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Glyco-epitope Diversity: An Evolving Area of Glycomics Research and Biomarker Discovery

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Although the term "glyco code" has only recently come into use [1-4], recognition of the carbohydrate signatures of microbes was documented nearly a century ago. In 1917, Dochez and Avery [5] found that when *Pneumococci* were grown in fluid media, there was a substance in the culture fluid that precipitated specifically with antisera to the same *Pneumococcus*. Heidelberger and Avery [6] showed that the substance recognized by the antibodies was a carbohydrate molecule and not a protein, as previously thought. It was later found that almost every microorganism expresses such glyco codes that are recognized by the host immune systems and are effective in stimulating specific antibody responses [7,8]. Such immunogenic carbohydrate moieties often serve as key targets for development of vaccines against infectious diseases [9-13]. Tumor glycomics research in recent years has uncovered a large panel of tumor-associated carbohydrate antigens [14,15] and evidence of immune recognition of tumor-derived aberrant carbohydrates [16-20].

Carbohydrates are capable of generating structural diversity in multiple ways and are prominently displayed on the surfaces of cell membranes or on the exposed regions of macromolecules. Unlike proteins, which are connected solely by a peptide bond, carbohydrates utilize many possible glycosidic linkages so as to extensively diversify their structures. Two amino acid residues, such as two alanines, can produce only one possible dipeptide; however, two molecules of glucose have the potential to generate 11 different disaccharides. A trimer of any of the nine common sugar residues of the human body theoretically can give rise to 119,736 different structural isomers; this is in striking contrast to the maximal construction of 8,000 tripeptides using 20 different amino acid residues. Theoretically, sugar chain structures can have unlimited variation.

Importantly, there are multiple carbohydrate-recognition systems in living species that "read" the biological information of complex carbohydrates. Two examples are the numerous anti-glycan antibodies produced by many animal species that play key roles in protecting a host from microbial infections [13,21,22] and the families of lectin-like glycan-binding proteins (GBPs) that are evolved for carbohydrate-mediated cell-cell communication [23-25]. Thus, carbohydrates are uniquely suitable for storing biological signals in the forms that are identifiable by other biological systems.

In the immunological and glycobiological literature, "glyco-epitope" is often used to specify the carbohydrate moiety that is recognized by an antibody or by a GBP. The antibody-binding glyco-epitopes are also classified as B cell epitopes or antigenic determinants. Conceptually, "glyco-epitome" refers to the entire repertoire of glyco-epitopes, including the B cell epitopes and those that are recognized by GBPs. Differing from "glycome," which covers all the existing carbohydrate molecules in living organisms, glyco-epitome refers to a unique subset of carbohydrates that serve as the sugar signatures for molecular recognition and bio-signal transmission. "Glyco-epitomics" is, thus, an evolving area of glycomics research focusing on identifying, characterizing, and understanding the carbohydrate moieties that serve for multiple levels of bio-communication.

The structural aspects of glyco-epitomics focus on the elucidation of the glycan structures that display glyco-epitopes. This research area has been substantially enhanced by the development of advanced profiling and structural characterization strategies. Notably, these include high-resolution chromatography methods coupled with exoglycosidase digestions [26,27], modern mass spectrometry [28-30] and nuclear magnetic resonance spectroscopy analyses [31-33] of carbohydrates, and the state-of-art methods of glycan structural modeling [34,35].

However, availability of carbohydrate structural information alone is not sufficient in defining a glyco-epitope unless its specific binding by an antibody or a GBP is also demonstrated immunochemically and/or crystallographically. For example, chemical determination of a tetrasaccharide that decorates the spore of *Bacillus anthracis* appears to be an important discovery in microbial glycomics [36]. Based on the past knowledge of immunogenic carbohydrate moieties, this structural glycomics progress may suggest that this unique sugar moiety may have potential in an immunological application [37,38]. However, whether such a carbohydrate moiety preserves a B cell epitope or a potent antigenic determinant must be determined immunologically, including at least demonstration of its antibody binding specificity and capacity in eliciting immune responses *in vivo* [10]. It was the integrated structural and immunological investigation with the support of carbohydrate microarray technologies [10,13] that has revealed anthrose-tetrasaccharides as key immunological targets of *B. anthracis*. Its applications may include identification of the presence of *B. anthracis* spores, surveillance and diagnosis of anthrax infection, and development of novel vaccines targeting the *B. anthracis* spore.

Modern carbohydrate microarrays emerged in 2002 [39-42] and introduced new glycomics tools to decipher the biological information content in the glycome. These technologies are especially useful in exploring the repertoire of glyco-epitomes. Given the structural characteristics of the carbohydrates displayed on chips, carbohydrate microarrays are classified into monosaccharide chips, oligosaccharide chips, and microarrays of carbohydrate-containing macromolecules. The latter includes polysaccharides and various glycoconjugates. These different sugar chips or arrays were developed to accommodate multipurpose applications in carbohydrate research. For example, the mono- and disaccharide microarrays are suitable for screening and characterizing carbohydrate-binding proteins or carbohydrate-

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catalyzing enzymes and for identifying inhibitors of carbohydrate-protein interaction [43,44]. However, some lectins and many anti-glycan antibodies recognize larger and more complex carbohydrate ligands or antigenic determinants. The mono- and disaccharide sugar chips are not sufficient for investigations involving such molecular targets. The oligosaccharide [10,45,46], polysaccharide [47,48], and glycoconjugate [49-53] microarrays come to fill this gap by displaying carbohydrates of complex structures or longer sugar chains on the chips.

One of the important research areas in glyco-epitomics is the understanding of the nature and characteristics of the immunogenic sugar moieties that render them key targets for immunological and clinical applications. Figure 1 illustrates an example that common sugar residue glucose can form either non-immunogenic or highly immunogenic polysaccharide molecules. The α(1,4)-linked glucosyl polymer illustrated is the digestible, non-immunogenic glycogen. A microbe-produced α(1,6)-dextran molecule is, however, immunogenic in human and many animal species. This is owing to the fact that α(1,6) dextran, but not α(1,4)glycogen, is resistant to the host enzymatic digestion and persists *in vivo* to stimulate B cell responses. Thus, whether a carbohydrate molecule is immunogenic is determined by a complex process of antigen processing, host recognition, and the regulated immune response to a target molecule.

A carbohydrate antigen, such as α(1,6)dextran (Figure 1, upper panel), may display different types of epitopes, such as the terminal and internal chain glyco-epitopes [8,54], on its solvent-accessible surface. This can be attributed to the hydrophilic property of carbohydrates, which makes them strikingly different from proteins. In aqueous solution, proteins tend to fold to bring their hydrophobic side chains together, forming an oily core with polar side chains exposed. Surface moieties of a protein antigen may serve as antigenic determinants interacting with B-cell Ig-receptors; interior residues are generally not accessible to such interactions. Carbohydrates are built up by monosaccharides, whereby the enriched hydroxy groups readily interact with water molecules by hydrogen bonding. Their glycosidic linkages are more flexible than the peptide bonds in proteins, and protein-like folding patterns are not seen in polysaccharides. Thus, not only are the terminals of the carbohydrate chains accessible for molecular recognition but residues in the internal chain are also exposed in solvent and are frequently reactive. Many carbohydrate-based vaccines focus on the terminal non-reducing end epitopes, leaving a large class of internal chain epitopes unexplored.

Identifying the immunogenic carbohydrate moieties of HIV-1 is, perhaps, one of the current challenges to carbohydrate researchers. Since the early 1980s when the acquired immunodeficiency syndrome (AIDS) and its etiologic agent, human immunodeficiency virus (HIV), were first described, the immunological complexity of this infectious disease has been recognized [55]. For example, discovery of an oligomannose cluster as the epitope of a broadly HIV-1–neutralizing antibody, 2G12 [56], has stimulated substantial interest in such a carbohydrate moiety for the development of HIV-1 vaccines. However, induction of 2G12-like HIV-1 neutralization antibodies by active immunizations has been proven difficult [57]. For example, Ni et al. [58] immunized rabbits using the synthetic oligomannose clusters that express 2G12 glyco-epitopes and found that this 2G12-positive antigen elicited only a small fraction of antibodies to the carbohydrate moieties, with the majority of the IgG-type antibodies being directed to the linkers in the conjugates. The rabbit anti-sera showed weak cross-reactivity to HIV-1 gp120.

However, demonstration of the poorly immunogenic 2G12-oligomannose antigen does not necessarily lead to a conclusion that the oligomannose antigens are generally non-immunogenic *in vivo*. This is because of the fact that the same sugar chain may generate different glyco-epitopes when the sugar moiety is presented in different cluster configurations. In essence, the same sugar chains can be assembled into different antigens; these carbohydrate antigens may interact with immune systems in different ways. Figure 2A illustrates three examples of such glyco-epitomics diversity. (Man9)n-KLH (2.) is similar to (Man9)n-BSA (3.) in the linkage used for coupling oligomannoses to a protein carrier and in the molar ratio between the Man9 unit and corresponding carrier. By contrast, [(Man9)4]n-KLH (1.) was constructed by introducing a defined scaffold to display the tetra-valent oligomannose clusters. Only the latter mimics the high-density Man9-clusters expressed by the gp120 glycoprotein of HIV-1 [58] and binds selectively to an HIV-1 neutralization monoclonal antibody (mAb) 2G12 (Figure 2B**,** blue column). However, a tumor vaccine-elicited mAb, TM10 [59], is highly reactive with the three Man9 clusters (Figure 2B, red column). Thus, these glycoconjugates express the TM10 epitope in common but differ in expression of the 2G12 epitope.

Interestingly, the TM10-positive and 2G12-poorly reactive oligomannose antigens appear to be immunogenic in human and mouse under certain conditions. This is evidenced by the fact that autoantibodies targeting these oligomannose antigens were found to be significantly elevated in the blood circulation of men with aggressive prostate cancers [19,20], in the cerebrospinal fluid of patients with multiple sclerosis, and in the serum of mice with experimental autoimmune encephalomyelitis (EAE) [60]. Interestingly, Wang et al. [60] found that co-immunization of SJL/J mice with a Man9-KLH conjugate (2.) at the time of EAE induction elicited significant levels of anti-Man9-cluster autoantibodies (IgG and IgM). Nevertheless, this anti-glycan autoantibody response was associated with a significantly reduced clinical severity of EAE. Thus, much remains to be learned regarding the molecular and cellular mechanisms underlying the

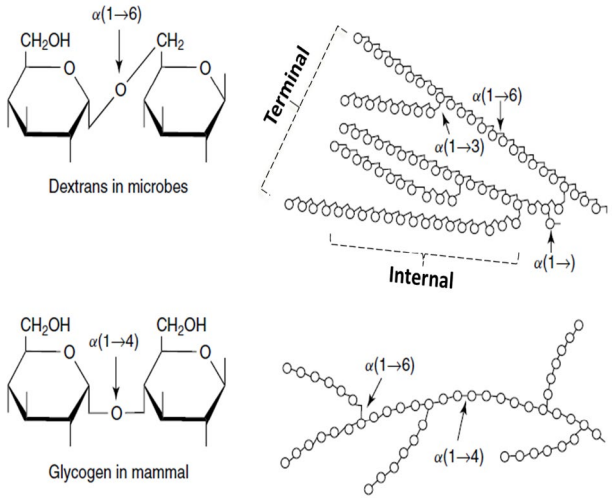

Figure 1: Schematics of microbial dextran and mammalian glycogen: glycosidic linkages make the difference. The only source of structural diversity in homopolysaccharides is the glycosidic bonds linking the monosaccharides. This figure illustrates two commonly found polysaccharides – one in microbes and another in mammals. The polysaccharide with *α*(1→6) linkage is dextran, which is found in many bacteria, including *Lactobacillaceae*. The polysaccharide with the *α*(1→4) is glycogen and it is found in mammals as one of the biological energy-storage molecules.

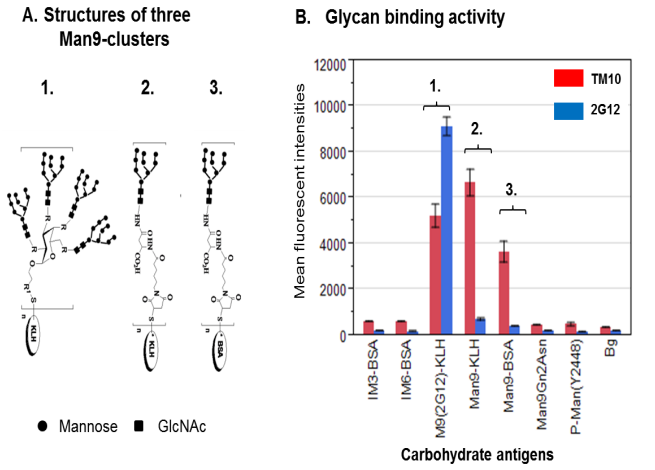

Figure 2: Carbohydrate microarrays identify tumor-associated oligomannosyl antigens that differ from the 2G12-specific HIV-1 carbohydrates by the Man9-cluster configuration. Panel **A** illustrates the structures of three Man9-conjugates: 1) M9(2G12), 2) Man9-KLH, and 3) Man9-BSA. The glycan-binding activities of antibodies are shown as the mean fluorescent intensities of triplicate detections of each antigen with red and blue column for TM10 and 2G12, respectively. Each error bar is constructed using 1 standard deviation from the mean.

differential antibody responses to oligomannose antigens of different cluster configurations.

It is noteworthy that "glyco codes," the molecular targets of glyco-epitomics study, are not limited to the glyco-epitopes that are defined by anti-glycan antibodies or GBPs. Conceptually, any carbohydrate moiety that plays a role in molecular recognition and bio-communication belongs to this family of bio-communicators. These may include, but are certainly not limited to, the carbohydrates that serve as host receptors of microorganisms [61-64] and those that are specifically recognized by toxins of various origins [65-68]. Glyco-epitope diversity is, therefore, an evolving area of glycomics research and biomarker discovery.

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