Tumor Antigens and Proteomics from the Point of View of the Major Histocompatibility Complex Peptides*

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The major histocompatibility complex (MHC) peptide repertoire of cancer cells serves both as a source for new tumor antigens for development of cancer immunotherapy and as a rich information resource about the protein content of the cancer cells (their proteome). Thousands of different MHC peptides are normally displayed by each cell, where most of them are derived from different proteins and thus represent most of the cellular proteome. However, in contrast to standard proteomics, which surveys the cellular protein contents, analyses of the MHC peptide repertoire correspond more to the rapidly degrading proteins in the cells (i.e. the transient proteome). MHC peptides can be efficiently purified by affinity chromatography from membranal MHC molecules, or preferably following transfection of vectors for expression of recombinant soluble MHC molecules. The purified peptides are resolved and analyzed by capillary high-pressure liquid chromatography-electrospray ionization-tandem mass spectrometry, and the data are deciphered with new software tools enabling the creation of large databanks of MHC peptides displayed by different cell types and by different MHC haplotypes. These lists of identified MHC peptides can now be used for searching new tumor antigens, and for identification of proteins whose rapid degradation is significant to cancer progression and metastasis. These lists can also be used for identification of new proteins of yet unknown function that are not detected by standard proteomics approaches. This review focuses on the presentation, identification and analysis of MHC peptides significant for cancer immunotherapy. It is also concerned with the aspects of human proteomics observed through large-scale analyses of MHC peptides.

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LOOKING AT THE PROTEOME FROM THE POINT OF VIEW OF THE IMMUNE SYSTEM

The proteome resembles in many ways a Japanese rock garden; looking at it from different viewpoints reveals its different intricacies. One of the many focuses of proteomics is the search for the changes in the patterns of protein expression associated with the onset of diseases. Searching for such changes is not merely a matter of scientific curiosity but also a means to develop new drugs and treatments modalities for these ailments. The immune system, likewise, attempts to identify these minute changes in the cellular proteome, while looking for the presence of telltale amounts of abnormal or foreign proteins. These serve as the alarm signals, indicating to the immune system the onset of infections and diseases. One of the ways the immune system attempts to eliminate pathogens is by killing infected cells with its cellular arm, thus preventing further spread of disease.

The major histocompatibility complex (MHC) molecules are peptide shuttles, transporting peptides from the cytoplasm to the cell surface. MHC class I molecules are products of the MHC-A, -B, and -C genes expressed in most of the nucleated cell types in the body (in humans the MHC molecules are called human leukocytes antigens, HLA). MHC class I molecules are loaded in the endoplasmic reticulum (ER) with peptides derived from proteasomal proteolysis of both normal and pathogenic proteins, most of which are 8–10 amino acids long. The MHC molecules are then shuttled to the cell surface with their peptide cargo, which are presented for scrutiny by CD8+ T lymphocytes through their T cell receptor (TCR). To prevent autoimmunity, high-affinity T cells specific for MHC molecules presenting “self” peptides are normally eliminated in the thymus by the end of the embryonic stage (central tolerance). Therefore, in healthy individuals the majority of the T cell population is thought to be composed of those T cells recognizing at high-affinity “non-self” peptides. Tight and long-lasting binding of T cells to cells presenting MHC molecules with non-self peptides elicits a cytotoxic response that results in the killing of the infected cells and prevents further spread of the disease (1–5). The body is left with T cells capable of binding to self epitopes at low affinity, and these are down-regulated by peripheral tolerance mechanisms including “ignorance” in order to prevent autoimmunity (6). Vaccination in the presence of strong adjuvant helps to overcome

1 The abbreviations used are: MHC, major histocompatibility complex; sMHC, soluble major histocompatibility complex; HLA, human leukocytes antigen; TCR, T cell receptor; TSA, tumor-specific antigens; TAA, tumor-associated antigens; SEREX, serological identification of antigens by recombinant expression cloning; ER, endoplasmic reticulum; LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; ORF, open reading frame.
tolerance by stimulating “anti-self” T cells through presenta-
tion of epitopes on activated dendritic cells (reviewed in Ref. 7).

Thus, immune recognition is tightly connected to the cellular
schemes of protein degradation. It is also affected by the
processing of the degradation products into MHC peptides
and by the properties of individual MHC molecules as peptide
receptors and mediators of immune recognition. Protein degra-
dation thus serves not only as the “end-of-life” of proteins,
aimed at recycling of their amino acids into new proteins, but
also as a means of communicating to the immune system the
health state of the cells. It puts MHC peptides at the cross-
road of proteomics and immunology, where the MHC pep-
tides can serve as indicators for aspects of the cellular pro-
tomes relevant to cancer, autoimmune, and viral diseases.
This knowledge can be exploited for developments of new
therapeutics for these diseases and also to ascertain which
proteins are expressed and degraded in the cells. The large-
scale analyses of MHC peptides thus offer a novel viewpoint
into the parts of the cellular proteome often overlooked by
other proteomics approaches. This review focuses on the
aspect of human proteomics that can be observed by large-
scale analyses of MHC peptides and their impact on cancer
immunotherapy.

TUMOR IMMUNOLOGY

Research on tumor immunology aims on one hand to find
ways to exploit the capacity of the immune system to kill
tumor cells (immunotherapy) and on the other hand to identify
new tumor markers for diagnostics, prognostics, or as target-
ing molecules. The immune system can be induced to reject
cancer cells by augmenting the existing weak anti-tumor im-
une response through therapeutic vaccination with tumor
antigens. Preferred antigens for immunotherapy are tumor-
specific antigens (TSA), which are proteins and peptides es-
sentially absent from all normal cells in the body (reviewed in
Refs. 8–18). The most desired TSA for immunotherapy are
mutated proteins, preferably those needed to maintain the
transformed state of cancer cells. Other useful TSA are em-
byronic proteins, which are expressed as a result of the loss
of cellular control prevalent in cancer cells (19). Tumor-asso-
ciated antigens (TAA) are proteins that are not unique to
cancer cells but are expressed to a larger extent in tumor
relative to normal cells, some of which are potentially useful
for immunotherapy. TAA include cancer testis antigens, which
are proteins normally expressed in immune-privileged tissues
(such as testis and placenta) and differentiation antigens,
which are normally expressed in nonessential tissues (such as
prostate, ovaries, and melanocytes). Both cancer testis anti-
gens and differentiation antigens were extensively tested for
their immunotherapeutic potential, and some of them have
been shown to induce powerful response with measurable
benefit to the patients (20).

In cancer patients, spontaneous anti-tumor immune re-
sponse are apparently too weak to cause spontaneous rejec-
tion of the tumors; otherwise cancer would not occur (13).
Therapeutic vaccination with specific tumor antigens was at-
ttempted in many clinical trials, resulting in partial response in
some of the treated patients, while a complete tumor regres-
sion was observed in only a small fraction of the patients. It
was suggested that the preferred time for immunotherapy is
at the minimal residual disease stage, because micro-metas-
tases are expected to be more responsive than large tumors
with their immune suppressing microenvironment (21, 22,
reviewed in Ref. 23).

SEARCHING FOR TAAS BY PROTEOMICS

The majority of the known TAAs were identified by different
immunological, genetic, and proteomic methods. Proteomics
was used for comparisons between tumor and normal cells
using two-dimensional PAGE, liquid chromatography (LC-
LC-mass spectrometry (MS), and surface enhanced laser de-
sorption/ionization (SELDI)-MS (24–26, reviewed in Ref. 27).
Serological identification of antigens by recombinant expres-
sion cloning (SEREX) and serological proteomics are more
recent approaches for identification of proteins against which
antibodies are present in the serum of people inflicted with
cancer. Some of the TAAs identified this way were selected
for further immunologic tests (28–30, reviewed in Refs. 24, 31,
and 32). Tumor antigens were also identified by related ge-
netic and reverse immunology approaches based on search-
ing for genes overexpressed in cancer cells using serial anal-
ysis of gene expression (SAGE) or DNA micro-arrays data
(33–35) and then further testing them for their immunogenic
properties (36, 37).

Identification of useful new tumor antigens is limited by a
number of obstacles. First, “the problem of the needle in a
haystack”: TAAs are often minor components of the cells,
hidden among other abundant proteins, which makes their
identification a demanding task (38). Second, there is no
simple way to ascertain the uniqueness of TSA expression in
tumor cells, raising the concerns that their administration
might cause autoimmune diseases inadvertently. SAGE or
micro-arrays data of all human tissues were used for screen-
ing for tumors-specific expression. However, it is possible
that the concern of causing autoimmunity was not justified in
most cases. Vaccination with antigens that are expressed
also in normal tissues, such as mucin, p53, and even total
tumor mRNA or unfractionated protein or peptide extracts,
did not cause the feared intolerable autoimmunity in most
(39–41) but not in all cases (42, 43). Third, the safety and
immunogenic potential of each tumor antigen identified needs
to be evaluated in human trials, because laboratory animals
are insufficient models in this regard. Normal human proteins
are by definition non-self in mice and therefore every identified
TAA needs to be tested for its immunogenic potential and
efficacy with humans. However, human trials with large num-
bers of different antigens are neither practical nor ethical,
making large-scale screenings impractical. Fourth, for effective immunotherapy, TAAs need to be expressed in tumor cells of the majority of patients and in the different tumors of the same patients. Cancer cells escape immunotherapy by down-regulating the expression of tumor antigens used for vaccination; it is therefore assumed that proteins that are needed to maintain the transformed state are less likely to be down-regulated by the cancer cells (44, 45, reviewed in Refs. 14, 17, and 37).

MHC Peptides as TAAs

MHC peptides are potentially very useful TAAs, and some of them were indeed demonstrated to be recognized by circulating cytotoxic T cells and then further tested with laboratory animals and by in vitro, ex vivo, and in human clinical tests (15). Most cancer immunotherapy trials failed to bring about long-lasting cure due to several reasons. In some cases the cause for tumor progression has been shown to be associated with the emergence of resistant tumor cells that either down-regulated the expression of the antigens or reduced the display of the MHC alleles (46, 47). Induction of anergy or apoptosis in the specific T cells was also observed (48). To overcome these obstacles, administration of multiple MHC peptides was attempted, either as mixtures, as parts of mini-genes, as full-size proteins, or even as mRNAs or cDNAs. Vaccination with combinations of MHC class I and class II peptides seemed to be beneficial (49, 50). Dendritic cells turn out to be the most effective antigen-presenting cells for immunotherapy. After loading them with the tumor antigens, they stimulated T cells both ex vivo and in vitro, and when grafted back to the patients they induced anti-tumor immune response, which were sufficiently strong to cause rejection of established tumors more often than the injection of the TAA alone (7, 16, 18, 51).

Identification of HLA Peptides as Cancer Vaccine Candidates

Identification of MHC peptides of potential value for tumor immunotherapy was approached using different methodologies (reviewed in Refs. 12 and 37). One of the approaches is the genetic approach, based on transfections of expression libraries made from cDNA of tumor cells into cells expressing the desired MHC haplotype. The clones of interest were selected by their ability to elicit immune response in T cells of individuals with the same MHC type. This approach was pioneered by Boon and colleagues and resulted in the identification of TAAs such as the testis antigens MAGE and the melanoma antigens Melan-A/MART and tyrosinase (52–58, reviewed in Refs. 59–61).

Another approach is the consensus peptide binding motif approach, which is based on identification of sequences that correspond to MHC peptides within the amino acid sequences of known tumor antigens (also known as the epitope deduction or reverse immunology approach). The binding motifs were first established by pooled Edman sequencing of MHC peptides recovered from immunoaffinity-purified specific MHCs (62). The amino acids observed at high levels at particular positions along the peptides were defined as the consensus residues. These were later determined to be the anchor residues of the peptides to the binding pockets in the groove. The motifs were later confirmed and refined by tandem mass spectrometry (MS/MS) sequencing of purified peptides. Dedicated software tools were used to predict the sequence of MHC peptides from amino acid sequences of proteins known to be TAA using the previously established consensus (63–72, reviewed in Ref. 73). This was only possible with well-studied MHC haplotypes, such as HLA-A2, that have well-established consensus peptide binding motifs. The predicted peptides were chemically synthesized and tested by reconstitution into empty MHC molecules at the cells' surface or into soluble recombinant MHC molecules, followed by testing their stabilization. The peptides were also tested for eliciting T cell responses. Presently, the binding motifs of most MHCs are not sufficiently defined even for the well-studied alleles. Many MHC peptides of tumor antigens such as p53, MAGE, mucin, telomerase, and NY-ESO1 were designed and tested. Some were observed to elicit immune responses in mouse models or in cancer patients (11, 15, 74). However, most of the available programs for predicting MHC class I peptides are successful in predicting only a subset of the peptides, and therefore large numbers of different alternatives need to be tested in peptide binding assays to evaluate their usefulness (75). The T cell-independent approach for identification of MHC peptides is somewhat different from the previous one. It is based on predicting the sequences of MHC peptides based on the sequences of proteins expressed in the cells or tumors. This is followed by purification of peptides from cells and their LC-MS/MS analysis while searching among them for those with the masses and fragmentation patterns similar to those predicted (33).

There are about 10 to 10 MHC-peptide complexes on the surface of each cell, and most of the cellular proteins contain a few different amino acid sequences that fit the binding motifs of known MHCs. Finding which peptides are actually presented on the cells can be done only by direct biochemical analyses. Peptides were recovered from immunoaffinity-purified MHC molecules after detergent extraction of cancer cells. The MHC molecules were denatured and the peptides were separated from the large subunits by ultrafiltration and resolved by reverse-phase high-pressure LC (RP-HPLC) into fractions. The peptides pools in each fraction were tested for their ability to stimulate T cells. The peptides in the active fractions were individually identified by re-fractionations with capillary RP-HPLC combined with electrospray ionization (ESI)-MS/MS. This method, developed by Hunt and colleagues for MHC peptide analysis, serves as the basis for the use of capillary RP-HPLC-ESI-MS/MS in today's proteomics (76–79, reviewed in Refs. 80–82). Similar to the other ap-
proaches, identified peptides of interest were chemically synthesized and tested for T cell responses. The synthetic peptides were tested for their elution time from the capillary columns and MS/MS fragmentation patterns that should be identical to those of the natural peptides, thus indicating the correct assignments of their amino acid sequences. Another advantage of the direct biochemical approach for analysis of MHC peptides is the ability to identify peptides with post-translational modifications. Peptides modified by deamidation, dimethyl-Arg, phosphorylation, and disulfide-bonded cysteine were identified by this approach (83–86, reviewed in Ref. 82).

Because MHC molecules are membrane proteins that are not present in high copy numbers per cell, they are hard to purify in large amounts as needed for peptides analyses. Two other approaches were developed to improve the yields of MHC peptides from a limited number of cancer cells. The first is based on treating cancer cells with mild acid, resulting in partial denaturation of the cell-surface MHC and the release of the bound peptides to the buffer solution, while leaving the majority of the cells intact (87). This way, the cells remain viable, and could be regrown and treated similarly a number of times, each time releasing another aliquot of peptides. Such peptide preparations were largely free of detergents and most of the cellular debris, which facilitated the subsequent LC-MS/MS analyses of MHC peptides.

Another approach for improved MHC peptide preparations was taken by Hildebrand and colleagues using HLA-B15 (88–91) and by us using HLA-A2, -B7, and -Cw4 (92, 93) based on the early experiments of Margulies and colleagues (94–99). The approach was based on transfection into cancer cells of expression vectors coding soluble, secreted MHCs lacking a functional transmembrane domain. The cells’ growth medium was collected multiple times with minimal stress to the cells, and the soluble MHC molecules (sMHC) with their bound peptides were subsequently immunoaffinity purified. With this approach, relatively large amounts of MHC peptides were purified from relatively small numbers of cells. Most cancer cell lines could be transfected with any of the different MHC haplotypes, regardless of their innate MHC haplotypes. The resulting peptide preparations were free of cellular debris or detergents, which simplified the subsequent LC-MS analyses, resulting in the identification of very large numbers of both cancer-related and normal MHC peptides (88, 90, 92, 93). These features make this method amenable for true proteomics-scale analyses of MHC peptides. The identified peptides were searched for those derived from tumor antigens, and some were synthesized and tested for their immunogenic potential. MHC peptide identification by the direct biochemical approach depends on recovery of peptides from cultured cancer cells. This raises the issue whether the peptide repertoire of these cells corresponds to the authentic repertoire of MHC peptides presented on tumor cells. To overcome this, peptides were recovered and identified from patients tumors (33–35).

BIOASSAYS: TESTING THE IMMUNOGENICITY OF MHC PEPTIDES

After being identified, the MHC peptides of interest should be tested for their potential usefulness as vaccine candidates by evaluating both their binding affinity to the MHC and their immunogenicity before and after vaccination. Usually the available amounts of natural purified peptides are not sufficient for these bioassays. Therefore, subsequent tests are performed with synthetic peptides. First, the peptides are reconstituted into empty MHC molecules, either at the cell surface or as soluble molecules, and tested for their binding affinity to MHC molecules and their stabilization. Empty MHC molecules are available for reconstitution on mutant cell lines, such as RMA-S or T2, which are defective in their peptide loading process. The binding of the peptides and the resulting stabilization of the MHC are followed by fluorescence-activated cell sorter analysis using an anti-native MHC monoclonal antibody such as W6/32 (100). Alternatively, an assembly assay can be performed by adding the peptides to detergent extracts of RMA-S or T2 and following the conformational change in the class I heavy chain and stable association of the heavy chain with β₂–microglobulin (101). The peptides can also be reconstituted into recombinant sMHC and assayed as dimers (102) or tetramers (103).

T cells respond to in vitro stimulation or vaccination by peptides presented on antigen-presenting cells or on MHC tetramers is tested by following the synthesis or the secretion by T cells of cytokines, such as interferon γ or interleukin-2. The assays include enzyme-linked immunosorbent spot, intercellular cytokine expression, and cytotoxic T cell assays. Immunogenicity can also be tested by delayed-type hypersensitivity or by establishing cytotoxic T cell lines from peripheral blood lymphocytes and following their ability to kill target cells that are presenting the studied peptides. Unfortunately, none of these assays is amenable to high-throughput screening of large numbers of different MHC peptides or of their source proteins (103–110, reviewed in Refs. 15 and 111). Testing the immunogenic potential of the MHC peptides is complicated even more by the problem of degeneracy of recognition of multiple peptides by some T cell clones (112). The specificity of T cells to a particular peptide is not always one-to-one. In reality, different peptides can activate one T cell clone. This may limit the use of MHC peptides in the clinic due to concern of inducing autoimmune disease inadvertently (109).

LOOKING AT THE CELLULAR PROTEOME FROM THE POINT OF VIEW OF THE MHC PEPTIDES

Now, as large datasets of MHC peptides are being established, they can be used as a new approach for shotgun or comparative proteomics. Indeed, the data obtained by large-scale analyses of MHC peptides points to the source proteins of these peptides. Among these, there are many novel and yet unstudied proteins, many of which were not observed by routine proteomics. A possible explanation is that even
though every MHC peptide represents a protein and most cellular proteins contribute one or more peptides to the MHC peptide repertoire, MHC peptides mirror more the patterns of protein degradations rather than the proteome of the cells. The cellular proteome is established by the rate of synthesis of the proteins and by their half-lives in the cells (113, 114). It is conceivable that some slowly synthesized proteins with relatively long half-lives accumulate in cells to a larger extent than other proteins synthesized at a high rate, which are degraded rapidly. MHC peptides are generated only after their proteins of origins have degraded. Therefore, MHC peptides are actually “memories” of past existence of their source proteins. However, once bound to the MHC molecules at the cell surface or on sMHC, the peptides resist degradation for hours. Therefore, it is likely that some relatively abundant MHC peptides originate from proteins that are almost absent from the cells due to their rapid degradation or to incomplete synthesis. It was suggested that significant portions of the proteins synthesized in the cells are rapidly degraded as a normal process in the cell and not only due to errors in their synthesis or improper folding. These proteins, termed “defective ribosome products,” are possibly a major source of the MHC peptides repertoire (114–117, reviewed in Refs. 118–121). This may explain some of the discrepancies between the cellular transcriptome and proteome (113, 122). Moreover, short-lived proteins were demonstrated to be the source of many of the peptides transported by TAP, the transporter associated with antigen processing (123). It was suggested that relatively rapid degradation of some of the newly synthesized proteins allows improved efficiency of protein synthesis by degrading defective ones rather than investing a significant effort in high-fidelity synthesis. Moreover, speeding the response to pathogens by rapidly degrading a fraction of all newly synthesized proteins and displaying the resulting MHC peptides to the T cells confers health benefits that can compensate for the seeming inefficiency of the protein synthesis machinery (114, 119). Studying the cellular proteome by large-scale analyses of the MHC peptides may help to identify such rapidly degrading proteins, some of which may actually be rare proteins that are undetectable by other methods. Among these proteins that did not fold properly or did not join their appropriate functional protein complexes and were subsequently degraded rapidly there may be dysfunctional tumor suppressors and cell cycle regulatory proteins. Such dysfunctional proteins can be identified among the pools of MHC peptides.

A new spin-off from proteomics, called degradomics, is a new research direction aimed at analyzing the cellular proteases and their substrates (124). Because MHC peptides are products of proteolysis, their large-scale analysis offers an insight into the subset of cellular proteases of most interest to immunologists (125). The final size of MHC peptides actually presented at the cell surface reflects the efficiency of proteolysis of the source protein, at the proteasome or elsewhere (126–129, reviewed in Refs. 2, 120, 121, and 130–132). The presentation of peptides by the MHC molecules serves also as an indication of their transport efficiency into the ER by TAP (123, 133, 134) or by other transporters (135), the efficiency of their amino-peptidase trimming to the final length after binding to the binding groove on the MHC in the ER, and their binding affinity to the MHC molecules (125, 136, 137). Those peptides most effectively processed, transported, and displayed are also better represented among the peptides recovered from cell surface or from soluble MHCs. Such peptides possess some of the desired attributes needed for immunotherapy.

The patterns of cellular MHC peptides change in response to external stimulation not only in accordance with the cellular proteome but also with changes in the proteolytic processing machinery. Stimulation of the immune system due to infections or inflammation leading to elevated cytokines (such as interferon γ), induces a transition of the proteasome to the immunoproteasome that contains different catalytic subunits (2, 138–141). The immunoproteasome is more active in proteolyzing cellular proteins. It has an altered preference for peptide cleavage sites and is capable of more efficient transport of peptides into the ER (reviewed in Refs. 142 and 143). Therefore, the presentation of specific MHC peptides need to be ascertained separately in healthy cells expressing the normal proteasome and in disease cells expressing the immunoproteasome (140, 144).

The inherent technical difficulties of analyzing MHC peptides make proteomics based on these analyses a more torturous route to take compared with other proteomics approaches based on two-dimensional PAGE or two-dimensional LC. There are close to a thousand different MHC haplotypes in the human population, each possibly displaying a different set of peptides due to the polymorphism of their binding groove (www.ebi.ac.uk/imgt/hla/stats.html). Moreover, in most cases only one MHC peptide is detected from each individual protein. In routine proteomics, proteins are trypsinized and therefore each protein is represented by many peptides, all contributing to its correct identification. Also, in most cases MHC peptides are not preceded or terminated by a particular amino acid (such as Lys or Arg in tryptic peptides). Thus, the numbers of all the possible MHC peptides in the protein databanks are very large. The MS/MS analyses are further complicated due to the fact that many of the MHC peptides are singly charged, often causing their MS/MS spectra to be suboptimal for identification. The molar amounts of MHC peptides are significantly lower than the amounts of proteins obtainable from the same number of cells. Even with the relative high-yield method of purifying peptides from sMHC, there is still a need to grow about 10- to 100-fold more cells to obtain sufficient amount of peptides for MHC peptide identification by μLC-MS/MS than the number of cells needed for identification of the same number of proteins. Moreover, similarly to cellular proteins, MHC peptides are presented at
varying copy numbers at the cell surface (also with sMHC), thus complicating the analyses even further (145).

The shortage of dedicated software tools is another limiting factor for large-scale analysis of MHC peptides, even more than for regular proteomics. The available software tools such as Sequest and Mascot are optimized for protein analyses (146–150). They combine MS and MS/MS data of a number of different peptides derived from each protein for the identification of the protein. However, MHC peptides analyses calls for the identifications of each of the proteins represented as an MHC peptide separately. Significant computer resources are therefore needed to identify each of the peptides detected in the LC-MS/MS run. Proteomics-scale projects of MHC peptides often include dozens of LC-MS/MS runs, resulting in hundreds of thousands of different MS/MS spectra. To overcome this problem, Beer et al. have developed a new software tool called Pep-Miner to cluster MS/MS data from different LC-MS/MS runs.² It uses the clustered spectra to identify the peptides in the protein data banks and to compare patterns of MHC peptides between runs. Because every cluster combines many MS/MS spectra of one peptide, it has a better signal-to-noise ratio and a better accuracy than each of the individual MS/MS spectra members of the cluster. We have recently purified and analyzed MHC peptides from the soluble HLA haplotypes (A2, B7, and Cw4) and have observed the presence of a few thousand different MS/MS clusters each possibly representing an MHC peptide. Out of these, a few hundred MHC peptides were identified at high certainty (92, 93).

Databanks containing listings of MHC peptides are available on the internet with software resources for T cell epitope predictions (151, 152). Links to these websites are: syfpeithi.bmi-heidelberg.com/, www.cancerimmunity.org/peptideDatabase/TcellEpitopes.htm, and more are listed in MHCligand.ouhsc.edu. The sequences resulting from our large-scale analyses of peptides that were identified by the Pep-Miner software are listed at: www.technion.ac.il/~admon/MHCpeptides.htm. The Pep-Miner software is described at www.haifa.il.ibm.com/projects/verification/bioinformatics/.

FUTURE DIRECTIONS

Novel Gene and Open Reading Frame Identifications Based on Sequencing of MHC Peptides—The identification of every MHC peptide indicates the expression of its source protein that can be traced back to its open reading frame (ORF) and gene. This opens the possibility of using the amino acid sequences of identified MHC peptides to identify their coding sequences. It turned out that many of these are expressed from introns, novel ORFs, or genes that were overlooked by other approaches (153–157). New DNA sequences of very small ORFs or genes with unusual consensus can be identified this way. Such assignments of new genes and ORFs are likely to be straddled with artifacts due to the immense size of the genome with its six reading frames and the myriad possible alternative splicings, combined with the usual less-than-optimal MS/MS data of MHC peptides. As high-accuracy MS and MS/MS analyses become possible, the confidence of assignments of MHC peptides to DNA sequences will be improved, making such assignments a viable possibility (158–160).

The Human MHC Peptide Project—One of the most ambitious proposed research projects concerning MHC peptides involves the determination of the repertoires of all the MHC peptides displayed by the different cell types in the context of the different MHC haplotypes (161). The expected investments and technical obstacles are enormous but the outcome can be very rewarding.

The vast majority of MHC peptides identified so far were from cultured cells rather than from tissues or tumors (the same issue arises with proteomics analyses of culture cells versus cells in their tissues of origin). Which peptides are displayed by cells in body tissues still remains largely unanswered. Acid extractions of MHC peptides from murine tumors were attempted but only a few peptides were identified (162). Immunoaffinity purification of MHC from human tumors, followed by subsequent analyses of the purified MHC peptides, has not been performed as often as with MHC molecules purified from culture cells (33, 34).

Viral and Autoimmune MHC Peptides—MHC peptides can be identified from cells after viral infections, and the identified peptides may serve as new vaccines (163). It is possible to infect cells with viruses and to follow the appearance of MHC peptides derived from the viral proteins. Such vaccines made from peptides or from mini-genes containing the peptides may be safer for use and less expensive to produce and administer than inactivated viruses (111). Autoimmune diseases are often mediated by MHC peptides, and some diseases are linked to specific MHC haplotypes. The MHC peptides that mediate the autoimmune diseases may serve as frameworks for construction of homologous peptides in order to induce anergy in the abnormally activated T cells or loaded on soluble multimers to target the specific disease causing T cell clones. Analysis of MHC peptides presented on particular haplotypes on cells that are targets of the autoimmune T cells may help to identify such disease causing peptides (164).

Personalized Medicine with MHC Peptides—The use of MHC peptides in the clinic as therapeutics for cancer, autoimmunity, or infectious diseases requires a shift to personalized medicine in order to adjust the MHC peptides used as therapeutics to the genetic MHC types of the patients. Because peptide sequences and consensus binding motifs are available for only a small subset of the different MHC haplotypes, only those patients with these haplotypes will be treatable (34, 165, 166). It may become possible to use tumor antigens to vaccinate people at risk of developing specific

² Beer, I., Barnea, E., Ziv, T., and Admon, A., submitted for publication.
cancers as immuno-prevention, again matching the peptides to be used for preventive vaccination with the MHC types of the individuals (167).

**TCR-like Antibodies May Help to Bypass the Need to Vaccinate**—MHC molecules displaying specific peptides on cancer cells can be targeted even if the displayed peptides are not very immunogenic. There are many more known displayed cancer-specific MHC peptides than cancer-specific cell surface proteins. MHC molecules displaying cancer-specific peptides, including those that are not sufficiently immunogenic, are valid targets for recombinant antibodies or recombinant TCRs directed against cell surface MHC-peptide complexes (168–174). Such anti-MHC-peptide antibodies (TCR-like antibodies) have been developed and tested for their ability to target cancer cells (175, 176). This opens up a whole new field of targeting cancer cells by recombinant TCRs and TCR-like antibodies. TCR-like antibodies specific for cancer MHC peptides were made as chimeras with the fragment crystallizable parts of the TCR and transfected into already stimulated T cells, providing them with a new anti-cancer specificity (169, 170).

**CONCLUSIONS**

The significance of the analysis of MHC peptides is now well established for development of therapeutica, including new useful tumor vaccines. However, the repertoire of MHC peptides can facilitate the identification of their source proteins, whose rapid degradation is significant to cancer progression and metastasis. Moreover, the identified MHC peptides can help identify new proteins of yet unknown function that are not normally detected by standard proteomics approaches.

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