Mimotopes of Tumor-Associated T-Cell Epitopes for Cancer Vaccines Determined with Combinatorial Peptide Libraries

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Abstract

Cytotoxic T-cells are the most important effector cells in immune responses against tumors. The identification of tumor-associated epitopes for these cells, therefore, has become a key aspect of the development of cancer vaccines. Here, we describe a new approach to the determination of tumor-associated T-cell epitopes which employs combinatorial peptide libraries with singly defined sequence positions in a randomized context. The analysis of the responses of a T-cell clone to these libraries yields the amino acid constituents of the epitope which can be combined to obtain mimotopes that are suitable as vaccine antigens for the induction of tumor-specific responses.

Index Entries: Brefeldin-A; combinatorial peptide library; SEREX, serological analysis of tumor antigens by recombinant cDNA expression cloning; TAA, tumor-associated antigen; TATE, tumor-associated T-cell epitope.

1. Introduction

The identification of tumor-associated antigens (TAA) is of major importance for the development of tumor-antigen-specific therapeutic and prophylactic vaccines, and is a prerequisite for the immune monitoring of tumor-specific immune responses, including the immunologic effects of therapeutic interventions. Knowledge about TAA provides insights into the physiologic state of tumor cells and information about the interrelationship of tumors and the immune system, and thereby adds to the understanding of cancer. Cytotoxic T lymphocytes (CTL) play a central role in the immune surveillance for malignant disease. They can utilize different mechanisms to destroy tumor cells, including perforin- and granzyme-mediated cytolysis. The cytolytic responses of CTL are triggered by the recognition of peptides—T-cell epitopes—derived from antigens by limited proteolysis and bound to and presented at the surfaces of cells by molecules of the major histocompatibility complex (MHC). In recent years a number of tumor-associated T-cell epitopes (TATE) have been identified *(1)*. Different strategies have been developed for the detection and study of TATE, including: (l) direct isolation, by high-pressure liquid (HPLC), of MHC-bound peptides that stimulate TAA-specific T-cells and determination of their amino acid sequences by mass spectrometry *(2)*; (2) T-cell-guided epitope mapping of known or suspected TAA with overlapping peptides or peptides predicted to bind to the associated MHC molecules *(3)*; (3) identification of the genes encoding TAA by screening, with T-cells, cDNA expression libraries transfected into appropriate target-cells *(4)* and performing subsequent epitope mapping; and (4) identification of potential antigens by using serological techniques such as serological analysis of tumor antigens by recombinant cDNA expression

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cloning (SEREX), followed again by epitope mapping *(3)*. Albeit successful in a number of cases, these strategies are hampered by several disadvantages. All of these approaches require tumor material as well as tumor-specific T-cell clones or at least T-cell lines; moreover, tumor material is often unavailable in amounts sufficient for the direct identification of T-cell epitopes, and the establishment of stable long-term human T-cell clones is still a major challenge. Additionally, all of these approaches are very time consuming and require tumor-specific T-cells throughout. Here we describe a new approach to the determination of TATE that utilizes combinatorial peptide libraries and yields potent T-cell epitopes with just two consecutive T-cell assays *(5)*. Tumor material is required only in small amounts to establish the tumor specificity of the T-cell clones employed.

1.1. Strategy

Epitopes for cytotoxic T-cells are in most cases nonapeptides that conform to MHC allele-specific sequence motifs *(6)*, (i.e., they bear amino acids of restricted variability at sequence positions that anchor the peptide into the peptide-binding grooves of MHC molecules). These motif amino acids vary with the MHC allomorph. Combinatorial peptide libraries can be designed around these features to consist of mixtures of peptides in which either all sequence positions are randomized or one sequence position is defined and the remaining positions are randomized *(7–9)*. A complete set of nonapeptide libraries for mapping the specific sequence requirements for a T-cell epitope contains an X_9 library and 171 OX_8 libraries for 19 proteinogenic amino acids and 9 sequence positions, or, theoretically, 19^9 (i.e. about 3.23×10^{11}) different peptides (**Fig. 1**). Cysteine is usually omitted from such libraries to avoid problems with oxidation and crosslinking of peptides. The combinatorial peptide chemistry strategy for determining a T-cell epitope consists of the following four steps (**Fig. 2**).

 l. Identifying the critical amino acids for the induction of a specific T-cell response for every sequence position, by testing the response of a

T-cell clone to the combinatorial peptide libraries. This scan yields the potential epitope amino acids.
 2. Designing and synthesizing potential epitopes that include these active amino acids as well as the MHC allele-specific motif residues.
 3. Identifying the active epitopes in a second T-cell assay.
 4. Validating the mimotope as a T-cell epitope through the use of independent T-cells.

2. Materials

2.1. Cells

Mimotopes can be determined for all T-cell types. Since the recognition of antigens by T-cells is degenerate, it is important to use T-cell clones rather than T-cell lines. The peptide libraries used for identifying a T-cell epitope are presented to the T-cell clones by antigen-presenting cells (APC), which are used as target or stimulator cells in the T-cell assays. B-lymphoma cell lines generated by the Epstein-Barr-virus (EBV)-induced transformation of B lymphocytes from peripheral blood are suitable targets. Because allogenic MHC molecules can induce strong T-cell responses by themselves, it is preferable to use B-lymphoma cell lines established from the same donor that provided the T-cells. The tumor-specificity of the T-cell clones needs to be established beforehand.

2.2. MHC Haplotyping

The MHC restriction for the antigen recognition by T-cells must be considered when T-cells and target cells are chosen. Knowledge of the MHC allele that controls the TATE recognition of the test T-cell clone is also required for the selection of suitable anchor residues for the mimotopes. It is recommended that the MHC haplotypes of the patients for whom a vaccine is being designed be determined at least by serological techniques. In some cases molecular genetic subtyping might be necessary to discriminate among specific MHC types. The MHC allele-specific sequence motifs for the anchor amino acids of T-cell epitopes that have been identified so far are listed in the SYFPEITHI database at http://www.syfpeithi.de.

sequence position

	P1	P2	·	·	·	Pn-1	Pn
P1	O	X				X	X
P2	X	O				X	X
.							
.							
.							
Pn-1	X	X				O	X
Pn	X	X				X	O

(left axis label: **scan position**)

X: randomised sequence positions containing mixtures of the amino acids A, D, E, F, G, H, I, K, L, M, N, P, Q, R, S, T, V, W, Y

O: defined sequence positions with one of the above amino acids

Fig. 1. Combinatorial peptide libraries.

2.3. Combinatorial Peptide Libraries

The synthesis of the combinatorial peptide libraries used in tumor-vaccine development has been described in **ref. 10**. The quality of the peptide libraries is crucial for the success of the combinatorial peptide-library approach. A nearly equimolar representation of the individual peptides is theoretically desired but is practically impossible. It is important that the representation of the amino acids in the randomized positions is indeed random, that the defined sequence positions are occupied only by the one defined amino acid, and that the terminal main-chain charges are free for MHC class I-restricted epitopes.

The peptides and peptide libraries should be dissolved in ultrapure dimethyl sulfoxide (DMSO) at a concentration of 20 mg/mL. For long-term storage they should be kept at –80°C. For the T-cell assays the libraries should be diluted in cell-culture medium containing 0.1 % bovine serum albumin (BSA). The concentration of DMSO in the assays should not exceed 1 %, in order to avoid artificial results of the bioassays. Because DMSO can be oxidized, it should be used under nitrogen and kept carefully sealed. For long-term storage the DMSO should be kept under nitrogen at 4°C.

2.4. Buffers, Media, and Stock Solutions

2.4.1. Buffers and Media for the Peptide Library Scan by Cell-Mediated Cytotoxicity Assays

1. ^{51}Chromium (^{51}Cr), 1 Ci/mL in saline.
2. Dulbecco's modified Eagle's medium (DMEM)/ BSA: DMEM supplemented with 2 mM glutamine; 30 µM 2-ME; 100 U/mL penicillin; 100 µg/mL streptomycin with 0.1 % BSA (type V).
3. DMEM/fetal calf serum (FCS): DMEM with supplements as above and 20% FCS.
4. NP40 (1% in 10 % acetic acid).
5. Concavalin A (20 µg/mL in DMEM/BSA).

2.4.2. Buffers and Media for the Intracellular Cytokine Assays

1. Ficoll-Plaque (Pharmacia, Freiburg, Germany).
2. DMEM/BSA (*see* **Subheading 2.4.1.**).
3. DMEM/FCS (*see* **Subheading 2.4.1.**).
4. Brefeldin A (BFA) (20 µg/mL in DMEM).
5. Phosphate-buffered saline (PBS) (Dulbecco's) without Ca^{2+} or Mg^{2+} with EDTA (1 mM).
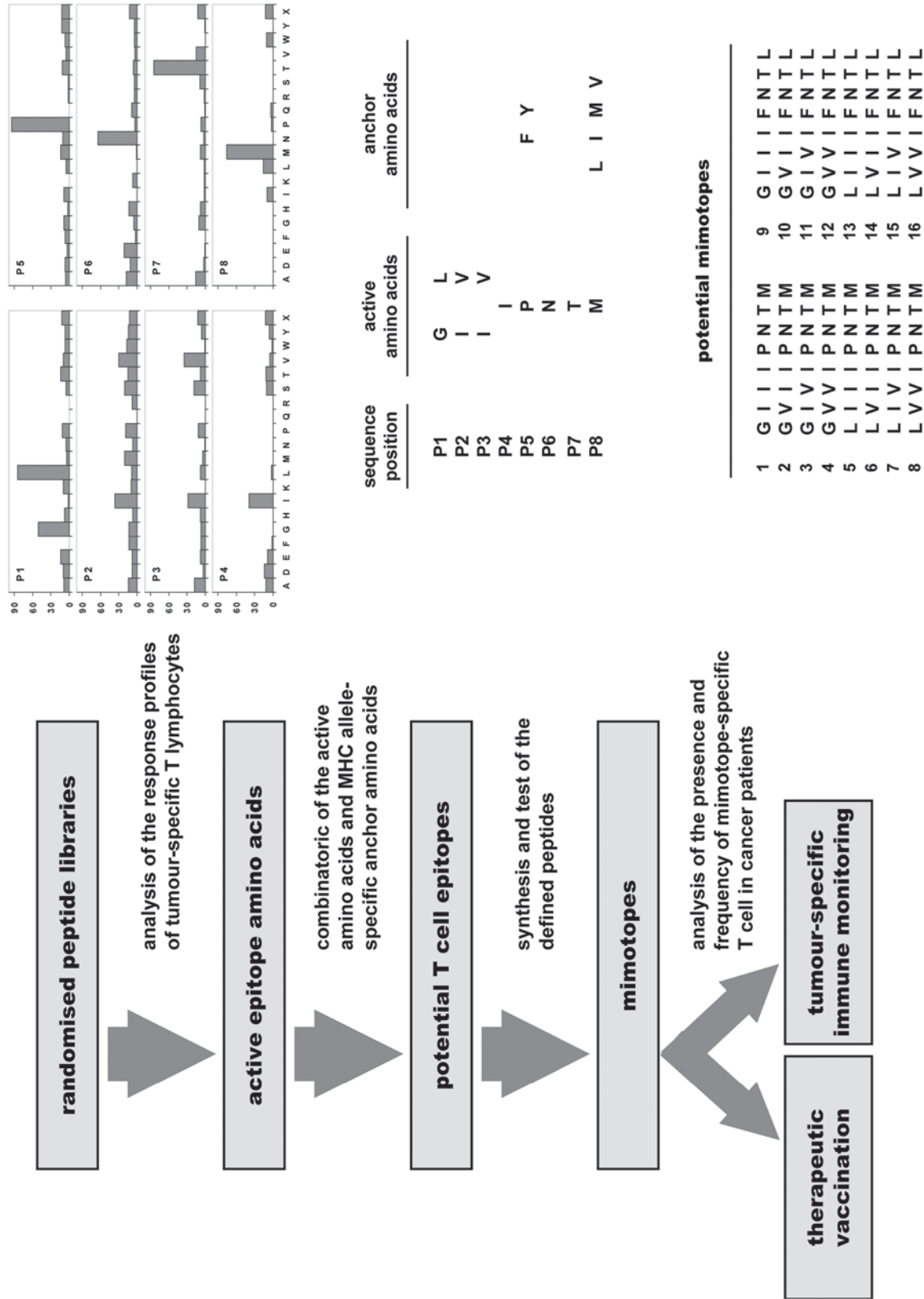6. Paraformaldehyde (4% in water).

Fig. 2. The combinatorial peptide library approach to the identification of epitopes for tumor-specific T-cells, illustrated with the example of a mouse cytotoxic T-cell clone (*15*).

7. FACS permeabilization Solution (Becton Dickinson, Heidelberg, Germany).
8. FACS lysing solution (Becton Dickinson).
9. Anti-interferon-γ (IFN-γ) clone 25723.11, fluorescein isothiocyanate (FITC) labeled (Becton Dickinson).
10. Anti-CD69 antibody clone L78, PE labeled (Becton Dickinson).
11. Anti-CD8 antibody clone DK25, PE-Cy5 labeled (Dako, Hamburg, Germany).
12. Anti-CD3 antibody clone SK7, allophycocyanin labeled (Becton Dickinson).
13. Phorbol myristyl acetate (PMA), 500 ng/mL.
14. Ionomycin, 500 ng/mL.
15. PBS/BSA. PBS as above, with 0.1 % BSA and 0.01% NaN_3.

3. Methods

3.1. Screening of Combinatorial Peptide Libraries with T-Cell Clones

Identification of the amino acids critical for T-cell recognition is done with standard T-cell assays, using T-cell clones. Any type of T-cell response, whether cytotoxicity, proliferation, cytokine secretion, or others, is suitable for screening (*see* **Subheading 4.**). All assays should be done at least in duplicate in microtiter plates. The following description is given for ^{51}Cr release cytotoxicity assays, which are the most sensitive T-cell assays that can be used for the scanning of peptide libraries.

3.1.1. Preparation of the Peptide Libraries

1. Dilute the sublibraries in cell culture medium containing 0.1% BSA to a final concentration of 100 μg/mL.
2. Pipet 50 μL of these peptide library solutions into the microtiter plate wells.
3. Use as controls the completely randomized X_9 peptide library, a complete absence of peptides as negative controls, and a nonspecific T-cell stimulator, such as concavalin A at a concentration of 20 μg/mL as a positive control. The controls should be used at least in triplicate. It is recommended that six replicates be prepared for the controls.

4. Store the dissolved peptides at –20°C until the assay is performed.

3.1.2. Preparation of the Target Cells

1. Label the target or stimulator cells with ^{51}C (100 μCi per 100 μL cell suspension) in cell-culture medium containing 10 % FCS for 1 h at 37°C and an incubate with 5–8% CO_2 in a humidified atmosphere.
2. Wash the cells 3–5 times with the culture medium containing 0.1% BSA, and resuspend them in the same medium at a cell density of 1 × 10^5 cells per mL.

2.1.3. Cytotoxicity Assay

1. Dispense 50 μL of this cell suspension into the microtiter plate wells with the peptide solutions to yield 5000 cells per well and 50 μg peptide/mL.
2. Incubate the cells with the peptides for 20–30 min at room temperature in a damp box.
3. Suspend the T-cells at a density of 5 × 10^5 cells/ mL in cell culture medium containing 20% FCS.
4. Dispense 100 μL of this cell suspension to the peptide-pulsed target or stimulator cells to obtain a final FCS concentration of 10% and 50,000 T-cells per microtiter plate well. This gives an effector-to-target-cell ratio of 10:1.
5. Include three wells each with no T-cells as a control for the spontaneous ^{51}Cr release, with the detergent NP40 at a concentration of 1% in a 10% solution of acetic acid in water to determine the total ^{51}Cr release.
6. Incubate for 5 h at 37°C in an incubator with 5–8% CO_2 and a humidified atmosphere.
7. Collect 100 μL of the supernatant from each well and measure the radioactivity.
8. Calculate the percent specific ^{51}C release as:

(cpm ^{51}Cr release with the peptide library or controls – cpm spontaneous ^{51}Cr release)/ (cpm total ^{51}Cr release – cpm spontaneous ^{51}Cr release) × 100.

9. Plot the percent specific ^{51}Cr release for all the 19 peptide libraries for every sequence position plus the percent specific ^{51}Cr release obtained both with the X_9 peptide library and without peptides.

3.1.4. Identification of the Active Epitope Amino Acids

Peptide libraries that induce greater cytolysis than the X_9 library plus twice the standard deviation for these controls can be expected to define amino acids that contribute to the activation of the T-cells used in the assay. These amino acids are considered in the design of the potential epitopes. Peptide libraries that yield cytolysis values below those of the background controls lacking peptides may define potential antagonists.

3.2. Epitope Design

1. To derive the sequences of potential mimotopes, the active amino acids as determined in the peptide library scan are combined including or excluding the canonical MHC allele-specific epitope motifs *(6)* for the MHC molecules expressed by the patient from whom the T-cells in the selected clone were obtained.
2. The resulting set of peptides, with defined sequences, is synthesized.
3. In the case that different MHC molecules expressed by the patient's cells can be relevant for the specific T-cell response, and the specific MHC restriction is not known, the respective anchor amino acids for these different MHC molecules should be included in the design of potential mimotopes.

3.3. Identification of the Epitope

In a second T-cell assay, the specifically active mimotopes can be identified from the set of putative epitopes designed by the combination of the amino acids identified by the peptide library scans and the amino acids in the MHC allele-specific motif.

The peptide concentration in this assay should be titrated to fall within the range of 1 pg/mL and 100 ng/mL. Excessive peptide concentrations may cause false-negative results. The same assay should be used to identify the mimotopes as was used for the initial screening. The amino acid sequences of the active mimotope may also reveal the HLA restriction of the T-cell clone being tested.

3.4. Database Analysis

Genome and protein database searches with the sequence information obtained from the peptide library scans may reveal information about the natural counterpart of the mimotope, the TATE, and thus give access to the natural source protein or TAA. However, the sequence of the synthetic analogue of a TATE may also deviate substantially from the sequence of the natural epitope, with the result that the database searches are often futile. Since the natural T-cell epitope is not always the most efficient inducer of T-cell responses, the database searches should be done with all of the different amino acids identified with the peptide library scan.

3.5. Validation of the Mimotope

Independent validation of the mimotope is required, and can be done with peripheral blood mononuclear cells (PBMC) from the patient from whom the T-cell clone was isolated, and/or with T-cells from independent but HLA-matched patients with the same disease as the patient. Several assays can be used for these validation experiments, but, they should be chosen to allow the detection of mimotope-specific T-cell responses in a complex T-cell population, without the need for previously established T-cell clones. As an example of such assays, we describe a flow cytometric assay to detect T-cells that, upon incubation with a specific peptide, produce cytokines (e.g. IFN-γ). For this assay PBMC are incubated with the mimotope, and the T-cells that respond specifically to the mimotypes are enumerated by flow cytometry after staining of intracellular cytokines with fluorochrome-labeled monoclonal antibodies *(11)*.

The steps in the procedure are as follows:

1. Isolate at least 1×10^7 PBMC from the peripheral blood of the tumor patients by Ficoll density centrifugation.
2. Resuspend the cells in cell-culture medium/ 0.1% BSA at a density of 1×10^7 cells/mL.
3. Dispense the cells into 5-mL-fluorescence activated cell sorting (FACS) vials, each with 200

μL of cell suspension. At least five tests, including the controls, are required as follows: a control without immunostaining, a stained control but without mimotope stimulation, an isotype control, a PMA-plus-ionomycin control as a positive control, and the mimotope test sample.

4. Add the peptide (final concentration: 10 μg/mL) or PMA (final concentration: 500 ng/mL) plus ionomycin (final concentration: 500 ng/mL) to the cell suspensions.
5. Incubate for 30 min at RT.
6. Incubate for 30 min at 37°C in a cell-culture incubator with 5–8 % CO_2 and a humidified atmosphere at a 5° slant.
7. Add 200 μL culture medium containing 20% FCS and 20 μg/mL BFA to each sample (final concentrations: 10% FCS, 10 μg/mL BFA).
8. Incubate for 5 h in a cell-culture incubator with 5–8 % CO_2 and a humidified atmosphere at a 5° slant.
9. Centrifuge for 5 min at 500g at RT to pellet the cells.
10. Resuspend the cells in 0.5 mL PBS without Ca^{2+} or Mg^{2+} and with 1 mM EDTA and incubate for 10 more minutes.
11. Resuspend each sample carefully with a pipette to detach adherent cells.
12. Centrifuge for 5–10 min at 500g at RT.
13. Fix the cells by incubation for 5–10 min with FAC Lysing Solution (Becton Dickinson).
14. Centrifuge for 5 min with 500g at RT.
15. Permeabilize the cells by incubating for 10 min with FAC Permeabilization Solution (Becton Dickinson).
16. Centrifuge for 5 min at 500g at RT.
17. Stain the cells with fluorochrome-labeled antibodies (αCD3, αCD8, αCD69, and αIFN-γ) at a concentration of 0.1–1 μg/mL antibody for 30 min at RT in the dark.
18. Wash and resuspend the cells in PBS with 0.1% BSA and 0.01% NaN_3.
19. Analyze up to 500,000 cells per sample by flow cytometry for IFN-γ-producing cells expressing CD3, CD8, and CD69. Determine the frequency of these cells in comparison to the controls. The greater the fraction of patients who respond to the mimotope assay, the more likely the mimotype represents a shared TAA and TATE.

Frequencies below 0.03% for a mimotype are difficult to reproduce. Significant responses are expected to occur at values above this frequency. The sensitivity of this assay, is therefore 1 in 3000 mimotope-specific cells in the T-cell population tested.

4. Notes

4.1. Combinatorial Peptide Libraries

The design of the necessary combinatorial peptide libraries is crucial for the identification of synthetic epitopes. Usually the 19 proteinogenic amino acids except for cysteine are used for the construction of these libraries. Cysteine can cause problems by crosslinking peptides or because of oxidation, and is therefore usually excluded from the libraries. The peptide libraries are designed in scanning format (i.e., they have one defined sequence position, with all other positions being randomized [*see* **Fig. 1**], and the defined amino acid is varied through all sequence positions. Theoretically, every library that defines a fixed sequence position contains 19^n different peptides (where n is the number of randomized sequence positions). The longer the peptides the more complex the mixtures and the lower the likelihood of specific T-cell responses to such libraries. Cytotoxic T-cell responses are best analyzed with octa- and nonapeptide libraries *(7,12)*. There are some initial reports of the identification of T-helper-cell epitopes *(13,14)*, which are longer than the MHC class I-restricted epitopes that elicit T-cell cytotoxicity. These libraries are often designed with fixed anchor amino acids to reduce the complexity of the peptide mixtures.

4.2. Identification of Active Epitope Amino Acids

4.2.1. T-Cell Degeneracy

T-cells are, depending on the T-cell clone, more or less degenerate in their antigen recognition *(15–18)*. For this degeneracy, the number and kind

of amino acids identified for the different sequence positions of the epitope sequences can vary with the T-cell clone. Different combinations of the active amino acids can yield epitopes that may differ substantially in sequence and in potency. Every T-cell clone can lead to different mimetics of the same natural epitope. Also the primary structure of the natural epitope and its mimotope may differ greatly from one another (7,18). Without knowledge of the natural epitope itself, no conclusion can be derived from the mimotope with respect to the structural relationship of the natural epitope and its synthetic counterparts. Usually, synthetic epitopes are more potent, sometimes by several orders of magnitude, than the corresponding natural epitopes (7,18–21). T-cell degeneracy also allows the selection of variant epitopes that fulfil different additional requirements, such as optimal MHC binding (22,23) or high resistance to degradation. Peptide libraries that define amino acids that suppress the response of the T-cell clone as compared to the response obtained with the no-peptide control might lead to the identification of antagonistic variants of the mimotope being investigated.

4.2.2. MHC Binding Motifs

The recognition of peptides by T-cells is restricted by specific MHC molecules (i.e., the peptides have to bind to the MHC molecules in order to be recognized by the T-cell). Every MHC allomorph has special requirements regarding the amino acids in the peptide that anchor it in the peptide-binding groove of the MHC protein. Usually, two or three sequence positions of the peptide serve as anchor residues for MHC binding (6). Given that more than 1000 MHC class I allomorphs have so far been identified for the human population, the MHC-restricted nature of peptide recognition by T-cells significantly complicates the identification of active epitopes. The anchor positions are often ill-defined by combinatorial peptide library scans because those peptide libraries that carry the anchor amino acids in the anchor positions contain a high fraction of competing peptides that can bind efficiently to the MHC molecule without being recognized by the T-cell (6).

Therefore, in addition to the amino acids defined for the anchor positions by combinatorial peptide library scans, the amino acids of the canonical motif should be considered for the design of potential epitopes. The MHC allele-specific epitope motifs are being collated in the SYFPEITHI database.

4.3. Heterogeneity of the T-Cell Responses

The mimotopes identified by combinatorial scans can induce differential responses in T-cells. Some mimotopes can induce cytolysis, others cytokine production, and still others can induce both. The assay chosen for the peptide library scan can therefore influence the definition of the sequence of the mimotope (24,25).

4.4. Immune Monitoring of Tumor Patients

The validation of a mimotope by finding mimotope-responsive T-cells in the testing of PBMC from MHC-matched cancer patients not only confirms that the mimotope is indeed recognized by tumor-specific T-cells in these cancer patients, but also indicates that it is relevant for the disease. When mimotope-specific responses are found in a high fraction of the patients tested, it can be assumed that the mimotype is a mimetic of a shared TAA, and is therefore a potential vaccine antigen for patients with the corresponding MHC alleles. Moreover, the analysis of cytokine production induced in the T-cells specifically responding to a mimotope also provides information about the frequency and physiologic status of TAA-specific T-cells in the cancer patients from whom the cells were taken, and thus makes mimotyping a useful tool for monitoring of the tumor-specific immune response capacity of these patients.

References

1. Renkvist, N., Castelli, C., Robbins, P. F., and Parmiani, G. (2001) A listing of human tumor antigens recognized by T-cells. *Cancer Immunol Immunother.* **50,** 3–15.
2. Castelli, C., Storkus, W. J., Maeurer, M. J., et al. (1995) Mass spectrometric identification of a naturally processed melanoma peptide recognized by CD8+ cytotoxic T lymphocytes. *J. Exp. Med.* **181,** 363–368.
3. Tureci, O., Sahin, U., Schobert, I., et al. (1996) The SSX-2 gene, which is involved in the t(X; 18) translocation of synovial sarcomas, codes for the human tu-

mor antigen HOM-MEL-40. *Cancer Res.* **56,** 4766–4772.

4. De Plaen, E., Lurquin, C., Brichard, V., et al. (1997) Cloning of genes coding for antigens recognized by cytolytic T lymphocytes. In: *The Immunology Methods Manual.* (Lefkovits, I., ed.), Academic Press Ltd., NewYork, pp. 692–718.

5. Walden, P., Wiesmüller, K. H. and Jung, G. (1995) Elucidation of T-cell epitopes: A synthetic approach with random peptide libraries. *Biochem. Soc. Trans.* **23,** 678–681.

6. Rammensee, H. G., Bachmann, J. and Stevanovic, S. (1997) MHC *Ligands and Peptide Motifs.* Landes Bioscience, Georgetown, TX.

7. Gundlach, B. R., Wiesmüller, K. H., Junt, T., et al. (1996) Determination of T-cell epitopes with random peptide libraries. *J. Immunol. Methods* **192,** 149–155.

8. Walden, P. (1996) T-cell epitope determination. *Curr. Opin. Immunol.* **8,** 68–74.

9. Rubio-Godoy, V., Ayyoub, M., Dutoit, V., et al. (2002) Combinatorial peptide library-based identification of peptide ligands for tumor-reactive cytolytic T lymphocytes of unknown specificity. *Eur. J. Immunol.* **32,** 2292–2299.

10. Furka, A. (1996) Chemical synthesis of peptide libraries. In: *Combinatorial Peptide and Nonapeptide Libraries.* (Jung, G. ed.),VCH Verlagsgesellschaft mbH, Weinheim, Germany.

11. Kern, F., Surel, I. P., Brock, C., et al. (1998) T-cell epitope mapping by flow cytometry. *Nat. Med.* **4,** 975–978.

12. Linnemann, T., Tumenjargal, S., Gellrich, S., et al. (2001) Mimotopes for tumor-specific T lymphocytes in human cancer determined with combinatorial peptide libraries. *Eur. J. Immunol.* **1,** 156–165.

13. Hiemstra, H. S., Duinkerken, G., Benckhuijsen, W. E., et al. (1997) The identification of CD4+ T-cell epitopes with dedicated synthetic peptide libraries. *Proc. Nat. Acad. Sci. USA.* **94,** 10,313–10,318.

14. Wilson, D. B., Pinilla, C., Wilson, D. H., et al. (1999) Immunogenicity. I. Use of peptide libraries to identify epitopes that activate clonotypic CD4+ T-cells and induce T-cell responses to native peptide ligands. *J. Immunol.* **163,** 6424–6434.

15. Gundlach, B. R., Wiesmüller, K.-H., Junt, T., et al. (1996) Specificity and degeneracy of minor histocompatibility antigen-specific MHC-restricted cytotoxic T lymphocytes. *J. Immunol.* **156,** 3645–3651.

16. Boesteanu, A., Brehm, M., Mylin, L. M., et al. (1998) A molecular basis for how a single TCR interfaces multiple Ligands. *J. Immunol.* **161,** 4719–4727.

17. Hemmer, B., Fleckenstein, B. T., Vergelli, M., et al. (1997) Identification of high potency microbial and self ligands for a human autoreactive class II-restricted T-cell clone. *J. Exp. Med.* **185,** 1651–1659.

18. Udaka, K., Wiesmüller, K.-H., Kienle, S., et al. (1996) Self-MHC restricted peptides recognized by an allo MHC-responsive CTL clone. *J. Immunol.* **157,** 670–678.

19. Dressel, A., Chin, J. L., Sette, A., et al. (1997) Autoantigen recognition by human CD8 T-cell clones: enhanced agonist response induced by altered peptide ligands. *J. Immunol.* **159,** 4943–4951.

20. La Rosa, C., Krishnan, R., Markel, S., et al. (2001) Enhanced immune activity of cytotoxic T-lymphocyte epitope analogs derived from positional scanning synthetic combinatorial libraries. *Blood* **97,** 1776–1786.

21. Hemmer, B., Pinilla, C., Gran, B., et al. (2000) Contribution of individual amino acids within MHC molecule or antigenic peptide to TCR ligand potency. *J. Immunol.* **164,** 861–871.

22. Udaka, K., Wiesmüller, K. H., Kienle, S., et al. (1995) Tolerance to amino acid variations in peptides binding to the major histocompatibility complex class I protein H-2Kb. *J. Biol. Chem.* **270,** 24,130–24,134.

23. Jung, C., Kalbus, M., Fleckenstein, B., et al. (1998) New ligands for HLA DRB1 *0301 by random selection of favourable amino acids ranked by competition studies with undecapeptide amide sublibraries. *J. Immunol. Methods* **219,** 139–149.

24. Agnes, M. C., Tan, A., Jordens, R., et al. (1998) Strongly increased efficiency of altered peptide ligands by mannosylation. *Int. Immunol.* **10,** 1299–1304.

25. Ostankovitch, M., Guichard, G., Connan, F., et al. (1998) A partially modified retro-inverso pseudopeptide modulates the cytokine profile of CTL specific for an influenza virus epitope. *J. Immunol.* **161,** 200–208.