Peptide Arrays for the Analysis of Antibody Epitope Recognition Patterns

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Abstract: One of the first applications of peptide arrays in general and those prepared by SPOT synthesis in particular was the mapping of antibody epitopes. In this article the diverse applications in this field are described. Different types of peptide libraries such as peptide scans, substitution analyses, truncation and deletion libraries as well as combinatorial and randomly generated libraries are briefly covered. Furthermore, their applications for antibody epitope mapping are described. These are: (i) identification of antigenic determinants within protein sequences for monoclonal and polyclonal antibodies, (ii) identification of key residues for binding, mapping of linear and discontinuous binding sites, (iii) paratope mapping, (iv) identification of mimotopes, and finally (v) the profiling of complex (auto)antibody signatures in biological fluids such as human or mice sera to identify novel biomarkers for e.g. cancer, allergy, infectious and autoimmune diseases.

Keywords: Antibody, antigen, diagnostics, epitope mapping, peptide library, peptide microarray, peptide scan, SPOT synthesis.

I. INTRODUCTION

The investigation and characterization of antibody binding events is among the most important fields of use for peptide libraries. Specific applications range from epitope mapping, identification of key residues for antibody-antigen interactions, characterization of monoclonal and polyclonal antibodies and paratope mapping to high content analyses of antibody signatures in biological fluids such as the identification of specific antibody epitope patterns in sera from patients with infectious diseases or the utilization of peptide microarrays for companion diagnostics and patient stratification.

In section II the variety of different peptide library types based on protein sequences by dissecting or modifying the primary structure of a protein antigen is described. Conceptually different peptide library types are based on *de novo* approaches exploring the entire or at least a significant and sometimes biased part of the potential sequence space (section III). Section IV addresses general principles of antibody binding assays for peptide arrays. Specific applications of peptide arrays for antibody characterization are described in section V. Finally, most recent developments describing high content analyses of antibody signatures in biological fluids are reviewed in section VI.

II. PROTEIN SEQUENCE-DERIVED PEPTIDE LIBRARIES

Protein sequence-derived libraries provide the basic tool to analyze interactions between antibodies and antigens by providing detailed information about the ligand binding site.

1. Scans of Overlapping Peptides

The standard library to identify an antibody binding site on an antigen is a scan of overlapping peptides, also called a peptide scan or simply pepscan (Fig. **1**) [1, 2]. The entire protein sequence, or a certain part of it corresponding for example to a particular domain, is synthesized as short, overlapping, linear peptides that are subsequently tested for antibody binding. Usually, this involves 6- to 15-mer peptides since most linear binding sites do not exceed this range [3-5]. However, to identify discontinuous binding sites longer peptides are considered an advantage if the peptide covers a folding motif comparable to the native protein structure. In addition to the peptide length, another important parameter of peptide scans is the number of overlapping amino acids between two consecutive peptides. Usually, the peptides are shifted by one to three positions along the protein sequence. With shorter overlaps important peptides may be overlooked. In peptide scans derived from proteins containing disulfide bonds or free cysteine residues, these residues are commonly exchanged by similar amino acids, such as serine, to avoid dimerization and oligomerization of the peptides or covalent linkage to thiols in the ligands [6].

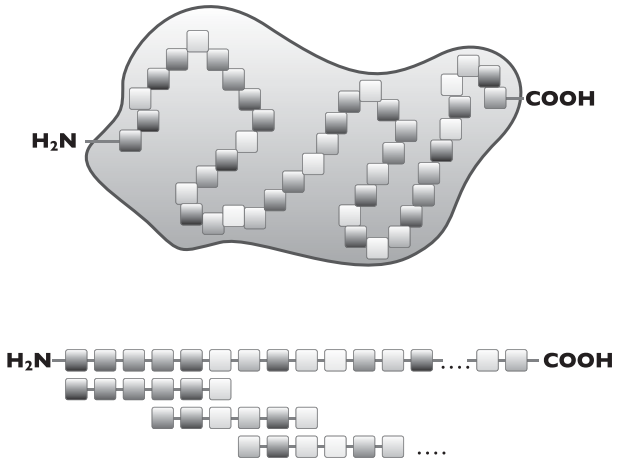

Fig. (1). Scan of overlapping peptides. The amino acid sequence of the protein under investigation is used to generate short linear overlapping peptides (peptide scan).

2. Amino Acid Substitution Scans

The interaction of a peptide with an antibody usually relies upon a limited number of amino acid residues that are effectively in contact with the binding partner. These amino acids contribute either to the binding free energy or to the specificity of the interaction, and are referred to as key residues. If the peptide has to adopt a certain conformation upon or prior to binding, amino acids facilitating these conformations can also be regarded as critical. The concept of "alanine scanning" introduced to map protein-protein interactions by site-directed mutagenesis has been used to identify these residues (Fig. **2**) [7]. Here, residues that cannot be exchanged

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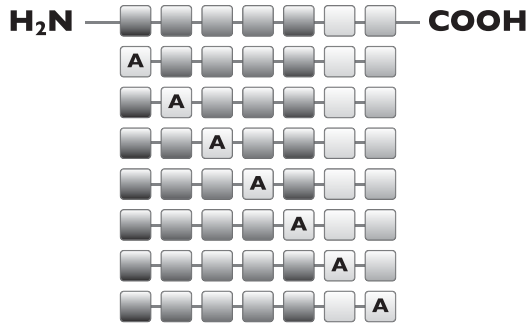

Fig. (2). Amino acid substitution scan (alanine scan) of a 7-mer peptide.

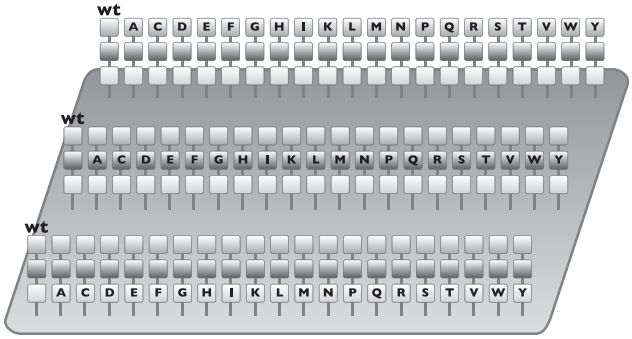

Fig. (3). Complete substitutional analysis of a 3-mer peptide.

without loss of binding are regarded as key residues for the interaction. Scans with other amino acids are similarly used to explore the effect of charged residues, for example. It has to be considered that this only reveals effects that depend on the amino acid side chains unless one incorporates building blocks that lead to a modified backbone. Among the naturally occurring amino acids proline plays a special role in amino acids substitution scans (synonym: replacement scan) and is therefore often used as a substitute. It can influence the pre-binding conformation by inducing a turn structure or preventing helical structures, providing indirect information about binding modes.

3. Substitutional Analyses

If, for example, the amino acid substitution scanning approach employs all genetically encoded amino acids it is called (complete) substitutional analysis (synonyms: mutational analysis as referred to in some former publications, replacement analysis, analoguing). These experiments explore the effects of all possible single site substitutions of the starting peptide (Fig. **3**) [3, 8-10]. This identifies key residues meaning that they cannot be substituted at all or only by physicochemically similar amino acids (e.g. leucine/isoleucine). Usually, other positions are not sensitive to substitutions and some may even lead to increased binding activity. Complete substitutional analyses are a rapid and effective way to delineate the structure-activity relationship of peptides and to simultaneously optimize the starting sequences with respect to affinity. In addition to substitutional analyses using the genetically encoded amino acids, D-amino acids [11], other unnatural amino acids [12, 13], or peptoidic building blocks [14] are used to increase the diversity of side chain functionalities or backbone modifications. This often results in identifying substitution analogs that are stabilized against proteolytic degradation.

4. Truncation, Deletion, and Combinatorial Deletion Libraries

Peptides comprising an antibody epitope which are identified for example using a peptide scan or by other types of peptide libraries, including chemical and biological approaches, often contain a well-defined core of key residues. In addition, these peptides include other dispensable positions resulting from the predefined peptide length used in the library design. In order to narrow down the peptide to the minimal epitope three different types of libraries are useful: (1) Truncation libraries (synonyms: size scan, window scan) comprise peptides omitting one or more N-, C- or N- and C-terminal amino acids (Fig. **4A**) [8], (2) Peptides from libraries of deletion analogs (Fig. **4B**) have one or more consecutive amino acids deleted at all possible positions, (3) Compared to deletion libraries, combinatorial deletion libraries additionally cover peptides with two or more positions omitted independently all over the sequence (Fig. **4C**).

5. Cyclization Scans

A widespread strategy to optimize the binding free energy of a peptide, interacting for example with an antibody, is to stabilize the binding conformation. This is often achieved by cyclization, for example via disulfide bonds [15, 16]. However, the binding conformation is usually unknown. Therefore, a large number of cyclic peptide analogs have to be synthesized and screened to seek out the proper conformation of a biologically active peptide. A systematic approach is the "cyclization scan" comprising all possible combinations of two oxidized cysteine residues within the starting peptide (Fig. **5**). An example of this approach is a library of 466 cyclic peptide analogs for mimicking a discontinuous interleukin-10 epitope [15]. Cyclization of peptides by disulfide bonds via cysteine residues is the most amenable strategy and often applied for peptide arrays especially for stepwise *in situ* peptide array production. However, several other chemical cyclization strategies can be similarly applied as shown for amide bonds [16] and the entire chemical repertoire for peptide or peptidomimetic cyclization can be used for array production technologies with pre-synthesized compounds.

6. Specialty Libraries

A specialized peptide library type was applied to elucidate evolutionary transition pathways between three completely unrelated peptides recognized by the anti-p24 (HIV-1) mab CB4-1 [17]. These different peptide ligands were identified previously using combinatorial and deconvolution libraries [18]. The question of whether the different CB4-1 peptide ligands can be reciprocally converted into each other was answered by synthesizing and analyzing all possible (7,620,480) single step transition pathways (i.e. sequential conversion of one amino acid after the other) between the three ligands. The library comprising all 2560 possible transition peptides was designed with the software PepTrans. Complete L-amino acid substitution analyses of all intermediates from the best transition pathways were performed in order to better understand the structural mechanisms involved in the sequence transformation. In this study the exceptional synthesis capacity of the SPOT method was exploited to analyze the sequence space between functionally related peptides with no sequence similarity.

III. *DE NOVO* GENERATED PEPTIDE LIBRARIES

If the antigen is not known, one has to use combinatorial libraries with peptide mixtures or randomly generated libraries of single individual sequences. It should be mentioned that most of the library types described in this section were pioneered at the beginning of the combinatorial chemistry era and in the field of chemical libraries on beads [19-21] or by using biological display techniques such as phage display [22-24]. Michal Lebl has published a very lively historical review, with personal comments by the authors, of "classical" papers form the beginning of combinatorial chemistry [25].

The main problem for *de novo* identification of peptides is how to handle the immense number of potential peptide sequences, referred to as "combinatorial explosion". Even if only using the ge-

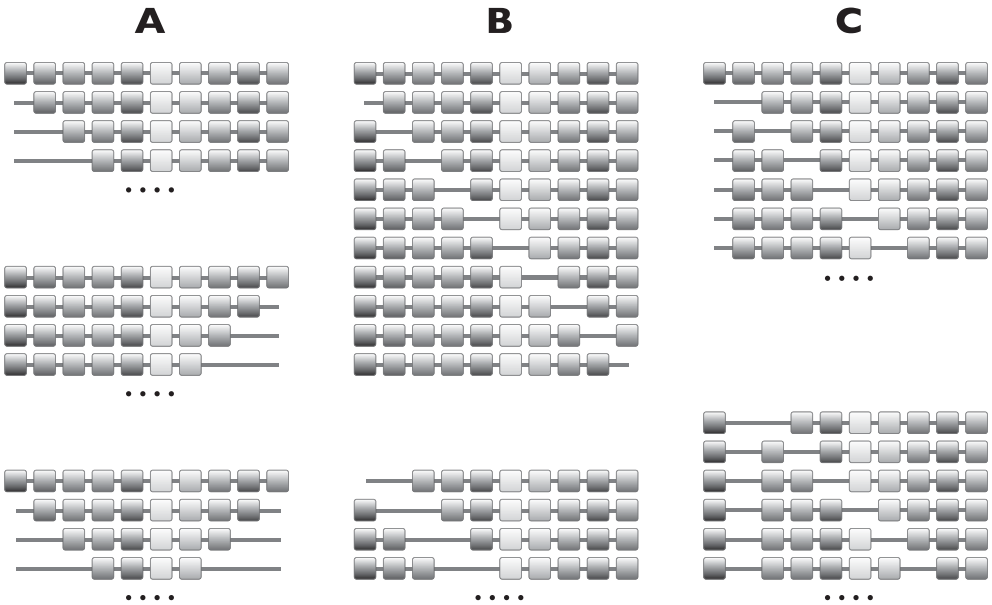

Fig. (4). Analysis and optimization of peptide length. (**A**) Truncation library with N-terminal, C-terminal and bi-directional stepwise truncations; (**B**) deletion library (one or more consecutive amino acids deleted at all possible positions), and (**C**) combinatorial deletion library comprising all peptides with two or more positions omitted independently all over the starting sequence.

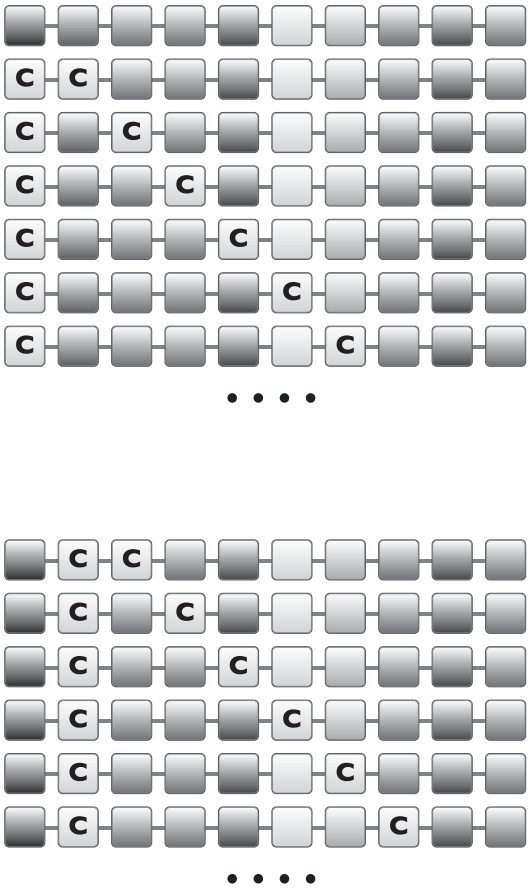

Fig. (5). Disulfide cyclization scan. The library covers all possible combinations of two cysteine residues within the starting sequence that are subsequently oxidized for cyclization.

netically encoded amino acids the number of possible sequences dramatically increases with the peptide length (dimers: 20^2 = 400; trimers: 20^3 = 8,000; tetramers: 20^4 = 160,000; pentamers: 20^5 = 3,200,000; hexamers: 20^6 = 64,000,000).

Using combinatorial libraries the aim is to completely cover the potential sequence space. Although peptide arrays can be prepared with a high spot density (> 40,000/cm^2) [26] there is no technology yet available to synthesize and handle billions of different compounds individually. The solution is to synthesize peptide mixtures with degenerated or randomized positions by statistically incorporating amino acids of a certain set (Fig. **6**). Defined amino acids are only used at a limited number of positions. This results in a manageable number of peptide pools screened on the peptide arrays. The randomized positions of active pools must then be deconvoluted iteratively using deconvolution libraries individually designed for the project, ultimately selecting the active compounds. Since deconvolution is a time-consuming process, arrays of randomly generated peptides have also been applied. Since such libraries only

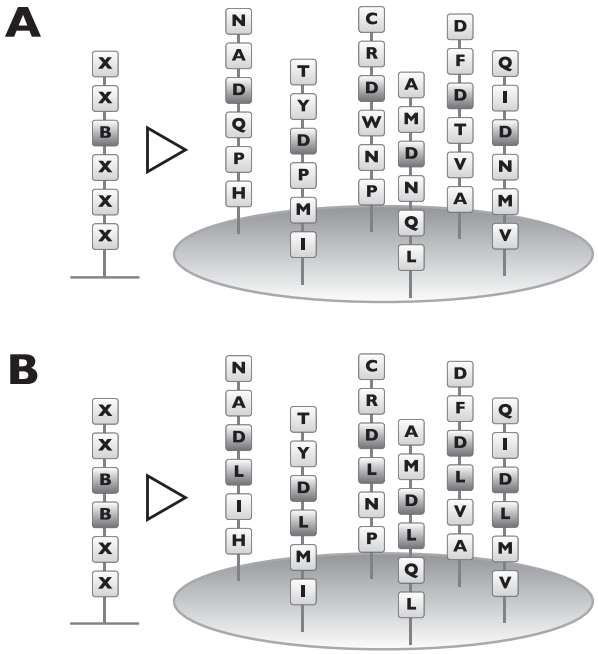

Fig. (6). Peptide mixtures. Peptide mixtures with one (**A**) and two (**B**) defined positions (B) and randomized position (X).

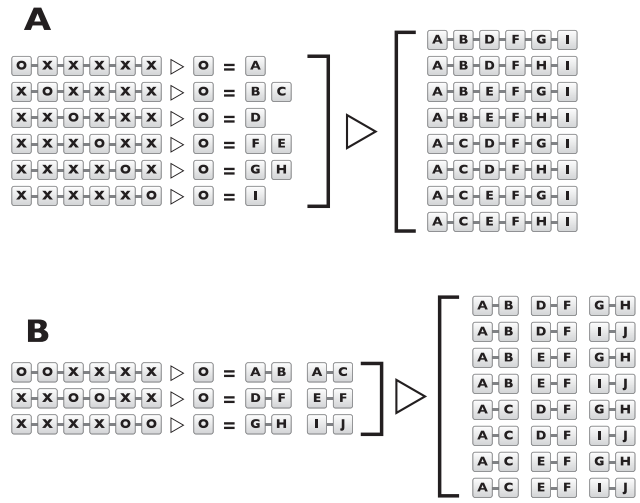

Fig. (7). Deconvolution of active peptide mixtures. (**A**) In the positional scanning approach the most active amino acids at each position are identified from the initial library with peptide mixtures (X = randomized position; O = defined position with an individual amino acid). The deconvolution library consists of individual peptides representing all possible combinations of the most active amino acids. (**B**) In the dual positional scanning approach two positions are defined interdependently in the starting library.

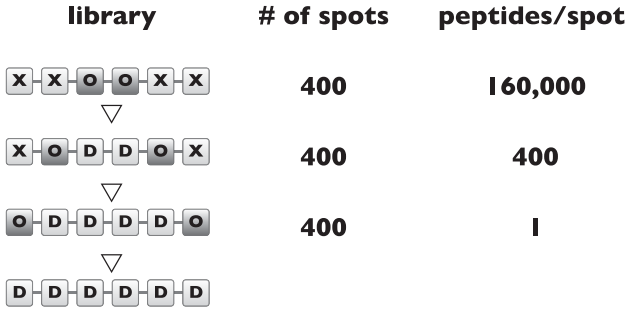

Fig. (8). Iterative deconvolution process of active peptide mixtures. A starting hexamer library of the type XXOOXX (X = randomized position; O = defined position) is screened and the best dipeptide combination OO is selected for the first deconvolution library (XODDOX; D = defined position identified from the preceding library). Subsequently, the second deconvolution library ODDDDO is based on the best tetrapeptide motif ODDO from the preceding library and leads to a single peptide.

cover a small percentage of the potential sequence space, initially selected peptides often have low affinities to the binding partner and must subsequently be optimized, for example using substitutional analyses.

1. Combinatorial Libraries

The five most critical parameters for identifying peptide ligands from combinatorial library arrays are: (1) the number of peptide mixtures tested, (2) the number of defined positions, (3) the ratio between defined and randomized positions, (4) the appropriate spacing of the defined positions within the entire sequence length, and (5) the overall length of the peptides. These parameters determine the ratio between active and inactive compounds in the peptide mixtures and consequently the signal to noise ratio and likelihood of identifying bioactive peptides.

The development of multiple peptide synthesis robots has enabled a continuous increase in peptide library complexity. The first arrays to be developed were hexamer libraries with two defined positions (mostly abbreviated as “O” or “B”) and four randomized, or mixed positions (X): e.g. XXO_1O_2XX [27]. Initially, these libraries were used to map linear antibody epitopes, but several other applications emerged.

The randomized positions have to be deconvoluted to obtain single active peptides. Two general procedures have been described: (1) In the positional scanning approach (Fig. **7A**) the entire library is subdivided into a small number of peptide mixtures that have single amino acids at certain positions: O_1XXXXX, XO_2XXXX, XXO_3XXX, $XXXO_4XX$, $XXXXO_5X$ and $XXXXXO_6$ (O and X as defined above). If the 20 naturally encoded amino acids are used for the defined positions (O) this library comprises 6 x 20 = 120 separate mixtures that are screened for binding [28]. Subsequently, individual peptides representing all possible combinations of the most active amino acids at each position are synthesized and screened. Alternatively, two (dual positional scanning approach) (Fig. **7B**) [8, 29] or even more positions are defined in the first library. Although two defined positions involve greater synthesis efforts ($20^2 = 400$ peptide mixtures) the chance of successful primary screening is significantly better due to interactions with higher affinity and specificity. All randomized positions have to be deconvoluted in a second step based on the results with the starting library. Whereas the initial library is not predefined for a given screening molecule and can be applied universally, the follow-up libraries are tailor-made for specific purposes. The positional scanning approach assumes that the contributions of preferred amino acids at each position are additive or at least not interfering. However, this cannot be taken for granted in every system. (2) In order to circumvent this limitation, the randomized positions can be deconvoluted by an iterative process (Fig. **8**). Each deconvolution library is designed based on results from the starting or precursor library [27, 30]. Finally, a re-evaluation is recommended since there might be other amino acids at positions defined early in the process that have a more positive effect on those defined later in the deconvolution.

A dramatic increase in the effectiveness of peptide and peptide mixture multiple automated syntheses paved the way for more complex libraries of the type $XXXXO_1O_2O_3XXXX$ (8000 peptide mixtures) [31]. For example, the most complex library prepared by the SPOT technique described so far is of the type XXXX[3O3X]XXXX. The internal core [3O3X] is an abbreviation for three defined and three randomized positions arranged in all combinations $XXXX[O_1O_2O_3XXX]XXXX$; $XXXX[O_1O_2XO_3XX]XXXX$ and so on [18]. This library comprised 68,000 spots and was used to identify not only antibody epitopes but also other peptides that bind to the antibody's paratope in a different way, referred to as mimotopes [32, 33]. This was shown by structure determination of the peptide/antibody complexes by X-ray crystallography [34]. In many cases such complex libraries are essential for identifying peptide ligands that may require a certain number of key residues in a distinct pattern.

An alternative way to reduce the number of peptide mixtures that have to be prepared, yet match as many defined positions as practicable uses so-called combinatorial clustered amino acid peptide libraries [35, 36]. Each cluster contains physicochemically similar amino acids. The rationale of this approach is based on the assumption that physicochemically related amino acids contribute similarly to binding. For instance, grouping the amino acids into six clusters would lower the number of peptide mixtures in a combinatorial library containing four non-random positions from 20^4 (160,000, with four defined positions) to 6^4 (1,296, with four cluster positions). Kramer *et al.* described the epitope mapping of anti-transforming growth factor α (TGFα) mab Tab2 using a library of the type $XC_1C_2C_3C_4X$ (C = one of six amino acid clusters [APG], [DE], [HKR], [NQST], [FYW], [ILVM]) in comparison to phage display techniques [36]. The peptide library array identified several motifs unrelated to known TGFα-derived linear epitope sequences,

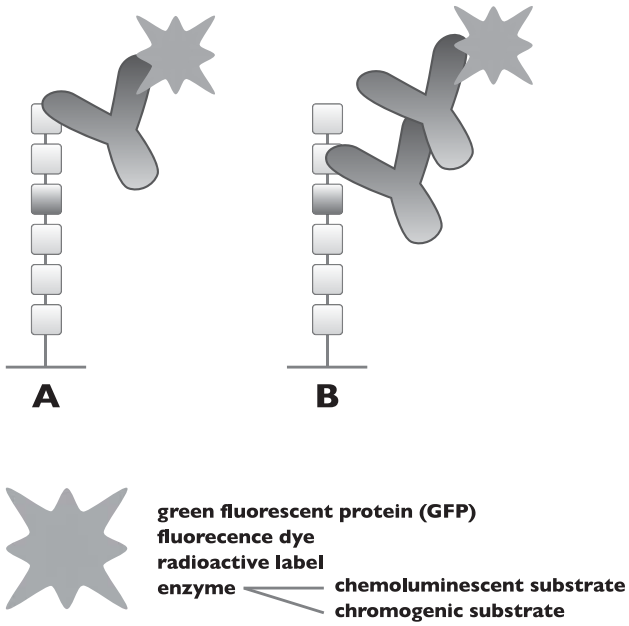

Fig. (9). Binding assays and detection methods to identify peptide-protein interactions. (**A**) Antibody epitope mapping with a directly labeled antibody; (**B**) antibody epitope mapping using a labeled secondary antibody.

whereas the phage display technique only revealed peptide ligands closely related to the wild-type epitope.

A very interesting technique worth mentioning here is the so-called orthogonal library concept [37, 38]. The principle is that the same compound is represented in two different mixtures. Comparative activities of different mixtures observed after screening enables identification of the compound responsible for activity.

2. Random Libraries

An alternative to protein sequence-derived or combinatorial peptide array libraries is to use sets of randomly generated peptide sequences. A peptide array approach was described using a library of 5520 randomly generated individual 15-mer peptide sequences prepared by SPOT synthesis and incorporating all genetically encoded amino acids except cysteine [5]. Even though this only covers an extremely small fraction of the potential sequence repertoire the peptide library array was successfully used to identify specifically binding peptide epitopes and mimotopes of three different antibodies (anti-IL-10 mab CB/RS/13, anti-TGFα mab Tab2, anti-p24 (HIV-1) mab CB4-1). Initially identified peptide ligands mostly had very low affinities for the antibodies with dissociation constants around 10^{-4} M. However, subsequent substitutional analyses revealed several analogs with dissociation constants in the low micromolar and high nanomolar range in a one step process. In two other studies 4450 randomly generated 12-mer peptides prepared on 10 "mini-Pepscan cards" (455 peptides per card) as well as a tripeptide library comprising the genetically encoded amino acids in all possible combinations were used to identify peptides binding to monoclonal antibodies. Peptides were identified as either homologous to the wild-type epitope [39] or unrelated mimotopes [40].

In addition to randomly generated peptide library arrays, this approach was also used for peptidomimetics. Heine *et al.* described an 8000 membered hexapeptoid and hexapeptomer peptidomimetics array for the anti-TGFα mab Tab2 [41]. The best compound had a dissociation constant of 2.7 μM. The same antibody was used to probe an array of 8000 1,3,5-trisubstituted triazines, with the best hit having a dissociation constant of ~ 400 μM [42].

IV. ANTIBODY BINDING ASSAYS FOR PEPTIDE ARRAYS

This section is divided into the sections "Screening" and "Read-out" addressing either the molecular recognition event, or how one observes which peptide was bound and/or converted by an interaction partner or enzyme.

1. Screening

Peptide arrays are most frequently applied to study peptide binding by polyclonal or monoclonal antibodies. This can be achieved with directly labeled primary antibodies or with labeled secondary antibodies (Fig. **9A**, **9B**). For all immunological detection systems it is critical to rule out direct, nonspecific binding of the detection antibodies (or protein A/G) to the peptides. Incubation procedures as well as control experiments for peptide arrays have been described and are analogous in principal for all types of arrays [43].

2. Read-out

Quantification of peptide-bound antibodies is described with chemoluminescent, fluorescent, chromogenic, and label-free read-out methods such as surface plasmon resonance (SPR), mass spectrometry (MS), or atomic force microscopy (AFM).

Detection of peptide-antibody binding often employs *chemoluminescence read-out* since a huge number of peroxidase-labeled antibodies are commercially available. Very high sensitivity is achieved using a chemoluminescence substrate combined with either imaging systems or X-ray or photographic films. Luminol-based substrates can be mixed very cheaply or purchased. In addition, ultra-high sensitivity substrates are commercially available. Fortunately, peroxidase itself shows almost no detectable binding to the peptides. Signal amplification and increased sensitivity is achieved by coupling more than one enzyme molecule to the detection antibody, e.g. a second antibody or protein A or G.

The sensitivity of *fluorescence read-out* depends on the number of fluorescent moieties coupled per analyte molecule, the quantum yield of the fluorescent dye, the peptide loading, and the amplification achieved, for example by a sandwich assay with primary and secondary antibody. Fortunately, many secondary antibodies labeled with different fluorescence dyes are available. However, background fluorescence from the array support can be a severe drawback. Such background signals could be almost completely suppressed using planar waveguide technology in combination with fluorescence-based detection methods [44-46]. Glass chips usually have significantly lower intrinsic background compared to the membranes commonly used for arrays prepared by the SPOT technology. These porous membranes usually contain traces of fluorescent substances left over from the production process. Another source of background fluorescence can arise from the peptides, peptidomimetics or often side products from the synthesis process, e.g. side chain protecting groups. Fluorescent dyes with longer emission wavelengths, (e.g. Texas Red®) are preferable to avoid interference with background fluorescence from substances in complex mixtures such as cell lysates.

Several examples for *chromogenic read-out* and densitometric quantification on peptide arrays probed with antibodies are described. These are precipitating substrates, e.g. nitroblue tetrazolium (NBT)/bromochloroindolyl phosphate (BCIP) catalyzed by alkaline phosphatase [27] or bromochloroindolyl-β-D-galactopyranoside catalyzed by β-galactosidase [47]. No imager system is required for this type of read-out. Visual inspection is sufficient for quantification and documentation only requires a scanner. However, the dynamic range and sensitivity are far worse than achieved by chemoluminescence or fluorescence read-out.

Label-free read-out systems are the ultimate goal for screening peptide arrays. The screening molecule needs no modification,

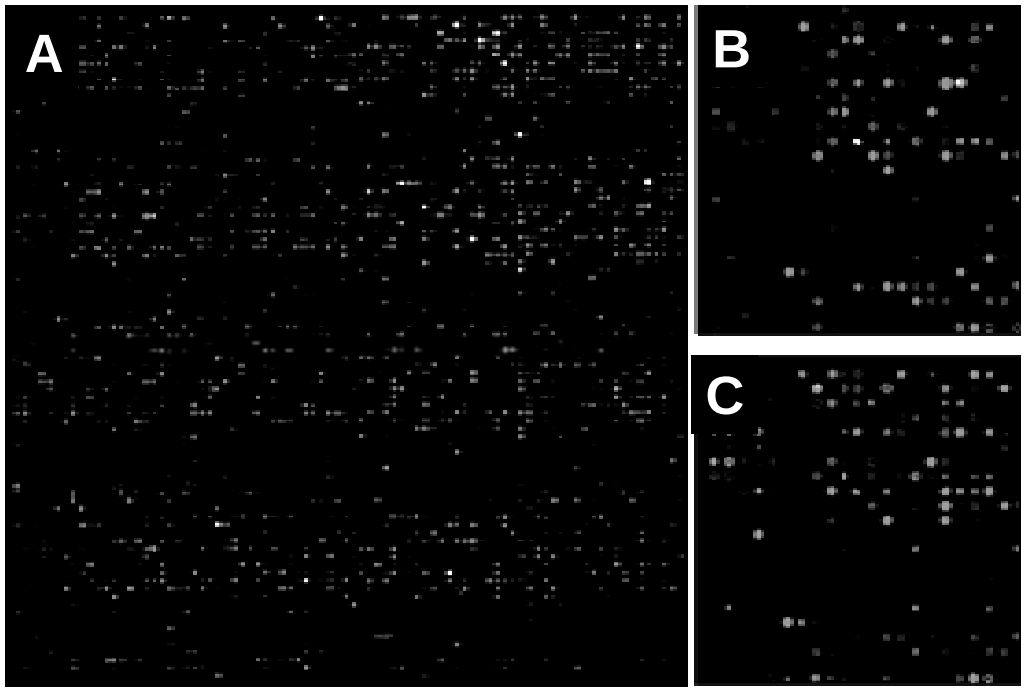

Fig. (10). A 6912 peptide microarray presenting overlapping peptide scans through 46 human tumor associated antigens. (A, B) incubation with serum of a healthy donor, (C) incubation with serum of a cancer patient. For detection an anti-human IgG Cy5-labelled antibody was used. (A) One of three identical subarrays. (B) Block 5 of 16 within this subarray. (C) The same block as in B incubated with a patient sample. Even though the general signal pattern between B and C is similar, single signals are different.

which is usually very tedious and may affect the biological activity of the analyte. Moreover, this excludes artifacts associated with the detection molecule, e.g. secondary antibodies or fluorescent markers, as discussed above. The most important label-free read-out systems are SPR, which can record kinetic data of the binding event [48, 49], MS, which can identify a certain molecule out of a crude mixture with high sensitivity [50] and AFM resulting in a three-dimensional image of the screening molecule bound to the array. So far, these technologies have mostly been used for DNA, protein, or small molecule arrays but it is just a question of time until they are used to a similar extend with peptide arrays.

V. APPLICATIONS OF PEPTIDE ARRAYS FOR ANTIBODY CHARACTERIZATION

The most frequent application of peptide arrays described so far is the mapping of antibody epitopes. This can be ascribed to the fact that antibody-antigen interactions are often used as model systems for the evaluation of novel peptide library techniques, for several reasons: (1) Usually, antibodies bind to their antigens with high affinity and specificity (disregarding cross-reactivity), (2) many antibodies raised against proteins bind to linear epitopes, (3) antibodies can be easily detected using commercially available enzyme- or fluorescence dye-coupled secondary antibodies, (4) antibodies are stable and easy to handle, and (5) state-of-the-art techniques allow rapid and cheap preparation of sufficient antibody amounts.

1. Monoclonal Antibody Epitope Mapping: Linear Epitopes

In linear epitopes (also referred to as continuous or contiguous epitopes) [51] the key amino acids mediating antibody contacts are located within one part of the antigen's primary structure, usually not exceeding 15 amino acids in length. Peptides covering these sequences have affinities to the antibody within the range shown by the entire antigen.

Three-dimensional structures of antibody-antigen complexes obtained from X-ray crystallography reveal relatively large contact surfaces in a range between 500 and 1000 $Å^2$ with more than 15 amino acids in contact with the binding partner. This led to the definition of the "structural epitope" comprising all contact residues as observed in the complex structure without considering their energetic contribution. On the other hand, extensive site-directed mutagenesis studies have shown that only a few residues effectively contribute to the binding free energy. These residues are summarized as defining the "energetic epitope" or "hot spot of binding" [52]. Here, it should be pointed out that linear peptide epitopes identified by protein sequence-derived peptide scans comprise the amino acids of the energetic epitope, as well as a few linking residues, rather than the structural epitope. Today, experiments to identify and characterize linear antibody epitopes using peptide scans, amino acids scans, substitutional analyses, truncation libraries, deletion libraries, cyclization scans, all types of combinatorial libraries and randomly generated libraries of single peptides are standard techniques widely applied even in non-specialized laboratories [53]. Most of the peptide array applications for mapping antibody epitopes are based on the SPOT synthesis technique. However, the earliest publication describing antibody binding to arrays of short linear peptides, in 1991, utilized light-directed spatially addressable peptide synthesis [26]. Furthermore, several papers have been published where microarrays were prepared by directed immobilization [54-57] or co-polymerization of pre-synthesized peptides [58, 59].

2. Monoclonal Antibody Epitope Mapping: Discontinuous Epitopes

Compared to linear epitopes, discontinuous epitopes are much more difficult to map. In discontinuous (or conformational) binding sites the key residues are distributed over two or more binding regions separated in the primary structure [51]. Upon folding, these binding regions are brought together on the protein surface to form a composite epitope. Even if the complete epitope mediates a high affinity interaction, peptides covering only one binding region, as synthesized in a scan of overlapping peptides, have very low affinities, which often cannot be measured by normal ELISA or SPR experiments. Therefore, very sensitive detection procedures have to

be applied to map discontinuous epitopes using conventional peptide scans. Peptide arrays on cellulose membranes prepared by SPOT synthesis are especially suited for this purpose. This is mainly due to the extremely high peptide loading [60], which correlates to a concentration in the millimolar range assuming an equal distribution of peptides over the membrane area. This extremely high peptide density facilitates detection of even weakly binding peptides due to avidity effects. These peptides often have dissociation constants in the high micromolar or even millimolar range. Such sensitive read-out can of course lead to the detection of unspecific peptide-protein interactions. This has to be ruled out by control incubations, inspection of the three-dimensional antigen structure, competitive ELISA experiments or SPR studies [10, 61].

There are considerably fewer publications describing the mapping or mimicking of discontinuous epitopes due to the obstacles mentioned above. Here, three outstanding publications are worth mentioning: (1) Korth *et al*. identified a three-segmented binding site for mab 15B3, which specifically recognizes the disease form of bovine prion protein [62]. (2) An interleukin-10 (IL-10)-derived peptide scan was used to identify a discontinuous epitope on IL-10 composed of two segmented regions. Connection of these binding regions and further optimization by substitutional analyses followed by a disulfide cyclization scan resulted in an IL-10 mimicking peptide with a dissociation constant in the lower nanomolar range [15, 63]. (3) A special type of discontinuous epitope appears in the hinge region of antibodies where the analogous sequences of the two heavy chains have a parallel orientation with several disulfide bridges, depending on the species and the antibody class and subclass. Welschof *et al*. were able to identify and mimic epitopes of scFv antibodies that simultaneously bind to both heavy chains in the hinge region of IgG antibodies [64]. These sites were mimicked by branched peptides with two identical hinge region-derived sequences coupled to the α- and ε-amino group of a Lys residue immobilized on a cellulose membrane.

3. Antibody Paratope Mapping

Alternatively, epitope mapping approaches can be applied to paratope mapping or to identify active peptides from antibody complementarity determining regions. For example, three peptides with CD4-binding capacity and HIV antiviral activity were identified using a scan of overlapping peptides covering the V_H and V_L domains of the murine IgG1κ anti-CD4 mab ST40 [65]. The "paratope dissecting" approach was reviewed in detail [66].

4. Polyclonal Antibody Epitope Mapping

Protein sequence-derived peptide scans are similarly applied to mapping polyclonal antibody epitopes. The only difference compared to monoclonal antibodies is that there is no way of distinguishing between linear epitopes and single binding regions of discontinuous antigenic determinants. In an outstanding publication Valle *et al*. mapped the epitopes of a polyclonal serum raised against the *Bacillus subtilis* bacteriophage Φ29 connector [67, 68]. Eleven immunodominant regions were identified. The membrane-bound spots were used to purify fractions of the serum [69]. Since only a low resolution structure of the Φ29 connector is available, these epitope-specific polyclonal antibodies proved extremely useful for topographical assignment of the epitope sequences using electron microscopy of Φ29 connector-antibody complexes.

VI. PROFILING OF ANTIBODY SIGNATURES IN COMPLEX BIOLOGICAL FLUIDS

Peptide arrays based on solid supports such as cellulose membranes are successfully used in a multitude of applications, including the profiling of antibody signatures in serum (reviewed in [53]). The development of peptide microarrays overcame a number of limitations of the cellulose based arrays most importantly the requirement for high sample volume and the generation of only one replica per synthesis. The impressive development and improvement of DNA-microarray technologies fertilized the development of alternative biochips such as protein, antibody, carbohydrate and peptide microarrays immensely. Virtually all machinery and materials used for the production, incubation and evaluation of DNA microarrays can be used for the generation of biochips and incubation experiments therewith. Today, biochips can be produced in quantity with thousands of individual molecules allowing parallel multiplex assays of a multitude of samples against high content libraries (Fig. **10**). Antibody and protein microarrays are used for the detection of antigens and the mapping of antibody signatures in biological fluids, respectively (reviewed in [70-72]). These biochips are promising in biomarker discovery and validation as well as *in vitro* diagnostics. However, protein microarrays do not enable the detection of individual epitopes at single proteins. Peptide microarrays enable the identification of linear epitopes at the sequence level and thus, higher resolution data. Consequently, peptide microarrays are increasingly used for the detection of novel biomarkers and the classification and stratification of patients in a growing number of studies in different disease areas.

1. Antibody Epitope Patterns in Infectious Diseases

Detailed knowledge of target epitopes from pathogens is valuable for diagnosis of and rational development of vaccines against infectious diseases. High content peptide microarrays have been used to profile immune responses to *M. tuberculosis* epitopes and allowed a general insight on the immune status of patients suffering from active pulmonary tuberculosis compared to healthy donors. Microarrays with 7446 peptides of scans covering 61 *M. tuberculosis* proteins were incubated with sera from 34 patients and 35 healthy donors [73]. Peptides derived from cell wall proteins or proteins connected to the pathogenicity of *M. tuberculosis* were frequently present in three selected groups of peptides with different epitope properties in the diseased (TB+) *vs.* healthy (TB-) group (1) exclusively recognized as epitope in TB+ group, (2) exclusively recognized as epitope in TB- group and (3) differentially recognized in TB+ and TB- group. A recognition pattern of 89 peptides for IgG-epitopes was detected and could be verified using sera of infected patients and healthy donors form different ethnic and geographical background.

In another study, mice were infected with the intestinal helminthic parasite *Heligmosomoides polygyrus* [74]. Serum samples taken before and two weeks after infection were incubated using a peptide microarray with 255 14-mer peptides of random sequence (see Section III.2). Additionally, the results were compared to mice of a different strain. Using principal component analysis (PCA), linear discriminant analysis (LDA) and potential support vector machines (P-SVM) for data analysis, peptides for the reliable classification of the different mouse strains and the healthy/infected mice were selected. This shows the applicability of random peptide microarrays for cases, where no or insufficient information on the protein sequence of the pathogen is available.

2. Identification of Epitopes in Food Allergens

The identification of antigenic determinants in food allergens is one of the early applications of peptide microarrays. Different new epitopes of peanut allergens were identified and the polyclonal IgE and IgG4 response was monitored in a double-blind placebo-controlled peanut challenge study in children [75, 76]. The clinical sensitivity was positively related to a more diverse IgE response. These results are seen as a basis for the development of prognostic tests for severe reactions in children with peanut allergy. Similarly, epitope regions of milk allergenes were identified using peptide microarrays presenting scans through five milk proteins [77, 78].

Here, results were compared to earlier investigations using peptide macroarrays and found to be mostly consistent.

3. Identification of Autoantibody Reactivities

Peptide microarrays displaying overlapping peptides based on potential autoantigens are a powerful tool for the detection of autoantibody signatures in autoimmune and other diseases. A detailed description of such assays was published recently [79].

Interestingly, autoantibodies to a number of self antigens are already present in the blood of newborn infants [80]. This is a result from a study where an antigen microarray loaded with peptide and protein antigens was used. An additional study showed close details of the development of the humoral immune system of individuals [81].

In a number of different studies autoantibody signatures provided new insights in the role of autoantibody responses of diseases such as rheumatoid arthritis (RA), multiple sclerosis or Alzheimer's disease. Using samples from 77 patients suffering from rheumatoid arthritis, psoriatic arthritis or ankylosing spondylitis and 19 healthy donors for incubations with microarrays revealed an association of blood levels of autoantibodies targeting citrullinated antigens and proinflammatory cytokines [82].

A microarray with a mixture of 363 immobilized antigens, peptides, proteins, lipids and mixtures, allowed the discrimination between patients with different types of multiple sclerosis, healthy donors and patients with other autoimmune or neurodegenerative disorders such as lupus erythematosus, adrenoleukodystrophy and Alzheimer's disease [83].

Peptide microarray-based characterization of autoantibody reactivities in patients with Alzheimer's disease supported the concept of autoantibodies that may protect from toxic amyloidogenic peptides [84]. This involvement of neuroprotective antibodies in the pathogenesis of Alzheimer's disease could open new therapeutic options.

4. Peptide Microarrays for Companion Diagnostics and Patient Stratification

Expectations for peptide microarrays include the identification of predictive biomarkers for the early diagnosis of diseases, the characterization of diseases for an optimal choice of therapy and patient stratification and companion diagnostics for the monitoring of treatment success. The applicability of peptide microarrays for these aims was already shown in a number of model studies, but also in clinical phase 1/2 studies.

In a model study where diabetes was induced in healthy mice it could be shown that patterns in the IgG reactivity of the mice were predictive for the susceptibility of mice to stimulation with cyclophosphamide [85]. The IgG reactivity was measured using microarrays with 266 antigens of which around 100 were peptides. The superparametric clustering algorithm (SPC) proved robust and effective in data evaluation.

Anti-TNF therapy is an effective treatment for rheumatoid arthritis. However, one third of the patients proves insensitive towards treatment with anti-TNF agents. A microarray with more than 500 peptides and proteins was used for the identification of a 24-biomarker signature for the prediction of positive clinical response to an anti-TNF drug [86]. In an earlier study with RA patients peptide microarray experiments provided diagnostic information and allowed stratification of the patients with early RA into disease subsets [87].

Peptide microarrays are also useful for monitoring the status of the antibody response during vaccination studies. Simian-human immunodeficiency virus infected macaques were vaccinated with three different vaccines [88]. Mixed arrays with 410 pathogen derived peptides and 20 proteins enabled the vaccinated animals to be distinguished from the challenged ones, the identification of three novel viral epitopes, the surveillance of antiviral antibody response and the prediction of survival. In a clinical study for the evaluation of BHT-3009 in relapsing-remitting multiple sclerosis the development of autoantibody titers against the target protein, myelin basic protein and related proteins was monitored by peptide microarray experiments with cerebrospinal fluids of patients [89, 90]. The peptide microarray experiments allowed a closer look at changes in autoantibody titers for myelin-related proteins. The results were in agreement with the end point measurement using magnetic resonance imaging.

5. Identification of Autoantibody Reactivities in Cancer

The use of specific autoantibody signatures for early and companion diagnostics would be an attractive new route for the management of cancer. Up to now mainly protein arrays were used in this research field (reviewed in [91, 92]). Less frequently, peptide microarrays were used for the characterization of autoantibodies in cancer. For an animal study mice were inoculated with tumors and serum samples tested on microarrays with 327 antigens, including proteins, peptides, nucleotides and phospholipids [93]. No single autoantibody could be detected which would have been unambiguously indicative for the tumor. However, selected collectives of about 10-15 antigens allowed a full separation between the healthy and tumor bearing repertoires. Individual mice inoculated with the same tumor cells develop common autoantibody reactivities. These reactivities are not limited to known tumor associated antigens but also to self antigens associated with autoimmune diseases. The results suggest that only complex autoantibody reactivity patterns for a high number of antigens can be of diagnostic use. Further collection of data is necessary for the determination of feasibility. However, peptide microarrays proved powerful tools for the deconvolution and characterization of biological relevant signals in complex biological fluids.

VII. CONCLUSION

Peptide arrays have become standard tools for the identification and characterization of linear antibody epitopes. The increasing use in screening of animal or patient samples gives rise to new applications. The multiplex assay format and the opportunity for using established equipment from the DNA microarray world make peptide microarrays attractive tools for biomarker identification and patient stratification.

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Received: April 06, 2010 Revised: July 20, 2010 Accepted: August 06, 2010