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Current Strategies for the Identification of Immunogenic Epitopes of Tumor Antigens

*Ludmila Müller, Stephanie McArdle,
Evelyna Derhovanessian, Thomas Flad,
Ashley Knights, Robert Rees,
and Graham Pawelec*

SUMMARY

Peptide-based cancer immunotherapy relies on the identification of epitopes recognized by T-lymphocytes. Because of the high degree of polymorphism of human leukocyte antigens and issues of tumor escape from the immune response, the availability of a wide choice of diverse epitopes is essential for the therapist. There are a number of different approaches for identifying new class I- and class II-restricted target antigens appropriate for immunotherapy and as discussed in this volume, several of these are complimentary. The strategy of “reverse immunology,” which is presented in this chapter, is applied for prediction of tumor-associated antigens by *in silico* screening sequences of selected proteins for peptides with high binding affinity to different human leukocyte antigen molecules. Subsequently, these peptides are synthesized and tested experimentally. Here, we outline some of the most prominent current algorithms and methods for assessing the immunogenicity of the predicted peptides *in vitro* and *in vivo*. We also describe the complimentary approach of isolating major histocompatibility complex-associated peptides from target cells followed by sequencing using reverse phase high-pressure liquid chromatography fractionation and mass spectrometric analysis. We conclude by discussing some of the potential advantages and disadvantages of these methods and problems associated with their application.

Key Words: Tumor immunology; immunoinformatics; T-cell sensitization; reverse immunology; tumor antigens; transgenic mouse model; immunoaffinity chromatography; mass spectrometry.

1. INTRODUCTION

The idea of the immune system recognizing and responding to tumors goes back to the end of 19th century, when rare spontaneous tumor regression events were observed after infectious episodes. The modern era of tumor immunology began decades later. The concept of immunosurveillance of cancer was put forth in 1970 by Burnet (1). This idea was

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later to be discredited because of several observations that the incidence of tumor development was not significantly higher in athymic nude mice compared with wild-type mice (2) or in immunodeficient individuals compared with immune competent hosts (3). It was accepted that tumors are not different from normal tissue for the immune system, i.e., they do not express any tumor antigens (4). The turning point was in 1982 when van Pel and Boon (5) showed that protective immunity could be established against nonimmunogenic tumors when the immune system is properly activated. They explained the inability of the immune system to fight cancer as an inability of the tumors to activate the immune system, rather than lack of tumor-rejection antigens. It is now widely accepted that the immune system indeed can be manipulated to specifically recognize and eliminate tumor cells as demonstrated in animal models and illustrated in some clinical trials (reviewed in refs. 6 and 7).

Since the molecular cloning of melanoma-associated antigen, the first identified human tumor antigen recognized by cytotoxic T-cells in 1991 (8), and identification of the first tumor-specific CD8⁺ T-cell epitope from the same antigen 1 yr later (9), molecular identification and characterization of novel tumor-associated antigens (TAAs) has evolved rapidly. With the identification of the first human leukocyte antigen (HLA) class II-restricted epitope in 1994 (10), much attention has also been paid to the role of CD4⁺ T-cells in antitumor immunity.

Despite the fact that a large number of T-cell defined TAA epitopes are now identified, the majority are class I-restricted and are limited to few HLA alleles, widely expressed in the Caucasian population. Moreover, there has been a strong focus on melanoma, and few TAA epitopes have been identified in other tumors. To overcome such limitations, the search for new TAAs from histologically different tumors and containing peptide epitopes restricted to less-frequent HLA alleles remains of crucial importance. With the human genome sequenced, novel proteins have been identified, some of which may be potential targets for induction of an immune response against cancer.

2. CLASSIFICATION OF KNOWN TUMOR ANTIGENS

Tumor antigens can be divided in two major groups: those unique to an individual tumor and those shared between different tumors. *Unique antigens* commonly result from a mutation in the coding region of a ubiquitously expressed gene. They can arise through different mechanisms, such as point or frameshift mutations, which have been described in a wide range of malignancies like melanoma (for examples, *see* refs. [11], renal cell carcinoma [12,13], lung [14], colorectal [15], head and neck [16], and bladder cancer [17]). Another mechanism, often observed in different kinds of leukemia, involves translocation of chromosomes, which results in fusion of distant genes and the synthesis of a novel fusion protein that may contain new T-cell epitopes, usually spanning the fusion junction (18).

Various HLA class I and HLA class II epitopes have already been reported that arise from these mutated gene products ([19]; www.cancerimmunity.org). Such tumor-specific antigens could play an important role in the natural antitumor immune response of individual patients. An immune response to these antigens seems to be associated with a good prognosis (20–22). However, most of them cannot be widely used as immunotherapeutic targets, because they are not shared by tumors from different patients.

Shared tumor antigens are present on many independent tumors and can be divided, in turn, into three groups according to their expression pattern (www.cancerimmunity.org):

1. *Cancer-germline* or *cancer-testis (CT) antigens* arise from reactivation of genes that are normally silent in adult tissue and become activated in different tumor histotypes (23). Their expression on normal tissue is limited to placental trophoblasts and testicular germ cells. Because these cells lack major histocompatibility complex (MHC) class I and class II molecules, no epitopes of these antigens should be expressed on the surface of these tissues. These antigens can therefore be regarded as tumor-specific or tumor-associated antigens. More than 20 different CT antigens have been shown to be expressed in bladder, breast, colon, non-small cell lung, prostate, and renal cancers, as well as melanoma. In contrast, esophageal, gastric, head and neck, and ovarian cancers, as well as leukemia/lymphoma, hepatocellular carcinoma, and sarcoma, appear to be moderate expressors of CT antigens, with the expression of 10–20 CT antigen families (24). With 44 gene families already identified, 19 of which encode an immunogenic gene product, this group of antigens is the largest and best-characterized group of TAA. Because of their immunogenicity and restricted expression, CT antigens seem to be ideal for use as cancer vaccines. These TAA have indeed been one of the main components of the antitumor vaccines tested in the clinic during the last decade.
2. *Differentiation antigens*. The expression of this group of antigens is restricted to the tumor itself and the normal tissue from which the tumor arose. So far, most TAA of this type have been reported in melanoma and normal melanocytes (25). Many of these melanocyte lineage-related proteins are involved in the biosynthesis of melanin. Other differentiation antigens have also been reported in prostate (26,27) and gut carcinoma (28). Because the expression of these antigens is not restricted to tumor tissue, their use as targets for cancer immunotherapy may result in autoimmunity toward the corresponding normal tissue. Nevertheless, this group of tumor-associated antigens has been, and is, commonly used in current cancer vaccination trials, often together with CT antigens.
3. *Overexpressed antigens* are expressed in a wide variety of normal tissues and overexpressed in tumors. Genes encoding widely expressed TAA have been detected in histologically different types of tumors, as well as in many normal tissues, generally with lower expression levels. It is difficult to make predictions regarding the safety of targeting these overexpressed antigens with tumor vaccines. Because a minimal amount of peptide is required for cytotoxic T-lymphocyte (CTL) recognition, the low level of expression of these genes in normal tissues may result in an inadequate amount of epitope presentation on the surface of these normal cells and render the latter resistant to CTL recognition. Among the most interesting TAAs of this group are the antiapoptotic proteins (livin, survivin), human telomerase reverse transcriptase, and tumor suppressor proteins (e.g., p53).

3. REVERSE IMMUNOLOGY APPROACH AS A STRATEGY FOR IDENTIFICATION OF TAA

3.1. Epitope Selection

The “reverse immunology” approach for antigen discovery implies the use of *in silico*, or computer-aided, prediction of antigenic determinants from tumor antigens that the investigator believes may represent an attractive target for immune therapy, for example, if an antigen is only expressed in tumor tissue or if it is expressed in a wide array of tumor types. Following selection of the target antigen and the prediction of potential T-cell epitopes, these are then synthesized and screened for their immunogenicity and their natural processing and presentation in the context of MHC, either in *in vitro* assays or with *in vivo* models. This method has largely replaced the technique involving the production

of a panel of overlapping peptides that span the whole of the target protein; a method that is costly in terms of both the quantity of synthetic peptides required and the amount of time required to screen the peptide library. The reverse immunological method, in theory, should reduce both the cost of investigation, as well as the workload required to identify novel T-cell epitopes.

T-cell epitopes derived from the target protein and presented in the context of MHC are usually between 9 and 20 amino acids in length, depending on whether they are restricted to MHC class I or class II molecules. For a peptide to be presented in the context of an MHC molecule, the protein must first undergo a number of steps that will result in the transport and cleavage of this peptide. These steps vary considerably, depending on the origin of the protein. Intracellular proteins, such as those resulting from viral infection or oncogenic transformation, are processed by the cell in a way that leads to presentation by the MHC class I molecule, present on essentially all normal nucleated cells. Extracellular proteins such as those internalized or engulfed by antigen-presenting cells (APCs) are processed in a manner resulting in MHC class II presentation. More is known currently about the mechanisms of class I processing than class II; however, the whole story for both pathways is probably far from complete. To complicate matters further, nonclassical pathways of MHC processing are also involved and may represent an overlap between these two pathways; these have been reviewed elsewhere (29).

The number of computer-aided tools available for the prediction of T-cell epitopes reflects somewhat the current level of understanding of antigen processing, and hence, the tools for CTL epitope prediction outnumber those for T-helper (Th) epitope prediction. Furthermore, the current array of “immunoinformatic” tools for CTL prediction only represents a few of the known processes important for epitope generation. Briefly, this involves the degradation of proteins within the cytoplasm to peptide fragments by proteases and the proteasome, where a selection of peptide fragments is then transported via transporter associated with antigen processing (TAP) proteins to the endoplasmic reticulum (ER), where newly synthesized MHC molecules are located. Within the ER, peptides undergo further N-terminal trimming before their subsequent loading into the empty MHC-binding groove ready for transport to the cell surface. Despite this complexity, computer algorithms representing two of these steps, peptide–MHC interactions, and more recently, proteasomal degradation, have enabled the successful identification of many T-cell epitopes in recent years.

3.1.1. PREDICTION OF MHC–PEPTIDE BINDING

The first of these prediction tools, and the major criteria used to date for initial selection of epitopes, was based on the discovery that MHC molecules of one type would bind peptides with similar structural characteristics, or “motifs” (30). Such fundamental discoveries have led to the ability to use computer-based algorithms to screen protein sequences for peptides of defined lengths that should bind to an MHC molecule of the investigator’s interest. These algorithms fall into several categories based on the rules that govern the programs. The simplest of these approaches are based on anchor-motif patterns, where the information that determines if a peptide is going to bind into the groove is simply determined by the presence or absence of key anchor residues in positions known to be favorable for interactions with the particular MHC-binding cleft of interest. These algorithms tend to have low prediction accuracy, as they do not take into account the other amino acids within the peptide and their potential interactions with the MHC pocket. For

example, if a peptide were to have the small and hydrophobic anchor residues in positions 2 and 9 required for a favorable binding, but also a large bulky amino acid that would interfere with binding at another site, this would not be taken into account. Therefore, models that consider every amino acid within a peptide and assign it a positive or negative value, depending on the characteristics of the MHC groove with which it will interact, give much greater accuracy. These algorithms are based on matrices and are known as motif-matrices or quantitative matrices; an example of one of the most commonly used is SYFPEITHI ([31]; www.syfpeithi.de). This algorithm is an evidence-based motif matrix, as the data used within the algorithm are derived from knowledge of actual natural MHC ligands and can predict both class I and class II epitopes. A similar tool called BIMAS (<http://bimas.cit.nih.gov/>) generates results expressed as estimated peptide dissociation values. Another matrix-based prediction tool is TEPITOPE ([32]; www.vaccinome.com), where matrices are constructed, again, based on the interaction of every amino acid within the MHC binding cleft. However, rather than determining this empirically for each HLA allele, TEPITOPE combines these data with HLA sequence variation data to form virtual matrices. This program is restricted to only MHC class II predictions, though allowing for the prediction of highly promiscuous peptides within one search. Another method utilizes the power of artificial neural networks. These are self-training systems that will predict results with very high accuracy, although only after initial training with a large data set, and therefore, their major disadvantage is in the required amount of training data. Examples for this method of epitope selection are such programs as PREDICT (<http://sdmc.lit.org.sg:8080/predict/>) and nHLAPred (<http://www.imtech.res.in/raghava/nhlapred/>).

3.1.2. PROTEASOMAL CLEAVAGE PREDICTION

Although a peptide's ability to bind MHC is the overwhelming factor in terms of whether the epitope can be presented to a T-cell and hence, the primary selection criterion, one must also consider whether this peptide is likely to be available to the empty MHC molecule for binding in the first place. The degradation of proteins into TAP-transportable fragments for transport into the ER is carried out by the proteasome, which is charged with the task of recycling proteins that either are no longer needed within the cell or are damaged; these are then broken down into short peptide subunits before subsequent degradation into their single-amino acid components by other peptidases. A proportion of these peptides are rescued from recycling by their uptake into the ER by TAP. Current data support the hypothesis that for a peptide to bind MHC class I, the C terminus of the peptide must be correctly cleaved within the proteasome, and the remaining N-terminal amino acids that need to be removed to produce the peptide of 8–10 amino acids result from cleavage within the ER by aminopeptidases. To further strengthen the interaction between the proteasome and its role in providing peptides for class I MHC presentation, the immune effector molecule interferon (IFN)- γ can result in the modulation of the proteins comprising the proteasome and an additional regulator protein, resulting in an "immunoproteasome" that generates different peptide fragments to that of its constitutive relative. The immunoproteasome is currently believed to be responsible for the generation of the majority of CTL epitopes (33,34); however, this is still a point of debate.

Once a list of MHC class I-binding peptides has been produced using one of the aforementioned prediction tools, investigators can refine this to include only those that are likely to result from the correct proteasomal cleavage. Furthermore, for defining CTL

epitopes, only peptides that will have either the exact length peptide produced within the proteasome or the correctly cleaved C-terminal with flanking N-terminal residues are included. Several computational tools have been developed that are based on proteasomal *in vitro* cleavage data. All of these tools, including PaProC ([35]; www.paproc.de), NetChop ([36]; [<http://www.cbs.dtu.dk/services/NetChop/>]), and FragPredict ([37]; www.mpiib-berlin.mpg.de/MAPPP/), will allow the user to predict cleavages based on the human 20S proteasome. The PaProC server now also allows users to select whether they wish to predict cleavage using the constitutive human proteasome or base predictions on the immunoproteasome (33). This is a valuable tool, as there are currently data suggesting that some tumor epitopes would only be produced by the immunoproteasome, such as the melanoma-associated antigen 3 (MAGE-3) epitope identified by Schultz et al. (38). Moreover, whereas Dissemond et al. showed that the presence of components of the immunoproteasome in spontaneously regressing tumors were associated with the presence of tumor-infiltrating lymphocytes (39), Meidenbauer et al. demonstrated that a lack of immunoproteasome components was responsible for a tumor-specific CTL's inability to lyse autologous tumor (40). This suggests that the loss of immunoproteasome components by tumors may act as an immune escape mechanism. Consequently, as the jury is still out on the exact role of the immunoproteasome and its interactions in tumor immunology, selection of epitopes produced by both types of proteasome and their subsequent testing either *in vitro* or *in vivo* should shed more light on which epitopes are more suitable for inclusion in immunotherapeutic protocols.

Two publicly available tools, MAPPP ([41]; www.mpiib-berlin.mpg.de/MAPPP/) and RANKPEP ([42,43]; <http://mif.dfci.harvard.edu/Tools/rankpep.html>) offer the ability to combine both the prediction of MHC-binding peptides and screen these for proteasomal cleavage leading to the generation of the correct C-terminal motif, within one search. Combining both of these parameters should result in an increase in accuracy and the significance of the predicted epitopes. However, any model is only as good as the data used to generate it, and as these models are currently based on restricted data sets from either limited *in vitro* studies or lists of natural T-cell epitopes that are currently known, the accuracy of the results will certainly still be improved. A comparison of three of these methods has been compiled by Saxova and colleagues (44), who assessed the ability of these programs to predict the correct cleavage of known natural MHC class I ligands. Values ranging from 40 to 80% accuracy were determined using this model, and this value was described as the sensitivity. However, a specificity value for each method was also determined, and this represents the likelihood of the program to predict an epitope that would contain a cleavage site within the peptide with a higher probability than the C-terminal cleavage. The three programs compared varied in both of these values, and generally, the programs with the higher sensitivity had the lower specificity, although updated versions of some of the models are now available.

3.1.3. PROBLEMS ASSOCIATED WITH *IN SILICO* PREDICTION

Although many epitopes have now been defined using reverse-immunology prediction, and several of these have been implemented in vaccine protocols, the methodology is not without drawbacks. The epitopes that are selected with this method tend only to be high-affinity epitopes, as the aim of the prediction software is to select the top few percentages of strong binding peptides. However, often many of these high-affinity peptides on lengthy testing *in vitro* do not demonstrate immunogenicity. As many tumor antigens

fall into the category of overexpressed self-antigens, and some of these, such as human telomerase reverse transcriptase, are interesting candidates because of their wide expression-pattern in tumors, there is a desire to evade the tolerance that CTLs naturally have high-affinity peptides from these self-antigens. One method is to target the low-affinity “cryptic epitopes” contained within these antigens—those that are normally neglected during epitope prediction. Another disadvantage of the method that has been recently highlighted is that some peptides presented in the context of MHC are not necessarily derived from classical proteasomal cleavage. It has been demonstrated that peptide splice variants, short MHC-restricted peptides composed of two non-contiguous sections of the same protein, are produced within the proteasome (45,46). It is currently not known what proportion of the peptides displayed by MHC result from such peptide-splicing events, and their general importance in tumor recognition by T-cells is unclear. Moreover, peptide splicing can be included among several other posttranslational modifications that can occur to a potential T-cell epitope and that we are currently unable to model *in silico*.

3.1.4. UTILIZATION OF EPITOPE PREDICTION TOOLS

Questions regarding which antigens should be targeted for use in tumor vaccine protocols—how many epitopes should be used, and whether these should comprise MHC class I epitopes alone or in combination with class II motifs to engage T-cell help—are the subject of many ongoing studies and have been covered in numerous review articles (47,48). However, several clinical trials that have focused on using single MHC class I epitopes to induce an antitumoral CTL response have been disappointing. The reasons for this may be multiple, but one factor that is likely to have contributed to the poor outcome is the lack of T-cell help provided by CD4 T-cells. Several studies are currently aimed at using multiepitope vaccines (49), including Th epitopes (50), combinations of both multivalent vaccines and Th epitopes (51), and class II-restricted epitopes that contain class I motifs (52), among others, to circumvent some of the shortcomings. We have recently applied the combination of MHC peptide binding predictions with proteasome processing algorithms to select several peptides from the novel cancer/germline antigen HAGE. One of these peptides, a class II-binding 15-mer selected because it contains a class I-binding 9-mer with a correctly predicted C-terminal cleavage, demonstrates immunogenicity in both human *in vitro* assays and in transgenic mouse models. Furthermore, sensitization with dendritic cells (DCs) pulsed with the 15-mer results in both CD8 and CD4 T-cells specific for the class I and class II motifs, respectively (Knights et al., unpublished data).

3.2. Screening Immunogenicity of Predicted T-Cell Epitopes

3.2.1. IN VITRO

Once peptides are identified using one or a combination of the database searches, they can then be synthesized and further assessed for their ability to bind to the appropriate HLA allele, using, for example, T2 cells. This HLA stabilization assay can be used in conjunction with other assays to assess peptide binding. After the respective candidate peptides are synthesized, their immunogenicity can be examined using different approaches of reverse immunology. The synthetic peptides can be used for T-cell sensitization experiments *in vitro* or for immunization of transgenic mice *in vivo*. The source of T-lymphocytes for priming of naïve T-cells or restimulation of precursor T-cells *in vitro* might be the peripheral blood of tumor patients or healthy donors. The peptide-specific T-cell

lines or T-cell clones that are obtained then have to be tested for tumor-cell recognition. The main obstacle in using patient-derived T-lymphocytes for activation experiments is that the majority does not respond or responds very weakly to tumor antigens. Different strategies are required to overcome the difficulties of nonresponsiveness by providing an optimal activation environment *in vitro*. On the other hand, most TAA represent self-proteins and as result of tolerance, are poorly immunogenic. Immunomodulation with cytokines may break tolerance and allow tumor antigen-reactive T-cells to recognize their respective tumor antigen or to activate naïve T-lymphocytes *de novo*. Many cytokines have demonstrated immunomodulatory activities in this context. As reported for breast cancer, melanoma, renal cancer, and neuroblastoma, a combination of interleukin (IL)-2 and IL-12 *in vitro* may strongly enhance the development of tumor-specific CD8⁺ CTL and prevent overgrowth of nonspecific, less-effective lymphokine-activated killer cells (reviewed in ref. 53). In our priming experiments, we have also used IL-12, because it has been reported that regulation of IFN- γ production in stimulated peripheral blood mononuclear cell (PBMC) cultures requires direct involvement of IL-12. Such cytokine modulation of the priming conditions gave rise to antigen-specific, granulocyte/macrophage colony-stimulating factor (GM-CSF)- and IFN- γ -secreting cells. Synergistic effects of IL-4 with IL-12 on IFN- γ production by DC have also been reported (54,55). IL-12 is known to promote a potent cellular immune response in which tumor-specific CTL and Th cells are induced to secrete Th1 cytokines (56–58). Consistently, part of the modulatory activities of several other well-recognized Th1- and Th2-driving factors, such as IFN- γ , IL-4, IL-10, prostaglandin E2, and IFN- α , is mediated by regulating either the production of IL-12, or the responsiveness to this cytokine (59). In cytokine-modified mixed lymphocyte-tumor cell cultures, we have developed culture conditions for the expansion of CD4 and CD8 effector cells from PBMCs of chronic myeloid leukemia (CML) patients by the addition of GM-CSF, IL-4, and in some cultures, IL-7 and IL-12. Our data showed enhanced T-cell responses in IL-12-supplemented mixed lymphocyte-tumor cell, particularly secretion of IFN- γ (60).

APCs play an essential role in the generation of tumor-specific immune responses. The most potent of them, the DCs, are capable of activating both antigen-specific cytotoxic and Th cells. Consequently, it is now accepted that for the optimal implementation of an immunotherapeutic approach to cancer therapy, both CD8 cytotoxic cells and CD4 helper cells, predominantly the Th1 polarization, should be targeted. Numerous investigations have involved the use of DCs as APCs in an attempt to stimulate both primary and secondary immune responses, even to poorly immunogenic tumor antigens. We have shown that using monocyte-derived DCs for peptide presentation, in addition to B-cell receptor (bcr)/Abelson leukemia virus (abl), in CML the reciprocal abl/bcr fusion products may also be immunogenic, and that certain breakpoint peptides contain both MHC class I- and II-binding peptides that stimulate CD4 helper cells, as well as CD8 CTLs. The latter respond not only to peptide but also to primary CML tumor cells, indicating that these TAAs are indeed expressed on the cell surface and could represent novel targets for immunotherapy (61,62).

DCs can be easily generated from the monocytic fraction of peripheral blood by culture in GM-CSF and IL-4, followed by maturation, for example, with tumor necrosis factor α . According to a consensus protocol established by the European Union Concerted Action on Peptide Sensitization Consortium (an EU-sponsored collaboration, active 1999–2001, see <http://www.medizin.uni-tuebingen.de/eucaps/>), in addition to this cytokine as

a maturation signal, we also use polyribonucleosinic-polyribocytidylic acid (poly I:C), a synthetic molecule composed of double-stranded RNA (to induce maturation) for generating DCs in serum-free medium. We tested the validity of the inclusion of this factor (63), and phenotypic analysis of these cells revealed that exclusion of poly I:C resulted in DCs with a lower expression of HLA-DR, CD86, and CD1a, with little to no expression of CD83 when compared with cells matured in the presence of poly I:C. Another group has also previously described the use of this molecule to induce stable maturation of DCs and described DCs with a phenotype consisting of high levels of HLA-DR, CD86, and CD83 surface expression, as well as functional characteristics comparable to DC generated in monocyte-conditioned medium (64). It is therefore likely that inclusion of this factor will be beneficial for functional DC generation.

Autologous irradiated PBMCs loaded with an appropriate peptide could also be used in priming experiments, as well as for rechallenge of activated T-cells. To provide an enhanced population of APCs, some protocols recommend the use of the plastic adherent fraction of autologous PBMCs. However, following several attempts to restimulate cells in this manner, this protocol in our hands led to the repeatedly observed induction of apoptosis, even despite the addition of both IL-7 and IL-12 during the restimulation stage, known for their ability not only to drive cells toward a Th1 polarization, but also for their T-cell growth factor and antiapoptotic properties. An attractive tool applied recently for activation and expansion of peptide-specific T-lymphocytes *in vitro*, helping to overcome many of the obstacles associated with limitations in current approaches, is artificial APCs, known as aAPCs (65,66). These artificial cells have several distinct advantages over cellular APCs, including DCs: they can be prepared and stored for long-term use without loss of activity and, remarkably, they can be adopted easily using different HLA complexes for different HLA-specific responses (67). The aAPC system allows the exact control of the MHC density, enabling selective elicitation of high- or low-avidity antigen-specific CTL responses with high efficiency from healthy individuals (66).

Once T-cell lines or T-cell clones are established, they need to be screened for their specificity. A number of sophisticated technologies are available to quantify peptide-specific and tumor-cell-specific activity of generated T-cells, even at the single-cell level. The most common techniques to validate their specificity include the enzyme-linked immunospot assay, which is based on the detection of cytokine secretion in response to antigen-specific restimulations, and standard ^{51}Cr release or lactate dehydrogenase release assays, both of which determine the capability to kill peptide-pulsed target cells in a peptide-specific manner. Another attractive method described recently for identification of antigen-specific CD8⁺ T-cells with cytolytic activity is a flow cytometric assay for degranulation, a novel technique which measures the exposure of CD107, present in the membrane of cytotoxic granules, at the cell surface as a result of degranulation—a necessary precursor of cytotoxicity (68). During the process of cell killing by release of cytotoxic mediators, such as perforin and granzymes, CD107a becomes transiently mobilized to the cell surface, allowing rapid identification and isolation of antigen-specific cytolytic T-cells (69). We used this method in our experiments on defining the immunogenicity of HAGE antigen- and heme oxygenase 1-derived peptides for screening specificity of the generated T-cells. The most important characteristic of this test is that it provides information on not only the frequency and phenotype of the T-cells in question, but also their functional status.

Such tests as cytokine-secretion assays, intracellular cytokine analysis, HLA tetramer, and multimer staining have been thoroughly validated and established recently as

methods to quantify the induction of antigen-specific T-cells. Thus, recent developments in this field now allow combinations of technologies not only to enumerate antigen-specific T-cell responses, but in parallel, to define the phenotype and functionality of the detected T-cells (reviewed in ref. 70).

Once peptide specificity of the generated T-cells is verified, the critical step is to demonstrate the recognition of HLA-matched tumor cell lines or patients' tumor cells. It is also important to show if the peptide epitopes, which were previously identified by means of prediction algorithm methods, are naturally processed and presented on tumor cells. Peptides could be identified by elution from tumor cells using methods described herein, or tumor recognition by the generated T-cells could be screened by using tumor cells expressing, or not expressing, the antigen of interest and coexpressing (or not) the required MHC restriction element. A tool enabling investigators to locate tumor cell lines bearing particular selected combinations of HLA alleles, TAA, cytokine secretion, accessory molecule expression, and so on, is now available for on-line searching at <http://www.ebi.ac.uk/ipd/estdab>. This EU-supported research infrastructure (*see* <http://www.medizin.uni-tuebingen.de/estdab/>) provides these certified tumor cell lines to qualified investigators (so far limited to melanoma lines).

3.2.2. In Vivo

The immunogenicity of the predicted peptides can also be tested in vivo using mice transgenic for different HLA antigens. For the most part, HLA-A*0201, HLA-DRB*0101, and DRB*0401 transgenic mice have been used for this purpose, because these are the most common class I and II alleles, respectively, in European and North American Caucasian populations.

3.2.2.1. HLA-A2 Transgenic Mouse Model, HHD II. The HHD II mouse provides a good animal model for the study of HLA-A2-restricted CTL responses in vivo (71,72). These animals express a single MHC class I molecule consisting of the human HLA-A2.1 a1 and a2 domains, the murine H-2Db α 3, transmembrane, and cytoplasmic class I heavy chain regions, covalently linked to human b2-microglobulin, allowing for HLA-A2-restricted antigen presentation (72). The HHD II mice have been used extensively for the identification of HLA-A2.1-restricted CTL responses and may be superior to the "conventional" HLA-A*0201/Kb transgenic mouse model (71,73). We have shown that these animals respond to human p53 peptides by mounting HLA-A2-restricted responses to epitopes previously defined in humans, and that they are indeed superior to the HLA-A*0201/Kb transgenic mouse model (Fig. 1). We also identified a novel HLA-A2 prostatic acid phosphatase-derived peptide using this model. (The peptides used are shown in Table 1.) At 1 wk post-immunization, cytotoxicity was assessed using RMA-S cells transfected with the HHD II gene (RMA-S-A2) as targets, and IFN release monitored. Prostatic acid phosphatase- and p53-derived peptide immunization resulted in CTLs capable of specifically killing relevant peptide-pulsed RMA-S-A2⁺, in an HLA-A2-restricted manner

Fig. 1. (*Opposite page*) Mean cytotoxic T-lymphocyte (CTL) activity of splenocytes cultured in vitro following immunization with peptide. At 1 wk postimmunization, the CTL activity of splenocytes cultured for 5 d in vitro with the peptide was assessed using RMA-S/A2 transfected cells pulsed with peptides (relevant PAP135, irrelevant GP100) for (A) C57 human leukocyte antigen (HLA)-A2.1 and (B) HHD II transgenic mice transgenic mice. (**Note:** Results presented are representative results \pm SEM.) * Denotes significance compared with irrelevant. (C) To confirm that the cytotoxic responses observed following immunization with PAP135 are restricted to HLA-A2.1, a blocking

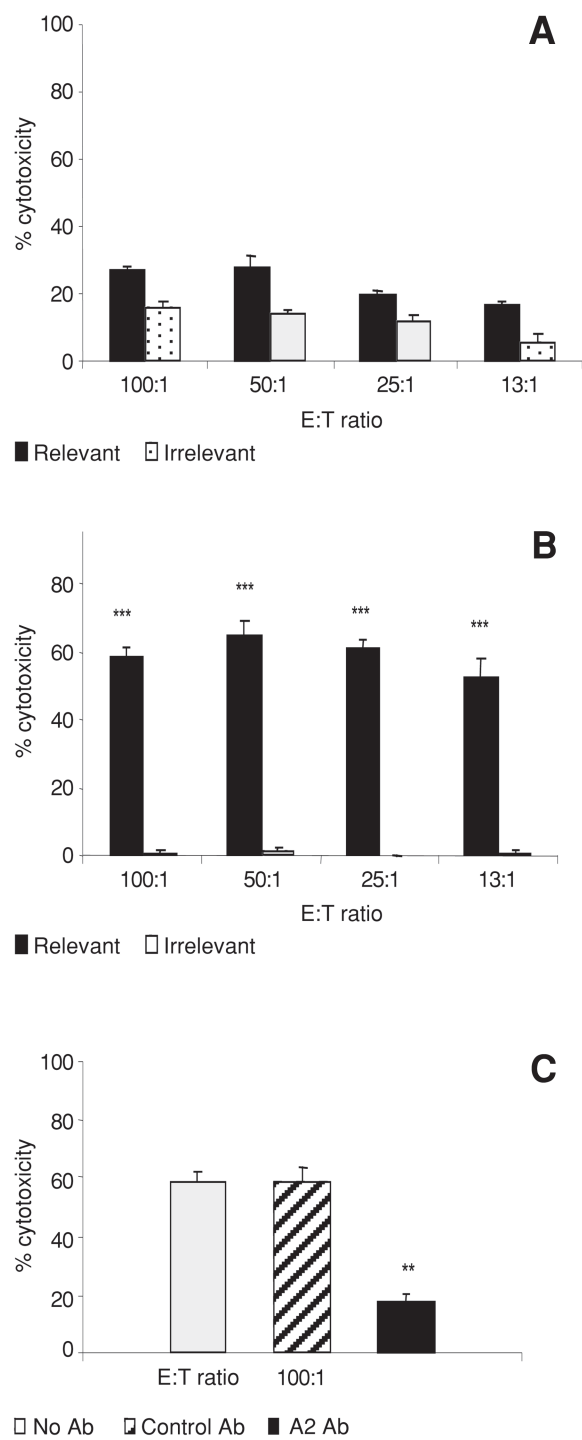


Fig. 1. (continued) antibody specific for A2.1 was used in the chromium release assay to block any A2.1 mediated responses. Results demonstrate that the addition of 3 μ g of the A2 antibody was sufficient to reduce target cell cytotoxicity from 59% (control and untreated) to 18% (A2), a significant reduction of CTL activity by 41%.

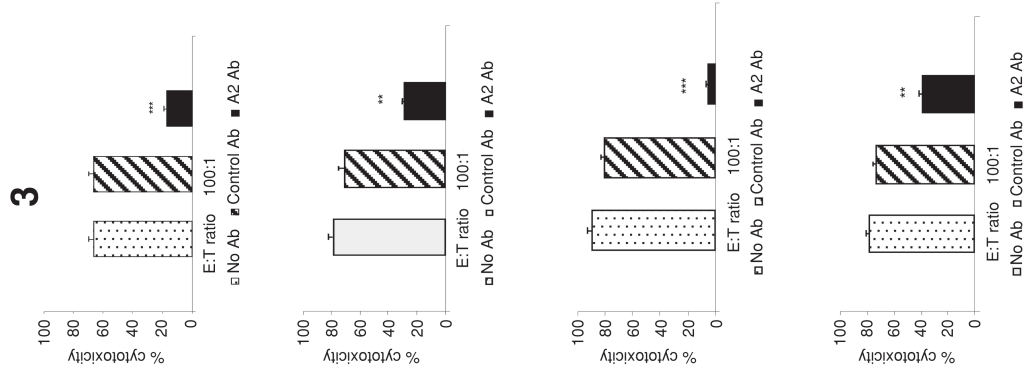
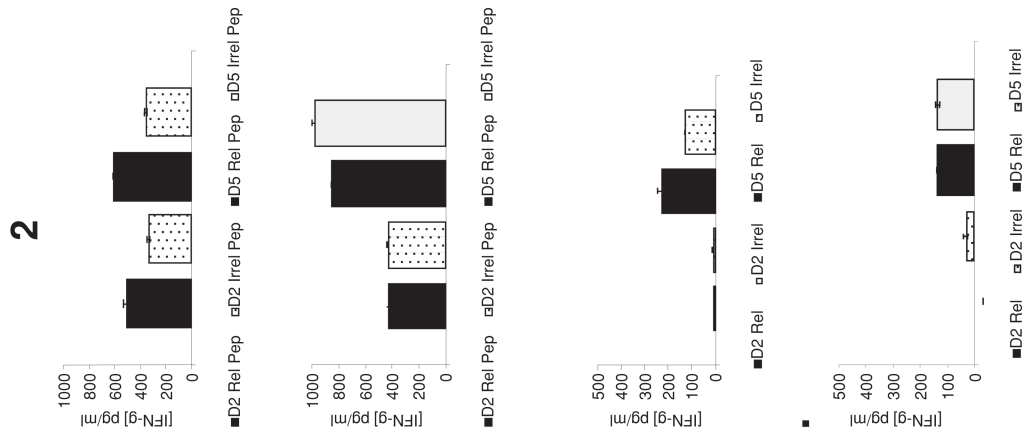
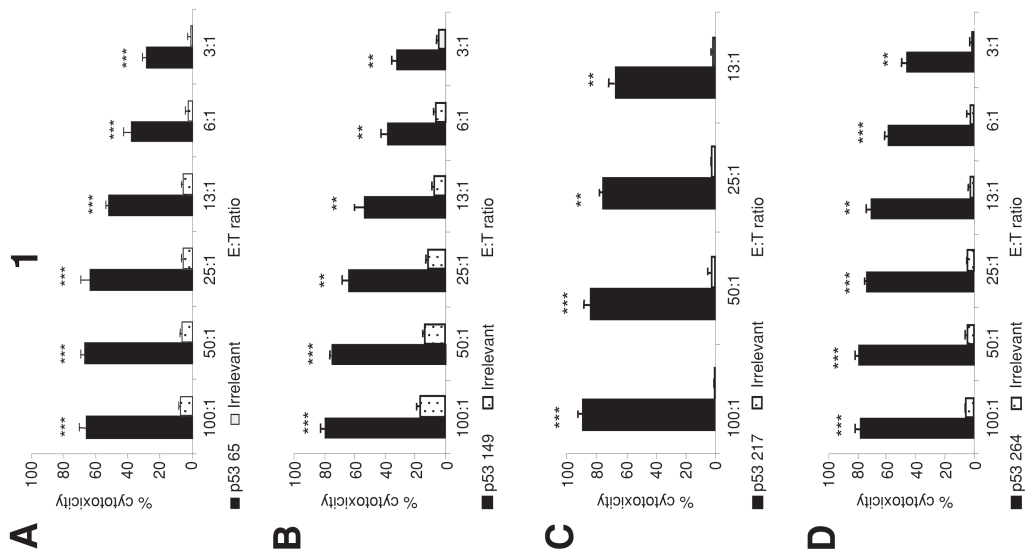


Table 1
Peptides Used for Experiments, Synthesized at Alta Bioscience (Birmingham, UK)
and Dissolved in 100% Dimethyl Sulfoxide at a Concentration of 10 mg/mL

| <i>Protein</i> | <i>Peptide region</i> | <i>Sequence</i> | <i>SYFPEITHI scoring</i> |
|----------------------------|-----------------------|-----------------|--------------------------|
| p53 | 264–272 | LLGRNSFEV | 24 |
| | 65–63 | RMPEAAPPV | 21 |
| | 149–147 | STPPPGTRV | 19 |
| | 217–225 | VVPYEPPEV | 17 |
| Prostatic acid phosphatase | 135–143 | ILLWQPIPV | 24 |

Table 2
Summary of Peptide Immunization in HHD II Mice

| <i>Peptide used for immunization</i> | <i>Strain</i> | <i>Cytotoxicity: responders/no. immunized</i> | <i>Ab block: responders/ no. immunized</i> | <i>Specific IFN-γ: responders/no. immunized</i> |
|--|---------------|---|--|---|
| p53 65 | HHD II | 2/3 | 2/3 | 1/3 ^a |
| p53 149 | HHD II | 3/3 | 3/3 | 0/3 |
| p53 217 | HHD II | 4/6 | 3/3 | 1/3 |
| p53 264 | HHD II | 3/6 | 2/3 | 0/3 |

^aNegative animal in the cytotoxicity assay was negative for IFN- γ production as measured by enzyme-linked immunosorbent assay. Ab, antibody; IFN- γ , interferon- γ .

(Figs. 1 and 2). A difference of almost 40% of killing was obtained in the HHD II mice compared with the conventional HLA-A*0201/Kb transgenic mice (Fig. 1). This was usually accompanied by a strong IFN- γ production (Figs. 1 and 2; Table 2).

Several in vitro stimulations are, however, needed to obtain murine CD8⁺ T-cells capable of killing human cancer cells (74). This is probably because of the fact that, unless CD8⁺ T-cells generated are of very high avidity for the peptide, the recognition of the target cells by CD8⁺ cells requires not only contact between the MHC/peptide and T-cell receptor, but also between the CD8⁺ chain and the $\alpha 3$ loop of the MHC class I molecule from the same species. Still, using the HHD II mice as a screening model, it has been possible to assess the effect of various modifications within the peptide sequence, as well

Fig. 2. (Opposite page) Summary for p53 peptide-immunized splenocytes. Cytotoxicity responses of cytotoxic T-lymphocytes (CTL) generated from HHD II splenocytes immunized with 100 μ g of relevant class I peptide and 140 μ g of class II helper peptide in phosphate-buffered saline in a 1:1 ratio with incomplete Freud's adjuvant. At 1 wk post immunization, the CTL activity of splenocytes cultured for 5 d in vitro with lipopolysaccharide blasts pulsed with 100 μ g/mL of relevant or irrelevant peptide was assessed by standard chromium release assay using RMAS-A2 cells pulsed with peptide as the targets. (1) Mean cytotoxicity T-lymphocytes (CTL) activity of splenocytes cultured in vitro after DNA immunization, against RMAS-A2 cells pulsed with relevant (black bars) p53 peptide-65 (A), 149 (B), 217 (C), or 264 (D), or pulsed with irrelevant PAP135 peptide (open bars). (2) Effect of human leukocyte antigen (HLA)-A2.1 blocking antibody on HHD II-immunized splenocyte cytotoxicity. Three microliters per well of either blocking antibody or isotype control was added to some wells set aside in the chromium release assay. (3) Supernatants collected on day 2 (D2) and day 5 (D5) of in vitro culture were assayed for interferon- γ concentration by enzyme-linked immunosorbent assay, values expressed as pg/mL. ***Denotes significance in (A1), (B1), (C1), and (D1) compared with irrelevant peptide, in (A3), (B3), (C3), and (D3) compared with isotype control/without antibodies.

as the immunogenicity of different fractions of peptides eluted from tumor cells (75,76). CTLs generated with some altered peptides have been shown to not only recognize and kill target cells pulsed with modified peptide, but also to recognize and kill target cells pulsed with the wild-type sequence (75). Moreover, HHD II mice have proven to be a very useful model to study, assess, and compare the efficiency of different vaccine forms, such as naked plasmid DNA encoding either multi-epitopes, containing class I and class II, or whole-protein sequences either as single agents or in a prime-boost regimen (77,78). CTL activities of splenocytes from HHD II mice, immunized three times with gold-labeled DNA encoding the entire human mutated p53 protein at position 273 at weekly intervals and stimulated once in vitro with peptide 65, 149, 264 and 217, could be generated against RMA/2 cells pulsed with the same peptides, with the exception of RMA/2 cells pulsed with the 264 peptide that were not killed (Fig. 3). These results are in accordance with previous published work where peptide 264 was reported not to be produced from p53 protein mutated at position 273 (79).

We provide the above concrete examples in a little more detail in order to illustrate that it is therefore possible to use HHD mice not only to identify immunogenic peptides, but also, and more importantly, to test whether they are endogenously processed. Thus, the HHD II mouse provides an ideal model for the identification of novel immunogenic peptide but can also be used for preclinical evaluation of vaccine constructs as potential immunogens in vivo (*see* Figs. 1–3). Some investigators, however, have reported that the peptide repertoire resulting from the endogenous processing of human papilloma virus-derived proteins by HHD II mice differs from humans, and that caution is required when interpreting data obtained using these mice. In any event, results from HHD II mice will always need verification using in vitro cultures from healthy donors and cancer patients.

3.2.2.2. HLA-DR4 and HLA-DR1 Transgenic Mouse Models. HLA-DR1^{+/+}/IA^{+/+} and HLA-DR4^{+/+}/IE^{0/0} transgenic mice provide good models of the human CD4⁺ T-cell immune response (80) and can be used for the identification of MHC class II peptides. Analogous to class I above, MHC class II peptides derived from tumor-associated proteins, such as p53, can be synthesized and used to immunize DR4 and DR1 transgenic mice. We have employed this approach for the identification of a novel p53 class II peptide (81). Mice were immunized twice with peptides in incomplete Freund's adjuvant, and 7 d after the last immunization, splenocytes were stimulated in vitro with the same peptide. Seven days later, CD4⁺ T-cells were tested for their ability to respond specifically to peptide-pulsed DCs, tumor lysate-pulsed DCs, or tumor cells directly. We were able to show specific DR-restricted proliferation and cytokine production by splenocytes derived from p53 peptide-immunized animals. Moreover, mature DCs prepulsed with lysate of p53 overexpressing tumors were also recognized. This work was then translated into the human system using HLA-DR4/DR1 PBMCs from healthy donors, with similar results (81). Furthermore, in the same way that HHD II mice have proven superior to the conventional A2/Kb transgenic mice (*see* Subheading 3.2.2.1), Pajot et al. have recently demonstrated the superiority of HLA-DR1^{+/+}/IAβ^{0/0} transgenic mice (82) over HLA-DR1^{+/+}/IAβ^{+/+} mice for this purpose.

3.2.2.3. Double Transgenics. Many groups are now looking into the generation of mice transgenic for both human class I and class II molecules and knockouts of the murine class I and class II molecules. Some groups have been able to generate mice transgenic for class I molecules as well as a tumor antigen (83). Both of these models will prove extremely useful in the investigation of the role of CD4⁺ T-cells in the generation of CTLs and

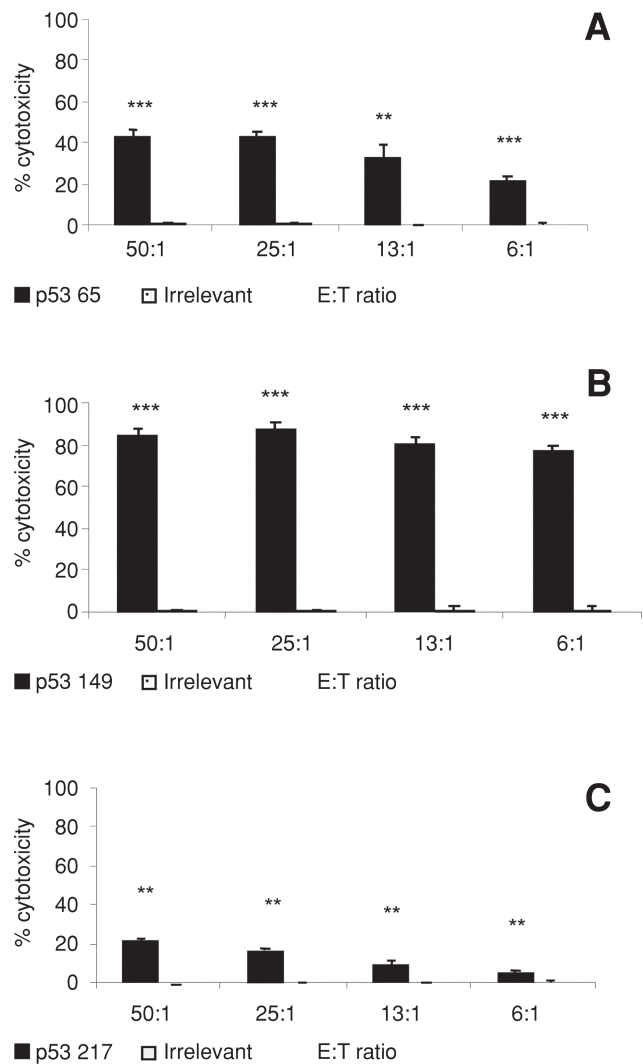


Fig. 3. Cytotoxic T-lymphocyte (CTL) activity of splenocytes derived from mice immunized with naked DNA coding for the entire human mutated p53 protein and stimulated in vitro once with p53 peptides. CTL activity of splenocytes from HHD II mice immunized three time using gold-labeled DNA encoding the entire p53 protein at weekly intervals. Each gold “bullet” contained 3 μ g of DNA, prepared in advance, administered intraperitoneally with a gene gun using compressed helium. CTL activity was measured 5 d after in vitro restimulation with peptide p53 65, 149, and 217—(A), (B), and (C), respectively—by chromium release assay using RMA/S cells pulsed with peptide as target cells. Representative traces, $n = 4$ for each peptide. ***/**Denotes significance when compared with irrelevant peptide pulsed target cell killing.

their antitumoral effect. These mice will also allow the study of tolerance toward a particular tumor antigen and how to understand the antigen’s effect(s).

3.3. Direct Isolation and Analysis of MHC-Associated Peptides

As discussed above, reverse immunology approaches are a powerful means for the definition of unmodified, high-affinity MHC-binding peptides as potential T-cell epitopes.

However, MHC peptides with low MHC receptor-binding affinities or those carrying posttranslational modifications can hardly be predicted with this approach. Among posttranslational modifications of MHC peptides, phosphorylation (84), glycosylation (85, 86), deamidation of asparagine to aspartic acid (87), and deimination of arginine to citrulline (88) are known examples. Just recently, a novel posttranslational modification of MHC peptides was described: the generation of antigenic peptides by slicing and splicing of noncontiguous protein fragments via the proteasome (45,46). All these modification possibilities lead to a tremendous diversity of MHC ligands. Therefore, the arduous approach of direct biochemical isolation for identifying potential T-cell epitopes remains an indispensable tool. As sources for the isolation of MHC peptides, either cell lines or native tumor material (solid tissue or blood from leukemia patients) can be used. The advantage of using cell lines is their nearly unlimited expansion capability *in vitro*, which allows higher yields of isolated peptides and makes the subsequent sequence analysis simpler. However, after many culture passages, it is not clear how representative the MHC peptide repertoire of a cell line is compared with the originating tumor. Where it is feasible, direct analysis of uncultured material is preferable. Two main methods based on either immunoaffinity chromatography or acid stripping of cell surfaces are normally used for the isolation of MHC peptides.

3.3.1. ISOLATION BY IMMUNOAFFINITY CHROMATOGRAPHY

The immunoaffinity chromatography method for the isolation of MHC complexes was already described in the late 1970s (89) and is based on the solubilization of the MHC complexes and their subsequent specific isolation by immobilized monoclonal antibodies (MAbs). Most of the yet known MHC peptides were isolated by this method or modifications thereof. A comprehensive list of peptides with their associated MHC alleles can be found in the SYFPEITHI database (www.syfpeithi.de). Because of the central importance of this technique, we describe our own approach in detail.

The source material for this isolation approach is normally frozen tissue, blood cells, or pellets from cultured cell lines. Requirements for providing good amounts of peptides for sequence analysis are about 10^9 cells; however, with improvements made in mass spectrometry (MS), this amount is continuously decreasing. This is very important for the analysis of native tumor tissue samples, where often only very small samples are available after surgical excision. Solid tissue is first mechanically crushed, e.g., using a ceramic mortar filled with liquid nitrogen. As soon as the material is thawed for the lysing process, cellular proteases must be blocked by protease inhibitors to avoid any cleavage of the MHC receptor complexes, which could lead to their loss. Apart from protease inhibitors, the lysis buffer normally contains between 0.5 and 2% of a detergent to solubilize the MHC receptor complexes by covering the insoluble lipophilic transmembrane regions. During the whole lysing process, it has to be ensured that the pH value is held constant between 7.0 and 8.0, because lower pH values cause release of the MHC peptides from their receptors, leading to their loss. The lysis is performed for several hours by gentle stirring of the tissue with lysis buffer. After centrifugation and filtering, the lysate is passed over MHC-specific MAbs immobilized on sepharose beads either by covalent chemical bonds or on protein A or G sepharose beads by specific noncovalent interaction with the antibody Fc part. The antibody specificity can be either for a certain single MHC receptor type (e.g., HLA-A2) or for many different types in parallel. The two most frequently used MAbs to isolate human MHC complexes (HLA complexes) are the pan-HLA

class I (i.e., all types of HLA-A, -B, and -C) antibody W6/32 (90) and the pan-HLA-DR antibody L243 (91). Technically, the binding step can be performed by cycling the lysate over antibody columns with peristaltic pumps or in batch mode by coincubating the lysate with the antibody and protein G sepharose beads in one tube. After the binding of the MHC complexes, the beads are washed with high-pressure liquid chromatography (HPLC)-grade water to remove all detergent from the lysis buffer, which interferes with subsequent mass spectrometric analysis. The release of the MHC complexes from the antibodies is normally accomplished by treatment with acid, typically trifluoroacetic acid, at pH 3.0, which causes the breakage of all noncovalent bonds. In addition to the released MHC peptides, the eluate therefore also contains MHC proteins (i.e., MHC class I: α -chain and β 2-microglobulin, MHC class II: α - and β -chain) and the antibody itself (if not covalently immobilized). The peptides can be separated from the proteins by ultrafiltration using a membrane with a molecular cutoff of 10 kDa. The flowthrough containing the MHC peptide pool is lyophilized before fractionation and sequence analysis.

The disadvantages of this approach are the requirement for large amounts of MHC-specific antibodies (10–30 mg per isolation), the complexity of the protocol, and the impossibility of distinguishing between intracellular MHC complexes and those from MHC actually expressed at the cell surface. The unequivocal advantage is the high purity of the MHC peptide isolate.

3.3.2. ISOLATION BY ACID ELUTION OF CELL SURFACES

The acid elution method was first described in 1993 (92) and is based on the release of MHC class I peptides from the cell surface by a short (15–300 s) acid treatment at pH 3.3 with a mixed citrate–phosphate buffer. This method is not used as frequently as the immunoaffinity chromatography, but it has been successfully employed, e.g., to identify T-cell epitopes from melanoma cells (93) and an immunogenic peptide deriving from the bcr–abl fusion protein (94).

As a source of material for this isolation approach, trypsinized adherent cells can be used, as well as suspension cells. These cells are accessible for the acid treatment, in contrast to solid tumor tissue, where protocols for this approach still have to be established. The crucial requirement of the method, but also its main advantage, is that the cells remain intact during the acid treatment. Cell damage would cause the release of proteases generating peptide fragments from highly abundant cell proteins and of cytoplasmic peptides and proteins. After elution, the peptides are mostly concentrated on cation exchange or reversed-phase cartridges before they are further analyzed. The apparent advantages of this method are its simplicity, cost-effectiveness, and the outlook to obtain mainly cell surface MHC peptides, which are the relevant ones for T-cell recognition. The big disadvantage is that the acid treatment is a rather unspecific method of isolation, cell damage during the process can hardly be avoided, and therefore, the resulting MHC peptides are always contaminated by non-MHC peptides and proteins (e.g., proteolytic fragments, cytoplasmic peptides, peptides from other peptide receptors).

3.3.3. FRACTIONATION AND SEQUENCE ANALYSIS OF ISOLATED MHC PEPTIDES

The sequence analysis of MHC peptide pools is a colossal challenge. On the one hand, the peptide pools are highly complex and estimated to consist of tens of thousands of different peptides even from only one cell type (95). On the other hand, the yield of peptides after isolation is low: from only several picomoles of the most dominant peptides

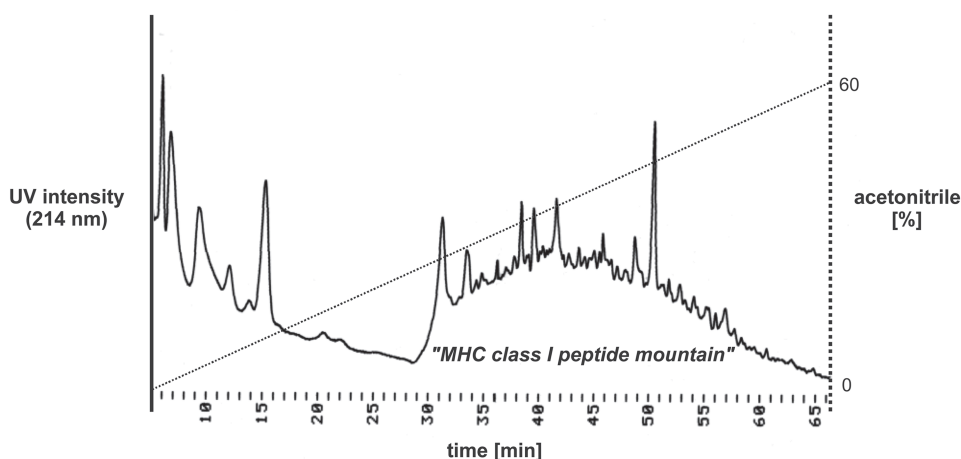


Fig. 4. Typical reversed-phase high-pressure liquid chromatography ultraviolet chromatogram of the fractionation of a major histocompatibility complex (MHC) class I peptide pool isolated by immunoaffinity chromatography with the monoclonal antibody W6/32. The MHC peptides are eluting from 30 to 65 min, giving the characteristic mountain shape.

to less than a few attomoles of less abundant peptides. Additionally, the physicochemical properties of the different peptides are rather similar (more so for MHC class I peptides with a typical length of 8–10 amino acids than for class II peptides with different lengths of approx 9–30 amino acids), which makes their purification difficult. Purification is most commonly performed by liquid chromatography, particularly by reversed-phase (RP)-HPLC, where the peptides are separated according to their lipophilicity. Ultraviolet chromatograms of such fractionations recorded at 214 nm (absorption maximum of the peptide bond) typically appear in the shape of a mountain (Fig. 4). The reason for this is that before a peptide completely elutes from the column (which would cause the respective peak to reach the baseline) another, slightly more lipophilic peptide coelutes. To improve the separation of MHC peptide pools, multidimensional liquid chromatography approaches are applicable. These are already standard in proteomics analyses (96), e.g., of tryptic pool digests of cell lysates or serum. Once the peptides are fractionated, the sequence analysis is the next difficult task. At the beginning of the 1990s, MHC peptides were frequently sequenced by automated Edman degradation (97), which is based on the stepwise chemical degradation of a peptide from the N terminus by phenylisothiocyanate and the identification of the cleaved amino acids by HPLC. The sensitivity of the method is in the low picomole range, so that only high-abundant MHC peptides can be identified. If several peptides are mixed, which frequently occurs in MHC peptide fractions, the data interpretation is very difficult or impossible because of an overlap of degraded amino acids. Since the first time that MS was used for the characterization of MHC class I (98) and class II (99) associated peptides in 1992, there has been rapid development in MS technology, driven not only by immunological questions, but also especially by proteomics projects. The basis of MS is an accurate determination of molecular masses of analyte molecules. For sequence analyses, peptides are fragmented (MS/MS) by collision-induced dissociation through a collision gas (e.g., argon) or by postsource decay, induced after ionization of the analyte by an ultraviolet laser. The mass spectra of the fragment ions generated are used to deduce the peptide sequence. Even peptides from complex mixtures can

be sequenced by MS because of the capability of modern instruments to select single precursor molecules for fragmentation (precursor selection). State-of-the-art MS instruments provide a sensitivity for MHC peptides in the subfemtomole range with a mass accuracy of lower than 5 ppm (*100*). MS can today be regarded as the gold standard for the analysis of MHC peptides. To sequence a maximum number of peptides from one isolation, the peptides can be directly transferred to a mass spectrometer from the RP-HPLC system for fragmentation analyses (online liquid chromatography-MS/MS). With this technique, hundreds of peptide fragment spectra can be recorded in one run. However, sequence data interpretation is still the bottleneck in the identification of the peptides. Several computer algorithms are available to automatically deduce peptide sequences from peptide fragment spectra (e.g., SEQUEST[®]: <http://fields.scripps.edu/sequest/>), but as yet, no one algorithm is providing high enough confidence to accept the suggested sequences without checking output by hand, which is highly time-consuming. To obtain the highest reliability of a sequence, the postulated peptide can be synthesized and fragmented and the spectra of the original and the synthetic peptide compared like a fingerprint (*101*).

4. CONCLUDING REMARKS AND PERSPECTIVE

Although the results on identification of tumor-associated antigens are very encouraging, there is still a requirement for more T-cell epitopes to enable at least the most frequent tumors and the most common HLA-phenotypes to be covered by vaccines providing optimal targets for specific and effective immune intervention in cancer. Despite the enormous progress made in the last decade toward discovery of novel tumor antigens, new strategies are still needed. A novel method for their identification is the comparative expression profiling of a tumor and the corresponding autologous normal tissue at mRNA or DNA level by DNA microarray technology (*102–104*). It is worth noting that some of the widely expressed/overexpressed TAAs were discovered by DNA microarray technologies, combined with new immunological tools such as reverse immunology and tetramer staining (*105*). Another promising approach for the differential analysis of MHC peptides from cancerous tissue and the benign tissue counterpart was described recently (*106*). The basis of the method is the labeling of peptides from the benign tissue with a light, and the peptides from the cancer tissue with a heavy, stable isotope tag or vice versa. The difference of the tags consists of an exchange of four hydrogen atoms (H_4 , light) by four deuterium atoms (D_4 , heavy), which can easily be distinguished ($\Delta 4$ Da) by MS. Importantly, the light- and heavy-labeled corresponding peptides retain the same physicochemical properties, namely, that they coelute from the RP-HPLC and appear as ion pairs with a mass difference of 4 Da. The intensities of the respective pairs can be used for the relative quantification of MHC peptides being present in both normal and cancer tissue. Despite the fact that several hundred peptides can now be identified from the isolation of a single tumor tissue because of recent hardware and methodological improvements, we are still far from the clarification of the entire MHC peptide repertoire, on demand from each individual tumor.

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