

MINI REVIEW

Carbohydrates and antigen recognition by T cells

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T Lymphocytes (T cells) recognize short antigenic peptides bound to either MHC I or II molecules, in contrast to antibodies which can bind to native antigen. The mechanism by which antigens are processed into peptides, and the nature of the interactions of antigenic peptides with MHC molecules and with the T cell receptor have now been defined in some detail. Of significance to glycobiologists is the recent appreciation that the carbohydrate of glycoprotein antigens can contribute to the T cell recognition of epitopes presented by MHC molecules. Experiments using model T cell epitopes have demonstrated that carbohydrate can modulate T cell responses in a variety of ways; for example, there are a number of cases where glycopeptide-specific T cell responses have been identified. Many of these glycopeptide-specific T cell responses involve a peptide bearing a single glycosyl residue, thus it appears very likely that both glycan and peptide make contact with the T cell receptor binding site. Significantly, glycopeptide-specific T cell responses have also been detected to native glycoproteins. The ability of carbohydrate to influence T cell recognition of antigen has important consequences for a wide range of immune responses as well as the current strategies for mapping T cell determinants.

Key words: T cell recognition/antigen processing/glycoprotein/MHC molecules

Introduction

Understanding the nuances of T and B lymphocyte recognition is important in considering the role of glycoconjugates as antigens. B Lymphocytes can recognize carbohydrate antigens, either as carbohydrates, glycoproteins, or glycolipids. However, the recognition of carbohydrates by T lymphocytes or T cells is more problematic in view of the very different way T lymphocytes recognize antigen compared with B lymphocytes. In this review, we initially summarize the pathways for processing of antigen and presentation to T cells as this is of central importance to the understanding of T cell recognition.

T Lymphocytes or T cells form essential cellular components of the adaptive immune response. This lymphocyte subset consists of two functionally distinct populations; the cytotoxic T lymphocyte (CTL) and the helper T cell (Th cell) groups of cells. CTLs recognize and kill cells expressing new

antigenic components such as those derived from replicating infectious virus. Th cells, by contrast, primarily exert their effect by secreting immunomodulators called cytokines which modify the immune function of nearby cells. For example, Th cells involved in inflammatory immune responses to bacterial pathogens secrete the cytokine interferon- γ which activates adjacent macrophages and promotes their effective destruction of phagocytosed bacteria.

Given this, it should be clear that T cell recognition shows two characteristic hallmarks. Firstly, T cells exhibit clonal specificity for foreign antigen. For example, influenza-specific CTLs will lyse target cells infected with this virus but will ignore those that are either not infected or contain some other nonrelated virus (Townsend and Bodmer, 1989). Secondly, this antigen recognition is never seen in isolation but always involves a cognitive interaction with an adjacent cell. In other words, the influenza-specific CTLs mentioned above will only recognize virus infected cells and ignore free virus. Indeed the actual entity recognized by a CTL or a Th cell is never an intact antigen, be it bacteria, virus or even protein subunit. Instead, T cells recognize small peptide fragments that are derived from these larger antigenic components. These peptides are bound by a highly specialized group of cell surface molecules encoded by the highly polymorphic major histocompatibility complex (or MHC) which act as combined targets for the intercellular interactions involving the T cells and scaffolds for the binding of the foreign peptide antigens. The peptide antigen is therefore said to be "presented" by the MHC and the overall phenomenon involving peptide binding to MHC for effective T cell recognition is termed "antigen presentation."

There are two "classes" of MHC molecules involved in these events. CTLs recognize foreign peptides bound to the class I MHC molecules while Th cells recognize peptides bound to the class II MHC molecules. In both cases these peptides are derived from intracellular proteolysis of a larger antigenic moiety such as a protein encoded by an infecting virus. This intracellular degradation is termed "antigen-processing" and is the key determinant of whether a peptide will ultimately bind and be presented by the class I or class II MHC molecules and as such, whether the antigen will call into play a helper or a cytotoxic T cell response.

It is now well established that peptides that ultimately bind class I MHC molecules have their origins in the cytoplasmic compartment of the target cell. They are produced by the normal turnover of cytosolic proteins via the action of a multicatalytic protease complex called the proteasome (Glynne *et al.*, 1991; Monaco, 1992). It should be kept in mind that all cell products commence their synthesis on free ribosomes, regardless of whether their ultimate subcellular fate is the cell membrane, nucleus, or cytoplasm. There is considerable evidence that proteins with many different intracellular targeting potentials can all give rise to MHC class I presented peptides prob-

ably as a consequence of the cytoplasmic degradation of a subset of "failed" ribosomal products (Yewdell *et al.*, 1996). Once formed, the cytoplasmic peptides derived from proteasomal action are actively transported into the endoplasmic reticulum by the action of the transporter associated with antigen-processing, or TAP, a member of the ATP-binding cassette family of transporter proteins (Monaco *et al.*, 1990; Spies *et al.*, 1990; Trowsdale *et al.*, 1990). Here they come into contact with the nascent MHC class I protein which, on binding to the peptide antigen, is then free to progress along the secretory pathway. It remains controversial whether class I-binding peptides are further trimmed within the endoplasmic reticulum compartment. In addition, it is not clear whether peptides can also be generated within the endoplasmic reticulum by degradation of proteins translocated into this site. Regardless, it can be stated with some certainty that the majority of class I-bound peptides have their origins within the cytoplasmic compartment of the presenting cell as depicted in Figure 1.

From the above description it is clear that peptides associated with MHC class I molecules are derived from proteins originating in the presenting cell. They are therefore termed endogenous and include viral antigens as well as tumor and minor transplantation antigens (Bevan, 1987; Yewdell and Bennink, 1990). In contrast, MHC class II presentation in-

volves antigens that largely originate outside the cell. They are derived from larger components that are taken up by endocytosis and degraded within an acidic endosomal compartment (Figure 1). These peptides can come from large particulate antigens, such as bacteria taken up by phagocytic cells, or proteins taken up during pinocytosis. Consequently, such antigens are termed exogenous as is the processing pathway involved in MHC class II-restricted presentation. Despite these terms, it should be noted that certain membrane-bound surface proteins and even ligands bound to surface receptors can be targeted to the supposed "exogenous" MHC class II-processing pathway by endocytosis.

Both MHC class I and class II molecules have similar biological function, notably the binding and presentation of peptide for T cell surveillance. There are certain common elements to the binding that are fundamentally important to this discussion. Firstly, the MHC forms one of the most polymorphic genetic loci found in most species and their products bind a wide range of peptide sequences having only a few key residues in common. However, these common residues are crucial and form distinctive allele-specific motif patterns that stabilize peptide association via favorable interactions with pockets found within the binding cleft of the MHC proteins (Garrett *et al.*, 1989; Fremont *et al.*, 1992; Stern *et al.*, 1994; Figure 2).

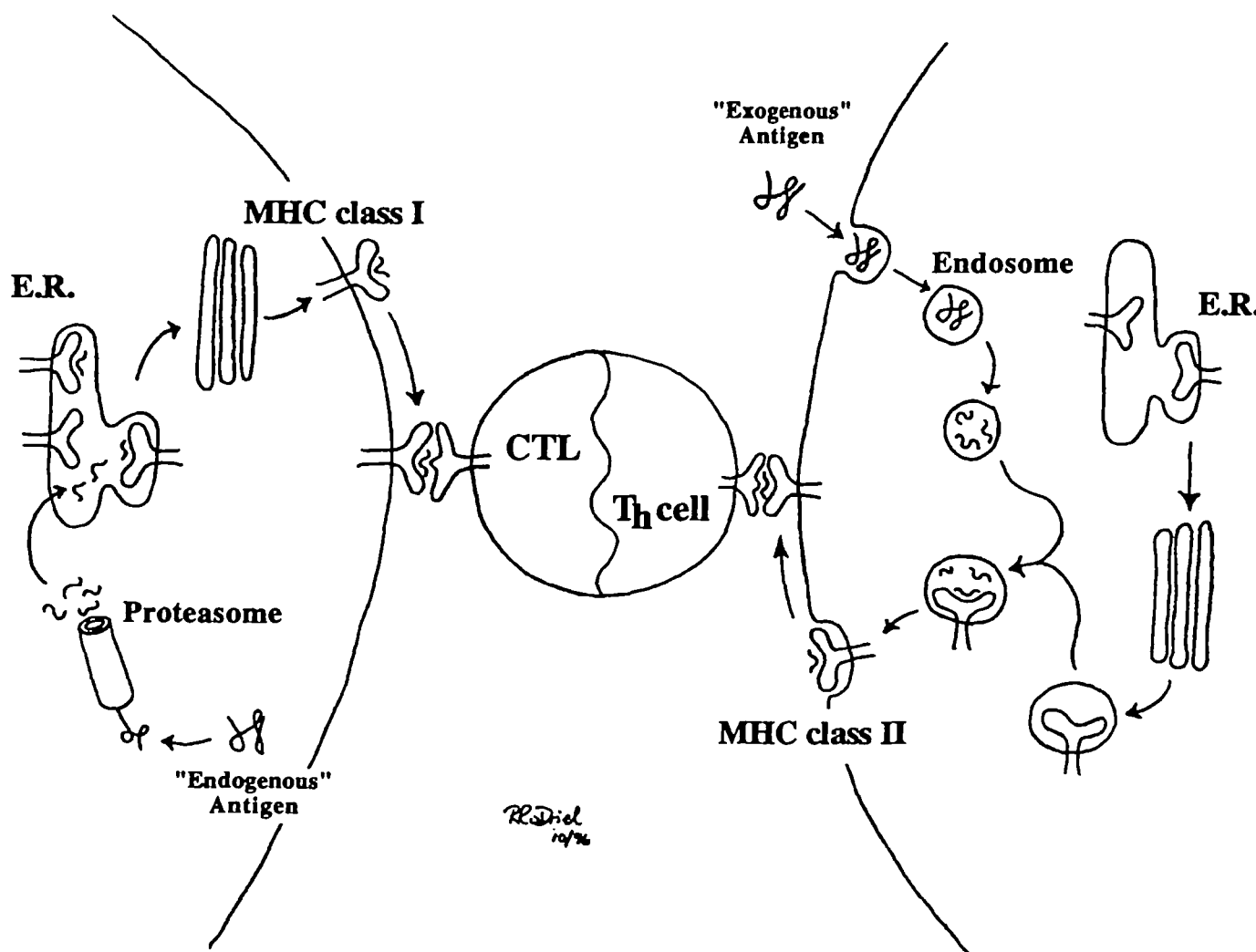


Fig. 1. Outline of the MHC class I presentation pathway (left) and the MHC class II presentation pathway (right).

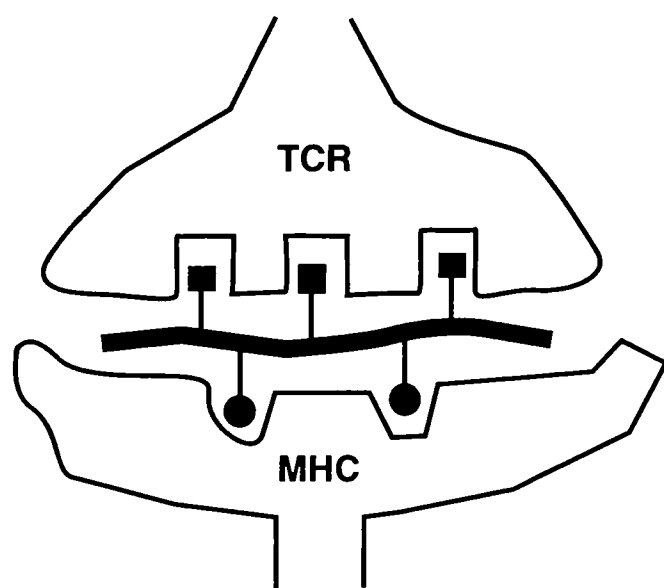


Fig. 2. Binding of peptide to MHC molecules and the recognition of the MHC/peptide complex by the T cell receptor (TCR). The diagram illustrates the interaction of anchor residues of the peptide with the binding groove of the MHC molecule (●), and the interaction of peptide side chains with the TCR (■). It should also be noted that the TCR also makes contact with residues of the MHC molecule (not shown).

While there are many differences between class I and class II MHC proteins, such as their ability to bind peptides of varying lengths, both classes have a common requirement for key conserved residues which effectively anchor the peptide within the groove.

In addition to the anchor residues which are buried deep within the peptide-binding groove of the MHC products, the antigenic peptides have other residues with largely exposed side-chains. These form the sites that are involved in T cell recognition and it is the composition and variability of these exposed side-chains that determines the specificity of T cell recognition (Garcia *et al.*, 1996). Recognition is mediated by a clonally distributed cell surface T cell receptor (TCR) which is closely related to the antibody molecule. Like antibodies, TCR molecules consist of variable and constant domains and recognize a complex of peptide embedded within the binding groove of a MHC class I or class II molecule. Antigenic peptide side-chains therefore contribute to MHC binding, where they act as anchor residues, and to TCR interactions as depicted in Figure 2.

Carbohydrates and antigen recognition by conventional T cells

The conventional α/β T cell population recognizes a diverse array of antigens; this T cell lineage provides the majority of the T cell repertoire. Most of the studies carried out to date which have examined the potential of T cells to recognize carbohydrate have focused on α/β T cells. Currently, there is no evidence for binding of oligosaccharides by the groove of MHC molecules (Harding *et al.*, 1991; Ishioka *et al.*, 1992). Thus, a direct recognition of exclusively sugar epitopes by conventional α/β T cells, although not excluded, seems most improbable. However, there is increasing evidence that the carbohydrate of glycosylated protein antigens may contribute

to T cell recognition. There are a number of possible ways carbohydrate can influence T cell recognition and these are discussed below and are summarized in Table I.

Effect of CHO on antigen processing

As discussed above, antigen processing is fundamental to the presentation of antigenic peptides by MHC class I and II molecules for recognition by α/β T cells. For the class I pathway, processing is mediated by the proteasome particle found in the cytosol and the resulting peptides then actively transported from the cytosol to the lumen of the endoplasmic reticulum. Analysis of peptides eluted from purified class I/peptide complexes has shown that in many cases MHC class I-bound peptides are derived from cytosolic and nuclear proteins (Rammensee *et al.*, 1995). This location of antigens destined for the class I pathway means that they are excluded from the glycosylation machinery of the endoplasmic reticulum and Golgi apparatus during their synthesis and, therefore, the native antigens of the class I pathway will not be modified with N-glycosylated or Ser(Thr) O-glycosylated oligosaccharides. However, it remains a formal possibility that processed peptides could be N-glycosylated after TAP-mediated transport into the endoplasmic reticulum, prior to binding to MHC class I molecules. More important is the identification of a novel O-linked glycosylation mechanism, which occurs almost exclusively on nuclear and cytosolic proteins (Holt and Hart, 1986), as this is highly pertinent to MHC class I antigens. The O-glycans of cytosolic and nuclear proteins involve substitution of serines or threonine residues with single O- β -linked N-acetylglucosamine residues (Haltiwanger *et al.*, 1992; Hart *et al.*, 1989).

For the class II pathway, exogenous antigens are internalized by antigen-presenting cells and degraded by proteases found in an acidic "lysosomal-like" membrane bound compartment. In addition, it is now clear that endogenous self-antigens of the secretory pathway can also be presented by class II molecules (Chicz *et al.*, 1993). It is not surprising then that many MHC class II antigens are glycoproteins bearing either N- or O-glycans on the mature protein.

The presence of a glycan side group on antigens of either the class I or class II pathway could theoretically limit the access of proteolytic enzymes and thereby inhibit the generation of an otherwise antigenic peptide. However, at this stage there is little information available on the effect of carbohydrate on antigen processing, although a few studies do indicate that carbohydrate can influence the processing of glycoproteins. For example, in a study by Drummer *et al.* (1993) T cell clones to a defined class II restricted determinant of influenza hemagglutinin failed to respond when N-glycans were attached to an asparagine residue just outside the T cell determinant. This

Table I. Possible consequences of glycosylation of antigens on T cell recognition

1. Inhibition of antigen processing
2. Reduced binding of glycopeptide epitopes to MHC molecules $\rightarrow$ loss of an epitope
3. Increased binding of glycopeptide epitope to MHC binding $\rightarrow$ creation of a neoepitope
4. Reduction in immunogenicity
5. Lack of cross-reactivity of primed T cells raised to the non-glycosylated counterpart
6. Generation of glycopeptide-specific T cell responses

finding shows that the presence of oligosaccharides can convert an immunodominant T cell determinant or epitope into a hidden or cryptic determinant. The modulation of the hemagglutinin T cell response by carbohydrate could well occur at the level of antigen processing, although this has yet to be directly demonstrated.

Effect of carbohydrate on MHC binding of glycopeptides

Once glycopeptides are produced by either the class I or II processing pathway the next hurdle, prior to T cell recognition, is that they must bind to MHC molecules. The carbohydrate of glycoproteins can influence the ability of glycopeptides to be accommodated in the MHC peptide-binding groove. A number of studies involving glycosylated analogs of defined immunodominant peptides have been carried out. These studies have included both class I and II binding peptides and are summarized in Table II. Defined T cell epitopes were glycosylated synthetically, either with natural or unnatural oligosaccharides, and the effect of glycosylation on MHC binding examined directly. The presence of carbohydrate on defined epitopes resulted in either (1) reduced binding to MHC molecules, (2) no effect on MHC binding, or (3) increased binding affinity to MHC. Not surprisingly, glycosylation of the MHC-contact residues of the epitope invariably resulted in reduced or loss of binding of the glycopeptide (Ishioka *et al.*, 1992; Haurum *et al.*, 1995; Jensen *et al.*, 1996). On the other hand there are many cases where the presence of a glycan within the determinant was tolerated (Ishioka *et al.*, 1992; Harding *et al.*, 1993; Haurum *et al.*, 1994; Jensen *et al.*, 1996). These include the glycosylation of either non-MHC contact residues or residues that extend outside of the peptide binding groove of the MHC class II molecule. The studies carried out so far, although not comprehensive, also indicate that the smaller O-glycans on peptides may be more readily tolerated than larger N-glycans. In two cases, the presence of a glycan on an epitope actually increased the binding affinity to mouse MHC class I molecules (Mouritsen *et al.*, 1994; Haurum *et al.*, 1995). Haurum *et al.* (1995) employed a mutant epitope from the Sendai virus nucleoprotein which no longer bound to class I MHC; O-linked

glycosylation of the nonbinding epitope with GlcNAc residues (i.e., the cytosolic O-glycan type) partially restored the binding of the variant peptide to the MHC class I allele, H-2D^b (Haurum *et al.*, 1995). Although an isolated case, this is an important observation as it indicates that glycans have the potential to create a neo-epitope.

How do these studies involving synthetic glycopeptides relate to natural glycoproteins? There are a few examples where naturally glycosylated epitopes have been reported which bind MHC molecules. Firstly, a well-defined tissue-specific protein which is glycosylated is type II collagen. The posttranslational modifications of the immunodominant peptide (residues 256–270) involve O-linked hydroxylysines. Michaëlsson *et al.* (1994) have demonstrated that the naturally glycosylated immunodominant epitope can bind directly to rat MHC class II molecules. Secondly, the characterization of naturally processed peptides bound to human MHC class II molecules has identified a glycopeptide derived from LAM (Chicz *et al.*, 1993); this glycopeptide contained only a single N-acetylglucosamine residue on asparagine 104, indicating that considerable degradation of the complex N-linked glycan had taken place, presumably by lysosomal glycosidases, prior to loading on class II molecules. And thirdly, MHC class II restricted T cell responses to the bee venom allergen, phospholipase A2, has been shown to be dependent on the presence of N-glycans (Dudler *et al.*, 1995). Although the location of the glycosylated asparagine in relation to the peptide epitope(s) has not yet been mapped, the dependence of N-glycans on phospholipase A2 for a class II restricted T cell response strongly indicates a glycopeptide epitope is bound by MHC molecules (Dudler *et al.*, 1995).

Carbohydrate dependent T cell recognition

The above clearly shows that glycopeptides can bind to MHC molecules and the glycans can be located within the MHC peptide binding region. Thus, in these cases, both peptide and glycan would be presented to interacting T cells. Given this, there is no reason *a priori* that the glycans could not be included in the recognition by T cell receptors. Indeed a number

Table II. Glycosylated analogs of defined T cell epitopes

Origin of determinant	MHC restriction	Carbohydrate substitution	Major findings ^a	Reference
Sendai virus nucleoprotein	Class I (K ^b)	β-D-GlcNAc attached to Ser/Thr substituted analogs	2, 3, 5, 6	Haurum <i>et al.</i> (1994) Haurum <i>et al.</i> (1995)
Influenza A virus nucleoprotein	Class I (D ^b)	Variety of di- and tri-saccharides coupled to either N- or C-terminal or to internal residues	4	Abdel-Motal <i>et al.</i> (1996)
VSV nucleoprotein	Class I (K ^b)	Variety of di- and tri-saccharides coupled to either N- or C-terminal or to internal residues	6	Abdel-Motal <i>et al.</i> (1996)
Sendai virus nucleoprotein	Class I (K ^b)	Variety of di- and tri-saccharides coupled to either N- or C-terminal or to internal residues	6	Abdel-Motal <i>et al.</i> (1996)
Mouse hemoglobin	Class II (I-E ^k)	α-D-GalNAc (Tn antigen) attached to Ser or Thr substituted peptideanalogs	2, 4, 5, 6	Jensen <i>et al.</i> (1996)
Ovalbumin (residues 323–339)	Class II (I-A ^d)	β-D-GlcNAc attached to Asn peptide analogs	2, 5, 6	Ishioka <i>et al.</i> (1992)
Hen egg lysozyme (residues 81–96)	Class II (I-E ^k)	(1) N-terminal substitution with mono-, tri- and penta-saccharides (2) Central Ser or Asn analogs substituted with penta-saccharide and GlcNAc, respectively	2, 3	Mouritsen <i>et al.</i> (1994)
Hen egg lysozyme (residues 52–61)	Class II (I-A ^k)	Galα1-4Galβ attached to amino terminus	6	Harding <i>et al.</i> (1993)
		Galα1-4Galβ attached to Ser analogs	6	Deck <i>et al.</i> (1995)
Rabies virus glycoprotein	Class II	β-N-GlcNAc-Asn and α-D-GalNAc-Ser	2	Orvos <i>et al.</i> (1995)

^aRefer to Table I for explanations.

of studies using defined MHC binding glycopeptides have demonstrated glycopeptide-specific T cell responses.

Collectively, the studies utilizing glycosylated analogs of model T cell epitopes which are presented by MHC molecules resulted in three patterns of T cell reactivity: (1) reduction in immunogenicity (Abdel-Motal *et al.*, 1996; Jensen *et al.*, 1996), (2) minimal effect on T cell reactivity (Ishioka *et al.*, 1992; Mouritsen *et al.*, 1994; Jensen *et al.*, 1996), and (3) carbohydrate dependent glycopeptide-specific T cell responses (Ishioka *et al.*, 1992; Harding *et al.*, 1993; Haurum *et al.*, 1994, 1995; Deck *et al.*, 1995; Abdel-Motal *et al.*, 1996).

The third group is the most interesting and there are a number of examples where T cell response have been demonstrated to be glycopeptide-specific; in other words, a response is detected only in the presence of the carbohydrate. Two studies have used glycosylated peptides with the unnatural carbohydrate, galabiose (Gal α 1,4Gal β). Studies by Unanue and colleagues used a class II restricted T cell epitope of hen egg lysozyme (HEL) (residues 51–62) which was glycosylated with galabiose at either the N-terminus or Ser 56 (substitution of Leu from wild type sequence; Deck *et al.*, 1995; Harding *et al.*, 1993). Position 56 of the wild-type determinant is known to be a T cell receptor contact site. Glycopeptide-specific T cells were identified in both cases. As the galabiose oligosaccharide of the Gal $_2$ N-terminal HEL peptide is outside the MHC peptide binding region, it is likely that the carbohydrate is influencing the conformation of the bound peptide and T cell recognition is peptide conformation dependent. On the other hand, T cell recognition of Gal $_2$ -Ser 56 HEL peptide may involve recognition of both the disaccharide and the peptide. Although, these studies have used peptides substituted with an unnatural oligosaccharide, they demonstrate, nonetheless, that T cells have the potential of recognizing epitopes which are partially defined by glycans.

Of more biological significance are studies by Haurum and colleagues, involving the cytotoxic T lymphocyte recognition of the class I-restricted epitope from Sendai virus (FAP-GNYPAL) modified to include a serine with a substituted O-linked N-acetylglucosamine residue (Haurum *et al.*, 1994). This glycan is found on nuclear and cytosolic proteins and therefore represents a naturally occurring posttranslational modification of proteins. Based on the known crystal structure of the FAPGNYPAL peptide with the MHC class I molecule, K^b, carbohydrate modifications were made at positions most likely to point out of the peptide-binding groove and interact with the T cell receptor. A glycopeptide, bearing a Ser-O-GlcNAc substitution at position 3, was found to elicit CTL responses which were glycopeptide-specific as there was little cross-reactivity with the nonglycosylated peptide. Further, the cytotoxic T lymphocyte recognition was shown to be dependent on the structure of the glycan and the position of the glycan on the peptide, suggesting that the glycan is involved in a specific contact with the T cell receptor.

T Cell hybridomas have been raised to type II collagen which recognize the glycosylated immunodominant determinant (residues 256–270; Michaëlsson *et al.*, 1994). The hydroxylsines of this epitope are glycosylated with either the monosaccharide Gal β or the disaccharide Glc α 1,2Gal β . T Cell reactivity was abolished on removal of the hydroxylsine linked carbohydrates. This clearly demonstrates that carbohydrate can influence T cell recognition of natural glycoproteins. However, it is unclear whether the carbohydrate is directly interacting

with the T cell receptor or is altering the conformation of the peptide structure.

The examples of glycopeptide-specific T cell responses discussed above all involve small glycans (either mono- or disaccharides); furthermore, in a number of cases these glycans are linked to residues within the peptide antigen which have been defined as T cell receptor contact sites. It would appear highly likely that these small glycan moieties can be accommodated within the T cell receptor site and contribute directly to the specificity of the T cell response. Of relevance is that the majority of the glycoprotein antigens of the class I pathway are likely to be glycosylated with only a monosaccharide (GlcNAc), whereas the glycoprotein antigens of the MHC class II pathway carry oligosaccharides of varying sizes. If glycopeptides bearing large oligosaccharides (e.g., undegraded N-glycans) can bind to MHC molecules, the bulky carbohydrate is likely to block access of the T cell receptor to the contact sites of the MHC molecule. Hence, the extent of degradation of the oligosaccharide chains of glycoprotein antigens in the class II processing pathway becomes a significant factor in the potential of Th cells to recognize class II/glycopeptide complexes. As yet, we know very little about oligosaccharide degradation in the class II pathway.

Nonconventional T cells

Recently, human α/β T cells have been detected that are stimulated by nonpeptide antigens. These T cells recognize antigen presented by the nonclassical MHC molecule, CD1, which is distantly related to MHC class I molecules (Bendelac, 1995). The human CD1b isotype has been shown to present lipoglycans, namely lipoarabinomannan and mycolic acid derived from mycobacterium cell walls, to α/β T cells (Beckman *et al.*, 1994; Sieling *et al.*, 1995). Presentation of the lipoglycan antigens required intracellular processing, however, the nature of the interaction between the lipoglycan and CD1b has not been defined (Sieling *et al.*, 1995). T Cell recognition of the lipoarabinomannan antigen appears dependent on the glycan and the phosphatidylinositol component (Sieling *et al.*, 1995). These findings are important as they extend the potential repertoire of antigens recognized by α/β T cells beyond the paradigm of (glyco)peptides that bind to the classical MHC class I and II molecules, and have important implications in immune responses to infectious organisms.

Practical considerations

The influence of oligosaccharides on T cell recognition has very important practical consequences. Firstly, although it has been appreciated that exogenous antigens presented via the MHC class II pathway are often glycosylated, it has not been widely appreciated that many of the cytosolic and nuclear protein antigens presented by class I molecules may be glycosylated with an O-linked N-acetylglucosamine residue. Secondly, as recombinant antigens are commonly used in T cell assays and as immunogens, the source of the recombinant antigen (prokaryotic or eukaryotic) is an important consideration in generating a glycosylated molecule which is similar to the native antigen. Thirdly, the standard technique of using overlapping (nonglycosylated) peptides to map T cell epitopes is potentially limiting as they are devoid of posttranslationally modifications. And fourthly, changes in site-specific glycosylation (for example, point mutations affecting glycosylation

of a viral antigen) may influence immunogenicity of T cell epitopes by either the loss of an epitope or the creation of a neo-epitope.

Conclusions

It is clear that carbohydrate can influence T cell recognition in either a positive or negative manner. The modulation of the T cell response by carbohydrate may occur at the level of antigen processing, presentation or recognition. The experiments discussed in this review show that the presence of oligosaccharides on glycoproteins can convert an immunodominant T cell determinant or epitope into a hidden or cryptic determinant. This has important ramifications in autoimmunity as T cells specific to such cryptic determinants will not be tolerized but will be present within the adult T cell repertoire. In the event of exposure to a nonglycosylated form of the protein the relevant T cells will be able to respond, resulting in the activation of an autoimmune response. On the other hand, also discussed was the important finding that the presence of oligosaccharide can result in the creation of a neo-epitope. As the glycosylation of proteins can vary, especially under conditions of stress and associated with tumorigenesis, this scenario needs further consideration. Clearly, the role of oligosaccharides in the processing and presentation of peptide epitopes needs to be more fully explored.

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Abbreviations

MHC, major histocompatibility complex; TCR, T cell receptor; CTL, cytotoxic T lymphocyte; Th cell, helper T cell.

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