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The contributions of mass spectrometry to understanding of immune recognition by T lymphocytes

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Abstract

Over the last 15 years, the ability of mass spectrometry to analyze complex peptide mixtures and identify individual species has provided unprecedented insights into the repertoire of peptide antigens displayed by MHC molecules and recognized by T lymphocytes. These include: understanding the peptide binding specificity of MHC molecules; understanding of roles of different intracellular components of the antigen processing pathways in determining the peptide display; and identification of a large number of individual peptide antigens associated with infectious diseases, cancer, and transplant rejection that have provided the basis for new immunologically based therapies. This review will summarize the impact that the application of mass spectrometry has had on these advances, with particular attention to the contributions of Professor Donald Hunt and members of his laboratory, and point out the opportunities for future work.

Keywords

antigen processing; post-translational modification; peptides; MHC molecules

1. THE NATURE OF ANTIGEN RECOGNITION BY T LYMPHOCYTES

T lymphocytes are a branch of the immune system concerned with recognition of antigens that are displayed on the surface of host cells. These antigens are produced through intracellular proteolytic mechanisms that create small peptides, which are then rescued and presented at the cell surface by adaptor proteins called MHC molecules [reviewed in [1–6]. The binding site of each MHC molecule enables it to bind to a wide range of different peptides, and results in the display of a repertoire of such antigens, each recognized by a distinct T lymphocyte. This “antigen processing and presentation” mechanism results in the display of peptides derived from the proteins of pathogens that have infected the cell or been taken up by endocytosis. Their recognition by T lymphocytes results in the development of an immune response, and results in clearance of the pathogen and infected cells from the body. However, antigen processing and presentation pathways operate constitutively on normal cellular proteins as well, resulting in the display of peptide antigens that can be distinguished on tumors and transplanted tissues. Some of these “self-peptides” are also involved in the development of T lymphocytes in the thymus, and their recognition also controls the balance between self-tolerance and autoimmune disease.

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There are two different classes of MHC molecules, distinguishable by their structures, the intracellular pathways through which they encounter peptide antigens, and their recognition by different kinds of T cells. The peptides presented by class I MHC molecules are typically generated from proteins that are degraded by proteases in the cytosol. They are then transported into the lumen of the endoplasmic reticulum (ER) via the transporter associated with antigen processing (TAP), become associated with newly synthesized MHC class I molecules, and the complexes are trafficked to the cell surface. This pathway operates constitutively in all host cells. The antigens presented are recognized by CD8 T lymphocytes, which typically kill the displaying cell or secrete cytokines such as interferon- γ or tumor necrosis factor- α , resulting in local recruitment of other immunological effectors. The peptides presented by class II MHC molecules are typically generated from proteins that are expressed at the cell surface or captured by cell surface receptors. They are internalized into endosomes and degraded by endosomal proteases, after which the peptides associate with newly synthesized class II MHC molecules. This pathway usually operates only in a subset of cells called antigen presenting cells (APC), which includes dendritic cells, macrophages, and B lymphocytes. Peptide antigens displayed by class II molecules on these cells are recognized by CD4 T lymphocytes, which regulate the development of immune responses. Based on the relatively promiscuous binding of MHC molecules, both of these pathways result in the display of information derived from a large fraction of proteins that are either made inside the cell or internalized from the extracellular environment [7,8]. Infection or encounter with foreign extracellular agents results in the display of new information derived from foreign proteins and consequent stimulation of immune responses.

In most individuals, molecules of each MHC class are expressed from 3 genetic loci that encode structurally similar but non-identical proteins. At the level of the human species, each of these loci shows tremendous genetic polymorphism, with as many as 400 different allelic forms having been defined for some loci [9,10]. This creates a very large genetic pool of structurally distinct class I and II MHC molecules, from which a small subset are represented in any individual human. Most of the polymorphic differences among MHC molecules encoded by different genes and alleles alter the structure and specificity of the peptide-binding site. Thus, the polymorphism of MHC molecules results in an increase in the overall information content displayed on each cell for inspection by the immune system.

2. STRUCTURAL CHARACTERISTICS OF PEPTIDES DISPLAYED BY MHC MOLECULES

The first application of mass spectrometry to analysis of MHC associated peptides occurred soon after the demonstration by Rammensee and colleagues that individual peptide antigens, already known to be recognized by T cells when presented by certain class I MHC molecules, could be extracted from whole cells using acid and fractionated by HPLC [11,12]. They also demonstrated that Edman degradation of the entire peptide mixture showed dominant amino acids in cycles 2 and 9, and that the amino acids found in these two cycles were dependent on the MHC molecule from which the peptides were isolated [13]. This suggested that all of the peptides associated with a single allelic class I MHC molecule shared a common length and that the conserved amino acids were “anchor residues” determining a binding motif.

Using microcapillary HPLC/electrospray ionization/tandem mass spectrometry on a triple quadrupole instrument, Hunt and co-workers analyzed and sequenced peptides isolated from single allelic class I MHC molecules [14–16]. This data gave a more complete picture of these peptides than had been predicted by Edman degradation of peptide mixtures [13]. As expected, all of the peptides were derived from normal cellular proteins. About 70–80% of class I associated peptides are 9mers, but others range up to 14 residues. These longer peptides can bind with high affinity [17], and in some cases extend outside of the binding site [17,18].

Although more amino acid variation was found at anchor positions than was previously appreciated, this work also established the existence of sequence conservation at additional positions. This was later shown to be important by molecular modeling and direct binding studies. Overall, these results significantly extended the understanding of the factors that control the binding of peptides to class I molecules.

Mass spectrometry also was used to analyze peptides associated with class II MHC molecules. In contrast to class I MHC molecules, the peptide-binding site of class II molecules is not constrained at the ends. This allows considerable heterogeneity in the lengths of the associated peptides, and complicates localization of binding motif elements because they do not have a common phasing with respect to the peptide ends. Edman degradation analysis provided information about a few dominant peptide sequences [19]. Mass spectrometry analysis of class II MHC associated peptides provided a much more robust sequence database and established directly that many peptides occurred in nested sets with common core sequences and variable ends [20–24]. This information enabled provisional assignment of motif elements. It also established that the lengths of these peptides were substantially larger than necessary to occupy the peptide binding site defined from X-ray crystallographic studies – such “minimal length” peptides were not significant components of the extracted peptide mixture [24]. This suggested that the peptide sequence initially associated with the class II MHC molecule was contained within a larger peptide or protein fragment that was subsequently trimmed by proteases. A final observation was that the peptides primarily originated from source proteins made by the cell itself, and usually with a membrane or endosomal location. This was unexpected, in that the prevailing immunological models at that time were based on of model protein antigens added extracellularly in large quantities. Thus, this analysis suggested that class II MHC molecules would more readily present peptides from extracellular proteins if they were concentrated into endosomal compartments through receptor-mediated uptake.

Mass spectrometry also enabled estimates of the representation of individual peptides and the complexity of the overall repertoire. By standardizing against the ion current of known amounts of model peptides doped into extracts, many MHC-associated peptides are present at 1 copy or less per cell [14,25]. More typically, peptides are present at 10–400 complexes per cell [14,15,26], but a single viral peptide from cells infected with measles virus has been found at the 100,000 copies per cell [27]. How does this wide variation affect their ability to be recognized by the immune system? The answer is not altogether clear. The number of peptide-MHC complexes required for T lymphocyte recognition varies from several thousand per target cell to as few as one [25,28–33], although weak recognition of other peptides may contribute to the T cell activation process [34]. Some studies have shown a direct correlation between cell surface densities of individual peptide antigens and the magnitude of the immune response against them [35–39], but other studies have shown exactly the opposite [25,27,40]. Higher doses of peptide antigen can actually reduce the magnitude of an immune response in vivo [27,41]. Finally, there are examples of self-peptides that become antigens on cancer cells [42,43], suggesting that the immune response may be initiated because their level of presentation is altered. A more systematic examination of how antigen cell surface density affects the immune response is still needed.

By using off-line HPLC to achieve greater peptide separation and subsequent enumeration of ions in representative fractions, early analyses yielded an estimate of 10,000–20,000 different peptides displayed at greater than 1 copy per cell by individual class I and II MHC allelic forms on B lymphoblastoid cells [14]. Given the relatively low-resolution separations employed and the subsequent demonstration that peptides at lower copy numbers are also evident, this seems certain to be an underestimate. Peptide mixtures with a similar apparent degree of complexity have been isolated from several other cell types with somewhat lower levels of MHC molecules. Since most fully heterozygous individuals will express between 3 and 6 different

class I MHC molecules, this represents a potential display of 30,000–120,000 bits of information on the surface of every cell. A similar level of complexity is evident for class II MHC molecules. This repertoire represents a status report to the immune system on protein expression and degradation in each cell.

3. IDENTIFICATION OF PEPTIDES RECOGNIZED BY SPECIFIC T LYMPHOCYTES

A major challenge for immunologists is the identification of the small number of new peptide species that are presented by MHC molecules on cells that have become infected or transformed. When a source protein can be readily identified, the peptide antigen can usually be determined by using overlapping peptides spanning the sequence. These are incubated with a cell that expresses the appropriate MHC allele, and the correct peptide will bind and create a target for T cell recognition [44]. This approach has been enhanced by the development of algorithms to predict the ability of peptides to bind to individual MHC molecules [45,46]. These algorithms have been substantially improved by making use of the database of naturally occurring MHC-binding peptides that have been identified by mass spectrometry [47]. However, this approach is of limited value in identifying peptide antigens displayed on tumor cells, or those infected with complex pathogens, where the source protein cannot be readily identified.

In principle, the ability to sequence peptides in HPLC fractions that had been established to have immunological activity should allow direct identification of peptide antigens without reference to the original source protein. However, even after multiple rounds of HPLC fractionation, these fractions still contain over 100 peptides, and the active species is frequently not among the most dominant. A major advance was the development, by Hunt and colleagues, of the “splitter” technique. In the approach, the effluent from a microcapillary HPLC column coupled to the mass spectrometer was split, allowing a high precision correlation to be made between the elution profile of individual peptides and biological activity in an antigen reconstitution assay [43,48–50]. This approach has been used to identify a large number of individual peptide antigens that are presented by class I MHC molecules and recognized by CD8 T cells. These include: transplantation antigens recognized by T cells during the course of tissue graft rejection and graft versus host disease [26,49–57]; antigens expressed on melanoma [43,58–60], lung carcinoma [61], colon carcinoma [62], breast and other epithelial carcinomas [63], and UV-induced sarcoma [64]; antigens derived from intracellular bacteria [65,66]; and antigens recognized during the development of autoimmune diabetes [67]. A similar approach has enabled the identification of peptide antigens presented by class II MHC molecules and recognized by CD4 T cells [68]. These antigens now serve as the basis for development of immunologically based therapies, particularly for cancer and infectious diseases. Several have been incorporated into clinical trials [69–71]. The identification of the source protein from which the antigen originates has also stimulated new questions about how the immune system is able to distinguish cancer cells from their normal counterparts.

4. BEYOND PEPTIDES: LIPIDS AND CD1 MOLECULES

CD1 molecules are structurally similar to conventional class I MHC molecules, but do not exhibit extensive allelic polymorphism. Instead, there are 4 such molecules (CD1a, CD1b, CD1c, and CD1d), each encoded by a distinct gene [72]. Their function remained mysterious until it was demonstrated that they presented lipid molecules derived from bacterial pathogens to unusual subsets of T cells [73–76]. The structures of the presented lipids have largely been inferred from the wealth of information about pathogen-based lipids, coupled with synthesis and confirmation. However, mass spectrometry was recently used to establish that the CD1a molecule presents a previously unidentified family of lipopeptides derived from

Mycobacterium tuberculosis [77]. In addition, it has also been shown that CD1 molecules present lipid antigens derived from host cells as well as pathogens [78,79]. These provide another means to recognize tumor cells, and they also seem to mediate early activation of T cells in situation of cell stress or inflammation. These lipid-based antigens remain relatively poorly characterized, despite their potential importance: an opportunity for mass spectrometrists.

5. THE IMPACT OF ANTIGEN PROCESSING PATHWAYS ON THE DISPLAY OF MHC ASSOCIATED PEPTIDES

The peptides presented by class I MHC molecules are typically generated from proteins that are degraded by proteases in the cytosol, one of which is the proteasome. These degradation products are transported into the lumen of the ER via the transporter associated with antigen processing (TAP) [reviewed in [1]. This suggests that peptide cleavage specificities of the proteasome and transport specificities of TAP should play an important role in determining which peptides are available for binding to class I MHC molecules, and thus displayed at the cell surface. In keeping with this idea, the quantitation of naturally presented peptides using mass spectrometry established that their cell-surface densities were not directly correlated with their class I MHC binding affinities [17,80].

The identification of several human minor histocompatibility antigens (mHAg)s by mass spectrometry led to further insight into this issue. Minor histocompatibility antigens are MHC-associated peptides that are derived from allelic forms of the same protein expressed in different individuals. During tissue transplantation, these can be distinguished as “foreign”, leading to graft rejection or graft-versus-host disease [11,81]. In most cases, the allelic forms of the peptides differ by 1–2 residues. In some cases, both peptides bind well to the relevant MHC molecule, but the substitutions are distinguishable by T lymphocytes [50,52,53]. For other mHAg)s, both allelic peptides bound well to the MHC molecule and were well recognized by the same T lymphocytes when added as exogenous synthetic peptides [53,55,56,82]. However, mass spectrometry analysis failed to identify the “negative” peptide at the cell surface when it was produced by intracellular degradation of the source protein. In one case this was due to the failure of the negative peptide to be efficiently transported by TAP [55], while in another, the amino acid substitutions in the negative peptide enhanced its destruction by the proteasome [56]. This work helped to establish the importance of the class I MHC antigen processing pathway in controlling the display of structurally similar peptides.

Subsequently, mass spectrometry has been used to demonstrate how variations in the structure and activity of proteasomes and TAP transporters affect the repertoire of peptides displayed on cells by class I MHC molecules [83–85], and to analyze the products of proteasome proteolysis in vitro [86–89]. The latter efforts have led to the creation of algorithms that predict proteasome cleavage sites [90,91]. These algorithms have been combined with those for class I MHC peptide binding [92], and in some cases for TAP transport [93], to predict the peptide antigens most likely to be displayed. This approach has led to the successful identification of several antigens [94–96].

6. DEFINITION OF ANTIGEN PROCESSING PATHWAYS FOR MHC ASSOCIATED PEPTIDES

6.1. Involvement of HLA-DM/H-2M in antigen presentation by class II MHC molecules

Newly synthesized class II MHC molecules bind to peptides in endosomal compartments, rather than in the endoplasmic reticulum. A protein called invariant chain (Ii) binds to class II molecules immediately after synthesis, occluding the peptide-binding site and diverting the

complex to endosomal compartments within the cell. The Ii molecule is degraded at this point, allowing the class II MHC molecules to move to the cell surface. In the early 1990s, mutant cell lines were discovered in which the class II MHC molecules appeared partially folded, suggesting that there was a defect in some part of this pathway. By using mass spectrometry [97] and Edman sequencing [98], it was discovered that the peptides associated with these molecules were dominated by a nested set derived from one portion of Ii, called CLIP. Soon thereafter, several groups showed that a molecule called HLA-DM in humans and H-2M in mice catalyzed the displacement of these remnants of Ii to allow binding of other peptides [99–101], and that HLA-DM was defective in the mutant cells [102,103]. Subsequently, mass spectrometry analysis of peptide repertoires has demonstrated that HLA-DM/H-2M functions as a “peptide editor”, displacing not only CLIP peptides but poorly-fitting endogenous peptides [104]. A related molecule, HLA-DO/H-2O, serves a similar function [105,106], although its specificity is distinct. Thus, mass spectrometry has played a central role in uncovering the existence of molecules that fine-tune the peptide repertoire displayed by class II MHC molecules.

6.2. Involvement of tapasin in antigen presentation by class I MHC molecules

Peptide binding to class I MHC molecules in the ER is necessary for them to fold stably and be expressed at the cell surface. Several additional proteins assist in this folding process. Of particular interest is tapasin, which is associated with both TAP and partially folded class I MHC molecules in the ER, and dissociates upon peptide binding. One suggested role for tapasin is as an “editor” to augment removal of low affinity peptides, analogous to that of HLA-DM for class II MHC molecules. This hypothesis was addressed by using the high resolution accurate mass capabilities of Fourier Transform mass spectrometry instrumentation, coupled with software to display 3-dimensional peptide profiles from two samples and compare them [107]. The repertoire of peptides displayed by class I MHC molecules was substantially altered in cells that did not express tapasin. However, the class I MHC binding affinity of the peptides from the tapasin negative cells was actually higher than that from the tapasin positive cells. These results suggested that tapasin does not function as an editor, but instead stabilizes partially folded class I MHC molecules to accept a larger array of peptides with a broader range of binding affinities.

6.3. Processing and presentation of membrane proteins in the ER and cytosol

The use of mass spectrometry to analyze MHC associated peptides in mutant cell lines also led to the discovery of a distinct pathway of peptide generation, which has subsequently led to additional insights into the processing of peptides derived from membrane associated and secreted proteins. In cell lines lacking the TAP transporter, most class I MHC molecules are expressed at extremely low levels, indicative of the critical role of peptide in their folding. The remaining class I MHC molecules present peptides derived from the signal sequences domains responsible for co-translational translocation of proteins into the lumen of the ER [51,108]. These peptides are also presented in cells expressing TAP [51], indicating that they are a normal component of the peptide repertoire. Numerous antigenic peptides derived from signal sequences and presented in a TAP-independent manner have since been reported [48,109–112]. Conversely, presentation of several peptide antigens derived from signal sequences is dependent on TAP function [113–115] and carboxyl terminal trimming in the cytosol [116], indicating the existence of a still poorly characterized mechanism for retrograde transport of peptides from the ER to the cytosol. ER proteolytic activity is primarily that of aminopeptidase while carboxypeptidase activity is limited [117]. An interesting adaptation of this pathway came to light with the demonstration that two unusual class I MHC molecules, HLA-E in human and Qa-1b in mice, are specialized to present a signal sequence derived peptide that is highly conserved among all class I MHC molecules [113,118,119]. This system serves as a sensor for viruses that attempt to evade the immune response by inhibiting expression of class I MHC

molecules. Under these circumstances, the loss of HLA-E/Qa-1b expression renders the cells susceptible to an alternative mechanism of destruction mediated by NK cells.

7. IDENTIFICATION OF POST-TRANSLATIONALLY MODIFIED PEPTIDES PRESENTED BY MHC MOLECULES

The use of mass spectrometry for direct sequencing of MHC associated peptides also led to the surprising observation that many have undergone structural alteration or postranslational modification. The first such modification was detected by Hunt and colleagues as asparagine deamidated to aspartic acid in a peptide antigen derived from tyrosinase [58]. In melanoma cells expressing full-length tyrosinase, the deamidated (Asp-containing) form of this peptide is presented at high copy number by class I MHC molecules, while the Asn-containing form encoded by the gene is not detectable using either T cells or mass spectrometry [58,120]. Subsequently, 3 additional deamidated peptide antigens from viral proteins has been reported [121–123]. In all four cases, the modified residues are Asn-linked glycosylation sites, and it has subsequently been shown that deamidation is a consequence of the removal of the carbohydrate by peptide-N-glycanase [124], which normally accompanies protein degradation [125–127].

Additional work has identified a wide range of enzymatic modifications in peptides associated with class I and II MHC molecules (reviewed in [128]. Among these, O-linked glycosylation of Lys [129], N-linked glycosylation of Asn [124,130], N-terminal acetylation [131], methylation of Arg [130], and phosphorylation of Ser and Thr [61,129] have all been identified through the use of mass spectrometry. Hunt and colleagues have also identified several peptides containing cysteine residues that have been altered by the attachment of another cysteine residue via a disulfide bond [53,132,133]. This non-enzymatic reaction is due to disulfide exchange involving cystine, a normal component of serum. Most of these modifications have been shown to significantly affect recognition by T cells (reviewed in [128]. Thus, depending on the extent to which it occurs, and its influence on antigen processing, an individual post-translational modification may alter the repertoire of peptides displayed by MHC molecules by simply substituting one peptide for another, creating a more diverse collection, or enhancing the processing of a peptide sequence. Many post-translational modifications known to occur in proteins have yet to be identified on MHC-associated peptides, and others are represented as yet only by single examples. Mass spectrometry can play a central role in establishing the range of modifications present on these peptides, as well as their representation in the total repertoire.

Many of the modifications so far described occur constitutively (for example, N-linked glycosylation), but others (phosphorylation, methylation) are regulated by alterations in cell growth/signaling inflammation, necrosis/apoptosis, or disease [134–138]. This category of modified MHC-associated peptides is of particular interest. They may represent important antigens displayed on infected cells as a consequence of inflammatory reactions [139,140], or may create new targets for immune recognition of cancer cells [61,129,141,142]. They have been shown to be involved in the development of autoimmune diseases [143]. These create new possibilities for neoantigens to be presented, and new opportunities for the development of therapies against pathogens, transformed cells and autoimmune diseases.

8. FUTURE DIRECTIONS AND CHALLENGES

The direct analysis of naturally processed peptides displayed by MHC molecules has offered several important insights into the characteristics and origin of this repertoire. In moving forward, continued improvements in mass spectrometry instrumentation and bioinformatic software offer the possibility to push to new levels of comprehensiveness and understanding.

The use of ion trap mass spectrometers, coupled with data-dependent MS/MS analysis [144] and database searching algorithms such as SEQUEST [145], has enabled the identification of as many as 700 to 900 MHC-associated peptides in 1 or 2 runs [24,146], and unpublished]. The use of Fourier Transform instrumentation in combination with the ion trap promises to increase these numbers still further. Fourier Transform instrumentation also enables estimation of relative abundance of different peptides [24,25], providing an additional dimension of information for use in understanding how the immune system discriminates important antigens. Combined ion-trap and Fourier Transform instrumentation also makes it possible to compare the dominant peptides in two samples, identify differences, and sequence them – a true “differential peptide display” technique [66,85,147]. This approach has been substantially enhanced through the use of stable isotope labeling, enabling the identification of peptides selectively displayed by MHC molecules on cancer and infected cells [148–152]. It promises to yield substantial new information about how changes in cell status alter the overall peptide repertoire, and to identify a large cohort of candidate peptide antigens for vaccine and immunotherapy development.

Robust application of these new technologies requires overcoming hurdles in other areas. A most immediate problem is sample handling, which can result in significant losses of peptide when the amount of starting material is small. Automated sample handling, augmented by protein chip technologies, are an important avenue for further development. Also, because MHC associated peptides are relatively short, their fragmentation spectra frequently do not allow unambiguous matches to database sequences. Melding algorithms that predict the characteristics of MHC-associated peptides with programs such as SEQUEST may help to overcome this limitation. Finally, it is important to begin to understand how changes in expression of MHC associated peptides reflect the nature of their source proteins and their metabolism as a function of cell status. The development of bioinformatic tools that rapidly provide comprehensive information about the source proteins for MHC-associated peptides is important in assessing how these changes may enable effective immune recognition of cancer cells or underlie the development of autoimmune disease.

In the past 15 years, mass spectrometry has provided a wealth of information about the antigens recognized by T lymphocytes, illuminating fundamental immunological processes and identifying targets for therapeutic intervention in human disease. However, this information has led to a new round of outstanding immunological questions that require more robust analytical approaches. Ideally, the immunological questions help to drive the development of those technologies, and new capabilities in mass spectrometry rekindle old questions that had been dismissed as too difficult. That synergy develops in environments where both groups of scientists work to understand what the other has to offer. In such environments, the intersection of mass spectrometry and immunology should continue to be fertile scientific ground for many years to come.

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Abbreviations

ER	endoplasmic reticulum
TAP	transporter associated with antigen processing
APC	antigen presenting cell
mHAgs	minor histocompatibility antigens

II

invariant chain