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Immunologic mapping of glycomes: implications for cancer diagnosis and therapy

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1. ABSTRACT

Cancer associated glycoconjugates are important biomarkers, as exemplified by globo-H, CA125, CA15.3 and CA27.29. However, the exact chemical structures of many such biomarkers remain unknown because of technological limitations. In this article, we propose the "immunologic mapping" of cancer glycomes based on specific immune recognition of glycan structures, which can be hypothesized theoretically, produced chemically, and examined biologically by immuno-assays. Immunologic mapping of glycans not only provides a unique perspective on cancer glycomes, but also may lead to the invention of powerful reagents for diagnosis and therapy.

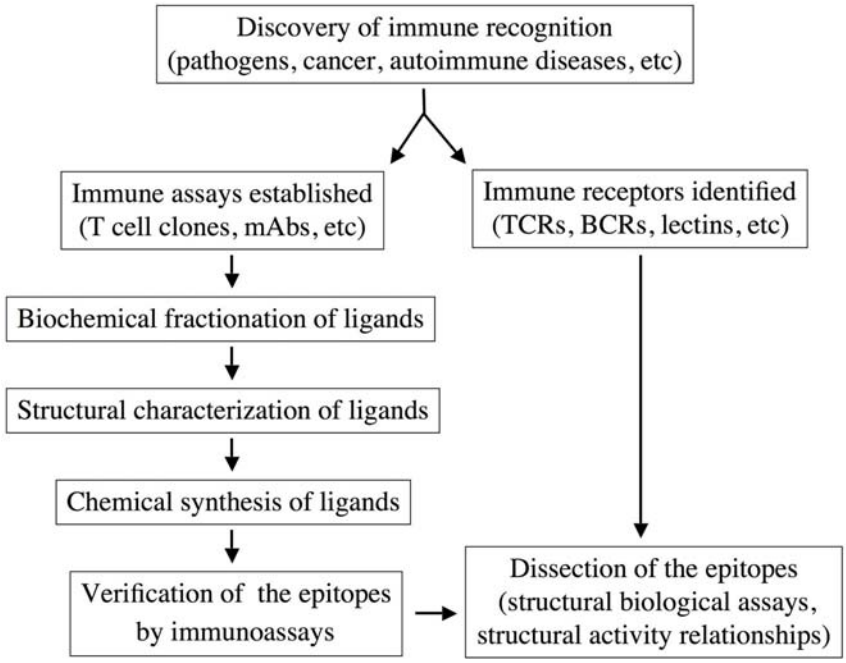

Figure 1. Molecular identification of an immune epitope. mAb, monoclonal antibody; TCR, T cell receptor; BCR, B cell receptor.

2. INTRODUCTION

The immune system identifies pathogenic microbes and tumor cells by recognizing pathogenic epitopes that originate from microorganisms and cancers (1, 2) and are not expressed by healthy tissues and cells. Because of the complex and dynamic nature of microbial and cancerous epitopes, multiple mechanisms have evolved to recognize and counteract the pathogens and cancers from which they arise. Defects in recognizing pathogenic immune epitopes result in lethal infectious diseases and cancer. On the other hand, immune rejection toward epitopes expressed by normal self tissues and cells can lead to autoimmune diseases such as allergies, asthma, rheumatoid arthritis, diabetes mellitus type 1, and lupus erythematosus. Identifying the chemical nature of pathogenic immune epitopes facilitates both vaccine development for cancer and pathogens, and invention of therapeutic approaches to prevent and treat autoimmune diseases.

Immune epitopes are expressed by diseased self tissues and cells. To improve the efficacy of recognizing immune epitopes, professional antigen presenting cells called dendritic cells (3) have evolved to process and present immune epitopes. Meanwhile, pathogens and cancer cells mutate to evade the attacks by cytotoxic immune components such as complement and cytotoxic T cells. Furthermore, pathogens alter their expression of epitopes at different stages in their lives. Thus identifying immune epitopes can be a very challenging task. In addition, biochemical identification of clinically relevant immune epitopes is often hindered by a lack of materials. It is not unusual that receptors for a cancerous or infectious epitope are identified in immunobiological studies, but the exact chemical nature of the epitope itself is only identified years after.

2.1. The role of immune receptors as signal sensors in both innate and adaptive immunity

Immune system receptors have evolved in both the innate and adaptive arms of immunity. Receptors of innate immunity (pattern recognition molecules) recognize structures of microbial and self origin, and transduce signals in a highly sensitive mode to activate adaptive immune responses. Recent progress in biochemical and structural biological studies has shed light on the mechanisms of interaction between innate receptors and their ligands (4). Receptors of adaptive immunity include B cell receptors and T cell receptors. B cell receptors recognize antigens and transduce activation signals that drive the differentiation of B cells and the production of opsonization antibodies. In identifying a ligand or epitope recognized by B cells, peptide fragments of candidate proteins may be purified and analyzed by mass spectrometry (MS), and candidate peptides can be chemically synthesized to test specific recognition using antibody binding assays (Figure 1). T cell receptors recognize antigens presented by major histocompatibility complex (MHC) molecules, which means they are restricted to self MHC molecules; the receptors' fine specificity is regulated by the recognition of antigens loaded on the surface of MHC molecules. T cell epitopes can be identified by purifying peptides bound to MHC molecules, and the identified candidate peptides can be examined by testing their recognition by T cell clones expressing specific T cell receptor genes (5, 6).

2.2. The impact of immune receptor signaling on the biology of immune cells

Receptors expressed on immune cells mediate important signaling functions that drive the cells' development and function.

2.2.1. T cells

T cell precursors originate from bone marrow and migrate to the thymus, where they undergo several stages of development. A critical step in the development of T cells is the expression of the T cell receptor on the cell surface, which allows them to receive positive selection signals mediated by self MHC molecules and putative self, low-affinity TCR ligands (7). After they mature in the thymus, T cells migrate to peripheral lymphoid organs, where they are activated by foreign (pathogen-derived) antigens. For conventional T cells, the self ligands that mediate positive selection are never the same as the foreign ligands they recognize in the peripheral organs. However, some non-conventional T cells, such as natural killer T (NKT) cells, show evidence that when they are in peripheral organs they can be activated by ligands expressed in the thymus. Such auto-reactivity (8) may be responsible for the important immune regulation function of NKT cells.

2.2.2. B cells

It is not clear whether the B cells require ligand recognition during their development. When mature B cells enter lymphoid organs and encounter pathogens there, B cell receptors receive antigen stimulation, and turn on a series of signal transduction pathways responsible for their differentiation, class switching, and immunoglobulin secretion.

2.2.3. Dendritic cells and macrophages

Dendritic cells and macrophages express surface and cytosolic receptors that transduce microbial signals. The chemical nature of the microbial ligands determines the pro-inflammatory or anti-inflammatory cytokine profiles, as well as organelle functions and the fate of dendritic cells and macrophages. For example, the mycobacterial cell wall component lipoarabinomannan (LAM) inhibits phagosome maturation and autophagosome formation in macrophages (9). LAM- regulated proteins responsible for such changes have been studied using a proteomics approach.

3. IMMUNOBIOLOGY OF RECEPTORS RECOGNIZING GLYCOCONJUGATES

In the past several decades, many immune receptors specific for complex glycoconjugates have been molecularly identified and characterized, including C-type and other animal lectins, B cell receptors, and T cell receptors.

3.1. C-type lectins, Siglecs, galectins, and other animal lectins

Multiple animal lectins (including C-type lectins, Siglecs, galectins, and other newly discovered lectins) are expressed by immune cells or by tissues that interact with immune cells bearing lectin ligands. For example, C-type lectins are calcium-dependent carbohydrate-binding animal proteins (10). The role of C-type lectins as immune receptors was established after lectins expressed by dendritic cells were found to sense the environmental carbohydrate structures and regulate the signaling events of dendritic cells. In addition to serving as signaling receptors of dendritic cells and other antigen presenting cells such as macrophages, C-type lectins are also expressed by natural killer cells, T cells, and plasmacytoid dendritic cells, and their functions are currently being studied closely. Interested readers are urged to consult other extensive and excellent reviews about this field (10, 11). Siglecs are another family of lectins widely expressed on immune cells and exert signaling functions upon encountering their ligands (12-14). Some lectins (such as galectins) are mainly expressed by tissues that interact with immune cells bearing glycan ligands (15). Galectin expression in the microenvironment of cancer and infectious diseases may regulate the function of immune cells (16-19).

The development of glycan-array technologies by the Consortium of Functional Glycomics has played an essential role in identifying the physiological ligands for lectins. In many cases such arrays also generate inconclusive results. It is well-known that lectin binding to glycans is dependent on multivalent interactions of ligands. Technologies are being developed to measure these multivalent interactions. For example, Kurmyshkina et al. designed "glycoprobes" in which fluorescence-labeled polyacrylamide was conjugated to multivalent glycan ligands (20). Such glycoprobes were used to determine the expression of galectins in tumor cells by immunohistochemical staining. However, whether the multivalent glycans displayed by polyacrylamide are similar to those displayed by native glycoproteins remains to be studied. In another study, Zhang et al. displayed glycan ligands on a carrier protein, bovine serum albumin (21). Such "neoglycoproteins" were used as tools to evaluate binding to more than 150 antibodies and lectins. However, it is unclear whether the density and 3-dimensional conformation of multivalent glycans displayed in "neoglycoproteins" are similar to those of native glycoproteins (21). Thus there is an urgent need to develop technologies to determine the density and 3-dimensional conformation of displayed glycans.

3.2. B cell receptors

B cell receptors and monoclonal antibodies have been identified for a series of glycoconjugates: 1) receptors only specific for carbohydrate moieties, such as the blood group antibodies (A and B), which recognize blood group A or B antigens present in glycoproteins or glycosphingolipids (GSLs), and the xeno-reactive antibodies toward the Galα1,3Gal expressed on both glycoproteins and GSLs (22); and 2) receptors specific for a carbohydrate moiety plus an additional structural motif, such as a peptide. This second type of B cell receptor is exemplified by antibodies that require both the sugar part and the peptide part for recognition (23).

3.3. Special concerns for glycoconjugate-specific antibodies

Many antibodies "specific" for a glycan are known for their cross-reactivity with structurally related

glycoconjugates. Whether staining by such an antibody, like immunohistochemical staining of tissue sections, should be recognized as a standard for biochemical relevance remains controversial (24, 25). Several questions remain unanswered: 1) whether such antibodies bind to yet-unknown glycan structures; 2) whether such antibodies bind to other glycans with much stronger affinity than to the target glycan structure; 3) what the sensitivity of such antibodies is in immunohistochemical staining or flow cytometry analysis.

3.4. T cell receptors for glycans

T cell receptors have been identified for glycopeptides, GSLs and non-GSL glycolipids.

3.4.1. Glycopeptides

T cell epitopes that recognize both the sugar and peptide parts of a glycopeptide have been reported, such as Galα1,3 Gal-HEL (hen egg lysosome, 26). Glycopeptide epitopes have also been found in pathogens, such as bacteria-derived glycopeptides recognized by CD4-positive T cells restricted to mouse MHC class II molecules (27).

3.4.2. GSLs

GSL epitopes of T cells were first discovered for CD1-restricted T cells. A marine sponge-derived GSL, α-galactosylceramide, was found to be a superagonist antigen for invariant NKT cells (28). Similar structure of GSLs were later discovered in *Sphingomonas* (29-31), a bacterium related to an autoimmune disease (primary liver cirrhosis), in which the bacterial GSL α-glucuronic acid ceramide is recognized by the immune system simultaneously with bacterial mitochondria proteins that share homology with human hepatocyte membrane proteins. Thus, the bacterial GSL-induced NKT activation elicits autoimmune antibodies toward hepatocytes (32). Recognition of self GSLs by CD1-restricted T cells was first discovered by the De Libero group in multiple sclerosis patients (33, 34). Another self GSL, isoglobotriaosylceramide (iGb3), although reported by several groups to be a stimulatory antigen for invariant NKT cells with characteristics of natural ligands mediating NKT cell development, has unclear physiological functions (35-39).

3.4.3. Non-GSL lipids

Non-GSL lipid epitopes for CD1-restricted T cells were first discovered in mycobacteria (40, 41). Furthermore, mycobacteria-derived lipopeptides were reported as T cell epitopes as well (42). Their physiological relevance to disease progression is unclear. Mycobacterial lipids, which induce CD1-restricted adaptive T cell responses in animal models, have been proposed as vaccine candidates for *Mycobacterium tuberculosis* (43).

α-Galactosyl diacylglycerol, a non-GSL structure expressed by the pathogenic bacteria *Borrelia burgdorferi* which causes Lyme disease (44, 45), is another bacterial glycolipid antigen for CD1d-restricted invariant NKT cells. These glycolipids constitute toll-like receptor-independent activation of innate immunity and may play important roles in the human immune defense against these bacteria.

4. GENETICS AND BIOCHEMISTRY FOR GLYCAN IMMUNE EPITOPES

4.1. Glycoproteins and glycolipids are metabolically unique and structurally challenging

Complex carbohydrate structures bear an important function of information storage. Therefore, it is not surprising that these chemical structures are epitopes recognized by the immune system. The most well-known of this type of epitope is that defining the blood group ABO system, which was discovered in 1900 (46). However, it was not until one century later that the system's chemical and genetic basis was elucidated. The technical difficulties of carbohydrate biochemistry are obvious and are due to the unique feature of glycosidic linkage: one typical hexose sugar may have five hydroxyl (OH) groups in different positions with which another sugar can form glycosidic linkages. Furthermore, sugars have anomers and form branches (Figure 2). The identification of a complex carbohydrate structure from biomaterials thus is often hampered by two layers of barriers: 1) the need to separate the multiple isomers into individual homogenous fractions; and 2) the limited material available to analyze the sugar identities, sequence, and linkages of the glycan structure.

Complex lipid structures include GSLs and non-GSL structures such as phospholipids, the major components of the bilayers of the plasma membrane. The heterogeneous nature of GSLs (47, 48) is also caused by: 1) variations in the length of their fatty acid components (typically from C16 N-fatty acyl to C26 N-fatty acyl); 2) unsaturation of the N-fatty acyl chain; and 3) hydroxyl modification of the N-fatty acyl chain or sphingosine chain (Figure 3). Taking the trisaccharide-ceramide GSL iGb3 as an example, the ceramide part of a chemically synthesized iGb3 is d18:1/C26:0, while iGb3 in a leukemia cell line (RBL) has a ceramide core mixed with d18:1/C24:0 and d18:1/C24:1. When iGb3s with different forms of ceramide are separated in thin layer chromatography, they appear as different bands and are often misunderstood as different glycans by non-experts.

4.2. Gene chips and microarrays cannot be used to detect glycan epitopes

Unlikely polynucleotides and proteins, glycans are synthesized by glycosyltransferases and glycosidases according to a different central dogma, characterized as an "assembly line" by Rosemarie and Stuart Kornfeld (49). Glycan structures are not copied or translated from any existing template. Instead, each individual glycan is assembled by glycosyltransferases and glycosidases sequentially (Figure 4). Therefore, the expression level of glycosyltransferase or glycosidase genes is not predictive of the presence or absence of related glycan structures because they are subjected to further modification by other enzymes.

4.3. Assembly of glycan immune epitopes in unique cellular compartments

Immune epitopes can be generated from both exogenous phagocytosed pathogens and endogenously assembled structures. Particular pathogens, such as

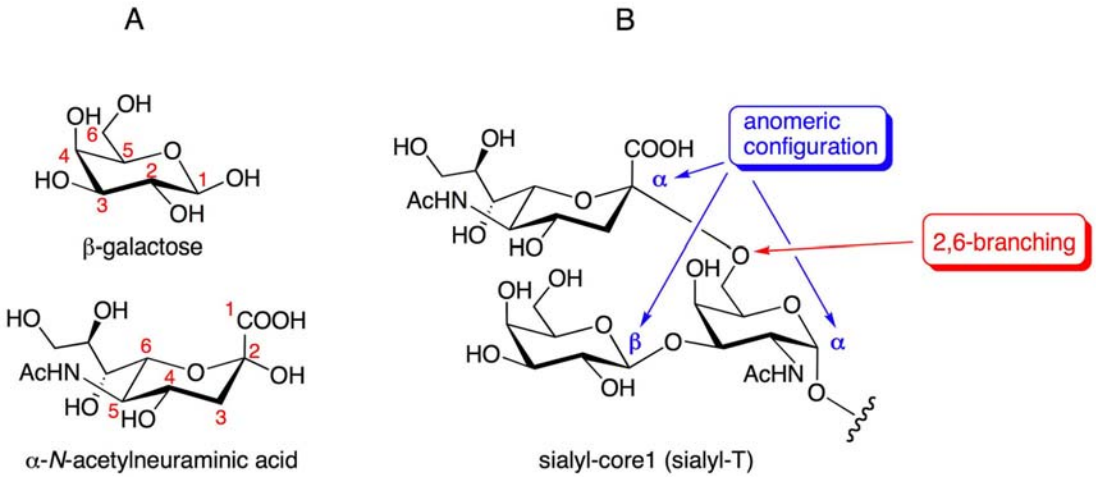

Figure 2. Structural basis of diverse glycosidic linkages. A. Numbering of representative hexose sugars (galactose and N-acetylneuraminic acid). Hydroxyl groups at different positions (OH-2, OH-3, OH-4, and OH-6) of a typical hexose acceptor may be involved in glycosidic linkages. B. Two possible anomeric configurations (α versus β) and branching are the basis for further structural diversity. In this example, the T antigen (Galβ1,3GalNAc) is branched with an α-N-acetylneuraminic acid moiety at position 6 of N-acetylgalactosamine.

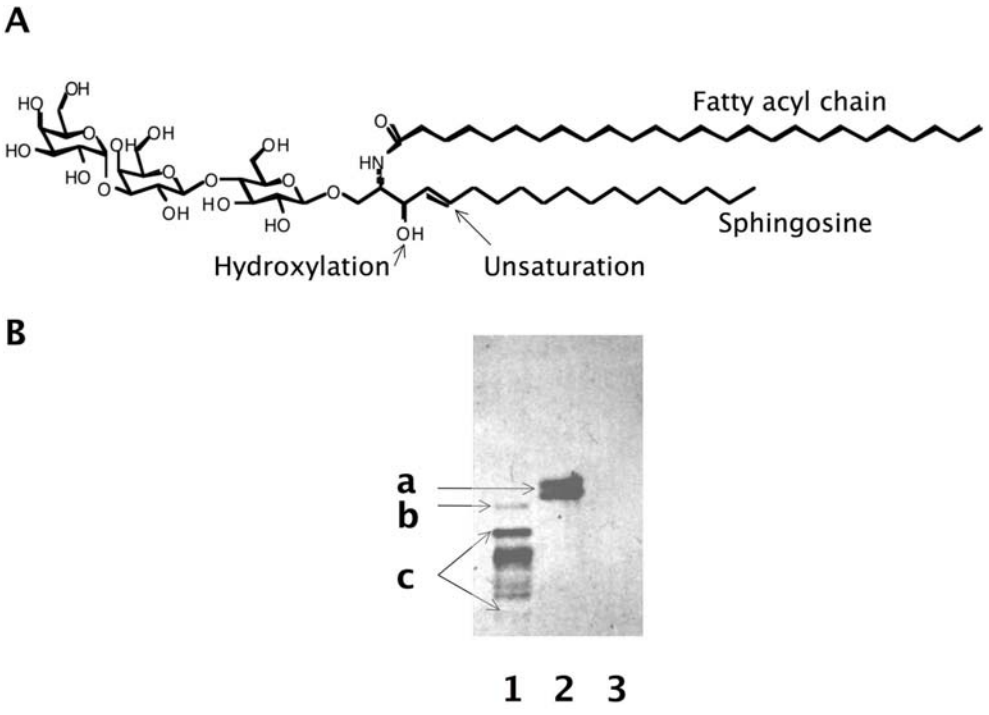

Figure 3. GSLs are heterogeneous because of variations in their ceramide parts. A. The ceramide part of GSLs includes a sphingosine and an N-fatty acyl chain. Both the sphingosine and N-fatty acyl chains may be modified by hydroxyl groups and unsaturation. B. Immunostaining of GSLs separated by thin layer chromatography (TLC). Lane 1, leukemia cell line RBL, which expresses iGb3 and other α1,3Gal-terminated GSLs, as stained by a monoclonal antibody specific for Galα1,3Gal; Lane 2, a chemically synthesized iGb3 from Alexis Biochemicals, CA; Lane 3, a chemically synthesized Gb3 from Alexis Biochemicals, CA. The retention factor of GSLs on thin layer chromatography is dependent on its sugar moiety (hydrophilic) and ceramide moiety (hydrophobic). The hydroxylation and unsaturation of a GSL also influences its retention factor. a, a chemically synthesized iGb3 (h18:1/C26:0, which means it contains one hydroxylation and one unsaturation in the sphingosine chain, and no unsaturation in N-fatty acyl chain); b, iGb3 expressed in a leukemia cell line, which has a different ceramide part (mixed d18:1/C24:0 and d18:1/C24:1); c, multiple bands representing other α1,3Gal-terminated GSLs expressed by RBL cells.

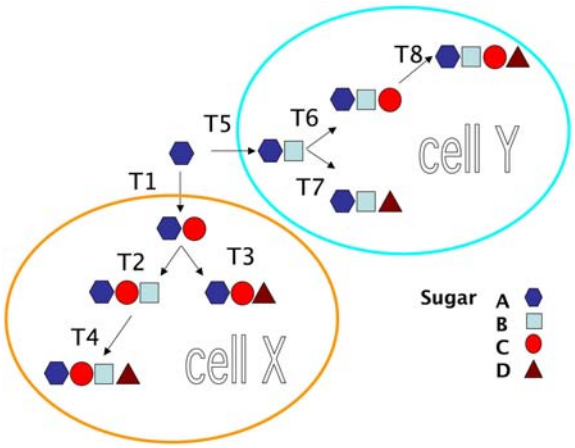

Figure 4. The assembly line of glyco-enzymes. Glycans are assembled in a stepwise manner catalyzed by glycosyltransferases, instead of from DNA or RNA templates. In different cells (here represented as X and Y), different glycan structures are formed by different glycosyltransferases (T1-T4 in cell X, and T5-T8 in cell Y). The presence of specific glycan structures cannot be predicted by gene expressions, because each glycan can be converted to another structure by downstream glyco-enzyme modification.

bacteria, are typically phagocytosed and digested in the endolysosomal pathways of professional antigen presenting cells, resulting in peptide epitopes presented to CD4 T cells (through the MHC class II antigen presenting pathway) and B cells. Viruses hijack the host's protein synthesis pathway and assemble viral proteins in the endoplasmic reticulum compartment, which is surveyed by the MHC class I antigen presenting pathway, and trigger CD8 responses.

The generation of glycan immune epitopes may involve multiple cellular compartments. For endogenous antigens, glycans can be synthesized in the endoplasmic reticulum and Golgi compartments and subsequently processed in the lysosomal compartment (50). For external antigens, bacterial glycan and lipid antigens can be processed in the endolysosomal compartment, similar to protein antigens (51). In contrast to peptide antigens which are generated by proteases, lipid and glycan epitopes are generated by glycosidases and lipidases (50, 51). For CD1-restricted T cell glycolipid antigens, chaperone proteins, such as saposins, are required for antigen loading (50, 52-53). These chaperone proteins are responsible for removing lipids loaded to CD1 when it is synthesized and secreted to the cell surface, and facilitate the loading of self and foreign glycolipid antigens in the lysosome.

4.4. Conventional biochemical purification approach for immune epitope discovery

Successful identification of a biologically active structure from biological samples is dependent on the establishment of a detection system, the availability of sufficient initial samples for detection and characterization, effective structural analysis methods, and the ability to chemically synthesize a structure for verifying the natural structure's biological activity.

4.4.1. Detection system

An absolute prerequisite for identifying a biologically active epitope is a reliable and robust detection system, which must have 1) sufficient sensitivity to detect the biologically active epitope, thereby allowing further downstream enrichment; 2) a specificity that discriminates the biologically active epitope from the vast array of non-active epitopes; and 3) a reproducibility reliable enough for repeated fractionation and enrichment of the immune epitope.

For B cell epitopes, the availability of a specific and sensitive monoclonal antibody itself will allow the detection of the antigen by technologies such as enzyme-linked immunosorbent assays (ELISA). For T cell epitopes, it is much more difficult to directly use T cell receptor protein to measure the binding to an antigen presented by MHC molecules or MHC-like molecules such as CD1. T cell clones must be generated based on their specific recognition of certain antigen epitopes. Such T cell clones produce cytokines or exert cytotoxicity when recognizing antigen presenting cells that present the epitope of interest. Pippa Marrack invented T cell hybridoma technology, which uses cell fusion between fresh T cells and a myeloma tumor cell line. T cell hybridomas can be repeatedly sub-cloned to select those producing high amounts of cytokines upon stimulation by a T cell antigen, and thus can have a sensitivity 2 to 3 orders of magnitude higher than that of non-fused T cell clones.

4.4.2. Sufficient initial samples for detection and characterization

To successfully identify a biologically active epitope, one must acquire a considerable amount of biological material that contains the structurally unknown immune epitopes. The concentration of a desired structure in samples can be estimated quantitatively before large-scale fractionation. Furthermore, a desired structure may suffer considerable loss in every step of fractionation; thus the percentage of recovery in every purification step should be estimated quantitatively as well. For example, according to our previous studies, we can typically recover 160 μg of acidic GSLs and 80 μg of neutral GSLs from each thymus of 4-week-old C57B6 strain of mice (54). From 2000 mouse thymuses, we expect to purify 320 mg of acidic GSLs and 160 mg of neutral GSLs. Assuming 5 μg of a natural ligand is required for biological assays, a ligand must have an abundance among total GSLs of higher than 0.001% to be detectable, assuming its 100% recovery. Using a conservative estimation of recovery for a GSL ligand of 50%, we would need to start with 4000 mouse thymuses.

4.4.3. Analytic method to prove the chemical nature of the fraction of interest

A bottleneck in immune epitope discovery lies in the lack of analytical technologies available for analyzing the epitopes' chemical compositions and structures. A highly sensitive and specific technology would not only

precisely determine the chemical nature of an immune epitope, but would also significantly reduce the amount of purified epitope required for structural determination, and the amount of starting material initially needed for fractionation and purification. In general, structural analysis requires a highly purified fraction that contains only the structure representing the immune epitope; contaminants or mixtures are not allowed. This is a particularly challenging task for identification of carbohydrate-containing epitopes because of heterogeneity in both the O-glycans and N-glycans of naturally occurring glycoconjugates.

4.4.4. Synthetic method to verify the chemical nature of an immune epitope

After a candidate structure of an immune epitope is established, larger quantities of such a potential epitope can be synthesized by chemical or biochemical methods, and tested for their immunogenicity. Such synthesis may also provide the basis for the manufacturing of vaccines and diagnosis reagents. Purity of a desired synthetic immune epitope is essential for biological assays. It is well-known that less than 1% of contaminants introduced during chemical synthesis can be recognized by other immune receptors, and trigger potent undesired immune responses.

4.5. Limitations of the conventional biochemical purification approach

Conventional biochemical purification may be hampered by the nature of the immune epitope, the resolution of separating technologies, and the power of analytic technologies.

4.5.1. Weak antigenicity or affinity

Biochemical isolation will be difficult if an immune epitope has weak affinity. C-type lectins expressed by dendritic cells are known to bind to sugar ligands with low affinity (with Kd values in the micromolar range). A natural ligand for invariant NKT cells, iGb3, also binds to T cell receptors with Kd values in the micromolar range and serves as a weak agonist.

4.5.2. Low abundance

A good example of low-abundance epitopes is the natural ligands for NKT cells (40). Although such natural ligands exist in the mouse and human thymus, lipidomic and glycosphingolipidomic studies have found no natural ligands among the known lipids (including GSLs) other than iGb3. We have estimated that the ratio of iGb3 to Gb3 in mouse thymus is 0.4% (54). If each mouse thymocyte expresses about 20,000 copies of Gb3 (38), 80 copies of iGb3 will be present. If iGb3 is not the ligand responsible for NKT cell development, it is likely that the abundance of the major ligand would be above 80 copies per thymocyte.

4.5.3. Resolution of separation technologies

Most technologies used to separate an immune epitope from others are based on chromatography. These include size exclusion chromatography, hydrophobic interaction chromatography, hydrophilic interaction chromatography, and affinity chromatography. Currently, peptide immune epitopes can be separated in proteomics core facilities in most academic or industrial institutions. However, the separation of glycoconjugate and lipid immune epitopes remains challenging because of the presence of multiple glycoconjugate isomers, which are similar in size and polarity, and the difficulty in separating lipid species with subtle structural variations.

4.5.4. Power of analytic technologies

It is generally accepted that mass spectrometry (MS) is the standard technology for immune epitope identification. Although high-performance liquid chromatography (HPLC) is still occasionally used for identifying immune epitopes, it cannot provide exact structural information, nor can it reveal possible contaminants that are invisible to the conventional detectors used in HPLC, such as ultraviolet and fluorescence detectors. Current U.S. Food and Drug Administration regulations related to peptide vaccines require MS data, which may disclose low levels of contaminant peptide even though the HPLC profile may show a single “pure” peak. Modern liquid chromatography (LC)-MS instruments combine separation and mass spectrometric analysis of mixtures and provide information about the retention times as well as the masses of individual compounds if the peaks are base-line resolved.

The invention of matrix-assisted-laser-desorption/ionization time-of-flight MS (MALDI-TOF MS) and electro-spray ionization mass spectrometry (ESI-MS) has revolutionized the field of immune epitope discovery. The ionization methods in these technologies are highly efficient and relatively mild, which allows for the detection of molecular ion peaks of unfragmented species. For more detailed characterization of biomacromolecules, MS instruments have been invented for selecting individual molecular ion species and breaking them into smaller fragments (product ions) that provide structural information about the parent ions. This fragmentation process is normally done by collision-induced dissociation, which involves activating the biomacromolecules (ionized as parent ions) through collision with inert gas molecules. The increased energy induces bond breakage, and systematic analysis of the resulting fragments provides information about the molecular structure of the biomacromolecules. Often, "signature" fragments that are diagnostic for particular structural features can be generated, in addition to the molecular mass of the original parent biomacromolecules. These fragments can sometimes be sufficient for identifying biomacromolecules, particularly if standards, often chemically synthesized, are available. Multiple rounds of low-energy fragmentation, which can be effectively carried out in an ion-trap mass spectrometer (IT-MS), further expand the information yield from mass spectral analysis (55). With four or five rounds of fragmentation (which can only be done usng IT-MS, but not other MS instruments), it is possible to determine the structure of an immune epitope.

Two other analytical methods useful for the accurate structural determination of immune epitopes are nuclear magnetic resonance (NMR) spectrometry and X-ray crystallography. These methods allow for the distinction of constitutional isomers and stereoisomers,

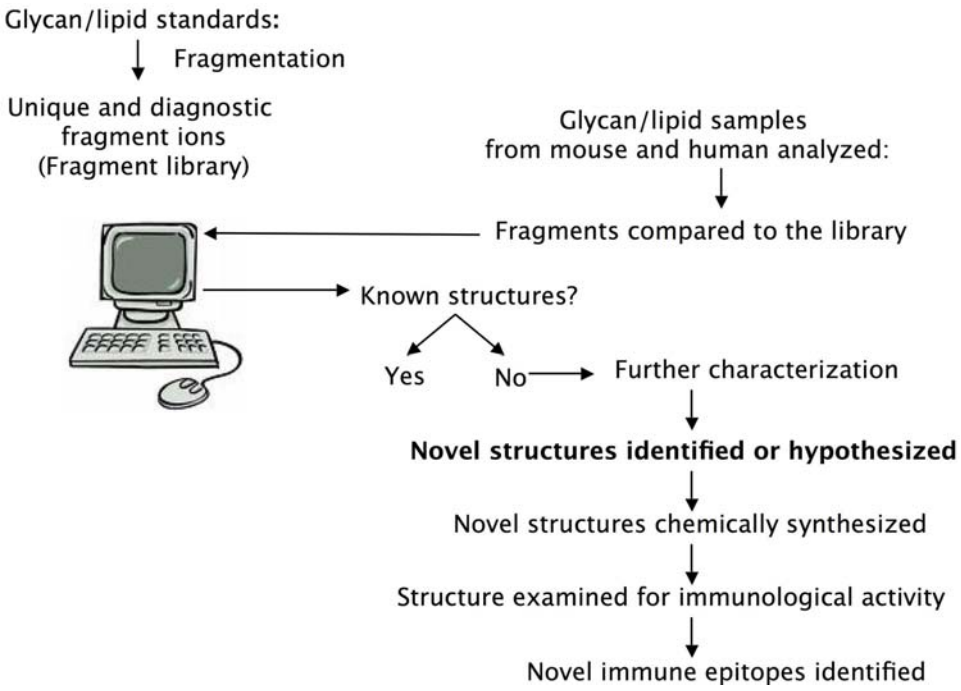

Figure 5. Generation of hypotheses in immunologic mapping of the glycome. Glycan structures are collected from immune cells and compared to a database of known glycans. Candidate novel glycans are isolated and their structures hypothesized. Novel structures are then chemically synthesized and examined for immunological activity.

which can be a difficult task using MS, even with sophisticated MS capacities, particularly when no reference material is available. A drawback of immune epitope determination by NMR spectrometry and X-ray crystallography is that larger sample quantities (micrograms to milligrams) are required.

5. PROPOSING, TESTING AND VERIFYING HYPOTHETICAL GLYCAN IMMUNE EPITOPES

5.1. Why hypothetical epitopes

Because of the limitations of conventional approaches to biochemical purification, it may be impossible to identify those immune epitopes with weak immunogenicity or low abundance. When conventional biochemical purification approaches do not lead to unambiguous structural information of an immune epitope composed of sugar units and/or lipids, we propose that such epitopes may be identified through a "reverse" approach. In this process, the existing glycan structures from biological samples may be hypothesized based on limited structural information and candidate immune epitopes synthesized by methods of bio-organic chemistry. Chemically synthesized candidate epitopes would then be examined for their immunogenicity, revealing novel immune epitopes. For example, in our recent studies, we identified a candidate novel glycolipid structure from mouse thymus by MS analysis, but the abundance of this structure does not allow for extensive analysis. Based on partial structural information, we hypothesized it to be Galα1,3(Fuc α1,2)Galβ1,4Glcβ1,1Cer (fucosylated iGb3). Chemically synthesized fucosylated iGb3 was found to be stimulatory to mouse NKT cell clones (unpublished data). Thus fucosylated iGb3 serves as a candidate natural ligand for NKT cells.

5.2. Interdisciplinary nature of immunologic glycomics: synergized efforts by immunobiology, analytical chemistry, and synthetic chemistry

The success of the immunologic glycomics approach is based on advances in the fields of immunobiology, analytical chemistry, and synthetic chemistry. Sensitive immunoassays, such as those that use reporter genes fused to immune signaling molecules, may allow large-scale screening of candidate immune epitopes. In addition, chemo-enzymatic synthesis of glycans, glycolipids, and glycopeptides provides candidate immune epitopes for immunoassays in quantities often impossible to purify from natural materials.

The essential driving force for this synergized effort is the development of specific, sensitive, and generally applicable analytical technologies. A complete picture of a cellular glycome is possible only after known structures are viewed (Figure 5). For identifying candidate unknown structures (the hypothesis-generating step), powerful analytical technologies allow finding of more structures that may not be visible by insensitive methods like HPLC. More importantly, such analytical technologies are essential to verify the structures and purities of chemically synthesized immune epitopes. Chemical synthesis will unavoidably introduce other byproducts, which may not be visible by HPLC but may interfere with immunoassays even at very low abundance.

6. ESSENTIAL ROLE OF ANALYTICAL METHODS AND TOOLS IN THE HYPOTHESIS-GENERATING PHASE

6.1. IT-MS as a unique tool in the study of glycomics and lipidomics

The presence of multiple isomers sharing the same molecular masses is a longstanding impediment to their structural characterization. Tandem MS (MS-MS) technology has significantly helped in solving part of the problem in that it permits identifying characteristic fragment ions generated by instruments with tandem MS capacity, such as MALDI-TOF-TOF and triple quadruple/time-of-flight (Q-TOF) instruments. However, tandem MS analysis often does not reveal the complex linkage information of glycans. The Reinhold group first demonstrated that multiple rounds of low-energy fragmentation, which can be effectively carried out in IT-MS, greatly improves the information yield from glycan mass spectral analysis (55).

6.1.1. Glycolipid analysis

Levery and coworkers combined all the potential advantages of IT-MS methodology, including the detection of isomeric structures using signature diagnostic ions that are observable only in MS^4 and MS^5 spectra (which involve three and four rounds of fragmentation, respectively), for the highly sensitive identification and quantitation of GSLs present in the form of multiple isobaric mixtures (56-57). In theory, this method may be applied to identify every existing glycolipid structure, pending the availability of standard glycolipids, which can be chemically synthesized.

6.1.2. N-glycans, O-glycans, and O-glycopeptides

The power of the IT-MS^n method has also been demonstrated for N-glycan and O-glycan analysis (58-60), as well as the analysis of glycopeptides modified by O-glycosylation (60). The powerful multi-stage fragmentation (up to MS^5 or MS^6) function of ion trap instruments allows investigators to diagnose highly complex sugar linkages, which they can not do using other mass spectrometers.

Although IT-MS instruments can dissect the structure of complex sugars, for analyzing a glycopeptide, they may be limited in their ability to determine where the sugar modification is because the fragmentation induced by collision with high-energy gas cleaves the weakest bond in the molecule first, and this bond is often the glycosidic bond linking the glycan to the peptide backbone. This problem can be overcome by an alternative fragmentation method, electron transfer dissociation (ETD), which can cleave the peptide backbone stepwise without damaging glycosylated amino acids. Using this powerful technique, two independent groups recently generated a complete glycoprofile for alpha-dystroglycan (α-DG), an O-glycosylated protein related to congenital muscular dystrophies (61, 62).

6.2. Sample preparation and separation technology

Sample preparation and separation in glycomics studies critically influence the quality, reproducibility, and reliability of data. The combination of MS and liquid chromatography is now routinely practiced for proteomics studies. Efforts in combining these technologies for glycomics and glycolipidomics have also been made.

Separating glycans by HPLC after cleaving them from glycoproteins or glycolipids has proved to be an effective method to generate glycan profiles (48). Recently, native glycans have also been separated by means of hydrophilic interaction without labeling with fluorescence groups (63, 64). Several groups of investigators have reported separating certain categories of lipid structures by reverse-phase HPLC in combination with MS (65).

6.3. Controversies on the detection of immune epitopes in low abundance

Immune epitopes, such as T cell antigens, may function at extremely low abundance. For example, α-galactosylceramide, formulated by Kirin Pharma Co. as KRN7000 using a patented solvent technology, may stimulate mouse NKT cells in vivo at a serum concentration of 10 pg/mL, whereas current available MS technology can only measure α-galactosylceramide at a serum concentration of 12.5 ng/mL (66). Controversies may arise regarding which standard should be recognized as evidence of a chemical structure, if such a structure could not be detected by any chemical measurement such as HPLC or MS. So far, biological assays remain the most sensitive assays for detecting T cell epitopes.

The super-agonistic activity of some immune epitopes, such as α-galactosylceramide as mentioned above, also raise the question of which standard should be used to conclude that an immune epitope is absent. For example, in our own experience, we have not been able to develop an assay based on MS to detect less than 1% contamination of α-galactosylceramide in the presence of its β-isomer. Thus, when we prepare such β-glycosylceramides, the only reliable method we can use to exclude contamination by α-isomers is bioassays.

7. EARLY INSIGHT INTO CLINICAL APPLICATIONS

7.1. Demands and markets

Demand for lipidomics and glycomics in clinical research endeavors is emerging. Glycans and glycolipids are recognized as targets for immunodiagnosis and immunotherapy in multiple diseases; examples include 1) Tn antigens, a family of under-glycosylated mucin proteins expressed in more than 80% of cancer patients, which are being used both as cancer markers (CA125, CA15.3 and CA2.29) and vaccine candidates (67); 2) GM2, GD2, GD3, and other gangliosides, which are being evaluated as vaccine candidates in melanoma and neuroblastoma clinics (68, 69); and 3) GbH, which is being evaluated as a biomarker and vaccine candidate in breast cancer clinics (70). Important clinical applications have already been developed in several areas: 1) cancer vaccines, such as Tn glycans and GM2; 2) tumoricidal antibodies or toxins, such as anti-GD2 and anti-MUC1; and 3) diagnostic reagents for pathogens, such as the monoclonal antibodies for anthrax spore glycans (71, 72) and Chaga's disease (73).

7.2. Future directions

Research trends in the field of immunologic glycomics are likely to include the following: 1) more precise, accurate, and complete structural determination of epitopes will be demanded: for example, not only should the sugar moiety of a glycoprotein or glycolipid be characterized, but also the sequence of the protein and the lipid moieties. 2) More sensitive analytical methods suitable for clinical samples will be needed: for instance, MS technology has been recently applied for detecting Gb3 from the urine of patients with Fabry disease (74). 3) Assays will be highly focused on detecting specific structures, while the extensive profiling of glycans and lipids in small samples may continue to encounter technical difficulties and provide little value for studying disease mechanisms or in developing products for clinical use. 4) Advances in synthetic bioorganic chemistry may improve the commercial availability and accessibility of homogeneous standards, which will help definitively unravel mechanisms in biological processes.

7.3. Five-year view

The last few years have seen much progress in our understanding of glycan and glycolipid functions. The next five years are likely to continue to reveal functional aspects of this type of biomarcromolecules. Synergistic efforts in immunology, biochemistry, and synthetic chemistry will be routine practice for future discoveries. Question-driven studies on important diseases such as cancer, allergies, and asthma may provide clues for ground-breaking discoveries. Furthermore, hypothesis-generating studies may also lead to interesting findings. Major goals for the next five years will be to complete the picture of the spectra of functionally specialized glycan and lipid structures, to improve our understanding of related genetic pathways responsible for synthesizing of functional glycan and lipid structures, and to use such information to develop preventive and therapeutic approaches for personalized therapy.

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9. FINANCIAL DISCLOSURE

DZ is a consultant for BioTex, Houston, TX, and an inventor involved in patents related to technologies mentioned in this article, issued or in application.

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