andere, of dat het beide keren goed uitvalt.
Het licht is zalig en het is een weldaad voor de ogen de zon te zien.
Hoe lang iemand ook leeft, laat hij genieten van elke dag en bedenken, dat er nog genoeg donkere dagen zullen zijn en dat alles wat daarna komt ijdel is.

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Aan mijn ouders
Aan Jaap

ABBREVIATIONS

(general)

AA	Amino acid
AFC	Antibody forming cell
ALL	Acute lymphoblastic leukemia
ApoE	Apo-lipoprotein E
α_1-AT	α_1-antitrypsin
AP	Alkaline phosphatase
AR	Androgen hormone receptor
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BCP	Branched chain peptide
BD	B cell determinant
BMH	Bismaleimidohexane
BPH	Benzoic acid para hydroxylase
BSA	Bovine serum albumin
CD	Cluster of differentiation
CFA	Complete Freund's adjuvant
CGG	Chicken gamma globulin
CML	Chronic myeloid leukemia
CS	Circumsporozoite
CSP	Circumsporozoite protein
CTL	Cytolytic T cell lysis
DDA	Dimethyldioctadecylammonium bromide
DTH	Delayed type hypersensitivity
EAE	Experimental autoimmune encephalomyelitis
EDC	1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide
ELISA	Enzyme linked immuno-sorbent assay
FCS	Fetal calf serum
FMDV	Foot-and-mouth disease virus
Fmoc	9-Fluorenylmethoxycarbonyl
β-Gal	β-galactosidase
GA	Glutaraldehyde
Gp120	glycoprotein 120 kDa (HIV-1)
hAR	Human androgen receptor
HEL	Hen egg lysozyme
HIV-1	Human immunodeficiency virus type 1
HRP	Horse radish peroxidase
HSA	Human serum albumin
HuIgG	Human immunoglobulin G
IEF	Isoelectric focusing
IFA	Incomplete Freund's adjuvant

IFN-χ	Interferon gamma
IL-#	Interleukin-#
i.p.	Intraperitoneal
ISCOM	Immune stimulating complex
i.v.	Intravenous
KLH	Keyhole limpet hemocyanin
MAbs	Monoclonal antibodies
MAP	Multiple antigen peptide
MBS	*m*-maleimidobenzoyl-N-hydroxysuccinimide ester
MHC	Major histocompatibility complex
MLA	Monophosphoryl lipid A
NBT	nitroblue tetrazolium
NK	Natural Killer
OPD	o-phenylendiamine
PAbs	Polyclonal antibodies
PBS	Phosphate-buffered saline
PNP	p-Nitrophenylphosphate
PPD	Purified protein derivative (of tuberculin)
P3C	tripalmitoyl-S-glycerylcysteine
RAM-PO	Rabbit anti mouse Ig conjugated with horseradish peroxidase
RAM-AP	Rabbit anti mouse Ig conjugated with alkaline phosphatase
R1881	[17α-*methyl*-^{3}H]methyltrienolone
SAF	Syntex adjuvant formulation
s.c.	Subcutaneous
SE	Staphylococcus enterotoxin
SP	Synthetic peptide
SPDP	N-succinimidyl-3-(2-pyridyldithio)-propionate
t-Boc	tertiary-butyl-oxycarbonyl
TCR	T cell receptor
TD	T cell determinant
Th	T helper
TT	Tetanus toxoid
TFE	Trifluoroethanol
TFA	Trifluoroacitic acid
WIO	Water in oil emulsion
X-Gal	5-bromo-4-chloro-3-indolyl-galactosidase

Componds used in peptide synthesis

Acm	Acetamidomethyl
Boc	Butoxycarbonyl
Bop	Benzotriazole-1-yl-oxy-tris(dimethylamino)-phosphoniumhexafluorophosphate
2-Br-Z	2-Bromobenzyloxycarbonyl
Bzl	Benzyl
2-Cl-Z	2-Chlorobenzyloxycarbonyl
DCC	N,N'-Dicyclohexylcarbodiimide
DCM	Dichloromethane
DIPCDI	Di*iso*-propylcarbodiimide
Dnp	2,4-Dinitrophenyl
DVB	Divinylbenzene
Fmoc	α-fluoreneylmethyloxycarbonyl
HBTU	2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate
HMP	4-(Hydroxymethyl)phenoxymethyl
HMPB	4-hydroxymethyl-3-methoxy-phenoxybutyric acid
HOBt	1-hydroxybenzotriazole
HONSu	N-hydroxysuccinimide
HOOBt	3-hydroxy-3,4-dihydro-1,2,3-benzotriazin-4-one
MBHA	Methylbenzylhydrylamine
4-MeBzl	4-Methylbenzyl
Mts	Mesitylene-2-sulphonyl
OBzl	Benzyloxy
ODhbt	3-Hydroxy-4-oxo-3,4-dihydro-1,2,3-benzotriazine ester
ONp	Nitrophenyl ester
ONSu	N-hydroxysuccinimide ester
OPfp	pentafluorenyl ester
OtBu	t-Butoxy
PAM	phenylacetamidomethyl
PEG	Polyethyleneglycol
Pmc	2,2,5,7,8-Pentamethylchroman-6-sulphonyl
PSA	Performed symmetrical anhydrides
PyBOP	Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphoniumhexafluorophosphate
SPPS	Solid phase peptide synthesis
tBu	t-Butyl
TBTU	2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate
TFA	Trifluoracetic acid
Tos	Tosyl
Trt	Trityl
t-Boc	tertiair-Butoxycarbonyl
Z	Benzyloxycarbonyl

AMINO ACID CODES

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

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Chapter 1

General Introduction

GENERAL INTRODUCTION

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I. INTRODUCTION

Synthetic peptides are useful tools for the generation of antibodies. The use of antibodies as specific reagents in immunochemical assays is widely applied. In this chapter, the application of synthetic peptides for the generation of antibodies is described. The different steps that lead to the unique reagents, such as the selection of the peptide sequence, the construction of the immunogen, immunization procedures and assays for the selection of the antibodies, are discussed.

I.1. Antibodies as reagents

Antibodies can serve as powerful reagents in immunochemical assays for the specific detection of many different molecules, termed antigens. Immunochemical detection is rapid, simple, cheap and specific, and has the additional advantage that purification of antigens from a matrix is not required. Immunochemical detection is not only applied for diagnostics in the clinical laboratory, but also, e.g., in the food industry, in health research and therapy.

Generation of antibodies is carried out in animals by immunization with the antigen. The serum contains many different antibodies that are specific for many different antigens. Seldom are more than 10% of the circulating antibodies specific for one antigen. Antibodies in serum are called *polyclonal* antibodies (PAbs) since they are secreted by different B lymphocyte clones. Each B cell clone secretes a different type of antibody. The use of these mixed populations of antibodies may create a variety of different problems in immunochemical techniques. With the advent of the hybridoma technique (Köhler and Milstein, 1975), production of *monoclonal* antibodies (MAbs) became available. Hybridomas are the result of a somatic cell fusion between a myeloma cell and an antibody-secreting cell. Hybridomas can be easily cultured. After selection of those hybridomas with antibody secretion of a defined specificity and high affinity for the antigen, cloning of the selected hybridoma yields an unlimited production of the monoclonal antibodies. Monoclonal antibodies are all identical and possess a unique chosen specificity.

Monoclonal antibodies are more time-consuming and costly to prepare than polyclonal antibodies. Producing the right set of monoclonal antibodies is often a difficult and laborious job. Consideration must be given beforehand to whether monoclonal antibodies are required or whether polyclonal antibodies will suffice.

I.2. Peptides as immunogens

Antibodies recognize spatial structures (*epitopes*) which may be build up from fragments of a larger molecule, termed the *antigen*. The antigen may be, e.g., a protein. These epitopes can interact, in their turn, with the amino acids forming *the antigen binding site* of the antibody. Antibodies may recognize antigenic fragments or sites which are interconnected (*continuous* or *linear epitopes*) or, on the other hand, widely spaced fragments which may form a configuration complementary to an antigen binding site (*discontinuous epitopes*)(Arnon, 1973; Atassi, 1984). Ideally, antibodies will bind to the antigen which was used to generate the antibody production. Clearly, the degree of degeneration in reactivity (*crossreactivity*) observed in practice offers the advantage that synthetic structures can be used for immunization. These synthetic antigens mimic antigenic structures to which antibodies can be generated. The antibodies which react with the synthetic structures may also crossreact with the native structures from which the synthetic structures was derived.

In the case of proteins, linear epitopes can be mimicked by synthetic peptides (SP). With these peptides, antibodies can be raised which recognize the native protein (Atassi, 1984). There is no strict consensus on what might be the size of an antigenic determinant recognizable by antibodies. Sufficient data are available to suggest that short peptides [7-10 amino acids (AA)] may represent such a determinant (Atassi, 1984; Geysen et al., 1987). It could be shown with lysozyme that up to 16 amino acids can be involved in, or contribute to, the interaction of the antibody (17 AA) and a surface loop of the antigen (16 AA) (Amit et al., 1986). In the antibody-antigen complex, these residues form a tightly packed interface from which water molecules are excluded. For actual antigen-antibody complexes of neuraminidase of influenza virus however, a more flexible interaction was observed (Colman et al., 1987). Binding occurs by an induced-fit mechanism.

Under certain conditions, application of synthetic peptides provides the first choice of approach to the generation of antibodies specific for a certain protein (Table 1):

(1) Antibodies for application in selective diagnostic test systems need to be able to discriminate between molecules which express a high degree of homology. In order to generate useful specific reagents, it is most efficient to generate the antibody responses with protein fragments or selected peptide sequences as immunogens. These peptides include only those parts of a molecule which determine the differences between the structures to be discriminated (Boersma et al. 1989a,c; Kleine et al., 1990; Gerritse et al. 1992; Zegers et al., 1991a,b).

(2) For the production of highly specific polyclonal reagents by immunization, intact proteins or fragments need extensive purification. When purification is not successfully achieved or when this results in the denaturation of the antigen, the selection of sequences of interest can be based on the primary structure. When the protein product of a gene is not

TABLE 1
Diagnostic antibody production using synthetic peptides

❐	To discriminate between the members of a homologous protein family *Search for sequences with differences*
❐	To discriminate between variant proteins up to one amino acid difference *Restricted to the sequence including the substitution*
❐	To be able to detect a group of homologous proteins *Search for sequences with complete homology*
❐	To produce antibodies when a protein can not be sufficiently purified *Search for antigenic determinants*
❐	To produce antibodies when a protein is not available but can be predicted from the DNA base sequence *Search for antigenic determinants*
❐	To produce antibodies to various domains of a protein; immunodominant determinants can thus be avoided *Search for domain specific antigenic sequences*
❐	To produce antibodies against autologous sequences *Search for antigenic determinants and use appropriate adjuvant*
❐	To circumvent pathogenic or toxic proteins *Search for harmless sequences*
❐	To evaluate sequences for use in synthetic vaccines or vector-based vaccines

known, the amino acid sequence can be predicted from the DNA base sequence. Synthetic peptides thus can be used to elicit native protein-specific antibody responses in these cases (Papkoff et al., 1981; Chomyn et al., 1983; Van Denderen et al., 1989, 1990; Gerritse et al., 1990).

(3) Antibodies to mapped determinants are efficient tools in dissecting the function of protein structural elements (Atassi, 1975; Amit et al., 1985; Altschuh et al., 1985; Voskuilen et al., 1987; Zegers et al., 1991a).

TABLE 2
Strategy for the production of antibodies to synthetic peptides

☐	Selection of antigenic sequence
☐	Identification of region of interest and limitations in choice
☐	Synthesis and purification of peptide
☐	Coupling of peptides to a carrier protein
☐	Immunization with proper adjuvant in target animal
☐	Development of the appropriate assay for evaluation of the anti-native protein reactivy
☐	Evaluation of immune response in serum sample
☐	Production and selection of monoclonal antibodies
☐	Characterization of the monoclonal antibody in the proper assay
☐	Large scale production, purification and labelling if necessary

(4) Under normal physiological conditions, antibodies directed to autologous molecules (i.e., 'self' molecules) are seldom found. Immunization of SP's in a very strong adjuvant and via a proper route may result in an antibody response against 'self' molecules (Jemmerson and Margoliash, 1979; Jemmerson et al., 1985; Cooper et al., 1986). For example, immuno-castration (Remy et al., 1993; Meloen et al., 1994) or contraceptive methods (Aitken et al., 1993) can thus be achieved. Similarly, experimental auto-immune encephalomyelitis (EAE), an animal model for multiple sclerosis, can be induced with a peptide derived from PLP (proteolipid protein)(Tuohy et al., 1989).

(5) Pre-selection of immunogenic sites with SP may provide a basis for the more efficient engineering of vaccines. Due to limitations in the construction of recombinant or synthetic vaccines, it is necessary to include only the most important and representative immunogenic sites, which are able to elicit, e.g., neutralizing antibodies (Steward & Howard, 1987; Zanetti et al., 1987). Avoidance of pathogenic sequences and of dominant epitopes is one major advantage of the peptide strategy.

The basis for the successful application of the synthetic peptide approach is found in the appropriate application of a combination of a multitude of techniques (synthesis,

antigenicity calculations, immunochemistry methods (assays like enzyme linked immunosorbent assays (ELISA), dotblot, immunoblot, somatic cell fusion, immunocytochemistry). In addition, insight into the interaction of cells in immune responses with respect to processing (selection of sequence), presentation (immunization, carrier system etc.) and T-B cell interaction is indispensable. In the following sections, the influence these various parameters have on the different aspects of applications of peptides will be briefly discussed. They affect the generation as well as the analysis of the specificity of monoclonal and polyclonal antibodies for diagnostic use (Table 2).

I.3. Immunogenicity

Exactly what determines the capacity of an antigen or structure to induce an immune response (immunogenicity) and to be recognized by an antibody is a subject of discussion (Hopp, 1986; Van Regenmortel, 1986, 1989). Two major sets of features determine the result of immunizations.

Firstly, a large number of physical parameters governs the outcome of an antibody response to specific parts of a protein. Nevertheless, there are strong indications that almost every single peptide sequence (Geysen et al., 1984, 1985) with only a few exceptions (Briand et al., 1985; Gerritse et al., 1992) depending on the length, amino acid distribution and the method of immunization, can serve to elicit antibody responses, irrespective of its localization in the intact protein (Arnon et al., 1971, Green et al 1982). With overlapping synthetic peptides covering about 75% of influenza hemagglutinin, Lerner and co-workers observed that > 90% of the peptides elicited antibodies cross-reactive with the native protein (Lerner, 1981; Green et al., 1982). Also, when short peptides are utilized for immunization anti-peptide responses are generally obtained, although however, the recognition of the native protein in these cases is not always warranted (Boersma et al., 1988a).

The level of cross-reactivity of anti-peptide antibodies with the antigen from which the peptide is derived, depends strongly on the sequence chosen and the micro-environmental (i.e., *in vivo* or *in assay*) conditions. The structure of a given antigen is subject to change dependent on the micro-environment where the antigen is encountered. Antigen recognition in various immunoassays therefore is dependent on the assay and a major source of pitfalls (Milstein et al., 1983; Haaijman et al., 1984; Boersma et al., 1988b, 1989b).

Secondly: after immunization with an intact protein regulatory fine tuning, selection and response modulation lead to the production of responses to the immunodominant sites only (Van der Drift et al., 1991; Van Noort et al, 1990; 1991). Immunogenicity, especially with respect to antibody formation, is influenced by the spatial structure of a protein or peptide. Immunodominant sites may be formed by linear as well as by discontinuous epitopes. Linear epitopes can be mimicked by short peptides or protein fragments. For the discontinuous epitopes, intact proteins, combinations of peptides in a tertiary structure or, in the case of micro-organisms and viruses, particle-sized antigens are generally required.

When synthetic epitopes are used to elicit anti-native protein antibodies, the question arises whether the affinity for the native antigen will be such that the antibodies elicited become useful reagents. The large number of diagnostic anti-peptide antibodies used in many

investigations, and in the clinic, makes clear that this is certainly the case. With synthetic peptides, monoclonal antibodies and polyclonal antibodies with similar or even better affinities for native proteins as generally observed for anti-protein responses could be produced (Bahraoui et al., 1986; Laman et al., 1991).

PAbs raised with synthetic peptides have the additional advantage of being monospecific for the native protein. They lack the common disadvantages of PAbs raised with proteins or impure preparations of proteins, such as unwanted crossreactivity with other proteins. Crossreactivity of PAbs can cause background problems or false-positive detection in immunochemical assays. Peptide-induced PAbs may preclude the need for preparation of MAbs.

In the sections below, the variables will be subsequently discussed which have their impact on eliciting and selecting diagnostic antibodies. The steps to be taken in the strategy using synthetic peptides to achieve to antibodies applicable in various assays is outlined in Table 2.

II. SEQUENCE SELECTION

The first step when using peptides for the generation of antibodies specific for a protein will be the selection of a peptide sequence. It is obvious that, if free to choose, one will decide upon a stretch of the protein which is highly immunogenic. The antigenicity can be predicted by taking the weighted average of various physico-chemical parameters of a short sequence of amino acids. These parameters will be briefly discussed in this section. In addition, some examples of restricted choices will be given.

II.1. Analysis of parameters which determine antigenicity

The influence of physical parameters on antibody responses can be predicted in part on the basis of common sense reasoning. Antigenic sites of intact proteins are accessible to large molecules, like antibodies, when exposed on the outside of a protein. Hydrophillic sequences, readily soluble in aqueous solutions, are thus most likely to be antigenic. Flexibility might enhance the fitting of antigen and antibody (Westhof et al., 1984; Karplus & Schulz, 1985; Fieser et al., 1987) and hence segmental mobility of the epitope may enhance antigenicity (Novotny et al., 1986). Loops of the protein may protrude from the globular form of a protein and as a result of that they may be more readily engaged in binding to a circulating antibody (Kyte & Doolittle, 1982). However, it was shown that an antigenic site contained one or more highly exposed and accessible critical side chains and one or more largely buried and inaccessible critical side chains within the native protein structure. Initial binding of an antibody to solvent-exposed critical amino acid residues may promote local side chain displacements and thereby allow the participation of other, previously buried, critical residues (Getzoff et al., 1987).

Various algorithms have been developed on the basis of the aforementioned type of reasoning. Averaging of physical and chemical properties of individual amino acids over a

certain sequence length (window) leads to a mean value which predicts the behaviour of an entire sequence. First, methods were developed to describe the tendency to form secondary structures: α-helix or β-sheet or β-turns (Chou & Fasman, 1978; Garnier et al., 1978; Hopp & Woods, 1981; Emini et al., 1985). Furthermore, to each amino acid a relative value is given which represents its ranking in hydrophillic behaviour (Hopp & Woods, 1981, Kyte & Doolittle, 1982). Computational methods to attribute helical character to amino acid sequences to detect transmembrane fragments have been developed from hydrophobicity plots (Kyte & Doolittle, 1982). When mean hydrophobicity for varying windows was calculated, most hydrophobic parts were attributed to transmembrane segments. This method was improved by combining the hydrophobic moment of a helical sequence with its amphiphillic character. (Amphiphillic helix: one side along the helix consists of residues with a hydrophillic character while the other side along the helix contains residues with a hydrophobic character). In this way the "surface seeking" tendency or affinity for the membrane interior was computed (Eisenberg et al., 1984a,b). Normalized hydrophobicity values have been specially developed, adapted for the prediction of transmembrane sections of proteins which function as membrane spanning transport proteins (Eisenberg et al., 1984a,b). The mean value for a number of these physico/chemical parameters, combined in an antigenic index, derived from amino acid sequence only gives an estimate of the antigenic properties (index) of a protein segment (Wolf et al., 1988). The outcome of such predictions is determined to a large extent by the number of amino acids of which the average is taken (window size)(Bangham, 1988). Over a short distance, amino acids influence each other. In addition, amino acid sequences which, on a linear scale are far distant, may interact by forming backfolding loops. It is of great importance to take this into account when choosing the length of peptides used for immunization (Jacob et al., 1986; Horiuchi et al., 1987). High (Green et al., 1982; Niman et al., 1983) as well as low (Jemmerson, 1987) frequencies of responses have been reported dependent on the length of peptides used to elicit the production of antibodies.

The computer prediction leads to a set of putative antigenic sites (Wolf et al., 1988). These predictions of epitopes using an algorithm for the antigenic index of an amino acid sequence were successfully applied to a high number of viral and other proteins (Jameson and Wolf, 1988; Modrow and Wolf, 1990).

One should realise that the structural parameters are not the only factors that determine the antigenicity of a site on the protein. The function of the protein under investigation has to be kept in mind. Participation of functionally active regions of a domain in interaction with other molecules, e.g., ligand-receptor interaction or interaction with DNA molecules, prevents the binding of antibodies to the protein. Peptides probably can have multiple conformations of which only a fraction will mimic the native protein. A selection from such a set does not account for immunodominance as will be encountered when intact protein is used for immunization. Each species mounts a different antibody response against antigens. This means that each species has its own antibody repertoire. Monoclonal antibodies can only be produced in certain mouse -or rat- strains due to the histocompatibility

of available plasmacytoma fusion lines. These mice and rats however may not cover the whole repertoire of antibodies desired.

In conclusion, selection of peptide sequences can be based on predictions based on structural parameters combined with the knowledge of the function of the domains of the protein.

II.2. Restricted choices

If antibodies are required for detecting a protein, it is clear that one chooses a peptide sequence for antibody generation which offers the best chance of being highly immunogenic. More often, however, antibodies specific for a certain domain of a protein are desired, e.g., for studying domain functions, or antibodies against sites of a protein which differ from sites in homologous proteins are desired in order to be able to discriminate between those proteins. For these selected cases, the peptide approach is extremely valuable. Computer predictions for antigenicity are of limited value in these selected cases.

III. PEPTIDE SYNTHESIS

Merrifield introduced in 1963 a method for peptide synthesis, based on reactions on a solid phase, for which he would receive the Nobel prize (Merrifield, 1963). The solid phase peptide synthesis (SPPS) is very suitable for automation. Formation of a peptide bond by coupling two amino acids needs on the one hand an activated carboxyl and a protected amino group of the N-terminal amino acid and on the other hand a protected carboxyl group of the C-terminal amino acid. The latter is achieved in SPPS by coupling the C-terminal amino acid to a functionalized insoluble resin. Functional groups of the side chains of the amino acids are also protected but with another type of protection groups than the one used for the α amino group. After deprotection of the α amino function, the next amino acid is coupled. When coupling is complete, all reactants are washed away, leaving a protected dipeptide on the resin. The coupling reaction can be checked for completeness by, e.g., the Kaiser test (Kaiser et al., 1970; 1980) which detects unsubstituted free amino groups. Upon detection of amino groups, one can decide (1) to couple again the same amino acid, (2) to acetylate the amino group by, e.g., acetic anhydride/base or acetylimidazole leaving abrogated peptides, (3) to continue with the next amino acid resulting in a part of the peptide molecules with a deletion. Following the required number of these cycles, the peptide is detached from the resin. The side chain protecting groups are usually simultaneously removed with the cleavage of the peptide from the resin. The peptide chain thus grows from the C-terminus to the N-terminus. The synthesis scheme is shown in figure 1.

III.1. Protection groups for α amino group

The tertiair-butyloxycarbonyl group (t-Boc) is an acid labile group used for protection of the α amino group of the amino acid. After deprotection with 25% trifluoroacetic acid (TFA), the resulting protonated amino group is neutralized using the base di*iso*propylethylamine. The

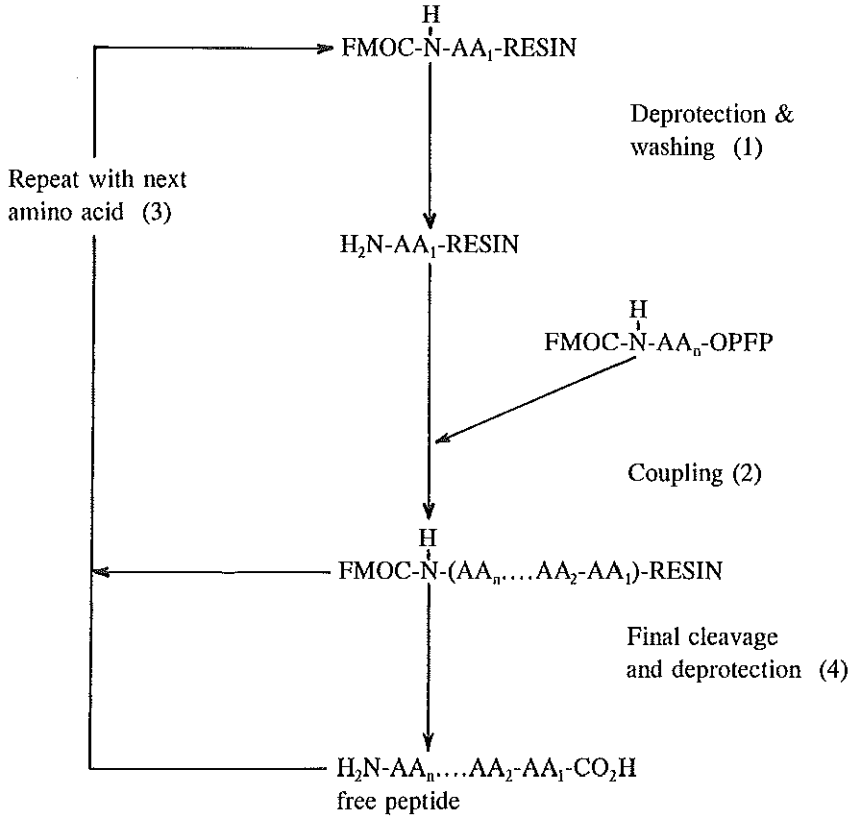


Fig. 1. Synthesis scheme for peptides using Fmoc strategy

side chain protecting groups should resist these acid and base treatments. Barany and Merrifield (1977) defined the principle of orthogonality to describe how the nature of protective groups should be chosen to arrive at the cleanest end-product. One protective function can be removed under conditions in which a second is absolutely stable. Usually groups that need strong acidolysis are used for side chain protection in combination with the mildly acid labile t-Boc group. Deprotection and cleavage are achieved by treatment with HF or trifluoromethanesulphonic acid.

A major improvement was the introduction of the α-fluoreneylmethyloxycarbonyl group (Fmoc), a base labile protecting group (20 % piperidine is necessary for deprotection)

for the α amino group. This enables a change to complete orthogonality resulting in a combination with mild acid labile side chain protection cleavable by TFA.

III.2. Comparison of t-Boc and Fmoc chemistry

Peptide synthesis can be carried out in a batchwise or continuous flow manner. In the latter the resin is contained in a column through which reagents and solvents are pumped. Local high concentrations of reactants are achieved. Recirculation of the reagents improves the efficiency of coupling. Only the Fmoc strategy is fully compatible with the continuous flow method, which allows for real time spectrophotometric monitoring of the progress of coupling and deprotection.

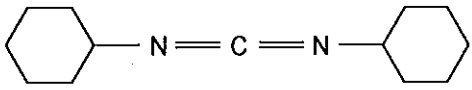
In the past, the costs of Fmoc reagents did not encourage the usage of the Fmoc SPPS. Today, prices are more competitive. Repetitive TFA acidolysis of t-Boc deprotection could lead to alteration of sensitive peptide bonds as well as acid catalyzed side reactions. In Fmoc strategy, the growing peptide chain is subjected to mild base treatment using piperidine during Fmoc group deprotection and TFA is only required for the final cleavage and deprotection of peptide resin. Cleavage and deprotection in t-Boc strategy requires the use of dangerous HF and expensive laboratory apparatus which is not always readily available.

Recently published results obtained for the synthesis of difficult peptides, for example those containing very hydrophobic sequences, demonstrated clearly that Fmoc strategy and especially continuous-flow are superior in these cases (Rovero et al., 1991; Smith et al., 1991).

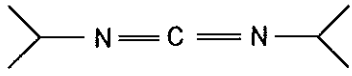
Forty research groups synthesized the same peptide containing 16 amino acids in a sequence with sites for problematic or slow couplings using their own protocols with Fmoc or t-Boc chemistry. Crude peptides were compared (Smith et al., 1991). Over 33% of the crude peptide preparations made by using t-Boc chemistry did not contain any of the desired peptide and over 44% were unable to achieve greater than 25 % purity. In contrast, 31 % of the samples made by using Fmoc chemistry had over 75 % of the desired compound. These results proved the superiority of the Fmoc strategy. However, the purity of the best peptides made by t-Boc chemistry was comparable with that of the best made by Fmoc chemistry. This suggests that, in skilled and experienced hands, either method can give good results.

III.3. Activation of the carboxyl group

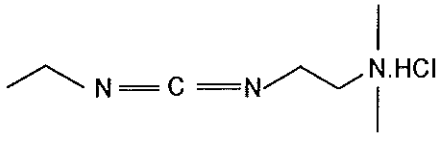
For peptide bond formation, the carboxyl group of the amino acid requires the activation of an electron-withdrawing group. The activated carboxyl group is attacked by the free amino function. There are basically four major types of coupling techniques currently employed for stepwise introduction of N-α-protected amino acids in SPPS. Carbodiimides (Fig. 2) have been some of the most popular *in situ* activating reagents in peptide synthesis. The most successful coupling reagent, dicyclohexylcarbodiimide (DCC) was introduced by Sheehan and Hess (1955). It is a particularly appropriate choice for the apolar environment of polystyrene resins. Di*iso*-propylcarbodiimide (DIPCDI) is used to prevent insoluble acylureas



DCC



DIPCDI

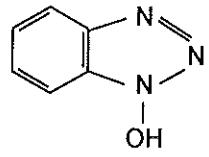


EDCI

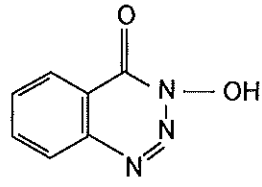
Fig. 2. Some examples of carbodiimides

incompatible in column synthesis (Sheehan, 1960). The principal limitation in using carbodiimides is the dehydration of Asn and Gln residues. The addition of 1-hydroxybenzotriazole (HOBt) to the reaction mixture will prevent dehydration and has the benefit of acting as a catalyst in speeding up sluggish reactions. Carbodiimides introduce racemization at the highly activated carboxyl terminal residue. The racemization can be suppressed by the addition of excesses of HOBt, N-hydroxysuccinimide (HONSu), or 3-hydroxy-3,4-dihydro-1,2,3-benzotriazin-4-one (HODhbt) (Fig. 3). These acidic nucleophiles reduce the lifetime of reactive intermediates by conversion of the overactivated *iso*uronium esters into less reactive and thus more selective active esters.

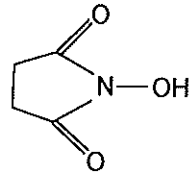
Activation through active esters, which have a long "shelf life", have found widespread application in SPPS. Several active esters are described: nitrophenylesters (ONp), N-hydroxysuccinimide esters (ONSu), N-hydroxyphtalimide esters, pentafluorenyl esters (OPfp), 1-hydroxybenzotriazole esters (OBt) and 3-Hydroxy-4-oxo-3,4-dihydro-1,2,3-



HOBt



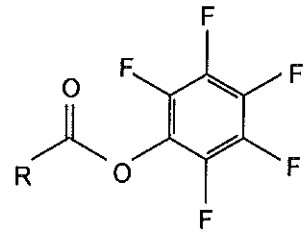
HOOBt



HONSu

Fig. 3. Some examples of additives for suppression of racemization

benzotriazine esters (ODhbt). OBt esters are easily formed *in situ*. These OBt esters are not isolated. *In situ* formation has become widely applied because of their ease of use, fast reactions (even between sterically hindered amino acids) and their general lack of side reactions. For this purpose, generally phosphonium or uronium salts are used which, in the presence of a tertiary base, can smoothly convert protected amino acids to a variety of activated species. The most commonly employed, Benzotriazole-1-yl-oxy-tris(dimethylamino)-phosphoniumhexafluorophosphate (BOP), Benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphoniumhexafluorophosphate (PyBOP), 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and 2-(1H-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU) generate HOBt esters. OPfp esters are also very efficient acylating agents and their chemical structures provide little opportunity for side



OPfp ester

Fig. 4. The OPfp-ester

reactions (Fig. 4). They react somewhat slower than symmetrical anhydrides. The addition of HOBt significantly increases the rate of the reaction. OPfp esters are commercially available. However Fmoc-Ser(tBu)-Opfp and Fmoc-Thr(tBu)-Opfp do not crystallize and are difficult to purify. The ODhbt esters provide suitable alternatives in these cases. A favourable property of ODhbt esters is the appearance of a bright yellow colour during acylations which can be used to monitor coupling efficiency.

Preformed symmetrical anhydrides (PSA) have been used by many research groups because of their high reactivity. They are generated *in situ* using two equivalents of protected amino acid and one equivalent of DCC in DCM. The urea formed is removed by filtration. PSA are mainly used with t-Boc chemistry. Fmoc-amino acids PSA have some undesirable properties. The intermediates formed during PSA generations can undergo rearrangement. Some Fmoc amino acids such as Gly, Ala, Cys(ACM), Gln(Mbh) and Asn(Mbh) a.o. are not readily soluble in DCM and require DMF for solubilization, but DMF slows down the rate of activation. Another drawback is that the use of PSA is quite wasteful, since two equivalents of protected amino acids are required to form one equivalent of activated species and the latter has to be used in excess.

III.4. Resins

The Merrifield resins have been used for more than twenty years for the synthesis of peptides and are the standard resins for SPPS of small or medium-sized peptides using the t-Boc strategy (Fig. 5). The resin consists of polystyrene beads (200-400 mesh, 1% divinylbenzene) functionalized with chloromethyl groups onto which the first amino acid of the peptide is

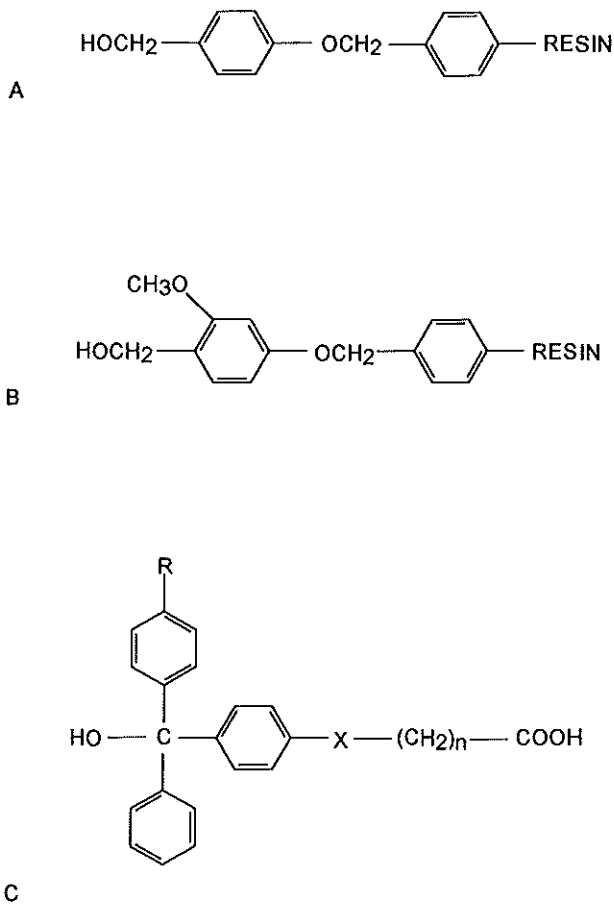


Fig. 5. A). The structure of the Merrifield resin. B). The structure of the Wang resin. C). The Fmoc-Rink handle.

attached. The use of resins with a phenylacetamidomethyl (PAM) group for the synthesis of large peptides has become increasingly popular. This is due in part to the stabilizing effect of the PAM function on the ester linkage which helps to reduce losses during acidolysis (Merrifield, 1963).

Both Merrifield and PAM resins are used for production of peptide acids. For peptide amides and t-Boc chemistry, alternative resins were developed, such as the MBHA resin (Matsueda and Stewart, 1981). The resin consists of polystyrene beads acylated with methylbenzoyl chloride to introduce an additional phenyl ring thus being a ketone resin. This intermediate

is then reductively aminated to yield MBHA resin. The additional phenyl ring renders the peptide-resin linkage labile to HF, giving peptide amides upon cleavage.

For Fmoc chemistry, the KA (Kieselguhr/amide) resins were developed, which can be used in continuous-flow synthesizers. They are based on a composite of polydimethylacrylamide and a inorganic (kieselguhr) support matrix activated with ethylenediamine and functionalized with an acid labile linker (4-hydroxymethylphenoxyacetic acid). For batch procedures the Wang resin is used throughout, also called the HMP resin (Wang, 1973) (Fig. 5). It consists of a polystyrene bead (1% DVB) onto which a acid-labile linker has been attached. Several resins are available for peptide amides based on the acid-labile (Fmoc)Rink handle (Fig. 5) (Rink, 1987).

A new range of resins concerns the resins based on tentagel (registered trademark of Rapp Polymere; Bayer and Rapp, 1986) which is a polystyrene-polyoxyethylene support. They usually consist of a polystyrene backbone to which are attached derivatized polyethyleneglycol (PEG) spacers. Advantages of these resins include very favourable physicochemical properties, in particular their swelling behaviour, physical stability in flow systems and resistance to abrasion and mechanical pressure and improved chemical efficiency. High flow rates have been reported to increase the acylation and deprotection rates. Nowadays, we apply this resin for our Fmoc peptide synthesis.

More resins are nowadays available for special applications. For example, for production of protected fragments with Fmoc chemistry use can be made of the super acid labile 4-hydroxymethyl-3-methoxy-phenoxybutyric acid (HMPB) linker of Riniker (Flörsheimer and Riniker, 1991). Also for protected peptides made with t-Boc chemistry several resins are available. Production of cyclic peptides, peptide libraries and multiple antigenic peptides find their origin in chemically adapted resins.

III.5. Protection groups for side chain functions

A large variety for side chain functions compatible with t-Boc or Fmoc chemistry have been described. The most suitable ones are listed in table 3 and figures 6 and 7. For details with respect to strategies of chemical peptide synthesis, deprotection and cleavage protocols the reader is referred to the huge amount of literature which exists in this field (Bodanszky and Bodanszky, 1984; Atherton and Sheppard, 1989; Stewart and Young, 1984).

III.6. Purification

When peptides are used as immunogens, the selection of the antibodies specific for native protein is performed using the target antigen in the assay in which the antibodies are to be applied. In that case, for immunization purposes, the purity of the peptides is of limited importance (a purity of 75-90% will be sufficient). For peptides with a lower degree of purity coupling methods may give odd results since part of the impurities may consist of shorter homologous peptides which will, though not all, contribute to coupling results. If, on the other hand, peptides are used for screening, then the highest degree of purity should be used.

TABLE 3
Recommended protection groups for amino acid side chains

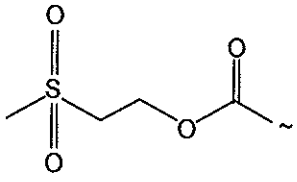
Amino Acid	With Fmoc chemistry	With t-Boc chemistry
Arginine	Pmc	Tos
Asparagine	Trt; Tmob	
Aspartic acid	OtBu	OBzl
Cysteine	Trt[1]; Acm[2]	4-MeBzl[3]; Acm[4]
Glutamine	Trt; Tmob	
Glutamic acid	OtBu	OBzl
Histidine	Trt	Dnp
Lysine	Boc	2-Cl-Z
Serine	tBu	Bzl
Threonine	tBu	Bzl
Trp	Boc	Formyl
Tyrosine	tBu	2-Br-Z

1 Recommended for the routine preparation of cysteinyl peptides
2 Stable to TFA. Can be removed with I_2 to form cyclic disulfide peptides in a single step. Has been used in combination with Trt to prepare peptides containing multiple disulfide bonds
3 Generates a cysteinyl peptide on HF treatment
4 Stable to HF. Enable peptide to be purified prior to liberation of air sensitive thiol groups

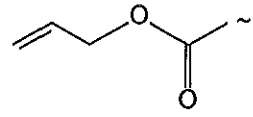
Apart from incomplete coupling and side reactions during synthesis, the process of cleavage and deprotection is always hampered by deleterious side-reactions involving certain side-chain protection groups which are liberated as stabile cations reacting with sensitive amino acids. Scavengers are added during cleavage to trap the reactive carbonium ions. The crude product will exist of a mixture of peptide molecules containing also the desired peptide. The concentration of the desired peptide can vary from roughly a few per cent to 90% in a crude preparation depending on the nature of the peptide and sequence length.

The crude product can be purified by gelfiltration, HPLC, or ion-exchange chromatography. HPLC is used most frequently for purification today. Following deprotection and cleavage from the resin and subsequent lyophilization of the crude product after ether precipitation, the peptide mixture is dissolved in water with 0.1% TFA. The peptide mixture is separated by reversed phase HPLC using a C-18 stationary phase and eluted with a gradient of acetonitrile and 0.1% TFA as counter-ion. If a peptide cannot be dissolved in water, diluted acetic acid or formic acid or other organic solvents can be tried. For acidic peptide, solution in diluted ammonia may be necessary. Sometimes, alternative eluants are needed. To confirm the peptide composition, amino acid analysis can be performed on the hydrolysed peptide or the mass spectrum can be verified.

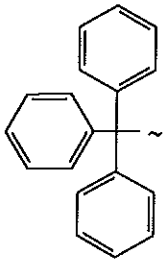
AMINO PROTECTIVE GROUPS



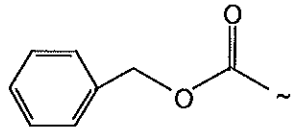
Msc: 2-(methylsulfonyl)ethyloxycarbonyl
β-elimination
base labile



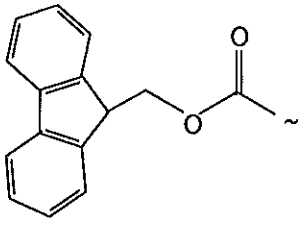
Aloc: Allyloxycarbonyl
allyl transfer by Pd^0 $(Ph_3P)_4$



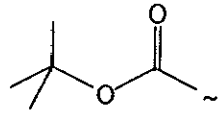
Trt: trityl
mild acidolysis
side chains: Asn/Gln, His, Cys



Z: benzyloxycarbonyl
catalytic hydrogenation
strong acidolysis



Fmoc: 9-fluorenylmethyloxycarbonyl
β-elimination
base labile



Boc: *tert.*-butyloxycarbonyl
mild acidolysis
side chain: Lys

Fig. 6. Protective groups for amino groups. Some protective groups can also be used for other groups as indicated in the figure.

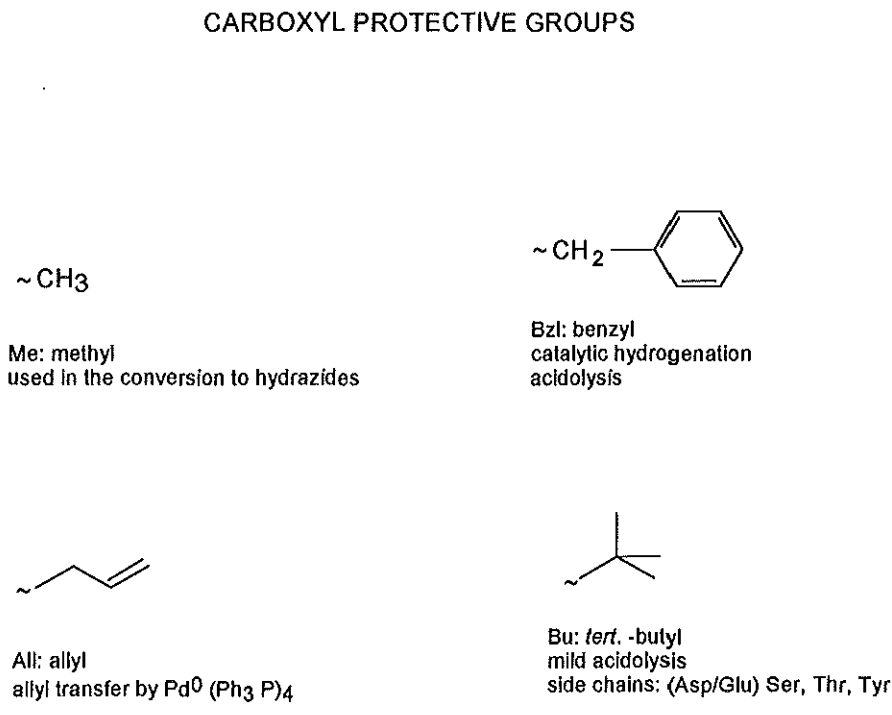


Fig. 7. Protective groups for carboxyl groups.

IV. COUPLING OF PEPTIDES TO CARRIERS

An important clue to the successful use of peptide immunogens is the mode of presentation of the selected peptide to the immune system. Using SP, the antigen usually is an assembly which consists of a carrier protein, a peptide and bridging coupling reagents. Here, the role of these different components in the development of an immune response will be discussed.

Synthetic antigenic determinants have a wide variety of applications. The actual role of a determinant can best be judged from the results of immunizations, i.e., the anti-peptide antibody responses in sera, the recognition of antigenic determinants by antibodies in various

immunoassays, each with its typical micro-environmental conditions, and the specificity of the MAbs selected.

For non-immunogenic peptides, (i.e., B cell epitopes only), coupling to a carrier protein to provide T cell help is required. This is not a matter of molecular mass since immunogenicity of peptides in general is not increased by homo-polymerization with, e.g., glutaraldehyde (Zegers, unpublished results). Polymerization using carbodiimides led to encouraging results though in part based on the coupling of T-determinants (Borrás-Cuesta et al., 1988). Straightforward covalent elongation with a selected T-cell determinant is an alternative option (Hackett et al., 1985; Francis et al., 1987b).

A method was developed for the synthesis of branched peptides (Tam & Zavala, 1989). This multiple antigen peptide method was developed for sensitive detection of anti-peptide antibodies but, in addition, the multimeric peptides show enhanced immunogenicity as compared to free peptides, peptide conjugates or peptides still attached to their solid support (McLean et al., 1991). A more detailed discussion on multiple antigen peptides is given in chapter 10.

IV.1. Choice of carrier

The properties of a carrier protein determine to a large extent the outcome of immunizations with a peptide conjugate. Size, structure, homology with mammalian proteins and relative frequency of certain amino acids are important points to consider.

Larger proteins (> 60 KDa) are preferred as carriers because they contain a sufficient number of functional groups (-NH2, -SH, arginine etc.) for coupling to generate satisfactory peptide/protein ratios. In principle most proteins will do if derived from a non-self source. However, the more genetically distant a protein is to the animal to be used for immunization experiments, the better the chances for immunogenicity. Foreign proteins induce an antibody response more easily than proteins which are homologous to 'self' proteins. Keyhole limpet haemocyanin (KLH), a large aggregated protein from a gastropod, is therefore often used as a carrier protein in mice, rabbits, goats, sheep etc. Other widely used carriers are of bacterial origin: tetanus toxoid (TT), purified protein derivative of tuberculin (PPD), and diphtheria toxoid. Less efficient in mammals are ovalbumin (OVA) and bovine serum albumin (BSA) (Geerligs et al., 1989), probably due to tolerance to these highly conserved proteins.

Chicken gamma globulin (CGG) is rather immunogenic in mice, but has a major drawback. CGG probably contains a major immunodominant epitope which elicits antibodies crossreactive with immunoglobulins (human and mouse)(Boersma et al., 1992).

A carrier protein which is very immunogenic may contain immunodominant epitopes which render the response to attached peptides negligible (Neurath & Kent, 1985). In practice, however, this problem is only very seldom encountered or recognized. It turns out that the immunogenicity of the carrier is changed by the coupling of new determinants. Apparently a new ranking of immunogenicity of B cell determinants is reached which results in antibodies to the newly introduced (peptide) determinants. The size of a carrier is important since a larger protein can accommodate more SP. However, coupling of relatively large numbers of SP, depending on the protein, can have a negative influence on the

properties of a conjugate. Overloading will lead to precipitation and decreased immunogenicity (Peeters et al., 1989). Also crosslinking agents like diazo compounds and glutaraldehyde can drastically decrease the solubility and immunogenicity of a carrier-SP conjugate.

Most large aggregated proteins, like KLH (> 600 kDa), have intrinsic adjuvant activity. They support long-lasting responses. Consequently, a booster immunization in a normal four week time-schedule will lead to a state of hyperimmunization. The use of virus particles (HbsAg) as a carrier for peptides with a hydrophobic tail that integrates in the outer-membrane has been proposed as an alternative for both carrier and adjuvant (Neurath et al., 1989).

KLH is the most suitable carrier protein because of its properties: a large molecule with adjuvant properties, many available groups for coupling and genetically distant from the proteins of the recipient. It appeared to possess T cell epitopes which can be bound by MHC class II molecules of different animal species. The complexes of MHC class II molecules and T cell epitope, presented by the antigen presenting cell, are recognizable by their T cells.

IV.2. Peptide conjugation: possibilities and choices

A number of relatively simple methods have been developed and evaluated for the coupling of labels to peptides and for the coupling of peptides to carrier proteins or fragments (for a review see Blair and Ghose, 1983)(Table 4).

Coupling methods need to be selected carefully. The coupling controls the site where the peptide is linked, the exposition of C- or N-terminus (orientation), the level of deformation by intra-chain coupling, and the level of crosslinking which also may lead to the deformation and inaccessibility of the putative epitope.

Coupling of peptides to carriers, though being an immunological prerequisite, is not just connecting the two molecules. The way a peptide is coupled to a protein may introduce unexpected new structural elements but also may lead to the disappearance of determinants. The orientation of the peptide with respect to the carrier backbone influences exposition to the environment. Since in the carrier protein all or most amino acids are available, selectivity of a coupling method is in part determined by the amino acids present in the SP sequence. It will be necessary to avoid the use of certain amino acids for coupling purposes because of their important contribution to the antigenicity or structure of the peptide or when they determine native protein specificity as in mutant and genetic variants of proteins (Zegers et al., 1991b; Gerritse et al., 1991, 1992).

For a coupling site of choice additional amino acids (cysteine, arginine) can be introduced at the C-or N-terminus of the SP. Addition of a cysteine residue yields a -SH group which can be used for coupling and the addition of arginine yields a guanido group also usable for coupling. In addition, various methods have been described for the introduction of -SH groups for coupling (Blair & Ghose, 1983). The method using N-succinimidyl-3-(2-pyridyldithio)-propionate (SPDP) (Carlsson et al., 1978) in this respect is probably the most widely used. It can be used to introduce -SH groups in both peptide and protein but it may also be used to introduce active -SH in the protein only for coupling to

TABLE 4
Homo- and bifunctional coupling reagents for peptides and proteins

Reagent	Group	Amino acid	References
Active -SH	-SH	Cys, Met	Traut et al. (1973) Lin et al. (1990)
Maleimide	-SH	Cys, Met	O'Sullivan et al. (1979) Fujiwara et al. (1988)
Carbodiimide	-COOH, $-NH_2$	Asp, Glu, Lys and α-NH_2 Cys, Tyr	Goodfriend et al. (1964) Deen et al. (1990)
N-Hydroxysuccinimide	$-NH_2$	Lys and α-NH_2	Carlsson et al. (1978)
Glyoxal	Guanido	Arg	Atassi (1977)
Azide Compounds	Peptide bonds Aromatic rings $-NH_2$ -COOH	 Tyr, Trp, His Lys, Arg Glu, Asp	Glazer et al. (1975)
Glutaraldehyde	$-NH_2$ -SH and -OH (weak)	Lys and α-NH_2 Cys, Tyr, His	Habeeb et al. (1968); Claassen and Adler (1988) Zegers et al. (1990)

a peptide cysteine. Introduction of -SH can be monitored spectrophotometrically. Introduction of -SH can also be achieved by the reaction of 2-iminothiolane with available -NH2 groups (King & Kochoumian, 1978). A peptide that does not contain a sulfydryl group can be functionalized during its synthesis at the amino terminus by acylation with S-acetylthioacetic acid or the succinimidyl ester of the latter compound (Duncan et al., 1983). Treating the protected peptide with base, even hydroxylamine, results in the liberation of the sulfydryl group. A method was described to introduce -SH via the carboxyl groups of a peptide (Lin et al., 1990).

The advantage of -S-S- bridge coupling may on other occasions prove to be the weak point. -S-S- bridges are relatively unstable under physiological conditions and at low pH. But on the other hand the acid lability of -S-S- bridges in conjugates allow dissociation of the coupling. In general however, the stability of -S-S- bridged conjugates is sufficiently stable to elicit immune responses.

Most widely used coupling agents are carbodiimides (Goodfriend et al., 1964; Bauminger and Wilchek, 1980; Deen et al., 1990), bis-diazo compounds (Glazer et al., 1975), SPDP for introduction of active -SH to produce -S-S- bridges (Carlsson et al., 1978) and compounds including maleiimides and N-hydroxysuccinimides all with a large variation in linkers (Liu et al., 1979, Green et al., 1982; Briand et al., 1985) and glutaraldehyde (Avrameas, 1969; Korn et al., 1972; Reichlin, 1980; Zegers et al., 1990)(see also Table 4).

Carbodiimides couple through -COOH and -NH2 groups, which results in at least two orientations of the peptide. Acetylation of the peptide leads to preferential coupling at the carboxy terminus.

Homo-bifunctional reagents (SPDP, glutaraldehyde) use the same (-NH2) group both in carrier and in peptide.

In principle, hetero-bifunctional coupling agents can be used in two ways since they consist of two different reactive groups connected by an inert linker sequence. Maleimide-N-hydroxysuccinimide (O'Sullivan et al., 1979; Ishikawa, 1980; Kitagawa et al., 1981) and maleimide-diazo (Fujiwara et al., 1988) compounds are mainly used to couple peptides with a terminal cysteine to protein -NH2 groups. When a protein is used which is relatively cysteine-rich, the method can be used the other way around. For this purpose thyroglobulin is a suitable carrier (Ernst et al., 1989). Introduction of maleimides and -SH separately requires two coupling procedures but may have some advantages for specific, covalent acid stable linkage of two amino groups. This approach avoids crosslinking and is more specific and better controlled than GA coupling. Therefore, the maleimide and -SH respective succinimide-derivatives were employed as has been described by Yoshitake et al. (1979).

Various possibilities of coupling methods using different chemistry exist. The method of choice largely depends on the composition and sequence of the peptide and its contribution to the function of the protein from which the peptide is derived (see also section IV.5).

IV.3. Peptide coupling efficiency

IV.3.1. Analysis of coupling efficiency

Coupling efficiency depends very much on the nature of the coupling method and conditions applied. Covalent linkage leads to stable conjugates.

The chemical stability of coupling agents in general is limited. Especially maleimide and N-hydroxysuccinimide based compounds (MBS-type), which are widely used, have limited stability due to hydrolysis. Sulfo-variants of these compounds lead to improved efficiency and 2-4 times higher yield (Myers et al., 1989). Though more stable and efficient conjugation is obtained with the aromatic linkers in bi-functional coupling agents, for immunization purposes the non-aromatic linkers are to be preferred due to their lower immunogenicity (Peeters et al., 1989).

Coupling efficiency is not easy to quantitate. Efficiency can be determined accurately only with labelled peptides. Fluorescence, biotin or radio-isotope (^{125}I,^{3}H) labelling are most frequently used. Fluorescence or biotin labelling generally utilizes one amino group per peptide and thus may influence the efficiency of the coupling. In addition, steric hindrance may be introduced (Von Grüningen & Schneider, 1989). Incorporation of a unnatural amino acid such as norleucine at one of the termini belongs to one of the possibilities to determine the coupling ratio. The substitution rate thus can be established from the amino acid composition of the conjugate. Other methods rely on the determination of available -SH or -NH2 groups before and after coupling (Jones et al., 1989; Peeters et al., 1989).

For short non-labelled peptides substitution can be estimated on the basis of changes in relative amino acid frequency after coupling (Briand et al., 1985). As was shown by Ernst et al. (1989) using radioactive labelling (^{125}I), amino acid frequency determinations for longer non-labelled peptides is too inaccurate.

In an intact protein sequence the immune response pattern is determined by immunodominance rather than by the frequency of a determinant in the protein. Peptides like haptens have an intrinsic antigenicity. From investigations using rigid hapten structures it is known that over-substitution leads to a decrease in immune responses (Claassen and Van Rooijen, 1985). Peeters et al. (1989) and Stevens et al. (1981) investigated coupling of peptides to Tetanus toxoid (TT) in various ways. Optimal substitution (10-20 SP/TT) was about half the maximal substitution rate (40-50 SP/TT).

Determination of the peptide to carrier ration in conjugates relies on the efficient separation of bound and unbound peptides. To achieve this separation, the reaction mixture is dialysed to remove the unbound peptides. We observed that not all unconjugated peptide molecules are removed by dialysis. Sometimes, peptide molecules adhere to carrier molecules by non-covalent forces forming a so-called "pseudo-conjugate".

IV.3.2. Pseudo-conjugates

We have repeatedly encountered non-covalent association of peptides with carriers which could not be disrupted using extensive dialysis or mild detergent treatment. Other more stringent treatments might not permit discrimination between covalent coupling and high affinity binding to the carrier. Pseudo-conjugates did not lead to antibody formation. On the basis of these observations, we doubt whether chemical evaluation of conjugation is relevant since all methods mentioned measure covalent as well as pseudo-conjugates. The quality of a conjugate as an immunogen cannot be estimated by determining the conjugation efficiency. Therefore evaluation of conjugation quality is best performed by the evaluation of immune responses (Peeters et al., 1989, Boersma et al., 1988a; Deen et al., 1990; Zegers et al.,1990).

IV.4. Immunogenicity of coupling neo-determinants: influence on anti-peptide responses

Homo-bifunctional coupling reagents, glutaraldehyde, carbodiimides and bis-diazo compounds have all been found to generate coupling agent specific antibodies. Most hetero-bifunctional coupling agents include a spacer segment. This segment often functions as a hapten and includes immunogenic structures. The length and rigidity of a linker may influence the response to the coupled SP (Peeters et al., 1989; Myers et al., 1989). In general, aliphatic-chains will be less immunogenic than linkers with aromatic structures. In addition, the structures involved in the binding (maleimides, N-hydroxysuccinimide, -SH from activated disulfides etc.) with the functional groups of the protein form neo-determinants which (as in the case of acyl-urea adducts formed in carbodiimide coupling) can be highly immunogenic. To evaluate for proper antibody responses, screening and immunization in general is performed using two conjugates of the same peptide with different carrier proteins and

different coupling methods. Even then unwanted antibody responses to neo-determinants can be erroneously interpreted as demonstrated by Edwards et al.(1989).

IV.5. Orientation of a peptide in conjugates and epitope specificity

Orientation of the peptide in a conjugate may determine to which side of the peptide a response will be elicited. Both C- and N-termini are highly represented among immunogenic sites of proteins. This is probably influenced by the flexibility of the termini and the orientation in the solution. In general this will be true for peptides as well. According to most authors there is no preference for coupling via the N-terminus or C-terminus. However, Dyrberg and Oldstone (1986, 1987) showed that for an octa-peptide coupling via the C-terminus led to better immune responses. Schaaper et al.(1989) applied PEPSCAN determinant mapping to evaluate the antisera produced by different coupling agents for either terminus. They observed that antibody responses were mainly directed to the non-conjugated terminus of a peptide. These findings should be taken into account when antibodies are desired against the N- or the C-terminus of a protein. Coupling of the N- or C-terminal peptide of a protein to a carrier protein could best be performed via the terminus of the peptide which is situated in the chain of the protein.

V. IMMUNIZATION

Immunization requires the introduction of an antigen at a certain site of the body. The form in which the antigen is presented determines the outcome of the response and may be, e.g. a purified protein, a virus particle or a construct in which segments of an antigen are sufficiently represented, etc. A large number of parameters influences the result of each immunization: a) the site at which the antigen is introduced, b) the vehicle which retains it at the chosen localization, c) the type of adjuvant applied, d) the doses of antigen, e) the genetic make-up of the recipient, f) genetic distance between recipient and antigen. These parameters will be discussed in the following sections. Immunization of peptide conjugates results in responses to the peptide, the coupling agent or what remains of it, the carrier protein and all combination-determinants. To understand the immune responses to peptide conjugates therefore requires differential analysis.

V.1. A vehicle can deposit larger amounts of antigen at pre-determined sites

The vehicle to which the antigen is coupled or is dissolved in, determines the localization of an antigen. Sepharose beads, pieces of nitrocellulose, aluminium hydroxide gels and oil emulsions, a.o., can be used for the purpose of delivery in the peritoneum, under the skin or even in the spleen. Some of these vehicles such as alum and oil emulsions have adjuvant properties, while others, such as sepharose beads and nitrocellulose are relatively inert in an immunological sense. Specol, a water in oil emulsion, shows a reasonably good performance in supporting a good immune response with mild side effects (Bokhout et al., 1981).

Haptens bound to various carrier structures such as proteins and carbohydrate polymers readily induce antibody responses (Claassen and Van Rooijen, 1985). This suggested that similarly small peptides bound to a polymeric carrier could be used for immunization. Fischer et al. (1989) synthesized peptides according to the Fmoc technique which were subsequently immunized as a peptidyl-resin. The immunogenicity of peptides still attached to the resin was compared to free peptides and KLH and OVA conjugates. KLH and OVA conjugates performed much better than the same peptide bound to the resin irrespective of the co-administration of adjuvant (Freund's) (Fischer et al., 1989).

For antibody production against synthetic peptides, not meant as a vaccine, the use of a mineral oil can be recommended. Mineral oils, such as specol or Freund's complete adjuvant, perform, both as a vehicle, and also as an adjuvant (see also section V.4). Since peptides by themselves are rather weak immunogens, the antibody response against peptides needs immunopotentiation by adjuvants. Specol deserves preference above Freund's adjuvant since specol provokes less side-effects in animals.

V.2. Specific routeing of antigen determines localization of antigen

The site where the antigen is deposited in part determines which lymphoid organs are activated and determines to some extent the isotype of the antibodies generated. Intraperitoneal (i.p.) and subcutaneous (s.c.) classical immunizations activate mainly the spleen and peripheral lymph nodes respectively and result in IgG responses.

The immunization route is limited by the formulation of the antigen. Insoluble antigens cannot be administered intravenously (i.v.). In order to avoid antigen wasting in the periphery, the antigen can be applied topically in the spleen, either free or attached to a vehicle (nitrocellulose, sepharose beads). Doses 50-100x lower than with intraperitoneal immunization could be used on intrasplenic immunization (Spitz, 1986; Hong et al., 1989). Despite the low antigen doses needed, intra-splenic immunization has not found widespread application because of major drawbacks. The method is not easy to employ since the spleen can easily be damaged and immunization is therefore not very reproducible unless in skilled hands. In addition, this method is animal-unfriendly.

Peptides which appear to possess low immunogenicity upon routine i.p. or i.v. administration may be given locally to sensitize peripheral lymph node cells. In the mouse, immunization in the footpad or subcutaneously in the dorsal flanks may activate regional lymph nodes very efficiently when strong adjuvants are applied [Complete Freund's Adjuvant (CFA)].

Targeting of antigens to certain molecular structures on cells is a form of specific routeing of antigen. Enhanced responsiveness has been shown to occur when peptides are targeted to surface immunoglobulin or class I molecules (Casten, 1988). For BSA, Lees et al. (1990) showed that targeting with anti-IgD especially led to strong potentiation of antibody responses. Immunological targeting to, for example, MHC molecules acts as a replacement for the adjuvant in that it targets the antigen and at the same time acts as an activation for the antigen presenting cells. To this end, MHC-specific antibodies were applied (Carayanniotis et al., 1988; Carayanniotis & Barber, 1990). Experiments carried out so far

with antigen-targeting have shown variable results. The effectiveness corresponds to the construction of the antigen linked to the targeting molecule. Targeting with anti-IgD led to a polyclonal antibody response in the sense that also aspecific B cells are stimulated. Conventional immunization using strong, animal-friendly adjuvants can be recommended.

V.3. Scheduling of immunization

Timing of responses is dependent on the mode of antigen presentation. Slow release of antigen from emulsions or crystalline deposits may introduce a delay.

A general observation with respect to scheduling of immunization schemes is, that it proved to be worthwhile to allow a long period (more than four weeks) between the first and following immunizations. In these cases booster immunizations evoked higher antibody responses. This suggests that in these cases the activation of resting B memory cells is synchronized. In some examples we used mice for hybridoma production after a third immunization which was given six months after the second. A high number of specific clones were found compared to the number of specific clones found after immunizations with an interval of four weeks between booster injections.

V.4. Adjuvant

V.4.1. The use of adjuvant (general)

Adjuvants are substances that aspecifically potentiate immune responses when given simultaneously with the antigen. Most applications of peptides are found in experimental immunization for antibody production and the testing of the efficacy of peptide vaccines. Peptides are thought to be only slightly immunogenic. Adjuvant formulations are needed for stimulation of a high antibody response using peptides (Boersma and Claassen, 1995). That is why in most experimental protocols the most potent adjuvant, complete Freund's adjuvant (CFA), is used. Conjugates are emulsified in CFA and immunized in experimental animals like mice and rabbits. For the production of PAbs however, this has the drawback of the contamination with antibodies to bacterial antigens and DNA which are present in CFA. Therefore adjuvants without bacterial antigens were developed which in addition provide milder treatment for the laboratory animal (Bokhout et al., 1981; Barteling and Vreeswijk, 1991).

The precipitation of peptide conjugates on aluminum hydroxide gels also leads to good anti-peptide responses (Geerligs et al., 1989; Boersma et al., 1988a, 1989b,c). Comparison of results of immunization of conjugates of the same peptide using six different adjuvant protocols showed insignificant differences only, when tested for anti-peptide reactivity or reactivity to the coupling reagent dependent neo-determinant. Though it has been claimed that alum precipitated antigens may sub-optimally stimulate cell mediated immunity, they do elicit delayed type hypersensitivity reactions. Until now alum hydroxide gels are the only adjuvants generally approved for use in humans and primates (Bomford, 1986; Allison & Gregoriadis, 1990). A large number of substances (lipid derivatives, liposomes: Tamauguchi et al., 1983; non-ionic block polymers: Zigterman et al., 1987; Hunter et al., 1981; virosomes, micelles

and the like: Morein & Simons 1985) have been promoted as a promising adjuvant for use in vaccine formulations and certainly are worthwhile of being used in the generation of diagnostic reagents (Warren et al., 1986, Geerligs et al., 1989).

In most adjuvant formulations, certainly water in oil or double emulsions, antigen presentation is influenced by the hydrophobic character of various components of the antigen or conjugate. For FMDV vaccine the use of various adjuvant formulations led to a different outcome of the vaccine trials (Barteling & Vreeswijk, 1991). Certainly for peptides with hydrophobic and hydrophillic regions antigen presentation may be dependent on the vehicle and adjuvant formulation chosen (Fieser et al., 1987). For the generation of diagnostic antibodies we favour the use of adjuvants, which are relatively mild for experimental animals and do not result in crossreacting antibodies, i.e., water in oil emulsions without the addition of micro-organisms.

V.4.2. Derivatization of peptides with integrated adjuvanticity

Derivatization of peptides for the purpose of improved immunogenicity is a rapidly developing field. Therefore only a few applications are mentioned. Derivatization of peptides with hydrophobic tails as proposed by Neurath et al. (1989) may facilitate incorporation into liposomes. Derivatization with an aliphatic anchor enabled Neurath and Strick (1987) into incorporate synthetic peptides into ISCOM-like structures.

Important for the development of vaccines is the use of purified synthetic bacterial cell wall components, muramyl-dipeptides, which can be added to and even synthesized onto peptides and protein fragments (Leclerc et al., 1985). For the priming of virus-specific cytotoxic T helper cells, Deres et al. (1989) prolonged a peptide representing an MHC class-I restricted epitope of influenza nucleoprotein (AA 147-158) by covalent linkage to a tripalmitoyl-S-glycerylcysteinyl-seryl-serine (Jung and Bessler, 1995). This type of integrated adjuvanticity is most promising. Such lipopeptide-antigen conjugates have a number of advantages. They remain stable in heat, light and solvent. They are fully biodegradable. No toxic side-effects have been seen thus far.

VI. CHARACTERIZATION OF POLYCLONAL AND MONOCLONAL ANTIBODIES

The antibody responses elicited with synthetic peptides should be evaluated in time after each administration of the antigen. In this section, methods for the assignment of the antibodies are described. In addition, it will be emphasized that the choice of the evaluation method implies the reactivity of the antibodies found.

VI.1. Performance characteristics of antibodies are assay specific

As has been discussed (Haaijman et al., 1988), MAbs tend to show a specific, assay dependent, recognition pattern towards the antigen to which they are directed. The assay conditions exert their influence not only on the antigen but also on the diagnostic antibody.

This phenomenon is regularly encountered even in routine diagnostics. Choy et al. (1988) using antibodies specific for *Trichinella spiralis,* demonstrated assay dependent variation in the results of eight different immuno-assays tested for the detection of *Trichinella spiralis* antigens. This indicates, that antibody selection has to be performed in the assay, or under the conditions, in which the antibody should to be applied.

In the past we produced a large number of specific MAbs for various antigens. For each antigen only a few proved to be multi-assay applicable. The general rule, however, is that the specific antibodies perform well only under certain assay conditions. This could mean that selection of MAbs in ELISA may preclude finding antibodies that perform optimally on, e.g., fixed tissues. In the case of the selection of antibodies produced upon immunization with peptides, the initial selection may be performed on conjugates of the peptide in ELISA. In that case, conjugates of the peptide are used which are prepared with a different carrier and different coupling agent than those used for immunization. It is assumed that in such conjugates a peptide will show various possible conformations. Some of these may mimic the conformation which is found in the assay of choice. With selected anti-peptide antibodies one may proceed to the more specific antigen detection methods for which the antibodies were produced (Zegers et al., 1991a). The scheme for a standard strategy of the synthetic peptide approach including performance analysis is represented in Table 2. A feature that is incorporated in all assays (ELISA, immunoblotting, immunocytochemistry) is the specificity control of native protein and individual determinants by inhibition with the antigen or the peptide (with the relevant antigen and with an irrelevant antigen as a negative control), respectively. These inhibition assays are usually performed as a pre-incubation of the antibody with various concentrations of the appropriate antigen before application *in assay*. A true competition between free and bound antigen *in assay* can be performed as an alternative.

VI.2. Assay dependent conformation of the antigen

The conformation of the antigen is dependent on its actual micro-environment and treatment before and during the assay. In ELISA systems the antigen adheres to the plastic surface of a microtiter plate. This may introduce some conformational stress which may result in a deformation of an epitope present in the antigen as it was used for immunization.

Similarly, the treatment of proteins in biochemical methods for detection and analysis results in different levels of denaturation and therefore different conformations of a protein. For instance, when a protein is treated with SDS it loses most of its conformation.

VI.3. Analysis of anti-peptide antibody responses in various assays

VI.3.1. ELISA

Antisera elicited with a carrier-peptide conjugate may contain antibodies directed to the peptide, the carrier, the coupling reagent, and neo-determinants. The search will be for antibodies that crossreact strongly with the native protein. Proper screening procedures allow discrimination between the specificities mentioned. Free peptides are used for detection as

well as the conjugates of these peptides made with a different protein carrier and a different coupling agent than those used for the conjugate applied for immunization. This procedure allows discrimination between the various immunogenic determinants. Antibody responses are analysed in a direct ELISA procedure on plates with a coating of peptides, peptide conjugates and the native antigen, or, alternatively, in an indirect or in a capture ELISA with, e.g., antibodies that capture the native antigen. In competition assays, competition between the peptide and the native antigen can be analysed.

Capture and competition ELISA are most sensitive. Competition is preferred when denaturation of the antigen is expected by the direct coating procedure. Titration is needed of all reagents used for coating as well as for detection for proper evaluation of the antibody specificity. The orientation of peptides in conjugates used for immunization and for response analysis may definitely cause confusion in the interpretation of results (Boersma et al., 1992). The orientation of the peptide in the conjugate used for immunization may be different from the orientation of the peptide in the conjugate used for screening or even from the free peptide adhered to the plastic surface. Elicited antibodies may not recognize the peptide in the assay system, but, by contrast, they can bind to peptides free in solution.

According to some authors peptides shorter than 15 amino acids do not bind to plastic surfaces (Briand et al., 1985). In our experience all unmodified peptides can be coated directly when the proper conditions are applied (see Chapter 12). Peptides which have been acetylated or peptides with an amide terminus will show a binding behaviour to plastic which is pH dependant. Variation in buffer pH can provide suitable conditions for ELISA assays. In addition, peptides can be coated to polystyrene plates using glutaraldehyde pre-activation (Klasen et al., 1983). This however may lead to antigen presentation *in assay* with preferential exposure of the non-coupled site. The advantage of the application of covalent coating is that more stringent conditions can be used in the assay, such as the use of urea (Klasen et al., 1983). Similarly coupling of antigens with carbodiimides can be used for *in assay* antigen presentation. Other methods include indirect coating through poly-L-lysine (see Chapter 12) or copolymers of lysine and alanine (Hobbs, 1989).

Also other ways of presenting peptides, e.g., coated beads etc. applied in ELISA format have been developed (Modrow et al., 1989).

VI.3.2. Blotting to nitrocellulose or other supports

Sensitive direct immuno-blotting assays such as have been described by Radl et al. (1988) and Boersma et al. (1989b) are easy and rapidly performed assays for the detection of antibody specificity. The procedure performed with the strips of nitrocellulose is similar to that in ELISA. The amount of antigen needed is extremely low. Antigen detection limits are in the ng order. Dilutions of ascites fluid of 1:50.000 are sufficient for detection. Adsorption onto nitrocellulose may introduce an assay characteristic recognition pattern as any other assay.

VI.4. PEPSCAN: epitope mapping as the counterpart of anti-peptide antibodies

Synthetic peptides selected according to various (theoretical) criteria may lead to the generation of specific antibodies. By their very nature, it is known to which site these antibodies are directed. Vice versa, peptides can be used for the screening for antigenic epitopes along the entire sequence of a protein. This concept was first implemented by the group of Leach (Smith et al., 1977; Hurrell et al., 1978). Using the same support for peptide synthesis and RIA, they showed that, in principle, it was feasible to delineate antigenic determinants. On a solid support, short overlapping peptide sequences were synthesized together representing an entire protein sequence. Subsequently, antibodies generated to the protein were tested for binding to these peptides using an RIA-like approach. In this manner antigenic sequences were detected. This method has been refined and made available for large scale applications (over 400 peptides per protein) by Geysen et al. (1984, 1985) and Meloen and Barteling (1986).

Peptides of characteristically 8-9 amino acids spanning the entire protein were used to demonstrate interaction between antibodies and synthetic peptide sequences. In this PEPSCAN method, the antigenicity scan is performed as in ELISA. At present, 2000 peptides can be tested daily in ELISA, while a batch of 3000 pin-bound peptides can be produced in 4-6 weeks. A major advantage is the reusability of the pin-bound peptides for screening. Following an ELISA, bound antibodies are eluted from the peptides using SDS and β-mercaptoethanol. Synthesis of peptides to paperdiscs allowing screening of multiple antisera simultaneously has been described by van 't Hof et al. (1993).

The drawbacks of the PEPSCAN method are that with short sequences (hexamers) in combination with the ELISA type of system extremely low binding affinities can be determined of which the physiological relevance is questionable. In addition, the method of synthesis does not allow for a control of the integrity of the peptide sequences on the solid support during synthesis. The density of the peptides on the rods may interfere with antibody binding. This means that a negative PEPSCAN remains inconclusive (Geysen et al., 1987), whereas a positive signal has to be confirmed using longer peptides (nonamers). According to Geysen et al. (1985) the binding of antibodies to peptides derived from distant segments of a protein is an indication that even discontinuous determinants can be detected using the PEPSCAN method. On the other hand all synthetic peptides antigenically may be discontinuous since not all amino acids in a peptide necessarily contribute to the antibody binding (Geysen et al., 1987).

The PEPSCAN method can also be used for the detection and mapping of T cell epitopes by applying a proliferation assay rather than the ELISA (Van der Zee et al., 1989). Peptides must be cleaved off from the solid support. The method has been refined (Schaaper et al., 1992)

The PEPSCAN method can be used for selecting immunogenic sequences in a practical approach, instead of selection based on theoretical considerations, for the purpose of, e.g., vaccine development. Exact determination of the epitope in a peptide to which the selected antibody binds is possible.

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Chapter 2

Introduction to experimental work

I. INTRODUCTION TO EXPERIMENTAL WORK

The aim of the studies presented in this thesis was to investigate and optimise the parameters which play a role in the generation of antibodies using synthetic peptides. The most important parameters are: a) selection of the peptide sequence that is going to be used for immunization and which represents a part of the protein against which antibodies are desired; b) the synthesis chemistry; c) the construction of the immunogen: choice of the carrier or incorporation of intrinsic T cell epitopes, choice of coupling chemistry which influences the orientation of the peptide to the carrier; d) the immunization protocol which influences both the level of the immune response and the affinity of the antibodies; e) the evaluation method of the antibody response; f) the method used for the selection of the hybridomas.

Eliciting antibodies with part of the amino acid sequence instead of with the entire protein is an alternative if the protein of choice is pathogenic, lethal or not available. In addition, antibodies specific for a pre-selected site of the protein can only be generated by using peptides. The success of the generation of specific antibodies using synthetic peptides heavily depends on the careful application of the different steps that control the method. The peptide method therefore often fails, since the steps are carried out without the proper considerations. It was our intention to carefully investigate these steps and combine them to formulate a strategy.

Two different proteins were chosen for which site-specific antibodies were desired and for which antibodies could not be obtained using classical immunization with the entire protein. The human androgen receptor was chosen because of its known homology with other steroid receptors and as an example of a protein which was not available, but the amino acid sequence could be predicted from the cDNA sequence. Human α_1-antitrypsin Z was studied as an example of a protein with a single amino acid substitution resulting in dramatically altered biological properties. Although model systems, antibodies against both proteins could be employed directly in a clinical research setting. The generation and characterization of antibodies specific for these two proteins, with the aid of synthetic peptides, serves as a model system to optimise the parameters that govern the specificity of the selected antibodies and is described in part I and part II.

In chapter 1, the strategy followed for eliciting and selecting antibodies using synthetic peptides is outlined. The different factors, which have a decisive impact on the successful generation of the desired antibodies, are introduced and discussed briefly. This chapter is quite comprehensive and is also meant to be a concise review and guideline for those entering the field of antibody generation using peptides.

Efficient coupling of a synthetic determinant to a carrier protein is generally a prerequisite for adequate antigen presentation to the immune system. A new method for conjugation of a peptide to a carrier protein using glutaraldehyde as coupling reagent is described in chapter 3. The advantage of this method lies in controlled coupling of the peptide in a convenient two-step dialysis procedure preventing the formation of homodimers of the peptide and preventing crosslinking of carrier protein molecules.

In part I, the generation and characterization of antibodies, specific for a protein for which the amino acid sequence was not known, but could be predicted from the cDNA sequence, are described. The human androgen receptor is a member of a protein family of steroid receptor showing considerable homology. Immunization with a peptide sequence derived from the androgen receptor, which avoids homology with sequences from the other steroid receptors, may lead to antibodies specific for the androgen receptor provided that the proper choices in the entire process are made. The selection of the epitope in such a case is crucial for success. An example of epitope selection is given in chapter 4 describing the generation of monoclonal antibodies which are specific for the different domains of the human androgen receptor and do not crossreact with the other steroid receptors. These antibodies have opened a wide field of possibilities for studying the function, localization and regulatory mechanisms (of the domains) of the receptor in biochemistry and histochemistry. In addition, these antibodies offer the possibility of investigating prognosis and behaviour of prostatic tumours under hormonal therapy.

In part II, the generation of antibodies, which are able to distinguish proteins with only one amino acid difference, are described. In such a case, one is restricted to a fixed localized sequence of the protein: the substitution site. Exact delineation of the peptide sequence then becomes very important. Flanking sequences of the substitution site strongly influence the immunogenicity and may therefore be decisive in obtaining the proper antibodies. Immunodominant flanking sequences, e.g., may prevent the generation of antibodies directed to the substitution site. An overview of the strength and limitation of the usage of peptides for single amino acid specificity is given in chapter 5. In chapter 6 and 7 and partly in chapter 5, the generation and application of monoclonal antibodies for the detection of genetic variants of α_1-antitrypsin is described. Antibodies specific for the Z-variant of α_1-antitrypsin (a $Glu^{342} \rightarrow Lys$ substitution) offer the possibility of easily and rapidly detecting carriers early in life. Z homozygous individuals develop lung emphysema at an early age. With early detection, preventive life-style measures and/or specific therapy can be given. The generation and selection of antibodies which specifically recognize variants of α_1-antitrypsin offer an unique model system to investigate the factors involved. In these chapters, the importance of sequence selection, construction of the immunogen, route and timing of immunization, selection assays for monoclonal antibodies and accessibility of the epitope in α_1-antitrypsin is emphasized.

In part III, the use of synthetic T cell epitopes in a hybrid construct as an alternative to a peptide-carrier complex is discussed. A synthetic T cell epitope may be advantageous for use in a synthetic vaccine. In addition, also for antibody production, synthetic T cell epitopes may deserve preference over the use of a carrier protein, e.g., when a selected epitope is a weak immunogen. Immunodominance from the carrier protein can be circumvented in this way. Chapter 8 gives an overview of all the parameters involved with T cell epitope selection and the relative localization with respect to the B cell epitope for inducing proper B cell-epitope specific antibody responses. In literature there is some confusion as to whether covalent linkage between a T and a B cell determinant is necessary for an antibody response against the B cell epitope. A covalent linkage between a T and a

B cell epitope is a requirement for achieving cognate B-T cell interaction, yielding a memory antibody response, as shown in chapter 9. An alternative format for a peptide construct consists of a branched poly-lysine core to which linear epitopes are synthesized. These multiple antigen peptides proved to be very suitable as antigens in various immuno-assays. In literature, they were initially proposed as immunogenic with every B cell epitope sequence. We show in chapter 10 their usage as immunogen and that incorporation of a T cell determinant into a multiple antigen peptide construct is essential for induction of antibodies with a high affinity for the native protein.

A general discussion on the use of peptides as antigens is given in chapter 11. Besides epitope selection, selection assays for screening hybridomas proved to be decisive for the outcome of the specificity of the monoclonal antibodies. Straightforward instructions on the usage of peptide antigens, abstracted from the experience with synthetic peptides, are given. A detailed description of the methods used is given in chapter 12.

PART I

Chapter 3

An improved conjugation method for controlled covalent coupling of synthetic peptides to proteins using glutaraldehyde in a dialysis method

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AN IMPROVED CONJUGATION METHOD FOR CONTROLLED COVALENT COUPLING OF SYNTHETIC PEPTIDES TO PROTEINS USING GLUTARALDEHYDE IN A DIALYSIS METHOD

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Key words: Synthetic peptide; Conjugation; Glutaraldehyde two-step reaction

Abbreviations: KLH, keyhole limpet hemocyanin; MBS, succinimidyl *m*-maleimidobenzoate; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide); SP, synthetic peptide; BSA, bovine serum albumin; GA, glutaraldehyde.

SUMMARY

Controlled and efficient conjugation of synthetic peptides to proteins, for use in immunization or in assay procedures, is a prerequisite for the immunological applications of synthetic peptides. This study describes a new method of conjugating synthetic peptides to proteins in such a way that no homopolymers of synthetic peptides or proteins occur. To achieve this, the protein is first activated with glutaraldehyde and subsequently excess glutaraldehyde is removed. Then coupling of the synthetic peptide to the activated protein occurs while subsequently the surplus reactive glutaraldehyde groups on the protein are blocked with lysine. Excess free peptide and lysine is then removed by dialysis. This improvement not only results in better defined conjugates when compared to classical glutaraldehyde coupling, but also in the consumption of smaller amounts of synthetic peptide during conjugate formation. When used for immunization we obtained similar and sometimes even better responses with the glutaraldehyde based conjugates than with succinimidyl (MBS) conjugates of the same peptides. The performance of the modified conjugates in ELISA procedures, immunization and immunocytochemistry suggests that they are superior to conjugates formed by classical glutaraldehyde coupling.

INTRODUCTION

Synthetic peptides (SP) have become important tools in the production of antibodies (cf., Van Regenmortel, 1989). They are used when there is no purified protein available (Van Laar et al., 1989), when the amino acid sequence is unknown but can be predicted on the basis of the DNA sequence (Van Denderen et al., 1989) and when there is a strong homology between proteins that differ only in one or a few amino acids (Boersma et al., 1989). In most

cases SP are too short to be immunogenic and coupling to a carrier protein, which can provide T cell help, is a necessary prerequisite for antibody production. A broad range of coupling reagents that make use of different kinds of functional groups, can be used. For immunization and screening of anti-peptide antibody responses two different and independent methods of conjugation are required. Two conjugates, of which the one used for detection must be different from the one used for immunization, must be available, in order to evaluate the immune response in, e.g., ELISA. This permits discrimination between the specific peptide response and the response against the coupling (neo)-determinant which has been introduced. Alternative procedures to the maleimide-based reagents and carbodiimide methods become available.

Richards and Knowles (1968) first proposed glutaraldehyde as a protein cross-linking reagent. Avrameas (1969) demonstrated the conservation of immunological and enzymatic activity of different enzymes coupled to proteins with the aid of glutaraldehyde in a one-step reaction. It is this one-step reaction that is used for the conjugation of SP to proteins by many investigators. A major drawback of this method is the crosslinking of peptides in an uncharacterized way, with the formation of homopolymers. The method is inefficient since SP are able to cross-link to each other and great excess SP must be added to the carrier protein to ensure that enough peptide molecules are coupled to the carrier.

We report here an alternative conjugation method making use of a two-step-reaction with glutaraldehyde, which produces peptide carrier conjugates without the formation of homopolymers of either component.

MATERIALS AND METHODS

Peptide synthesis, purification and characterization

Peptides (SPEK13, SPEK14, SPEK15, SP13 and SP68) were synthesized essentially as described by Merrifield (1963) with *t*-Boc protected amino acids using a Biosearch Sam II automatic peptide synthesizer. Deprotection was performed using hydrogen fluoride. Peptides (SP59, SP60, SP61, SP66) were synthesized on RapidAmide resin beads using F-moc protected amino acids (Ramps System, Dupont, Medical Products, U.S.A.), and deprotected with piperidine. Elongation was controlled at each step (Kaiser et al., 1970, 1980). Final deprotection and cleavage was performed using a mixture of trifluoroacetic acid (TFA), phenol, and ethane-dithiol followed by precipitation and filtration from diethyl ether. Tertiary butyl protection groups were removed using mercuric (II) acetate.

Peptides were purified as described in detail by Van Denderen et al. (1989). Amino acid analyses were performed using the method described by Janssen et al. (1986). Free thiol groups were determined using DTNB (5',5'-dithiobis(2-nitro-benzoic acid) according to Ellman (1959).

TABLE I

Antibody responses [a] in BALB/c mice immunized with various peptides conjugated using GA or MBS to a KLH carrier

		GA	MBS
SPEK13	NH_2-KAVLTIDKKGTEAASAC	-	+
SPEK14	NH_2-KAVLTIDEKGTEAASACA	+	+
SPEK15	NH_2-KLSKAVHKAVLTIDKKC	-	+
SP59	NH_2-CQQQEAVSEGSSSGRAREASG	+	+
SP60	NH_2-EGSSSGRAREASGAPTSSKDNYC	+	+
SP61	NH_2-EDTAEYSPFKGGYTKGLEGEC	+	+
SP66	NH_2-CVQVPKILSGKVKPIYFHTQ	+	Nd[b]
SP13	NH_2-APPVAGGPSVC	+	+
SP68	NH_2-TKAKRRVVQREKRAVGIGALC	+	+

[a] Qualitative expression of serum antibody responses in ELISA.
[b] Not determined.

Synthetic peptides

The sequences of the peptides are given in Table I. SP13 is derived from HuIgG2 (Boersma et al., 1988). SP59, SP60, SP61 and SP66 were from the human androgen receptor (Van Laar et al., 1989). SPEK13, SPEK14 and SPEK15 were from human α_1-antitrypsin. SP68 is a peptide from gpl20 of the human immunodeficiency virus type 1 (HIV-1).

Coupling methods

Glutaraldehyde

The (carrier) protein was dialysed at a concentration of 10 mg·ml^{-1}against 200 ml 0.2% glutaraldehyde (GA, 'Baker' grade 25% in H_20, J.T. Baker Chemicals, Deventer, The Netherlands) in PBS (0.01 M phosphate-buffered saline, pH 7.2) for 16 h at 4°C. The activated (carrier) protein was then dialysed against PBS (three times in 500 ml for several hours), to remove excess GA, and transferred to a reaction vessel. The peptide (10 mg·ml^{-1} in distilled water) was added to the GA activated carrier in a molar ratio of 100 molar equivalents and the mixture stirred for 16 h at 4°C. The remaining active GA groups were blocked by 0.2 M lysine-HCl (0.1 ml) during a 2 h incubation. The excess peptide and lysine molecules were removed by dialysis.

MBS

Essentially the method as described by Van Denderen et al. (1989) was used. Briefly, MBS (succinimidyl *m*-maleimidobenzoate) was dissolved in dimethylformamide (20 mg·ml^{-1}). MBS

was added in three equal portions at 5 min intervals using a molar ratio of 200 to 1, relative to the (carrier) protein (10 mg·ml^{-1}in PBS). Excess MBS was removed over a PD-10 column (Pharmacia). Finally the peptide (10 mg·ml^{-1})was added in 100 times molar excess and incubated for at least 1 h at room temperature.

EDC

The modified method described by Deen et al. (1990) was used. Briefly, the carrier protein (10 mg·ml), EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide, 200 mg·-ml) and the peptide (10 mg·ml) were dissolved in 0.5 M *N*-methyl-imidazole pH 6.0 (Aldrich, M5, 083-4, Brussel, Belgium). The peptide was added to the carrier to give a 100 times molar excess. After the addition of EDC (protein: EDC ratio of 100:1) the mixture was stirred for 30 min at room temperature followed by dialysis against PBS.

Immunization

Conjugates (25 μg) of the different peptides and keyhole limpet hemocyanin (KLH) (Calbiochem, 374811, San Diego, CA, U.S.A.) emulsified in Specol (Bokhout et al., 1981), were injected intraperitoneally into groups of three or four 12-week-old BALB/c female mice and boosted after 5 weeks with the same dose of conjugates. 7 days after each immunization a blood sample was taken from the tail vein.

Immunoassays

ELISA procedures were performed essentially as described by Deen et al. (1990). PVC microtiter plates (Titertek, 77-172-05) were coated overnight with protein or peptide (50 μl of 5 μg·ml^{-1}in PBS) at 4°C and blocked with 0.5% gelatin in PBS. Sera were incubated for 1 h at different dilutions in 0.1% gelatin and 0.05% Tween 20 in PBS. Goat anti-mouse IgG conjugated to alkaline phosphatase (KPL) was used as second antibody, and *p*-nitrophenyl phosphate (1 mg·ml^{-1})in 0.01 M diethanolamine + 1 mM $MgCl_2$ (pH 9.8) was used as substrate. Immunoprecipitation was performed as described by Van Laar et al. (1989). All of the values were corrected by subtraction of the pre-immune serum background.

RESULTS

Coupling of SP to carriers

All peptides (Table I) were coupled with either GA or MBS to KLH and with EDC to BSA (for ELISA). The activation of the KLH with GA using the dialysis method did not result in any significant degree of cross-linking. The solution of activated KLH did not contain precipitates. After conjugation the resulting solutions remained clear. After storage in the freezer for several months the GA conjugate still showed no precipitation.

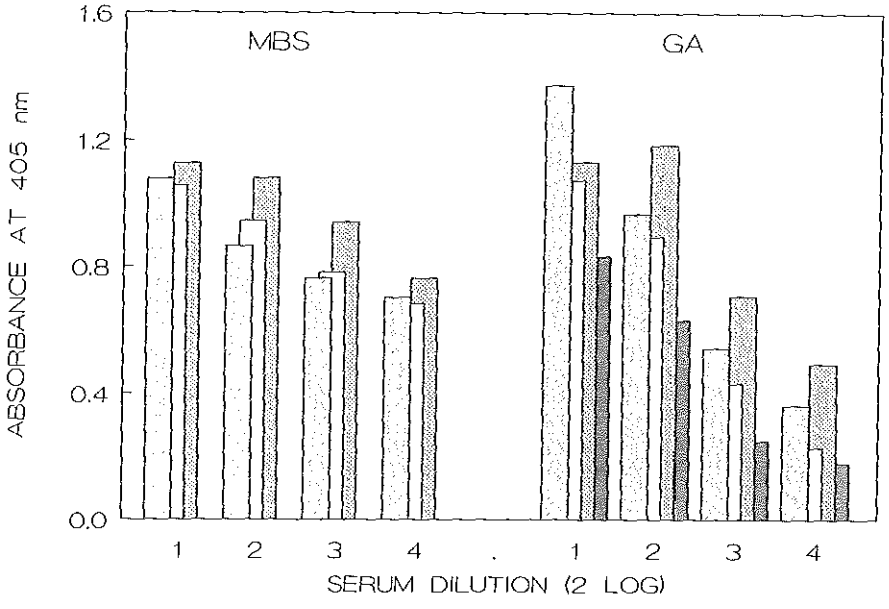

Fig. 1. Serum antibody responses to unconjugated SP61 determined in direct ELISA. Mice were immunized twice with KLH-MBS-SP61 conjugates (left) or KLH-GA-SP61 conjugates (right). SP61 was coated directly to a 96-well plate. The initial serum dilution was 1/100. Results are shown for three (MBS) or four (GA) individual mice.

Polyclonal sera against conjugates produced with GA and MBS

Mice, three or four per group, were immunized with 25 μg of the peptide conjugates coupled with GA or MBS to KLH. The antibody response in the sera was measured in a direct ELISA. The specific response to the relevant peptide was assayed. The peptide was coated directly to the plate or indirectly when coupled to a non-relevant protein (BSA) through a carbodiimide coupling using EDC. This was in order to be able to discriminate between the antipeptide and coupling agent specific responses. Table I shows the results of analyses of the sera from the different groups. Sera from mice immunized with the peptide conjugates coupled with MBS gave a positive response to the free peptide coat as well as to the coating of peptide in the BSA conjugate. Seven out of nine mice immunized with GA coupled conjugates gave a positive response both to the peptide and to the SP-BSA conjugate.

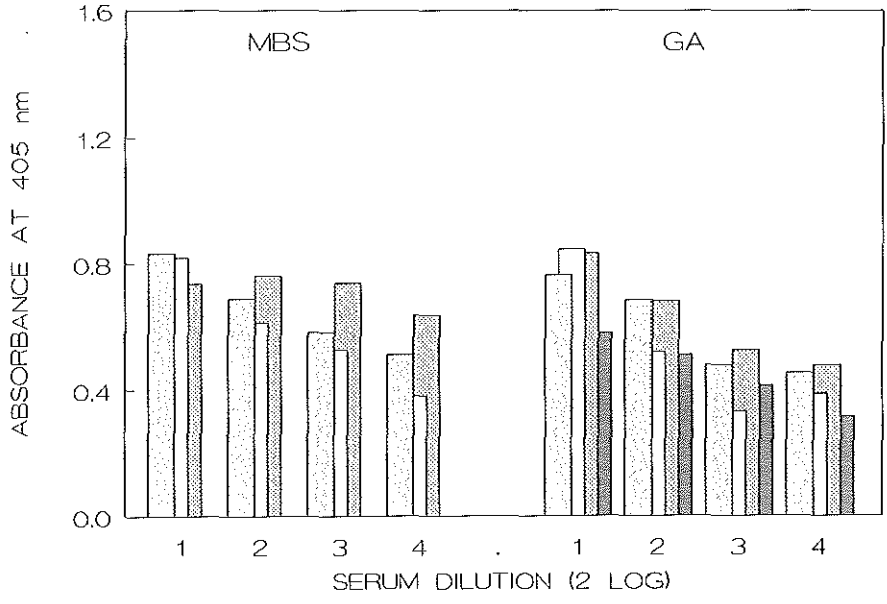

Fig. 2. Serum antibody responses to SP61 presented in BSA-(EDC)-SP61 conjugates. Data obtained using an ELISA procedure (see materials and methods section) to assay the same sera as used in Fig. 1. BSA-(EDC)-SP61 was directly coated to a 96-well PVC plate. The initial serum dilution was 1/100. Results are shown for three (MBS) or four (GA) individual mice.

Reaction of polyclonal anti-SP61 sera with SP61

The antibody responses to SP61 are shown as a representative example in more detail (Fig. 1). The antibodies elicited with KLH-MBS-SP61 conjugate (three animals) were compared to those elicited with KLH-GA-SP61 conjugate (four animals). At lower serum dilutions (1 and 2) the responses to SP61, coated directly to the plate, were similar. However, the amount of SP specific antibodies in the sera elicited with the GA based conjugates was much lower at higher serum dilutions (3 and 4) when compared with the MBS based conjugates.

Reaction of polyclonal anti-SP61 sera with SP61 conjugate

Recognition of SP61, present in a conjugate with EDC coupled BSA, by antibodies elicited with KLH-GA-SP61 was compared with recognition by antibodies generated with

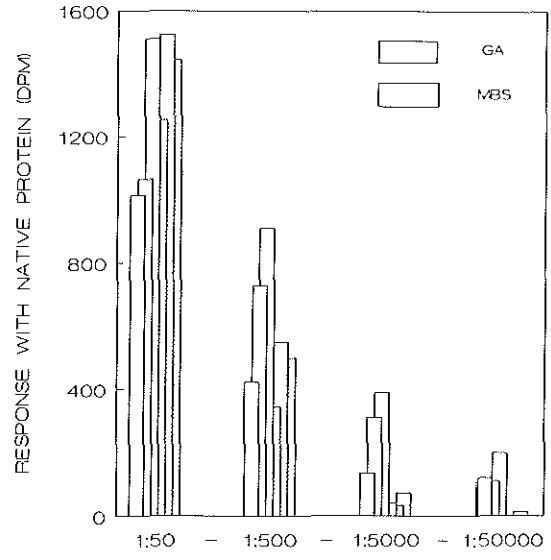

Fig. 3. Serum antibody responses to the native protein of the human androgen receptor were determined by agarose immunoprecipitation. The results obtained using the same sera as in Fig. 1 are shown. The initial serum dilution was 1/50. Note serum dilutions are tenfold. The amount of radioactive hormone was expressed as desintegrations per minute (DPM).

KLH-MBS-SP61. As shown in Fig. 2 the responses were similar for all serum dilutions. Responses generated with GA based conjugates were comparable to the response obtained with the sera generated with MBS coupled conjugates to the free peptide coated directly to the plate. Thus the conformation of the peptide in the BSA-EDC conjugate resembled the conformation in both the KLH-GA and the KLH-MBS conjugates.

Reaction of polyclonal sera with androgen receptor

Antibodies directed against the native protein (the human androgen receptor) were detected in an immunoprecipitation assay. The results are shown in Fig. 3. The antibody responses towards the native protein in sera from mice immunized with both the GA and the MBS conjugate were similar at lower serum dilutions (1 and 2). At higher serum dilutions (higher

than 1/5000) it was obvious that the sera elicited with KLH-GA-SP61 gave a native protein specific response, whereas the signal had disappeared in sera from the mice immunized with the MBS conjugate. It should be noted that ^{10}log serial dilutions were used in the immunoprecipitation assay whereas in the ELISA ^{2}log serial dilutions were used.

DISCUSSION

In this study we have shown that a novel two-step dialysis method with GA as a coupling agent for covalent linkage of SP to carrier proteins is a very efficient method for the production of immunogenic SP-carrier conjugates. Apart from the advantage of conjugation with lower amounts of SP in comparison with the classic method, and the absence of homopolymers of SP or carrier, we also found a larger proportion of antibodies against SP61 crossreacting with the native protein when this new method was used.

For both immunization and assay purposes it may be necessary to couple SP to proteins and this can be done by making use of several functional groups on either molecule. The functional groups most frequently used are amino, carboxyl and thiol groups. EDC is a heterobifunctional reagent that couples an amino group with a carboxyl group to form a peptide bond (Goodfriend et al., 1964; Deen et al., 1990). MBS (Boersma et al., 1988), MHS (succinimidyl-6-(*N*-maleimido)-*n*-hexanoate) and SMCC (succinimidyl-4-(*N*-maleimido-methyl)-cyclo-hexane-1-carboxylate) (Peeters et al., 1989) are widely used when an amino group and a thiol group are available. When cysteine has a conformational role in the sequence, it is preferable to avoid coupling via this cysteine.

Glutaraldehyde is a homobifunctional reagent that couples two proteins or peptides via amino groups. The one step GA method often fails to evoke peptide specific antibodies because of inefficient conjugation (i.e., unwanted polymerization or even precipitation during coupling) and it is for this reason that one resorts to a more defined method. We have previously described a two-step GA dialysis method (Claassen and Adler, 1988; modified after Boorsma, 1984) for the conjugation of a polyclonal antiserum to an enzyme (alkaline phosphatase) and this conjugate has proven to be of value in immunocytochemistry.

In the present study peptide specific responses were obtained with seven out of nine peptides, derived from different proteins. This indicates that selective use of GA leads to efficient SP coupling. In two cases (SPEK13 and SPEK15) it was not possible to raise antibodies to these peptides. A striking feature of these two SP was the fact that they were the only examples with two adjacent lysines (see Table I).

Two or more lysine residues in the peptide do not need to be inhibitive for elicitating antibody responses as shown in peptides 61, 66, 68 and SPEK14. It is a remarkable feature that SPEK14 differs from SPEK13 only in one amino acid and yet this small variation results in a different response. It should be noted, however, that this difference involves a lysine which is probably part of the epitope against which the antibodies are directed.

As has been shown by Schaaper et al. (1989) and Boersma et al. (1988) antibodies are elicited particularly against that part of the SP most distant from the coupling site.

Different orientations of the peptide relative to the carrier, as may be the case when the sequence has one or more lysines, could be a very useful feature. Thus, at least one of the several possible orientations, resulting from GA conjugation, mimics the epitope in the native protein.

As can be seen from the results with SP61 a higher antipeptide response does not always correlate with an enhanced response to the native protein. Indeed the opposite seems to hold true in this case. The sera elicited with the GA conjugates have a higher titer against the native protein than sera elicited with the MBS conjugate (Fig. 3) in spite of a weaker response against the peptide (Fig. 1). The responses to the peptide presented in a protein conjugate were similar. This suggests that conjugation efficacy should always be tested in an assay specific way, i.e., with the native protein as described in the case of EDC conjugation (Deen et al., 1990).

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Chapter 4

Epitope prediction and confirmation for the human androgen receptor: Generation of monoclonal antibodies for multi-assay performance following the synthetic peptide strategy

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EPITOPE PREDICTION AND CONFIRMATION FOR THE HUMAN ANDROGEN RECEPTOR: GENERATION OF MONOCLONAL ANTIBODIES FOR MULTI-ASSAY PERFORMANCE FOLLOWING THE SYNTHETIC PEPTIDE STRATEGY

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Key words: Androgen receptor; Synthetic peptide; Antibody; MAb performance; Immunohistochemistry, Diagnostic; (Human)

Abbreviations: SP, synthetic peptide(s); hAR, human androgen receptor; ELISA, enzyme-linked immunosorbent assay; KLH, keyhole limpet hemocyanin; BSA, bovine serum albumin; GA, glutaraldehyde; MAb, monoclonal antibody; PAb, polyclonal antibody; R1881, [17α methyl-^{3}H]methyltrienolone.

SUMMARY

The human androgen receptor (hAR) is an important regulatory protein particularly in male sexual differentiation. The investigation of hAR functionality has been hampered by the lack of AR specific monoclonal antibodies recognizing the functional domains of the receptor. Therefore production of high affinity mono-specific polyclonal (PAbs) and monoclonal antibodies (MAbs) directed to the hAR was initiated following the synthetic peptide (SP) strategy. Five hAR specific peptides were selected on the basis of their predicted antigenic properties avoiding homology with other steroid hormone receptors. Peptide specific polyclonal antisera were obtained following selected immunization protocols. Mono-specific polyclonal antibody responses were elicited to all peptides in mice and rabbits. Crossreactivity of the peptide specific antisera with the native hAR in various biochemical assays was observed with two out of five peptides. Peptide SP61 (hAR residues 301-320) was used for the generation of site-directed MAbs specific for the hAR. Specificity for the hAR was established by immunoprecipitation, immune-complex density gradient centrifugation and immunohistochemistry on human prostate tissue sections. The multi-assay performance of the selected high affinity antibodies proved the usefulness of the straight forward peptide approach and opens a wide field of possible biochemical and physiological investigations into questions related to androgen action.

INTRODUCTION

Androgens play a major role in male sexual differentiation and they are essential for prostate gland development. They also play a role in tumour genesis in the human prostate. Most human prostatic tumours are at least initially androgen dependent. Androgens exert their action via binding to the intracellular androgen receptor. The activated androgen receptor (AR) subsequently modulates specific gene transcription by interaction with cis-acting hormone responsive elements present in target genes. Steroid hormone receptors consist of three domains: the N-terminal part, which might be involved in the regulation of gene transcription, a DNA binding domain, and a steroid-binding domain at the C-terminus [1,2]. Recently the cDNA sequence of the human androgen receptor (hAR) was elucidated [3,4]. This information provided a basis for the production of new tools to study the hAR system. Since a considerable homology with other steroid receptors (progesterone, oestrogen and glucocorticoid receptors) exists and in addition the purification of a sufficient amount of the protein was not obtained, we decided to use the synthetic peptide (SP) approach to generate site specific, hAR specific, polyclonal and monoclonal antibodies (MAbs). Especially the availability of hAR specific antibodies directed to the different parts of the molecule: the N-terminal regulating part and the DNA and steroid binding domains could be very helpful for immuno-affinity purification of the receptor. Furthermore, site specific antibodies would facilitate detailed investigation of the AR structure, distribution and its physico-chemical properties. Specific antibodies open new possibilities for histochemical localization of the receptor in tissue sections, for diagnostic purposes and to monitor, e.g., AR expression during endocrine therapy for prostatic cancer.

In previous studies, it was shown that when a protein is not available for immunization, for instance for reasons of purification, but the DNA sequence has been determined, synthetic peptides (SPs) become very important tools to generate specific antibodies [5,6]. The use of SPs allowed us to circumvent the difficulty in generating specific antibodies when there exists a strong homology in a family of proteins [7]. SPs are frequently applied in model studies of antigenicity [8-14]. Furthermore, SPs are used to assign the importance of certain amino acid residues (in replacement studies) with respect to the function of a protein domain [15]. SPs may become important as specific subunits in the production of vaccines [16,17].

As the antigenic determinants of the hAR were not known, putative continuous epitopes were selected on the basis of theoretical considerations. In this report, we describe the selection and synthesis of the peptides and the immunization and screening procedures to evoke hAR specific monoclonal and polyclonal specific antibodies. We show that the MAbs are widely applicable in various biochemical and histochemical techniques and that they do not crossreact with other steroid receptors. These properties make them useful diagnostic tools.

MATERIALS AND METHODS

Peptide synthesis, purification and characterization

Peptides (SP59, SP60, SP61, SP65 and SP66) were synthesized on RapidAmide resin beads using Fmoc protected amino acids (Dupont, U.S.A.) following the procedure as described for the RAMPS System (Dupont, Medical Prod., Biotechnology Syst., U.S.A.). Piperidine was used for deprotection. Elongation was checked each step [18]; the method described by Kaiser et al. [19] was used in case of a proline. Final deprotection and cleavage was performed using a mixture of trifluoroacetic acid, phenol, and ethanedithiol followed by precipitation and filtration from diethyl ether. Mercuric (II) acetate was used to deprotect the peptides containing cysteine with a tertiary butyl protection group. Cysteine containing peptides were reduced with ß-mercaptoethanol for 1 h at pH 8. This procedure also allows to restore the N to O migration in case of serine or threonine. Peptides were purified using liquid chromatography on G-15 Sephadex (Pharmacia) in 5% acetic acid. Fractions were analyzed on a Beckman Ultrosphere 5 μm reversed-phase C18 column using a gradient of acetonitrile with 0.1% trifluoroacetic acid. Fractions with the same major compound and with a high purity were pooled and lyophilized twice. Amino acid analyses were performed to confirm the peptide composition of the hydrolysed peptide using pre-column derivatization of the amino acids [20]. The amount of free thiol groups was determined using DTNB (5',5'-dithiobis(2-nitrobenzoic acid)) [21].

Coupling methods

All peptides were coupled to immunogenic carrier proteins to enhance antigen presentation (*in vivo*) or to be used in screening (antigen presentation *in assay*) [12].

Glutaraldehyde

Essentially the method described by Zegers et al. [14] was applied. The (carrier) protein was dialysed at a concentration of 10 mg·ml^{-1}against 200 ml 0.2% glutaraldehyde (GA) ('Baker' grade 25% in H_20, J.T. Baker Chemicals, Deventer, Holland) in 0.01 M phosphate-buffered saline, pH 7.2 (PBS) for 16 h at 4°C. The activated (carrier) protein was then dialysed against PBS (three times in 500 ml for several hours) to remove excess GA and transferred to a reaction vessel. The peptide (10 mg·ml^{-1} in distilled water) was added to the GA activated carrier in a molar ratio of 100 molar equivalents. The mixture was stirred for 16 h at 4°C. Remaining active GA groups were blocked by 0.1 ml lysine-HCl (0.2 M) during a 2 h incubation. The excess peptide and lysine molecules were removed by dialysis.

MBS

m-Maleimidobenzoyl-N-hydroxysuccinimide (MBS) ester (Pierce 22310, Rockford, IL. U.S.A.) coupling, conjugating -NH_2 and -SH groups, was performed as described earlier [12]. The MBS was dissolved in dimethylformamide at a concentration of 20 mg·ml^{-1}. To the (carrier) protein (10 mg·ml^{-1}in PBS) MBS was added in a molar ratio of 200:1, in three equal portions at 5 min intervals. After incubation for another 20 min at 4°C, under stirring,

the mixture was centrifuged to remove any insoluble salts. The supernatant was purified over a PD-10 gel filtration column (Pharmacia, Sweden) to remove excess MBS. To the MBS-activated protein the peptide (10 $mg \cdot m^{-1}$) was added in 100-times molar excess and incubated for 1 h at room temperature. The conjugate was then purified by dialysis against PBS.

EDC

For 1-ethyl-3(3-dimethylaminopropyl)carbodiimide (EDC; Sigma E-7750, St Louis, MO, U.S.A.), conjugating -COOH and -NH2 groups, the modified method as described earlier [13] was used. Briefly, the carrier protein (10 $mg \cdot m^{-1}$), EDC (200 $mg \cdot ml^{-1}$) and the peptide (10 $mg \cdot ml^{-1}$) were dissolved in 0.5 M *N*-methylimidazole pH 6.0 (Aldrich, M5, 083-4, Brussels, Belgium). The peptide was added to the carrier in a 100 molar excess. After addition of EDC (molar ratio protein/EDC is 1:100) the mixture was stirred for 30 min at room temperature followed by dialysis against PBS.

Conjugates of peptides and proteins for screening were made with bovine serum albumin (BSA) and for immunization purposes with keyhole limpet hemocyanin (KLH, Calbiochem, 374811, San Diego, CA, U.S.A.). The conjugates were produced with the aid of various coupling agents (MBS, GA or EDC) and were designated as, e.g., KLH-MBS-SPxx or BSA-EDC-SPxx.

Immunization

Rabbits (Flemish Giant random bred, MBL-TNO, Rijswijk, The Netherlands) were injected subcutaneously three times at 4 week intervals, with 250 μg of the conjugate. First immunization and booster conjugates were emulsified in Freund's complete adjuvant. For the third immunization the conjugate was emulsified in incomplete Freund's adjuvant. Serum was analyzed 14 days after each immunization in ELISA or immunoprecipitation reaction followed by sucrose density gradient centrifugation [22]. Pre-immune sera of the same rabbits served as (negative) controls.

Conjugates of the peptide and KLH were emulsified in Specol [23], and 25 μg was injected intraperitoneally into groups of four (12-week-old female BALB/c) mice. The mice were boosted after 5 weeks with the same dose of conjugates and adjuvant. Seven days after each immunization a blood sample was taken from the tail vein. The serum was assayed in a direct ELISA (in which the antigen was coated directly to the plate by passive adsorption) or in an immunoprecipitation assay.

ELISA

PVC-microtiter plates (Titertek, 77-172-05 highly activated, Flow Laboratories, Irvine, U.K.) were coated overnight at 4°C with 50 μl PBS containing 5$\mu g \cdot ml^{-1}$ protein, peptide or alternatively the peptide conjugated to BSA (not used for immunization) with a reagent not used in the immunogen. Plates were blocked with 0.5% gelatin in PBS during a 30 minute incubation at room temperature. Sera were diluted in 0.1% gelatin and 0.05% tween-20 in PBS and incubated for 1 h. Alkaline phosphatase conjugated to goat-anti-mouse IgG or

swine-anti-rabbit Ig (Kirkegaard and Perry Laboratories, MD, U.S.A.) were used. *p*-nitrophenyl phosphate (1 mg·ml^{-1})in 10 mM diethanolamine + 1 mM $MgCl_2$ at pH 9.8 was used as substrate. After 30 min the absorbance was read at 405 nm in a Titertek Multiskan reader (Flow Laboratories, Irvine, U.K.) against normal mouse serum or pre-immune rabbit serum responses as a blank. The titre in the ELISA was chosen arbitrarily as that dilution which gave an absorbance of 1.0 after 30 min of incubation. A direct ELISA was used to determine the isotype of the MAbs with specific rabbit-anti-mouse-immunoglobulin-subclass anti-sera (Miles Laboratories, Kankakee, IL, U.S.A.).

Immunoprecipitation

The response to the native androgen receptor was analyzed in an immunoprecipitation assay. Sera were incubated at different dilutions for 2 h at 4°C in roller tubes with anti-mouse-IgG conjugated to agarose (Sigma). After centrifugation and washing of the pellet with PBS, 25 μl of a nuclear extract from LNCaP cells (a human cell line of a lymph node carcinoma of the prostate) [24] in which the receptor was labelled with [17α-*methyl*-^{3}H]methyltrienolone ([^{3}H]R1881, a synthetic androgen, NEN-Dupont, Dreieich, F.R.G.), was added. Subsequently 400 μl PBS was added and the mixture was incubated for 2 h at 4°C while rotating [22]. After centrifugation and washing of the pellet, the sample was assayed for the amount of radioactivity. Values are given corrected for background.

Sucrose gradient centrifugation

Essentially the method described by De Boer et al. [25] was followed. Briefly, 50 ml of [^{3}H]R1881-labelled nuclear extract (0.15 M NaCl) from LNCaP cells was incubated for 6 h at 4°C with antiserum or RPMI 1640 culture medium (Flow, Irvine, U.K.) supplemented with 7.5% FCS and 1% NMS as a control and subsequently layered on 10-30% sucrose gradients in the presence of 0.15 M NaCl and centrifugated for 20 h at 370 000 x *g* at 4°C.

Monoclonal antibodies

Cell fusion was performed essentially as described by Haaijman et al. [26]. Briefly, a spleen cell suspension was prepared 4 days after the second boost. Spleen cells and SP2/0 cells in logarithmic growth were fused at a ratio of 5:1 in 40% poly(ethylene glycol) 4000 (Merck, Darmstadt, F.R.G.) + 5% DMSO in PBS. The fused cells (10^5/well in 0.2 ml) were cultured in RPMI 1640 selection medium, containing 1 μg·ml^{-1}azaserine and 0.1 mM hypoxanthine, 15% FCS, 2 mM glutamine, 0.1 mg·ml^{-1}streptomycin, 100 E·ml^{-1} penicillin, 1 mM sodium pyruvate, and $5 \cdot 10^{-5}$ M ß-mercaptoethanol. After 1 week of culture the azaserine was discontinued and the FCS concentration was lowered to 10%. Selected cell cultures were subcloned by limiting dilution at a density of 0.5 cells/well.

BALB/c mice were injected intraperitoneally with 0.5 ml pristane (2,6,10,14-tetramethylpentadecane 96%, Ega-chemie, Steinheim, F.R.G.). Seven days later the mice were injected with 10^6 monoclonal hybridoma cells in 0.25 ml PBS. Ascites fluid was collected under anaesthesia.

Immunohistochemistry

Samples of prostatic tissue with glandular hyperplasia were removed from prostatectomy specimens immediately after surgery, snap-frozen by immersion in cooled isopentane (-150°) and stored in liquid nitrogen. Immunohistochemistry was performed on cryostat sections of 5 μm thickness using the method described by Ruizeveld de Winter et al. [27]. After fixation and rehydration, the slides were incubated overnight with appropriate dilutions of MAbs in PBS (pH 7.8) at 4°C. After several washing steps the reactivity was visualized using horseradish peroxidase conjugated to rabbit-anti-mouse immunoglobulin (DAKO, Denmark) diluted 1:100 in PBS containing 5% non-immune human serum and 5% non-immune rabbit serum. Diaminobenzidine and hydrogen peroxide were used as substrate. Control slides were incubated with pre-immune sera or PBS.

RESULTS

Peptide selection

The amino acid sequence of the human androgen receptor [3,4] was used to select stretches of about 20 amino acids, with a high antigenicity index [28,29] with the aid of a computer prediction program (Genetics Computer Group, Wisconson; [30]). This selection was based on analysis of primary and secondary structure parameters such as hydrophilic character, protein surface probability and expected flexibility [31-36]. Preferable sequences including putative "turn" sequences were selected based on earlier experiences. The cut-off places in sequences were chosen in such a way that amino- and carboxy-termini had a relatively high hydrophilicity index. Because of a strong homology between the hAR and other steroid hormone receptors, peptides were chosen such that amino acid sequences similar to those in other steroid hormone receptors, like progesterone-, oestrogen- and glucocorticoid receptors, were avoided [37-41].

The selected peptide sequences were matched with protein sequences in the protein sequence database PIR (Protein Identification Resource, National Biomedical Research Foundation, Washington, D.C., U.S.A.) to determine whether the selected sequences showed homology with proteins stored in the database. Even when a mismatch of ten residues (50% homology) in the peptide sequence was allowed, we did not find similar peptide sequences from putative crossreactive (human) proteins except the hAR fragment itself.

The position of the five selected peptides is shaded in Fig. 1. An extra cysteine (at the N-terminus for SP59 and SP66, at the C-terminus for the other peptides) was added for coupling purposes. SP59, SP60 and SP61 are situated in the N-terminal domain and SP65 and SP66 in the steroid binding domain of the protein (Table 1).

Generation of monospecific polyclonal antibodies

Each peptide conjugated to KLH with MBS was injected in two rabbits. SP59 was also coupled to KLH with glutaraldehyde. Therefore one rabbit was immunized with KLH-MBS-SP59 and one rabbit with KLH-GA-SP59.

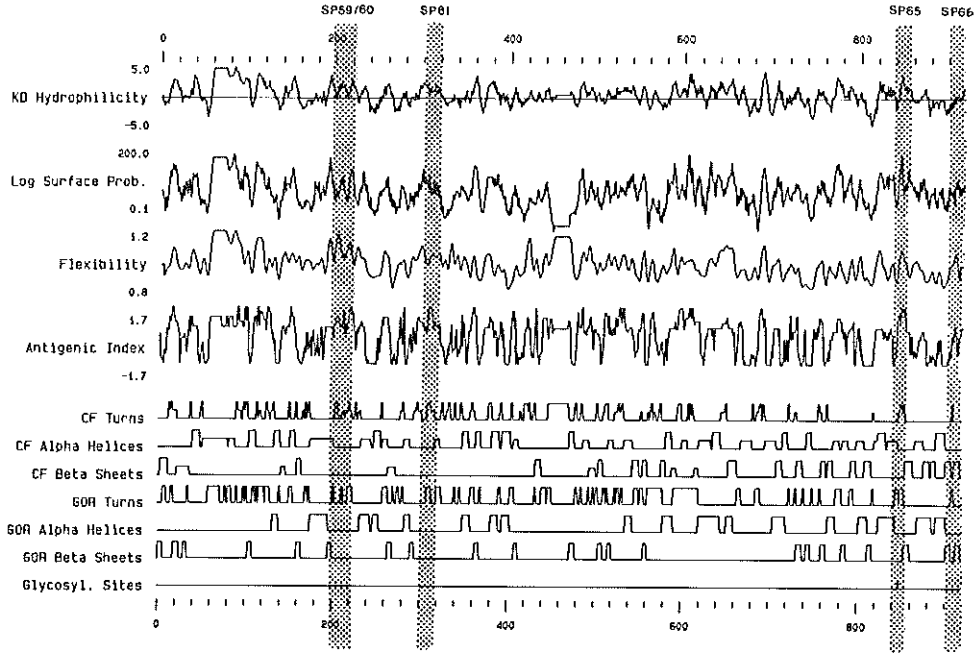

Fig. 1. Physical parameters of the androgen receptor protein predicted on the basis of the amino acid sequence deduced from the DNA sequence. In this plot hydrophilicity data are calculated according to Kyte and Doolittle [34]. CF: Chou and Fassman (1978), GOR: Garnier, Osguthorpe and Robson (1978). See Materials and Methods section.

After the second boost, sera from all rabbits as tested in ELISA gave a positive response to the coating of free peptide as well as to the same peptide in a BSA conjugate (Fig. 2). The response towards SP65 was low compared to the response to other peptides. Pre-immune sera of rabbits did not show any response to the peptides. SPs immunized in mice gave essentially similar polyclonal responses as observed in rabbits. All responses were read against non-immune serum as a blank.

Effect of conjugation on antibody responses in mice

At higher serum dilutions, the reactivity of antibodies to SP61 was much lower in sera of mice immunized with conjugates of SP61 prepared with glutaraldehyde (KLH-GA-SP61) than was determined after KLH-MBS-SP61 immunization (Fig. 3, right). The responses towards

TABLE I

Selected amino acid sequences of the human androgen receptor

SP	Residues	Sequence
SP59	(AA194-213)	CQQQEAVSEGSSSGRAREASG
SP60	(AA201-222)	EGSSSGRAREASGAPTSSKDNYC
SP61	(AA301-320)	EDTAEYSPFKGGYTKGLEGEC
SP65	(AA834-853)	KELDRIIACKRKNPTSCSRRC
SP66	(AA899-917)	CVQVPKILSGKVKPIYFHTQ

Underlined Cysteine was not a part of the hAR sequence, but was added at one of the termini of the peptides for coupling purposes.

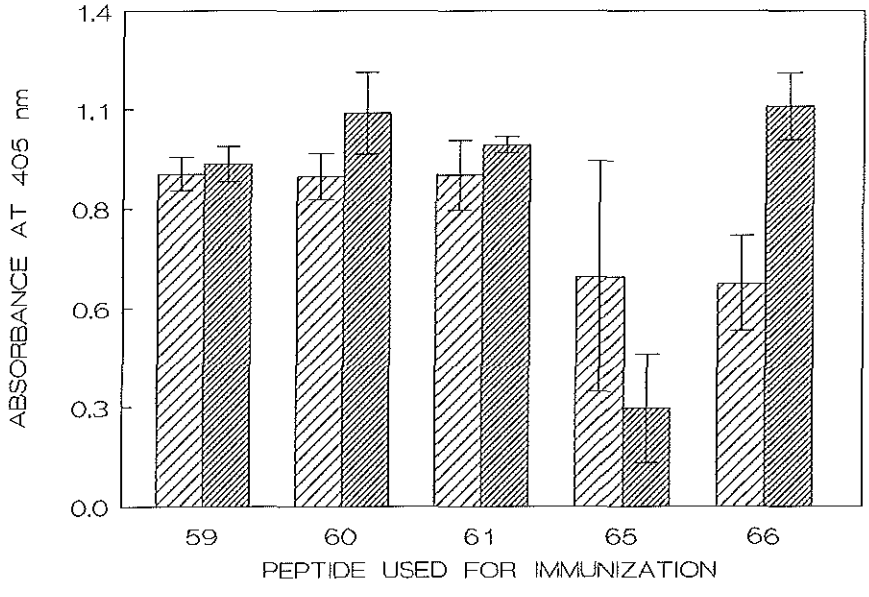

Fig. 2. Response to peptide and conjugate. Reactivity of antibodies in rabbit sera with free peptide or with the peptide conjugated to BSA via EDC determined in ELISA. Rabbits were immunized twice with KLH-MBS-SP conjugates. The serum dilution was 1: 500. Results are shown as the mean value with standard bars for two rabbits. Coatings are: peptide, striped bars; BSA-peptide, solid bars.

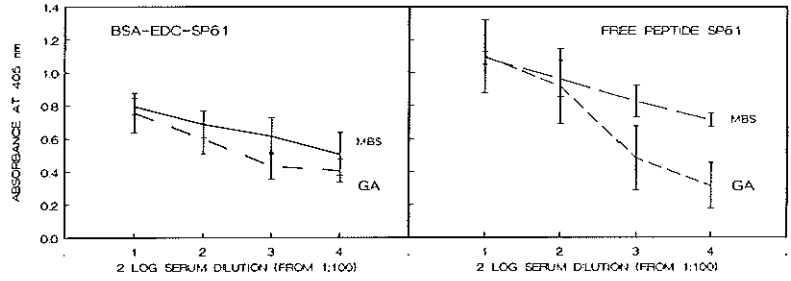

Fig. 3. Reactivity of antibodies in mouse sera with free SP61 (right) and BSA-EDC-SP61 (left) in ELISA. Mice were immunized twice with KLH-MBS-SP61 or with KLH-GA-SP61. The initial serum dilution was 1:100. Results are shown as the mean value with standard deviation bars for three (MBS) or four (GA) mice. The response to the free peptide differs significantly for the two last dilutions ($P < 0.05$ and $P < 0.01$ respectively for a final dilution 1: 400 and 1: 800)

SP61 conjugated with EDC to BSA were similar for both groups (Fig. 3, left). Sera from these mice did not react with other peptides or with BSA. In Fig. 4, the responses towards the human androgen receptor of the sera elicited with the GA and the MBS based KLH-conjugates of SP61, as measured in an immuno precipitation assay, are shown. At higher serum dilutions (higher then 1: 5000), the sera from the mice immunized with KLH-GA-SP61 still gave a native protein specific response, while for the sera elicited with MBS conjugate the signal has disappeared.

Recognition of the native protein

The hAR specific antibody response in the rabbit and mouse anti-sera was demonstrated for the peptide SP61 and for SP60 with rabbit sera only, in several independent techniques: sucrose gradient density centrifugation, immunoprecipitation, Western blotting and immunohistochemistry (data not shown).

Generation of monoclonal antibodies

Groups of three or four mice were immunized with KLH-MBS-SP61 and KLH-GA-SP61. Two different conjugates were used in order to present the peptide in various orientations to

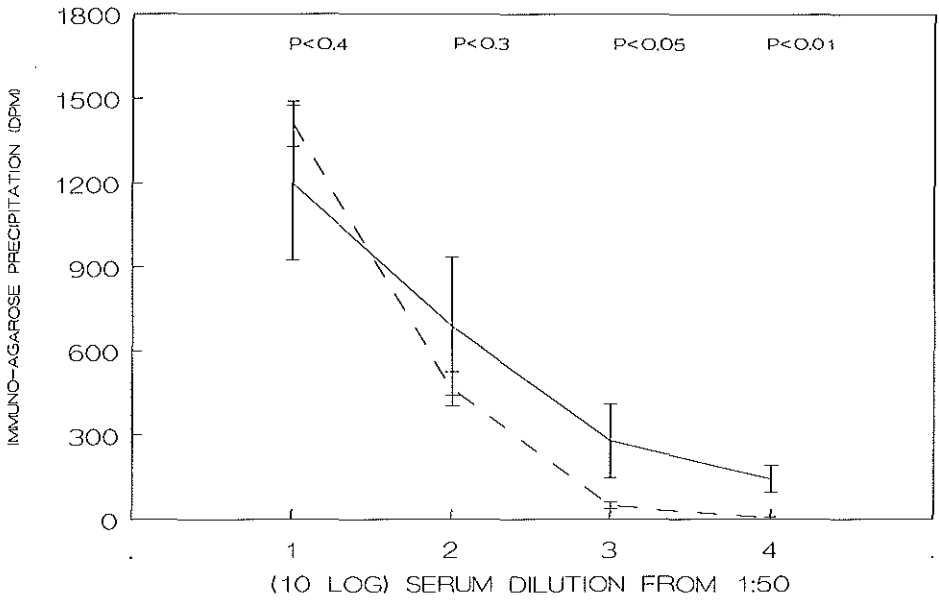

Fig. 4. Reactivity with androgen receptor. The reactivity of antibodies in mouse sera with hAR-steroid complex using immuno-agarose precipitation. Mice were immunized twice with KLH-MBS-SP61 or KLH-GA-SP61. Results are shown for ^{10}log serial dilutions of the sera with an initial serum dilution of 1: 50. The mean value with standard deviation bars for three (MBS) or four (GA) mice are given. Solid line represents glutaraldehyde and the dotted line represents MBS.

the immune system [12,42]. Spleen cells of mice immunized with KLH-GA-SP61 were used for cell fusion. The first fusion was highly efficient as all the wells (768) contained proliferating hybridomas. SP61 specific antibody producing clones were identified in a primary selection in ELISA. Anti-SP61 responses were found in 107 out of 768 wells. A second selection was performed in immunoprecipitation and gave 15 positive anti hAR clones. Seven wells containing clones with the highest reactivity were selected for subcloning. Three out of seven lost their activity during subcloning. Four monoclonal cell lines, F39.3.1, F39.4.1, F39.5.1 and F39.6.2, derived from different wells in the original 96-well fusion plates, were selected for ascites production.

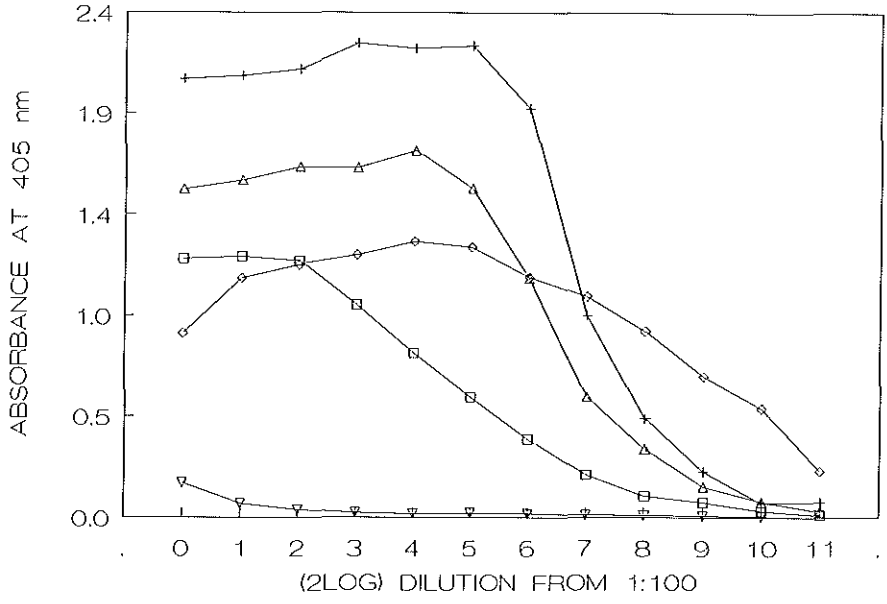

Fig. 5. Titration curve of MAbs to SP61 in a direct ELISA. The titre was expressed as the dilution at which the absorbance is 1.0 after 30 min of incubation. The initial ascites dilution was 1:100 (point 0). □, F39.3.1; +, F39.4.1; ◇, F39.5.1; △, F39.6.2; and ▽, background.

Characterization of hAR specific monoclonal antibodies

Immunoprecipitation

Specificity for the human androgen receptor was determined by assaying the crossreactive response to other hormone receptors (progesterone, oestrogen and glucocorticoid receptors) in immuno precipitation assays. The four MAbs showed a positive response with the hAR only. The selected MAbs crossreacted with rat AR and calf AR in immunoprecipitation and Western blots. In a comparison of the reactivity of the four anti-AR MAbs, in the agarose precipitation assay, the highest titre was obtained with F39.4.1 ascites (Fig. 5; Table II). Ascites fluid F39.4.1 could be diluted ten times more than both ascites F39.5.1 and F39.6.2. The affinity of MAb F39.3.1 for hAR is very low, the titre being lower than 1:100.

TABLE II
Properties of hAR specific monoclonal antibodies

MAb	Isotype	hAR titre[a]	SP61 titre[b]	Sedimentation rate constant[c]	Immuno-histo-chemistry[d]
F39.3.1	IgG2b κ	<1:100	1:918	4.5S	-
F39.4.1	IgG1 κ	1:13000	1:12800	6.5S	+
F39.5.1	IgG1 κ	1:1300	1:18100	6.5S	-
F39.6.2	IgG2b κ	1:600	1:7900	6.5S	-

[a] The titre is expressed as the dilution at which the antibody binding to the hAR-steroid complex is 50% of maximum, determined in immuno agarose precipitation.
[b] The titre is expressed as the dilution at which the absorbance is 1.0 after 30 min of incubation with substrate at 25°C determined by ELISA
[c] Complex formation of antibody binding to R1881-hAR induces a shift in sedimentation constant
[d] Only F39.4.1. gave strong immunostaining (+) in sections of hyperplastic prostatic tissue. The other MAbs were negative (-). Detailed information in Fig. 7 and in the text.

Anti-hAR MAb F39.4.1 in this assay crossreacted with the rat-AR (not shown) but the affinity as judged from titration curves might be a factor ten lower than observed for the hAR.

To precipitate 25 fmol hAR, 0.13 μg MAb F39.4.1 was needed. The concentration of IgG was established by measurement of the total protein concentration and from scanning agarose gels to assign the proportion of total protein of the IgG band.

ELISA

The curves of MAbs F39.4.1 and F39.6.2 (Fig. 5) with SP61 at comparable protein concentrations showed a steep slope in a direct ELISA. The slope of MAb F39.5.1 decreased more slowly and showed a prozone effect at lower dilutions. This might indicate that the affinity of F39.5.1 for SP61 is lower than the affinity of F39.4.1 and F39.6.2. The affinity of F39.3.1 for SP61 is very low as can be derived from both the slope and the titre (Table II).

The isotype of the MAbs (Table II) was determined in a direct ELISA. Both F39.4.1 and F39.5.1 were IgG1κ and the MAbs F39.3.1 and F39.6.2 were IgG2bκ.

Antigen-antibody complex formation

Formation of antibody-receptor complexes was analyzed by sucrose density gradient centrifugation. In Fig. 6 the precipitation of the androgen receptor in presence or absence of the MAbs is shown. For MAbs F39.4.1, F39.5.1, and F39.6.2 the buoyant density of the steroid-hAR complex in a sucrose gradient shifted approx. from 4.6S to 6.5S due to the

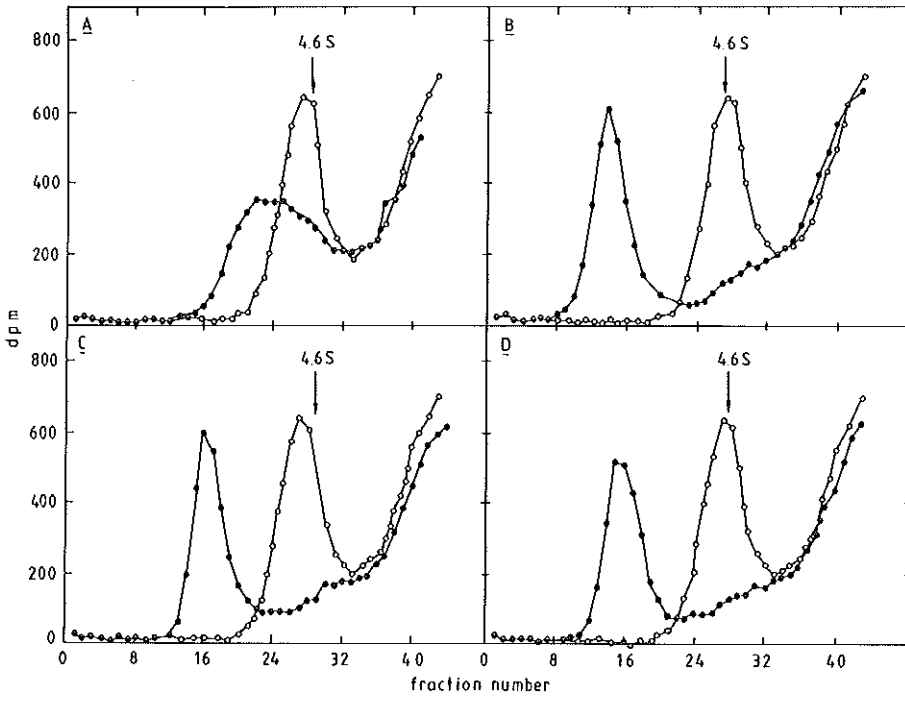

Fig. 6. Complex formation of selected MAbs with the hAR. Complexes were demonstrated by a shift in the sedimentation profile of the [^{3}H]R1881-labelled hAR. Appropriate antibody dilutions were established in separated titration experiments. Sedimentation profiles in 10-30% sucrose gradients of [^{3}H]R1881-labelled hAR in LNCaP nuclear extracts incubated with (A) MAb F39.3.1 (●); (B) MAb F39.4.1 (●); (C) MAb F39.5.1 (●); (D) MAb F39.6.2 (●); or without antiserum (○). The total volume was 100 μl in which the ascites dilution was 1:100. MAbs F39.4.1, F39.5.1 and F39.6.2 show a characteristic sedimentation shift. With MAb F39.3.1 there is an interference of the MAb with the sedimentation properties of the receptor not characteristic for immune-complex formation.

binding of the antibody to the complex. For MAb F39.3.1 the shift was less pronounced, only broadening of the 4.6S peak was seen (Fig. 6A).

Immunohistochemistry

Supernatants from 15 cell cultures (tested positive in immunoprecipitation) of anti-SP61 specific MAbs were applied for the indirect immuno-enzymatic staining of human prostate tissue sections. Only MAb F39.4.1 gave strong staining in immuno-histochemistry. Fig. 7 shows hyperplastic prostatic glands surrounded by stromal cells. A strong and specific

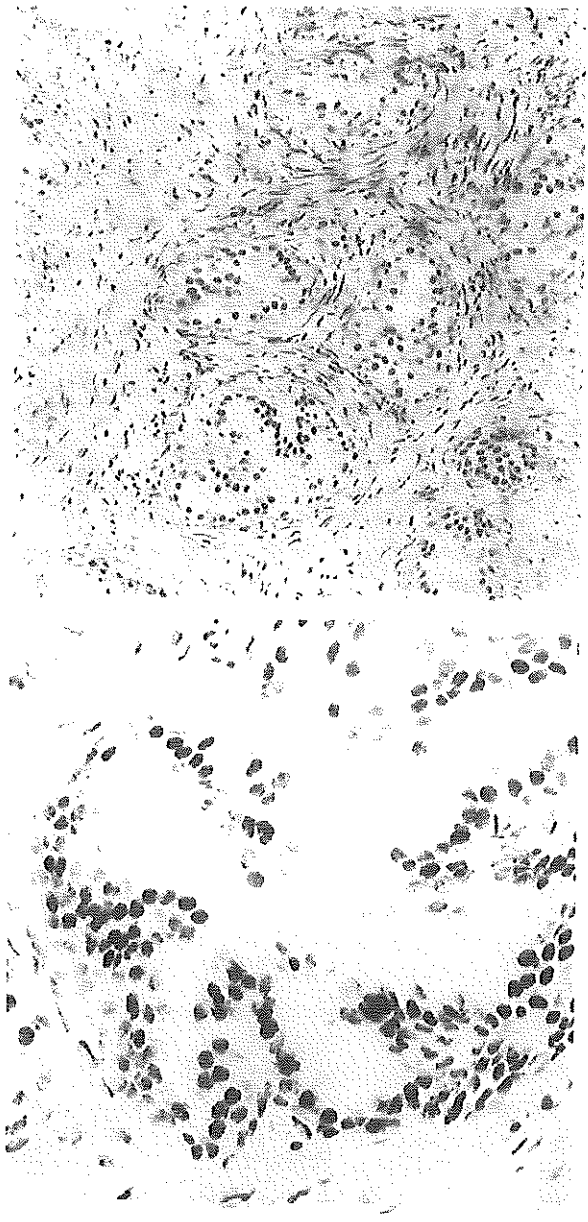

Fig 7. Top panel: low-power magnification of hyperplastic prostatic tissue immunostained with F39.4.1. Prominent nuclear staining of prostatic glands and a large proportion of stromal cells. No nuclear counterstaining (x 100). Lower panel: hyperplastic prostatic gland immunostained with F39.4.1. Intense nuclear staining of secretory epithelial cells lining the glands. Variable staining of stromal nuclei. The basal cells do not show reactivity with F39.4.1. No nuclear counterstaining (x 250).

immunostaining of the nuclei of secretory epithelial cells and some of the stromal cells is obtained using the anti-SP61 MAbs.

DISCUSSION

In this study we demonstrate, that the synthetic peptide strategy leads to the generation of highly specific and desirable reagents that could not be produced following classical approaches. Human androgen receptor antibodies were produced with: known determinant specificity, high affinity and multi-assay performance. The properties of the high affinity hAR specific poly- and monoclonal antibodies are summarized in Table II. Calculation and prediction of putative antigenic sites of the AR to which the MAbs are directed was based on the amino acid sequence derived from the cDNA sequence. Peptides were selected by exclusion of sequences with a large homology with other steroid receptors (oestrogen, progesterone, glucocorticoid) and selection of putative immunogenic peptides, based on the prediction of the antigenicity index. This approach is limited to prediction of continuous linear epitopes, against which antibodies can be evoked with synthetic peptides. We elicited a peptide specific response to all peptides selected with a computer algorithm. In two out of five cases antibodies crossreacted with the intact hAR. These two peptides, SP60 and SP61 are both located in the same N-terminal domain of the protein. MAbs were only developed with the peptide that generated the highest response. This N-terminal domain of the hAR showed a relatively low degree of homology (approx. 80%) with N-terminal sequence of the rat AR as compared to the homology observed in the DNA binding domain and the steroid-binding domain [46]. Homology of hAR with the rabbit AR is not known, but the DNA sequence as present in the hAR may be conserved in rabbits as well. For SP61 17 out of 20 amino acids are homologous in human and rat androgen receptors. A putative high degree of homology between hAR and rabbit AR did not prevent the generation of specific hAR antibodies. This can be explained by the fact, that the receptor in general is not or only to a limited degree exposed to the immune system.

Peptides SP65 and SP66 are hydrophilic stretches derived from the C-terminal steroid binding domain. Anti-SP65 and anti-SP66 antibodies were not crossreactive with the hAR as shown with immuno-precipitation and in immuno-histochemistry. Apart from exposed sites, hinges and protein edge strands, the hydrophilic stretches in a protein may have a function in protein-protein, protein DNA-RNA or protein-steroid interactions. The reason for anti-SP65 and SP66 antibodies being not crossreactive with the hAR might originate in either a difference in 3D structure of the peptide or in the assay systems applied. The sera were screened for the presence of hAR specific antibodies with agarose immuno precipitation. In this assay the hAR is complexed first with a labelled steroid analogue ([^{3}H]R1881) and subsequently the antisera are added. It cannot be excluded that the putative antigenic sites of the hAR are shielded and/or drastically changed by the steroid binding, such that the antibodies cannot recognize the altered hAR. It is also possible that only denatured hAR may be recognized by anti SP65 or anti SP66 antibodies. However, this is unlikely since the

immuno-precipitation method and the sucrose gradient centrifugation method applied make use of soluble hAR extracted with 0.15 M. NaCl without any detergents and only mild fixation was used for immuno-chemistry.

Four MAbs with different isotype (IgG1κ (2 x) and IgG2bκ (2 x)) and with different affinity for the hAR (varying from moderate to high) were isolated in one fusion after immunization with SP61. The MAbs obtained are site specific for AA301-320 situated in the N-terminal part of the hAR. Anti-SP61 MAbs show a broad applicability as was shown in our performance testing namely in immuno-histochemistry, in immunoblotting assays and in immuno-precipitation assays. MAb F39.4.1 has the highest affinity for the hAR (0.0052 μg/fmol hAR) (Table II) but not the highest affinity for SP61. MAb F39.5.1 has the highest affinity for SP61 but hAR binding is moderate (Table II; Fig. 5). Differences in isotype are wellcome since this enables double staining studies in immunohistochemistry with different second step (anti-isotype) conjugates, MAbs with low affinity can also be used, e.g., in competition studies.

PAbs against a synthetic peptide of 15 amino acids at the N-terminal end of the DNA binding region of the rat AR, that do recognize the ratAR in immunohistochemistry have been described [43,44]. Chang et al. [45] produced PAbs and MAbs obtained after immunization with ß-galactosidase fusion proteins of fragments of the hAR (A: AA331-572 and B: AA 544-822). One of the MAbs raised with the fragment A fusion protein has the highest affinity, and precipitates an amount of 25 fmol AR with 4 μg monoclonal antibody. In contrast, only 0.13 μg was needed for MAb F39.4.1 (this paper) for precipitation of a similar amount of AR. Although non-identical assay conditions could have affected these data it suggests, however, that MAb F39.4.1 has a very high affinity for hAR.

In the present study we have shown, that the strategy using synthetic peptides for MAb production may lead to qualitative and quantitative results, that otherwise only could be obtained at the expense of considerably greater effort, e.g., production of purified fusion proteins. Especially the properties of the hAR: instability, large, not available in purified form made it a good candidate for this type of approach.

Excellent site specific performance of the mono-specific polyclonal antibodies as well as for the monoclonal antibodies was demonstrated in different biochemical assays: immunoprecipitation, sucrose density gradient centrifugation and immuno-histochemistry. The antibodies probably recognize the native hAR in immuno-precipitation since the extraction method applied only uses 0.15 M NaCl without use of detergents and only mild fixation was used for tissue sections in immuno-histochemistry. The MAbs have high affinity for hAR (ascites F39.4.1 still effective at 1:13000 dilution) and they crossreact with the homologous rat AR and bovine AR.

The hAR-specific antibodies produced are reactive with the N-terminal part of the receptor and are supposed not to interfere with DNA- and steroid-binding and allowing, therefore, biochemical and cell-biological investigations at the molecular level. Furthermore, the immuno-histo-cytochemistry at light microscopic and electron-microscopic level should enable now the generation of data on expression of the receptor during growth and development as well as in malignant processes.

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PART II

Chapter 5

The potency of peptides in immune responses and in immune response analysis: single amino acid specificity

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THE POTENCY OF PEPTIDES IN IMMUNE RESPONSES AND IN IMMUNE RESPONSE ANALYSIS: SINGLE AMINO ACID SPECIFICITY

Netty D. Zegers, Eric Claassen and Wim J.A. Boersma

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I. INTRODUCTION

Synthetic peptides are nowadays very potent tools for a broad spectrum of applications in biological research. Anderer and Schlumberger (1965) used a synthetic peptide derived from the C-terminal end of the tobacco mosaic virus coat protein for raising native protein-specific and virus neutralizing antibodies. In the 1970s, more investigators elicited antibodies against identified antigenic epitopes from proteins with synthetic peptides (Arnon et al., 1969, 1971; Fearny et al., 1971; Langbeheim et al., 1976). In that decade, it was a common belief that the production of antisera with peptides against native proteins required the prior identification of antigenic determinants. After demonstration of the possibility to produce antibodies against peptides which were chosen based on knowledge only of the nucleotide sequence from which the amino acid sequence was derived (Walter et al., 1980), an ever-faster moving production of antibodies using synthetic peptides started. By then, synthetic peptides also became favorite tools for vast fields of research, such as mapping proteins for antigenic determinants and T cell determinants, ligand specificity of numerous cellular and nuclear receptors, probes for determining protein structure, immunomodulation, vaccines, autoimmunity, biochemical mechanisms and more. Peptides are used for investigation of peptide-MHC interaction. Sequence analogs can mimic or inhibit protein function. Dramatic changes with peptide analogs were observed in agonist-antagonist character.

Many inheritable diseases are the result of a mutant protein, often with substitution of only a single amino acid, which may lead to dramatic changes in protein structure and function. Tracking carriers with such protein disorders may be a matter of life and death. Virus mutants can be screened with variant- specific monoclonal antibodies (MAbs). Synthetic peptides are indispensable for the production of MAbs with single amino acid specificity. These MAbs are important tools for detection of protein variants. In addition, synthetic peptides are also very useful for induction of antibodies which can discriminate between individual members of a protein family sharing a high degree of homology, e.g., hormone receptors. These antibodies may contribute to the investigation of regulatory mechanisms.

In this chapter, we will discuss the single amino acid specificity of antibodies and the use of peptides as probes to prove single amino acid specificity in biochemical reactions, illustrated with examples from the literature and from work of our own group. A detailed description of the generation of MAbs specific for one amino acid will be given in Section VI. Peptide sequences will be given in a single letter code for amino acids, otherwise the three letter code for amino acids will be used.

II. DETECTION OF VIRUS MUTANTS

Retroviruses show a large mutation frequency, which occurs during reversed transcription or replication. Mutations are the result of various factors. Reversed transcriptase lacks exonucleolytic (proofreading) activity and the error rate is 10^{-4} per generation. DNA polymerase from the host works more accurately with an error rate of 10^{-5}. The error rate for polymerase II is not known, but it probably will not be different from reversed transcriptase. Major rearrangements of sequences, a high frequency of genetic recombination together with the ability of oncogene capture of the virus genome, contribute further to the high mutation rate. The human immunodeficiency virus (HIV), a retrovirus, is a striking example. Proteins from HIV show extensive variation among isolates, which are expressed in the primary amino acid sequence. These variants may have different biological activity (replication rate, cell tropism, and syncytia induction), which may be related to sequence and to the structural differences in the surface proteins. The third variable domain (V3 domain) of HIV-1 external glycoprotein gp120 evokes a major fraction of neutralizing antibodies during a natural infection. Several studies using MAbs have confirmed the role of the V3 domain in viral infectivity. The V3 domain is therefore considered to be important for candidate vaccines. Polyclonal and monoclonal antibodies directed against preselected epitopes of the V3 domain may be useful reagents to characterize structure-function relationships of viral proteins. Such antibodies may be used to probe epitopes that can be included in candidate vaccines for induction of neutralizing antibodies. In addition, variant-specific antibodies may have valuable diagnostic and therapeutic application.

We used synthetic peptides to generate rabbit polyclonal antibodies (PAbs) and murine MAbs against relatively conserved sequences from IIIB and MN variants of HIV-1 (Laman

et al., 1992). The two variants both contain the immunodominant sequence GPGRAF. In the flanking sequences some differences exist: a few amino acid substitutions and one deletion of two amino acids (IIIB variant: IRIQRGPGRAFVTIG; MN variant: KRIHIGPGRAFYTTK). A IIIB derived peptide overlapping the tip of the V3 domain, IRIQRGPGRAFVTIG, was used to generate MAbs. Two MAbs neutralized IIIB but not MN and inhibited syncytium formation induced by IIIB. The MAbs showed high affinity for gp120 with K_ds of 6.8 $*10^{-11}$ M and 1.6 $* 10^{-10}$ M respectively. PEPSCAN analysis mapped the binding site to the sequence IRIQRGPGR. In addition, two non-neutralizing MAbs were raised with peptides derived from the N- and C-terminal side of the loop. A similar MN derived peptide was used to produce a rabbit PAb. This PAb inhibited syncytium formation induced by HIV-1 IIIB and four field isolates. These findings showed that variant-specific and HIV-1 neutralizing antibodies can be obtained with synthetic peptides and that high affinity anti-peptide MAbs can be generated. Moreover, the PAbs generated through single peptide immunization crossreacted with four different viral strains (field isolates) which indicate major implications for vaccine development.

Another application of variant-specific synthetic peptides was developed in our group (Laman et al., 1991). Peptides derived from the neutralizing epitope of gp41 of HIV-1 (residues 586–608) were conjugated to the enzyme alkaline phosphatase. These enzyme-peptide conjugates may be used for detection of the epitope specificity of antibody forming cells in tissue biopsies, e.g., lymph nodes, from HIV infected individuals. Cryostat sections of lymph nodes were incubated with these conjugates. Enzyme-peptide conjugates bound by antigen-specific antibodies present in antibody forming cells stained with an appropriate substrate. In addition, the antibody subclass isotype (IgG1, IgG2, IgG3, IgM, IgE or IgA) and the specificity of the antibody forming cell could simultaneously be demonstrated in double staining experiments using peptide-alkaline phosphatase conjugates (using a blue substrate) together with isotype-specific antibody-peroxidase conjugates (using a red substrate) incubation. Violet stained (blue + red) cells thus produce peptide-specific antibodies with the isotype under study. Blue stained cells produce peptide-specific antibody with a different isotype, and red cells visualize antibodies directed against different antigens. This method allows correlation of *in vivo* function of B cells with lymph node pathology, clinical stage of the disease and serological data after HIV infection.

III. IMMUNODETECTION OF GENETIC VARIANTS OF PROTEINS

Nowadays more than 4000 inherited disorders are known. New molecular techniques have accelerated the discovery of the molecular defects of the disease. Partly thanks to the Human Genome Project, the genes causing these diseases are tracked. In the last decade, the genes for cystic fibrosis (Rommens et al., 1989; Riordan et al., 1989; Kerem et al., 1989), neurofibromatosis (Wallace et al., 1990), Duchenne's muscular dystrophy (Monaco et al., 1986), Huntington's disease (The Huntington's Research Group, 1993) and others have been located. Some of the disorders are listed in Table 1. (For an exhaustive review, see Cooper

TABLE 1
Some inherited disorders

Disorder	Protein	Defect	Location	Incidence	References
Hemophilia A	(Factor VIII)	Deletion	Several exons	1:10000 males	Furie and Furie, 1988
		Nonsense mutations	Several exons		Furie and Furie, 1988
Hemophilia B	(Factor IX)	Deletion	Several exons	1:30000 males	Furie and Furie, 1988
		Point mutation	Arg^{145}→His		
		Point mutation	Arg^{1}→Ser		
		Point mutation	Arg^{4}→Gln		
		Point mutation	Asp^{47}→Gly		
Hemoglobinopathies (thalassemias)	Hemoglobin			1:600 certain ethnic groups	
Familial hyperlipoproteinemia	Apo-E	Point mutation	Arg^{158}→Cys	1:500	Weisgraber et al., 1982
Inherited emphysema	α_1-Antitrypsin	Point mutation	Glu^{342}→Lys	1:3500	Bollen et al., 1983; Carrell et al., 1982
Severe combined immunodeficiency	Adenosine deaminase	Deletion		Rare	Valerio et al., 1983; Orkin et al., 1983
		Point mutation	Arg^{101}→Gln		Bonthron et al., 1985
		Point mutation	Leu^{304}→Arg		Valerio et al., 1986
Duchenne muscular dystrophy	Dystrophin complex	Deletion		1:3500 (males)	Monaco et al., 1986; Koenig et al., 1987
					Ervasti et al., 1990
Becker muscular dystrophy				1:35000	
Cystic fibrosis	CFTR protein	3 Base deletion	ΔPhe^{508}	1:2500 (Caucasians)	Rommens et al., 1989; Riordan et al., 1989
					Kerem et al., 1989
		Point mutation	Gly^{542}→X		Ng et al., 1991
		Point mutation	Gly^{551}→Asp		
		Point mutation	Arg^{553}→X		
		Point mutation	Asn^{1303}→Lys		
		Point mutations	Several, rare		Dean et al., 1990
Neurofibromatosis	NF1 gene product (chromosome 17)	Inserts	Several exons	1:3500	Wallace et al., 1990
		Deletions	Several exons		Viskochil et al., 1990
		Point mutations	Leu→Pro; Arg→stop		Cawthon et al., 1990
Huntington's disease	IT15 gene product	Inserts	Multiple Gln	1:10000	Gusella et al., 1983; Huntington's Res. Group, 1993
Phenylketonuria	Phenylalanine hydroxylase	Point mutations, deletions, inserts		1:10000	Woo et al., 1983; Dilella et al., 1986a, 1986b

and Schmidtke, 1991, 1992.) The majority of the inherited disorders are caused by a mutation in the DNA sequence, by deletion of a single base or of part of the DNA sequence, or by alternative splicing. Point mutations may lead to a single amino acid substitution or to an aberrant protein product if the mutation is a nonsense mutation. Even single amino acid substitutions in the protein product may have dramatic effects, e.g., the $Glu^{342}\rightarrow Lys$ substitution in α_1-antitrypsin predisposes the variant homozygous carriers for risk of developing lung emphysema at a relatively young age or the $Arg^{158}\rightarrow Cys$ substitution in apolipoprotein E (Apo-E) which leads to hyperlipoproteinemia. Especially for these two examples, preventive lifestyle measures like no smoking and avoiding areas with smog (α_1-antitrypsin) or a low fat diet (Apo-E) delay onset and severity of the disease.

With development of lifestyle advices and new therapeutic approaches, e.g., replacement therapy and gene therapy, reliable and simple diagnostic assays for tracking variant carriers at risk become very important. Though polymerase chain reaction assays are now broadly applied for screening mutated genes, they are rather expensive, time consuming and not fully reliable. For some disorders, diagnosis early in pregnancy is required, especially when a severe disease develops very early in life. But for other inherited diseases, which can be restored or delayed by therapy or certain lifestyle measures, postnatal screening may be chosen. In that case, diagnostic assays based on antibodies are simpler and cheaper. For specific diseases, these variant protein products may even be detected in serum by monoclonal antibodies specific for the variant amino acid region of the protein. The monoclonal antibodies required can be obtained by immunizing animals with synthetic peptides which contain the variant region. They have been developed in our laboratory for Apo-E variants (Gerritse et al., 1992) and for α_1-antitrypsin variants (Zegers et al., 1991). A detailed description of raising variant-specific antibodies for α_1-antitrypsin will be given in Section VI.

Though most valuable for single amino acid substitutions, the synthetic peptide approach can be applied for deletions also, especially when a single amino acid has been deleted (rare), but also when a part of the sequence has been deleted. A nice demonstration thereof has been described by Ris-Stalpers et al. (1990). An aberrant form of the human androgen receptor, missing 41 amino acids in the N-terminal region caused by a point mutation abolishing normal RNA splicing at the exon4/intron4 boundary leading to a disturbed male sexual differentiation and development in an individual with a 46,XY karyotype but a female habitus, could not be detected with a monoclonal antibody specific for the N-terminal region of the human androgen receptor.

Another demonstration of the powerful technique of the synthetic peptide approach was the elicitation of antibodies specific for tumor-specific *bcr-abl* joining regions in chronic myeloid leukemia (CML) as well as in acute lymphoblastic leukemias (ALL). The reciprocal translocation between chromosome 9 and chromosome 22 as observed in CML and ALL results in a $22q^-$ chromosome, the so-called Philadelphia chromosome. The translocation events create on the Philadelphia chromosome a fusion between two genes: *bcr* and *abl*. Depending on the localization of the breakpoint in the *bcr* gene, different chimeric *bcr-abl* genes are generated, each encoding their own tumor-specific protein. The amino acid

sequences at the point of the junction represent unique tumor-specific determinants. Since the fusion of the *bcr* and *abl* genes occurs within a coding triplet, a newly generated amino acid emerges. For example, the b2a2 fusion protein has the sequence INKEE*ALQRP in the junctional region and the b3a2 fusion protein has the sequence KQSSK*ALQRP in the junctional region. The first four residues are from the second (b2) or third (b3) exon of the *bcr* gene, respectively, and the last five residues are from the second exon (a2) of the *abl* gene. These sequences are not unique for the individual with cells with a Philadelphia chromosome, since they are also present in cells expressing the normal *bcr* and *abl* gene encoded proteins. The residues marked with an asterisk (E and K), however, are newly formed. The junctional regions are the aimed determinants for raising specific diagnostic antibodies. Detection of these tumor-specific proteins has a strong clinical significance. We therefore immunized rabbits and mice with peptides spanning these junctional regions. Antibodies thus raised were site specific and could discriminate, using a protein kinase cell assay, between the different Philadelphia proteins in various CML and ALL cell lines and in blood samples of patients (van Denderen et al., 1989, 1990, 1992, 1994).

IV. VARIATION OF AMINO ACID SUBSTITUTION

In this section we will describe examples of investigations with synthetic peptides for which substitution of single residues has been applied. The reader is also directed to the chapters 2 and 3 on PEPSCAN methods. Peptide sequence variation is widely employed in various biochemical research topics.

IV.1. Modulation of ligand function

A synthetic peptide derived from hen egg-white lysozyme (HEL) (1-18) and an analogous peptide in which Phe at position 3 is substituted by Tyr are both immunogenic in (C57Bl/10 x DBA/2)F1 mice. In the parent C57Bl/6 mice, genetically non-responders to HEL, the analogous peptide itself (Tyr^3) induces anti-peptide antibodies that also bind to the wild-type peptide (Phe^3), whereas the wild-type peptide itself is not immunogenic. Thus, a single amino acid substitution of aromatic amino acids — Phe is a non-polar and Tyr is a polar residue — in a peptide may convert the peptide from silent to immunogenic or virtually a non-responder mouse strain into a responder one (Sette et al., 1986).

Amino acid substitution in a synthetic peptide substrate may convert selective activity from agonistic to antagonistic or vice versa, or may convert the substrate susceptible for another receptor, as will be clear from the following examples. Substance P (RPKPQQFFGLM-(NH_2)) is an undecapeptide that acts as a neurotransmitter. It is part of a family known as tachykinins. Three main types of tachykinin receptors have been suggested to exist in the periphery: SP-P, SP-E and SP-N receptors. The hexapeptide [$pGlu^6$]SP_{6-11} is equipotent with the undecapeptide in SP-P systems. Substitution of Pro for Gly^9 in [$pGlu^6$]SP_{6-11} discriminates between two receptor subtypes in the same tissue preparation (Laufer et al., 1986).

Replacement of Pro^7 in the bradykinin sequence with D-Phe is the essential change used to convert kinin analogs into antagonists. The mechanism likely depends, at least in part, on the aromatic and basic character of the peptide (Lawrence et al., 1989). The impact of aromatic and basic amino acids was also demonstrated with analogs of bombesin (Saeed et al., 1989). His^{12} was replaced by several residues. Replacement of His^{12} by Phe resulted in an agonist with a 100-fold decrease in activity. Substitution by D-Phe yielded an antagonist, but with a 10^4-fold decrease of affinity. Substitution by a larger and more hydrophobic residue, D-β-naphtylalanine, produced an analog with complete loss of antagonist activity. On the other hand, substitution by a more basic hydrophillic group but with similar size to D-Phe, D-pyridylalanine, or D-Arg^{12} (basic residue) generated very weak agonists. D-Trp^{12} converted the analog inactive, and D-Tyr^{12} rendered a very weak antagonist, 100-fold less potent than the D-Phe^{12} analog.

Wild-type and mutant synthetic peptides conjugates were used as signal peptides of the SV-40 large T antigen to assay their binding to proteins of rat liver nuclei on Western blots. Proteins of 140 and 55 kD were exclusively recognized by wild-type peptide conjugates and less efficiently by conjugates of a mutant peptide which differed by a single amino acid from the wild-type (Wolff et al., 1988; Finlay et al., 1989; Meier and Blobel, 1990). Specific amino acid sequences have been suggested to serve as nuclear transport signals. Most of these sequences consist of a stretch of basic amino acids preceded by a Pro or Gly. Replacement of Lys^{128} of the SV-40 large T antigen by any amino acid but Arg completely abolished its nuclear targeting ability and conferred cytoplasmic localization. The stretch of basic amino acids (Lys or Arg) was thus found to be a prerequisite for the transport signal sequence.

A peptide (ASQKRPSQRHG) derived from myelin basic protein (AA 1-11), which is involved in the induction of experimental autoimmune encephalomyelitis (EAE), with a single amino acid substitution ($Lys^4 \rightarrow Ala^4$) appeared to show completely different characteristics than the normal peptide. The mutant peptide (Ala^4) binds to MHC class II molecules and stimulates encephalitogenic T cells *in vitro* better than the normal peptide. It is non-immunogenic and non-encephalitogenic *in vivo* in (PL/JxSJL)F1 mice and prevents EAE when administered before, at the time of immunization with the normal peptide or near the time of disease onset (Smilek et al., 1991). Thus, a change of charge (from positive to neutral) at position 4 in this peptide likely confers a different character to the peptide: from disease inductive to disease suppressive.

Actin polymerization can be inhibited by small synthetic peptides LKHAET and LKKTET. Substitution of the third position (His or Lys, both positively charged amino acids) by a Glu (a negatively charged polar amino acid) modulated the action from G-actin to F-actin (Vancompernolle et al., 1992).

IV.2. Analysis of protein structure

Synthetic peptides were used to investigate the structure of two adjacent zinc finger domains of the yeast transcription factor ADR1. These peptides readily bound zinc in a tetrahedral structure, while the mutant peptide containing a deletion for a single amino acid did not.

Cys^{134}, Cys^{137}, His^{150} and His^{155} are part of the metal binding site in the second zinc finger ^{130}KPYPCGLCNRCFTRRDLLIRHAQKIHSGNL^{159}.The residues between Cys^{137} and His^{150} form a loop. Deletion of one residue, Asn^{138}, prevents the folding of the peptide into a zinc finger domain. Ligand spacing is thus a major factor for zinc finger folding and has been revealed by use of a peptide analog (Párraga et al., 1990).

The influence of the flanking amino acid sequence on the O-glycosylation of a single threonine was investigated *in vitro* by examining a series of 52 related peptide substrates (O'Connell et al., 1992). Each residue of the parent peptide (derived from the human Von Willebrand factor) was substituted with different amino acids. Substitution of any amino acid tested at position +3, -3 and -2 relative to the threonine markedly decreased O-glycosylation, as did the presence of a charged residue at position -1. The substitutions of amino acids at the other positions of the peptide substrate had little effect.

Single amino acid variation was applied to investigate sequence-structure and structure-function relationships in proteins by various investigators. An elegant study was carried out with peptide analogs from myohemerythrin (Getzoff et al., 1987). The antibody binding determinants of myohemerythrin (MHr) were mapped using 113 overlapping hexapeptide homologs of the MHr sequence. The roles of individual side chains in each determinant were characterized by measuring the reactivities of antisera with sets of 120 peptides that included all possible analogs (replacement nets) differing in sequence from the parent peptide by replacement of a single amino acid residue. Such a replacement net study made it possible to assign each residue of a peptide to one of the following four categories: essential, selected, partially replaceable or generally replaceable. It was shown that each antigenic site contained one or more highly exposed and accessible critical side chains and one or more largely buried and inaccessible critical side chains within the native protein structure. Antibody binding was analyzed combined with three-dimensional crystallographic structure determination. With these techniques, it was found that initial binding of an antibody to solvent-exposed critical amino acid residues may promote local side chain displacements and thereby allow the participation of other, previously buried, critical residues.

Single amino acid deletions at position 19, 20, 21 or 22 in the C-terminal part of the α-helical region (residues 9–22) of salmon calcitonin did not greatly affect either the conformational (measured by circular dichroism) or the biological properties of this protein (Epand et al., 1988). The amphipathic character of this region was kept despite single amino acid deletions.

Ni et al. (1989) used nuclear magnetic resonance (NMR) to study the structural basis for the bleeding disorder caused by the single mutation of Gly^{12} to Val^{12} in the Aα-chain of human fibrinogen. Val^{12} was found to disrupt the type II β-turn involving Glu^{11} and Gly^{12} in normal protein. The positions of Gly^{13} and Gly^{14} were also displaced in this mutant protein. This altered geometry presumably affects the positioning of Arg^{16}–Gly^{17} bond in the active site of thrombin. As a result, the cleavage of the Arg^{16}–Gly^{17} peptide bond in the mutant peptide by thrombin is much slower than the cleavage of the same bond in normal peptides.

IV.3. Optimalization of ligand sequences

Pharmacological industries need to improve and to optimize therapeutic agents. Especially for peptides, used as substrates, improvement of their affinity for receptor binding is rather easy. Each residue is subsequently replaced by one of the coded or non-coded amino acids, and the mutant peptides are assayed for their biological activity and are compared with the original peptide and previous versions of mutant peptides.

For aprotinin, the Kunitz trypsin inhibitor from bovine mast cells, a series of homologs have been prepared by use of peptide-chemical procedures to modulate the inhibitory specificity. Lys^{15} at the P1 position of its reactive site was replaced by any other amino acid. Especially, Val^{15} aprotinin was the most powerful inhibitor in the series for leucocyte elastase, having a dissociation constant which ranks among the smallest values reported for protein inhibitors of this proteinase. Thus, this mutant of aprotinin may be beneficial in the treatment of human diseases which are associated with the destructive potential of leukocyte elastase. Aprotinin itself is an excellent inhibitor of trypsin due to the positively charged side chain of its P1, but a relatively weak inhibitor for chymotrypsin and shows no detectable inhibition with leukocyte elastase. This investigation demonstrated the individual contribution of a single residue to the inhibition of a particular target proteinase and enabled specific inhibitors to be designed (Tschesche et al., 1987).

Six analogs, single amino acid modifications, of the highly δ-opoid receptor selective, conformationally restricted, cyclic peptide [D-Pen2, D-Pen5] enkephalin were evaluated for opoid activity in rat brain receptor binding and mouse vas deferens (MDV) smooth muscle assays. The enkephalin peptide, Tyr-D-Pen-Gly-Phe-D-Pen, contains two D-Pen residues (penicillamine, containing a thiol group in the side chain) which are cyclized via a disulfide bond. The N-terminus was modified by substitution of Tyr by *N,N*-diallyltyrosine and resulted in a very low potency, low affinity agonist despite apparent overall conformational similarity with the original peptide. The low affinity could probably be attributed to a local effect of the diallyl substitution. Modification of the carboxy terminal group from a carboxylic acid to a carboxamide resulted in reduction in δ-receptor selectivity due to enhanced μ-receptor affinity. Substitution of Gly^3 with sarcosine or substitution of Phe^4 with D-Phe or phenylglycine all lead to drastic losses of binding affinity and MDV potency. Particularly noteworthy was the high δ-receptor affinity, potency and selectivity for the analog in which Gly^3 was replaced by α-aminoisobutyric acid. This analog displayed a quite different conformation than the original peptide (Haaseth et al., 1990). This example demonstrates the effects found by replacement of amino acids by non-coded amino acids.

A peptide, which was 2000-fold more reactive with a transmission blocking monoclonal antibody against *Plasmodium falciparum Pfs25* than the peptide derived from the 25 kD surface protein, could be obtained by subsequent amino acid substitution (van Amerongen et al., 1992). The reactivity of the MAb with the peptide sequence LDTSNPVKT (derived from the surface protein of *P. falciparum*) was low. The improved peptide with the sequence FDDTDPIKK resulted from the amino acid variation.

Besides the examples mentioned above, also other investigations on substrate selectivity and optimalization have been frequently described. Only the subjects of these

studies will be mentioned here. Lottenberg et al. (1986) described the amino acid variations at different positions P1–P4 of peptide substrates for factor Xa. Ho et al. (1988) studied the effects of substitutions in β-endorphin on analgesic potency. An increase of antagonistic activity of parathyroid hormone analog was found by replacement studies by Goldman et al. (1988). Prorok and Lawrence (1989) studied the influence of amino acid insertions to peptide substrates for the cAMP-dependent protein kinase.

V. ANTIBODY SPECIFICITY

Poly- and monoclonal anti-peptide antibodies with a unique fine specificity for even single amino acids are applicable to distinguish members of a protein family with high homology or even to discriminate between variant proteins differing in only a single point mutation. Here we review some results from the literature.

A monoclonal antibody against hemoglobin S was produced with a synthetic peptide derived from the N-terminal β-chain of hemoglobin S, where the single amino acid difference between hemoglobins A and S occurs. Hemoglobin A contains the sequence VHLTPEEV and the S variant contains the sequence VHLTPVEK. The negatively charged glutamine has been replaced by the neutral valine, and two residues downstream a second change in charge has occurred: valine has been replaced by the positively charged lysine. With this MAb, one heterozygotic AS cell in a mixture with one million AA cells could be detected. (Jensen et al., 1985).

In human cells, the *ras* protein family consists of several members including normal (proto-oncogene) and mutant (oncogene) forms. The mutant forms which result from somatic mutations of the normal *ras* genes appear to be responsible for the loss of normal growth control. The mutant form typically contains a single amino acid change at position 12. Antibodies were raised against the *ras* gene product p21. Anti-p21 Ser^{12} (Ser is polar, non-charged) was raised with synthetic peptides and recognized only v-Ki-*ras* protein, but not v-Ha-*ras* protein (containing Arg^{12}, polar, positive charge) nor the normal *ras* protein containing Gly (neutral) at position 12. Micro-injection of antibodies specific for Ser^{12} of the oncogenic v-Ki-*ras* protein into cells transformed by this protein caused a transient reversion of the cells to a normal phenotype (Feramisco et al., 1985).

Also Clark et al. (1985) raised polyclonal anti-peptide antibodies against the *ras* gene product p21. These antibodies distinguished the normal p21, containing glycine at amino acid position 12, from the oncogenic form with serine at position 12. This remarkable specificity was achieved by affinity purification. The polyclonal antibodies were adsorbed to a column to which a Gly^{12}-containing peptide was attached and the flow-through was subsequently adsorbed to the Ser^{12}-containing peptide column.

Schoofs et al. (1988) raised antibodies against a synthetic peptide corresponding to the carboxy terminal 24 amino acids (^{305}CPKYVKQNTLKLATGMRNVPEKQT^{328}) of the heavy chain of the hemagglutinin molecule of influenza virus A/X-31 (H3). This peptide represents a region of the hemagglutinin molecule which is buried in the molecule. When

intact virus or protein was used as the immunogen, no antibodies against this region were found. The peptide appeared to possess three antigenic sites, identified with polyclonal mouse anti-peptide sera in a PEPSCAN. Subsequently, two monoclonal antibodies were selected with the 305–328 peptide. One MAb was found to be specific for the most immunodominant epitope of the peptide ^{314}LKLAT^{318}. In a "replacement set" PEPSCAN, it was shown that all five amino acids are essential for the interaction and most probably are contact residues. In addition, no amino acid substitutions were allowed at either site of the epitope. The MAb was highly specific for the H3 virus subtype and did not bind to the H2 subtype. Subtype H3 differs from subtype H2 at position 315 (Lys vs Val), which is included in the ^{314}LKLAT^{318} epitope. A second MAb recognized the ^{322}NVPEKQT^{328} epitope of which N^{322}, E^{325} and Q^{327} using PEPSCAN technology were implicated as contact residues. This study elegantly shows that for influenza virus subtypes, in which the subtype-specific residues are buried in the protein or alternatively are not immunogenic, subtype-specific monoclonal antibodies can only be developed with the help of synthetic peptides.

Antibodies specific for the VP1 protein type O foot-and-mouth disease virus were raised with a synthetic peptide comprising residues 141–160. This region contains a highly conserved RGD (145–147) sequence which contributes to neutralizing antibody binding sites. Anti-peptide antibodies neutralized a wider range of type O variant isolates than the anti-virion serum. Elongation of this peptide at the amino terminus reduced the number of O-variant strains which could be neutralized by the anti-peptide sera. Selected substitutions of a single amino acid at position 148 altered the specificity of the raised antibodies. In particular, the Leu→Ser substitution induced antibodies which neutralized both type O (Leu^{148}) and type A (Ser^{148}) virus-equally (Parry et al., 1989).

Polyclonal site-specific antibodies were raised to synthetic peptides with a length of 12 to 13 amino acids representing HLA-DQw7 and -DQw8 allelic sequences, differing only by one amino acid in position 57 being Asp and Ala, respectively. This position is the least conserved one. The difference investigated is substantial, being a negatively charged Asp and a neutral Ala. These peptide-specific PAbs distinguished single amino acid substitutions in HLA-DQ β-chain alleles associated with insulin-dependent diabetes (Atar et al., 1989).

Immunological discrimination between human apolipoprotein E2 (Arg 158→Cys) and E3 isoforms differing in amino acids was described (Gerritse et al., 1992). A monoclonal antibody was selected after using a panel of several variant-specific peptides. The peptide sequences used were homologous to human Apo-E2 and in all but one amino acid homologous to the murine Apo-E2. The mouse immune system appeared to be tolerant to most of the selected sequences. Immunization with only one of the peptides (AA 154–172) evoked an anti-protein-specific response despite a low predicted antigenicity index. This study clearly showed the utmost frontiers of the peptide approach.

The foregoing examples all demonstrate the possibility and usefulness of raising variant-specific antibodies with the restriction that the substitution has a major impact on the protein, in general implying a change of charge. These examples have been summarized to indicate the limits of the presently available peptide technology. In addition, another example will be extensively described in the next section including all important aspects to be taken

into consideration when generating site directed monoclonal antibodies which can discriminate between proteins differing in a single amino acid residue.

VI. RAISING MONOCLONAL ANTIBODIES SPECIFIC FOR GENETIC VARIANTS OF α_1-ANTITRYPSIN

Human α_1-antitrypsin is a serum protease inhibitor which inhibits mainly neutrophil elastase in the lungs. Nowadays more than 70 different isoforms of the protease inhibitor are known, most of them being point mutations in the DNA sequence leading to amino acid substitutions. Severe α_1-antitrypsin deficiency results from a single amino acid substitution (Glu^{342}→Lys) in the molecule which causes α_1-antitrypsin accumulation in the liver where it is synthesized. The change of charge from negative to positive in the Z molecule induces an alteration in the three-dimensional structure of the Z variant protein which, as a consequence, also showed a reduced activity.

Homozygous carriers with the Z type substitution (Glu^{342}→Lys) are at risk to develop lung emphysema at a relative young age. Also, heterozygous carriers with the SZ phenotype may be at risk. Early detection of such carriers offers the possibility to prescribe lifestyle measures, which delay the disease, or to start with replacement therapy. Presently applied methods for detection of variants are not suitable for routine screening since they are difficult to interpret. Simple assays based on monoclonal antibodies, raised using synthetic peptides, which can distinguish between the Z and S variant and the normal M protein, have been developed.

For this purpose, we synthesized several peptides (Table 2) with different length and various overlapping sequences derived from the M and the Z α_1-antitrypsin. Peptides mostly contain only a B cell determinant and are therefore not immunogenic themselves. Hence, peptides were coupled to keyhole limpet hemocyanin (KLH) as a carrier to provide T cell determinants with the bifunctional heterogenous coupling agent m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS) via a Cys added at one of the termini or via their functional amino or carboxyl groups with glutaraldehyde (GA) (Zegers et al., 1990) or a carbodiimide, (EDC: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide) (Deen et al., 1990). Balb/C mice were immunized i.p. or s.c. with the peptide-KLH conjugates or with free peptides both given with an adjuvant.

We earlier showed that also short peptides of about ten amino acids may induce native protein- specific antibodies (van Denderen et al., 1989, 1990). Short peptides (10–12 amino acids) (SPEK1A, SPEK1B and SPEK3) derived from the 342 region of α_1-antitrypsin in general did not elicit antibodies cross-reactive with the native protein. Based on our present experience and also on that of other investigators (Partidos and Steward, 1992), longer peptides of about 15 to 25 amino acids readily lead to protein-specific antibodies of higher affinity. Elongated peptides (SPEK11 to SPEK15) derived from α_1-antitrypsin with various positions of the substituted amino acid were more successful in raising α_1-antitrypsin antibodies.

TABLE 2
Peptides used to elicit anti α_1-antitrypsin variant-specific antibodies

SPEK1A	*CVLTIDKKGTE	342 position in Z-variant (AA 337–346)
SPEK1B	*CVLTIDEKGTEA	342 position in M-variant (AA 337–347)
SPEK3	*CHKAVLTIDKK	342 position in Z-variant (AA 334–343)
SPEK11	KLSKAVHKAVLTIDEKC*	342 position in M-variant (AA 328–343)
SPEK12	*CKLSKAVHKAVLTIDKK	342 position in Z-variant (AA 328–343)
SPEK13	KAVLTIDKKGTEAAGAC*	342 position in Z-variant (AA 335–350)
SPEK14	KAVLTIDEKGTEAAGAC*	342 position in M-variant (AA 335–350)
SPEK15	KLSKAVHKAVLTIDKKC*	342 position in Z-variant (AA 328–343)
MOUSE	KAVLTIDETGTEAAAV	Homologous sequence of mouse α_1-antitrypsin

Variant-specific residues have been underlined and in redline. Terminal cysteine residues with * superscript are added to the α_1-antitrypsin sequence for thiol coupling possibility. Mouse-specific residues have been underlined (Latimer et al., 1990).

SPEK11 (derived from the M variant) is almost completely homologous with the mouse α_1-antitrypsin. A Thr (mouse) in stead of a Lys (human) at position 343 at the carboxy terminus of the peptide is the only difference. Immunization of Balb/C mice with SPEK11 induced only weak α_1-antitrypsin-specific antibody responses. The complementary Z derived peptide SPEK15, with two residues different from the mouse sequence, induced stronger α_1-antitrypsin-specific responses. The polyclonal antibodies were not able, however, to discriminate between the M and the Z type. SPEK12, which was conjugated to a carrier protein with the distal end compared to SPEK15, also did not result in Z-specific antibodies.

Subsequently, we synthesized peptides SPEK13 and SPEK14 with the substitutions in the middle of the peptides and in addition two extra residues different from the mouse sequence at the carboxy terminus. Immunization, either i.p. or s.c., with KLH-MBS conjugates of the peptides induced polyclonal antibodies specific for α_1-antitrypsin (Table 3).

Free peptides only induced a peptide-specific response after s.c. immunization, but failed to induce protein-specific responses. KLH-MBS-SPEK14 even raised antibodies specific for the M variant which did not recognize the Z variant; KLH-MBS-SPEK13 induced polyclonal antibodies which also recognize the M variant, but with lower specificity or affinity than the Z variant.

Conjugates made by coupling with EDC or with glutaraldehyde failed to induce peptide- or protein- specific antibodies. These coupling reagents make use of amino groups (EDC or glutaraldehyde) or carboxyl groups (EDC). Peptides SPEK13, SPEK14 and SPEK15 possess several residues with these functional groups (Lys, Asp, Glu and end groups). The peptides probably will be conjugated to the carrier at more than one site. This probably will

TABLE 3
Antibody responses after immunization with various conjugates via different routes

		ELISA Coating		
Immunization	**Route**	**Free SP**	**α_1AT(M)**	**α_1AT(Z)**
Free SPEK 13	i.p.	-	-	-
KLH-MBS-SPEK 13	i.p.	+++	+	+
KLH-EDC-SPEK 13	i.p.	-	-	-
KLH-GA-SPEK 13	i.p.	-	-	-
Free SPEK 13	s.c.	++	+	-
KLH-MBS-SPEK 13	s.c.	++	-	+
Free SPEK 14	i.p.	-	-	-
KLH-MBS-SPEK 14	i.p.	+++	++	-
KLH-EDC-SPEK 14	i.p.	-	-	-
KLH-GA-SPEK 14	i.p.	+	-	-
Free SPEK 14	s.c.	++	-	-
KLH-MBS-SPEK 14	s.c.	+++	++	-
Free SPEK 15	i.p.	-	-	-
KLH-MBS-SPEK 15	i.p.	+++	++	+
KLH-EDC-SPEK 15	i.p.	-	-	-
KLH-GA-SPEK 15	i.p.	-	-	-
Free SPEK 15	s.c.	+	-	-
KLH-MBS-SPEK 15	s.c.	+++	+	+

TABLE 4
Specificity of anti-human α_1-antitrypsin monoclonal antibodies

		ELISA			
MAb	**Isotype**	**SPEK13**	**SPEK14**	**α_1-AT(Z)**	**α_1-AT(M)**
F43.8.1	IgG1 κ	-	+	-	+
F46.4.1	IgG1 κ	+	+	+	+
F50.2.1	IgG2b κ	+	-	+	-
F50.3.1	IgG1 κ	+	-	+	-
F50.4.1	IgG2a κ	+	-	+	-

modify the specific epitope to such an extent that antibody recognition of the free peptides or of the native protein will be hampered.

Variant-specific monoclonal antibodies were selected after immunization of mice with KLH-MBS-SPEK13 or KLH-MBS-SPEK14. The reaction pattern of the MAbs is shown in Table 4. Three classes of MAbs were obtained. MAbs F50.2.1, F50.3.1 and F50.4.1 specifically recognize only the Z variant of α_1-antitrypsin. MAb F46.4.1 recognizes all variants of α_1-antitrypsin, and MAb F43.8.1 recognizes all variants except the Z variant. With the combination of these MAbs, it is possible to discriminate between hetero- or homozygous Z carriers in different assays.

The selected MAbs have a high affinity for α_1-antitrypsin in ELISA systems in which the protein is coated and also for α_1-antitrypsin variants in immunoblots after isoelectric focusing (Zegers et al., 1991). Subsequently, a sandwich ELISA was designed with a polyclonal antibody to capture different variants of α_1-antitrypsin combined with our selected variant-specific MAbs for rapid and facile discrimination between homo- or heterozygous Z carriers. However, commercially available polyclonal antibodies interfered with the binding of our variant-specific MAbs. We therefore raised polyclonal antibodies in rabbits using an immune complex of α_1-antitrypsin and polyclonal rabbit Abs specific for α_1-antitrypsin (Sigma, St. Louis, MO). After absorbing these polyclonal antibodies to human serum albumin, they were suitable for use as capture antibodies in a sandwich ELISA (Zegers et al., 1993).

MAb F43.8.1 had a very low affinity for native α_1-antitrypsin in the sandwich ELISA, and it was not possible to detect all offered samples. The MAbs were selected in an

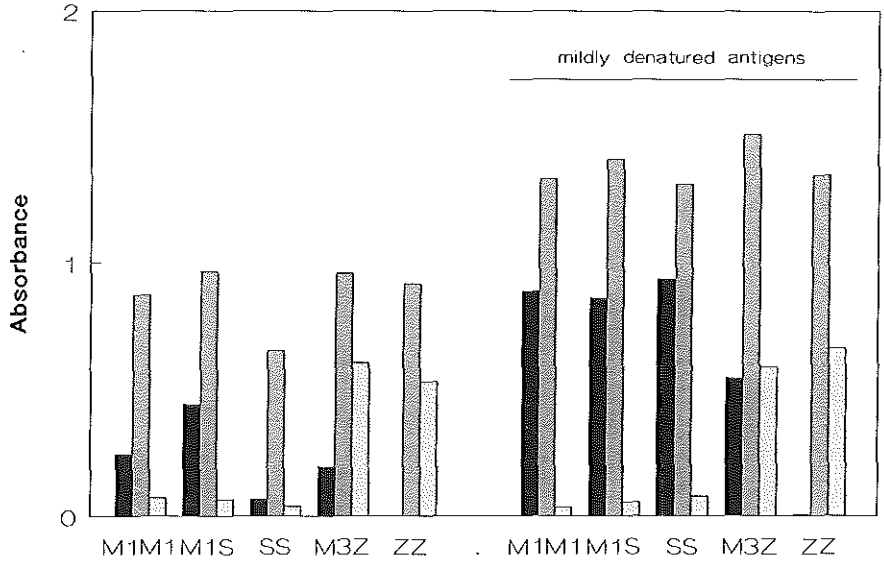

Fig. 1. Comparison of native and denatured α_1-antitrypsin variant-specific detection with a capture ELISA. Human plasma samples with known α_1-antitrypsin phenotype were diluted 1:50. Samples were denatured by heating at 56°C for 30 minutes (right panel) or used in native form (left panel). Variants were captured by variant-specific MAbs: non-Z-specific F43.4.1 (solid bars), all variant-specific F46.4.1 (cross-hatched bars) or Z-specific F50.4.1 (F50.2.1 was used for the right panel) (striped bars). Captured antigens were detected with a polyclonal rabbit serum W9/04 specific for α_1-antitrypsin and a second antibody specific for rabbit Ig conjugated to alkaline phosphatase. Reactivity was measured after 45 minutes for the right panel and after 90 minutes for the left panel.

ELISA with antigen coated to the surface of a plate. The MAbs therefore readily recognized, to some extent, denatured antigen coated to a plastic surface or coated onto nitrocellulose. In addition, MAbs F46.4.1 and F50.2.1 also recognized α_1-antitrypsin in solution, but MAb F43.8.1 had a much lower affinity for α_1-antitrypsin in solution. When the diluted human plasma samples were mildly denatured by heating at 56°C for 30 minutes, MAb F43.8.1. could detect non-Z α_1-antitrypsin in all samples (Fig. 1). Also MAbs F46.4.1 and F50.2.1 could still readily detect the captured and denatured α_1-antitrypsin. A dipstick method, based on the sandwich ELISA, was also quite suitable to distinguish homo- or heterozygous Z carriers (Fig. 2).

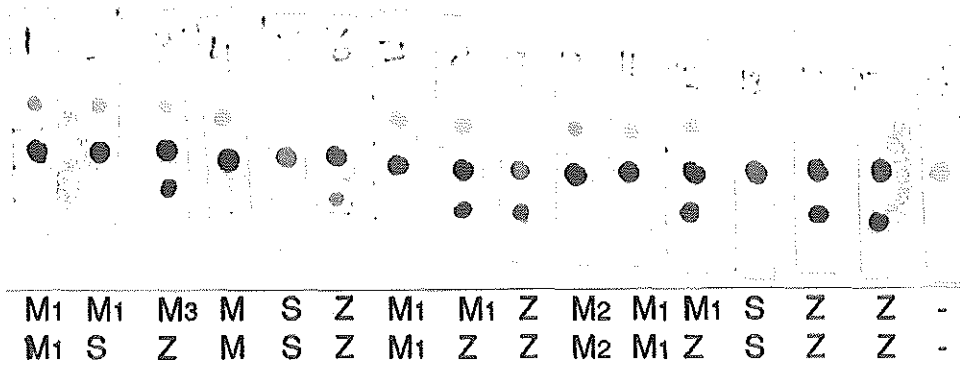

Fig. 2. α_1-Antitrypsin variant-specific detection with a capture dipstick. Human plasma samples with known α_1-antitrypsin phenotype were diluted 1:50. Samples were denatured by heating at 56°C for 30 minutes. Variants were captured by variant-specific MAbs spotted onto nitrocellulose (1 μl of a 2 mg.ml^{-1} solution): non-Z-specific F43.4.1 (upper dots), all variant-specific F46.4.1 (middle dots) or Z-specific F50.2.1 (lower dots). Captured antigens were detected with MAb F91.3.2 specific for α_1-antitrypsin conjugated to β-galactosidase. X-Gal was used as substrate to yield a clear blue spot.

Another set of peptides (Table 5) was synthesized encompassing the substitution of the S variant of α_1-antitrypsin (Glu 264→ Val). Mice were immunized with free peptides or with KLH conjugates made with MBS. With short peptides SP138 and SP139, no peptide- or protein-specific antibody response was found. Elongation of SP138 with three residues at either end, SP136, introduced solubility problems, and therefore the conjugation procedure was not effective. The homologous SP137 (M variant) was soluble and KLH-MBS-SP137 induced peptide- and α_1-antitrypsin (M)-specific antibodies.

Elongation of SP138 to SP190 with three residues only at the N-terminal side resulted in a soluble peptide. SP190 was coupled to KLH or N-terminally extended with a well-known T cell epitope (Hackett et al., 1985) termed SP191. Both KLH-MBS-SP190 and free SP191 or KLH-MBS-SP191 raised α_1-antitrypsin-specific antibodies. These polyclonal antibodies recognized, however, both the M and the S variant. Monoclonal antibodies resulting from these immunizations also could not discriminate between the S and the M variant. Inhibition ELISAs with peptides SP136, SP137, SP138, SP139 and SP190 indicated that the dominant MAb binding site probably is the DEGK sequence. This finding corresponds with a computer prediction algorithm program (Jameson and Wolf, 1988; Wolf et al., 1988) in which the sequence DEGK was found to be a stretch with a high antigenicity index (Table 6) as compared to the rest of the sequence. Especially for the S variant, the sequence LQHLVNE has a much more lower antigenicity index compared to the homologous sequence LQHLENE of the M variant.

SP256, with the deletion of the immunodominant sequence DEGK, was conjugated to KLH with MBS and injected in mice. We also synthesized the corresponding multiple antigen peptide (Tam, 1988), MAP255, 8 linear peptide sequences with a N-terminal

TABLE 5
Peptides used to elicit S or M variant-specific antibodies

Mouse	DDGKMQHLEQTLSKEL	Homologous mouse α_1-antitrypsin sequence
SP136	DEGKLQHLVNELTHDIC*	S variant AA 256–271
SP137	DEGKLQHLENELTHDIC*	M variant AA 256–271
SP138	KLQHLVNELTC*	S variant AA 259–268
SP139	KLQHLENELTC*	M variant AA 259–268
SP190	C*DEGKLQHLVNELT	S variant AA 256–268
SP191	FERFEIFPKE-DEGKLQHLVNELTC*	T cell epitope + S variant AA 256–268
MAP190	$(DEGKLQHLVNELT)_8$-core	S variant AA 256–268
MAP191	$(FERFEIFPKE-DEGKLQHLVNELT)_8$-core	T cell epitope + S variant AA 256–268
MAP250	$(DEGKLQHLVNELTHDI)_8$-core	S variant AA 256–271
MAP251	$(FERFEIFPKE-DEGKLQHLVNELTHDI)_8$-core	T cell epitope + S variant AA 256–271
MAP255	$(FERFEIFPKE-LQHLVNELTHDI)_8$-core	T cell epitope + S variant AA 260–271
SP256	LQHLVNELTHDIC*	S variant AA 260–271

Variant-specific residues are underlined. Terminal cysteine residues with * superscript are added to the α_1-antitrypsin sequence for thiol coupling possibility. MAP peptides are multiple antigen peptides (8 peptide sequences per molecule) branched to a core of 7 lysine residues according to Tam et al., 1988. The T cell epitope used for N-terminal extension of peptides was derived from influenza virus hemagglutinin (Hackett et al., 1985). Mouse-specific residues have been underlined (Latimer et al., 1990).

TABLE 6
Antigenicity index for α_1-antitrypsin M and S variant

Residue number	Amino acid	M	S
250	A	-0.600	-0.600
251	I	-0.600	-0.600
252	F	-0.600	-0.600
253	F	-0.600	-0.600
254	L	-0.600	-0.600
255	P	0.250	0.250
256	D	1.300	1.300
257	E	1.300	1.300
258	G	1.300	1.300
259	K	0.900	0.900
260	L	0.750	0.600
261	Q	0.750	-0.300
262	H	0.750	0.300
263	L	0.750	0.300
264	E/V	0.900	0.300
265	N	0.900	-0.300
266	E	0.900	-0.300
267	L	0.750	0.750
268	T	0.600	0.600
269	H	-0.300	-0.300
270	D	-0.600	-0.600
271	I	0.300	0.300
272	I	-0.300	-0.300
273	T	-0.600	-0.600
274	K	-0.600	-0.600
275	F	0.450	0.450
276	L	0.900	0.900
277	E	0.900	0.900

The antigenicity index was calculated according to Jameson and Wolf (1988).

TABLE 7
Antigenicity index for α_1-antitrypsin M and Z variant

Residue number	Amino acid	M	Z
330	S	-0.450	-0.450
331	K	0.450	0.450
332	A	0.450	0.450
333	V	0.600	0.600
334	H	-0.300	-0.300
335	K	-0.600	-0.600
336	A	-0.600	-0.600
337	V	-0.600	-0.600
338	L	-0.600	-0.600
339	T	-0.600	-0.600
340	I	0.000	0.000
341	D	0.900	0.900
342	E/K	1.100	0.900
343	K	1.100	0.900
344	G	1.100	0.900
345	T	0.900	0.900
346	E	0.750	0.750
347	A	-0.300	-0.300
348	A	-0.300	-0.300
349	G	-0.600	-0.600
350	A	-0.600	-0.600
351	M	-0.600	-0.600
352	F	-0.600	-0.600
353	L	-0.600	-0.600
354	E	-0.600	-0.600
355	A	-0.600	-0.600
356	I	-0.600	-0.600
357	P	-0.600	-0.600

The antigenicity index was calculated according to Jameson and Wolf (1988).

extension of the T cell determinant branched to a core of 7 lysine residues. MAP255 was also used for immunization. In ELISA, a peptide-specific response was found, but not an α_1-antitrypsin-specific response. We may conclude from these results that the sequence LQHLVNELTHDI has a low intrinsic immunogenicity and is too short to mimic the structure of native α_1-antitrypsin.

The experiments described in this section nicely demonstrate the attractiveness and the pitfalls of the peptide strategy for generating variant-specific antibodies. In these cases, one is constrained to a limited possibility of peptide sequence choice. To raise Z- specific antibodies, it was needed to carefully select the peptide sequences. Several overlapping peptides had to be tested. It proved to be essential to locate the substituted amino acid in the middle of the peptide (SPEK13). Also for this region the predicted immunogenicity corresponds with the findings. The residues surrounding the variant-specific amino acids have a higher predicted antigenicity than the preceding sequence (Table 7). If this part of the sequence is situated, however, adjacent to the coupling site, then the steric hindrance probably prevails over the immunogenicity. SPEK15 apparently is a less antigenic sequence. Also the mode of coupling of the peptide to a carrier protein is of importance. Only with conjugates made with MBS were α_1-antitrypsin-specific antibodies produced.

Sometimes peptides are not soluble in aqueous buffers and in DMF or DMSO which cause troubles with conjugation procedures. To overcome this problem, linear extension of the peptide with a T cell epitope or synthesis of a branched peptide may be advantageous. For some applications with MAbs generated with synthetic peptides, it is necessary to make the binding site of the protein for the antibody more accessible by denaturing the protein. Direct selection of MAbs in a sandwich ELISA instead of only selection on PVC surface coated protein will probably yield MAbs which have a higher affinity for the native protein in solution. Denaturing the protein may not be necessary. Until now, we did not succeed in generating S variant-specific antibodies, mainly due to the low antigenicity of the variant-specific domain and the immunodominant preceding DEGK sequence.

Experiments based on these considerations led to the production of variant-specific monoclonal antibodies which can discriminate between proteins with only one amino acid different in various immuno-assays.

VII. CONCLUSION

Synthetic peptides have proven to be essential means for investigational purposes in a numerous number of disciplines. Single amino acid specificity is encountered Variant-specific residues are underlined. Terminal cysteine residues with * superscript are added to the α_1-antitrypsin sequence for thiol coupling possibility. MAP peptides are multiple antigen peptides (8 peptide sequences per molecule) branched to a core of 7 lysine residues according to Tam et al., 1988. The T cell epitope used for N-terminal extension of peptides was derived from influenza virus hemagglutinin (Hackett et al., 1985). Mouse-specific residues have been underlined (Latimer et al., 1990).
with a large number of functionally diverse biological molecules. With well chosen sequences in synthetic peptides, the specificity of these molecules can be investigated and the function of ligands can be mimicked or inhibited.

MAbs which are single amino acid specific in general can only be produced with the aid of synthetic peptides. These MAbs are extremely useful for early, fast, reliable and cheap

detection of variant proteins, e.g., genetic variants or virus mutants. In order to generate variant-specific MAbs, a number of aspects should be carefully taken into account, such as peptide length, sequence, orientation relative to a carrier, coupling mode procedures, carrier, B and T cell epitope selection, immunization scheme and formulation and finally selection methods. In the example described, important aspects were highlighted. This example can serve as a guideline for production of other variant-specific MAbs in the future.

ABBREVIATIONS

AA	Amino acid
ALL	Acute lymphoblastic leukemia
Apo-E	Apolipoprotein E
α_1AT	α_1-Antitrypsin
CML	Chronic myeloid leukemia
EAE	Experimental autoimmune encephalomyelitis
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
ELISA	Enzyme-linked immunosorbent assay
HEL	Hen egg-white lysozyme
HIV	Human immunodeficiency virus
i.p.	Intraperitoneal
i.v.	Intravenous
KLH	Keyhole limpet hemocyanin
MAb	Monoclonal antibody
MBS	m-Maleimidobenzoyl-N-hydroxysuccinimide ester
MHC	Major histocompatibility complex
MHr	Myohemerythrin
PAb	Polyclonal antibody

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Chapter 6

Detection of genetic variants of α_1-Antitrypsin with site-specific monoclonal antibodies

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DETECTION OF GENETIC VARIANTS OF α_1-ANTITRYPSIN WITH SITE-SPECIFIC MONOCLONAL ANTIBODIES

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Abbreviations α_1-AT, α_1-antitrypsin; ELISA, enzyme-linked immunosorbent assay; KLH, keyhole limpet hemocyanin; MBS; *m*-maleimidobenzoylsuccinimide ester; IEF, isoelectric focusing; MAb(s), monoclonal antibody(ies); PBS, phosphate-buffered saline; and BSA, bovine serum albumin.

SUMMARY

The serum protein α_1-antitrypsin (α_1-AT) serves as the major inhibitor of neutrophil elastase. The most common allele of the α_1-AT gene is designated as Pi^M. The Z mutation is a single-base substitution of the normal M allele, causing a Glu→Lys change at position 342 in the molecule. The ZZ phenotype is associated with a severe deficiency of α_1-AT, serum concentrations of the protein being 10% of normal. Individuals with an α_1-AT deficiency are at an increased risk of developing emphysema. To generate antibodies that specifically detect the 342 position in the context of the flanking sequences, we synthesized several peptides that included the 342 position for both the M and the Z variant. Immunization with variant-specific peptide-carrier conjugates elicited α_1-AT variant-specific responses, as determined in a direct enzyme-linked immunoassay. Monoclonal antibodies (MAbs) were selected with different specificity for the 342 region: MAbs F43 recognize only the α_1-AT sequence with ^{342}Glu, i.e., all variant proteins that are non-Z, either from hetero- or homozygous individuals; MAbs F50 recognize only the sequence with ^{342}Lys, i.e., all Z-variant proteins in ZZ or heterozygous individuals; MAbs F46 recognize α_1-AT with either ^{342}Lys or ^{342}Glu, all variant proteins with sequences as in the peptides used. Z homo- and heterozygotes were detected with our MAbs in a rapid and simple immunoblot assay. Other variants (M, S, and F) can also be assigned on the basis of the electrophoretic pattern. This sensitive detection method is very easy, rapid, and straightforward and provides a powerful tool for diagnosis of the α_1-AT deficiencies, allowing early treatment (augmentation of α_1-AT) and proper advice on lifestyle practices.

INTRODUCTION

α_1-Antitrypsin (α_1-AT) is a serum protein that inhibits several proteases, primarily neutrophil elastase (EC 3.4.21.37) (*1*).[1] The most common allele of the α_1-AT gene is designated Pi^M. The Z-allele, Pi^Z, a genetic variant of α_1-AT, contains a single mutation such that a lysine instead of a glutamic acid is encoded at position 342 (*2, 3*). The Z-gene represents 1-2% of α_1-AT haplotypes in individuals of Northern European descent.

The ZZ phenotype is associated with a severe deficiency of α_1-AT, resulting in serum concentrations of the protein only 10% of normal (normal = 2-4 g/L). This deficiency is basically a decreased production of α_1-AT rather than production of a variant with decreased activity as a result of the amino substitution. Individuals with an α_1-AT deficiency are at an increased risk of developing emphysema (*4*). Carriers are at risk for lung disease when the α_1-AT concentration in serum is less than $\approx$35% of normal. Moreover, $\approx$15% of neonates with the ZZ phenotype develop hepatitis and are more likely to develop cirrhosis as adults. There is also an association between α_1-AT deficiency and various immune-mediated diseases, e.g., rheumatoid arthritis, anterior uveitis, systemic lupus erythematosus, and asthma (*5*).

Cigarette smoking, air pollution, and other situations leading to airway irritation contribute to a severe clinical course. Preventive lifestyle measures, e.g., non-smoking behaviour, are advised. Several clinical trials with substitution therapy with recombinant DNA-produced α_1-AT have been initiated recently (for an overview, see Crystal, *6*). Based on statistical calculations of the incidence of the Pi^Z allele, it is likely that a cohort of patients with clinically unrecognized α_1-AT deficiency exists. Because they are probably hidden, via a false diagnosis, in the group of patients with asthma or chronic aspecific respiratory diseases, they probably will not receive proper therapy. For obvious reasons of early diagnosis, easy screening of the population for the presence of the Z α_1-AT phenotype could be very helpful. α_1-AT is not phenotyped in all patients with chronic aspecific respiratory diseases, let alone in the whole population, because of the specialized character of the current detection methods for the different variants.

Current α_1-AT phenotyping is intricate, being based on analysis of complex isoelectric focusing (IEF) patterns to be read only by skilled personnel. Site-specific monoclonal antibodies (MAbs) would be of great value in straightforward detection assays. The generation of Z- or M-specific antibodies has been hampered because purified α_1-AT from MM or ZZ origin injected into animals does not generate antibodies specific for the 342 region. Thus an alternative approach is needed.

In our hands, synthetic peptides have been very powerful tools for evoking site-specific antibodies that cross-react with the native protein. They have been applied (e.g.) when no purified protein was available (*7*), when the amino acid sequence was unknown but could be predicted on the basis of the DNA sequence (*8, 9*), and when proteins were strongly homologous, differing in only a few amino acids (*10*).

We synthesized several synthetic peptides that contain the 342 position for both the M and the Z variant. Immunization of mice with peptide-carrier protein conjugates evoked

an antibody response in serum that was specific for the variant protein, as detected in enzyme- linked immunosorbent assays (ELISA). After fusion of immune spleen cells with SP2/0 myeloma cells, we selected several MAbs that were specific for the 342 region with either glutamic acid or lysine at this position.

Based on the use of these antibodies, a rapid, sensitive screening method that can easily be applied on a large scale has been developed. By using this immunoblot assay with IEF gels, one can detect homo- or heterozygous carriers for the Z-allele. Because application of blotting techniques has been rapidly advancing in clinical diagnostic laboratories, we envisage a quick introduction of our assay.

MATERIALS AND METHODS

α_1-AT(M) was obtained from Sigma Chemical Co., St. Louis, MO. α_1-AT(Z) was purified from 20 mL of plasma from a ZZ donor by use of an immuno-affinity column. Purified rabbit IgG directed against α_1-AT (Sigma) was coupled to 5 mL of Affigel^{R}HZ (Bio-Rad Labs., Richmond, CA) according to the manufacturer's protocol. The α_1-AT protein bound to the column was eluted with 0.1 mol/L glycine buffer pH 3.0.
Phenotyped human sera was obtained from Dr. R.R. Frantz (State University of Leiden, The Netherlands) and Dr. J.H. Kramps (Academic Hospital, Leiden, The Netherlands).

Peptide synthesis

We synthesized peptides by using a Sam II automatic peptide synthesizer (Biosearch, San Rafael, CA) according to the solid-phase synthesis method with *tert*-butyloxycarbonyl (t-Boc)-protected amino acids as described before (*8*).

Conjugates

Conjugates of peptides and proteins for immunization were made with keyhole limpet hemocyanin (KLH; Calbiochem, San Diego, CA) with the aid of *m*-maleimidobenzoyl-succinimide ester (MBS) (*11*). Briefly, MBS was dissolved in dimethylformamide (20 g/L), then added in three equal portions at 5-min intervals at a molar ratio of 200 to 1, relative to that of the carrier protein, 10 g/L in phosphate-buffered saline (PBS; 10 mmol of phosphate and 150 mmol of NaCl per liter, pH 7.2). Excess MBS was removed by passage through a PD-10 column (Pharmacia, Uppsala, Sweden). Finally, the peptide (10 g/L) was added in 100-fold molar excess and incubated for at least 1 h at room temperature. Conjugates for screening consisted of bovine serum albumin (BSA) and peptide, coupled by using a different reagent (a carbodiimide) (*12*) from the one used for immunization.

Immunization

Conjugates (25 μg) of the different peptides to KLH emulsified in "specol" (a water-in-oil suspension) (*13*) were injected intraperitoneally into groups of four 12-week-old female BALB/c mice. The mice were given booster injections after five weeks with the same dose

of antigen in specol. Serum antibody responses were analyzed by ELISA seven days after each immunization.

ELISA

We coated poly(vinyl chloride) microtiter plates (highly activated, Titertek; Flow Labs., Irvine, Scotland) with 50 μL of protein, peptide, or peptide conjugate (5 mg/L) in PBS overnight at 4°C and subsequently blocked the remaining binding sites with gelatin, 5 g/L in PBS, during a 30-min incubation at room temperature. Sera were incubated for 1 h at various dilutions in a solution of 1 g of gelatin and 0.5 mL of Tween 20 per liter of PBS. Goat IgG directed against mouse IgG conjugated to alkaline phosphatase (Kirkegaard & Perry Labs., Gaithersburg, MD) at a 500-fold dilution in the gelatin-Tween 20 solution was incubated for 1 h at room temperature to detect the bound antibodies to the coated antigen. *p*-Nitrophenyl phosphate in, per liter, 10 mmol of diethanolamine and 1 mmol of $MgCl_2$, pH 9.8, was used as substrate. The absorbance was read after 30 min at 405 nm against the signal from nonimmune mouse serum as a blank.

The specific antibody response was assayed on a coating of the peptide; on a coating of the peptide conjugated to BSA, a protein that was not used for immunization but was coupled with the aid of another reagent; and on coatings of the purified M and Z variants of α_1-AT protein.

Monoclonal antibodies

A spleen cell suspension was prepared four days after the second booster injections. Spleen cells and SP2/0 cells were washed twice with serum-free RPMI 1640 (Boehringer Mannheim, Mannheim, F.R.G.). Spleen cells and SP2/O cells were fused at a ratio of 5:1 in a reagent containing 400 g/L of Polyethylene Glycol 4000 (Merck, Darmstadt, F.R.G.) and 50 mL of dimethyl sulfoxide per liter of PBS. We resuspended the fused cells in selection medium and cultured 10^5 cells/well in a 96-well plate (total volume 0.2 mL/ well). The selection medium contained, per liter, 5.7 μmol of azaserine and 0.1 mmol of hypoxanthine supplemented with 150 mL of fetal calf serum, 2 mmol of glutamine, 69 μmol of streptomycin, 100 int. units/mL of penicillin, 1 mmol of sodium pyruvate, and 50 μmol of ß-mercaptoethanol in RPMI 1640. After one week of culture, we omitted the azaserine and decreased the fetal calf serum to 100 mL/L. Half the volume of the supernate was replaced by culture medium twice a week. Selected positive wells were subcloned by limiting dilution at a cell density of 0.5 cell/well.

BALB/c mice were injected intraperitoneally with 10^6 monoclonal hybridoma cells in 0.25 mL of PBS. Ascites fluid was collected under anesthesia.

IEF blotting

Samples of human sera (1 μL undiluted) were transferred to the middle of an IEF gel, pH 4-6.5. We used the protocol and system from the Phast System^R (Pharmacia). Immediately after gel electrophoresis, we blotted the protein bands by diffusion onto pre-wetted (with Tris/methanol/glycine, pH 8.3) 0.1-μm-thick cellulose-nitrate membrane (PH79; Schleicher

TABLE 1
Sequence of Synthetic Peptides

SPEK13	KAVLTID*K*KGTEAAGAC[a]	Positions 335-350 Z-variant
SPEK14	KAVLTID*E*KGTEAAGAC[a]	Positions 335-350 M-variant

[a]A C-terminal cysteine was added to the sequence for coupling through the thiol group with MBS to KLH.

and Schuell GmbH, Dassel, F.R.G.) at room temperature for at least 3 h or overnight. The cellulose-nitrate membrane was developed according to the ELISA protocol, but with 5-bromo-4-chloro-3-indolyl phosphate (Sigma), 165 mg/L, and nitroblue tetrazolium (Sigma), 330 mg/L, as substrate and stain, respectively. After blocking the unoccupied binding sites on the membrane, and between incubations with antiserum, we washed the blots three times with Tween 20, 0.5 mL/L in PBS, for 5 min. Finally, we rinsed the blots in distilled water and dried them between tissues.

RESULTS

Generation of Monoclonal Antibodies

We synthesized peptides from both the M and the Z variant (Table 1). An extra cysteine was added to the sequence for coupling purposes. Peptides were conjugated to KLH with MBS. Mice were injected intraperitoneally with peptide conjugates. All antigens were immunized with the aid of specol, a mineral oil, as adjuvant. Immune responses were measured by ELISA on coatings of free synthetic peptides, or of conjugates of synthetic peptide and BSA, α_1-AT(M), or α_1-AT(Z) after the first booster immunization.

The KLH-MBS conjugates of the peptides induced high antibody responses to the specific peptides. A cross-reacting antibody response to α1-AT(M), but not to α_1-AT(Z), was elicited with KLH-MBS-SPEK14. Immunization with KLH-MBS-SPEK13 gave an anti α_1-AT(Z)-specific response (data not shown).

We fused myeloma cells with the spleen cells from mice immunized with the KLH-MBS-SPEK14. For non-Z-specific α_1-AT, we selected Mabs F43.8.1, F43.17.1, and F43.20.1 after subcloning.

In two subsequent experiments, we fused with myeloma cells the spleen cells from mice immunized with KLH-MBS-SPEK13. MAbs F46.1.3, F46.3.1, and F46.4.1, being anti-α_1- AT(Z + non-Z)-specific, were selected after subcloning. We selected α_1-AT(Z)-specific MAbs F50.1.3, F50.2.1, F50.3.1, and F50.4.1 in a second fusion experiment.

TABLE 2
Specificity of Monoclonal Antibodies[a]

		ELISA			
MAb	**Isotype[b]**	**SPEK13**	**SPEK14**	**α_1-AT(Z)**	**α_1-AT(M)[c]**
F43.8.1	IgG1 κ	-	+	-	+
F43.17.1	IgG1 κ	-	+	-	+
F43.20.1	IgG1 κ	+	+	-	+
F46.3.1	IgG1 κ	+	+	+	+
F46.4.1	IgG1 κ	+	+	+	+
F50.1.3	IgG1 κ	+	-	+	-
F50.2.1	IgG2b κ	+	-	+	-
F50.3.1	IgG1 κ	+	-	+	-
F50.4.1	IgG2a κ	+	-	+	-

[a] Assayed in a direct ELISA with coating of peptides or proteins. +, a positive signal (with A_{405} >0.2 read against a blank after 30 min of incubation with substrate); -, a negative result (A_{405} <0.01).
[b]MAbs were assayed (about 1:50 000 dilution) on coatings of peptides in ELISA, with rabbit antibodies directed to different isotypes of mouse Ig. To make visible the bound rabbit antibodies, we used swine IgG directed to rabbit IgG conjugated to alkaline phosphatase, with *p*-nitrophenyl phosphate as substrate.
[c]The MAbs have been assayed on α_1-AT(M) coating, but from the blots from isoelectric focusing gels, it appeared that all variants except the Z variant were recognized by MAbs marked with + in the column headed by α_1-AT(M).

Specificity of the MAbs

The reactivities of the MAbs with peptides and proteins were measured in an ELISA. Table 2 shows results for a 1000-fold dilution of ascites fluid. MAbs F43.8.1, F43.17.1, and F43.20.1 recognized SPEK14 and α_1-AT(M) in the ELISA; MAbs F46.3.1 and F46.4.1 recognized SPEK13, SPEK14, α_1-AT(Z), and α_1-AT(M); and MAbs F50.1.3, F50.2.1, F50.3.1, and F50.4.1 appeared to be specific for SPEK13 and α_1-AT(Z).

Titration of the MAb F43 series, F46 series, and F50 series in the ELISA was performed on a coating of purified α_1-AT(M) and α_1-AT(Z). The initial dilution of the MAbs (ascites fluid) was 1000-fold, after which $\log_2$ serial dilutions were made (Figure 1). The titer of MAbs was arbitrarily chosen as the dilution of ascites fluid that gave an absorbance (A_{405}) of 0.5 after 30 min of incubation with substrate. MAbs F43 showed a high reactivity with α1-AT(M), with titers between 1:32 000 and 1:128 000, and no reactivity with α_1-AT(Z). MAbs F46 showed a titer of 1:64 000 to α_1-AT(M) and 1:32 000 to α_1-AT(Z). MAbs F50 showed a lower reactivity pattern with α_1-AT(Z), compared with the signal of the F43 and F46 series to α_1-AT(M). MAb F50.4.1 showed the greatest reactivity with α_1-AT(Z), with a titer of 1:16 000. The MAbs F50 series did not react with α_1-AT(M).

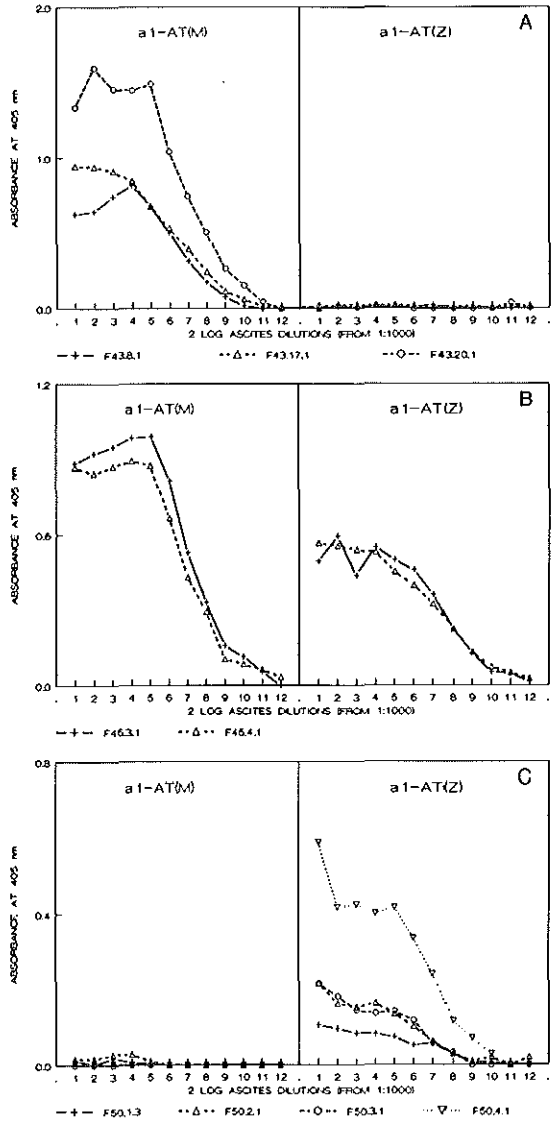

Fig. 1. Reactivity of MAbs with α_1-AT(M) and α_1-AT(Z) in ELISA. (*A*) Reactivity of MAbs F43 series with α_1-AT(M) and α_1-AT(Z). A $\log_2$ serial dilution of the MAbs was made. The first mark indicates a 1000-fold dilution, the second mark a 2000-fold dilution, and so on. The MAbs were assayed on coatings of purified α_1-AT. (*B*) Reactivity of MAbs F46 series. (*C*) Reactivity of MAbs F50 series

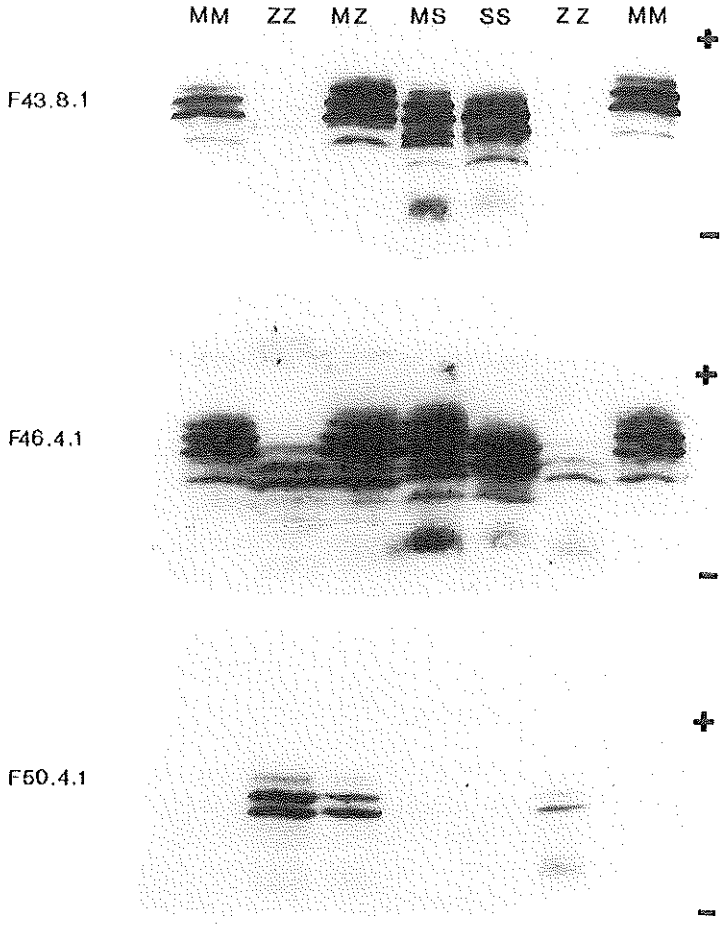

Fig. 2. Immunoblots from isoelectric focusing gels for human plasma with different α_1-AT phenotypes. The blots were developed with (*top*) non-Z-specific MAb F43.8.1 (diluted 500-fold), recognizing α_1-AT sequences with ^{342}Glu; (*middle*) MAb F46.4.1 (500-fold dilution), which reacts with all α_1-AT variants (^{342}Glu and ^{342}Lys); and (*bottom*) Z-specific MAb F50.4.1 (500-fold dilution) (^{342}Lys). + and -, anodal and cathodal electrodes, respectively

The isotype of the MAbs was determined in an ELISA with use of rabbit antisera that were specific for mouse immunoglobulin subclasses (Table 2).

Immunoblotting

The different variants of α_1-AT, as they occurred in human sera or plasma, could be detected in immunoblots for IEF gels (Figure 2). MAbs F46.3.1 and F46.4.1 all gave visible bands from reaction with all different variants of α_1-AT. Because of variation in glycosylation, the α_1-AT from one genotype shows multiple bands, and the pattern is characteristic of the phenotype. MAbs F43.8.1, F43.17.1, and F43.20.1 recognized all bands except the bands from the Z phenotype. From the difference between MAbs F43 and F46, we could detect homo- or heterozygous carriers for α_1-AT(Z). A direct evidence for the Z-positive sera was the visible band in an immunoblot with MAbs F50.1.3, F50.2.1, F50.3.1, or F50.4.1. Figure 2 shows a representative example for each of the three groups of MAbs.

We did not detect any bands from sera from 27 homozygous ZZ carriers in an immunoblot developed with MAb F43.8.1 (data not shown). The bands of these 27 ZZ sera were clearly visible after reaction with MAb F50.4.1 (data not shown). These experiments demonstrated the reproducible specificity of the MAbs, which can distinguish the two variants of the 342 position of α_1-AT.

Assay Sensitivity

To show the sensitivity for α_1-AT in immunoblots, we made $\log_2$ serial dilutions of human serum in PBS, then transferred 1 μL of undiluted or diluted human serum to an IEF gel. The protein bands were blotted to cellulose nitrate and were visible after reaction with MAb F50.4.1 (Z-specific) and with F46.4.1 (Z-specific and non-Z-specific) (Figure 3). The sample of human serum, which contained 0.36 mg of α_1-AT(Z) per milliliter, could be detected until an eightfold dilution with the Z-specific MAb F50.4.1. The normal range of α_1-AT concentration in human serum is 2-4 g/L and the mean value for α_1-AT(Z) is 0.2-0.4 g/L. About 50 ng of α_1-AT(Z) (0.045 g/L) is the detection limit in this immunoblot assay with F50.4.1. With MAb F46.4.1, a 64-fold dilution was still detectable, which contained 5 ng of α_1-AT(Z) (0.0045 g/L) on the gel.

DISCUSSION

We developed several MAbs that are specific for either α_1-AT(Z) or non-Z α_1-AT and that do not show unwanted cross-reactivity with the proteins assayed. These MAbs, with different isotypes and various affinities for α_1-AT, were raised against variant-specific synthetic peptides. Specificity for the variant-type native protein was established in an ELISA and verified in an immunoblot from IEF gels. Application of the combination of MAbs F50.4.1, F43.8.1, and F46.4.1 allowed us to detect respectively all the α_1-AT Z variants, i.e., those with ^{342}Lys homozygous and heterozygous; all non-Z ^{342}Glu variants that have the same 342 position sequence as M variants; and the M and Z cross-reactive sequences around the 342 position (^{342}Glu and ^{342}Lys). Having antibodies available with different reactivity patterns and isotypes could be very advantageous for competition studies or in double-labelling studies in histocytochemistry.

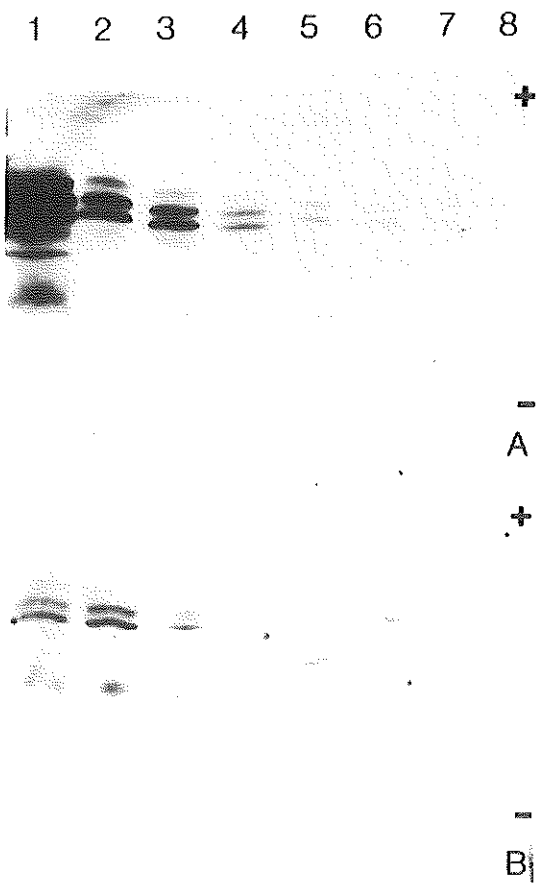

Fig. 3. Immunoblots from IEF gels for human plasma with ZZ phenotype of α_1-AT: 1 μL of plasma serially diluted with PBS. Lane 1, undiluted plasma. Subsequent lanes contain the serially diluted ($\log_2$) samples. Blot A was developed with MAb F46.4.1 (specific for all α_1-AT variants) diluted 500-fold. Blot B was developed with the Z-specific MAb F50.4.1, diluted 500-fold. Anodes and cathodes designated as in Fig. 2

We were able to assign the phenotype of the α_1-AT in human donor sera by immunoblotting from IEF gels. The Z phenotype could be detected very easily with MAbs of the F50 series in particular. Analysis of 27 typed ZZ sera from two different laboratories with our immunoblot method allowed us to calculate a detection limit of 50 ng for α_1-AT(Z) with MAb F50.4.1. This is sufficient to detect homozygous Z carriers, for which the mean α_1-AT concentration in serum is about 0.2-0.4 g/L, only 10% of normal. We applied 1 μL of serum onto the IEF gel and this appeared to contain saturating amounts of α_1-AT(Z) to be detected. In case of doubt, it is possible, with the Phast System, to apply threefold more sample at different places of the gel or even ninefold more sample if a different applicator is used. The detection limit for α_1-AT(Z) in the immunoblot system is 10-fold more sensitive (5 ng) with MAb F46.4.1.

Heterozygous Z carriers of α_1-AT could be detected with a second blot developed with the MAb F43 series (to make visible the additional bands) or with the MAb F46 series (to make visible the complete set of α_1-AT bands). Because all variants of α_1-AT except α_1-AT(Z) share the same sequence of amino acids as in SPEK14, all bands of α_1-AT except the ones from α_1-AT(Z) should be detectable with MAb F43 series. To date, only a few of the major types of α_1-AT — M1M1 (n = 7), M1Z (n = 1), SS (n = 1), M3Z (n = 1), M1S (n = 1), M2M2 (n = 1), and ZZ (n = 27) — have been examined. We did not observe differential staining for M1, M2, M3, S, or Z with MAb F46 series, although staining depends on the concentration of α_1-AT in serum. The same is true for MAb F43. All protein bands from all variants of α_1-AT thus far analyzed are detected with MAb F46 series.

The immunoblotting method is reproducible and so far has not produced any false-positive or false-negative results. This approach confirms that antipeptide antisera can be very powerful reagents by being very specific. Because the MAbs described were raised with synthetic peptides and were first selected with variant-specific synthetic peptides, the selected MAb can react with only proteins that contain the sequence of the peptide used for immunization. Human serum albumin, transferrin, immunoglobulins, and human proteins thus far listed in a protein sequence databank (Protein Identification Resource, National Biomedical Research Foundation, Washington, DC) did not contain the sequence contained in SPEK13 or SPEK14, even when a mismatch of eight residues (out of 16) was allowed. We show here the specificity of the MAbs that react with a variant protein only that differs in only one amino acid. Recently, we showed the specificity of an antipeptide MAb that could differentiate between the human androgen receptor and other homologous steroid receptors (7). We also selected a human IgG2-specific antipeptide antibody that showed no reactivity with human IgG1, IgG3, or IgG4 (14). These examples and the present data illustrate the unique power of antipeptide antisera.

We expect that our immunoblot method will be very useful for the routine laboratory to screen patients with chronic obstructive lung disease. Until now, only very specialized persons, usually only found in university hospitals, could interpret the IEF pattern and assign the phenotype of homozygous or, even more difficult, heterozygous carriers of α_1-AT Z variants. However, the method we describe here can be performed and interpreted rapidly and easily by every laboratory technician. This method may therefore become useful at least as an independent secondary confirmatory procedure. Also for differential diagnosis of neonatal jaundice, often seen in ZZ carriers, the immunoblot method may have great value because of its simplicity and rapidity. Application of blotting techniques are used more and more in the routine clinical laboratories. Especially, chromatographic procedures carried out with miniature electrophoretic systems (Phast System) are very rapid and easy. They mostly are provided with a simple blotting system. Carriers who are hetero- or homozygous for the Z allele may be detected in every hospital laboratory among patients with chronic obstructive lung disease by using miniature electrophoretic systems, combined with immunoblotting for variant-specific antibodies.

Current procedures are based on polyclonal antisera directed to α_1-AT, and typing is based on the banding pattern. However, variant-specific polyclonal antibodies are not easy to produce because they have to recognize a difference of only one amino acid. Monoclonal antibodies, however, are very advantageous over polyclonal antibodies in being reproducible reagents, always containing the same constant specificity and affinity.

Some other α_1-AT alleles have been associated with pulmonary disease: e.g., the S allele, when it occurs in the homozygous state or heterozygous with the Z allele; the null alleles

(heterozygous with other deficiency alleles or homozygous), which result in either no expression of α_1-AT or expression of unstable truncated α_1-AT which is not detectable in serum; and some rare deficiency alleles (15) such as $M_{heerlen}$, M_{malton}, $M_{procida}$, $M_{nichinan}$, $M_{mineral\ springs}$, P_{lowell}, and $Z_{augsburg}$, when they occur in the heterozygous state with the Z allele. Because a panel of variant-specific antibodies might become very helpful in screening programs for α_1-AT deficiency, an S-variant-specific antibody is currently being developed in our laboratory via the same synthetic peptide strategy.

In Northern Europe, the frequency of homozygous Z-carriers is about 1 in 2000. Homo- and heterozygous carriers of M, S, and Z alleles constitute >98% of the population in Northern Europe. The other 2% exists of hetero- or homozygous carriers of alleles that have not been associated with pulmonary diseases, e.g., the F, I, and P alleles. Null allele products cannot be detected in serum at all and are therefore found only by familybased screening in combination with DNA sequencing techniques. Rare deficiency alleles are detected from their deviant banding pattern in IEF or from DNA sequencing. But for the more common deficiency phenotypes (MS, SS, SZ, and ZZ), the variant-specific antibodies may be a welcome supplementary tool in α_1-AT typing.

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Chapter 7

Generation and selection of monoclonal antibodies to variants of α1-antitrypsin specific for one single amino acid using synthetic peptides: importance of conjugation of synthetic determinants

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GENERATION AND SELECTION OF MONOCLONAL ANTIBODIES TO VARIANTS OF α1-ANTITRYPSIN SPECIFIC FOR ONE SINGLE AMINO ACID USING SYNTHETIC PEPTIDES: IMPORTANCE OF CONJUGATION OF SYNTHETIC DETERMINANTS

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I. INTRODUCTION

α_1-Antitrypsin (α_1-AT) is a serum protein that serves as the major inhibitor of neutrophil elastase. The Z mutation is a single base substitution in the normal M allele causing a GLU to LYS change at position 342 in the molecule. The ZZ phenotype is associated with a severe deficiency of α_1-AT with serum levels 10% of normal. Individuals with an α_1-AT deficiency are at an increased risk of developing lung emphysema. Antibodies that specifically can detect the 342 position would be very helpful for reasons of early diagnosis. Pointmutations, however, are not readily detected by polyclonal antibodies and in addition the sequence that includes the mutation may not be located in an antigenic site of the protein. MAbs were therefore generated using synthetic peptides.

II. RESULTS AND DISCUSSION

We synthesized several synthetic peptides which include the 342 position for both the M and the Z variant. After conjugation of peptides to a carrier protein KLH with different coupling agents (MBS, EDC and glutaraldehyde), we found a protein specific response, detected in a direct ELISA, after immunization with the MBS conjugate only.
Location of the position of the mutant specific amino acid in the peptide appeared to be of importance with respect to the sequence of the B cell determinant which was recognized on the peptide. Position distal of the coupling site leads to readily recognition of the determinant.
In addition, the method applied for selection of the specific hybridomas was a crucial and point in the whole process. Following fusion of stimulated spleen cells with myeloma cells, we selected several MAbs specific for the 342 region with either glutamic acid or lysine at this position. MAb F43.8.1 (M-variant specific) was selected in an ELISA with the antigen coated to the plate. The affinity for α_1-AT in solution as used in a capture ELISA (fig 1) is low compared to α_1-AT adsorbed to an ELISA plate. Thus, when antibodies are selected with particular methods, then these antibodies do not necessarily have to recognize the same protein presented in a different method.

Polyclonal rabbit antibodies (PAbs) specific for α_1-AT shield or change the 342-region of α_1-AT. Using these PAbs as capture antibodies, we obtained MAb's which probably recognized a sequence situated N-terminal of the 342 position in α_1-AT.
The selected MAb F46.4.1 recognizes all variants and is not specific for the variant α_1-AT(Z). However, when we first incubated MAb's to be selected with variant α_1-AT(Z) and then transferred the mix to the wells coated with rabbit PAbs anti α_1-AT for the sandwich ELISA, we obtained Z-specific MAbs F50.4.1. These experiments underline the importance of the selection strategy in the production of MAbs. Development of polyclonal and monoclonal capture antibodies, which do not prevent binding of the variant specific MAbs, is at present in progress.
We demonstrated that it is possible to raise specific antibodies against strongly homologous proteins that differ only in one amino acid. We developed an immuno-blot assay using IEF gels in which it is possible to detect homo- or heterozygous carriers for the Z-allele [1] with MAb F50.4.1 in combination with MAb F43.8.1 or MAb F46.4.1. Our method applying immunoblotting is simple, sensitive and specific. The selected MAbs can also be used as capture antibodies in a sandwich ELISA for rapid detection of Z carriers. The synthetic peptide approach has shown to offer perspectives in the development of diagnostic assays with site specific MAbs that could discriminate between strong homologous proteins.

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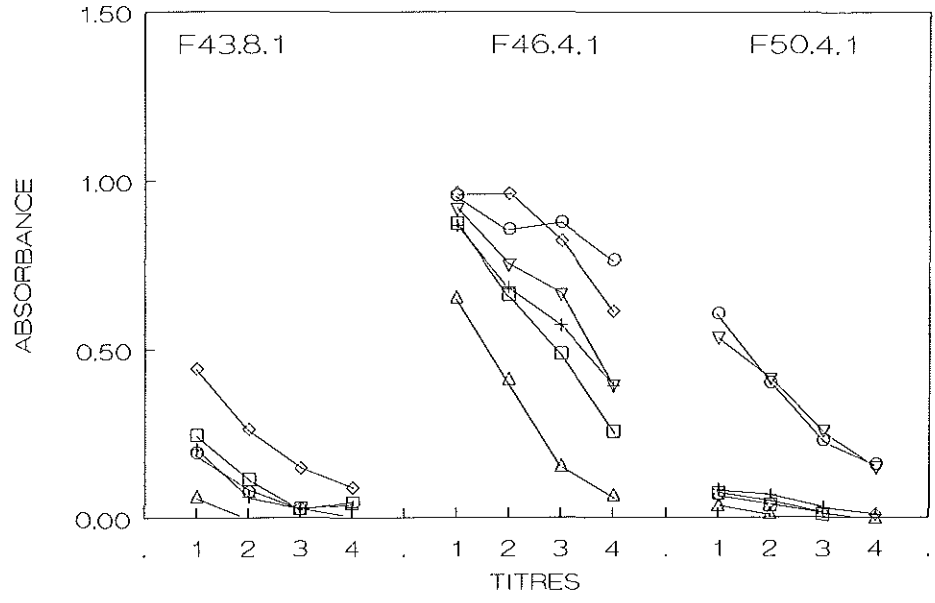

Fig. 1. Detection of α_1-AT variants in a capture ELISA. Non-Z α_1-AT specific MAb F43.8.1 (left), α_1-AT specific MAb F46.4.1 (middle) and Z α_1-AT specific Mab F50.4.1 were used as capture antibodies. ^{2}Log serial dilutions (initial dilution 1:50) of human sera with the M1M1 □, M2M2 +, M1S ◊, SS △, M3Z O and ZZ ▽ phenotype were incubated for 1 hour and subsequently detected with α_1-AT specific polyclonal rabbit serum, swine anti rabbit Ig conjugated to alkaline phosphatase and p-nitrophenylphosphate as substrate.

PART III

Chapter 8

Use of T cell epitopes in raising immune responses

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USE OF T CELL EPITOPES IN RAISING IMMUNE RESPONSES

Netty D. Zegers and Wim J.A. Boersma

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I. INTRODUCTION

Synthetic peptides are valuable tools to raise antibodies crossreactive with the native proteins from which the sequences were derived. Most randomly chosen short peptides consist of a B cell determinant which, by definition, does not induce an epitope-specific immune response by itself. Introduction of a T cell epitope is necessary to offer T cell help and memory for a humoral immune response. A T cell determinant can activate specific T cells to synthesize certain lymphokines necessary for T and B cell activation, interaction and proliferation. The T cell determinant needs to be presented to T cells bound in the groove of an MHC molecule at the cell surface of antigen presenting cells. The peptide-MHC complex is recognized by the T cell receptor (TCR) at the T cell. Therefore, a T cell determinant contains at least two domains: one which binds to the groove of the MHC molecule and one for interaction with the TCR. In peptide immunization, the T cell epitope is usually supplied by a carrier protein to which synthetic peptides are covalently coupled. Alternatively, a short synthetic determinant with a distinct sequence motif which can be presented by MHC class II to T cells can be linked directly to a B cell epitope.

Milich et al. (1986) were among the first to demonstrate that a synthetic peptide immunogen, encompassing one T cell epitope and one B cell epitope, could be used to raise anti-native protein antibodies. These epitopes were non-overlapping peptide bond linked determinants situated in the same region of the pre-S(2) protein of hepatitis B surface antigen. With constructs of a similar synthetic peptide containing a T cell determinant of the circum-

sporozoite protein of the *Plasmodium falciparum* and a major B cell determinant from the same protein covalently linked with MBS, it was possible to elicit high-titer antibodies against the B cell epitope (Good et al., 1987). Hybrid constructs of T and B cell determinants derived from different proteins made by linear synthesis (Francis et al., 1987a; Cox et al., 1988; Sinigaglia et al., 1988; Borras-Cuesta et al., 1987; Palker et al., 1989) or linked via glutaraldehyde (Leclerc et al., 1987; Jolivet et al., 1990) induced antibodies against the B cell determinant of the hybrid peptides which were crossreactive with the native protein. This proved that the anti-B cell determinant responses also may be elicited with foreign helper T cell determinants.

An immunogen consisting of only one B cell epitope and one T cell epitope may be advantageous over a large carrier-hapten complex immunogen. Since the number of antigenic sites in the peptide immunogen is restricted, implicating the lack of antigenic competition, such a construct may be useful to produce antibodies against weakly immunogenic regions of a large protein. Also immunodominance resulting from the carrier protein could be circumvented.

Here, we will describe some aspects concerning the use of T cell determinants for MHC class II presentation to T cells to raise high-titer native protein specific antibodies.

II. SELECTION OF T CELL EPITOPES

Dependent on the specific application of a synthetic antigen to be used for immunization, the choice of a T cell epitope sequence for incorporation in a synthetic antigen is more or less fixed. For vaccines, T cell determinants have to be derived from the pathogen sequence, since a pathogen-specific memory response is needed at the time a pathogen enters the body. Identification of strong T cell epitope fragments in protein sequences is therefore of importance. These T cell determinants preferably should be promiscuous, i.e., suitable for stimulation of multiple MHC haplotypes. A vaccine constructed with such a promiscuous T cell determinant then is active in a major part of the population.

To raise protein-specific antibodies applicable for research or diagnostic purposes, it is important to select a T cell determinant that is dominant in the target animal and restricted for the target animal's MHC class II haplotype. In this case, the T cell epitope sequence may be derived from any other protein than the protein from which the B cell epitope is derived. A limited number of known T helper cell determinants for use in different strains of mice is listed in Table 1.

A number of methods have been proposed for prediction of T cell determinants and are described in detail elsewhere in this edition (Chapters 1–6). A brief summary is made in this paragraph. Prediction only is possible if the exact amino acid sequence of this protein is known. On the other hand, T cell epitopes can be assigned in a more pragmatic approach by using T cell stimulation assays with protein fragments (see below). It may be clear that only linear T cell epitopes can be predicted this way.

TABLE 1
Murine MHC restricted T cell epitopes

MHC	AA residues	Sequence	References
Hen egg lysozyme			
H-2^a	46–61	NTDGSTDYGILQINSR	Gammon et al., 1991
H-2^d	20–35	YRGYSLGNWVCAAKFE	"
H-2^d	116–129	KGTDVQAWIRGCRL	"
H-2^b	74–90	NLCNIPCSALLSSDITA	"
H-2^b	30–53	CAAKFESNFNTQATNRNTDGSTDY	"
H-2^b	81–96	CSALLSSDITASVNCAK	"
I-E^k	1–18	KVFGRCELAAAMKRHGLD	Adorini et al., 1988b
I-A^k	51–61	TDYGILQINSR	"
I-A^k	112–129	RNRCKGTDVQAWIRGCRL	"
H-2^d	108–120	WVAWRNRCKGTDV	Gammon et al., 1991
H-2^d	105–129	MNAWVAWRNRCKGTDVQAWIRGCRL	Adorini et al., 1988a (2 apparent I-E^d sites)
Ovalbumin			
H-2^d	323–339	ISQAVHAAHAEINEAGR	Shimonkevitz et al., 1984
Sperm whale myoglobin			
H-2^k	26–40	QDILIRLFKSHPETL	Kim et al., 1990
I-A^d	102–118	KYLEFISEAIIHVLHSR	Cease et al., 1986
I-E^d	136–146	ELFRKDIAAKY	Berkower et al., 1986
Pigeon cytochrome *c*			
I-E^k	95–104	LIAYLKQATAK	Carbone et al., 1987
λ Repressor protein			
I-A^d; I-E^k	12–26	LEDARRLKAIYEKKK	Guillet et al., 1986 Lai et al., 1987
I-E^d	12–26	LEDARRLKAIYEKKK	Sette et al., 1990
Influenza hemagglutinin protein (A/PR8/48)			
H-2^d	111–119	FERFEIFPK	Hackett et al., 1985
I-A^k	129–140	NGVTAACSHEGK	Sette et al., 1989a
Plasmodium falciparum circumsporozoite coat protein			
I-A^b	365–380	NANPNANPNANPNANP	Good et al., 1987
Promiscuous	378–398	DIEKKIAKMEKASSVFNVVMS	Sinigaglia et al., 1988

T cells recognize peptide fragments presented in a groove of the MHC class II molecules at the cell surface of antigen presenting cells. These peptide fragments which originate from processing of a protein by an antigen presenting cell are usually 8–12 residues in length. T cells recognize only the primary and secondary structure of the protein, but not the tertiary structure of the native protein. As a result, prediction of T cell recognition sites can be based on secondary structure and the amino acid sequence of the native protein. Therefore, most theoretical prediction methods are merely based on the feasibility of peptide binding in the groove of the MHC molecule, ignoring the interaction with the TCR.

By analyzing functionally recognized T helper determinants, the hypothesis was formulated that T cell determinants tend to be amphipatic helices, α- helices with hydrophobic residues on one side and hydrophilic residues on the other side along the helix (DeLisi and Berzofsky, 1985; Berkower et al., 1986; Spouge et al., 1987). Based on this hypothesis, an algorithm called AMPHI was developed for predicting such T cell determinants (Margalit et al., 1987). The computer program identifies regions that tend to form amphipatic helices by scoring both the strength of hydrophobic moments as a function of the periodicity, within seven amino acids, and the lengths of positive segments.

An alternative method was developed by the group of Humphreys (Elliott et al., 1987; Stille et al., 1987). They demonstrated that the strip-of-helix hydrophobicity index (SOHHI) (mean hydrophobicity of residues at positions n, $n+4$, $n+7$, $n+11$, etc.) identified T cell-presented epitopes, presumably on the basis of quantifying the cooperativity among hydrophobic residues in such longitudinal hydrophobic strips. Based on the SOHHI, they developed the strip-of-helix hydrophobicity algorithm (SOHHA). The degree of hydrophilicity of the non-hydrophobic side of the helix was not relevant to the selection of the T cell-presented epitope. The algorithm was proposed to select both class I and class II restricted epitopes (Reyes et al., 1988).

A third method for selection of T cell determinants was the method developed by Rothbard and Taylor (1988). They formulated an algorithm based on the motif (charged or Gly)-(hydrophobic)-(hydrophobic)-(hydrophobic or Pro)-(polar or Gly). The motif was postulated on the basis of analysis of functional T cell epitopes. Sette et al. postulated a similar motif for interaction of peptides with I-A^d or I-E^d (Sette et al., 1987, 1988, 1989a, 1989b, 1989c). For I-E^d, the motif was composed of three basic residues. The first basic residue was found either in positions 1, 2 or 3. The other basic residues were found in position 4 and 6, whereas position 5 was usually occupied by a non-charged or somewhat hydrophobic amino acid. Using this motif based method, they predicted three overlapping I-E^d binding sites in dynorphin, an opioid peptide. The peptide 1–13 of dynorphin was found to bind with more than a 10-fold higher affinity than other good I-E^d binding peptides (Sette et al., 1990).

The efficacy of three methods for predicting T cell epitopes (AMPHI, SOHHA and the motif of Rothbard and Taylor) was evaluated (Reyes et al., 1990). By comparing functional T cell determinants and predicted sequences, the SOHHA methods scored higher in terms of overlap (ratio predicted over experimental sequence residues ≥ 0.5), sensitivity (correct predictions/number of reported T cell-presented sequences) and efficiency (correct

predictions/number of predictions). However, it is not clear which methods were used for tracing the known T cell determinants involved in this analysis (Reyes et al., 1990). The T cell epitopes used in this study for comparison with predicted sequences were from the literature communicated by multiple researchers which have used a variety of functional assays. The number and sequence of the experimentally determined known T cell epitopes is dependent on the assays and strains of mice used (Gammon et al., 1991). More extensive, precise, experimental localization of T cell epitopes may thus alter the outcome of the comparison of the three methods.

Prediction of T cell determinants does not allow for MHC polymorphisms. Many known T cell determinants are MHC restricted. Moreover, predicted sequences, capable of binding to MHC molecules, are not always immunogenic because of a deficient T cell repertoire. For these reasons, some investigators favor the empirical approach for localization of T cell determinants. Analysis of a protein is carried out by stimulation of protein primed T cells with overlapping peptides. The most comprehensive method uses the PEPSCAN strategy initially developed by Geysen et al. (1984) and since then many times improved and refined (van der Zee et al., 1989). The advantage of the PEPSCAN method using peptide synthesis onto pins is the possibility of synthesizing a high number of different peptides. Scanning a protein sequence in single amino acid steps with a series of sequential peptides allows exact localization of the T cell determinants present in the protein. Complete scanning of lysozyme using this method for three strains of mice with different haplotypes showed markedly different T cell determinants for the strains used (Gammon et al., 1991).

An alternative functional method for tracing MHC class II restricted T cell determinants was developed in our laboratory (van Noort and van der Drift, 1989). This method is of major importance when the amino acid sequence of the protein is not known, but it is also applicable for known sequences. The protein is mildly digested by cathepsin D, a lysosomal/endosomal enzyme involved with cellular antigen processing. Subsequently, the fragments are separated and used in a T cell stimulation assay. Using this method, several proteins were analyzed for the presence of T cell determinants (van Noort et al., 1991; Boots et al., 1991; Boog et al., 1993). Cathepsin D recognizes a certain motif of eight residues as substrate for digestion. Retrospectively, based on experimental data, the motif (hydrophobic)-(Leu)-(hydrophobic)-(charged, polar or small)-(hydrophobic)-(hydrophobic)-(charged, polar or small)-(hydrophobic) was postulated (Van Noort and Van der Drift, 1989; see also Chapter 7 in this edition). In 50% of all sequences cleaved, a Leu residue is found at position P1 (the residue prior to the scissile bond). The N- terminus of a processed T cell determinant is located adjacent to the cleavage site formed by cathepsin D. T cell determinants may be predicted in this way on the basis of the cathepsin D substrate motif.

Fundamental questions about the length of the T cell determinant are difficult to answer (see also the following paragraphs). For MHC class II with an open peptide binding groove, a variety of peptide lengths can be found, but the minimal length appeared to be 8–10 amino acids (Brown et al., 1993; Rudensky et al., 1991; Chicz et al., 1993).

In conclusion, it is clear that present prediction methods are not fully efficient due to the fact that they are based on peptide-MHC interaction. For a functional T cell determinant,

the interaction of this complex with the TCR is highly important. This interaction is, however, hard to predict. Selection of T cell determinants in a functional assay is most efficient, with the advantage that the target animal of choice can be involved. Especially for application in vaccines, experimental determination of useful sequences appears to be the method of choice.

III. T CELL EPITOPE RESTRICTION

Prediction methods for T cell epitopes do not take MHC polymorphism into account. Apart from MHC restricted T cell determinants (see Table 1), numerous promiscuous T cell determinants, capable of binding to different MHC haplotypes, were described also. Milich et al. (1988) demonstrated that two different MHC class II restricted T cell determinants combined with a B cell determinant incorporated in one construct yielded an immunogen which was capable of eliciting B cell determinant-specific antibodies in mouse strains with different MHC haplotypes (Table 2).

TABLE 2
Antibody responses to peptide constructs with MHC restricted T cell epitopes

Construct	Anti-BD antibodies in mice with MHC haplotype	Remarks	References
TD^{x}-TD^{y}-BD	x; y		Milich et al., 1988
$TD^{k}(1)$-BD	k; b	BD=TD in H-2^{b} mice	Rzepczyk et al., 1990
$TD^{k}(2)$-BD	k; b		
$TD^{k}(3)$-BD	k; b		
$TD^{k}(4)$-BD	k; b		
$TD^{k}(5)$-BD	k; b		
TD^{b}-TD^{k}-BD	b; k	Also Abs to TD^{b} and TD^{k}	Londoño et al., 1990
TD^{k}-BD	k; d	New H-2^{d} TD possibly formed in conjunctional sequence	
$TD^{promisc}$-BD	b; d; f; k; q; r; s		Sinigaglia et al., 1988

x and y can be any haplotype.

High-titer NANP-specific antibodies could be raised in both H-2^b and H-2^k mice after immunization with hybrid two-component peptides consisting of the NANP repeat from the immunodominant surface antigen of circumsporozoite protein (CSP) from *Plasmodium falciparum* and five different epitopes (T cell determinants) of the merozoite surface antigen 2 (MSA2) of *P. falciparum* (Rzepczyk et al., 1990). The NANP repeat itself is poorly immunogenic in H-2^b mice only (DelGuidice et al., 1986; Good et al., 1987). Even in studies using the $(NANP)_n$ repeat coupled to a carrier protein, only a low level of anti-$(NANP)_l$ was induced. This study also confirms the finding that genetic restriction can be overcome by addition of the proper epitope. A more striking finding was that the simple addition of a cysteine to the N-terminus of the peptide $(NANP)_6$ markedly enhanced the immunogenicity in H-2^b mice and generated antibodies in the normally non-responder H-2^k mice. By introducing a Cys, dimers could possibly be formed, which could account for better T cell stimulation (see also paragraph on multiple copies), or a new T cell epitope could be formed in the junctional sequence of the dimer. There appeared to be some proof for the formation of dimers, since blocking the free -SH group renders the peptide non-immunogenic. Enhanced immunogenicity after the addition of a cysteine residue was reported for poliovirus peptides, hepatitis B surface Ag peptides and foot-and-mouth disease viruspeptides (Ferguson et al., 1985; Dreesman et al., 1982; Brown et al., 1984; Francis et al., 1987b).

The action of different MHC class II restricted T cell epitopes together in one hybrid peptide construct was explored in studies described by Londoño et al. (1990). They synthesized a tri-component peptide composed of (1) an H-2^b restricted tandemly repeated tetrapeptide sequence (NANP) from the immunodominant surface antigen of circumsporozoite protein (CSP) from *Plasmodium falciparum*, (2) an H-2^k restricted T helper cell epitope (referred to as Th2R) from the non-repetitive part of the CSP sequence, and (3) a pure B cell determinant from *P. falciparum* liver stage Ag, the LSA, a 17 residue repeat sequence. Special attention was paid to the connection of the three sequences to obtain a reciprocal reinforcement of the helical potentials of the Th2R and LSA helical components. With this tri-component peptide $(NPNA)_4N$-Th2R-LSA_{27}, high and long lasting levels of antibodies were raised to all three components after a single immunization without adjuvant in mice with H-2^b (B6 and B10 mice), H-2^k (B10BR and C3H/He mice) and also H-$2^{d/b}$ (F1(BALB/cxB6) mice) background, but not in H-2^d mice (Table 2). The experiments indicated that each sequence containing an active T helper site induced antibodies against itself and helped antibody production to neighboring sequences, overcoming the genetic restriction of the immune response to the latter. Surprisingly, a di-hybrid peptide Th2R-LSA_{27} induced antibodies recognizing the LSA sequence in H-2^d mice in addition to antibodies against Th2R and LSA in H-2^k mice. A newly formed H-2^d epitope may be formed in the overlapping portion of the hybrid peptide. This H-2^d epitope was, however, not active in the tri-component peptide. Some hypotheses were proposed for this phenomenon. First, the NPNA repeats could induce in H-2^d mice a specific T cell mediated suppression of the antibody response against the rest of the determinants in the tri-component hybrid. Possible suppression in H-2^d mice might be overcome by conjugating the tri-component peptide to carrier proteins. Other explanations for the differences in the priming capacity between the

hybrids include differences at the level of the cognate interaction between T and B cells, differences in antigen processing and presentation or major alterations in the tri-hybrid peptide of the predicted α-helix secondary structure of Th2R-LSA_{27}. These experiments clearly demonstrate the necessity to carefully explore all possible actions of the constituting components of a hybrid construct.

Sinigaglia et al. (1988) localized a promiscuous T cell determinant in the CSP of the malaria parasite *P. falciparum* (residues 378–398 made with two Cys→Ala substitutions to prevent formation of a disulphide) which is recognized in association with most mouse and human MHC class II molecules. In mice with different genetic backgrounds (haplotypes b, d, f, k, q, r and s), antibody titers specific for $(NANP)_3$ were raised with a synthetic hybrid peptide composed of the $(NANP)_3$ sequence, which is a B cell determinant, and the T cell determinant AA 378–398. The elicited antibodies were also crossreactive with sporozoites. T cell clones from eight non-immune human donors with different haplotypes (DR1, DR2, DR4, DR5, DRw6, DR7 and DR9) were derived upon *in vitro* stimulation with that T cell determinant. Such a promiscuous T cell determinant may be valuable for incorporation in a synthetic anti-malaria vaccine, since individuals with different MHC class II alleles would respond to such a vaccine.

In a study performed by Su and Caldwell (1992), a chimeric peptide composed of a T helper and a B cell determinant of the *Chlamydia trachomatis* major outer membrane protein induced antibodies in strains of mice with different H-2 haplotypes. The peptide sequence used as the T cell determinant was rather long (25 residues) and might therefore contain more than one T cell determinant. The antibodies all recognized the B cell determinant regardless the strain of mice used, but neutralizing antibodies were only found in some strains. The fine specificity of the antibodies as determined with the PEPSCAN method (Geysen et al., 1984, 1987) did not appear to be MHC related, since both neutralizing and non-neutralizing antibodies were detected in strains of the same MHC haplotype. The difference in fine specificity may be a B cell related phenomenon, such as a "hole" in the B cell repertoire, or differences in genes that control the production of antibody to the epitope.

In conclusion, MHC restriction to a certain synthetic construct may be overcome by incorporating a T cell epitope with the proper MHC restriction (Milich et al., 1988; Rzepczyk et al., 1990; Londoño et al., 1990) or a promiscuous T cell epitope (Sinigaglia et al., 1988; Su and Caldwell, 1992). Special attention should be paid to the conjunctional site of two components, since new T cell epitopes may be formed or existing T cell epitopes may be lost (see also the following sections).

IV. INFLUENCE OF CHAIN ELONGATION ON T CELL EPITOPES

Ample evidence for the positive influence of elongated T helper cell determinants concerning T cell and antibody responses has been collected in several studies.

Childerstone et al. (1990) determined the minimal peptide sequence from the N-terminal region of a 3800 MW streptococcal antigen (residues 6–15) that stimulated T cells. Elongation of this T cell epitope with six residues (to residues 1–16) enhanced the proliferative T cell response roughly with a factor 2. These results were confirmed in a study in which an extended peptide was made to increase the amphipathic score of a T cell determinant, residues 26–40 from sperm whale myoglobin (Kim et al., 1990). These latter elongated peptide (residues 24–42) induced proliferation of specific T cell clones at a 30-fold lower concentration than the short peptide.

Ertl et al. (1991) showed that even very short peptides of 3–5 amino acids from the immunodominant region of the viral nucleoprotein of rabies virus (AA 404–418) were able to stimulate a T cell clone generated with intact rabies virus. They tested a series of peptides varying in length (3–32 amino acids long). The ability of peptides to induce a response was inversely correlated with their lengths. Short peptides had to be used at 10^6 times higher concentrations as compared to the long peptides (15 or 32 amino acids long). The specificity of the T cell response, however, was directly correlated to the length of the peptides. The response to 15 amino acids long peptides showed a high degree of specificity and the response to 3 to 5 amino acid long peptides showed a high degree of degeneration (flexibility). The long as well as the short peptides had to be presented in association with the same MHC class II haplotype ($I\text{-}E^k$).

In contrast to the studies described above, truncation of the N-terminus with one or two amino acids (Ser or Ser-Asp) of a minimal class I cytotoxic T lymphocyte (CTL) epitope (SDYEGRLI) from the nucleoprotein of influenza A/PR resulted in a higher binding affinity for a class I molecule than the epitope (Cossins et al., 1993). The minimal CTL epitope was determined in a functional ^{51}Cr release assay for recognition of a nested series of peptides by a specific class I restricted CTL clone in the presence of target cells. The relative binding affinities were measured using an indirect functional competition assay. Briefly, a suboptimal epitope (IEGGWTGMIDGW) and varying concentrations of competing peptides were incubated with specific (for IEGGWTGMI) effector cells and labeled target cells and subsequently ^{51}Cr release was measured. The N-terminal truncated peptides showed a higher inhibition of lysis than the minimal CTL epitope. The truncated peptides could not be recognized, however, by a class I restricted CTL clone. This study emphasizes the importance of choosing the proper functional read-out system when assigning the consequences of an engineered peptide construct on immune responses.

Differential effects of chain elongation of T cell epitopes on the specificity of the antibody response in the mouse appeared to be strain related (Partidos and Steward, 1992c). A peptide sequence derived from the fusion protein of measles virus (residues 240–252, GDINKVLEKLGYS(C)) induced both T and B cell reactivity in several strains of mice (C57BL/6, CBA, A/J and TO), but it elicited no reactivity in BALB/c and SWR mice. Elongation of this peptide sequence with six amino acids (GGDLLG(C)) at the C-terminus showed both positive and negative effects. Extension was based on the presence of residues of moderate hydrophilicity, flexibility and protrusion index along with the presence of a β-turn. A cysteine residue at the carboxyl end of each peptide was introduced to increase

immunogenicity (Francis et al., 1987b). The elongated peptide did induce an antibody response in BALB/c and SWR mice, but the B and T cell responsiveness disappeared in C57BL/6 mice. In addition, the antibody response was significantly higher after immunization with the elongated peptide than with the shorter one.

Using a major immunogenic T cell determinant of pigeon cytochrome *c* (residues 92–103), it was reported that N-terminal extension of the minimally required 12 to 23 residues (stepwise addition of one amino acid was carried out) maximally stimulated cytochrome *c*-specific T cell hybridomas (Srinivasan et al., 1993). The elongated peptide had the ability to bind directly to MHC class II molecules and did not require processing. Even the addition of a second T cell epitope of tetanus toxoid to the cytochrome *c* sequence, which resulted in a peptide of 51 residues, further enhanced stimulation of cytochrome *c*-specific T cells without processing. Thus, regions of the antigenic peptide which lie outside the proposed peptide binding groove of the class II molecule can influence presentation *in vitro* and subsequently the stimulation of specific T cells.

Elucidation of the structure of the MHC class I (Bjorkman et al., 1987) and class II molecules (Brown et al., 1993) together with binding studies revealed some striking differences between the two MHC classes apart from similarities. The class I peptide-binding groove is blocked at either end, implicating that only peptides with restricted size (8–10 residues) can bind. The class II binding groove is open at both ends and allows binding of a peptide which may stick out at both ends of the groove. Peptides varying in size (13–25 residues) could be extracted from class II molecules (Rudensky et al., 1991; Chicz et al., 1993).

In conclusion, interpretation of the results discussed with respect to determination of T cell epitope length is hampered, since in only a few experiments was the exact optimum T epitope determined. Elongation of T cell determinants may have a dual effect. On the one hand, increased stability of a helix as a result of a longer peptide chain may enhance peptide binding to class II binding. The composition of the sequences used for elongation is, of course, of importance in achieving a helix conformation. This is not the case for class I molecules, since the length of the bound peptide is fixed. On the other hand, elongation may more effectively result in an enhanced immune response in animals with a different MHC haplotype than with a shorter TD. Keeping this in mind, extension of a T cell determinant probably will be most effective for class II determinants. Elongation of T cell epitopes positively influences the strength of the immune response and may influence the restriction pattern to MHC binding.

V. POSITIONING OF T CELL EPITOPES IN CHIMERIC PROTEINS

Different combinations of a T cell determinant (residues 65–85) and a B cell determinant (residues 422–436), both derived from the mycobacterial 65-kD protein, were used to study whether it was possible to stimulate the antibody response against a poor immunogenic B cell determinant (BD) by coupling this determinant to a potent T cell determinant (TD) (Cox et

al., 1988). The orientation of the two determinants proved to have a profound effect on the magnitude and specificity of the antibody response. Only the BD-TD construct and not the TD-BD construct elicited antibodies specific for the B cell determinant (Table 3). Strikingly, both tested hybrid peptides stimulated T cell proliferation equally well. The TD monomer as well as the hybrid peptides (TD-BD and BD-TD) induced TD specific antibodies. The extent of the TD-specific antibody response was substantially decreased after immunization with the TD-BD construct. The TD, in monomer and hybrid, has a well-defined α- helical structure. The proline residue at the N-terminus of the B cell determinant is likely to disturb the α-helical nature of the hybrid TD-BD construct, resulting in an abrogated BD-specific antibody response in case of the TD-BD. The role of the proline at the N-terminus of the BD-TD construct is not important for the secondary structure of the construct, and antibodies specific for the B cell determinant can be elicited.

TABLE 3
Antibody responses elicited with hybrid TD-BD constructs

Immunogen	Anti-TD	Anti-BD	Remarks	References
TD-BD	+	-	Proline at N-	Cox et al., 1988
BD-TD	+	+	terminus BD	
TD-BD	-	+		Golvano et al., 1990
BD-TD	+	-		
TD-BD	-	+	Linear	Levely et al., 1990
BD-TD	+	-	Linker (BMH coupling)	
TD-BD		+		Partidos et al., 1992a
BD-TD	+		Appeared to be strain related	Partidos et al., 1992b
(151–174)-(133–143) =TD-BD	-	+		Milich et al., 1990
133–174 =BD-TD	+	-		

In another study, described by Golvano et al. (1990), combinations of two T cell determinants and two B cell determinants in hybrid constructs were evaluated for their ability to raise antibody responses. It was demonstrated that TD-BD constructs elicited antibodies specific for the B cell determinant and no, or low levels of, antibodies specific for the T cell determinant. Contrary to this, the BD-TD constructs induced antibodies specifically directed against the T cell determinant and not, or only at a low level, against the B cell determinant. It was hypothesized that enzymatic processing of the constructs by antigen presenting cells probably might be responsible for the difference in antibody responses. The lysine or arginine at the C-terminus of the T cell determinant may provide a cleavage site for certain proteases. Such a site is not present in the BD-TD constructs; here, the lysine or arginine is at the C-terminus of the complete construct.

Levely et al. (1990) compared a linearly synthesized construct yielding a TD-BD configuration with a construct in which the T cell and the B cell determinant were chemically coupled to each other via their terminal cysteines with the linker bismaleimidohexane yielding a BD-(linker)-TD construct. The TD-BD construct elicited BD-specific antibodies, but with the BD-(linker)-TD construct TD-specific antibodies were found only. Proliferative responses were similar for both constructs. The orientation of the TD appeared to determine the specificity of the antibody response. However, it is not quite clear whether this is due to the method used for coupling of the determinants or a mere consequence of the polarity of the hybrid peptide.

The importance of the mutual orientation of T and B cell determinants in one hybrid construct on the antibody response induced with such constructs was confirmed by Partidos et al. (1992a). Synthetic constructs were used with a T cell determinant (residues 288–302) and a B cell determinant (residues 404–414) from the F protein of measles virus. Chimeric peptides in which the T cell epitope was synthesized to the N-terminus of a B cell epitope induced primarily antibodies which recognized the B cell determinant. In contrast, localization of the T cell epitope at the C-terminus of a B cell epitope induced antibodies preferentially reactive with the T cell epitope. In a later study, it was found that positioning of the T cell epitope was strain related (Partidos et al., 1992b). In TO mice (H-2^v), the N-terminal orientation of the T cell epitope with respect to the B cell epitope was the most effective, whereas in CBA mice (H-2^k) the C-terminal orientation was the best in inducing high levels of antibodies specific for the B cell epitope. This may reflect differences in processing and presentation of the chimeras by the two mouse strains. The strain dependency was previously reported in a study using multiple antigen peptides with epitopes from the *P. falciparum* circumsporozoite (Munesinghe et al., 1991).

Both the orientation of the T and B cell determinants and the context of the T cell site within the larger composite peptide was found to influence both antibody fine specificity and T cell fine specificity (Milich et al., 1990). A hybrid peptide consisting of the dominant T cell determinant of the pre-S(2) region of the hepatitis B surface antigen (residues 151–174) positioned N-terminally to a dominant B cell determinant (residues 133–143) from the same protein yielded an effective pre-S(2) synthetic immunogen (151–174)-(133–143) = TD-BD. Antibody and T cell responses obtained with this hybrid construct were compared with the

responses obtained with a linear peptide from the native sequence (residues 133–174) in several strains of mice with different MHC class II haplotypes. The 133–174 peptide induced anti-peptide antibodies in all strains, but these antibodies were not crossreactive with the native protein. The level of antibodies specific for the 156–174 peptide (a minor antibody site after immunization with HBsAg) was significantly higher than the levels of antibodies specific for the 120–145 peptide, which contains two dominant group-specific pre-S(2) antibody sites 133–139 and 137–143. Strikingly, reversion of the T and B cell determinants induced a qualitatively different immune response. The (151–174)-(133–143) construct (=TD-BD) generated antibodies primarily specific for the 133–143 sequence. Above all, these antibodies recognized the native HBsAg protein. A low level of antibodies specific for the 156–174 region was found. Also T cell responses of lymph node cells from mice primed with one of the peptides qualitatively differed. Two overlapping T cell epitopes, 156–170 and 161–174, were found in the 151–174 region. The 133–174 primed T cells recognized the 156–170 site, but not the 161–174 site. Conversely, (151–174)-(133–143) primed T cells preferentially recognized the 161–174 site, and the 156–170 site was less well recognized. In addition, it was observed that differences in T cell specificity occurred after elongation or shortening of the T cell determinant (148–174, 156–174, 161–174).

Löwenadler et al. (1992) investigated how individual B and T cell epitopes are functionally linked to each other in a multi-determinant fusion protein. They constructed fusion proteins with two different B cell determinants derived from different proteins (Table 4). The two B cell determinants were separated by one, two or four copies of a dominant T cell epitope (ovalbumin residues 323–339) (Shimonkevitz et al., 1984; Buus et al., 1987) dominant in H-2^d mice (BALB/c). A peptide spacer (an IgG binding peptide) was positioned upstream or downstream of the T cell epitopes. The immunological relationship between T and B cell epitopes was examined by measuring specific T cell activation and antibody responses in animals immunized with chimeric peptides containing the inserts in varying numbers (1, 2 and 4 copies). The antibody responses against B cell determinants at the amino and the carboxy terminus, respectively, were differently influenced by the molecular localization of the inserted Th determinant (Table 4). All fusion proteins containing the Th epitopes induced antibody production against the B cell determinant at the amino terminal end irrespective of the positioning of the T cell determinants. Only a very weak antibody response was observed against the B cell determinant at the carboxy terminus after immunization with fusion proteins with single or multiple copies of the T cell determinant in the adjacent position. Similar results were obtained with constructs containing one or two copies of the T cell determinant in a distal position. This B cell determinant proved to be a minor epitope in these constructs. However, four copies of the T cell determinant in a distal position markedly enhanced the antibody response. The authors concluded that specific T cell help for antibody production was more effective when the T cell determinants were placed in a distal position. Furthermore, a fusion protein containing four copies of the ovalbumin (OVA) T cell determinant effectively elicited T cell help for high levels of antibody production against both B cell determinants examined, showing that activated Th cells recognizing a single epitope simultaneously provide help for distinct sets of B cells specific for widely

TABLE 4
Antibody responses elicited with fusion proteins

Immunogen	Anti-BD1	Anti-BD2
BD1-spacer-TD-BD2	+++	-
BD1-spacer-$(TD)_2$-BD2	+++	-
BD1-spacer-$(TD)_4$-BD2	++++	+
BD1-TD-spacer-BD2	++	-
BD1-$(TD)_2$-spacer-BD2	+++	±
BD1-$(TD)_4$-spacer-BD2	++++	+++

From Löwenadler et al. (1992).

spaced epitopes within a protein. Only the influence of inserting a spacer between the T cell determinant and the B cell determinant was investigated in these experiments. They did not investigate the influence of positioning the T cell determinant(s) N- or C-terminally of the same B cell determinant.

The examples above (Table 3) demonstrate that in most cases the antibodies are elicited against the C-terminal part of the hybrid constructs. The investigation of Cox et al. (1988) is an exception. With the TD-BD construct, TD-specific antibodies were found in their study. It was hypothesized that the conformation of the construct might play a role in directing an antibody response. The α-helical conformation in the TD-BD construct is disturbed by a proline situated at the N-terminus of the BD. Altered antigen processing and/or presentation may be the result. Differences in antigen processing and presentation were also assumed for the strain dependant antibody response, as was observed by Partidos et al. (1992b). Strikingly, the T cell proliferation was similar for both orientations of a hybrid construct (Cox et al., 1988; Levely et al., 1990).

In conclusion, a light preference for the N-terminal position of a TD seems to exist. No generals rules can be applied, however, for the best localization of a TD in a hybrid (TD/BD) construct with the aim of optimal BD-specific responses. Once again, to date, experimental testing of all possible combinations is the best approach in designing immunogenic constructs.

VI. IMMUNODOMINANCE

Despite the ability of each MHC molecule to bind many different peptides, the T cell response after immunization with a whole protein is directed to a few and often only to one of the processed peptide determinants (Roy et al., 1989). Several mechanisms might explain this observed immunodominance of certain T cell determinants: (a) the relative availability of the antigenic determinants after processing (Gammon et al., 1987; Adorini et al., 1988b); (b) the relative affinity of their binding to MHC molecules (Buus et al., 1987); (c) competition at the levels of antigen processing, binding and presentation among different determinants restricted to the same or different MHC molecules (Guillet et al., 1987; Adorini et al., 1988b); (d) the frequency in the individual's peripheral T cell repertoire of clones that can be stimulated by each determinant class II complex (Schaeffer et al., 1989; Adorini et al., 1988b; Gammon et al., 1990; Perkins et al., 1991b); (e) regulatory mechanisms such as suppression (Sercarz and Krzych, 1991).

Elongation of a selected T cell determinant with a sequence representing, e.g., a B cell determinant may strongly influence structural and physicochemical features of the T cell determinant with altered antigen presentation as a result. Immunodominance of a newly formed T cell determinant may emerge from such combined sequences (Bhardwaj et al., 1992). In fact, it is often seen that in chimeric peptides an overlapping new TD is formed (Perkins et al., 1991a; Wang et al., 1992a, 1992b). This newly formed junctional T cell determinant of a hybrid peptide was found to be immunodominant in several strains of mice with a different H-2 haplotype (Wang et al., 1992b). They also found that chimeric peptides did not require processing (Perkins et al., 1991a; Wang et al., 1992a; Bhardwaj et al., 1992), and hence, the enhanced immunogenicity of certain determinants may be due to competition for stimulation of T cells superimposed upon a hierarchy of competitive binding among different TD sequences of the complete peptides into the groove of the MHC molecule. When a peptide spanning the sequence of the overlapping new TD was covalently linked to a different previously defined TD, antigenicity of the immunodominant junctional region was silenced and a new epitope assumed immunodominance (Wang et al., 1992b). The immunodominant epitope is determined by complex interactions among the epitopes, which most likely depend on the structural conformation of the composite peptide.

For MHC class II, there seems to be a preference for binding of longer peptides at a central position rather than at either end. To avoid central binding, a hybrid peptide of 51 residues, composed of two known (α-helical peptides) T cell determinants joined by a β- turn to allow stable association of the α-helical peptides, was constructed (Srinivasan et al., 1993). This long peptide construct retained its ability to trigger a T cell response specific for one of the original T cell determinants. Therefore, conjunctions may be constructed keeping the two separated T cell determinants functional with the constraint that the structural units are taken into account.

Smolenski et al. (1990) found that several peptides from different proteins could compete *in vitro* for presented antigen binding sites on antigen presenting cells with processed cytochrome *c*. The cytochrome *c*- specific T cell response could be blocked in an MHC

unrestricted manner. They speculated that peptides may be bound to APC structures other than MHC class II molecules prior to the association with MHC class II. In addition, an important role in the immunodominance phenomenon may be attributed to the MHC class II associated invariant chain Ii. Recently, it was demonstrated that particular epitopes derived from hen egg lysozyme (HEL) could be preferentially presented probably due to Ii modulation (Momburg et al., 1993). Maybe the Ii is the APC structure speculated about by Smolenski and co-workers.

These studies demonstrate the so far unpredictable effect of flanking sequences on peptide recognition. Induced changes in peptide conformation, which either promote or inhibit appropriate interaction with the class II molecule or the TCR, may be the cause of this effect. Newly formed immunodominant T cell epitopes in the junction of a TD and BD are highly undesirable for application in vaccines. As demonstrated by Srinivasan et al. (1993), this effect can be avoided by keeping the structural units (α-helical peptides) intact. Keeping these investigations in mind, it is highly recommended that elongation of a selected T cell determinant with other T cell determinants or a B cell determinant with the aim to construct a strong and/or promiscuous immunogen should be accompanied by a thorough investigation into the effect on T cell determinant usage in the system under study.

VII. INFLUENCE OF MULTIPLE COPIES OF T CELL EPITOPES

Many investigators have demonstrated the beneficial effect of inserting multiple copies of a T cell epitope in a synthetic immunogen for raising a proper antibody response (Leclerc et al., 1987; Borras-Cuesta et al., 1988; Cox et al., 1988; Löwenadler et al., 1990; Munesinghe et al., 1991; Löwenadler et al., 1992; Partidos et al., 1992b). The mechanisms responsible for this property could be multiple and were the subject of many studies.

Linear homopolymers of peptides (the monomers consisted of only a B cell determinant or the B cell determinant was extended N-terminally with a T cell determinant), made by using a carbodiimide coupling with side chain protected peptides, were used to study immunogenicity (Borras-Cuesta et al., 1988). Two different B cell determinants were used. Three of the four polymers showed enhanced antibody responses. The generation of a new T cell epitope in the junction of the monomers was suggested by the investigators. They used several prediction methods to validate their assumption. One method (DeLisi and Berzofsky, 1985) indeed predicted a new T cell determinant for two of the three polymer peptides that showed an enhanced immunogenicity. With another method (Margalit et al., 1987), a predicted reinforced amphipathic pattern was found for all three polymer peptides, but this method could not predict a new T cell determinant.

Also, fusion proteins containing multiple copies of the major antigenic determinant of foot-and-mouth disease virus were shown to have enhanced immunogenicity (Broekhuijsen et al., 1987).

Löwenadler et al (1992) constructed fusion proteins with two different B cell determinants derived from different proteins. The two B cell determinants were separated by

one, two or four copies of a dominant T cell epitope. A peptide spacer was positioned upstream or downstream of the T cell epitopes (see Table 4). Multiple copies of the T cell determinant in any position led to increased levels of antibody production against the N-terminal B cell epitope, confirming earlier findings of a stoichiometric relationship between T and B cell epitopes in B cell activation (Löwenadler et al., 1990). Four copies of the T cell determinant in a distal position improved the poor antibody response to the C-terminal B cell determinant. Lymph node cells of BALB/c mice primed with the different chimeric fusion proteins proliferated, *in vitro*, to a similar extent in response to a synthetic peptide containing the T cell epitope and thus did not reveal any differences in priming efficiencies of the various constructs. T cell activation was also examined by measuring IL-2 production by a T cell hybridoma specific for the T helper determinant used in their constructs. The level of IL-2 production by this T cell line was correlated to the number of T cell epitopes in the fusion peptide. Furthermore, there was an absolute requirement for uptake and processing of the constructs since fixed antigen presenting cells failed to trigger IL-2 production. In contrast, presentation of the isolated synthetic T cell peptide was only partially inhibited in fixed antigen presenting cells. To exclude the possibility that any newly formed T cell epitope in the junction of two fused sequences contributed to T cell activation, the proliferative response to a junctional peptide spanning six amino acids on either side of the fusion point was examined. Lymph node cells of mice primed with any of the constructs did not recognize the junctional peptide.

These experiments demonstrate the stoichiometric relationship between the number of copies of T cell determinants and antibody production which proved to be based on an increased level of IL-2 production rather than on an enhanced T cell proliferation. The results, however, were obtained from *in vitro* proliferation data. It is not clear whether these conclusions are also valid under physiological conditions. The absolute numbers of specific T cells after immunization with the different constructs were not determined. On the other hand, the increase in antibody production also may be the result of increased T cell cytokine production rather than the expansion of specific Th cells by proliferation, since, *in vivo*, proteins with multiple copies of a T cell determinant may trigger increased production of other cytokines, e.g., IL-4, that are important in regulating B cell activity. Indeed, it was shown by Evavold and Allen (1991) that antigen recognition may result in IL-4 production by T cells even in the absence of proliferation.

Sette et al. (1990) incorporated the core region crucial for class II binding in multiple copies in the same peptide ("reiterative motif") and measured the binding capacity of this peptide for purified MHC class II molecules. They showed that the relative binding capacity increased a factor of 27.7 for the reiterative motif VHAAHAVHAAHAVHA derived from the I-A^d restricted T cell determinant of ovalbumin (AA 323–339) ISQAVHAAHAEINEAGR (Sette et al., 1987). This binding remained I-A^d specific, because the relative binding capacity for I-E^d was less than 1% of the binding capacity for I-A^d. When the peptide spacer EIN (AA 333–335) was inserted between two copies of the core motif, the relative binding capacity for I-A^d decreased to 1.6. In contrast, positioning the peptide spacer EIN at the end of two copies of the core motif yielded a binding capacity of 24.8. It may be questioned if two core

motifs subsequently repeated are sufficient for maximum binding capacity. Similar results were obtained with the I-E^d restricted T cell determinant from HEL (AA 105–120) and the I-E^d restricted T cell determinant from the λ-repressor protein (AA 12–26). These data showing enhanced binding capacity of the reiterative motif support the hypothesis that cooperative stabilization of peptide secondary structures is favorable for class II binding. Insertion of the tri-peptide spacer prevented the stabilization, but placing the spacer C-terminally of the two copies did not influence the stabilization. Since it was known from earlier studies (Sette et al., 1989b) that a Val → Ala substitution at position 327 did not affect the binding of the peptide to I-A^d, the analog reiterative motif peptide composed of only two amino acids (AHAAHAAHAAHAAHAA) was tested for I-A^d binding. This peptide even appeared to bind very strongly to I-A^d with an enhanced relative binding capacity of 34.

The OVA reiterative core peptide (VHAAHAVHAAHAVHA) also inhibited antigen presentation to an OVA-specific T cell line of the complete natural T cell determinant by fixed antigen presenting cells. This suggests MHC binding at the same site which is involved in T cell activation. Reiterative core motifs do not directly stimulate T cells since a part of the complete natural T cell epitope is missing.

Naturally occurring clustered class II binding sites have been identified. This implies a possible role for reiterative-like structures in the generation of immunodominant regions. Three overlapping I-E^d motifs have been found in dynorphin AA 1–13 (Sette et al., 1989b). The 327–332 OVA core motif is immediately preceded by another I-A^d motif 321–326. Other examples are the influenza virus hemagglutinin 121–146 region (two apparent I-A^k sites (Sette et al., 1989a); the HEL 105–129 region (two apparent I-E^d sites) (Adorini et al., 1988a); the pigeon cytochrome *c* 81–104 region (two apparent I-E^k sites)(Carbone et al., 1987); the staphylococcal nuclease 1–40 region (at least two independent I-E^k sites)(Schaeffer et al., 1989); and in some regions of the VP1, VP2 and VP3 capsid proteins of human rhinovirus (Hastings et al., 1993).

We think that for an estimate of the practical implications, these findings should be further investigated *in vivo* by analyzing qualitatively and quantitatively the antibody response after immunization of mice with peptide constructs composed of either (a) the complete natural T cell determinant, or (b) the repeated core motif extended with the necessary flanking sequence to obtain a complete T cell determinant, and both (a) and (b) combined with a B cell determinant.

Elongation of a T cell epitope sequence may increase the amphipatic score in a predictive algorithm (AMPHI) or may enhance the T cell response as measured in a proliferation assay (Ertl at al., 1991; Kim et al., 1990). These results are further extended evidence for increased conformational stability of the elongated T cell epitope and therefore for increased efficiency.

Using a multiple antigen peptide containing four copies of tandemly arranged T and B cell epitopes, from the repeat region of the *P. falciparum* circumsporozoite protein, optimal antibody responses were obtained (Munesinghe et al., 1991).

Both the level of specific antibodies and the affinity for the B cell determinant of these antibodies were enhanced after immunization with hybrid peptide constructs containing a double copy of a T cell determinant located at the N- or C-terminus of a B cell determinant as compared to constructs with a single copy of the T cell determinant (Partidos et al., 1992b). In these constructs, the B and T cell determinants were separated by a gly-gly dipeptide spacer. In this study, position of the T cell determinant at the N-terminus of the B cell determinant appeared to be more advantageous in terms of affinity.

The examples above demonstrate that multiple copies of a T cell determinant may lead to an increase of the immune response, which could be both quantitatively higher and qualitatively better in terms of affinity. Several mechanisms can contribute to this phenomenon. An increased conformational stability of the T cell determinant as a result of cooperative action of multiple copies within one molecule may lead to a higher binding constant between the MHC molecule and the T cell determinant. In addition, new potential T cell epitopes may be generated in the junction of the monomers. A stoichiometric relationship between copy number of T cell epitopes and the number of MHC molecules which are occupied with a Th peptide exposed at the cell membrane of the antigen presenting cell may lead to a more efficient activation of T cells at higher T/B cell epitope ratios. It is also conceivable that the level of IL-2 production and/or of other cytokines increases upon antigen presentation of a construct with multiple copies of a T cell epitope with more efficient activation of antibody forming cells as a consequence.

VIII. INFLUENCE OF COUPLING MODES ON T CELL EPITOPES

Linear constructs consisting of a selected T cell determinant and a B cell determinant can be made by linear synthesis. A linearly synthesized hybrid peptide often consists of more than 30 residues. An alternative method may be linking the two determinants after synthesizing them separately. This latter method offers the advantage of the combination of a stock of a well documented T cell determinant with various B cell determinants. A large batch of the T cell determinant ensures a constant quality when comparing different B cell determinants in hybrid constructs. Another advantage is that it is then possible to synthesize shorter peptides, since efficiency of peptide synthesis gradually decreases with length.

Coupling of the two determinants via their N-terminal amino group, their C-terminal carboxyl group or via a functional group of one of their amino acids, however, may influence the epitope orientation and the secondary structure of the composed peptides or may alter the immunogenic epitope. Examples from the literature making use of different coupling methods resulting in both effective or useless constructs will be discussed.

One of the first experiments using peptide constructs, which encompass both a T cell and a B cell determinant, made by covalent coupling via a linker, was described by Good et al. (1987). They coupled a predicted and subsequently proven T cell determinant from the malaria circumsporozoite protein via its C-terminal cysteine with the aid of MBS to the N-

terminal amino group of the $NP(NANP)_{15}NA$ peptide, a B cell determinant from the same protein. This construct elicited high-titer antibodies specific for $NP(NANP)_{15}NP$. However, in their experiments, they did not differentiate between the antibody response specifically directed to the T cell determinant or B cell determinant, nor did they analyze the functionality of the elicited antibodies in a neutralization assay. Nevertheless, this study is the first proof of the ability of a synthetic construct made by conjugation via a linker to elicit B cell determinant-specific antibodies.

Co-polymers of a B and a T cell determinant derived from different proteins, made by glutaraldehyde, elicited B cell determinant-specific antibodies (Leclerc et al., 1987). In a study performed by Jolivet et al. (1990), co-polymers of four different peptides of which at least one of the peptides represented a T cell determinant were made by glutaraldehyde. The polyvalent synthetic peptides induced antibodies directed against each of the four component peptides in outbred guinea pigs. Linkage via glutaraldehyde makes use of amino groups. Only one of the four peptides contained several lysines scattered through the whole sequence. But despite linkage via the lysine residues which may destroy the epitope, and despite random orientation of this peptide antibody, induction against this epitope was not prevented.

In a study from Levely et al. (1990), two methods of construction of hybrid peptides were compared. They linearly synthesized a peptide composed of a T cell determinant, at the N-terminal side of a B cell determinant both derived from proteins from the respiratory syncytial virus separated by a gly-gly spacer. These determinants were also chemically conjugated by means of a natural N-terminally located cysteine in the T cell determinant and an added N-terminal cysteine in the B cell determinant. The cysteines were coupled by the homo-bifunctional linker bismaleimidohexane (BMH). The chimeric peptides induced different specific antibody responses. Also, the free T cell determinant induced peptide-specific antibodies. The antibodies found after immunization with the linearly synthesized hybrid peptide were mainly directed to the B cell determinant, while the antibodies found after immunization with the chemically constructed peptides were principally directed against the T cell determinant. The consequences are discussed in Section V.

Similar results were found in experiments performed in our laboratory (Zegers et al., 1993). We constructed hybrid peptides by linear synthesis and by different chemical conjugation. The T and B cell determinants were coupled by the homo-bifunctional linker BMH or by the hetero-bifunctional linker MBS. Additional cysteines were incorporated in the sequences of the determinants for coupling purposes. The cysteine was placed C-terminally in the T cell determinant and C- or N-terminally in two different B cell determinants. First, with MBS conjugation, the thiol group of the B cell determinant was blocked with ethylmaleimide. After reaction of MBS with one of the amino groups of the B cell determinant, the T cell determinant was coupled. After immunization with these different constructs, only T cell determinant- specific antibodies were found with the chemical constructed peptides, while both T- and B cell determinant-specific antibodies were found with the linearly synthesized peptides.

Several explanations can be given why in this case we did not find B cell determinant-specific antibodies with chemically linked constructs. First, chemical modification of the B cell determinant, introduced during coupling procedures, might have altered or destroyed the immunogenic determinant. We therefore analyzed the antibody response in an enzyme-linked immunosorbent assay on isolated chemically modified B cell determinants. However, antibodies specific for a modified B cell epitope were not found. Previously, high-titer antibodies specific for the B cell determinant were elicited with conjugates of keyhole limpet hemocyanine (KLH) and the B cell determinants made by coupling with MBS. For these B cell determinants, the thiol group of an added cysteine to the B cell epitope was used. Thus, chemical crosslinking using thiol groups did not destroy immunogenicity by itself. However, antibodies specific for the B cell determinant could not be raised with a KLH conjugate made by coupling with glutaraldehyde. We then concluded that lysine residues in this specific B cell determinant are important for immunogenicity and therefore should not be used for coupling (Zegers et al., 1990). Second, antigen processing or presentation also may have impaired the antibody response against the B cell determinant after immunization with chemically linked constructs. It is conceivable that these TD-specific antibodies are elicited by the free TD molecules still present in the preparation of the hybrid construct. On the other hand, the chemically conjugated construct might not be processed properly which impairs binding of TD to MHC molecules. Antigen presentation is thus hampered, resulting in the absence of BD-specific antibodies. Several investigators have proposed that adjacent sequences may alter the conformation of an epitope, resulting in significant altered antigen processing or changes in T cell recognition (Perkins et al., 1991a; Wang et al., 1992a, 1992b; Kim and Jang, 1992).

As appears from the examples above, in appropriate designed experiments, chemically coupled TD-BD constructs may lead to the induction of BD-specific antibodies (Good et al., 1987; Leclerc et al., 1987; Jolivet et al., 1990). Glutaraldehyde or MBS are the linkers of choice. However, these coupling reagents did not appear to be useful if amino groups of residues present in the sequence of the BD were used for coupling (Zegers et al., 1993). Conjugation with BMH did not yield constructs which elicited BD-specific antibodies (Levely et al., 1990; Zegers et al., 1993).

IX. IS COVALENT COUPLING OF T AND B CELL EPITOPES NECESSARY?

In order to obtain an optimal humoral immune response, B and T cells must cooperate in close conjunction (Kupfer et al., 1986; Vitetta et al., 1987; Noelle and Snow, 1990; Clark and Ledbetter, 1994). The cognate interaction between helper T and B cells involves the T cell receptor which recognizes the antigen presented in association with MHC class II molecules at the cell surface of B cells. Also, the CD3 and the CD4 molecules are associated with this complex. Besides this cognate interaction, second co-stimulatory signals between T and B cells are necessary for activation of B cells to differentiate and proliferate. CD28

and B7 interaction plays a major role, and recently, the interaction between gp39 (activated T helper cells) and CD40 (B cells) was described (Noelle et al., 1992). Moreover, certain lymphokines produced by activated T cells are essential factors involved in antibody production. Lymphokines are active only in high local concentrations and are therefore optimally inductive only within short distances from their production site. Bartlett et al. (1989) showed *in vitro* that "bystander" B cells were less frequently induced into cell cycle than B cells in contact with T cells. Moreover, the B cells in physical contact with T cells had a higher RNA content and were larger than the bystander B cells present in the same cultures. Entry of B cells into the G_1 phase appeared to be antigen and T cell contact dependant, but not lymphokine dependant. Lymphokines were needed for cycle progression to S phase. Several other reports support the view that conjunction of B and T cells is required for B cell triggering (Kupfer et al., 1986; Vitetta et al., 1987; Noelle et al., 1983; Krusemeier and Snow, 1987). There are, however, also reports which describe lymphokine induced polyclonal B cell cycle entry in an MHC unrestricted manner and subsequent progression to S phase for 20% of the B cells (Leclercq et al., 1984, 1986; Defranco et al., 1984). A fraction of these cells differentiates and produces Ig, although at a lower rate than terminally differentiated plasma cells (Leclercq et al., 1986).

B cell activation via CD40 is required for specific antibody production and can also be induced *in vitro*, besides through T cell interaction, by a soluble recombinant gp39 or by an anti-CD40 antibody in the presence of cytokines (Hollenbaugh et al., 1992; Gascan et al., 1991; Nonoyama et al., 1993). Also, *in vivo*, the involvement of CD40-gp39 interaction was established to be essential for thymus dependent humoral immunity in our own laboratory (Van den Eertwegh et al., 1993).

Minimal requirements for antibody production to peptide antigens were defined in a study by Goodman-Snitkoff et al. (1990, 1991). They crosslinked a T cell as well as a B cell epitope to a hydrophobic anchor and complexed these epitopes into the phospholipid bilayer of liposomes resulting in a multivalent configuration. These immunogens were able to induce production of antibodies specific for the B cell epitope. Liposomes with a linear peptide comprising a T and a B cell determinant also induce B cell determinant-specific antibodies. These data suggest that Th epitopes do not have to be covalently linked to the B cell epitope to be active, but that intrastructural help is equal to intramolecular help.

Recently, some papers were published describing antibody production specific for B cell determinants after immunization with mixed uncoupled T and B cell determinants (Sarobe et al., 1991; Partidos et al., 1992d; Shaw et al., 1993). This antibody production can only be explained by assuming direct binding (or after internalization) of the T cell determinant to class II molecules of the B cell having recognized the B cell determinant. Direct binding to MHC class II molecules could take place by competition of the T cell determinant with a (self) peptide that is already present in the cleft of the MHC class II molecule. To accomplish this, the affinity of the T cell determinant for the class II molecule should be much higher than the affinity of the peptide bound in the MHC molecule. Stimulation via this pathway is probably very inefficient. A (locally) very high concentration

of the Th peptides is thus required. Binding of the TD to the class II molecule after internalization by the B cell favors the hypothesis of intermolecular/intrastructural help (Lake and Mitchison, 1976; Milich et al., 1987; Goodman-Snitkoff et al., 1990,1991). In the studies which describe co-immunization of the TD and the BD in a mixture, the peptides were emulsified in (complete) Freund's adjuvant. Both the TD and the BD peptide should be entrapped in the same micro-droplet to give intrastructural help. Evidence for the assumption of simultaneous presence of both T and B cell determinants in one droplet came from the study of Partidos et al. (1992d). B cell-specific antibodies only were detected after immunization with both BD and TD peptides mixed in the Freund's adjuvant emulsion. Intraperitoneal immunization of the TD combined with subcutaneous immunization of the BD at the same moment did not result in BD-specific antibodies. The possibility of associated, non-covalently bound TD-BD complex formation cannot be excluded. Co-immunization of a poor immunogenic peptide from the malaria circumsporozoite protein together with keyhole limpet hemocyanin raised peptide-specific antibodies (Good et al., 1988). In contrast to this finding, we observed that "pseudo conjugates", formed by non-specific adherence of peptides to carrier molecules, were not able to elicit a peptide-specific antibody response (Deen et al., 1990). Similarly, immunization with a mixture of the nucleocapsid (strong immunogen) of the hepatitis B virus with envelope antigen (not immunogenic) of the virus did not result in anti-envelope antigen-specific antibodies, whereas a particle (virion) encompassing both proteins did (Milich et al., 1987). These effects can hardly be explained as the result of bystander help as suggested by Partidos et al. (1992d) since only a small proportion of the B cells can be activated via this pathway (Bartlett et al., 1989). Moreover, the level and affinity of the antibodies to the B cell epitopes following immunization with mixed, non-covalently bound TD and BD peptides was lower than that obtained following immunization with an analogous chimeric TD-BD construct (Shaw et al., 1993).

In our laboratory, we were not successful in applying co-immunization with TD and BD peptides (Zegers et al., 1993). Our experiments indicated that induction of BD-specific antibodies with a mixture of free TD and BD peptides is certainly not possible with all combinations of T and B cell determinants. In contrast, a covalent bond between T cell and B cell determinants in general is required to induce high affinity anti-BD antibodies crossreactive with the native protein.

From the investigations described above, it is clear that for efficient antigen presentation and subsequent antibody production, a cognate interaction between B and T cells is required. This will assure antigen-specific T cell help resulting in a specific memory response. To accomplish this, a covalently bound TD-BD construct, or a liposome, a virion or another particulate structure with both a TD and a BD built in to give intrastructural help, is the most applicated and relatively effective manner. Also, a water-in- oil emulsion, in which a TD and BD are mixed, will be appropriate, if the TD and BD are entrapped in the same micro-droplet to give intrastructural help. However, entrapping in the same droplet is a chance process and will be less efficient. This might have caused the affinity and titer of

the antibodies raised with the mixed TD and BD to be lower than that obtained with a covalently bound TD-BD construct.

Taken together, to obtain a high level of BD-specific antibodies with a high affinity and crossreactive with the native protein, a covalently bound BD-TD construct should be used for immunization.

X. CONCLUDING REMARKS

Coupling of B cell determinants to carrier proteins may serve several goals. The carrier protein provides T cell help to anti-peptide antibody forming cells. Processing of a carrier-peptide conjugate by the antigen presenting cells generates different T cell determinants which can stimulate different T cells. The immunogenicity of a peptide-carrier may thus be increased by stimulating a larger T cell pool. Also through the size of a carrier-peptide conjugate, the capture by phagocytes may be increased.

On the other hand, it may be advantageous to work with a simple and precisely defined immunogenic construct. Using a rather small peptide containing one or several (the same or different) copies of a well selected T cell determinant and a B cell determinant for immunization provides the ability to manipulate the specificity, level and affinity of the immune response. Moreover, immunodominance of TDs from a carrier protein can be excluded when only TD-BD constructs are used for immunization. Especially for vaccines, such an approach offers the possibility to immunize with strong acting T cell determinants with a broad MHC haplotype spectrum. Cryptic T cell determinants hidden in a pathogen-derived native protein are circumvented in this way. In addition, it is highly desirable to stimulate pathogen-specific T cells for vaccine applications.

Selection of T cell determinants on the basis of prediction methods is not fully efficient. The action of a selected T cell determinant should be defined in a functional assay with cells from the target animal. For vaccine applications, it is recommended that a promiscuous T cell epitope is chosen, certainly for outbred populations and human use.

A light preference for the N-terminal position of a TD in a construct exists, since the antibody response is directed to the C-terminal part of a construct. However, this is not a general rule, since processing of a construct and antigen presentation largely depends on the sequence of the overlapping junction between the two determinants. In animals, this is strain dependent. Special attention, therefore, should be paid to the conjunctional site of the two components. New T cell epitopes may emerge, which is not advantageous since such a newly formed TD is not pathogen or protein derived and alternatively existing T cell epitopes may be lost. This effect can be avoided by keeping the structural units (α-helices) intact, e.g., separated by a helix breaker.

Chain elongation of a TD may increase the stability of a helix and, as a result, may enhance peptide binding to MHC class II molecules. Higher titers of antibodies were reported with a broader MHC restriction spectrum. On the other hand, the response in an animal with

a certain MHC haplotype may disappear. Also in this respect, special attention should be paid to the TD and BD junction.

Multiple copies of the TD may often lead to an enhancement of the immune response. An increased conformational stability of the TD favors binding to an MHC molecule. Processing of the constructs yields more molecules of the TD. A stoichiometric relationship between the copy number of TDs and the number of MHC molecules occupied with a TD and exposed at the cell membrane of the antigen presenting cell may lead to a more efficient activation of T cells. In addition, the level of Il-2 production may enhance upon better T cell activation with the more efficient activation of antibody cells as an effect.

Chemical coupling of a TD and a BD with linkers not always results in an efficient immunogen. Dependent on the availability of functional groups in the TD and BD, random orientation of the determinants may be generated. This may be disastrous for the immunogenicity. We are of the opinion that linear constructs with a natural peptide bond are most appropriate. This may be accomplished by linear synthesis, which becomes more difficult, however, with increasing length of the sequence. Condensation of fully protected fragments offers an alternative synthesis method.

In general, it is a prerequisite for an efficient immunogen to build the TD and BD in one construct or in a structural unit, such as a liposome or another particulate structure. Antibody production was reported upon immunization with just a mixture of free TD and BD, but the affinity and titer of the antibodies raised were lower than those obtained with a covalently bound TD-BD construct.

It is clear that careful analysis should be made of the structural influences of all components in designing immunogenic constructs. As shown above, many factors may play an important role. Just adding two sequences in one construct generally does not always lead to an optimally active immunogen. The construct which has been found most appropriate on the basis of theoretical choices still needs confirmation of the putative superiority in a functional assay where it is compared to alternative simple constructs.

ABBREVIATIONS

AA Amino acid
BD B cell determinant
BMH Bismaleimidohexane
CSP Circumsporozoite protein
CTL Cytotoxic T lymphocyte
HEL Hen egg lysozyme
KLH Keyhole limpet hemocyanin
MBS m-Maleimidobenzoyl-succinimide ester
MHC Major histocompatibility complex
OVA Ovalbumin
TCR T cell receptor

TD T cell determinant
Th T helper

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Chapter 9

Peptide-induced memory (IgG) response, cross-reactive with native proteins, requires covalent linkage of a specific B cell epitope with a T cell epitope

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PEPTIDE-INDUCED MEMORY (IGG) RESPONSE, CROSS-REACTIVE WITH NATIVE PROTEINS, REQUIRES COVALENT LINKAGE OF A SPECIFIC B CELL EPITOPE WITH A T CELL EPITOPE

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Abbreviations: TD: T cell determinant; BD: B cell determinant; MBS: m-Maleimidobenzoyl succinimide ester; α_1-AT: α_1-antitrypsin

SUMMARY

In order to raise antibodies synthetic peptides are often coupled to a carrier protein to provide the necessary T cell determinants. Alternatively, a short synthetic determinant with a distinct sequence motif which can be presented by major histocompatibility complex (MHC) class II to T cells, can be linked directly to a B cell epitope. Recently, it has been suggested that covalent linkage between a class II-presentable T helper peptide and a B cell epitope is not required to induce antibodies against a B cell determinant (Sarobe et al., *Eur. J. Immunol.* 1991. *21*: 1555). Therefore, we investigated the ability of an H-2^d-restricted T cell determinant (AA 111-120 FERFEIFPKEK) from the influenza virus hemagglutinin, to support B cell responses to different proven B cell determinant peptides, derived from human α_1-antitrypsin. Antibodies against B cell epitopes crossreactive with native α_1-antitrypsin could be raised only when these B epitope peptides were covalently coupled to the T cell determinant through a peptide bond. No antibodies were raised against the B cell epitope when the free peptides (T and B cell epitopes) were just mixed or when the T cell epitope was conjugated via m-maleimidobenzoyl succinimide ester or bis-maleimidohexane to the B cell determinant. Antibodies against the T cell determinant were raised in all cases, regardless of the mode of presentation: just mixed with or covalently coupled to the B cell determinant. The results indicate that a covalent bond between T cell and B cell determinants in general is needed to induce anti B cell determinant antibodies cross-reactive with the native protein.

I. INTRODUCTION

Most randomly chosen short peptides consist of a B cell determinant only, which, by definition, does not induce an epitope-specific immune response by itself. In that case, a T cell epitope is necessary to offer T cell help and memory for a humoral immune response. The T cell epitope is usually supplied by a carrier protein to which synthetic peptides are covalently conjugated. Using this approach, we successfully raised various antibodies cross-reactive with native proteins [1-3]. The methodology of chemical conjugation, with respect to the amino acid composition and orientation of the epitope used, proved to be of crucial importance [4-6].

Milich et al. [7] were among the first to demonstrate that a synthetic peptide, encompassing one T cell epitope and one B cell epitope, could be used to raise anti-native protein antibodies. With constructs of a similar synthetic hybrid peptide containing a T cell determinant and a B cell determinant from the same protein covalently linked with m-maleimidobenzoyl succinimide ester (MBS), it was possible to elicit high-titer antibodies against the native protein [8]. Hybrid constructs of T and B cell determinants derived from different proteins, made by linear synthesis [9, 10] or linked via glutaraldehyde [11], induced antibodies against the B cell determinant.

Recently, Sarobe et al. [12] described the induction of antibodies against a peptide hapten which does not require covalent linkage between the hapten and a class II-presentable T helper peptide . The current dogma, however, states that haptens and small peptides are poor immunogens to which antibody responses can be elicited only by coupling them to larger carrier proteins (for excellent review see [13]). We used the promising approach described by Sarobe et al. [12] to raise antibodies against genetic variants of α_1-antitrypsin (α_1-AT). Results presented in this report indicate that a covalent linkage between the T cell peptide and the B cell peptide as a rule is necessary.

II. MATERIALS AND METHODS

II.1. Peptides used for immunization and evaluation

SP149 (FERFEIFPKEKC) is a well-described T cell determinant (TD) from the influenza virus hemagglutinin [14]. SPEK15 (LSKAVHKAVLTIDKKC) is a peptide sequence derived from the genetic Z variant of α_1-AT (residues 329-343) and SP190 (CDEGKLQHLVNELT) is a peptide sequence derived from the S variant of α_1-AT (residues 256-268). Both SPEK15 and SP190 are B cell determinants (BD). SPEK15A (FERFEIF-PKEKLSKAVHKAVLTIDKKC) and SP191 (FERFEIFPKEKDEGKLQHLVNELTC) are hybrid peptide sequences of SP149) with SPEK15 or SP190, respectively, and therefore include at least one T cell epitope and one B cell epitope. Peptides were N- or C-terminally elongated with an extra cysteine for coupling purposes.

II.2. Peptide synthesis and coupling

Peptides were synthesized as free acids by the solid-phase method of Merrifield [15] using t-boc chemistry for peptides SPEK15 and SPEK15A as described elsewhere [1] or using Fmoc chemistry [16] for peptides SP149, SP190 and SP191 on an automated Milligen 9050 Continuous Flow Synthesizer (Millipore Co. Milford, MA). The cleaved and deprotected peptides were purified by gel filtration and analyzed as described before [1].

SP149 (TD) was covalently linked via MBS, by its C-terminal Cys to SPEK15 or SP190, representing B cell epitopes. Thiol groups in SPEK15 (C terminal) and SP190 (N terminal) were protected with ethylmaleimide. Remaining free thiol groups were measured with Ellman's reagent [17]. Equimolar amounts of MBS, 100 mM in dimethyl formamide (DMF), were allowed to react with the protected peptides and subsequently, SP149 was added in equimolar amounts to both reaction mixtures. Alternatively, peptides were covalently linked via their terminal-situated cysteines with 1,6 bismaleimidohexane (BMH). SP149 and SPEK15 were mixed in equimolar amounts. Subsequently, BMH dissolved in DMF was added at a concentration 1.5 times that of the total amount of free thiol groups. Coupling reduced the measurable amounts of free thiol groups by 90%. Statistically one third of the conjugates consists of the combination SP149-BMH-SPEK15. SP149 and SP190 were coupled under the same conditions as those described above. Conjugation of peptides and keyhole limpet hemocyanin (KLH) was carried out as described elsewhere [2].

II.3. Peptide modification

Thiol groups of SPEK15 and SP190 were blocked with ethylmaleimide in equimolar amounts. One of the five amino groups in SPEK15 and one of the two amino groups in SP190 were modified with MBS by adding MBS in equimolar amounts to the peptides. The maleimide moiety of MBS was blocked by addition of equimolar amounts of cysteine.

II.4. Immunization and evaluation

Groups of three 12-week-old female BALB/c mice were immunized by i.p. injection of 200 μl of an emulsion of 90 μl peptide solution in water and 110 μl "specol" (a mineral oil adjuvant [18]) containing 30 μg of each peptide used. Parallel groups were immunized with a mixture of peptides emulsified in complete Freund's adjuvant. Mice were given booster injections after 4 and 8 weeks with the same dose of antigen. For the groups administered with Freund's adjuvant, the booster injections were given in incomplete Freund's adjuvant. ELISA was carried out as described before [2].

III. RESULTS

III.1. Antibody response raised with peptide-carrier immunogens

First, we raised peptide- and native protein-specific antibodies with KLH-MBS-SPEK15 and KLH-MBS-SP190 (Table 1). Single B cell determinants (SPEK15 or SP190) did not elicit peptide specific responses.

TABLE 1
Relative levels of specific IgG reactivity in sera as determined in ELISA

IMMUNOGEN		COATING					
		SP149	SPEK15A	SPEK15	SP191	SP190	α_1-AT
SPEK15A	(TD-BD)	0.847 ± 0.158	1.181 ± 0.145	0.705 ± 0.002	nd[a]	nd	0.728 ± 0.193
SP149-MBS-SPEK15	(TD-MBS-BD)	0.689 ± 0.261	0.666 ± 0.345	0 ± 0.002	nd	nd	0 ± 0.004
SP149 + SPEK15 Specol	(TD + BD)	0.420 ± 0.248	0.556 ± 0.331	0.002 ± 0.005	nd	nd	0.035 ± 0.039
SP149 + SPEK15 Freunds	(TD + BD)	0.010 ± 0.088	0.156 ± 0.117	0 ± 0.005	nd	nd	0.002 ± 0.004
KLH-MBS-SPEK15[b]		nd	nd	0.314 ± 0.113	nd	nd	0.184 ± 0.119
SP191	(TD-BD)	0.233 ± 0.243	nd	nd	1.557 ± 0.023	0.251 ± 0.131	0.567 ± 0.065
SP149-MBS-SP190	(TD-MBS-BD)	0.391 ± 0.320	nd	nd	0.791 ± 0.456	0 ± 0.007	0 ± 0.005
SP149 + SP190 Specol	(TD + BD)	0.050 ± 0.320	nd	nd	0.131 ± 0.007	0.031 ± 0.027	0.011 ± 0.021
SP149 + SP190 Freunds	(TD + BD)	0.235 ± 0.342	nd	nd	0.536 ± 0.527	0 ± 0.017	0.041 ± 0.036
KLH-MBS-SP190[b]		nd	nd	nd	nd	0.586 ± 0.182	0.573 ± 0.294

a) not determined; b) Immunizations with KLH conjugates and ELISA evaluation were carried out in a separate study; TD: T-cell determinant; BD: B-cell determinant. The values in this table represent the average absorbances for sera of three mice ± standard deviation taken after the second booster immunization and diluted 1:400. With this dilution, plateau levels were not obtained (compare Fig. 1).

III.2. IgM and IgG antibody responses raised with peptide constructs

Four groups were immunized with (I) the TD-BD construct, (II) the TD-MBS-BD construct, (III) TD + BD mixed in specol and (IV) TD + BD mixed in Freund's adjuvant. The sequence SPEK15 was used as BD. At 5 days after the first immunization, both IgM and IgG responses were low to SP149 (TD), SPEK15A (TD-BD), SPEK15 (BD) and α_1-AT (native protein) and remained low for 28 days (data not shown). Seven days after a booster immunization, IgM responses in sera from all groups against the different antigens were low when compared to IgG responses (data not shown).

Since the anti-native protein responses were still relatively low after the second immunization, it was decided to boost the reaction a second time. Fourteen days later, the IgG antibody response to SPEK15A (TD-BD) after immunization with SPEK15A (TD-BD) was higher than the antibody responses after immunization with the other TD and BD combinations (Fig. 1A). The antibody response against SPEK15A after immunization with SPEK15A (TD-BD) probably is composed of the antibody response against the TD SP149 and the antibody response against the BD SPEK15, since the addition of the titration curves of the responses against TD and against BD is similar to the titration curve against TD-BD. The IgG antibodies in the sera of mice immunized with SPEK15A cross-reacted with the native protein α_1-AT (Fig. 1D). The immune responses against SPEK15A (TD-BD) elicited with the immunogens II to IV were directed only against the TD SP149. ELISA for the sera of these groups showed similar curves for SPEK15A and SP149; no contribution from the immune responses against the BD SPEK15 was added to the response against SP149 (see Fig. 1B and C). Only a response against SP149 (TD) was detected with mixtures of TD and BD.

III.3. Comparison of antibody responses from mice immunized with different BD

Constructs with SP190 as BD were immunized in four groups of mice as for SPEK15. Responses were essentially similar to the IgG and IgM responses after immunizations with SPEK15 (BD) constructs (data not shown). In sera from mice immunized with TD + BD mixtures or with TD-MBS-BD (with SPEK15 or SP190 as BD), no responses were found directed against the B cell determinants or against the native protein (α_1-AT). In these sera, only an antibody response against the TD could be found. In sera from mice immunized with linearly synthesized TD-BD peptides, antibody responses against the B cell determinants and against α_1-AT were detected (Table 1).

III.4. Role of chemical modification of B cell determinants

Since amino groups of SPEK15 and SP190 were used for linkage to the thiol group of SP149, the epitope of peptides SPEK15 and SP190 may be modified such that antibodies raised with these chemically linked constructs do not recognize the BD in the antigens used in ELISA (SPEK15, SP190 and α_1-AT). Responses (Ig total) against modified BD were compared with responses against BD and TD for SPEK15 as BD (Fig. 2). Similar results were obtained with SP190 as BD (not shown). In sera from mice immunized with SP149-MBS-SPEK15 or SP149-MBS-SP190 no antibodies were found against modified BD. Only antibodies against the TD in the TD-BD or TD-MBS-BD constructs were detected.

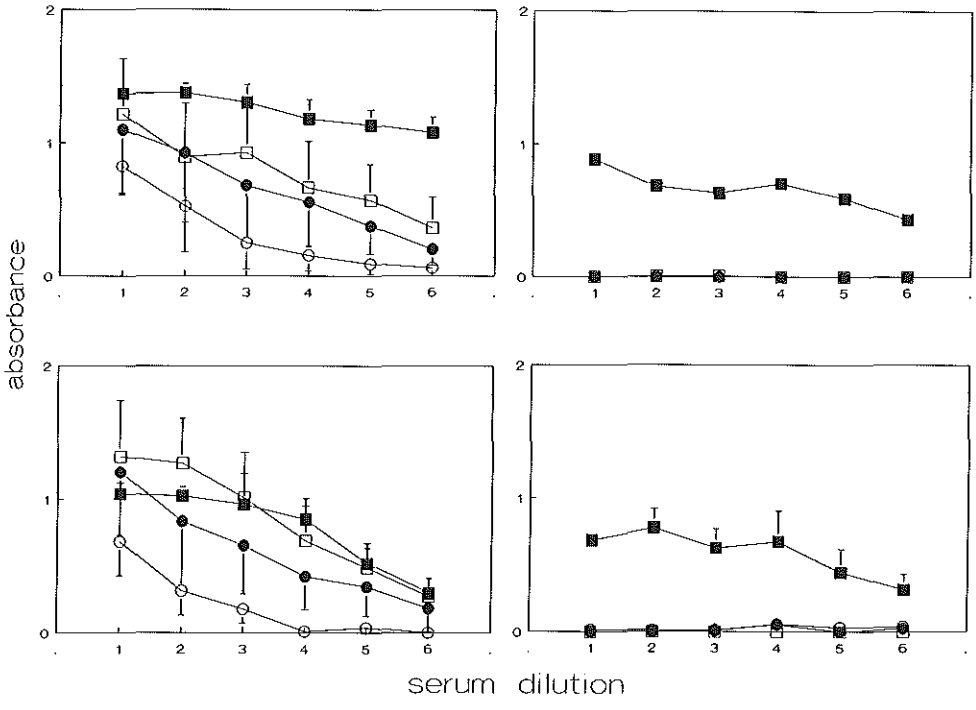

Fig. 1. Titration of IgG antibodies in serum 14 days after the third immunization determined in ELISA. Groups of three mice were immunized with SPEK15A ■, with SP149-MBS-SPEK15 □, with SP149 mixed with SPEK15 in specol ●, with SP149 mixed with SPEK15 in complete Freund's adjuvant ○. Serum IgG responses against SPEK15A (A) against SPEK15 (B), against SP149 (C) and against α_1-AT (D) are shown. Mean responses with standard error bars of groups of three mice using $\log_2$ serial dilution of serum with initial dilution 1:50 are shown.

We investigated whether it was possible to raise antibodies against the BD applying an alternative coupling method. TD and BD were chemically linked via their terminal thiol groups from cysteine with BMH. With these constructs, TD-BMH-BD, with SPEK15 and SP190 as BD, mice were immunized twice with a 4-week interval. The antibody response was evaluated in ELISA in sera taken 7 days after the second immunization. Only antibodies against the TD were found but not against the BD, modified BD and α_1-AT (results not shown).

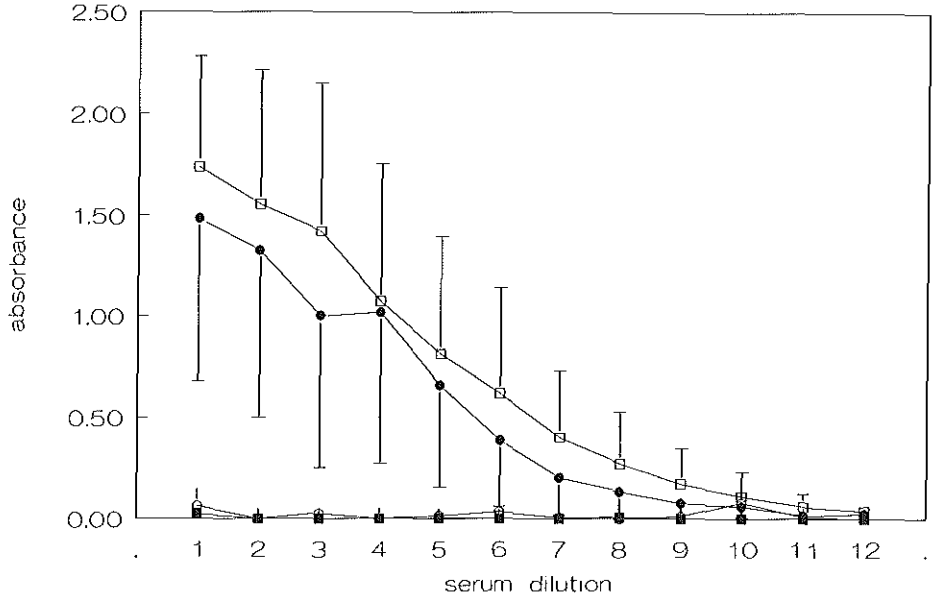

Fig. 2. Titration of antibodies (Ig) in serum 14 days after the third immunization determined in ELISA. Serum responses raised with SP149-MBS-SPEK15 are shown. A $\log_2$ serial dilution of serum with initial dilution 1:100 was made. The mean responses with error bars of three mice are shown. SPEK15 ■, SPEK15A □, with SP149-MBS-SPEK15 ●, with SPEK15 modified ○ were used as coating antigens in ELISA.

IV. DISCUSSION

As an alternative for a carrier protein, a synthetic T cell determinant (TD) which can be linearly synthesized to a B cell determinant (BD) was applied. We tried to verify another more simple alternative reported by Sarobe et al. [12] who just mixed a TD and a BD to raise anti-BD antibodies. This strategy offers a major advantage over the other methods mentioned, since coupling is time consuming, a coupling site must be introduced in the BD and, moreover, neodeterminants may be introduced. We used this attractive approach to raise anti-α_1-AT antibodies. Proven BD [5, 6] were synthesized linearly to a well described TD [14]. These BD, and TD were also chemically conjugated. In addition, BD and TD were mixed and immunized according to Sarobe et al. [12]. Using our immunization protocols,

all IgM titers were negligible for all groups. IgG antibodies which were cross-reactive with the BD and the native protein α_1-AT were induced only with linearly synthesized TD-BD peptides and with KLH-bound peptides. The TD appeared to include a BD which in all modes of presentation, linearly synthesized, chemically coupled or mixed TD and BD, leads to anti-TD antibodies. This, in addition, formed a buit-in-control for proper immunization.

With chemically (via MBS or BMH) linked TD-BD constructs, no humoral antibody responses were found cross-reactive with the BD or the native protein. Several interpretations might explain why we did not find anti-BD directed antibodies with chemically linked constructs: (a) chemical modification of the BD, (b) no conjugation between TD and BD, or (c) altered antigen processing or presentation.

Considering (a), previously, the BD SPEK15 and SP190 conjugated to KLH with MBS elicited high-titer anti-peptide antisera which were cross-reactive with α_1-AT. This proved that chemical cross-linking of these BD with MBS did not prevent immunogenicity by itself. For production of the KLH conjugate, the thiol group of SPEK15 was used for coupling as in the TD-BMH-BD conjugate. For coupling TD and BD with MBS, one of the amino groups of the BD was used, resulting in multiple constructs of different orientation. The immunogenic BD configuration might have been destroyed. Similar experience was encountered in the comparison between conjugates of KLH and several peptides coupled with MBS or with glutaraldehyde [5]. The immune response against SPEK15 totally disappeared when the glutaraldehyde conjugate was used as immunogen. Glutaraldehyde also uses amino groups for coupling. We concluded that lysine residues are extremely important for the immunogenicity of SPEK15. To analyze whether chemical modification of the BD generated during the coupling procedure might have elicited antibodies which only recognized the modified BD and not the unmodified BD, we measured the reactivity against modified BD in ELISA. This turned out to be negative.

Considering (b) above, linkage of TD and BD was checked by measuring free thiol groups in the construct combined with elution patterns of HPLC chromatograms. It could be estimated that over 50 % of the added BD was coupled to the TD with MBS. From remaining free thiol groups, it could be concluded that 90 % of the peptides were conjugated with BMH. Assuming random linkage between peptides, about one third of all conjugates may be expected to represent heteroconjugates. From other experiments [4], we learned that a dose of 10 μg (*i.e.*, one third of 30 μg) should be sufficient to induce a proper immune response, though it is lower than the dose given for the linearly synthesized BD-TD. In addition, antibodies cross-reactive with the TD were found after immunization with BMH-coupled constructs proving that the dose was sufficient.

Though it has been reported that chemical coupling of BD and TD [8, 11] could induce a memory immune response against the BD, our selected BD and TD did not induce antibody responses against the BD when chemically linked. Our results are consistent with a report [19] which showed that immune responses generated with a linearly synthesized TD-BD peptide are predominantly directed against the BD, whereas the BD-TD construct, chemically linked via BMH, elicited antibodies against the TD. In our study. there is no difference between responses to the chemically linked TD-BD or BD-TD construct when used

for immunization. Both constructs did not elicit antibodies against the BD. Thus, orientation of the TD in this case does not seem to be the only limiting factor as has been suggested by others [10, 20-22].

Considering (c), antigen processing, competition for MHC binding and hampered recognition by the T cell receptor: a "hole" in the repertoire may influence an immune response. Several investigators [23-25] have proposed that adjacent sequences may alter the conformation of an epitope resulting in significant altered antigen processing or changes in T cell recognition. For our chemically linked TD-BD constructs, altered conformation relative to linear synthesized TD-BD peptide may also account for the lack of antibody responses against the BD.

Though it has been reported that mixtures of free TD and BD [12] could induce a memory immune response against the BD, our experiments indicated that this is certainly not true for all combinations of T and B cell epitopes. Current views on the generation of a memory immune response recently stated that TD and BD must be localized in the same molecule [13, 26]. Peptides used in the studies by Sarobe et al. [12] might have associated to form a strong complex allowing antigen processing and recognition and subsequently cognate T and B cell interactions to elicit a humoral immune response. However, in previous studies [4], we found that "pseudo conjugates", nonspecific adherence of peptides to carrier molecules, were not able to elicit an immune response in contrast with peptides coupled to the same carrier molecules with carbodiimide.

Taken together, our results indicate that in order to induce a high-titer anti-peptide immune response, the peptide must be provided with a T cell epitope by covalent coupling to a carrier protein or by a peptide bond between TD and BD.

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Chapter 10

Multiple Antigen Peptides for immunization

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MULTIPLE ANTIGEN PEPTIDES FOR IMMUNIZATION

Netty D. Zegers, Eric Claassen and Wim J.A. Boersma

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I. INTRODUCTION

A new approach to generate anti-peptide antibodies has been initially developed by the group of Tam and colleages (Posnett et al., 1988; Tam, 1988). A branched core of lysine residues was constructed by making use of the α and ϵ amino groups of lysine. Subsequently, multiple copies of the peptide epitope of choice could be synthesized onto all arms of the core. The resulting structure has been called multiple antigen peptide (MAP) (Fig. 1).

Two categories of MAP application can be distinguished: (i) as a highly efficient immunogen and (ii) as an efficient antigen for detection in immunoassays. The MAP approach for immunization purposes offers several advantages over the conventional carrier-peptide construct. It is a construct of intermediate molecular mass with a defined orientation of the peptide. Most of the molecule (may be more than 90%) consists of the peptide antigen in contrast to the carrier-peptide conjugate, where the peptide in general is only part of the construct. Ad i. First, probable immunodominant determinants or even suppressor determinants of the carrier protein can be circumvented by applying a MAP. Second, purification is rather easy, and, dependent on the peptide sequence chosen, the construct can be used directly for immunization without coupling to a carrier protein. Third, the peptide dose of the administered MAP is exactly known, in contrast with the peptide dose administered as a peptide-carrier conjugate. In addition, the dendritic peptide chains on the MAP are probably mobile, which may contribute to enhanced immunogenicity. These characteristics make the MAP extremely useful for application as immunogen or as a synthetic vaccine.

Ad ii. MAPs were also shown to be very useful as antigens in solid phase immunoassays. Coating of MAPs to polymer surfaces leads to more efficient epitope exposition than is obtained with free monomeric peptides and thus are very effective for

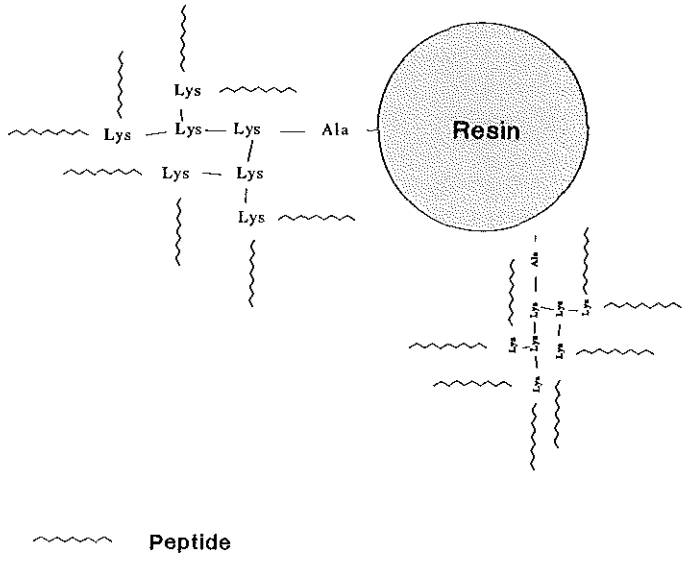

Fig. 1. Schematic drawing of a multiple antigen peptide. To the functional groups of the resin, the first amino acid (β-Ala as an internal standard amino acid) is coupled, and, subsequently, a Lys with two Fmoc protecting groups for the α and ϵ amino groups is coupled. After removing the Fmoc groups, two Lys residues can be coupled. This step can be repeated several times, until the branching of choice has been reached: the core. Four and eight branched cores are applied most frequently. The selected peptide is then synthesized onto the arms of the core. Here, two MAP molecules on a resin bead are shown.

sensitive detection in ELISA (Tam and Zavala, 1989; Habluetzell et al., 1991; Marsden et al., 1992; Marguerite et al., 1992).

Here, we will discuss the MAP immunogen properties only.

II. ALTERNATIVE SYNTHESIS

The basic design of a MAP allows alternative synthesis to offer investigators highly flexible possibilities to explore various abilities of the MAP approach for immunization purposes. By making use of two different protecting groups (Fmoc and tBoc) for lysine at the last level of

branching, two different peptide synthesis routes can be applied. Thus, two different peptides can be synthesized onto the core. (Tam and Lu, 1989).

Chemical coupling of a purified peptide with a N-or C-terminal cysteine and a chloroacetylated oligomeric lysine core matrix was described as an alternative for construction of the MAP (Lu et al., 1991). This method offers the advantage to study the influence of orientation of the peptide to the lysine core. It was found that antibodies raised with these MAPs were mainly directed against the distal (and most flexible) part, opposite of the conjugation site, of the peptide (Lu et al., 1991).

Drijfhout and Bloemhoff (1991) described a similar coupling method: the addition of a protected branched lysine core to a functionalized peptide. The lysine core is kept protected by N-(S-acetylmercaptoacetyl)-glutamyl residues. Removal of the S-acetyl groups just prior to the coupling procedure yields thiol functions which can be used for coupling to, e.g., S-(Npys)-cysteinyl peptides.

Virtually the same approach was followed by Baleux and Dubois (1992). Both the lysine core and the peptide antigen were provided with S-(Npys)-cysteine. Conjugation was carried out by removal of the Npys group of the lysine core with dithiothreitol and subsequent addition of the S-(NPys) peptide.

Coupling of a fully protected peptide, which contained a T cell determinant (TD), and a protected MAP, containing peptides with a B cell determinant (BD), proved to be an effective method for fragment condensation (McLean et al., 1992). The TD peptide can be synthesized in bulk in advance and thus does not require repetitive synthesis of the TD each time a di-epitope MAP is synthesized.

III. MAPS AS IMMUNOGENS

An octameric MAP was found to be highly immunogenic in mice (Balb/c) and rabbits in the first study carried out with MAPs. The peptide (14 residues) was derived from the human T cell antigen receptor β-chain constant region. The antibodies which were raised reacted with the peptide in its monomeric form as well as in its octameric form and with the intact β-chain protein (Posnett et al., 1988). The antibodies were mainly directed against the N-terminal residues of the peptide. No antibodies against the core were detected. The subclass distribution of the subsequently generated monoclonal antibodies was predominantly IgG1 and, to a lesser extent, IgA and IgG2a, which the authors attribute to the T cell dependent nature of the antigen. The authors did not demonstrate that the selected peptide sequence with which the MAP was constructed itself contained a T cell determinant, nor was it investigated whether a new T cell determinant had been formed upon assembly of epitopes by synthesis of the MAP. It is also conceivable that repeating epitopes on the same molecule may cause crosslinking of antigen-specific immunoglobulin receptors on B cells and thus behave as a relatively T cell independent immunogen which can induce isotype switching. However, known T cell independent immunogens as lipopolysaccharide (LPS) or ficoll activate B cells

to synthesize predominantly IgG3 besides IgG1. Posnett and colleagues did not investigate the IgG3-specific response.

In a subsequent study, five out of six different MAPs with octameric branched peptides derived from different proteins were highly immunogenic. The antibodies which were induced crossreacted with the native proteins (Tam, 1988). For one determinant investigated the analogous carrier-SP conjugate induced substantial lower levels of antibodies than was observed with the MAP construct.

Co-workers of Marsden compared the efficacy of six different MAPs with conventional carrier-peptide conjugates of the same peptide sequences as immunogens (McLean et al., 1991). The titers of sera from rabbits immunized with branched peptides were higher than those immunized with carrier-peptide conjugates. These findings are confirmed by others (Wang et al., 1991). However, Briand et al. (1992) have tested several peptides sequences, as a carrier-peptide conjugate or as a MAP, in immunization studies. They found that three out of four MAPs were immunogenic, but, most importantly, only one out of these four MAPs raised antibodies crossreactive with the native protein. It appeared that immunization with two MAPs, in which the sequence of the peptides was derived from the C-terminus of a protein, did not result in protein-specific antibodies. The orientation of the determinants with respect to the core matrix may have played a major role. From this, we tend to conclude that there is no advantage in using MAPs with respect to the generation of anti-native protein antibodies with synthetic immunogens.

With the $(NANP)_{10}$ repeat of *Plasmodium falciparum* circumsporozoite protein, it was demonstrated that a MAP construct with 4 branches could overcome genetic MHC restriction. The linear $(NANP)_{10}$ peptide is only immunogenic in $H\text{-}2^b$ mice (DelGuidice et al., 1986; Good et al., 1987), while with the $(NANP)_{10}$-MAP construct IgG antibodies were raised in various mouse strains with different MHC background (Pessi et al., 1991). However, MAP constructs with the repeat of other malaria parasites (*P. vivax* and *P. berghei*) did not overcome genetic MHC restriction. It is difficult to explain the finding that the MAP-$(NANP)_{10}$ construct overcame MHC restriction, since it seems a favorable exception. The authors speculated on three hypotheses. (i) A MAP might bind to MHC class II molecules as a multivalent ligand. However, it was shown that not all MAPs could overcome MHC restriction, thus this may only be found with special combinations of MAPs sequences and MHC haplotypes. (ii) (NANP)-specific B cells are activated with a MAP independently of T cell help through Ig crosslinking. However, IgG antibodies were observed and proliferation of lymph node cells from immunized mice was observed. (iii) A MAP follows different proteolytic pathways, as compared to linear peptides, leading to products that bind with higher affinity to Ia molecules. In addition, we think that it is conceivable that new T cell determinants may be formed in the MAP with the $(NANP)_{10}$ sequence.

IV. INCORPORATION OF A T CELL DETERMINANT

Enhancement of the antibody response by addition of a TD peptide in a MAP has been reported (Tam et al., 1990; Munesinghe et al., 1991; McLean et al., 1992; Nardelli et al., 1992a).

It was investigated whether incorporation of a TD in an essentially non-immunogenic BD-MAP would result in an immunogenic construct. MAPs containing 4 or 8 copies of a T cell determinant combined with 0, 1, 4 or 8 copies of a B cell determinant were compared for their efficacy to raise (neutralizing) antibodies (Tam et al., 1990). Also, the complementary MAPs in which the TD and BD were mutually exchanged (4 or 8 copies of the B cell determinant combined with 0, 1, 4 or 8 copies of the T cell determinant) were synthesized and immunized. Moreover, in the same study the effect of orientation of the determinants [$(BD\text{-}TD)_4$, $(BD\text{-}TD)_8$, $(TD\text{-}BD)_4$ or $(TD\text{-}BD)_8$] on the antibody response was investigated. The BD (residues 93–108, repeat domain) and the TD (residues 265–276) were derived from the circumsporozoite protein (CS) of *P. berghei*. The BD monomer and the 4 and 8 branched MAPs containing either the BD or the TD alone did not induce antibodies in mice. The BD-TD monomer induced only a very low level of CS-specific antibodies. In this study, 4 or 8 branched MAPs containing linearly synthesized tandem arrayed BD-TD or TD-BD peptides induced high levels of recombinant CS-specific antibodies and protected mice from challenge (50–80% protection). The $(BD\text{-}TD)_4$-MAP scored highest followed by $(TD\text{-}BD)_4$-MAP or $(TD\text{-}BD)_8$-MAP and $(BD\text{-}TD)_8$-MAP. This study showed that incorporation of a T cell determinant in a MAP indeed rendered the construct immunogenic as compared to the MAPs that only contained the BD. Four branches appeared to be sufficient, if not optimal, for eliciting a high immune response in case a TD is incorporated.

After mapping the *P. berghei* circumsporozoite protein for T cell helper activity, Migliorini et al. (1993) chose two apparent T cell determinants (residues 20–39 and 57–70) for constructing multiple antigen peptide constructs comprising only one of the T cell determinants or one of the T cell determinants co-linearly synthesized to a tandem repeat of B cell determinant of the CS protein. Mice immunized with one of the T-B constructs were protected after a challenge with infective sporozoites and a high level of B cell determinant-specific antibodies could be detected. Strikingly, also mice immunized with a MAP construct comprising only the 57–70 T cell epitope were protected, while peptide-specific antibodies were not found. These latter results indicate that protection can be obtained either by effector T cells or by high levels of antibodies. Thus, a double mechanism of protection can be acquired by a synthetic peptide vaccine. With evaluation of the antigen-specific antibody response alone, protection against infection after challenge with a pathogen may be missed.

We have investigated the effect of incorporation of a T cell determinant on the antibody response either in a linear peptide or in a MAP. The immunogens were compared with the linear peptide conjugated to a carrier protein, keyhole limpet hemocyanin (KLH). We determined whether or not a T cell dependent antibody response intrinsically is formed by a MAP without a TD sequence. The T cell responses were assessed in an *in vitro* proliferation assay after priming *in vivo* with the different immunogens.

TABLE 1
Peptide sequences

Peptide	Character	Sequence	Derived from
SP149	TD	FERFEIFPKEKC*	TD from influenza (AA 111–120)
SP190	BD	C*DEGKLQHLVNELT	α_1-Antitrypsin (AA 256–268)
SP191	TD-BD	FERFEIFPKEKDEGKLQHLVNELTC*	TD + AA 256–268 from α_1-antitrypsin
SP215	TD and BD	DKTVERKCCVECPPCPAPPVA	Human IgG2 (hinge) (AA 95–115)

* An extra cysteine was added to the sequences for coupling purposes.
TD: T cell determinant; BD: B cell determinant.

The peptides used for this study are listed in Table 1. Peptide SP190, a pure B cell determinant, was derived from the human S-variant of α_1-antitrypsin (residues 256–268). SP191 contained the sequence of SP190 which was N-terminally elongated with a TD from the influenza virus hemagglutinin (residue 111–120) (Hackett et al., 1985; Zegers et al., 1993). A third peptide, SP215, was derived from human IgG2 (residues 95–115) and contained an intrinsic TD and BD (Boersma et al., 1989). Peptides SP190 and SP191 were coupled to KLH via an extra added cysteine, and SP215 was coupled via EDC (using carboxyl and amino groups). These peptides were used for immunization in mice (BALB/c) as free peptide, MAPs or conjugated to KLH. Sera were analyzed for specific antibodies in ELISA.

From Fig. 2, it is clear that SP190 (BD) is not immunogenic. The KLH-SP190 conjugate induced peptide-specific antibodies which were crossreactive with α_1AT as expected. When SP190 was offered as a branched peptide (MAP), peptide-specific antibodies were detected and these antibodies were also reactive with the α_1AT protein in ELISA. The level of protein- specific antibodies raised with the MAP was lower than the level raised with the KLH-SP190 conjugate. It can be concluded from these results that a non-immunogenic peptide can be made immunogenic by offering it as a MAP or as a carrier-peptide conjugate.

From Fig. 3, it is clearly seen that incorporation of a TD renders the peptide immunogenic, though the antibody response induced with the free TD-BD peptide (SP191) is low as compared to the antibody responses induced with the MAP or the conjugate including the same sequences. Incorporation of a TD in a MAP therefore specifically enhanced the protein- specific antibody response. The level of the α_1AT-specific antibody response after immunization with MAP191 is similar to the response after immunization with the KLH-SP191 conjugate.

SP215 is immunogenic and induces peptide- specific antibodies crossreactive with the native protein (Fig. 4). Conjugation of this peptide with EDC to a carrier protein decreased the protein-specific response, probably due to modification of the epitope by the coupling procedure. Also with a conjugate made by coupling via the cysteines, protein-specific anti

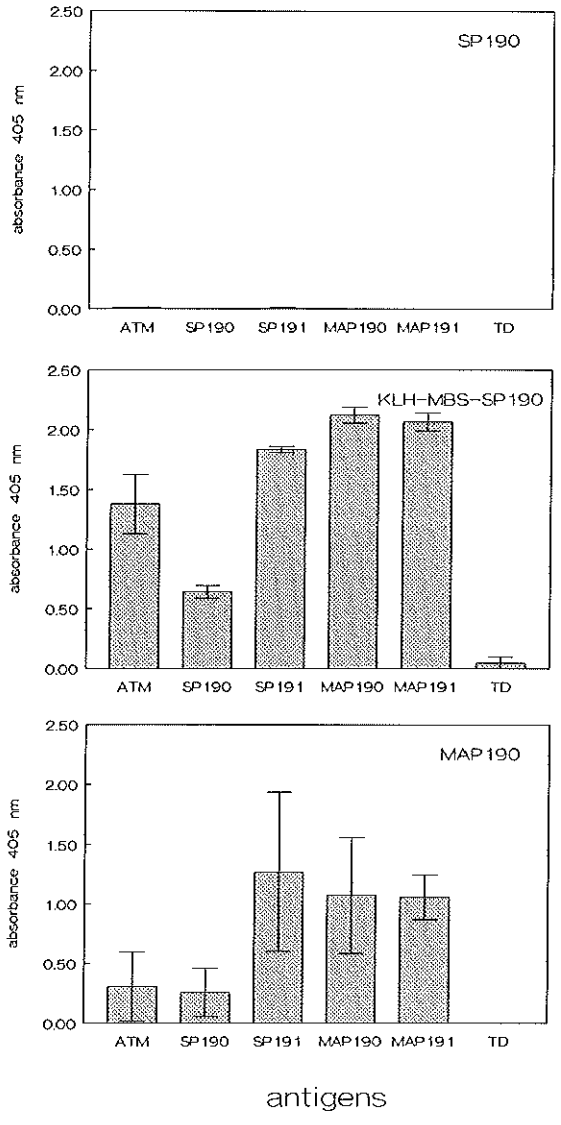

Fig. 2. Comparison of the specific reactivity of mouse sera after immunization with different peptide constructs containing a B cell determinant peptide. Mice were immunized twice with a 4 week interval with the linear peptide SP190 (upper graph), with a carrier-peptide construct KLH-MBS-SP190 (middle graph), or with a branched peptide MAP190 (lower graph). One week after the second immunization, sera were analyzed in ELISA for reactivity with linear peptides [SP190 and SP191, or the T cell determinant (TD)], with branched peptides [MAP190 and MAP191], or with the native protein α_1-antitrypsin [ATM]. Mean serum responses of three mice with standard deviation are shown. See Table 1 for sequences and character of peptides.

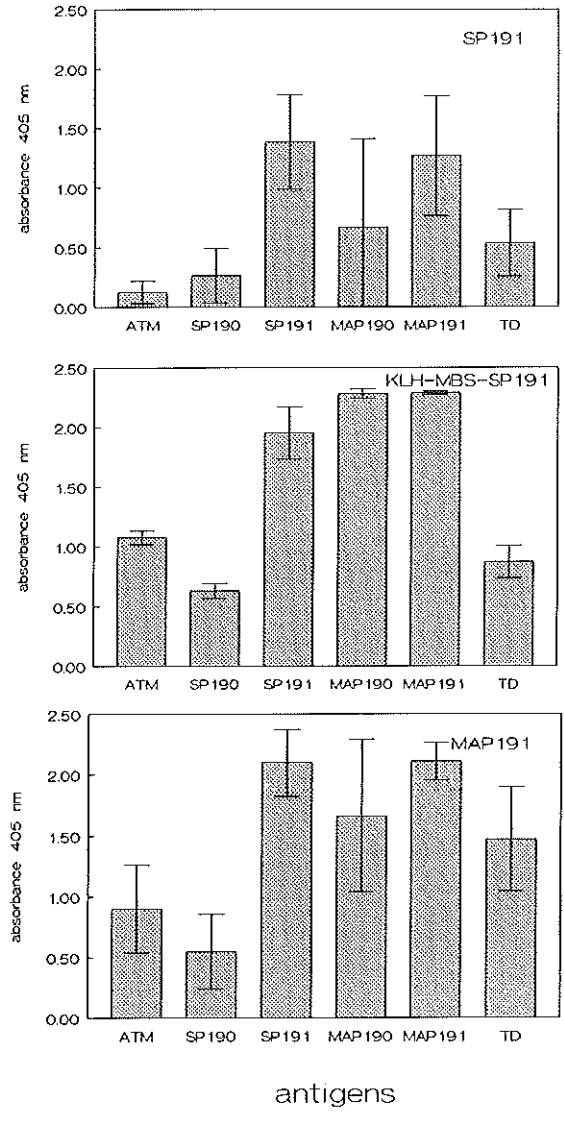

Fig. 3. Comparison of the specific reactivity of mouse sera after immunization with different peptide constructs containing a B and a T cell determinant peptide. Mice were immunized twice (4 week interval) with the linear peptide SP191 (upper graph), with a carrier-peptide construct KLH-MBS-SP191 (middle graph), or with a branched peptide MAP191 (lower graph). Serum immune responses were analyzed in ELISA as described for Fig. 2.

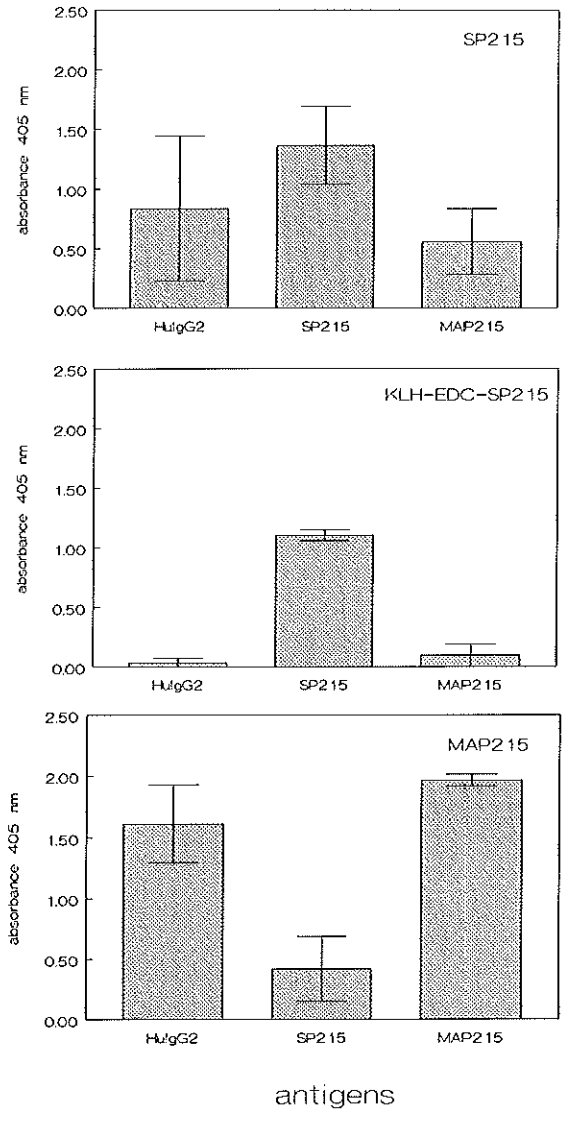

Fig. 4. Comparison of the specific reactivity of mouse sera after immunization with different peptide constructs containing a B cell and a T cell determinant peptide. Mice were immunized twice (4 week interval) with the linear peptide SP215 (upper graph), with a carrier-peptide construct KLH-EDC-SP215 (middle graph), or with a branched peptide MAP215 (lower graph). One week after the second immunization, sera were analyzed in ELISA for reactivity with the linear peptides, SP215, with branched peptide MAP215, or with the native protein human IgG2. See Table 1 for sequences and character of peptides.

bodies were not found (not shown). For this peptide, with many functional groups available for coupling, the MAP approach seems the most appropriate one. Indeed, high levels of protein-specific antibodies were raised with MAP215.

Lymph node cell proliferation assays were performed after *in vivo* priming with the immunogens. Most obviously, *in vitro* restimulation with a peptide or a MAP, which contains a TD, induced a specific enhancement of the proliferation (Fig. 5). Especially, the stimulation indices for restimulation with SP191, MAP191 or SP215 of cells primed with SP191, MAP191, SP215 or MAP215 were of the same order of magnitude as after restimulation with concanavalin A, a polyclonal mitogen (not shown). No proliferation was seen with SP190 and MAP190 (both contain only a BD) restimulation of cells primed with SP190 (BD), MAP190 (BD), SP191 (TD-BD) or MAP191 (TD-BD).

We found subclass distribution patterns (IgG1, IgG2a, IgG2b, IgG3) which were more or less similar for all immunogens used (data not shown). The only apparent difference observed was that the level of BD-specific IgG2b antibodies elicited with MAP190 (BD) was higher as compared to MAP191 (TD-BD). Also the ratio IgG2b/IgG1 was higher after immunization with MAP190 than that found with the other immunogens. It remains to be demonstrated whether a MAP without a TD in the peptide sequence can behave as a T cell independent immunogen capable of inducing isotype switching.

We showed that for strong and native protein-specific responses incorporation of a TD into a MAP can be an advantage. For the sequences used in our study, a MAP with only a BD did not stimulate T cells *in vitro* in a proliferation assay. Despite the absence of T cell activation, specific IgG antibodies (predominantly IgG2b and IgG1) could be detected. The antigen presentation as well as the B cell activation mechanism by MAPs still remains to be elucidated. In the present case, for a peptide which contained several residues sensitive to modification during coupling procedures, the MAP approach is most appropriate.

V. MAPS AS VACCINES

Major advantages of synthetic peptide vaccines are the avoidance of any pathogenic sequence which is present in the pathogen in its natural or attenuated form and the safe production of the vaccine. Furthermore, the immune response with MAP is directed only against the selected protective peptide sequence. MAPs are expected to be extremely useful for application as vaccines, since they present a multivalent construct with high potential immunogenicity. A MAP is of advantage, as compared to conjugation of a peptide to a carrier protein which is needed for immunogenicity, in that the immune response mounted against the carrier protein as is observed with a conventional carrier-peptide construct can be avoided. With a MAP, the immunodominant B cell determinant, relevant for elicitation of protective antibodies, together with efficient promiscuous T helper or cytotoxic T cell determinants, can be selected.

Several investigators explored the application of MAPs as vaccines in model studies. Mice could be protected from infection with rodent malaria after immunization with a MAP

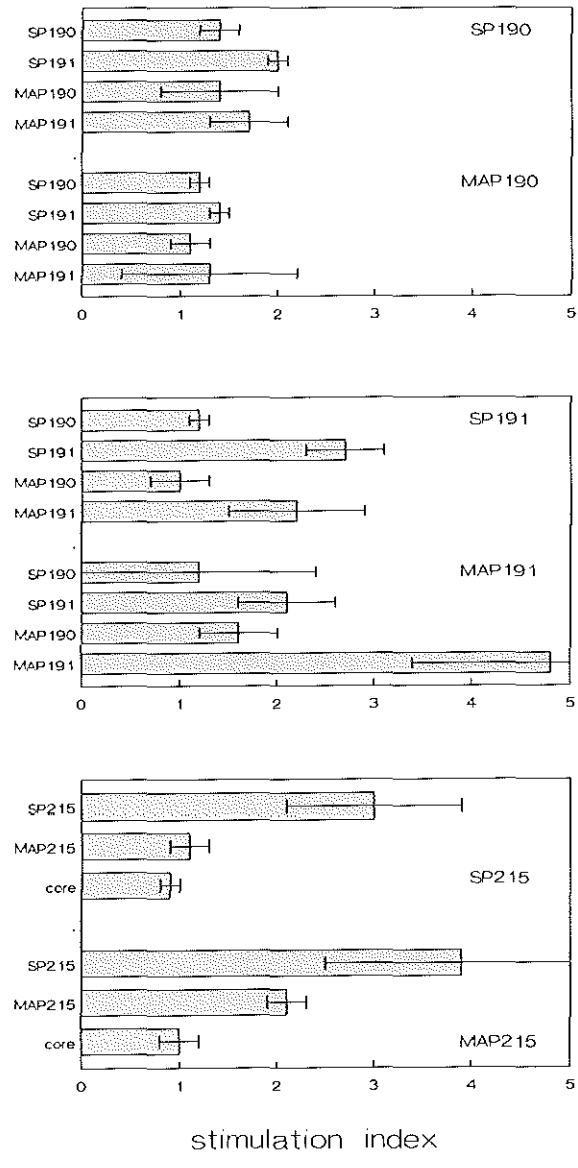

Fig. 5. Proliferation of stimulated lymph node cells primed *in vivo* with a linear peptide or with a MAP. Proliferation of stimulated lymph node cells primed with (Upper graph) SP190 or MAP190, (Middle graph)) SP191 or (Lower graph) MAP191, SP215 or MAP215. Cells were restimulated *in vitro* with different antigens (Y-axis). The mean stimulation index with standard deviation for three mice is shown on the X-axis after ^{3}H-Tdr incorporation and is calculated by dividing the cpm in cultures with antigen by the cpm in cultures without antigen. A stimulation index greater than 2 is significant.

containing a BD and a TD of the circumsporozoite protein of the parasite (Tam et al., 1990; Zavala and Chai, 1990; de Oliveira et al., 1994).

Neutralizing antibodies against the HIV-1 were elicited in guinea pigs after immunization with a MAP containing a rather long sequence (33 amino acids) from the third variable (V3) loop of gp120. The antibody response exceeded the response found with a peptide-bovine serum albumin conjugate prepared with glutaraldehyde with a factor 100. Moreover, the antibody response persisted more than 3.5 years (Wang et al., 1991).

Putative vaccines with intrinsic adjuvant properties were constructed by linking tripalmitoyl-S-glycerylcysteine (P3C) to a MAP with a peptide sequence from the V3 loop of gp120 from HIV-1 which includes a neutralizing B cell epitope, T helper and T-cytotoxic epitopes (Defoort et al., 1992; Nardelli et al., 1992b). The lipophilic membrane-anchoring group (P3C) can be used for inclusion in liposomes. Such complexes induced specific HIV-1 neutralizing antibodies and elicited cytotoxic T lymphocytes. Even with a single administration of the P3C-MAP construct without incorporation in a liposome, the same responses were obtained (Nardelli and Tam, 1994), demonstrating the promising potential use of MAPs as vaccines.

VI. CONCLUDING REMARKS

MAPs offer a very useful alternative for conventional immunogens (linear peptides and peptide-carrier conjugates), provided that a TD is incorporated in the peptide sequence or that a TD is formed by the sequences in the construct. Since a MAP is a spatial structure, conformational determinants also may be formed. MAPS are very flexible in use by various optional synthesis possibilities. In general, the specific antibody response raised with MAPs is higher than the antibody response raised with linear peptides and comparable with or even superior over the antibody response with peptide-carrier conjugate. Moreover, carrier-specific antibodies, immunodominant determinants of the carrier and carrier induced epitope suppression may be prevented by the use of MAPs, which is another important advantage for application in vaccine development.

In case the amino acid sequence of an antigen contains residues which are important for immunogenicity and of which side chains are sensitive to coupling reagents, coupling should be avoided. MAPs then offer an excellent alternative. Besides antibody production, T effector cell mediated protection also can be obtained by applying a MAP as vaccine including a TD.

From several studies it has become clear that a MAP with four arms of branching is sufficient to induce an optimal immune response.

ABBREVIATIONS

α_1AT	α_1-Antitrypsin
BD	B cell determinant
CS	Circumsporozoite
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
ELISA	Enzyme-linked immunosorbent assay
Fmoc	9-Fluorenylmethoxycarbonyl
KLH	Keyhole limpet hemocyanin
LPS	Lipopolysaccharide
MAP	Multiple antigen peptides
MBS	m-Maleimidobenzoyl-succinimideester
P3C	Tripalmitoyl-S-glycerylcysteine
t-Boc	*t*-Butoxycarbonyl
TD	T cell determinant

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Chapter 11

General discussion

I.

Detection of proteins using immuno-chemical assays is specific, reliable, simple, convenient, cheap and rapid. Furthermore, a lot of samples can be assayed simultaneously, this in contrast with many other, more conventional, biochemical assays. Specific antibodies are needed for immuno-chemical assays and have to be elicited in animals upon immunization with the protein of interest. Sometimes a protein is not available for immunization for different reasons (see chapter 1) or cannot be used because the protein is toxic or pathogenic. Immunization with part of the protein synthetically made is an alternative. Such synthetic peptides also offer the possibility for eliciting antibodies specific for proteins which are homologous to other proteins. The most optimal sequence, i.e., the part with the most mismatches to the homologous proteins, can then be selected for immunization. For some investigations domain-specific antibodies are a requirement. It is apparent that synthetic peptides are the most appropriate reagents for eliciting antibodies. It was the aim of the investigations presented in this thesis to develop a successful strategy for arriving at applicable antibodies with high affinity for the native protein. To realize the generation of antibodies, two model proteins were chosen, each with its own characteristics and reasons for applying the synthetic peptide approach. The first protein against which antibodies were desired, the human androgen receptor, had not been isolated before and the amino acid sequence was predicted from the DNA sequence. In addition, a considerable homology exists with other steroid receptors. The antibodies had to be applicable in various immuno-assays, such as ELISA, Western blotting, immuno-precipitation, or in histochemical procedures. The second protein, a variant of α_1-antitrypsin, differs in only one amino acid from the normal α_1-antitrypsin. It is obvious, that only antibodies specific for the substitution site can distinguish between the different variants. In these cases one is thus restricted in the choice of the synthetic peptide sequence.

Using immunizations with peptides, we were able to generate highly specific diagnostic antibodies, applicable in various immuno-chemical assays, with a high affinity for the different domains of the human androgen receptor and for the most important variant of α_1-antitrypsin. The generation, evaluation and applications of the antibodies will be discussed in view of the different parameters which have their impact on the complete route to success using the peptide strategy.

Based on our observations of more than 300 different peptide sequences derived from numerous proteins, suggestions will be given for the various steps in the route of the peptide strategy application (Table 1), meant for those who enter this field of research.

II. SEQUENCE SELECTION

The selection of the sequence to be used for the immunogen is the first and most important step in the generation of anti-peptide antibodies. The choice of the sequence is seldom a totally free one. Restriction to certain regions of the protein is often dictated by homology

TABLE 1

Strategy for the production of antibodies to synthetic peptides

- ☐ Selection of antigenic sequence
  - on the basis of physical and chemical structure parameters of the protein
  - Immunogenicity: Hydrophillic > hydrophobic sequences
    Surface probability
    Fragment flexibility
    Secondary structure (α-helix > β-sheet)
    Tertiary structure
  - Results in : Antigenic index
- ☐ Identification of region of interest and limitations in choice
  - Localization of mutations
  - Check for homology within protein families
  - Glycosylation or modification sites
- ☐ Check for homology with extracellular and/or serum proteins
- ☐ Synthesis and purification of peptide
- ☐ Derivatization
  - To improve immunogenicity
  - To include adjuvanticity
  - To include reporter molecule
- ☐ Coupling of peptides to a carrier protein
  - To incorporate a T cell determinant
- ☐ Immunization with proper adjuvant in target animal
  - Mostly 2 administrations with well selected time interval are needed
- ☐ Development of the appropriate assay for evaluation of the anti-native protein reactivy
  - Sometimes this step can only be carried out after induction of antibodies. This means after the next step.
- ☐ Evaluation of immune response in serum sample
  - Determination of anti-conjugate reactivity to check for proper immunization
  - Determination of anti-peptide reactivity
  - Determination of anti-carrier reactivity
  - Determination of anti-coupling determinant reactivity
  - Determination of anti-native protein crossreactivity in appropriate assay
  - Determination of specificity of the antibodies. Note: absence of specificity in a polyclonal serum is not necessarily a problem, since specific antibodies may be present in the mixture
- ☐ Production and selection of monoclonal antibodies
  - Selection with the assay in which the monoclonal antibody is going to be used. Preselection in ELISA with a peptide coating is possible in order to screen a huge number of clones.
- ☐ Characterization of the monoclonal antibody in the proper assay
  - Reactivity with native protein (inhibition assay) and specificity
  - Isotype
- ☐ Large scale production, purification and labelling if necessary

with other proteins. For instance, selection of a homologous peptide will generate family-specific antibodies which readily recognise shared epitopes. Alternatively, selection of heterozygous peptides will result in antibodies which are uniquely specific for a single member of the entire family. One should realise, however, that selection of a member-specific sequence can result in unwanted family-specific antibodies. Even restriction to a single position in a protein may be encountered. Thorough knowledge of the protein in relation to its characteristics such as structure, function and relationships with other proteins may substantially increase the rate of success. For example, structures which have a function in the binding of ligands often represent protein invaginations (hormone or DNA-binding pockets) which are difficult to reach for the relatively large antibodies (Novotny et al., 1986). In contrast, structures which form extensions of proteins such as zinc fingers or the V3 loop of the HIV-I might offer better accessibility. Searching for matches with other protein sequences in data bases and subsequently taking this information into account may prevent disappointments afterwards, e.g., unwanted crossreactions. Besides information about intrinsic characteristics of the protein, computer algorithms for prediction of antigenic sequences may provide additional help with varying success as we showed before (Zegers et al., 1991a, 1991b; Gerritse et al., 1990; Kleine et al., 1990; van Denderen et al., 1990, 1992, 1994; Boersma et al., 1989a).

As has been critically reviewed by Van Regenmortel & Daney de Marcillac (1988) none of the present computative methods for the selection of antigenic determinants leads to a satisfactory success rate in prediction of antigenic sites. It was demonstrated on the basis of a retrospective statistical analysis that segmental mobility (Karplus & Schulz, 1985) and hydrophillicity prediction algorithms led to the highest level of correct predictions of known epitopes. The lowest level of correct predictions was obtained using the method based on the relative frequency of specific amino acids in known determinants (Welling et al., 1985). However, the analysis of Van Regenmortel and Daney de Marcillac (1988) is based on literature data from a variety of different methods to assess antigenicity: e.g., (a). Some polyclonal antisera generated with the intact protein or fragments were subsequently tested for cross-reactivity with peptides. The outcome of this type of test system is influenced by the way the peptide is presented in the assay (free peptide, conjugated, bound to plastic surfaces, competition with labelled peptide etc.), (b). By inhibition assay using peptides of the binding to the native protein, (c). The data obtained by the PEPSCAN method. In this evaluation (Van Regenmortel & Daney de Marcillac, 1988) only the correct prediction was taken into account of known determinants of well-studied model proteins. These however represent only the laboratory animal selection of all possible antigenic sites. For a correct evaluation the ratio of the antibodies directed against the protein (peptide mimicking the protein structure under assay conditions) as a proportion of all anti-peptide antibodies (antigenicity of the peptide *per se*) might have given a more realistic estimate of the correctness of the various predictions.

We observed that only a proportion of the anti-peptide antibodies were crossreactive with the native protein. A peptide can adopt various conformations as well as in the immunogenic construct and in assay conditions dependent on the micro-environment. Only

one of these conformations resembles the conformation of the epitope in the native protein. And a proportion of these crossreactive antibodies are multi-assay applicable (Zegers et al., 1991b; Boersma et al., 1989b). Similar observations were reported by Jemmerson (1987) for antibodies elicited using peptides from horse cytochrome-c. Parry et al. (1988) demonstrated for the foot-and-mouth disease virus (FMDV) that guinea pig anti-peptide sera raised to a synthetic peptide of the AA 141-160 of FMDV, showed cross-reactivity with the virus particle dependent on the mode of peptide-presentation. About 60-70% of the activity in the sera was directed to the peptides only. Intact virus absorbed out 30-40% of the specific activity. This was irrespective of whether the peptide was immunized free or conjugated with KLH.

Short peptides (10 AA) are not always immunogenic or only yield peptide-specific antibodies not crossreactive with the native protein. Though we earlier showed that short peptides may induce protein-specific antibodies (Van Denderen et al., 1989, 1990), longer peptides of about 15 to 25 amino acids readily lead to native protein-specific antibodies of higher affinity. The explanation for these findings may again be found in the multiple conformations a longer peptide can adopt relative to the possibilities of a short peptide. Another explanation may be that the short peptide has too few contact residues for the IgM molecule on the surface of the B cell which initially bind the immunogen.

We suggested that, in general, short peptides tend to elicit antibodies which recognize the denatured form of the protein only (Boersma et al., 1988). Application of short peptides therefore may be of advantage for the elicitation of antibodies to be used *in assays* where the antigen is encountered in denatured form, e.g., in SDS-PAAGE, fixed tissue specimens etc. (Van Denderen et al., 1989, 1990). In contrast, Dyson et al. (1985) showed that an immunogenic nona-peptide which showed a preferential conformation in aqueous solutions elicited anti-native antibodies to hemagglutinin of influenza virus. Ultrastructural analysis showed that longer peptides indeed tend to mimic the native three dimensional structure of a protein much better since these peptides maintain a specific space-filling structure (Horiuchi et al., 1987).

Some, more general, recommendations for sequence selection are: Try to avoid "difficult sequences" to synthesize, e.g., sterically hindered amino acids such as Ile-Ile, Leu-Ile. Choose termini in such a way that they are relatively highly hydrophillic. Avoid residues such as Trp, His, Met, Tyr and Cys since they are sensitive to side reactions and/or oxidation during deprotection procedures. However, Cys is often incorporated for coupling purposes and aromatic residues may be advantageous for immunogenic reasons. Avoid synthesizing hydrophobic sequences or sequences that readily form β-sheet structures since solubility problems may arise. Choose the terminal sequences in such a way that they preferably contain hydrophillic residues. Include functional groups at one of the termini designed for coupling to a carrier protein by means of a proper amino acid residue for example. Avoid localization in the middle of the peptide sequence of residues of which the side chain might be involved (but not desired) in coupling procedures necessary for conjugation to a carrier protein. Localization of the substituted residue in the centre of the peptide sequence is most successful for generating variant-specific antibodies. Try to avoid

including immunodominant sequences as predicted with computer algorithms if site-directed antibodies are desired specific for a substituted residue.

Of course not all recommendations can be strictly followed and a compromise should be made. Fortunately, even in cases where selection choices were not fully optimal, appropriate antibodies could be found thanks to a flexible and partly degenerated immune system.

Computer algorithms for prediction of the antigenicity surely are helpful in the selection of putative immunogenic sequences. Crossreaction of the antibodies with the native protein induced with such selected peptides, however, is not guaranteed (van Regenmortel and Pellequer, 1994). But even if protein-specific antibodies should be elicited upon immunization with a peptide, they are just a fraction of the peptide-specific antibodies.

In conclusion, we show that several factors in the selection of sequences play a role and that from our experience the following rules can be proposed:

- search for antigenicity according to the computer predictions
- exclude sequences which are homologous to other proteins by searching data bases
- take into account other characteristics of the protein sequences, such as function and structure, that highly improve the success rate.
- incorporate useful coupling sites and avoid troublesome amino acids, with regard to synthesis as well as coupling aspects.
- select a sequence of at least 15 residues. The maximum number of residues is determined by the costs of the synthesis and the difficulty of synthesis which increase with the length of a peptide.
- locate the epitope in the centre of the peptide sequence or at the distal end from the coupling site.
- chose hydrophillic residues at the termini of the peptide for improving the solubility and, as a consequence, the efficiency of conjugation of the peptide and the carrier protein.

Together all these considerations will generally lead to the successful selection of the peptide sequence.

III. SYNTHESIS AND PURIFICATION

Synthesis of peptides is an intricate task despite the completely automatic synthesizers commercially available nowadays. It demands high investments in terms of money, knowledge and skill. Though synthesis seems a routine job, it definitely is not. Many protocols for protection, activation, coupling, deprotection and cleavage chemistry as well as for the kinetics of the reactions are available together with a wide range of reagents. These are all confusing to new researchers. If synthesis is a difficult job, the purification of peptides still is much more demanding. Already for a decapeptide, 20^{10} different sequences are possible, this is more than 10^{13}. All these sequences have their own characteristics which make it infeasible to work with one standard protocol for purification.

For immunization it is not necessary to achieve the highest purity, but the more pure a preparation is, the more homogenic the antibody response will be. It is conceivable, e.g., that a side-product in the preparation will be immunodominant which is not desired. For evaluation purposes, the peptides should be of the highest purity.

Many suppliers are nowadays offering custom-made peptides at reasonable prices and one can buy peptides with various grades of purity. Only if more than about a hundred peptide sequences per year are needed, could it be worthwhile to think of a synthesis unit with a technician trained well for the job.

IV. CONSTRUCTION OF THE IMMUNOGEN

IV.1. Free peptides

Immunization with free peptides in general does not lead to antibody responses, since most peptides selected with prediction algorithms for antigenicity only contain a B cell determinant.

According to some authors the success rate is unexpectedly high while using antigenic peptides all of sufficient length, ± 20 amino acids, for immunization as free immunogens (Lerner et al., 1981; Atassi & Webster, 1983; Briand et al., 1985). For lysozyme (Bixler et al., 1985) and for the FDMV (Francis et al., 1987b) it was found that only a selection of all peptides of a protein is able to generate an antibody response with the free peptide. Using a rather large number of peptides from various proteins selected by computer prediction methods, we found that only a minor proportion, ± 20%, is able to generate an anti-peptide response using the unconjugated peptide and in most cases, only when administered subcutaneously (chapter 5). McCormick et al. (1988) showed that irrespective of the T-cell epitope character of a peptide the response was dependent on the adjuvant, dose and timing as well as on the sex of the mice used. The generation of antibodies even with long peptides does not guaranteed the generation of anti-native protein antibodies as was shown for the insulin β-receptor. Four out of four peptides did not produce anti-native receptor antibodies (Pessino et al., 1989).

IV.2. Coupling procedures

Both for immunization as well as for evaluation of the immune response, conjugates of peptide and carrier protein are needed. Since upon immunization with a conjugate, antibodies are also raised against the linker between peptide and carrier, resulting from the conjugation, or against a neo-determinant introduced as a side product in the conjugation reaction, evaluation of the immune response should ideally be determined against a conjugate of the peptide with another carrier and a different coupling agent. Precise and conditioned coupling is a requirement for avoiding introduction of neo-determinants and for achieving controlled orientation of the peptide relative to the carrier. Amongst many homo- and hetero-bifunctional coupling reagents, MBS, EDC and glutaraldehyde are the most appropriate ones, since they are relatively simple to use, each with its own advantages and limitations. Improved protocols for these reagents were developed by us specifically for use with peptides

(Zegers et al., 1990; Deen et al., 1990). Conjugation by MBS by means of an introduced cysteine residue or a protected -SH function by SATA at one of the termini of the peptide is a favoured and controlled method. N-terminal conjugation is preferable because of two reasons. The first reason is that during synthesis the cysteine residue, which is sensitive to side reactions, is only exposed to possibly harmful chemicals during its own coupling cyclus. The second reason is that only completed sequences, in case the nascent chain is acylated after incomplete coupling, contain a cysteine residue. Of course, N-terminal conjugation of a peptide is not always an advantage (see under orientation). Addition of a non-native cysteine to a sequence in general does not disturb the immunogenicity of the conjugate and reaction pattern of the induced antibodies. On the contrary, enhancement of immunogenicity of free peptides was reported in some cases after incorporation of a terminal cysteine, probably by the forming of dipeptides (Francis et al., 1987a). When a cysteine residue is part of the peptide sequence, MBS conjugation cannot be used in most cases. Then a choice between EDC and glutaraldehyde should be made. EDC, which introduces the peptide in at least two orientations (N- or C-terminally) onto the carrier, should not be used when several Lys, Glu or Asp residues are scattered through the peptide sequence. The same holds true for the use of glutaraldehyde when several lysine residues, especially consecutive residues, are part of the sequence. That these rules, however, are not an absolute truth, has been proven by anti-hAR MAbs selected by us. Care should be taken, however, for the protocols employed, since neo-determinants and uncontrolled coupling are easily introduced.

It is not easy to predict the influence of coupling procedures on the immunogenicity of a peptide and the recognition of native protein. Application of different conjugation methods simultaneously and subsequent immunization with the different conjugates is the most pragmatic and fastest way of achieving antibodies with a broad reaction pattern. Evaluation of these antibodies should point out which method leads to success.

Poor solubility of the peptides negatively influences the efficiency of coupling. Synthetic peptides despite being selected from a hydrophillic sequence of a protein may show highly variable solubility behaviour. Peptides which do not readily dissolve in aqueous solution, DMSO or DMF (frequently used in coupling protocols) may be conjugated as described before (Gerritse et al., 1991). In this procedure, peptides were dissolved in high concentrations of urea and conjugated to alkaline phosphatase, which did not prevent the activity of the enzyme.

IV.3. Orientation of the peptide on the carrier

From the experiments conducted with peptides from α_1-AT, it can be concluded that the distal end of the coupling site is readily exposed to the immune system. The antibodies elicited are specific for the distal end.

The N- or the C-terminus of a protein in general is a flexible part of the protein implying probably a high immunogenicity. If antibodies are desired against one of the termini of a protein, by means of synthetic peptides, the termini should be exposed. In other words, the N-terminal part of the protein should be coupled at its C-terminus and the C-terminal part

at its N-terminus (Zegers, unpublished results; Boersma et al., 1992; Schaaper et al., 1989; Briand et al., 1992).

Most successful for eliciting Z-variant specific antibodies, were the peptides with the substitution in the centre of the sequence. Positioning the substitution site near the distal terminus (one residue from it) relative to the coupling site did not lead to the desired antibodies. The substituted amino acid probably has to be flanked with some residues in the synthetic peptide to mimic the structure in the α_1-AT protein. From the experiments with the peptides derived from the 342 site of α_1-AT, we decided to place the 264 substitution in the middle of all the peptides selected. With these peptides, we found that an immunodominant sequence, even when placed at the coupling site and thus badly presented to the immune system, in general, leads to antibodies against this immunodominant sequence overruling the presentation of the flexible distal end.

Cyclization of a peptide through a disulphide bridge of the termini may give good results when the native protein contains a loop. Such a cyclic peptide and structure-constraint peptide mimics the structure of a loop in the native protein. Antibodies raised with cyclic peptides may discriminate between a linear and a cyclized peptide and recognize the native protein instead of a denatured protein.

In conclusion, controlled coupling methods should be used for the conjugation of peptides and carriers, to avoid introduction of neo-determinants and to achieve a controlled orientation of the peptide relative to the carrier. The use of MBS, EDC or GA is dependent on the constituent amino acids, but can best be judged pragmatically. Multiple antigen peptides can be considered if conjugation is difficult, e.g., if the peptide is insoluble or if the residues involved in coupling are part of the epitope.

V. IMMUNIZATION

In general, a dose of 25-50 μg peptide-carrier conjugate (carrier-based calculations) or free peptide lead to a proper antibody response specific for the protein, from which the peptide sequence was derived, in mice. A higher dose of 150-250 μg may substantially enhance the fraction of the protein-reactive antibodies and can be employed in case a poor anti-protein antibody response is encountered. We usually immunize mice via an i.p. administration since the constructs often contain some aggregated material for which i.v. injection is not possible. Injection in the footpad stimulating the popliteal lymph node is a useful alternative, but in some cases only peptide specific responses were found (chapter 5). Moreover, this method is animal unfriendly and should be employed only in limited cases when other methods fail.

We compared different adjuvants for stimulating peptide immunization, from which specol, a water in oil adjuvant, emerges as useful with a good performance: convenient to prepare the immunogenic mixture, minor side effects for the animal, high and persisting antibody responses. Titermax*, Adjuvax*, Ribisystem* and Alhydrogel* or Lipovant* were compared to Freund's complete adjuvant by Bennett et al. (1992) using haptens and peptides.

Adjuvax®, Ribisystem® and Alhydrogel® or Lipovant® were compared to Freund's complete adjuvant by Bennett et al. (1992) using haptens and peptides. They demonstrated that Titermax® led to the highest titers when anti-peptide antibody titers were compared. In most experiments the Ribi® adjuvant was the second best. FCA induced the highest anti-peptide titers but with the highest adverse effects. This was also reported by Leenaars et al. (1994). The persisting antibody response can be a disadvantage, since for an optimal booster effect the response should be at a minimum level. In our protocols, at least a four week interval between immunizations is taken, leading to good results. Longer intervals between these injections (6-12 months) proved to give high numbers of stimulated B cells beneficial in cell fusion procedures for obtaining monoclonal antibody secreting hybridomas. An option could be: assay for specific antibody responses in serum at, e.g., weekly intervals and boost only when the antibody response level has returned to 10-20% or less of the maximum level.

To generate monoclonal antibodies, immunize at least 4 and at maximum 8 mice with a peptide conjugate. Following evaluation of the antibody response after the prime and booster immunization, only restimulate the mouse with the highest level of antibody responses for hybridoma production. Leave the other mice; these mice can be immunized at a later stage in case a second fusion protocol should be carried out, e.g., with an alternative selection procedure. Meanwhile, the antibody response in these mice will return at a minimum level. A strong memory-B cell response can be obtained by subsequent immunization.

In our experience peptide-specific PAbs (elicited in rabbits), which are mono-specific, provided useful reagents for use in various immuno-assays (ELISA, Western blotting, immuno-precipitation, immuno-histochemical detection and immuno-staining of cells) (Gerritse et al., 1990 ; Van Denderen et al., 1989; Van Laar et al., 1989). In addition, there appears to be a tendency that outbred rabbits recognize a broader repertoire than we regularly observe in the Balb/c mice.

In conclusion, intraperitoneal immunization with 25-50 μg of the peptide-carrier construct per mice given in a mineral oil suspension as a mild adjuvant generally leads to satisfying antibody responses, providing, of course, that the chosen sequence is immunogenic.

VI. EVALUATION, SELECTION AND CHARACTERIZATION

Upon immunization with synthetic peptides, the polyclonal antibody response has to be evaluated after each administration. Routine evaluation can be made 7 days after injection. As stated in section III, a booster injection can be given when the antibody level has decreased to a level below 20% of the peak level. Assay selection proved to be of major importance. Some of these are several kinds of ELISA formats, Western blot, immuno-precipitation, immunocyto- and histochemistry. The easiest method is assaying serum samples in ELISA on a coating of the native protein. In addition, the response to the peptide-carrier construct as a control for proper immunization, the response to the peptide and the response

to the peptide presented as a peptide-protein construct made by an alternative conjugation method should be determined.

It should be taken into account, however, that coating to the plastic may change the conformation of the protein in such a way that a denatured protein is mimicked. The antibodies which recognize protein coated to plastic in general also tend to recognize the protein applied in SDS-Western blotting procedures or in immunohistochemistry of freeze sections of tissue specimen. Recognition of proteins in formaldehyde treated paraffin sections of tissues is often hampered by the stringent fixation method. Formaldehyde modifies the protein to a large extent in such a way that antigenic determinants are destroyed. Retrieval of the antigenic determinant by heating the sections in acid solutions, in the presence of heavy metal ions necessary for some proteins, is possible in some cases following selected procedures (Jansen et al., 1994). Antibodies that recognize only the denatured form of the protein can still be useful reagents in diagnostic immuno-assays.

Recognition of the native protein in solution is possible with competition assays: pre-incubation of the antibodies with the native protein and subsequent transfer for incubation in ELISA, Western blot or immuno-chemistry (for review see Jemmerson, 1995). With polyclonal antiserum, a competition assay is not always predictive for the existence of native protein-specific antibodies, since these antibodies, especially those with high affinity, may only be a small proportion of total antibodies present (Pathak at al., 1995). With adequate stimulation and selection protocols, high-affinity monoclonal antibody secreting hybridomas can be found.

Upon detecting antibodies of the desired specificity in the serum of immunized animals, the next step often will be generating hybridomas which secrete monoclonal antibodies. The selection procedure to screen the hybridomas eventually determines the specificity of the antibodies and thus can be considered as one of the major steps. The MAbs should preferentially be selected with the method in which the MAbs are going to be used. For sandwich assays, this is not always possible, since a useful capture antibody may not be available. Polyclonal rabbit antisera can be very useful for this aim and should be raised with the protein, if possible, or with a peptide derived from a different region of the protein than the one that is used for MAb production. Care should be taken, if the protein is used for eliciting PAbs. As was demonstrated (Zegers et al., 1993), the major fraction of the PAbs could be directed against immunodominant regions which also have been used for peptide sequences. — Peptides often are selected because of their high antigenic indices. — A step by step sandwich assay then fails. A solution to this problem is a pre-incubation step of the supernatant from the culture wells with the native protein, hereby allowing the MAb to be selected to form an Ag-Ab complex. Subsequently, the Ag-Ab complex is transferred to the well with the capture antibody. The concentration of the native protein should not exceed the coating concentration of the capture antibody. Sometimes it is necessary to expose betterthe antigenic determinant in protein, e.g., by denaturing by mild heating in the presence of β-mercaptoethanol.

In conclusion, evaluation of the reactivity pattern in the polyclonal serum should be carried out after each administration. The reactivity should be assayed with the peptide, with the carrier, with the coupling determinant, with the native protein and with irrelevant peptides and proteins as negative controls. Crossreaction with the native protein should be carried out by competition or inhibition or sandwich assays. Only by selecting MAbs in the assay method that is going to be used or that is designed in such a way that it will resemble the ultimate assay, can specific, high-affinity antibodies be found.

VII. USE OF T CELL DETERMINANTS

Immunization with free hybrid peptides, i.e., which possess both a T cell determinant (TD) as well as a B cell determinant (BD), can be used to elicited BD-specific antibodies. The selection of the TD is important. The factors that play a role in the selection were exhaustively discussed in chapter 8. The conjunction between the TD and BD is very important. A natural peptide bond (instead of chemically linkage by, e.g., MBS or GA) yields the most immunogenic constructs, but the efficiency of synthesis of long sequences decreases with length. The conjunctional site may create a new TD or BD with positive or negative result. Consider the MHC restriction. Elongation of a TD can have a positive influence due to increased stability providing that the flanking sequences contribute to increased helix character. Multiple copies of a TD may lead to an increase of the antibody response. N-terminal position of the TD in a hybrid TD-BD construct is preferred.

Peptide-protein constructs in general are more immunogenic than free peptides, probably since these constructs possess various TDs. Free peptides could be advantageous for effective vaccines. A useful alternative for eliciting high-titer antibodies may be the MAP construct provided that a TD is incorporated into the sequence. Such a MAP is advantageous if a sequence can only with difficulty be conjugated to a carrier because of critical residues which might be involved in the conjugation reaction.

VIII. CONCLUSION

Peptides have proven to be useful tools in the production of antibodies. Though it seems that immunization with peptide antigens is straightforward, it is a delicate process to arrive at useful antibodies. On the basis of two model proteins, it was demonstrated in this thesis that only profound control of all individual steps of the peptide strategy eventually lead to the appropriate antibodies. Selection of the epitope sequence and the development of the selection assay for screening hybridomas together with the development of the assay in which the antibodies are going to be used are most important and determine the result of the affinity and specificity of the antibodies. Careful application of the peptide strategy following the recommendations abstracted and discussed in this chapter has led to hAR-specific MAbs and MAbs specific for α_1-AT and the Z-variant of α_1-AT.

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Chapter 12

Materials and Methods

CHAPTER 12

MATERIAL AND METHODS

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I. PEPTIDES

I. 1. Selection of peptide sequences for synthesis

For practical purposes, when there are no restrictions posed by homology etc., then the antigenicity prediction as described by a computer program is helpful. We used the program of the Genetics Computer Group, Wisconsin (Devereux et al., 1984). Terminal segments may be very useful since they loop out of the globular structure. Peptides are chosen at least 15 (15-25) amino acids long. Amino acids for coupling purposes may be added at strategic terminal positions. The sequence is chosen such, that both ends of the peptide do not represent a minimum in hydrophillicity. Peptides can be elongated either N- or C- terminally by a cysteine or arginine residue for coupling purposes.

I.2. Peptide synthesis

Peptides are synthesized with a Sam II Biosearch peptide synthesizer (San Rafael, Ca, USA) using the Merrifield (1963) solid phase technique with t-Boc-protected amino acids on a polystyrene resin (1% crosslinking). Deblocking and cleavage for t-Boc synthesis is performed by treatment with thioanisol and hydrogen fluoride (90 min, 0°C) as described by Bhatnager et al.(1983). Crude peptides are precipitated from ether followed by an extraction with acetic acid (5%). Subsequently, peptides are lyophilized and subsequently reduced with 10 equivalents of *threo*-1,4,dimercapto-2,3,butanediol, pH 8.0, 60 min at room temperature and lyophilized.

Some of the peptides (59-66) were produced semi-manually using the Dupont RaMPS system (Dupont Medical Products, Biotechnology Systems U.S.A.) and Fmoc protected amino acids (Dupont, Wilmington, USA) essentially as described elsewhere (Zegers et al. 1991a) according to the manufactorer's instruction.

Peptide synthesis using Fmoc-amino acids is performed on a Milligen 9500 machine (Milligen, San Rafael, CA, USA) using the protocol and amino acids from the manufacturer. Pepsyn-KA and PEG-PS resins (Milligen) are used substituted with the C-terminal Fmoc-amino acid, substitution degree 0.1-0.2 mmol/g. OPfp-esters are used for most amino acids if available. Otherwise, the Fmoc-protected amino acids (His, Asn, Gln, and Arg) are activated *in situ* using DIPCDI/HObt. Protecting groups are: Trt for Cys and His, tBu for Ser, Thr, and Tyr, OtBu for Asp and Glu, tBoc for Lys, Tmob for Asn and Gln, Pmc for Arg. Deblocking and cleavage is performed with a mixture of scavengers and trifluoroacetic acid according to the Milligen protocol. Standard 95% TFA/5% phenol/5% water. Dependent on the protecting groups used, additonal 2% anisol and/or 2% 1,2-ethanedithiol is used (see protocol Milligen). Relevant peptides containing Cys residues are reduced before purification using 10 equivalents β-mercaptoethanol for 30 min at 37°C and lyophilized.

I.3. Peptide purification

The crude peptide is dissolved in a minimum amount of 5% (v/v) acetic acid. Gelfiltration is performed on G-15 Sephadex (Pharmacia, Uppsala, Sweden) using 5% (v/v) acetic acid as eluent. Analysis of the fractions is assessed by reversed phase HPLC. A gradient of 0.1 % trifluoroacetic acid in acetonitrile (15-40%) into 0.1% trifluoroacetic acid in H_2O on a Beckman Ultrosphere C18 5μm column (4.6x250 mm) is applied. Fractions showing the same

homogenous peak at 214 nm, are pooled. Amino acid analysis is performed essentially as described by Janssen et al. (1986). The proportion of available -SH groups is determined using the method described by Ellman (1959) (See section I.4.3). All reagents for peptide synthesis or derivatization are HPLC quality.
Alternatively, peptides can be purified using HPLC as described above. For purification, a column for preparative scale Delta Pak C18 15 μm (19x300mm) is used.

I.4. Peptide derivatization

For an introduction into various amino acids, specific chemical protection techniques, which have been developed for proteins but in general can be applied to peptides as well, see Atassi (1977). For the selection of proper coupling reactions the following rules of the thumb: a) Use preferably bi-functional reagents, b) use specific coupling procedures for an amino acid that has a low frequency in the peptide, c) avoid amino acids that are crucial for recognition, d) elongate peptides with an amino acid for coupling purposes e.g. Cysteine or Arginine.
Frequently applied reagents: Sulfo derivatives of N-hydroxysuccinimide compounds are less susceptible to hydrolysis and water-soluble. In general they lead to better conjugate yields (2-4x). Most other coupling reagents are hygroscopic and readily hydrolyse. Storage -20°C. First equilibrate to ambient temperature before use. Tris, glycine and other amino-group containing reagents (ammonium sulphate) should be avoided. SP-protein conjugates stored at 4°C were not stable (Briand et al. 1985). It is advised to store all conjugates at protein concentrations > 1 mg.ml^{-1} at -20 °C.

I.4.1. Biotinylation (Long chain biotin, LCB)

Biotin binds extremely well to avidin. However, to prevent unwanted interactions by the avidin bound, due to the extreme depth of the binding pocket (4-5 amino acids deep), it is best to couple biotin via a, long aliphatic chain, linker (Suter et al. 1986). N-hydroxysuccinimidyl-6-(biotinamido)-hexanoate (Pierce) is bound equimolar to the peptide. The procedure is easiest to perform while the peptide is still bound to the resin with all its protecting groups still in place. Coupling to the unprotected -NH_2 terminal side is than easily obtained. Be sure that all protecting groups are removable using TFA.
Ingredients: DMF, amylalcohol, acetic acid, diethylether, a saturated solution of phenol in water, long chain biotin, imidazole (10 mg LCB in 10 μl imidazole).
Procedure: The resin-peptide is washed thoroughly with DMF. Leave the resin submersed in DMF. LCB is added in imidazole to equimolar concentration with the peptide. Incubate overnight (room temperature). Wash with DMF. Subsequently wash with tert.amylalcohol (2x), acetic acid (2x), tert. amylalcohol (2x), diethylether and dry in vacuum. With conjugated avidin the biotinylation can be monitored. Then cleave the peptide from the resin with 95% TFA, 5% phenol in water. Purification of peptides according to standard procedures.

I.4.2. Introduction of sulfhydrylgroups

When peptides or proteins do not contain free -SH groups, these easily can be introduced using Traut's reagent: methyl-4-mercaptobutyrimidate (iminothiolane) (Traut et al., 1973) This compound reacts preferentially with amino groups. On mild reduction the peptide or protein is tagged with free -SH groups. The -SH groups introduced can be used for coupling with a hetero-bifunctional coupling agent or they can be allowed to form -S-S- bonds (King et al., 1979).
Procedure: For the best result a 3-fold concentration of 2-iminothiolane (MW 140) over the peptide concentration is used. The reaction is allowed to take place in a sodium phosphate buffer (NaPi), pH 8.1. Iminothiolane is added dropwise to the peptide solution (5-10 mg.ml^{-1}) in a small reaction vial and allowed to react for 1 h at roomtemp. Subsequently the peptide is separated from iminothiolane by gelfiltration over G-15 Sephadex (Pharmacia). (Dialysis membranes with low mol. weight cut off. (1 kD) give disappointing results).

I.4.3. Determination of available -SH groups

Available -SH groups can be determined using Ellmans reagent (Ellman, 1959).
Ingredients: NaPi buffer 0.1 M pH 8.0.; 5,5'dithio-bis-(2-nitrobenzoic acid (DTNB, Ellman's reagent), 4 mg.ml^{-1} in NaPi 0.1M, pH 8.0.; solution of cysteine or β-mercaptoethanol as a standard.

Procedure: Briefly, add 0.1 ml of the sample to 0.1 ml of the reagent solution and mix with 4.8 ml NaPi pH 8.0 buffer. After a few minutes the reaction is completed. Read absorbance at 412 nm. $E_{412}=1.36.10^4 cm^{-1}.M^{-1}$ versus blank.

II. COUPLING PROCEDURES

II.1 Homo bi-functional coupling

II.1.1. Activated -SH

(coupling two active -SH which are incorporated into the protein/peptide)

Ingredients: N-succimidyl 3-(2-pyridyldithio)propionate (SPDP) solution 20-40 mM (MW 312.4; 1.56-12.5 mg.ml^{-1})in ethanol or DMSO; protein (carrier) 1-10 mg.ml^{-1} in NaPi buffer 0.1 M, pH 7.5, with 0.1 M NaCl; small reaction vial; sepharose G-25 column (PD-10, Pharmacia); acetate buffer 0.1 M, pH 4.5 with 0.1 M NaCl; dithiotreitol (DTT) 0.1 M; n-ethyl-maleimide 10 mM.

Procedure: Coupling principle: protein + SPDP (10 fold excess, minimum) → protein with protected -SH; idem for peptide. On reduction of one of the 2-pyridyldisulphides with DTT, preferably the protein, the protecting groups are removed. Mixing the peptide-2-pyridyldisulphide with the thiolated carrier protein yields an -S-S- coupling product. The coupling i.e. the release of the pyridyl-2-thione can be followed spectrophotometrically $E_{343}=8.08.10^{-3}M^{-1}.cm^{-1}$. Allow the protein to react with SPDP under stirring in a small reaction vial for 30 min at room temperature. For the determination of protein concentrations spectrophotometrically, a correction factor for the 2-pyridyl disulphide should be taken into account ($E_{280}=E_{280}$-[pyridyne-2-thione released, molair] x $5.1.10^3$, Pharmacia methodology SPDP method). To transfer to acetate buffer and to remove excess SPDP, bring protein-2-pyridyl disulphide over a G-25 column (PD-10). Reduce this disulphide with DTT (final concentration 50 mM, 30 min roomtemp.). Transfer to NaPi pH 7.5, 0.1 M NaCl and remove reagents over G-25 column (PD-10, Pharmacia). Similarly the peptide is treated with SPDP and excess of reagents removed by HPLC-chromatography. Finally, the 2-pyridyl disulphided peptide and the thiolated protein are allowed to form a disulphide bond (1h-overnight, room temperature). Thiolated protein (pH 7.0) may be stored at -20°C. Remaining free -SH groups if necessary can be blocked with n-ethyl-maleimide (10mM).

II.1.2. Glutaraldehyde

(coupling -NH_2)

Ingredients: Carrier protein: 10 mg.ml^{-1} in PBS; peptide: 10 mg.ml^{-1} in water; dialysis membrane cut off 10 kD); glutaraldehyde (Baker's); glycine 0.2 M; PBS; small reaction vials.

Procedure: Dialyse (4°C, overnight) carrier protein (1ml) versus 0.2% glutaraldehyde (1.6 ml) in 200 ml PBS. Dialyse two times versus 250 ml PBS (2 x 2h). Transfer activated carrier into reaction vial. Slowly add SP: 100 mol/mol carrier protein. Incubate overnight (4°C). Then add 0.1 ml 0.2 M glycine to block all active aldehyde groups left (2h, roomtemp.). Dialyse extensively to PBS. Store conjugate at -20°C (Zegers et al., 1990, adapted from Boorsma, 1984). For the conjugation of peptides which are not readily soluble a method has been described by Gerritse et al., 1991.

Peptides sometimes are not readily soluble in water or water solutions like borate or phosphate buffers etc. Alternatively DMSO or DMF can be used as a solute.

If peptides still are not dissolved urea may be used. Recently a modification of a glutaraldehyde coupling of peptides to alkaline phosphatase (Zegers et al., 1990) has been described by Gerritse et al. (1991), using 8 M urea to dissolve the peptides.

Procedure: Briefly, Dialyse AP (10 mg.ml^{-1}) against 0.2% glutaraldehyde in PBS for 16 h at 4°C. Remove excess glutaraldehyde by dialysation of AP against PBS. Transfer into a small reaction vial. Dissolve peptide (1mg.ml^{-1}) in 8M urea pH 7.0 by stirring for 10 min at roomtemp. Remove the non-dissolved material by centrifugation. Solubilized peptides are mixed with the activated AP (peptide/Ap = 100/1) for 16 h at 4°C. Block remaining active

glutaraldehyde groups by adding 100 µl 0.2 M lysine.HCl, incubate another 2h. Excess reagents are removed by dialysis to PBS.

II.2. Hetero-bifunctional coupling

II.2.1. Carbodiimide EDC

(coupling e.g. $-NH_2$, -COOH)

Ingredients: N-methyl-imidazole 0.5 M, pH 6.0; carrier 10 mg.ml^{-1} in N-methyl-imidazole; peptide 20 mg.ml^{-1} in N-methyl-imidazole; EDC (1-ethyl-3-(3-dimethylaminopropyl)cardodiimide hydrochloride, Pierce) 100 mg.ml^{-1} in N-methyl-imidazole; dialysis membrane cut off 10 kD; small reaction vials.

Procedure: Mix carrier and peptide solution in reaction vial: SP 100 mol/mol carrier (mol weight carrier 150 kD) for other carriers similar e.g. BSA (mol weight 60 kD): 50 mol SP/mol carrier protein. Subsequently add slowly (drop-wise) the EDC (MW 191.7) solution to 50 mol/mol SP. Mix 30' at roomtemp. Then dialyse vs PBS overnight, 4°C. Store at -20°C (Deen et al., 1990).

II.2.2. Maleimide/N-hydroxysuccinimide MBS

(coupling of -SH and -NH)

Ingredients: NaPi buffer 0.1 M, pH 7.0 (SPB); carrier protein 10 mg.ml^{-1} in SPB; Coupling agent m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS, mw. 314.2, Pierce) 20 mg.ml^{-1} in dimethylformamide (DMF); peptide: 20 mg.ml^{-1} in SPB; 2 small reaction vials; PD-10 column (Pharmacia).

Procedure: Carrier solution in vial is stirred on ice. MBS is added dropwise/slowly in 3 portions with 5 minutes intervals to 200 mol MBS/mol carrier (mol weight 150 kD). MBS with NaPi buffer gives a precipitate. This is discarded by centrifugation (Eppendorf, minifuge). Activated carrier is separated from unreacted MBS via a desalting column (Pharmacia, PD10). A little bit of precipitate can be discarded. The activated carrier is transferred to a clean reaction vial. The peptide is added to 100 mol SP/mol carrier (mol weight carrier 150 kD). Stir at roomtemp for 1 h. Store at -20°C.

II.2.3. Azidophenylglyoxal APG

(Arginine to Tyrosine, histidine; photoaffinitylabeling)

Glyoxal containing compounds react selectively (at pH 7-8) with arginine residues (Ngo et al., 1981) (for a review see Atassi 1977). Coupling arginine containing peptides to proteins and vice versa arginine containing proteins to any peptides containing tyrosine, histidine or lysine has been applied by us.

Ingredients: SP: 5.10^{-3}M (± 10 mg.ml^{-1}) in H_2O; APG: 1.10^{-2}M (1.9 mg.ml^{-1} in borate buffer 0.1 M, pH 7.9), prepared and kept in the dark; UV lamp (λ= 360 nm, socalled black ray 40W). $NaBH_4$ 10^{-2}M in H_2O. KLH 10 mg.ml^{-1} in PBS. Dark glass mini-reaction vial.

Procedure: The procedure starts in complete darkness! Incubate 0.2 ml SP with 0.2 ml APG (for one Arg in the peptide; APG: ARG= 2) at 37°C for 2 h. The course of the reaction can be followed spectrophotometrically: APG free has a maximum at 297 nm whereas the APG-Arg product has a maximum at 250 nm. 0.2 ml of the reaction mixture is mixed with 0.2 ml KLH solution. Mix well and place this solution in a smal petri-dish or 24 well tissue culture plate under the UV-lamp for 30 min. Subsequently the coupling product is reduced for stabilization with 0.4 ml $NaBH_4$ for 2 h on ice. Dialyse to PBS.

II.3. Coupling of peptides to enzymes

Peptide-enzyme conjugates can be applied for detection of receptors for peptide ligands (antibodies, peptide-hormone receptors etc.) (Laman et al., 1990, 1991a). In principle the methods as described for peptide coupling in general can be applied. In our hands GA and MBS-like reagents fulfil our criteria best.

III. IMMUNIZATION

III.1. Animals

For our experimentations,female BALB/c mice aged 12-16 weeks were used throughout. They were kept in macrolon cages under an 11 hr dark/13 hr light regimen at 20°C and were given acidified water (pH 3) and pelleted mouse food (Hope Farms, Woerden, The Netherlands) ad libitum. Flamish Giant rabbits rondom bred (MBL-TNO, Rijswijk, The Netherlands) were used to elicit polyclonal anti-peptide sera. Experiments were performed under the auspices of the Dutch Veterinary Inspection, according to the law on Animal Experimentation.

For the production of immunological reagents two routes are most widely used: 1) Injection of conjugate or peptide together with adjuvant intraperitoneally in mice or 2) Subcutaneously on the back of rabbits or subcutaneously on the upper side of the feet or in the inguinal and axillary regions of the mice. In both cases serum IgG responses are obtained after a booster immunization. The spleen and lymphe node cells can be used for the production of Mabs. With subcutaneous injections the doses should best be distributed over more places (four) for better results.

III.2 Doses

Peptides of 15-20 amino acids are immunized in doses between 25 and 100 μg per mouse or 100-200 μg per rabbit. Conjugates contain a number of peptides per carrier and in general a doses range between 50 and 150 μg of the conjugate, based on the carrier content, will be sufficient.

III.3 Adjuvants

III.3.1. Specol

Water phase (9/20 v/v) including the antigen is slowly pipetted onto the oil-phase (Specol) (Bokhout et al., 1981) under thorough mixing on a Vortex. The emulsion is relatively stable and can be used for administration.

III.3.2. Freund's adjuvant

Using Complete Freund's Adjuvant (CFA) thorough mixing of the components (incomplete, bacteria, antigen) until water-in-oil and oil-in-water emulsions are obtained is necessary for good results. Mixing is easily performed when two glass syringes are connected by a double luer-lock. In general 45/55 v/v of antigen solution and CFA is used. (Caution, the mixture of CFA is a skin-irritant).

In mice, 200 μl of the emulsion is administered intraperitoneally, while 50 μl per location is injected subcutaneously. In rabbits, 250 μl per location is used for water-in-oil emulsions as well as for CFA.

III.3.3. Alum precipitation

Precipitates of proteins or peptide-conjugates onto alum (Aluminium-potassium-sulphate gel) act as depot administrations.

Ingredients: 10% w/v solution of aluminium-potassium-sulphate in phosphate buffered saline (PBS) is prepared; 1M $NaHCO_3$ (2.1 g/25 ml PBS); mini-reaction vial.

Procedure: Dissolve 100 μg of protein to 210 μl PBS. Add dropwise the $NaHCO_3$ (90 μl) and mix. Subsequently 200 μl of the alum gel is added slowly under vigorous stirring (or vortexing). Leave the mixture overnight at 4°C, freshly prepared alum precipitates may be toxic! Mix before introduction. Use 200 μl of the mixture per mouse.

III.3.4. Bordetella Pertussis

Similar to mycobacteria in Freund's Complete Adjuvant, various other bacteria (mainly membrane/cell wall components) show adjuvant activity. For this purpose heat killed Bordetella Pertussis bacteria are frequently used. At the time of immunization or one day in advance $2x10^9$ bacteria are injected intraperitoneally in mice. For rabbits $8x\ 10^9$ bacteria are administrated subcutaneously. Do not mix Bordetella pertussis suspensions with alum precipitates since in that case large aggregates will develop.

III.4. Scheduling

The time-course of development of serum antibody responses may in part be dependent on the type of carrier protein used. When KLH is applied, then in mice serum antibodies (IgG + IgM) increase gradually up to at least 21 days. For an estimate of responses repeated tapping of the animals at various time points (14 and 21 days) after immunization is advised. In rabbits taking some serum 14 days after each immunization is sufficient. Immunizations with peptide-carrier conjugates are given with four weeks intervals. In general after two to three subsequent immunizations the maximum titers are obtained.

Best results following booster immunizations are obtained when priming titers have decreased. However, using KLH, which itself has adjuvant properties, anti-carrier titers will remain high for months. Nevertheless normal immunization schedules can be completed.

The timing of the immunization preceding spleen cell fusion depends on the routing: soluble antigens (conjugates) can be introduced intravenously three days before fusion. Less soluble antigens are given intra-peritoneally (i.p.) with IFA, as a water in oil emulsion or precipitated on aluminium hydroxide gel four days before an intended spleen cell-fusion. Though we prefer spleen cells for fusion, for fusion of lymph nodes regional nodes are activated by immunization with adjuvant (as for i.p. immunization). Mirza et al. (1987) performed fusion of lymphe node cells at day 14 after primary immunization for the production of anti-insulin (5.6 kDa) MAbs.

IV. EVALUATION OF ANTIBODY RESPONSES

IV.1. ELISA

ELISA is the most easy and rapid method for the analysis of experimental antibody reponses. In general optimal conditions for each ELISA are found by titration of coating and detector reagents. For regular ELISAs we followed the procedure as described in detail by Haaijman et al. (1988).

PVC-microtiter plates (Titertek, 77-172-05 highly activated, Flow Laboratories, Irvine, U.K.) are coated overnight at 4°C with 50 μl PBS containing 5μg·ml^{-1} protein, peptide or alternatively the peptide conjugated to BSA (not used for immunization) with a reagent not used in the immunogen. Plates are blocked with 0.5% gelatin in PBS during a 30 minute incubation at room temperature. Sera are diluted in 0.1% gelatin and 0.05% tween-20 in PBS and incubated for 1 h. Alkaline phosphatase conjugated to goat-anti-mouse IgG or swine-anti-rabbit Ig (Kirkegaard and Perry Laboratories, MD, U.S.A.) are used. *p*-nitrophenyl phosphate (1 mg·ml^{-1}) in 10 mM diethanolamine + 1 mM $MgCl_2$ at pH 9.8 is used as substrate. After 30 min the absorbance is read at 405 nm in a Titertek Multiskan reader (Flow Laboratories, Irvine, U.K.) against normal mouse serum or pre-immune rabbit serum responses as a blank. A direct ELISA is used to determine the isotype of the MAbs with specific rabbit-anti-mouse-immunoglobulin-subclass anti-sera (Miles Laboratories, Kankakee, IL, U.S.A.).

For various special applications other coating procedures were tested and practised.

IV.1.1. Passive coating

Coating of protein and peptides in PBS 5-10 μg.ml^{-1} overnight at 4°C or 1-2 h at room temp. Peptides are best coated according to this procedure. If the peptides do not adhere (a conjugate is the best control) then coating at lower or higher pH is advised. Citrate (pH 2.5) or borate (pH 9.5) provide conditions which will solve most problems.

IV.1.2. Glutaraldehyde

Antigens that do not adhere spontaneously to plastic surfaces can be coated actively onto glutaraldehyde pre-activated polystyrene plates (Nunc 2.69620). Procedure: Glutaraldehyde is diluted to 0.025% in Na_2CO_3 (0.1 M, pH 9.0). The wells of 96-wells polystyrene plates are filled with 100 μl. Leave the plates wrapped in aluminium foil for 120 min. at 56°C. After the incubation the plates are washed with water and subsequently dried with hot air in a short time (15 min). Then the antigen (5 μg protein.ml^{-1} in Na_2CO_3 (0.1 M, pH 9.0) is immediately added in a volume of maximum 100μl. The plates with antigen are incubated for 1-2 h at roomtemp. or at 4°C overnight. Pre-activated

plates cannot be stored (Klasen et al. 1983). Include proper controls since glutaraldehyde may introduce some background.

IV.1 Poly-L-Lysine

Proteins or peptides which do not adhere to polyvinylchloride plates, may adhere to poly-L-lysine.
Procedure: Poly-L-lysine (stock: 10 mg.ml^{-1} in PBS) is diluted to 10 μg.ml^{-1} in PBS. To each well 100 μl is added and incubated at 4°C overnight or 1-2 h at room temp.. After incubation the plates are washed in PBS (3x) and dried in air at room temp.. Plates can be stored at -20°C. This procedure has been shown to be useful for coating DNA to ELISA plates.

IV.1.4. Carbodiimide in imidazole

Various compounds can be coated to polystyrene plates using activation with carbodiimides. The procedure is carried out in imidazole (see also EDC coupling Deen et al., 1990).
Procedure: ELISA-plates are pre-coated according to the glutaraldehyde method with BSA (5 μl.ml^{-1}). The compounds to be coupled are pre-treated with carbodiimide (EDC, couples to NH_2 and COOH groups). For DNA: n ml of DNA 20 μl.ml^{-1} + n ml EDC (0.2 M in N-methylimidazole 0.1 M, pH 6.0) is mixed and incubated for 1 h at room temp. Of this mixture at max. 100 μl per well is incubated at 4°C overnight.

IV.1.5 Fixed antigen ELISA

Mimicking of antigens in fixed tissues can be accomplished with this coating. Antigen is coated as for standard procedure. Subsequently the coating is incubated with formalin/$HgCL_2$ for 1-2 h. Longer incubation does not lead to better discrimination. The plates are washed and the ELISA is developed as usual.
Ingredients: The fixative is prepared as follows: $HgCl_2$ (42 g) is added to a mixture of acetic acid 35 ml + aqua dest. 595 ml + formalin 40% 70 ml. The wells are incubated with this mixture for 1-2 h.

IV.2. Immunoblotting

Ingredients: Immobilon membrane (Millipore); Whatman 3MM filtrationpaper; PBS; DAB (20 mg DAB + 100 μl H_2O_2 (30%) in 100 ml PBS + 0.05% Tween 20 (PT-buffer); 0.1% BSA in PT-buffer. Electrophoresis in agar.
Procedure: First agar electrophoresis is performed. Immobilon is washed successively in methanol, aqua dest. and PBS. Dress Immobilon layer over the agar electrophoresis gel. Subsequently overlay with 3 layers of wet and 7 layers of dry filtrationpaper. Put on a glass plate and a pressure of 1 kg. Leave for one h. The blot is rolled up and put in a test tube (15 ml). 5 ml PTB buffer is added to block free sites at the immobilon membrane. Wash 2x with PT buffer. Apply antibody/serum appropriately diluted (5 ml). Incubate on a roller overnight at 4°C or 1-2 h room temperature. Wash 3x with PT buffer. Apply second antibody-conjugate (Peroxidase) diluted in PTB. Incubate on a roller as for the first antibody. Wash 3x with PT buffer. Staining with 5-10 ml substrate solution (for DAB in dark). Take the blot from the incubation tube and wash for 15 min in aqua dest. Dry under filterpaper and a glass-plate. Keep blots away from light. Store data as photonegatives (Radl et al., 1988, Nooij et al., 1990).
The BioRad immunodot apparatus is a very easy for direct immunoblotting. In ELISA format the antigen is deposited (1 h) on a pretreated Immobilon sheath. The pretreatment now includes a last wash with $NaHCO_3$ (0.5M, pH 8.0). The antigen applied is diluted (30 μg.ml^{-1}) in the same solution. The rest of the procedure is similar as described for blotting.

IV.3. Western blotting

We use the protocol and system from the Phast System^R (Pharmacia). The sample (1 or 3 μl) containing the proteins are transfered onto the gel (commercially available gradient or homogenous SDS polyacrylamide gels, or native polyacrylamide gels). Immediately after gel electrophoresis, the protein bands are blotted for 40 minutes at 70°C onto pre-wetted (with aqua dest.) 0.1-μm-thick cellulose-nitrate membrane (PH79; Schleicher and Schuell GmbH, Dassel, F.R.G.). The cellulose-nitrate membrane is developed according to the ELISA protocol, but with 5-bromo-4-chloro-3-indolyl phosphate (Sigma), 165 μg/ml, and nitroblue tetrazolium (Sigma), 330 μg/ml, as substrate and stain, respectively. After blocking the unoccupied binding sites on the membrane, and between

incubations with antiserum, the blots are washed three times with Tween 20, 0.5 μl/ml in PBS, for 5 min. Finally, the blots are rinsed in distilled water and dried between tissues.

IV.4. Blotting from IEF gels

Samples of human sera (1 μl undiluted) are transferred to the middle of an IEF gel, pH 4-6.5. We use the protocol and system from the Phast System® (Pharmacia). Immediately after gel electrophoresis, the protein bands are blotted by diffusion onto pre-wetted (with Tris/methanol/glycine, pH 8.3) 0.1-μm-thick cellulose-nitrate membrane (PH79; Schleicher and Schuell GmbH, Dassel, F.R.G.) at room temperature for at least 3 h or overnight at room temperature. The proteins are detected according the protocol described in section IV.3.

IV.5. Immunocytochemistry

The immunocytochemical procedure discussed in this thesis refers to frozen tissue sections. For this, tissues are taken from the animal or from patients and immediately immersed in liquid nitrogen. The tissues are stored at -70°C in airtight aluminium capsules containing a small amount of ice to prevent dehydration on prolonged storage.

IV.5.1. Fixation techniques

Staining techniques apply to thin cryostat sections (5-8 μm) of frozen tissues. The sections are kept overnight in a sealed box with relative high humidity, then they are air dried and stored at room temperature in sealed boxes over silicagel. Subsequently mildly fixed by submersion in acetone containing 0.02% (v/v) hydrogen peroxide (10 min) (Van Rooijen and Claassen, 1986). Then sections are air dried. Using this technique especially antibodies remain in good shape and keep their antigen specificity (Claassen and Van Rooijen, 1985; Van Rooijen et al., 1989).
The method was used to demonstrate the presence of HIV-1 determinant specific antibodies in immunized mice. Laman et al. (1991b) evaluated fixation inactivation methods that are applicable for situations of increased biohazard risks (HIV-infections). They showed that fixation in 37% (v/v) formaldehyde in PBS or in 0.5% paraformaldehyde (w/v) in PBS for 10 min at roomtemp. (Lifson et al., 1986) are the methods of choice, combining preservation of antigen binding sites (Fab) and membrane antigens in tissue with good morphology.
Alternatively, rapidly after surgery the tissue can be chilled in isopentane and stored in liquid nitrogen until use. Thin cryostat sections (5 μm) are air dried for 30 min and subsequently fixed in buffered 4% formalin for 10 min. Then immunocytochemistry can be performed. This method was used to reveal the distribution of the androgen hormone receptor (Polyclonal anti-peptide-antibodies: Van Laar et al., 1989; Ruizeveld-De Winter et al., 1990; Mabs: Zegers et al., 1991a).
Following fixations, the sections are incubated with the first antibody diluted in PBS/0.1% BSA for sufficient time, 1 hr RT or overnight at 4°C. The optimal dilution must be found in a pilot experiment. After washing the slides 3 times with PBS, the second antibody (specific for the first antibody) conjugated to an enzyme (e.g., alkaline phosphatase or horse radish peroxidase) is added onto the sections for 1 hr RT. After washing with PBS color staining with the appropriate substrate is accomplished.

IV.5.2 Alkaline phosphatase

Ingredients & procedure: Tris.HCl 0.1 M, pH 8.5, 37°C; To 40 ml Tris-buffer 5 mg naphthol-AS-MX phosphate in 250 μl *N,N*-dimethylformamide is added. 10 mg fast blue BB base is suspended in 250 μl 2 N HCl, add 4% $NaNO_2$ solution and mix for 1-2 min. This fast blue BB solution is added to the naphthol-AS solution. Finally 10 mg levamisole in 2 ml buffer is added to the substrate solution (inhibits endogenous AP). After mixing for 2 min (37°C) precipitates are filtered off. Slides are incubated vertically for 10-45 min. The reaction is stopped by transferring to PBS. If necessary slides can be incubated a second time in a fresh solution (Claassen et al., 1986)

IV.5.3. Horseradish peroxidase

Ingredients & Procedure: 40 ml Na-acetate (0.05M, pH 5.0). Add 16 mg 3-amino-9-ethylcarbazole (AEC) in 1 ml *N,N*-dimethylformamide to the buffer. Just before use add 200 μl 3% H_2O_2. Incubate slides vertically for 7-10 min. Stop reaction by transferring to PBS (Claassen et al. 1986)

Alternatively, diaminobenzidine (DAB) can be used for staining.
Ingredients & Procedure: 40 ml Tris-HCL buffer (0.05 M, pH 7.6). dissolve 20 mg 3,3'-diaminobenzidine-tetrahydrochloride into the buffer. Just before use add 50 µl 3% H_2O_2 and incubate as for AEC. Staining may be enhanced by incubation of the slides in 0.5% $CuSO_4$ in 0.9% NaCl.

IV.6. Immunoprecipitation

The response to the native androgen receptor was analyzed in an immunoprecipitation assay. Sera are incubated at different dilutions for 2 h at 4°C in roller tubes with anti-mouse-IgG conjugated to agarose (Sigma). After centrifugation and washing of the pellet with PBS, 25 µl of a nuclear extract from LNCaP cells (a human cell line of a lymph node carcinoma of the prostate) in which the receptor was labelled with [17α-*methyl*-^{3}H]methyltrienolone ([^{3}H]R1881, a synthetic androgen, NEN-Dupont, Dreieich, F.R.G.), is added. Subsequently 400 µl PBS is added and the mixture is incubated for 2 h at 4°C while rotating. After centrifugation and washing of the pellet, the sample is assayed for the amount of radioactivity. Values are corrected for background.

IV.7. Sucrose gradient centrifugation

Essentially the method described by De Boer et al. (1986) is applied. Briefly, 50 ml of [^{3}H]R1881-labelled nuclear extract (0.15 M NaCl) from LNCaP cells is incubated for 6 h at 4°C with antiserum or RPMI 1640 culture medium (Flow, Irvine, U.K.) supplemented with 7.5% FCS and 1% NMS as a control and subsequently layered on 10-30% sucrose gradients in the presence of 0.15 M NaCl and centrifugated for 20 h at 370 000 x *g* at 4°C.

IV.8. Dipstick method

The dipstick method is carried out as a sandwich assay for detection of antigens largely according to the ELISA protocol. Apply 1 µl of a catching MAbs 1 mg/ml in PBS as a spot onto 0.1-µm-thick cellulose-nitrate membrane (BA85; Schleicher and Schuell GmbH, Dassel, F.R.G.) in a convenient dipstick format. For easy handling of the cellulosenitrate, the membrane can be sticked to a plastic backing. We use plate sealers as backing (Costar 3095, Cambridge, MA, USA). Dry the spots and block free sites with PBS/0.5% gelatine. Immerse the dipstick in a solution with antigen (For α_1-antitrypsin detection, a serum dilution of 1:50 was made in PTG and heated for 30 min at 56°C for exposition of the determinant) and incubate for 1 hr on a rocking platform in a petridish or perform the incubation in a test tube in an end-over-end rotor. Wash with PBS/tween and incubate with a detecting MAb conjugated to β-galactosidase for 1 hr with mixing. Perform the staining with X-Gal (5-bromo-4-chloro-3-indolyl-galactopyranoside) substrate until the colour is clearly visible and rinse the dipstick with water. Alternatively, the reaction can be stopped with 0.1 M $NaHCO_3$. Dry them between tissues.
Substrate: Stock solution A: 10 mg X-Gal in 500 µl DMF; Stock solution B: Add 500 µl 50 mM Potassium ferricyanide and 500 µl 50mM Potassium ferrocyanide to 7 ml 1.1 mM $MgCl_2$ in PBS. Both solutions can be kept at -18°C. Working solution: Mix 125 µl of solution A with 5 ml of solution B.

V. PRODUCTION OF MONOCLONAL ANTIBODIES

A spleen cell suspension is prepared 4 days after a booster immunization. Spleen cells and SP2/0 cells in logarithmic growth were washed twice with serum-free RPMI 1640 (Boehringer Mannheim, Mannheim, F.R.G.). Spleen cells and SP2/0 cells are fused at a ratio of 5:1 in 1 ml 40% poly(ethylene glycol) 4000 (Merck, Darmstadt, F.R.G.) + 5% DMSO in PBS for 1 min. The suspension is slowly diluted with 9 ml RPMI 1640 culture medium and incubated for 20 min at room temperature. The fused cells are carefully centrifugated, 10 min 300g and resuspended in selection medium. The fused cells (10^5/well in 0.2 ml) are cultured in RPMI 1640 selection medium, containing 1 µg·ml^{-1}azaserine and 0.1 mM hypoxanthine, 15% FCS, 2 mM glutamine, 0.1 mg·ml^{-1} streptomycin, 100 E·ml^{-1} penicillin, 1 mM sodium pyruvate, and $5 \cdot 10^{-5}$ M ß-mercaptoethanol. After 1 week of culture the azaserine was discontinued and the FCS concentration was lowered to 10%. The culture medium is replenished 3 times a week by 100 µl at a time. The hybridomas can be tested for specific antibody production when the surface is overgrown for at least 30% with hybridoma cells, usually after about 10 days of culture for the fastest dividing cells. Selected cell

cultures are transfered to a 24-well culture plate and subsequently to a culture flask. They are collected for storage in liqued nitrogen as soon as possible. (A pellet of cells in logarithmic growth from a 25 cm^2 culture flask is suspended in 1 ml 10% DMSO in FCS and frozen via a programmed procedure in a a controlled rate freezer (Kryo 10 series II, Planer Biomed, Middx, UK) below -70°C and transfered immediately to the container with liqued nitrogen). Some of the most promising clones are subcloned at the earliest timepoint possible in culturing by limiting dilution at a density of 0.5 cells/well.

Ascites production: BALB/c mice are injected intraperitoneally with 0.5 ml pristane (2,6,10,14-tetramethylpentadecane 96%, Ega-chemie, Steinheim, F.R.G.). Seven days later the mice were injected with 10^6 monoclonal hybridoma cells in 0.25 ml PBS. Ascites fluid was collected under anaesthesia.

VI. MISCELLANEOUS

VI.1. Purification of α_1-antitrypsin variants

α_1-AT(Z) was purified from 20 ml of plasma from a ZZ donor by use of an immuno-affinity column. Purified rabbit IgG directed against α_1-AT (Sigma) was coupled to 5 ml of Affigel^{R}HZ (Bio-Rad Labs., Richmond, CA) according to the manufacturer's protocol. The α_1-AT protein bound to the column was eluted with 0.1 mol/L glycine buffer pH 3.0 and subsequently dialysed against PBS.

VI.2. Mild denaturation of α_1-antitrypsin

For binding of the variant-specific MAb F43.8.1 to the M variant, it is necessary to disrupt the salt bridge between residues Lys (AA 342) and Glu (AA 290) for exposition of the 342 site. This can be accomplished by heating the serum at 56 °C for 30 min.

VI.3. Conjugation of MAb and β-Galactosidase

β-Galactisodase is conjugated with MBS via its thiol groups to the amino groups of the immunoglobulin.
Ingredients: PBS; MAb 10 $mg.ml^{-1}$ in PBS; MBS 20 $mg.ml^{-1}$ in DMF; β-galactosidase 10 $mg.ml^{-1}$ (Boehringer Mannheim #745731); PD-10 column (Pharmacia), $MgCl_2$ 1 M; β-mercaptoethanol 1 M.
Procedure: Add the MBS solution in three equal portions every 5 min to 1 ml MAb at ice while stirring. Use 150 mol MBS per mol IgG. Stir for another 15 min. Separate the modified IgG from the excess MBS over a PD-10 column. Add slowly β-galactosidase to the IgG-maleimide and stir for 1 hr at room temperature. Add $MgCl_2$ and β-mercaptoethanol, both to an end concentration of 10 mM. Store with to parts (v/v) glycerol at -20°C.

VI.4. Conjugation of MAb and alkaline phosphatase

Ingredients: Alkaline Phosphatase (AP) 5 $mg.ml^{-1}$ in PBS; MAb 2 $mg.ml^{-1}$ in PBS: glutaraldehyde 0.2% in PBS; glycine 0.2 M in PBS.
Procedure: Dialyse 1 ml AP against the glutaraldehyde solution overnight at 4°C. Dialyse subsequently three times against PBS. Transfer the GA-activated AP to a reaction vial and add 2 mg of the MAb in 1 ml PBS and incubate overnight at 4°C. Add 0.1 ml glycine and incubate for 2 hr at room temperature. Dialyse against PBS. Store with 2 parts (v/v) glycerol at -20°C.

VI.5. Purification of MAbs with protein A

Ingredients: Protein A Sepharose CL-4B column (Parmacia Fine Chemicals # 17-0780-01), glycine 1.5 M/ NaCl 3 M pH 8.9; Tris-HCl 1.0 M pH 11; Phosphate (NaH_2PO_4/Na_2HPO_4) buffers 0.1 M pH 6.0, pH7.0 and pH 8.1; Citrate buffers 0.1 M pH 5.0, pH 4.5 and pH 3.0.
Procedures: For Mouse IgG1: Equilibrate column with glycine/NaCl. Dilute cleared ascites fluid 1:1 with glycine/NaCl and apply slowly (0.2 ml.min; IgG1 is poorly bound by protein A) over the column. Wash with glycine/NaCl. Eluate with NaPi buffer pH 6.0. Concentrate the eluate to > 1 $mg.ml^{-1}$ and dialyse against PBS. Store at -20°C.

For Mouse IgG2a, IgG2b and IgG3: Equilibrate column with NaPi buffer pH 7.0. Apply cleared ascites fluid over column (0.8 ml.min). Wash with NaPi pH 7.0. Eluate IgG2a with citrate buffer pH 5.0, IgG2b with Citrate buffer pH 4.5 and IgG3 citrate buffer pH 4.5. Immediately neutralise the fractions containing the immunoglobulin with 1.0 M Tris-HCl pH 11. Concentrate the antibodies to a concentration > 1 $mg.ml^{-1}$ and dialyse against PBS. Store at -20°C.
The column should be cleaned after each run with citrate buffer pH 3.0 and stored in NaPi pH 7.0 + NaN_3 (0.1% w/v).

MATERIALS

Buffers and media PBS: 0.01 M phosphate buffered saline 0.15 M NaCl, pH 7.4; PT: 0.05% Tween 20 in PBS; PTG: 0.1% gelatine in PT; PBS-GEL: 0.1-0.5% gelatin in PBS; DEA: diethanol amine 10mM (Merck p.a.)+ 1 mM $MgCl_2$, pH 9.8; N-methylimidazole (Aldrich, M5, 083-4, Brussel, Belgium); DMF, DMSO, TFE, amylalcohol, acetic acid, diethylether, TFA, ethanol and urea preferably all of p.a. quality or HPLC quality.

Adjuvants. A water-in-oil emulsion is simply made by thorough mixing of the antigen solution in PBS with Specol or span tween (the Netherlands Veterinary Instute, Lelystad, The Netherlands, Bokhout, 1981). Complete and incomplete Freunds can be obtained from Difco (inc.: 0639-60-6, com.: 06638-60-7).

Coupling reagents, derivatization MBS (Pierce, 223310), APG, HSAB, Biotin NHS, Iminothiolane can be obtained from Pierce. GA: J.T.Baker; DTT: Aldrich 14,046-0; N-ethylmaleimide: Sigma (E-3876); EDC: Sigma (E-7750). PD10 prepacked sephadex G25 colums to be obtained from Pharmacia, Uppsala, Sweden.

Antigens: KLH (Calbiochem, 374811, San Diego, Ca. U.S.A.); CGG: Sigma, G-6516; BSA: Sigma A-9647, St. Louis, MO., U.S.A.; α_1-AT(M) was obtained from Sigma Chemical Co., St. Louis, MO.

ELISA can be performed in polyvinyl plates (Falcon, 3911, Becton and Dickinson Labware, CA., U.S.A., or Titertek, 77-172-05) or polystyrene plates (NUNC, 2.69620).
Conjugates anti-mouse, anti-goat, anti rabbit conjugates can be obtained from various suppliers: Dakopatts, Sigma KPL-Labs. Alkaline phosphatase can be obtained from Sigma P-6774 Type VII-T; horseradish peroxidase (PO) RZ=3.0 (type VI; P-8375);
Substrates: PNPP: p-nitrophenylphosphate: Boehringer (107905); o-phenylenediamine-di-hydrochloride: OPD (1078054): Kodak, Rochester, USA; DAB: 3,3'-diaminobenzidine: Sigma D-5637; X-Gal (5-bromo-4-chloro-3-indolyl-galactopyranoside, Boehringer Mannheim #651745)
FITC (fluorescein Isothiocyanate, isomer 1) can be obtained from Nordic Immunology, Tilburg, The Netherlands.

Human sera phenotyped for α_1-antitrypsin were obtained from Dr. R.R. Frants (State University of Leiden, The Netherlands) and Dr. J.H. Kramps (Academic Hospital, Leiden, The Netherlands).

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SUMMARY

Antibodies are widely applied in immunochemical assays for the specific and sensitive detection of three dimensional structures in or on different cell-types, microorganisms and solutes. Immunization of experimental animals with an immunogen (a molecule, cell, or microorganism) generates a polyclonal antibody response against the immunogen. These polyclonal antibodies react with the immunogen but may also crossreact with other structures resembling the immunogen. Immunization with part of the immunogen, e.g., with a surface protein isolated from a cell, microorganism or virus, renders the antibody response more specific. Also, with the advent of the hybridoma technique, monoclonal antibodies which are more specific and do not demonstrate unwanted crossreactions can be selected. Sometimes, a protein is not available for immunization for different reasons: e.g., it cannot be purified, or the protein is toxic. Immunization with a synthetic peptide (SP), which represents a part of the sequence of the protein, is an alternative to immunization with the entire protein. SP can elicit antibodies that crossreact with the native protein. Such synthetic peptides are also powerful tools for the generation of antibodies against:

a) a protein which belongs to a protein family of which the members are homologous. Immunization with a unique sequence differing from the other members elicits antibodies which can discriminate a single protein from the rest of the family.
b) a protein that differs in only one amino acid from another protein, e.g., variants of a protein, or virus mutants. Antibodies elicited with an SP that contains the sequence with the substitution can specifically detect the variant protein.
c) functional domains of a protein. Immunodominant sequences from flanking domains can be circumvented by using an SP derived from the domain of interest. Antibodies specific for a domain are useful for unravelling the function of the domain.
d) a protein which has not been isolated before, but for which the amino acid sequence can be derived from the DNA base sequence.

It was the aim of the experiments described in this thesis to investigate and optimise the factors which play a role in the generation and evaluation of antibodies elicited with SP. These factors are: a) the selection of the peptide sequence; b) construction of the immunogen. Peptides themselves generally are not immunogenic since they often only contain a B cell determinant. Coupling of a peptide to a carrier protein, which provides T cell determinants, renders the peptide immunogenic. The choice of coupling chemistry and procedure, the ratio of peptide and carrier, and the orientation of the peptide relative to the carrier are important for the peptide construct; c) the immunization procedure; d) evaluation of the antibody response in pertinent assays; e) selection procedure of monoclonal antibodies. In chapter 1, these factors are introduced and discussed.

Immunization with synthetic peptides often results in disappointment. Only if all the conditions, which determine the outcome of the generation of antibodies upon immunization with SP, are carefully chosen and applied, can strongly specific (monoclonal) antibodies be selected which are useful in immunochemical assays. During the process of the generation

and selection of antibodies using synthetic peptides, the various parameters were investigated and optimised which resulted in straightforward suggestions.

Construction of the immunogen is an important step in antibody generation. In chapter 3, we describe a new and efficient conjugation method of a synthetic peptide to a carrier protein using glutaraldehyde. With this new method, the carrier protein is dialysed against a solution of glutaraldehyde. Most available ϵ amino groups of the carrier protein will be modified by glutaraldehyde. The excess glutaraldehyde is removed by subsequent dialysis against PBS. The peptide is added to the glutaraldehyde-modified carrier protein and will bind to it through its amino groups. Surplus reactive glutaraldehyde groups were blocked by lysine. This method prevents the homopolymerization of carrier proteins and the homopolymerization of peptides found in classical coupling by glutaraldehyde. Immunization with conjugates proved to elicit peptide-specific antibodies which were crossreactive with the native protein.

The human androgen receptor (hAR) is a member of the steroid receptor family. These steroid receptors are very homologous to each other. The steroid receptors consist of three functional domains: the N-terminal part which is involved in the regulation of gene transcription, a DNA-binding domain and a steroid-binding domain at the C-terminus. Domain-specific antibodies are helpful for research purposes. The antibodies can be helpful for immuno-affinity purification of the hAR, for investigation of the AR structure, distribution and its physico-chemical properties, for immuno-histochemical localization of the receptor, and for monitoring AR expression during endocrine therapy for prostatic cancer. Immunization with the hAR is not possible since the hAR has not been isolated, but the cDNA sequence has been elucidated. Therefore, immunization with SP is the only alternative. This method has additional advantages. With SP immunization, domain-specific sequences can be selected and sequences homologous with other steroid receptors can be avoided. Since the antigenic determinants of the hAR were not known, putative continuous epitopes were selected on the basis of theoretical considerations. The selection of the epitopes and the subsequent generation of polyclonal and monoclonal antibodies are described in chapter 4. The selected monoclonal antibodies were hAR-specific, not crossreactive with other steroid receptors (oestrogen, progesterone and glucocorticoid receptor), domain specific, not interfering with hormone and DNA binding, and could be used in multiple immunochemical assays.

Human α_1-antitrypsin is a serum protease inhibitor which mainly inhibits neutrophil elastase in the lungs. Nowadays more than 70 different isoforms of the protease inhibitor are known, most of them being point mutations in the DNA sequence leading to amino acid substitutions. Severe α_1-antitrypsin deficiency results from a single amino acid substitution ($Glu^{342}\rightarrow Lys$) in the molecule which causes α_1-antitrypsin accumulation in the liver where it is synthesized. The change of charge from negative to positive in the Z molecule (the normal protein is designated as M) induces an alteration in the three-dimensional structure of the Z variant protein which, as a consequence, also shows reduced activity. Homozygous carriers with the Z type substitution ($Glu^{342}\rightarrow Lys$) are at risk of developing lung emphysema at a relatively young age. Heterozygous carriers with the SZ phenotype may also be at risk. Early

detection of such carriers offers the possibility of prescribing lifestyle measures, which delay the disease. Replacement therapy with (recombinant) α_1-antitrypsin can be given to such individuals. Presently applied methods for detection of variants are not suitable for routine screening since they are difficult to interpret.

Generation of antibodies specific for the Z and S variant cannot be achieved by immunization with the variant proteins. Also, for these variant proteins, immunization with SP proved to be the only alternative. In part II (chapters 5-7), we describe the generation of variant specific antibodies. In doing this, we have investigated the influence of the length of the peptide, the position of the substitution site in the selected peptide sequence, the construction of the immunogen regarding the coupling chemistry and the orientation of the peptide relative to the carrier protein, the route of immunization, the immunization schedule, the selection method of the monoclonal antibodies, and the pretreatment of the protein. Simple assays, which can distinguish between the Z variant and the normal M protein, based on monoclonal antibodies, have been developed. Both homozygous and heterozygous carriers for the Pi^Z gene can be detected.

Most peptide sequences only contain a B cell determinant and do not contain a T cell determinant. For memory B cell responses, both T cells and B cells have to be activated. This can only be accomplished by making an immunogenic construct that contains both a T cell and a B cell determinant. Usually, such a construct is made by coupling an SP, which contains the B cell determinant, to a carrier protein, which contains one or multiple T cell determinants. In part III, the use of single selected T cell determinants in a construct with a B cell determinant is described. The use of a single T cell epitope can have advantages over the use of a carrier protein. Immunodominance resulting from the carrier protein could be circumvented. To produce antibodies against weakly immunogenic regions of a protein, a construct of that weakly immunogenic region combined with a T cell determinant will be useful. For synthetic vaccines, it is a requirement that the vaccine construct contains T cell determinants, in addition to B cell determinants, derived from the pathogen. The immune systems will mount a memory response to both the B and the T cell epitope. Challenge with the pathogen will lead to a prompt response. Chapter 8 gives a broad overview of the existing literature on this topic. Literature consensus guidelines for the use of T cell epitopes are: a) The T cell epitope should be selected in functional assay with cells from the animal species or from human origin for which the immunization is meant; b) attention should be paid to the conjunctional sites of a T and a B cell determinant, since new T cell determinants may arise with a positive or negative result; c) elongation of a strict T cell determinant may have a positive effect if the flanking sequences are chosen with the result that increased stability of a helix structure emerges; d) a light preference is given for the N-terminal position of a T cell determinant in a construct, but testing of all possible combinations is still the best approach in designing immunogenic constructs; e) multiple copies of a T cell determinant may lead to an increase of the antibody response; f) linkage of the determinants by peptide bonds is preferable to other chemical linkers; and g) covalent coupling of T and B cell determinants is a requirement for inducing a high level of antibodies with a high affinity towards the B cell determinant.

The last point of the aforementioned guidelines for the use of T cell determinants in peptide constructs, point g), was the subject of conflict in literature. Some investigators reported the generation of B cell determinant-specific antibodies with co-immunization of a T cell and a B cell determinant without a covalent bond between these two. However, these authors all used the same T cell determinant. We showed in chapter 9, that, in general, covalent linkage between a T and a B cell determinant is essential for generation of a bonafide memory (IgG) response.

In chapter 10, we investigated the role of T cell determinants in multiple antigenic peptides, a new format of peptide immunogens. A multiple antigenic peptide consists of a branch of coupled lysine residues (through α and ϵ amino groups) to which the selected peptide sequences can be synthesized. Branches with four or eight peptide sequences are most frequently used. These multiple antigen peptides appear to be highly immunogenic and were described in literature as applicable to every B cell determinant. We showed that incorporation of a T cell determinant is a requirement for strong and native protein-specific antibody responses.

In chapter 11, the main results from the different studies are discussed. Guidelines emerging from the different studies are given for the successful generation of antibodies using SP. The main points are:

a) *Selection of the sequence.* Choose a sequence of 15 to 25 amino acids. Avoid "difficult sequences" to synthesize. Choose the terminal sequences to contain hydrophillic residues. Avoid homology with other proteins by search in data bases. Place the epitope in the middle of the sequence or at the distal end of the coupling site. Search for antigenic sequences with the aid of computer prediction programs.

b) *Construction of the immunogen.* Apply controlled coupling methods for the conjugation of peptides and carrier proteins as described in this thesis. Use MBS, EDC or GA coupling dependent on the incorporated amino acids. Avoid coupling through amino acids when they are part of the epitope. Coupling through the N-terminus is preferable with an additional cysteine incorporated. Multiple antigen peptides can be considered if conjugation is difficult, e.g., if the peptide is insoluble or if the residues involved in coupling are part of the epitope.

c) *Immunization.* Intraperitoneal immunization with 25-50 μg of the peptide-carrier construct in a mineral oil as adjuvant leads to good results. For high-affinity antibodies, boost only after the level of antibodies has decreased below 20% of the highest level reached.

d) *Evaluation of the antibody response.* Evaluation of the reactivity pattern in the polyclonal serum should be carried out after each administration. Assay for reactivity with the peptide sequence, with the carrier protein, with the coupling determinant and with the native protein. Crossreaction with the native protein should be carried out by competition or inhibition or sandwich assays of the antibodies with the native protein. Sandwich assays are only possible if a catching antibody is available from another animal than the one generating the antibodies to be assayed.

e) *Selection of monoclonal antibodies.* Selection of the hybridomas is best performed in the assay that is going to be used for the eventual diagnostic assay. First screening on peptides can be done for cost-effective reasons.

SAMENVATTING

Antilichamen worden op grote schaal toegepast in immuno-chemische detectiemethoden. In deze technieken kunnen antilichamen specifiek en gevoelig drie-dimensionale structuren (antigenen) detecteren die kunnen worden aangetroffen in of op verschillende celtypen, micro-organismen en in oplossing. Immunisatie van proefdieren met een immunogeen (dat kan bijvoorbeeld een molecuul, een cel of een micro-organisme zijn) genereert een polyclonale antilichaam respons tegen dat immunogeen. Polyclonale antilichamen herkennen verschillende delen van hetzelfde antigeen doordat ze gemaakt worden door verschillende B lymfocyten. Deze polyclonale antilichamen reageren met het immunogeen, maar kunnen ook kruisreageren met andere structuren die gelijkenis vertonen met het gebruikte immunogeen. Immunisatie met een deel van het immunogeen, dat specifiek is voor b.v. een oppervlakte-eiwit geïsoleerd van een cel, een micro-organisme of een virus, beperkt de kruisreaktiviteit. Door de komst van de hybridoma techniek, kunnen monoclonale antilichamen gemaakt worden. Deze monoclonale antilichamen zijn afkomstig van nakomelingen van één B lymfocyt die onsterfelijk is gemaakt door fusie met een myeloma cel. De hybride cel blijft zich delen en produceert steeds dezelfde soort antilichamen. De monoclonale antilichamen, die zeer specifiek reageren met het immunogeen zonder ongewenste kruisreaktie met andere structuren, kunnen door een geschikte selectiemethode toe te passen, worden gevonden. Soms is het niet mogelijk om met een eiwit te immuniseren omdat het niet voorhanden is, b.v. omdat het niet gezuiverd kan worden of omdat het eiwit zelf toxisch is. Immunisatie met een synthetisch peptide (SP), dat een representatief deel van het eiwit vertegenwoordigt, is een alternatief voor immunisatie met het gehele eiwit. Een SP kan antilichamen opwekken die kruisreageren met het eiwit waarvan het SP is afgeleid. Zulke synthetische peptiden zijn ook belangrijke hulpmiddelen om antilichamen op te wekken tegen:

a) een eiwit dat tot een eiwit-familie behoort waarvan de leden homoloog zijn. Immunisatie met een unieke sequentie, specifiek voor één der leden, genereert antilichamen die een enkel eiwit van de rest van de familie kunnen onderscheiden.
b) een eiwit dat slechts in één aminozuur afwijkt van een ander eiwit, b.v. varianten van een eiwit of virusmutanten. Antilichamen, die zijn opgewekt met de sequentie die de substitutie bevat, kunnen gebruikt worden om specifiek het variant eiwit te detecteren.
c) functionele domeinen van een eiwit. Immunodominante sequenties die aanwezig zijn in omringende domeinen kunnen worden omzeild door een SP te gebruiken dat afkomstig is uit het gewenste domein. De antilichamen die specifiek een domein herkennen, zijn zeer bruikbaar om de functie van een domein te bestuderen.
d) een eiwit dat nog niet eerder is geïsoleerd, maar waarvan de aminozuur volgorde kan worden voorspeld uit de DNA base volgorde.

Verschillende factoren spelen een rol bij het opwekken van de antilichamen met SP en bij de karakterisering van de antilichamen. Het doel van de experimenten die beschreven zijn in dit proefschrift was om deze factoren te onderzoeken en te optimaliseren. Deze factoren zijn: a) de selectie van de peptide sequentie; b) de constructie van het immunogeen. Peptiden zijn zelf over het algemeen niet immunogeen daar ze slechts een B cel epitoop

bevatten. Koppeling van een SP aan een dragereiwit dat T cel epitopen bevat, maakt het SP immunogeen. De keuze van de koppelingschemie, de verhouding tussen SP en dragereiwit en de oriëntatie van het SP t.o.v. het dragereiwit zijn belangrijk voor de immunogeniciteit van het peptideconstruct; c) de immunisatieprocedure; d) de evaluatie van de antilichaam respons in de geschikte testmethoden; e) de selectieprocedure van de monoclonale antilichamen. In hoofdstuk 1 worden deze factoren geïntroduceerd en bediscussieerd.
Immunisatie met SP mondt vaak uit in teleurstelling in die zin dat het niet die antilichamen oplevert die bruikbaar zijn voor detectie van natieve eiwitten. Alleen als aan alle voorwaarden, die bij het vinden van de gewenste antilichamen belangrijk zijn, wordt voldaan, kunnen specifieke (monoclonale) antilichamen worden geselecteerd die bruikbaar zijn in de beoogde immunochemische detectie methoden. Gedurende het proces van het opwekken en selecteren van de antilichamen m.b.v. SP, werden de verschillende parameters onderzocht en bijgesteld. Dit onderzoek heeft geresulteerd in duidelijke randvoorwaarden voor de toepassing van SP als immunogenen.

De constructie van het immunogeen is een belangrijke stap in het gehele proces. In hoofdstuk 3 beschrijven we een nieuwe, efficiënte en handzame koppelingsmethode om een SP aan een dragereiwit te koppelen met behulp van glutaaraldehyde. In deze methode wordt het dragereiwit eerst gedialyseerd tegen verdund glutaaraldehyde. De meeste van de beschikbare ϵ-aminogroepen worden hierbij gemodificeerd door glutaaraldehyde. De overmaat glutaaraldehyde wordt verwijderd door het gemodificeerde dragereiwit vervolgens te dialyseren tegen PBS. Het peptide wordt dan toegevoegd en zal via zijn aminogroepen binden aan het dragereiwit. Overgebleven reaktieve glutaaraldehyde groepen op het dragereiwit worden geblokkeerd met lysine. Deze methode verhindert de homopolymerisatie van dragereiwit moleculen en de homopolymerisatie van peptiden zoals die worden gevonden bij de klassieke éénstaps glutaaraldehyde koppeling. Immunisatie met conjugaten die met deze nieuwe koppelingsmethode zijn gemaakt hebben peptide-specifieke antilichamen opgewekt die ook kruisreageerden met het natieve eiwit (waarvan het SP was afgeleid).

De humane androgeen receptor (hAR) is een lid van de steroid receptor familie. Deze steroid receptoren zijn onderling in hoge mate homoloog. De steroid receptoren bestaan uit drie functionele domeinen: het N-terminale deel dat betrokken is bij de regulatie van gentranscriptie, een DNA-bindend domein en een steroid-bindend domein aan de C-terminus gelegen. Domein-specifieke antilichamen zijn bruikbaar voor research doeleinden en diagnostiek. Deze antilichamen kunnen o.a. gebruikt worden voor de affiniteits-zuivering van de hAR, voor het onderzoeken van de hAR structuur en zijn physico-chemische eigenschappen, voor de immuno-histochemische localisering van de receptor, en voor het monitoren van de hAR expressie tijdens endocriene therapie bij prostaatkanker. Bij de aanvang van deze studie was immunisatie met de hAR niet mogelijk daar de hAR, ondanks vele pogingen, nog niet geïsoleerd was. Wel was de cDNA sequentie opgehelderd. Daarom leek immunisatie met SP het enige alternatief. Deze methode biedt belangrijke voordelen. Domein-specifieke sequenties kunnen worden geselecteerd en sequenties die homoloog zijn met sequenties van de andere steroid receptoren kunnen worden vermeden. Daar de antigene determinanten voor de hAR niet bekend waren, werden mogelijke epitopen geselecteerd op

basis van theoretische beschouwingen. De selectie van de epitopen en de generatie van poly- en monoclonale antilichamen worden beschreven in hoofdstuk 4. De geselecteerde monoclonale antilichamen zijn specifiek voor de hAR en kruisreageerden niet met andere steroid (oestrogeen, progesteron en glucocorticoid) receptoren. Ze zijn domein-specifiek en interfereren niet met hormoon en DNA binding, en ze kunnen worden gebruikt in verschillende immuno-chemische testen.

Humaan α_1-antitrypsine is een proteaseremmer in serum die hoofdzakelijk neutrofiel elastase in de longen remt. Tegenwoordig zijn er meer dan 70 verschillende isovormen bekend. De meeste isovormen zijn puntmutaties in het DNA die resulteren in substitutie van één enkel aminozuur in het eiwit. Een enkele aminozuur substitutie ($Glu^{342} \rightarrow Lys$) in het molecuul veroorzaakt α_1-antitrypsine accumulatie in de lever waar het wordt gesynthetiseerd. De verandering van lading van negatief naar positief in het Z molecuul (het normale eiwit wordt als M aangeduid) induceert een verandering in de drie-dimensionale structuur van het Z variante eiwit dat, als gevolg hiervan, een gereduceerde activiteit vertoont. Ernstige α_1-antitrypsine deficiëntie is hiervan het gevolg. Homozygote dragers met substitutie in het Z type ($Glu^{342} \rightarrow Lys$) ontwikkelen op jonge leeftijd longemfyseem. Heterozygote dragers van het SZ fenotype lopen ook dat risico. Vroegtijdige detectie van zulke dragers biedt de mogelijkheid om leefregels voor te schrijven die de ziekte kunnen uitstellen. Ook toedienen van (recombinant) α_1-antitrypsine kan als therapie worden gegeven aan deze individuen. Huidige detectiemethoden voor het opsporen van varianten zijn niet bruikbaar voor routine screening daar ze moeilijk interpreteerbaar zijn.

Het opwekken van antilichamen, die specifiek de M, Z en de S varianten kunnen onderscheiden, is niet mogelijk door immunisatie met het gehele eiwit. Ook voor deze variante eiwitten, is immunisatie met SP het enige alternatief. In deel II (hoofdstukken 5-7) wordt het generen van variant-specifieke antilichamen beschreven. Daarbij is de invloed van de lengte van het peptide onderzocht, de positie van de substitutieplaats in de gekozen peptide sequentie, de constructie van het immunogeen m.b.t. de koppelingschemie en de oriëntatie van het peptide t.o.v. het dragereiwit, de immunisatieroute, het immunisatieschema, de selectiemethode van de monoclonale antilichamen, en de voorbehandeling van het α_1-antitrypsine in serum. Eenvoudig uit te voeren testen die gebaseerd zijn op monoclonale antilichamen en die de Z variant kunnen onderscheiden van het normale M eiwit, zijn ontwikkeld. Zowel homozygote als heterozygote dragers van het Pi^Z gen kunnen worden gedetecteerd.

De meeste peptide sequenties bevatten alleen een B cel determinant en geen T cel determinant. Om geheugen B cel responsen te verkrijgen is het noodzakelijk dat zowel B als T cellen worden geactiveerd. Dit kan alleen worden bereikt door te immuniseren met een immunogeen construct dat zowel een B als een T cel determinant bevat. Gewoonlijk wordt zo'n construct gemaakt door een SP, dat een B cel determinant bevat, te koppelen aan een dragereiwit dat meestal meer dan één T cel determinant bevat. In deel III wordt het gebruik van losse T cel epitopen en een B cel epitoop in een construct beschreven. Het gebruik van een enkele T cel epitoop kan voordelen hebben boven het gebruik van een dragereiwit. Zo kan immunodominantie van B cel epitopen afkomstig van het dragereiwit worden vermeden.

Dus, om antilichamen op te wekken tegen zwak immunogene regionen van een eiwit zal een construct, gemaakt met een sequentie uit dat zwak immunogene deel gecombineerd met een T cel epitoop, bruikbaar blijken. Voor synthetische vaccins is het een vereiste dat het vaccinconstruct T cel determinanten naast B cel determinanten bevat die beiden afkomstig zijn van hetzelfde pathogeen. Na vaccinatie met dat construct, zal het immuunsysteem een geheugen respons opbouwen tegen de B en de T cel epitoop. Infectie met het pathogeen zal dan leiden tot een snelle en adequate respons.
Hoofdstuk 8 geeft een breed overzicht van de bestaande literatuur over T cel epitopen. Uit de literatuur zijn richtlijnen opgesteld over het gebruik van T cel epitopen. Deze zijn: a) De T cel epitoop moet geselecteerd worden middels een functionele testmethode m.b.v. cellen die afkomstig zijn van het dier of van de mens waarvoor de immunisatie bestemd is; b) aan de aansluiting van de epitopen moet aandacht worden geschonken daar nieuwe T cel epitopen op die plaats kunnen ontstaan met een positief of negatief resultaat als gevolg; c) verlenging van een strikte T cel epitoop kan een positief effect hebben als de flankerende sequenties dusdanig worden gekozen dat er een stabiliserende werking van uitgaat op de helix structuur; d) een lichte voorkeur wordt gegeven aan de plaatsing van een T cel determinant aan de N-terminus, maar het uittesten van alle mogelijke combinaties is nog steeds de beste benadering bij het ontwerpen van een construct; e) aanwezigheid van verschillende kopieën van een T cel determinant kan tot een toename leiden van de antilichaam respons; f) de verbinding van de determinanten door een peptide band verdient de voorkeur boven de verbinding via andere chemische schakels; en g) een covalente koppeling van de T en de B cel determinant is een vereiste voor het induceren van geheugen antilichaam (IgG) respons. Zo worden antilichamen met een hoge titer en een hoge affiniteit voor de B cel determinant opgewekt.

Over het laatste punt van de bovengenoemde richtlijnen voor het gebruik van T cel determinanten in peptide constructen (punt g) bestaat discussie in de literatuur. Sommige onderzoekers rapporteerden dat het opwekken van antilichamen die specifiek de B cel determinant herkennen, kan plaatsvinden door co-immunisatie van een T en een B cel determinant zonder een covalente band tussen deze determinanten. Deze onderzoekers gebruikten echter allemaal dezelfde T cel determinant. Wij laten in hoofdstuk 9 zien dat een covalente band tussen de T en de B cel determinant noodzakelijk is voor het opwekken van een bonafide geheugen (IgG) respons voor door ons geselekteerde determinanten.

In hoofdstuk 10 hebben we de rol van T cel determinanten onderzocht in 'meervoudig antigene peptiden' (MAP), een nieuwe peptideconstructie. Een MAP bestaat uit een vertakking van aan elkaar gekoppelde lysines (via α en ϵ amino groepen) waaraan het geselecteerde peptide kan worden gesynthetiseerd. Vertakkingen met 4 of 8 peptide sequenties worden het meest gebruikt. Deze MAPs blijken erg immunogeen te zijn en werden in de literatuur beschreven als toepasbaar voor iedere B cel determinant. Wij lieten zien dat de T onafhankelijkheid niet algemeen is en dat dus incorporatie van een T cel determinant in de MAP noodzakelijk is voor een sterke antilichaamrespons gericht tegen het natieve eiwit waarvan het peptide is afgeleid.

In hoofdstuk 11 worden de belangrijkste resultaten uit de verschillende studies bediscussieerd. Richtlijnen, die konden worden gedistilleerd uit de onderzoeken, voor het

succesvol opwekken van antilichamen met behulp van SP, zijn opgesteld. De belangrijkste zijn:

a) *Selectie van de sequentie.* Kies een sequentie van 15 tot 25 aminozuren. Vermijd "moeilijk" te synthetizeren sequenties. Kies de uiteinden zodanig dat ze hydrofiele residuen bevatten. Vermijd homologie met andere eiwitten door te zoeken in data bestanden. Plaats de epitoop in het midden van de sequentie of aan het andere eind van de koppelingsplaats. Selecteer antigene sequenties m.b.v. computer voorspellings programma's.

b *Constructie van het immunogeen.* Pas gecontroleerde koppelingsmethoden toe voor de conjugatie van peptiden en dragereiwitten. Gebruik MBS, EDC of GA koppeling afhankelijk van de geïncorporeerde aminozuren. Vermijd koppeling d.m.v. een aminozuur als dit deel uitmaakt van de epitoop.

c) *Immunisatie.* Intraperitoneale immunisatie van muizen met 25-50 μg van het peptide-dragereiwit construct in een minerale olie als adjuvans leidt tot goede resultaten. Herhaal de immunisatie pas nadat het niveau aan antilichamen weer is gedaald tot minder dan 20% van het piekniveau om antilichamen te verkrijgen met hoge affiniteit.

d) *Evaluatie van de immuunrespons.* Evaluatie van het reactiviteitspatroon van de antilichamen in polyclonaal serum moet worden uitgevoerd na iedere toediening van het immunogeen. Test op reaktiviteit tegen het peptide, tegen het dragereiwit, tegen de koppelingsdeterminant en tegen het natieve eiwit. Test op kruisreaktie met het natieve eiwit; dit moet worden uitgevoerd door competitieve, remmings of sandwich testen van de antilichamen met het natieve eiwit. Sandwich testen zijn alleen mogelijk als een invangend antilichaam van een andere diersoort beschikbaar is dan van de diersoort waarin de antilichamen die getest moeten worden gegenereerd zijn.

e) *Selectie van de monoclonale antilichamen.* Selectie van de hybridomas kan het best worden uitgevoerd in de test die uiteindelijk als diagnostische test gebruikt gaat worden. Een eerste screening kan met peptiden worden uitgevoerd uit overwegingen van kostenbesparing.

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CURRICULUM VITAE

Netty D. Zegers werd op 12 mei 1955 geboren te Terneuzen. Na het behalen van het HBS-B diploma in 1972 op het Zeldenrust College te Terneuzen, werd begonnen aan een HBO-B opleiding Analytische Chemie aan het IHBO te Breda. Tijdens de stages uitgevoerd bij het Koninklijke Shell Laboratorium Amsterdam en Dow Chemical Terneuzen werd resp. het temperatuurafhankelijk micel gedrag van visceuze oliën m.b.v. lichtverstrooingsmetingen bestudeerd (begeleider Dr. W. Mandema) en verschillende componenten in proceswater bepaald met diverse analytische methoden (begeleider Dr. L. Deij). Daarnaast werd de synthese van polystyrenen en de analyse van de molecuulmassa verdeling van polystyrenen met gelpermeatiechromatografie als afstudeeronderwerp gekozen.
Na het behalen van het diploma volgde oktober 1975 een aanstelling als research medewerker bij het Medisch Biologisch Laboratorium TNO (MBL-TNO) te Rijswijk op de afdeling Farmacologie (hoofd Dr. E. Meeter). Onder leiding van Dr. V.J. Nicholson werd het effekt van diverse neurotransmitters op het leergedrag bij ratten bestudeerd. Vanaf januari 1977 werd op de afdeling Immunologie (hoofd Dr. O. Brocades Zaalberg) o.m. het effekt van *in vivo* bestraling op helper T cellen van de muis en de invloed van loodverbindingen op het immuunsysteem bestudeerd. Monoclonale antilichamen gericht tegen diverse DNA addukten werden gegenereerd m.b.v. een rozettechniek speciaal ontwikkeld om te kunnen selekteren voor hoge affiniteit.
Naast deze werkzaamheden werd van september 1981 tot juni 1984 een avondopleiding MO-A Natuur- en Scheikunde gevolgd, afgesloten met het diploma in juni 1984. In het cursusjaar 1986/1987 gaf Netty Zegers de vakken Organische Chemie en Atoombouw en Chemische binding aan tweedejaars studenten van de chemische richting aan de Haagse Hogeschool, sectie Laboratorium Onderwijs. Daar heeft zij ook een geïntegreerde theoretische en praktische avondmodule Moderne Scheidingsmethoden opgezet voor HBO-studenten en heeft deze geleid van september 1987 tot juni 1990.
In 1985 werd een aanvang gemaakt met de studie Scheikunde aan de Rijks Universiteit te Leiden met als hoofdvak Biochemie. Het doctoraal diploma werd behaald in oktober 1989. De onderzoekstage werd uitgevoerd op de afdeling RecDNA, sektie Aspergillus (sektiehoofd Dr. C.A.M.J.J. van den Hondel, begeleider drs. P.J. Punt) van het MBL TNO, met Prof. Dr. Ir. P. van de Putte als mentor vanuit de RUL. Tijdens deze stage werd een bijdrage geleverd aan de bestudering van het moleculair mechanisme van de eiwitsecretie in *Aspergillus*.
In april 1990 volgde aanstelling als wetenschappelijk onderzoeker bij TNO-MBL afdeling Immunologie (hoofd Dr. E. Claassen). Onder leiding van Dr. W.J.A. Boersma heeft het in dit proefschrift beschreven onderzoek plaatsgevonden.
Sinds juni 1994 heeft de auteur in het nieuw gevormde TNO instituut Preventie en Gezondheid in de Divisie Immunologische- en Infektieziekten de leiding over een research groep die werkt aan de ontwikkeling van snelle immunologische en op DNA-probes gebaseerde detektiemethoden van toxinen alsmede pathogene microörganismen. Onder redactie van Netty Zegers, Wim Boersma en Eric Claassen is in samenwerking met een internationaal gezelschap collega's een boek getiteld "Immunological Recognition of Peptides in Medicine and Biology" gepubliceerd in 1995.

NAWOORD

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