

Review

Combining computer algorithms with experimental approaches permits the rapid and accurate identification of T cell epitopes from defined antigens

Markus Schirle, Toni Weinschenk, Stefan Stevanović *

Department of Immunology, Institute for Cell Biology, University of Tübingen, Auf der Morgenstelle 15, D-72076 Tübingen, Germany

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Abstract

The identification of T cell epitopes from immunologically relevant antigens remains a critical step in the development of vaccines and methods for monitoring of T cell responses. This review presents an overview of strategies that employ computer algorithms for the selection of candidate peptides from defined proteins and subsequent verification of their *in vivo* relevance by experimental approaches.

Several computer algorithms are currently being used for epitope prediction of various major histocompatibility complex (MHC) class I and II molecules, based either on the analysis of natural MHC ligands or on the binding properties of synthetic peptides. Moreover, the analysis of proteasomal digests of peptides and whole proteins has led to the development of algorithms for the prediction of proteasomal cleavages. In order to verify the generation of the predicted peptides during antigen processing *in vivo* as well as their immunogenic potential, several experimental approaches have been pursued in the recent past. Mass spectrometry-based bioanalytical approaches have been used specifically to detect predicted peptides among isolated natural ligands. Other strategies employ various methods for the stimulation of primary T cell responses against the predicted peptides and subsequent testing of the recognition pattern towards target cells that express the antigen. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: T cell; Epitope prediction; HLA; Peptide; Computer algorithm

Abbreviations: ANN, artificial neural network; CTL, cytotoxic T lymphocyte; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorting; HLA, human leukocyte antigen; MHC, major histocompatibility complex; MSMS, tandem mass spectrometry; RT-PCR, reverse transcriptase-polymerase chain reaction; TAA, tumor-associated antigen; TAP, transporter associated with antigen processing; TCR, T cell receptor; WWW, world wide web.

* Corresponding author. Tel.: +49-7071-2987645; fax: +49-7071-295653.

E-mail address: stefan.stevanovic@uni-tuebingen.de (S. Stevanović).

1. Introduction

Major histocompatibility complex (MHC) molecules are highly polymorphic cell surface molecules that present peptidic ligands to cells of the T cell compartment of the immune system. MHC class I ligands have a typical length of 8–12 amino acids and are derived from endogenously expressed proteins that are degraded by cytosolic proteases, most notably the proteasome. The proteolytic fragments are transported into the endoplasmic reticulum in an

ATP-dependent fashion by the transporter associated with antigen processing (TAP), where they bind to newly synthesized empty MHC class I molecules. The MHC-peptide complex is subsequently transported to the cell surface and can be recognized by the T cell receptor (TCR) of CD8⁺ cytotoxic T cells (CTL). MHC class II ligands have a more variable length of 9–25 amino acids and are derived mainly from exogenous or transmembrane proteins, but also from cytosolic proteins that are degraded by various proteases which originate from the lysosomal compartment. After binding to MHC class II molecules in the late endosomal/lysosomal compartment, the MHC-peptide complex is also transported to the cell surface, where it can be recognized by the TCR of CD4⁺ T cells.

Recognition of a peptide derived from a disease-associated protein, e.g., a viral or a tumor-specific protein, in the presence of a costimulatory signal leads to T cell activation and triggers a T cell-mediated immune response. Therefore, which peptide fragments of immunologically relevant antigens are available in the context of a certain MHC-molecule for recognition by T cells is crucial for the development of peptide-based or other defined antigen-based vaccines. Moreover, tools for monitoring specific T cell responses, such as MHC-peptide tetramers, intracellular cytokine staining or ELISPOT assays, depend on the identification of the relevant T cell epitope.

A major breakthrough in the identification of T cell epitopes was the discovery that ligands of a certain MHC-molecule carry chemically related amino acids in certain positions of their primary sequence, which permits the definition of a “peptide motif” for every MHC allele (Falk et al., 1991). This information was rapidly used to predict potential epitopes from protein sequences (Pamer et al., 1991; Rötzschke et al., 1991) and was the start signal for the so-called “reverse immunology” approach (Celis et al., 1994), which has since become the most successful strategy for the identification of T cell epitopes.

The first step of such studies is usually the computer-based prediction of potential MHC ligands from a protein of interest, which is followed by experiments to verify the natural processing of the predicted peptides and their recognition by T cells. The

classical and most straightforward approach relies on the stimulation of primary T cell responses against the predicted peptides *in vitro* or in (transgenic) animals and subsequent testing of the recognition pattern of the generated T cells towards target cells that endogenously express the antigen. Alternatively, patient-derived T cells, which might have been already primed *in vivo*, are used. This “pure” reverse immunology approach has been used extensively in the recent past for the identification of CTL and, to a lesser extent, helper T cell epitopes from a wide variety of immunologically relevant proteins. However, a major drawback of this labor- and resource-intensive approach is the high failure rate, even if the peptide in question binds well to the MHC molecule and the generated T cells recognize target cells loaded with the synthetic peptide. The reason for this is either that the peptide is not produced by the processing machinery of the cell, or the T cells raised by primary peptide stimulation *in vitro* are of low affinity, or a combination of both.

In consequence, various groups have made attempts to decrease the number of candidate peptides by applying additional criteria to epitope prediction. For example, the increasing knowledge about specificities of other cellular components of the antigen processing machinery, most notably the proteasome and TAP, has made it possible to apply these specificities as additional filters to the selection of candidate peptides. Moreover, experimental selection criteria, such as MHC-peptide binding assays and the bioanalytical verification of presentation of predicted MHC-ligands, have been applied successfully.

This review tries to give a comprehensive overview of available computer-based prediction algorithms and strategies that combine the predictive power of these theoretical approaches with experimental approaches for a reliable and rapid identification of T cell epitopes.

Since it has been written from an immunologist’s point of view, this review focuses mostly on prediction programs that have already been applied to the identification of novel epitopes. Furthermore, it should be noted that there are tremendous differences between different prediction softwares in terms of availability. Some programs are easily accessible for free on the world wide web (WWW); others are available from the authors of the corresponding pub-

lications; still others are available on a commercial basis only.

2. Computational predictions

2.1. Epitope prediction

The recognition of MHC-peptide complexes by the T cell receptor (TCR) is the first step in a series of cellular processes that lead to the initiation of T cell-mediated immunity. It is, therefore, not surprising that as soon as the peptidic nature of MHC ligands was discovered and before the rules for the binding of peptides to MHC molecules were elucidated, first attempts for the prediction of T cell antigenic structures from protein sequences were published. These predictions were based solely on comparisons of precursor peptide sequences known to contain T cell epitopes. The suggested characteristics of T cell epitopes were predicted secondary structures (amphipathic helices) (DeLisi and Berzofsky, 1985) or short primary sequence motifs (Rothbard and Taylor, 1988; Sette et al., 1989). It was the discovery of allele-specific motifs shared by eluted natural MHC ligands (Falk et al., 1991) that finally allowed the exact prediction of peptides from a given protein sequence presented on MHC class I molecules (Pamer et al., 1991; Rötzschke et al., 1991).

For MHC class II ligands, the elucidation of peptide motifs turned out to be more difficult due to the variable length of the ligands as well as the more degenerate anchor positions, which are sometimes not used at all. Several methods for the determination of MHC class II peptide motifs have been based on sequence information of natural ligands, including the alignment of natural ligands, pool sequence analysis, or substitution variants of known ligands (Rudensky et al., 1992; Chiczy et al., 1992; Hunt et al., 1992a,b; Falk et al., 1994; Leighton et al., 1991). In 1992, the first HLA-DR-motif based on binding assays using a phage peptide display library was published (Hammer et al., 1994a,b).

In the beginning of the 1990s, the selection of candidate peptides was done manually or using simple pattern search programs by scanning protein sequences for main anchor positions only. However,

the constant refinement of peptide motifs and knowledge about secondary anchor amino acids, disfavored amino acids and other chemical interactions important for binding to the MHC molecule quickly led to the development of more complex computational predictions.

Depending on the focus of research, the determination of peptide motifs has been approached from two sides.

On the one hand, the binding properties of particular MHC molecules have been analyzed by determining the binding of synthetic peptides from protein antigens using MHC-peptide binding assays (Ruppert et al., 1993; Kubo et al., 1994; Parker et al., 1994; Hammer et al., 1994a,b). The most refined descriptions of such “peptide-binding motifs” have been achieved by using positional scanning with random synthetic peptide libraries (Stryhn et al., 1996; Fleckenstein et al., 1996; Udaka et al., 2000).

On the other hand, the analysis of natural ligands by individual ligand analysis as characterized by Edman degradation (Falk et al., 1991; DiBrino et al., 1993; Corr et al., 1993; Falk et al., 1994) and tandem mass spectrometry (MSMS) (Hunt et al., 1992a,b) as well as Edman pool sequencing (Falk et al., 1991) leads to so-called “natural ligand motifs” that give information about the natural peptide repertoire presented by a certain MHC molecule. These motifs are, therefore, not only a result of the binding properties of this MHC molecule, but reflect also—to a certain extent—features of the antigen processing machinery, e.g., cleavage specificities of the proteasome or sequence requirements for transport into the ER by TAP. Indeed, MHC-binding motifs derived from random peptide libraries show significant differences, especially for the C-terminal amino acids, compared to motifs derived from natural ligands (Davenport et al., 1997). This raises the question of the *in vivo* relevance of motifs based on the binding properties of synthetic peptides.

2.1.1. MHC class I

Historically, MHC class I epitope prediction had a headstart over its MHC class II counterpart. The reasons for this include the defined length of MHC class I ligands of mostly nine amino acids and the more clearcut rules of MHC class I motifs that have been more clearly characterized. Currently, there are

two predictive algorithms available on the world wide web that allow unrestricted predictions free of charge: “BIMAS”, developed by Parker et al. (1994) (<http://www-bimas.dcrn.nih.gov/molbio/hla-bind/>) and “SYFPEITHI”, developed by our group (Rammensee et al., 1999) (<http://www.syfpeithi.de>). SYFPEITHI uses motif matrices deduced from refined motifs based on the pool sequence and single peptide analysis exclusively of natural ligands. Potential binders for various human, mouse and rat MHC class I molecules are ranked according to the presence of primary and secondary anchor amino acids as well as favored and disfavored amino acids.

BIMAS ranks potential human leukocyte antigen (HLA) binders according to predicted half-time dissociation of MHC-peptide complexes. While the motif matrix for HLA-A2 is based solely on binding studies, other matrices have been deduced from reported motifs, regardless of the method used for their identification. This might explain the similar results that were obtained with both algorithms in a recent study that identified HLA-B7-presented epitopes from the tumor-associated antigen (TAA) carcinoembryonic antigen (CEA) (Lu and Celis, 2000) since for HLA-B7 the reported motifs are based on natural ligands. Both algorithms have been used successfully during recent years for the identification of CTL epitopes from a wide range of antigens, e.g., from the human TAAs telomerase (BIMAS) (Vonderheide et al., 1999) and TRP2 (SYFPEITHI) (Sun et al., 2000).

Other prediction programs that have been used for the identification of MHC class I-presented epitopes are distributed on disk (e.g., “MOTIFS” (D’Amaro et al., 1995)), part of commercial websites [e.g., “EPIMATRIX” (De Groot et al., 1997) (http://www.brown.edu/Research/TB-HIV_Lab/epimatrix/epimatrix.html)] or are currently not publicly available (Sette et al., 1994).

The motif-based algorithms so far described are still relatively simple programs that consider every amino acid position in a peptide individually. For every position, a coefficient is assigned to each amino acid and the overall score or binding value of the peptide are determined by addition or multiplication of amino acid coefficients. Moreover, there are several reports employing artificial neural networks (ANN) (Brusic et al., 1994; Adams and Koziol, 1995; Gulukota et al., 1997; Milik et al., 1998) or the polynomial method (Gulukota et al., 1997). These approaches open up the possibility to consider amino acid preferences that depend on the properties of amino acids in other positions of the peptide.

Still another approach is based on structural information obtained by X-ray crystallography of peptide–MHC complexes (Altuvia et al., 1997; Schueler-Furman et al., 2000). While none of these latter, non motif-based approaches has actually been used to date for the identification of novel CTL epitopes, they could prove useful in the future.

Table 1 demonstrates the similarities and differences of some of the currently used MHC class I

Table 1

Comparison of MHC class I predictions

Epitopes identified using different epitope prediction programs were recalculated using programs that are available free of charge on interactive websites: the epitope prediction programs BIMAS and SYFPEITHI and a program for prediction of proteasomal cleavage sites PAPROC.

Sequence	Source (position)	HLA molecule	Program used for identification	Reference	SYFPEITHI rank/score	BIMAS rank/value	PAPROC *
ILAKFLHWL	human TERT (540–548)	A* 0201	BIMAS	(Vonderheide et al., 1999)	1/30	1/1745.714	No
KVLEYVIKV	human MAGE-A1 (278–286)	A* 0201	SYFPEITHI	(Pascolo et al., 2001)	3/26	1/743.189	Yes
ILHNGAYSL	human Her-2/neu (435–443)	A* 0201	Sette et al.	(Rongcun et al., 1999)	3/27	16/36.316	Yes
HLSTAFARV	human G250 (254–262)	A* 0201	MOTIFS	(Vissers et al., 1999)	19/22	38/4.493	No

* Prediction of proteasomal generation of C-terminus by human proteasome wild-type III.

prediction programs. It shows selected CTL epitopes that were identified using four different computer programs and also lists the corresponding results from SYFPEITHI, BIMAS and a prediction algorithm for proteasomal cleavage, “PAPROC” (see below), i.e., programs that are available at interactive websites at no cost. However, it should be noted that some studies do not give the results of the original prediction procedure, such as ranks or values, but only lists of peptides that were selected after performing additional tests.

2.1.2. MHC class II

The main obstacle for the prediction of MHC class II ligands has been the differing degree of degeneration of motifs. While some alleles, e.g., HLA-DRB1*0405, show a strong preference for certain related amino acids in the anchor positions that is comparable to MHC class I motifs (in this case D, E in P9) (Rammensee et al., 1999), other alleles make the definition of primary anchor amino acids virtually impossible (e.g., for HLA-DRB1*0401 all amino acids except G, P, F, W have been assigned anchor amino acids in P9) (Rammensee et al., 1999). To date, the focus has been on predictions of HLA-DR ligands, with only one report on predictions of HLA-DQ-restricted epitopes (Godkin et al., 1998). The first matrix-based prediction algorithms for HLA-DRB1*0401 were based on the results of side-chain scanning experiments using simplified, polyalanine-based peptide libraries (Hammer et al., 1994a,b; Marshall et al., 1995). The work by Hammer and colleagues resulted in the commercial program “TEPITOPE”, which focuses on promiscuous HLA-DR binding peptides, but also allows allele-specific predictions. It has been used in several studies, e.g., in the identification of an HLA-DR11-presented epitope from MAGE-A3 (Manici et al., 1999).

Another matrix-based HLA-DRB1*0401-prediction algorithm, also based on binding studies using synthetic peptides (Southwood et al., 1998) has been used for the retrospective calculation of an epitope from human gp100 (Touloukian et al., 2000) that had been identified previously (Halder et al., 1997). This program is available free on request from the Surgery Branch of the NCI.

Still another customized computer algorithm also based on published HLA-DR binding motifs has been used for the identification of HLA-DR-presented promiscuous T cell epitopes from *Plasmodium falciparum* (Doolan et al., 2000). The first program for prediction of MHC class II based on natural ligands of two HLA-DR13 alleles was the PAP program (Davenport et al., 1995). This program, which can be obtained free from the author, uses corrected amino acid yields from Edman pool sequencing directly as coefficients in motif matrices. One of the following publications also reports for the first time predictions for HLA-DQ2 and -DQ8 and the identification of a novel HLA-DQ2-presented epitope from gliadin, the antigen of celiac disease (Godkin et al., 1998).

Currently, SYFPEITHI is the only interactive website offering unrestricted MHC class II predictions free of charge. As for MHC class I, the available predictions for HLA-DR and several mouse MHC class II alleles are based on motif matrices deduced from natural ligands by single ligand analysis and Edman pool sequencing.

For HLA-DRB1*0401, predictions by artificial neural networks have already been applied successfully to the identification of helper T cell epitopes. Zarour and colleagues have used an ANN, based on the work of Honeyman et al. (1998) and Brusic et al. (1998), for the identification of epitopes from the TAAs Melan-A/MART-1 (Zarour et al., 2000a,b) and NY-ESO-1 (Zarour et al., 2000a,b). These prediction programs are currently not publicly available.

Finally, several publications have explored the possibilities of other more complex computational predictions. For example, Mallios (1999) has described an iterative algorithm to align and optimize an MHC class II-binding matrix based on sequences of known class II ligands alone or in combination with suggested motifs. Up to now, none of these predictions have been applied to the identification of T cell epitopes.

Table 2 shows examples of helper T cell epitopes that were identified by four different computer programs and also lists the corresponding results from SYFPEITHI and the HLA-DRB1*0401 prediction program from the NCI. However, in contrast to MHC class I, comparisons of MHC class II predictions can be even more difficult for several reasons.

Table 2

Comparison of MHC class II predictions

Epitopes identified using different epitope prediction programs were recalculated using programs that are available free of charge either on an interactive website (SYFPEITHI) or by email (NCI).

Epitope sequence	Source (position)	HLA molecule	Reference	Program used for identification	SYFPEITHI rank/source	NCI rank/value
WNRQ <u>LYPEWTEA</u> QRLD	human gp 100 (44–59)	DRB1* 0401	(Touloukian et al., 2000)	NCI	2/26	1/0.0195
RNGY <u>RALMDKSL</u> HVGTQCALTRR	human MART-1 (51–73)	DRB1* 0401	(Zarour et al., 2000)	ANN	1/22	– / –
TSYV <u>KVLHHMVK</u> ISG -----	human MAGE-A3 (281–295)	DRB1* 1101	(Manici et al., 1999)	TEPITOPE	8/22 * *	n.a.

Putative core sequences are underlined; * * best ranked peptides 284–298 (score 28) and 280–294 (score 24) also contain other putative core sequences (– – –); n.a., not available.

First, due to the variable length of MHC class II ligands, the identified epitopes may harbor more than one putative core sequence. Furthermore, some studies focus on promiscuous MHC class II-binding peptides, while others use allele-specific predictions.

2.2. Predictions of antigen processing

Much of the work combining computational predictions and applied immunology has focussed on MHC-peptide binding only. However, our increasing knowledge about the specificities of other cellular components involved in antigen processing, such as the proteasome and TAP, has been used to create comparable prediction algorithms with the future prospect of fusing them to epitope prediction programs.

The proteasome is a cytosolic multi-subunit protease that is, among other functions, involved in the generation of peptide ligands for MHC class I molecules and responsible for the generation of the correct C-terminus of CTL epitopes (Craiu et al., 1997; Stoltze et al., 1998). The proteolytically active core complex (20S proteasome) can associate with the 19S cap complex to form the 26S proteasome, which is responsible for the degradation of ubiquitin-marked proteins. The three different active sites show different proteolytic specificities, cleaving after large, hydrophobic amino acids, basic amino acids and acidic amino acids, respectively. Upon exposure of cells to interferon γ (IFN γ), the catalytically active subunits are replaced by three IFN γ -inducible subunits. This results in a shift in the overall cleavage specificity of this immunoproteasome towards hydrophobic amino acids, while cleavages after acidic amino acids are disfavored. This corresponds to the fact that almost all reported peptide motifs of vertebrate MHC molecules demand hydrophobic or basic C-terminal anchor amino acids.

An experimental approach was chosen by Kessler et al. (2001) for the identification of HLA-A*0201-presented CTL epitopes from the TAA PRAME by an extended reverse immunology strategy that incorporates proteasomal digest analysis. Predicted HLA-A*0201-binding peptides were tested in binding and stability assays, followed by an analysis of in vitro proteasome-mediated digestions of 27mer precursor

peptides encompassing candidate epitopes. For only 4 out of 19 high affinity binders, efficient generation of the correct C-terminus was observed and all four peptides were found to be efficient CTL epitopes.

The first published prediction algorithm for cleavages by 20S proteasomes, “FRAGPREDICT”, is based on published peptide cleavage data (Holzhütter et al., 1999; <http://www.mpiib-berlin.mpg.de/MAPPP/cleavage.html>). The PAPROC-algorithm (Nussbaum et al., 2001) <http://www.paproc.de>), developed by our group, is based on an evolutionary algorithm trained on cleavage data of 20S proteasomal digests of a whole protein substrate [enolase (Nussbaum et al., 1998)], and additional peptide digest data from the literature. While the currently available prediction programs are based only on semiquantitative data from human or yeast constitutive 20S proteasomal digests, more accurate predictions based on fully quantified protein digestion data as well as immunoproteasomal digestion data (Toes et al., 2001) are currently being developed. Another program, “NETCHOP” (Kesmir et al., manuscript submitted; <http://www.cbs.dtu.dk/services/NetChop/>) is an ANN that was trained on data from the analysis of residues at the termini and flanking regions of known natural MHC class I ligands. All these programs for the prediction of proteasomal cleavages are available free of charge on interactive websites.

The overall goal of combining proteasomal prediction algorithms with epitope prediction is already possible using combinations of FRAGPREDICT and PAPROC with SYFPEITHI and BIMAS at the website of the Max-Planck-Institute for Infection Biology (www.mpiib-berlin.mpg.de/MAPPP/). It should be noted that this program also predicts C-terminally extended precursor peptides.

An ANN-based device has also been reported for the prediction of human TAP peptide-binding affinities using a polyalanine-based peptide library (Daniel et al., 1998). The program predicted a higher affinity to TAP for eluted natural ligands compared to randomly selected peptides with similar binding affinities to the same HLA molecule, indicating that such predictions might be—at least for some HLA-alleles—a valuable addition for epitope prediction tools. However, this would probably require a more profound investigation of peptide translocation by TAP,

not only binding of peptides. The reported preferences of TAP for peptide translocations (Momburg et al., 1994) have not yet led to a prediction program.

3. Experimental verification of predicted epitopes

3.1. Analysis of binding properties

In most cases, epitope prediction is followed by binding studies with synthetic peptides in order to reduce the number of potential candidate peptides by discarding nonbinding peptides for the final analysis of recognition by peptide-specific T cells.

Binding of synthetic peptides to MHC molecules is mainly studied by assays based on competition (Sette et al., 1994; Olsen et al., 1994; Vogt et al., 1994), reconstitution of MHC-peptide complexes on the cell surface (Stuber et al., 1994; Zeh et al., 1994; Storkus et al., 1993), or stabilization of MHC-peptide complexes on TAP-deficient cells (Stuber et al., 1992).

In competition assays, the studied peptides compete with a labeled reference peptide of intermediate affinity for binding to MHC molecules. Such assays are performed either using whole cells or purified MHC molecules in solution. In both cases, the amount of labeled MHC-bound peptide is measured. For MHC class II-binding assays usually fluorescence dyes, like fluorescein or 7-amino-4-methylcoumarin-3-acetic acid (AMCA), are used for labeling the N-terminal amino acid of the MHC ligand. For MHC class I-binding studies, amino acid side chains have to be labeled by fluorescent dyes or by radioactive isotopes, such as ^{125}I without interfering with the more restricted binding properties of MHC class I molecules. Performing in-solution assays using purified MHC molecules, MHC-peptide complexes have to be separated from unbound free peptide by gel filtration (Sette et al., 1994), spun columns (Stryhn et al., 1997) or high-performance size exclusion chromatography (HPSEC) (Vogt et al., 1994). In addition, enzyme linked immunosorbent assay (ELISA)-based assays (Jensen, 1991; Hammer et al., 1995) or the more sensitive time-resolved fluorimetry in a europium fluoroimmunoassay (Tompkins et

al., 1993) have been used as the readout. Competition-based binding assays result in IC_{50} values, which do not represent an absolute dissociation constant since they are dependent on the reference peptide. However, they allow a better comparison of binding affinities of different peptides than the results obtained by reconstitution or stabilization assays. Competition assays using purified MHC molecules employ soluble MHC molecules, which might have a slightly different structure compared to native molecules embedded in the cell membrane. Whole cell assays overcome these problems by using MHC molecules on the surface of intact cells. As an additional feature, mild acid treatment of the cells can be used to remove peptides from the MHC-binding groove. Cells are then incubated with a fluorescence-labeled reference peptide together with different concentrations of the peptides of interest (van der Burg et al., 1995). The amount of bound reference peptide can be measured by FACS analysis. As peptide-depleted MHC molecules have a short half-life and β_2 -microglobulin dissociates rapidly, only membrane-anchored MHC- α -chains are left and, thus, this competition assay might also represent a reconstitution assay.

In classical reconstitution assays, cells expressing the appropriate HLA alleles are stripped by incubation at pH 3.2 for a short time, then the peptide of interest and a conformation-dependent monoclonal antibody are added. The difference in fluorescence intensity determined by FACS analysis between cells incubated with and without peptide after staining with fluorescence-labeled secondary antibody is used to determine peptide binding (Storkus et al., 1993).

The stabilization assay was first described by Stuber et al. (1992). Cell lines with defective peptide loading mechanisms, like the TAP-deficient murine cell line RMA-S (Ljunggren and Karre, 1985) or the human cell lines T2 (Cerundolo et al., 1990) or ST-EMO (de la Salle et al., 1994), display a low level of mostly empty MHC class I molecules on their surface due to the rapid turnover of empty MHC molecules. The addition of exogenous binding peptide to the cells, however, leads to stabilization and accumulation of MHC molecules, which can be determined using monoclonal antibodies and FACS analysis. Due to its simplicity, this assay can serve for screening large numbers of peptides. Moreover,

transfection of any human cell with cDNA of the immediate early protein ICP47 of the Herpes simplex virus generates a TAP-deficient phenotype and, thus, permits the extension of the stabilization assay to virtually any HLA allele (Gatfield et al., 1998).

3.2. Analysis of presentation

Since the first identification of a natural MHC class I ligand by tandem mass spectrometry (MSMS), this technique has become the bioanalytical method of choice for high sensitivity sequencing of natural MHC ligands. This has led to the development of approaches that combine epitope prediction and HPLC-MS and MSMS for specific detection and identification of disease-associated MHC class I ligands. Brockman et al. (1999) used a simple main anchor-based epitope prediction to identify masses of candidate H2-K^b-ligands from *Trypanosoma cruzi*. The corresponding mass chromatograms showed in two cases significant peaks and subsequent HPLC-MSMS analysis revealed indeed two naturally processed peptides. A variant of this approach is the “Predict, Calibrate, Detect” (PCD) method (Schirle et al., 2000), which permits a more rapid and sensitive screening of tumor samples by calibrating the HPLC-MS system with synthetic analogs of predicted potential HLA-A2 ligands from tumor-associated proteins. HLA-A2 ligands from TAAs p53, CEA and MAGE-A1 have been identified using PCD (Schirle et al., 2000; Pascolo et al., 2001); the MAGE-A1-derived peptide has been verified as a CTL epitope using an HLA-A2 transgenic mouse model (Pascolo et al., 2001).

These approaches are not a substitute for testing peptide-specific recognition of tumor cells by CTL; however, they allow further focussing of these efforts to those peptides that are not only binding to the MHC molecule, but are actually processed and presented.

3.3. Analysis of T cell recognition

The final experimental verification of a predicted epitope is achieved by the identification of T cells, which specifically recognize the naturally processed epitope in an HLA-restricted fashion. One major

source for T cells are peripheral blood mononuclear cells (PBMC) from patients or healthy donors, which are either already primed *in vivo* or which can be primed *in vitro*, respectively. In addition, immunization of various HLA class I and class II transgenic mice (reviewed in Faulkner et al., 1998 and Sonderstrup et al., 1999) can be used to generate specific T cells. T cell responses after stimulation can be measured by means of lytic activity, proliferation and cytokine production. Lytic activity of cytotoxic T lymphocytes has been analyzed since 1968 using the ⁵¹Cr-release assay (Brunner et al., 1968; Cerottini and Brunner, 1974); however, this assay requires the cumbersome *in vitro* expansion of specific T cells from PBMCs. Nevertheless, it is still widely used. As an alternative, T cell proliferation has been investigated by radioactive ³H-thymidine incorporation. To determine peptide-specific T cell responses, a sensitive proliferation assay was established in 1996. This assay avoids radioactivity and uses BrdU incorporation instead (Mayer et al., 1996). In our experience, this proliferation assay has a much higher sensitivity than the ⁵¹Cr-release assay. The first quantitative assay for IL-2 production by activated T cells described in 1978 is still used today (Gillis et al., 1978). This sensitive method uses an IL-2-dependent mouse T cell line, CTLL, as the target cell population, which proliferates and incorporates ³H-thymidine in response to IL-2 in culture medium. After IL-2- and IFN γ -specific antibodies became available, enzyme-linked immunosorbent assays (ELISA) were established to measure T cell responses (Gehman and Robb, 1984; Van der Meide et al., 1985; Troye-Blomberg et al., 1985). The advantages of ELISA procedures are their simple setup and the capacity to handle many samples in parallel.

None of the methods discussed so far is able to detect cytokine production by T cells at a single cell level. The first application to address this question was the ELISPOT assay reported in 1988 for enumerating IFN γ -secreting lymphocytes (Czerkinsky et al., 1988; Versteegen et al., 1988), originally developed for the detection of single specific antibody-secreting cells (Czerkinsky et al., 1983). In this assay, activated T cells are plated in anti-IFN γ antibody-coated wells; single IFN γ -secreting cells are detected as spots after staining trapped IFN γ with an insoluble dye generated by an enzyme coupled to the

second anti-IFN γ antibody. Peptide-specific T cells can be assessed in this way with higher sensitivity compared to the ^{51}Cr -release assay (Miyahira et al., 1995), even without the need for prior in vitro expansion, which may bias the result. ELISPOT has been used to measure TNF α secretion (Herr et al., 1996) or IFN γ secretion (Scheibenbogen et al., 1997; Schmittl et al., 1997) as markers for T cell activation. The tedious visual counting of spots was no longer necessary after the introduction of a computer-assisted image analysis system, which has made data analysis convenient, objective and suitable for handling large sample pools (Cui and Chang, 1997).

Another way to assess cytokine production at the single cell level is achieved by intracellular cytokine staining and flow cytometry analysis. After stimulation in the presence of monensin, T cells are fixed by paraformaldehyde and permeabilized by saponin to allow antibodies to penetrate through the cell membrane, the cytosol and the membranes of the endoplasmic reticulum and Golgi organelle. Monensin interrupts intracellular transport processes leading to an accumulation of the cytokine in the Golgi complex and, thus, an increase in the signal-to-noise ratio leads to a higher sensitivity (Sander et al., 1989, 1991; Jung et al., 1993; Mascher et al., 1999; Pala et al., 2000). Intracellular cytokine staining allows high throughput of samples and multiparameter characterization of cytokine production as well as parallel detection of cell surface markers on a single cell basis without the need for prolonged in vitro culture and cloning. However, the sensitivity is limited by the fluorescence-activated cell-sorting analysis.

Similar to the intracellular cytokine staining approach is the cell surface affinity matrix technology (Manz et al., 1995). Instead of detecting cytokines in the cytoplasm, secreted cytokines are measured on live single cells. After antigenic restimulation, secreted IFN γ or other cytokines are captured by a cell surface affinity matrix (Brosterhus et al., 1999). This matrix comprises bispecific antibody–antibody conjugates directed against CD45 for cell membrane anchoring and against the cytokine of interest. Detection of secreted, matrix bound cytokine is achieved by fluorescence-labeled antibody and flow cytometry. In contrast to intracellular cytokine staining, this technology allows enrichment and isolation of living cells, which can be further cultured and analyzed.

Visualization of antigen-specific T cells is possible by staining T cell receptors with MHC/peptide tetrameric complexes (Altman et al., 1996; Galimore et al., 1998; Murali-Krishna et al., 1998; Letvin et al., 1999). These tetramers are made by in vitro folding MHC heavy chain in the presence of β_2 -microglobulin and a specific peptide ligand. The heavy chain is engineered in a way that the purified MHC-peptide complex can be biotinylated and then tetramerized via fluorochrome dye-coupled avidin. Tetramerization enhances the affinity of the $\alpha\beta\text{TCRs}$ to soluble peptide-loaded MHC molecules. By this method, a quantitation of T cells displaying TCRs specific for the analyzed peptide is possible; however, nothing can be said about the functionality of these T cells. The tetramer approach is well established for MHC class I molecules; however, detection of antigen-specific CD4 $^{+}$ T cells with soluble MHC class II tetramers is now in progress (Crawford et al., 1998; Novak et al., 1999; Kotzin et al., 2000; Kuroda et al., 2000; Reichstetter et al., 2000; Kwok et al., 2000; Meyer et al., 2000). Application of MHC class I tetramers in the field of virus immunology has been reviewed by Doherty and Christensen (2000).

Probably, the most sensitive method for detecting T cell responses is the quantitation of induced cytokine mRNA after activation by quantitative real time reverse transcriptase-polymerase chain reaction (RT-PCR). This quantitative method is based on the 5' nuclease assay in which the 5' $\rightarrow$ 3' endonuclease activity of the Taq DNA polymerase cleaves a hybridized sequence-specific fluorogenic internal oligonucleotide probe during PCR. Relative increases in fluorescence emission from the reporter dye are detected online in an analytical thermal cycler (Heid et al., 1996). This assay has been used to quantify cytokine mRNA expression (IL-2, IL-4, IL-10, TNF α , IFN γ) in previously frozen whole blood samples (Kruse et al., 1997). More recently, this very sensitive and highly reproducible method was adapted by normalizing copy numbers of cytokine mRNAs to CD8 mRNA copy numbers in order to analyze specific T cell responses in fresh PBMCs without any further stimulation (Kammula et al., 1999, 2000). This way, it was possible to monitor the specific T cell response after peptide vaccination in melanoma patients. Though this is no single cell assay, it is very

sensitive, highly reproducible and permits a high sample throughput.

4. Outlook

While computational predictions have already become a familiar tool for many immunologists searching for T cell epitopes from immunologically relevant proteins, there is the constant need to use new data to update and extend the available programs. Reliable epitope prediction is still only available for a limited number of organisms and alleles because little or no information is available about the corresponding peptide specificities. However, several studies have shown both for MHC class I and II that the knowledge of which amino acids occupy those positions of the MHC polypeptide chain responsible for the interaction with peptide ligands make it possible, in some cases, to predict the peptide specificities for MHC molecules with as yet unknown motifs (Seeger et al., 1999; Sturniolo et al., 1999).

Furthermore, predictions for other characteristics of antigen processing, such as proteasomal cleavage specificities, are still in their infancy, with programs that are based on quantified cleavage data from immuno- and constitutive proteasomes not yet being publicly available. On the other hand, the development of prediction programs for MHC class II that are able to take antigen processing into consideration are still in the distant future.

Since the increasing usage of screening techniques, such as DNA microarrays (Lockhart et al., 1996) and serological analysis of recombinant cDNA expression libraries (SEREX) (Türeci et al., 1996), provide a rapidly growing pool of candidate protein antigens, interactive combinations of computational epitope predictions with these approaches, such as the recently described combination of DNA microarray analysis and TEPITOPE (Sturniolo et al., 1999), are expected to provide a rapidly growing number of epitopes for use in immunotherapy of a wide range of diseases.

However, while the described strategies combining computational prediction and experimental methods are likely to provide a rapidly increasing number of T cell epitopes as potential tools for therapeutic and diagnostic purposes, the identified epitopes still

have to pass the ultimate test: they have to prove to be useful in the *in vivo* situation.

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