

Exploring immunological specificity using synthetic peptide combinatorial libraries

Clemencia Pinilla*, Roland Martin†, Bruno Gran‡, Jon R Appel*, Cesar Boggiano*, Darcy B Wilson* and Richard A Houghten§

The definition of epitopes for human B and T cells is fundamental for the understanding of the immune response mechanism and its role in the prevention and cause of human disease. This understanding can be applied to the design of diagnostics and synthetic vaccines. In recent years, the understanding of the specificity of B and T cells has been advanced significantly by the development and use of combinatorial libraries made up of thousands to millions of synthetic peptides. The use of this approach has had four major effects: first, the definition of high affinity ligands both for T cells and antibodies; second, the application of alternative means for identifying immunologically relevant peptides for use as potential preventive and therapeutic vaccines; third, a new appreciation of the requirements for TCR interactions with peptide-MHC complexes in immunogenicity; fourth, the establishment of new principles regarding the level of cross-reactivity in immunological recognition.

Addresses

*§Torrey Pines Institute for Molecular Studies, 3550 General Atomics Court, San Diego, CA 92121, USA

†Cellular Immunology Section, Neuroimmunology Branch, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Building 10, Room 5B-16, 10 Center DR MSC 1400, Bethesda, MD 20892, USA

‡e-mail: rmartnib@box-r.nih.gov

§e-mail: houghten@tpims.org

Correspondence: Richard A Houghten

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Abbreviation

MAb monoclonal antibody

Introduction

Synthetic peptide combinatorial libraries have been found to be valuable tools for the study of protein–protein interactions. The development of such libraries is an extension of parallel peptide-synthesis approaches presented in the 1980s [1–3]; combinatorial libraries have greatly increased the effective number of peptides that can be synthesized and screened. Instead of testing individual peptide analogs, thousands to millions of peptides can be tested in a single assay in order to define the specificity of a given interaction. While a number of different synthetic approaches have been presented over the past 10 years, all use solid-phase methods in which peptides are synthesized individually or as mixtures on insoluble resins. Peptide libraries can be screened while bound to the resin or as free peptides in solution. Identification of individual peptides responsible for a given activity can be accomplished for resin-bound libraries by peptide sequencing [4]. For mixture-based libraries that con-

tain one or more positions defined with individual amino acids, the identification of active peptides is carried out by iterative or positional-scanning approaches [5,6].

The iterative approach uses a selection and enhancement process to identify individual compounds. Each library is made up of mixtures of sequences; these have one position that is defined by the presence of a particular amino acid while other positions have amino acids mixed together. The library is screened in an assay to identify the most active mixture for each defined position. The iterative process is carried out in which the other positions are successively defined.

The positional scanning approach simultaneously addresses each position of the library. The screening of a positional scanning library determines the most active amino acids at each position of the molecule. The combinations of the selected amino acids from each position of the library are synthesized as individual compounds and their final activities are determined. The advantage of the positional scanning library format is that iterations are not required and only a single synthesis is needed to obtain an active individual compound.

The first use of combinatorial chemistry in immunology was for the identification of high affinity peptides recognized by monoclonal antibodies (MAbs) [4–7]. More recently, MHC-binding motifs and T cell specificity have been examined using peptide libraries. In this review, we will discuss the most recent publications involving antibody specificity and cross-reactivity, highlight the latest developments in the growing field that investigates T cell specificity using peptide libraries and discuss the future use of peptide libraries for the identification of new ligands which may be used for the design of novel vaccines for pathogenic and cancer-related immunotherapies.

Antibody specificity determined by combinatorial libraries

B cell epitopes are typically classified as either continuous or discontinuous. It is believed that the majority of the B cell epitopes in proteins are discontinuous (i.e. they recognize only native, folded structures); however, there are exceptions in which antibodies that recognize discontinuous epitopes can cross-react with linear peptide fragments of the protein; therefore, while peptides rarely represent full epitopes of proteins, they can be used effectively to define antibody specificity.

In the first application of synthetic peptide combinatorial libraries, Geysen *et al.* [7] presented the mimotope approach,

Table 1

Recognition of peptides by MAbs identified from mixture-based libraries.

Type of library	MAb	Known determinant	Deconvolution method	Sequence	Activity	Reference
Pentapeptide (Diversity = 10^6) L-, D-amino-acids	3E7	YGGFM	Sequencing	YGGFQ	$K_i = 15$ nM	[4]
	3E7	YGGFM	Sequencing	YGAfF YGAaF	$K_i \sim 1$ μ M $K_i \sim 10$ μ M	[43]
Hexapeptide (Diversity = 10^7)	19B10	DVPDYA	Iterative PosScan	DVPDYA DVPDYA	$IC_{50} = 2$ nM $IC_{50} = 2$ nM	[5] [6]
	125-10F3	PYPNLS	Iterative	PYPNLP	$IC_{50} = 4$ nM	[44]
			PosScan	YPYPNL PYPNLR	$IC_{50} = 4$ nM $IC_{50} = 13$ nM	[45] [6]
	222-35C8	GRTTVFS	Iterative	GRTTVF GRWTVF RGIVFP QEFHAS	$IC_{50} = 25$ nM $IC_{50} = 2$ nM $IC_{50} = 4$ nM $IC_{50} = 68$ nM	[46]
	3E7	YGGFMT	Iterative PosScan	YGGFMT YGGFMP	$IC_{50} = 3$ nM $IC_{50} = 3$ nM	[47] [48]
	134B29	Fg A α ^{566-580*}	PosScan	GESTFE	$IC_{50} = 1.5$ nM	[49]
	155B16	Fg A α ^{87-100*}	Iterative	WWYESW	$IC_{50} = 40$ nM	[49]
	M1	DYKDDK	PosScan	DYKAKE	$IC_{50} = 3$ nM	[50]
	121-15B10	IGR	Iterative	IGRRYYD	$IC_{50} = 9$ nM	[51]
	172-12A4	AFGTIYK	PosScan	AYGPIDK	$IC_{50} = 120$ nM	[52]
	Anti-MHR	MHR	Iterative	DFLEKI	ND	[53]
	Tab2	TGF- α	Amino acid clusters	VSHFND	$K_D = 80$ nM	[54]
	Graves's IgG	TSRE†	PosScan/iterative‡	EEFDDA	$IC_{50} = 10$ μ M	[11*]
	12	HBsAg	PosScan	STTSMM SVGPPH	$IC_{50} = 170$ nM $IC_{50} = 170$ nM	[8]
	D-amino-acids	HGAC 39.G3	PosScan	yryygl	$IC_{50} = 200$ nM	[13**]
Octapeptide (Diversity = 10^{10})	15	FMDV	Iterative	WQMRHS	ND	[7]
	201/9	β -factor XIIa	PosScan/iterative	SFLQEN	ND	[55]
	19	RSV§	Sequencing	HWYISKPO	$K_a = 1$ nM	[56]
D-amino-acids	WEHI-231	Unknown	Sequencing	wGeyidvk	ND	[57]
Decapeptide (Diversity = 10^{12})	17/9	DVPDYA	PosScan	DDDDDVDPDYA	$IC_{50} = 0.6$ nM	[58]
	H9724	VQQEGA	Iterative/ MALDI-MS	PQEEGA	$IC_{50} = 45$ μ M	[59]
Dodecapeptide (Diversity = 10^{15})	A16	HSV-1*	Sequencing	- - -YGD- -RF-	ND	[60]
Tetradecapeptide (Diversity = $\sim 10^{17}$)	CB4-1	p24 (HIV-1)	Iterative	GATPEDLNQKLAGN	$K_i = 1.2$ nM	[9**]

*Peptide sequence from the A α domain of fibrinogen; †extracellular domain of thyroid-stimulating-hormone receptor; ‡mixture iterations were purified by high performance liquid chromatography and individual peptides were identified by amino acid analysis and sequencing; §respiratory syncytial virus F protein; *Herpes simplex type 1 virus

glycoprotein D. FMDV, foot-and-mouth disease virus; GlcNAc, *N*-acetyl-*D*-glucosamine; HBsAg, hepatitis B virus surface antigen; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; MHR, myohemerythrin; ND, not determined; PosScan, positional scanning; TGF, transforming growth factor.

in which a MAb known to recognize a discontinuous epitope was assayed against all the possible octapeptide sequences that were covalently bound to polyethylene pins. The individual peptide was deconvoluted by an iterative approach using the most active mixtures identified. The final peptide was termed a mimotope because this sequence, unrelated to the known antigen, was considered a mimic of the epitope recognized by the antiprotein antibody; however, the ability of this mimic to inhibit antibody binding to the antigen (a foot-and-mouth disease virus subunit) or a measurement of relative binding affinity were not presented.

Two other, differing, synthetic combinatorial library methods were simultaneously presented in 1991 [4,5]. Lam *et al.* [4] presented a new type of peptide library that was synthesized using 'one-bead, one-peptide' — also referred to as the split synthesis method — which ensures that each resin bead contains a single peptide. In the first application of this method, a library containing 2,476,099 (19^5) individual pentapeptides was synthesized and screened for recognition by a MAb against β -endorphin. Individual peptide beads that bound antibody were identified with a secondary label, retrieved manually and the peptide sequence was determined by microsequencing. While the native epitope (YGGFM; single-letter code is used for amino acids) was not identified, several peptide sequences having good homology with the β -endorphin determinant were found to have relatively good affinities.

At the same time, Houghten *et al.* [5] presented a synthetic peptide combinatorial library approach that differed from the methods of Geysen *et al.* [7] and Lam *et al.* [4] in that the libraries were cleaved from the solid support and tested in solution, thus these libraries could be used in virtually all existing assay systems. This approach was validated by screening the peptide library for inhibition of MAb binding to its peptide antigen, using a competitive enzyme-linked immunosorbent assay (ELISA). Since this library contained defined and variable positions, individual peptides were identified using an iterative synthesis and screening approach. The most active sequence that was identified was found to exactly match the antigenic determinant found in earlier studies to be recognized by this MAb. A more efficient approach for the deconvolution of mixture-based libraries, known as positional scanning, was first presented in 1992 [6]; the antigenic sequences recognized by two MAbs were identified.

Other advantages of using synthetic peptide combinatorial libraries for the study of antibody specificity are that no prior specificity information is required and that one library can be used for many MAbs that have a variety of different specificities and affinities. Table 1 summarizes the published work to date using synthetic combinatorial libraries for the study of antibody specificity. Peptides that bind with high affinity have been identified for anti-peptide, anti-protein and anti-carbohydrate antibodies. When the epitope recognized by an antibody was known, at least some of

the peptides identified corresponded to this sequence. Additionally, in other instances, highly active peptide sequences have been identified having little or no resemblance the known immunogen. It has been proposed that since antibodies have flexible hypervariable loops, each antibody's binding sites are not unique for a single antigen but can be considered functionally polyspecific. Molecular mimicry is more likely to be established by a functional conformation rather than identical primary structure.

The first identification of high affinity, cross-reactive peptides recognized by an antiprotein MAb was presented by Appel *et al.* [8]. Two different hexapeptides were found from the screening of a positional scanning library composed of 50×10^6 sequences. The STTS motif of one peptide (STTSMM) and the GP motif of the other (SVGPPH) share some homology with the antigen, the surface antigen of hepatitis B virus at residues 114–120 (STTSTGP). The two peptide mimics, when synthesized as a single peptide, were recognized by the MAb with a relative affinity 50-fold better than the protein antigen. Kramer *et al.* [9**] identified five unrelated peptides that compete for binding of MAb CB4-1 (this recognises p24 of HIV-1) using a synthetic positional scanning library prepared by spot synthesis. The molecular basis of the observed polyspecificity of MAb CB4-1 was studied in competitive binding assays using a complete set of substitution analogs of the five peptides. X-ray crystallographic studies carried out on three peptides with the antibody showed that the unrelated peptides bind to the same region of the antibody [10], thus offering more evidence that structurally different binding motifs can be recognized by individual MAbs. Park *et al.* [11*] used a hexapeptide positional scanning library to identify peptide sequences that inhibit the binding of IgG antibodies (from patients with Graves' disease) that recognized the thyroid-stimulating-hormone receptor (TSHR). The peptides identified from the library also inhibited the cAMP synthesis that is induced by the IgGs in some patients. The peptides do not resemble the linear sequence of TSHR and thus may mimic a spatial arrangement of the key antigenic residues.

Whether peptides can act as structural mimics of nonprotein antigens remains unclear. It may be assumed that cross-reactive antigens share structural similarity that is recognized by the antibody. While several peptide motifs — composed mostly of aromatic residues — have been identified from phage-display libraries recognized by anticarbohydrate antibodies, these mimics often have low affinity and little specificity [12]. In a recent study, Pinilla *et al.* [13**] described the use of a positional scanning library composed of D-amino acid hexapeptides for the identification of a mimic of *N*-acetyl glucosamine that is recognized with relatively high affinity ($K_a = 40$ nM). The peptide sequence yryygl (lower-case letters indicate D-amino-acids) shares little homology with the carbohydrate antigen, suggesting that the peptide interacts differently with the MAb. From analysis with substitution analogs, it

Table 2

Recognition of peptides by MHC molecules identified from mixture-based libraries.

Library	MHC	Assay	Deconvolution method	Activity*	Reference
Octapeptide (Diversity = 1.7×10^{10})	H-2K ^b	Stabilization	PosScan	Anchor residues	[61]
	H-2K ^k , H-2K ^b , HLA-A0204	Competitive binding	PosScan	Anchor residues	[62]
	H-2L ^d	Stabilization	PosScan	Anchor residues	[63]
Nonapeptide (Diversity = 3.2×10^{11})	HLA-A2, HLA-B7	Refolding	Sequencing	Anchor residues	[64]
	HLA-A2, HLA-B8, HLA-B53	Refolding and competitive binding	Elution [†]	Anchor residues	[14*]
	RT1-A ^a , RT1-A ^u , RT1-1A ^c	Refolding	Elution [†]	Anchor residues	[15*]
Undecapeptide (Diversity = 2×10^{14})	HLA-DR1	Competitive binding	PosScan	IC ₅₀ = 1.2 μ M	[16]

*Anchor residues were identified and compared with binding motifs obtained from previously published data. [†]MHC-bound peptides were subjected to acid elution and pool sequencing. PosScan, positional scanning.

was shown that the tyrosine residue at position 3 is highly specific and may mimic one of the hydroxyl groups of the carbohydrate. Additionally, the highly specific arginine of the peptide mimic may have been involved in a salt bridge with a negatively charged aspartic acid found in the CDR2 region of the variable domain of the MAb heavy chain.

MHC specificity determined by synthetic combinatorial libraries

MHC molecules are capable of binding to endogenously synthesized and exogenous peptides in cellular compartments and transporting them to the plasma membrane. This MHC-peptide complex may be the ligand for a specific TCR expressed on the surface of a T lymphocyte. MHC class I molecules are composed of two subunits (the heavy chain and β_2 -microglobulin). The peptide-binding site is formed by the heavy chain, allowing peptides of 8–10 amino acids in length. The groove is closed and this largely prohibits longer peptides from binding. On the other hand, MHC class II molecules bind peptides which are 10–25 amino acids in length. Knowledge about the rules of peptide-MHC binding is crucial for the development of new vaccines and the study of T-cell-mediated immunopathology.

Table 2 summarizes the published studies in which combinatorial libraries have been used to identify the properties of the peptide-MHC interaction. It can be seen that different MHC molecules and different methodologies have been used in these studies. Davenport *et al.* [14*] used a pool of randomly synthesized nonapeptides to address the peptidic residues necessary to bind HLA-A2, -B8 or -B53 heavy chains and β_2 -microglobulin. Refolding and stabilization of these class I complexes and purification by gel filtration, followed by elution and sequencing of the

peptides as a pool, allowed the identification the anchor residues of these three different class I molecules. Upon comparing the synthetic with the naturally bound peptides (isolated by elution and sequencing in early studies), some differences were found — probably due to transportation and processing. Similar discrepancies between synthetic and natural peptides were observed in other experiments done on rat class I [15*].

The elucidation of class II (human DRB1*0101 molecules; HLA-DR1) specificity has been presented by Fleckenstein *et al.* [16] using an undecapeptide positional scanning combinatorial peptide library. The deconvolution of the library screening permitted the identification of optimal peptide ligands for HLA-DR1. The main advantage of specificity studies on class II that are conducted with synthetic combinatorial libraries is the uniformity in the length and concentration of the peptides. The natural peptides, with different lengths that overhang on the groove of the class II, make the identification of contact residues difficult.

Using peptide combinatorial libraries to identify T cell epitopes

T lymphocytes orchestrate both the cellular and — through ‘help’ in the activation of B lymphocytes — the humoral arms of our specific immune system. The various subpopulations, such as CD8⁺ cytolytic T cells and CD4⁺ helper T cells, act as effectors during viral infections or inducers of T and B cell functions, respectively; thus they are involved in both physiological and pathological immune responses.

Understanding the rules that govern antigen recognition by T cells has been a major challenge for immunologists

Table 3**Recognition of peptides by T cells identified from mixture-based libraries.**

Library	T cell clone	MHC restriction	Deconvolution method	T cell response	Activity	Reference
Octapeptide (Diversity = $\sim 10^{10}$)	2C	H-2K ^b	PosScan	Cytotoxicity	IC ₅₀ = 10 pM	[30]
	269/3	H-2K ^b	PosScan	Cytotoxicity	IC ₅₀ = 12 pM	[31]
	H3-16, H3-30 H3-35	H-2K ^b	PosScan	Cytotoxicity	IC ₅₀ = 1 pM	[65]
Nonapeptide (Diversity = $\sim 10^8$)	EL4	H-2K ^b	Iterative	Cytotoxicity	IC ₅₀ = 50 pM	[32]
Nonapeptide (Diversity = 34,560)	10F4	HLA-A2	Dedicated PosScan	Cytotoxicity	IC ₅₀ = 2.5 nM	[33**]
Decapeptide (Diversity = $\sim 10^{12}$)	GDBP	DRB1*1302	PosScan	Proliferation	Contact residues*	[36*]
Undecapeptide (Diversity = $\sim 10^{14}$)	TL5G7	DR2b	PosScan	Proliferation	IC ₅₀ = 1 nM	[35**]
Tridecapeptide [†] (Diversity = $\sim 10^6$)	2.102	I-E ^k	Iterative	IL-2 production	0.3 ng/ml	[39*]
Tetradecapeptide [‡] (Diversity = $\sim 10^6$)	1c10	DR1	Sequencing	Proliferation	5 nM	[37*]
	MT1	DR3	Sequencing	Proliferation	1000 nM	[38*]
	MT2	DR3	Sequencing	Proliferation	50 nM	
	HG	DR3	Sequencing	Proliferation	10 nM	

*Contact residues were identified and compared with previously published data. †Only five positions were prepared as mixtures. ‡ Three positions contained less than the 19 amino acids tested in other positions. PosScan, positional scanning.

during the past two decades. It is only recently that structural studies of the bimolecular interactions between peptides and MHC molecules were elucidated [17] and, even more recently, that the first crystal structures of the trimolecular complexes of TCRs, antigenic peptides and MHC molecules have been obtained [18–20]; furthermore, there have been elaborate studies with antigenic peptides that have been systematically modified at amino acid positions that serve as contact residues with the TCR; these studies not only revealed a previously unexpected flexibility in T cell recognition but also demonstrated that such altered peptide ligands are capable of eliciting a variety of different functional responses — these range from TCR antagonism to partial agonism or full agonism [21,22]. Despite this progress, many questions remain unresolved. Systematic studies of T cell recognition still need to be conducted, first, to identify the best candidates for vaccines, second, to study the target antigens in various cancers as well as in infectious and autoimmune diseases and, third, for therapeutic purposes — to dampen immune responses in autoimmunity or to enhance it for specific tumor therapies.

T cell recognition was previously considered exquisitely specific for single peptides or close homologues with similar amino acid sequences. As mentioned before, it is now clear

that this stringent view is not correct [21]. Major constraints for recognition are imposed by the requirement of a peptide to bind to the presenting MHC molecule and thus form a bimolecular complex that serves as a ligand for interacting with TCR molecules. This MHC–peptide complex is achieved by the presence of anchor amino acids which mediate contact to the specificity pockets of the MHC class I or class II molecule in question [23]. On the other hand, recognition of this ligand by the TCR requires not only contacts with polymorphic and nonpolymorphic residues of the α -helical domains of the MHC molecule which flank the peptide-binding groove [17] but also specific amino acids that interact with the TCR and are referred to as primary and secondary TCR contacts [21,24,25]. While these requirements are now accepted as general rules for interactions between the TCR and the MHC–peptide, it has also been shown recently that the need for MHC and TCR anchor/contact amino acids is relative in that it can be compensated for by amino acids in other positions of the peptide [26]. In extreme cases, peptides which share no single amino acid with a known ligand may nevertheless be able to stimulate a T cell clone [26]. For numerous reasons it has been difficult to dissect the various aspects that contribute to the flexibility of T cell recognition, let alone the simpler problem of defining a single peptide ligand for MHC presentation to a single T cell clone. A major problem

(among others) is the low affinity of the interaction between the TCR and the MHC-peptide, which is in the lower micromolar range [27].

Thus far, several approaches have been used with variable success for studies of TCR-MHC-peptide interactions [28]: these include, first, epitope mapping conducted with a series of overlapping peptides in cases where the antigenic protein is known; second, biochemical analysis of pools of peptides which have been eluted from MHC molecules — derived, for example, from tumor cells that express a potential target peptide — using the high performance liquid chromatography (HPLC) separation technique and microsequencing of individual peptide fractions that are recognized by an indicator T cell; third, molecular biology approaches using expression libraries from an organism or tissue of interest; and fourth, the use of MHC-binding motifs to screen a protein of interest for potential areas that might be available for T cell recognition, combined with the subsequent synthesis of peptides and assessment of their activity in T cell stimulation assays. All these approaches present inherent technical biases, require an infrastructure that is available only in dedicated laboratories, are laborious to conduct and thus are very costly. The development of soluble and bead-bound peptide combinatorial libraries offers significant advantages over these methodologies, although combinatorial libraries are not routinely used in each of the above mentioned applications. We will briefly describe the combinatorial libraries and screening strategies that have been shown so far to determine T cell epitopes (Table 3).

Class-I-restricted T cell clones first received significantly more attention not only because they often show higher antigen affinities but also because the class I binding groove accommodates shorter peptides (8–10 amino acids) [29]. Thus, relatively less complex peptide libraries can be used to determine class-I-restricted specificities. An octapeptide positional scanning library has been used to identify an antigenic peptide recognized by an allospecific clone in the context of self MHC [30] and a peptide-specific T cell clone [31]. Others have reduced the complexity by eliminating certain amino acids in the mixture positions to avoid solubility problems and to increase the relative concentration of each compound [32]. These strategies have been modified further by fixing those amino acids in the libraries which had been identified as MHC anchor amino acids by pool sequencing of eluted peptides or of single, defined, self peptides [32,33**]. These libraries are referred to as MHC-biased, or dedicated, peptide libraries.

In one version of this strategy, Münz *et al.* [33**] synthesized mixtures composed of peptides having only those amino acids in every position of the peptide (9-mers) which had been identified previously from sequencing data of peptide pools eluted from HLA-A2 and -A3. These low complexity libraries (25,000–35,000 compounds as opposed to billions in a positional scanning library) were successfully used to pulse allogeneic class I alleles (HLA-A2 and -A3, respectively) and

generate alloreactive T cell clones in order to understand whether their specificity is predominantly directed to MHC determinants, the antigenic peptide, or both. Subsequent deconvolution of the peptide specificity was achieved by synthesizing a biased positional scanning library that was composed of sublibraries with single, defined amino acids in each position; these defined amino acids were derived from the set that was originally chosen to create the library.

In a study of tumor-specific peptide ligands defined from library scans of tumor-specific T cell clones, Blake *et al.* [32] demonstrated that such synthetic ligands can be employed effectively to inhibit tumor growth by direct immunization with the peptide itself, or following adoptive transfer of peptide-specific T cell clones. In summary the various peptide library approaches with class-I-restricted, antigen-specific or allogeneic T cell clones proved to be highly useful in determining single peptides that are recognized with high affinity or in determining sets of peptides recognized with various degrees of lower affinity.

Addressing the same questions in CD4⁺ class-II-restricted T cell clones appeared more difficult, mainly for two reasons: first, the affinity of class-II-restricted T cell clones is usually lower than described for class-I-restricted cytotoxic T cell clones [22,27]; second, class-II-associated peptides are longer (typically 12–15 amino acids) [34]. Thus, the use of peptide combinatorial libraries faces the problem that longer libraries are considerably more complex, resulting in lower concentrations of individual peptides in the mixture (complexity increases by 20-fold with each additional position) and at the same time the responding T cell may recognize peptides with lower affinity; nevertheless, three groups succeeded in adapting the use of peptide combinatorial libraries to class-II-restricted systems, although by considerably different strategies [26,35**,36*–39*].

Hiemstra *et al.* [37*] employed MHC-biased, bead-bound, 14-mer libraries (one-bead, one-peptide) to determine the peptide specificity of class-II-restricted T cell clones in a sequential identification process in which peptides were released from decreasing amounts of beads until sequencing from a single bead revealed the target sequence. The facts that the library represented only a fraction of the total possible sequences (8×10^6 versus more than 19^{12} theoretical possibilities) and that an MHC bias was introduced (towards HLA-DR1 or -DR3) permitted a relatively high concentration (5 nM) of each individual peptide for MHC binding. Combining this approach with truncation variants and with alanine-scans of the identified peptides permitted a determination of the minimal epitope followed by database searches for synthetic peptide mimics. In a recent study, the same group refined this approach further and included iterative steps with omission mixtures and substitutions of single amino acids [38*]. By combining these steps, they were able to identify the natural T cell antigen among a series of potential mimics; however, one should keep in mind that the antigen specificity for each of these clones

was known. Another group, Hemmer *et al.* [35^{••},36[•]] employed completely randomized 11- and 10-mer synthetic combinatorial libraries arranged in a positional scanning format to determine potential mimics for autoreactive T cell clones established from multiple sclerosis patients. For one such clone they demonstrated three features: first, responses to completely randomized libraries of 10 positions or longer — indicating that T cell recognition must be highly degenerate, since under the conditions tested the concentration of each peptide within the library is less than 1×10^{-17} M; second, the autoantigenic peptide was not the optimal ligand, since numerous synthetic peptide mimics that were 5–6 orders of magnitude more active in stimulating this clone could be identified; third, this approach allows a very extensive identification of molecular mimics of foreign and self antigens. Although the latter strategy is the least biased and offers the greatest potential for a thorough deconvolution of TCR specificity, it is currently applicable only to a small number of clones — most probably due to the fact that the complexity of the peptide mixture is too high and the concentration of individual peptides is too low.

Although still in its infancy, the application of peptide combinatorial libraries has tremendous potential for the study of T cell biology. This includes the identification of immunodominant epitopes in infectious or autoimmune diseases, the determination of tumor antigens and — eventually — the targeted modifications of such antigens to develop immunotherapies for infections, autoimmune diseases and malignant tumors. Many questions still need to be answered, particularly how to avoid biasing the libraries too much when reducing their complexity while at the same time allowing the screening of as many specificities as possible. Based on the flexibility of T cell recognition [21,40[•],41] it will be interesting to see whether such optimized approaches can be developed in the near future.

TCR degeneracy

One surprising finding to emerge from the use of large synthetic combinatorial libraries to identify candidate sequences that stimulate a given T cell clone is that the extent of T cell degeneracy (the number of different peptide ligands that can associate with the appropriate MHC molecule and stimulate a single CD4⁺ T cell clone) is considerably greater than has generally been appreciated.

T cell recognition of peptide–MHC molecules has been considered in the past to be exquisitely specific, the potential to respond to a vast number of different antigenic peptides being achieved by the diversity of genes for the TCR chains and the process whereby these are rearranged during the assembly of mature TCR $\alpha\beta$ heterodimers to generate an extensive repertoire of T cell specificities [42]. To a large extent this notion, combined with the process of negative selection during T cell ontogeny in the thymus, appeared to be a solution to the problem of avoiding cross-reactive immune responses that might also result in autoimmunity.

One problem with this scenario, however, is that the numbers of possible, different peptide epitopes of T cells are several orders of magnitude greater than the numbers of T cells comprising the lymphoid system of an individual. This leads to the notion that the expressed T cell repertoire of an individual can respond to only a small fraction of peptides from potentially pathogenic organisms. The number of different, possible, class I associated, nonamer, L-amino-acid peptides available for recognition by CD8⁺ T cells is large ($20^9 = 5 \times 10^{11}$) and, because of their greater length, the number of possible peptide sequences, for example 12- to 16-mers, available for presentation to CD4⁺ T cells by MHC class II molecules is significantly larger (20^{12} to $20^{16} = 4 \times 10^{15}$ to 6.5×10^{20}); however, the total number of T cells in a mouse is of the order 10^8 (for humans it is 10^{11}) and the number of different clonotypic specificities in these T cell repertoires is less. Because of this discrepancy between (on the one hand) the number of different and potentially antigenic peptides and (on the other) the number of different T cell clonotypes available for immune reactivity, it seems clear that Mason's argument in a recent article [41] provides a solution to this paradox: namely, T cell specificity must be highly degenerate to allow for adequate protective immune responses to any T cell epitope.

We have recently demonstrated ([26,35^{••},36[•],40[•]]; DB Wilson *et al.*, unpublished data) that a synthetic decapeptide or undecapeptide combinatorial library used at a concentration of 100 μ M is capable of stimulating activation and function of selected T cell clones in microwell cultures. This decamer library consists of all possible combinations of L-amino-acid peptide ligands in approximately equimolar concentration, systematically arranged in a positional scanning format with 200 mixtures. Suppose, for example, that a given decapeptide mixture having lysine defined in the fifth position (K₅X₉) stimulates a significant proliferative response by a given T cell clone in culture that is four-fold above background stimulation levels. This mixture has 3.2×10^{11} (19^9) different decamer peptides and, at a library concentration of 100 μ M, each different peptide is present at a concentration of 3×10^{-10} μ M. Typically, a CD4⁺ T cell clone requires its native peptide ligand at a concentration of 10^{-2} μ M for detectable stimulation, a concentration 30×10^6 -fold greater than that of a single peptide in the library mixture. Thus, finding that the K₅X₉ mixture causes a detectable clonal response implies that as many as 30×10^6 different peptides present in the library mixture contributed to this process.

The finding that T cell specificity may be so promiscuous raises the paradoxical problem of why cross-reactive autoimmune responses are not as prevalent as might be expected for such an extent of TCR degeneracy. For activated CD4⁺ T cells that recognize complexes of peptide and MHC class II, part of the answer may be that expression of class II molecules is limited to relatively few cells of an individual, a circumstance that would diminish opportunistic autoimmune cross-reactivity. For CD8⁺ T cells, on the other hand,

MHC class I molecules are expressed almost ubiquitously and the possibility of autoimmune cross-reactivity would seem to be significant. Studies currently in progress suggest that TCR degeneracy of the CD8⁺ subset may be several orders of magnitude less than for CD4⁺ T cells, thus providing one possible solution to this apparent paradox.

Conclusions

The identification of high affinity ligands for antibodies and T cells using synthetic peptide combinatorial libraries suggests that this approach is a powerful tool for the analysis of immunological specificity. Current studies are using these libraries to identify unknown natural ligands of disease-associated antibodies, MHC molecules and TCRs; these are important first steps in the design of therapeutic vaccines for the treatment of metastatic and infectious diseases. Individual peptides derived from the screening of libraries represent an extensive set of related peptides that permit basic analysis of the molecular interactions. The identification of these new antigens will not only further the understanding of the disease process but should also lead to the design of new diagnostics, therapeutics and prophylactic strategies. Future studies using synthetic peptide combinatorial libraries combined with newly optimized strategies will lead to a greater understanding of immunological specificity.

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