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Protein glycosylation in infectious disease pathobiology and treatment

Mini-Review

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Abstract: A host of bacteria and viruses are dependent on O-linked and N-linked glycosylation to perform vital biological functions. Pathogens often have integral proteins that participate in host-cell interactions such as receptor binding and fusion with host membrane. Fusion proteins from a broad range of disparate viruses, such as paramyxovirus, HIV, ebola, and the influenza viruses share a variety of common features that are augmented by glycosylation. Each of these viruses contain multiple glycosylation sites that must be processed and modified by the host post-translational machinery to be fusogenically active. In most viruses, glycosylation plays a role in biogenesis, stability, antigenicity and infectivity. In bacteria, glycosylation events play an important role in the formation of flagellin and pili and are vitally important to adherence, attachment, infectivity and immune evasion. With the importance of glycosylation to pathogen survival, it is clear that a better understanding of the processes is needed to understand the pathogen requirement for glycosylation and to capitalize on this requirement for the development of novel therapeutics.

Keywords: *Virus • Bacteria • Glycosylation • Infectious disease*

1. Introduction

Glycomics is rapidly becoming an emerging field of importance in understanding the biology of the cell. The glycome of an organism is the repertoire of glycan modifications that are made to DNA, lipid and protein and until recently, the significance and role of these modifications has been under appreciated. It is now believed that the post-translational modification of proteins provides for a series of unique regulatory and functional activities in both eukaryotic and prokaryotic cells. According to a recent review by Witze, there are perhaps 300 forms of post-translational modification of proteins [1]. Of these 300 modifications, glycosylation events may be some of the most important molecular modifications of cellular proteins. Glycosylation is a ubiquitous post-translational event where glycans are attached to amino side chains. In the past few years, research has demonstrated that glycosylation is essential to cell adhesion, receptor activation, signal transduction, molecular trafficking and endocytosis. In prokaryotic and eukaryotic cells there are 13 different monosaccharides and 8 amino acids that are involved in glycoprotein assembly and more than 40 glycosidic bonds are known to link glycans to protein targets in N-linked, O-linked, C-linked, phosphoglycosyl and glypiation events [2].

Four types of glycosylation have been described thus far, N-linked, O-linked, C-linked and the newly described cysteine S-linked (Figure 1) [3]. The most common form in regard to infectious disease is N-linked glycosylation where a high mannose core is attached to the amide nitrogen of asparagine in the context of the conserved motif Asn-X-Ser/Thr. This modification occurs early in protein synthesis and is followed by a complex process of trimming and remodeling that culminates in transit of the protein through the endoplasmic reticulum and Golgi. The result is a glycoprotein with complex oligosaccharide structures. N-linked glycosylation is important for the folding of many proteins occurring widely in eukaryotic organisms but very rarely in bacteria. Viruses commonly use this host cell process to modify proteins present on

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their surface in a host-specific fashion which ultimately impacts the viral glycoproteins in roles such as stability, antigenicity, and host cell invasion.

O-linked glycosylation is a post-translational modification of secreted and membrane bound proteins that take place in the cis-Golgi compartment. O-linked glycans play important roles in protein localization and trafficking, protein solubility, antigenicity and cell-cell interactions. Modification of the oxygen in serine or threonine on proteins occurs with the addition of N-acetyl galactosamine (GalNAc). O-glycans can extend into long chains similar to N-glycan, but are usually less branched and can result in the formation of mucin-like molecules. O-glycans can function in lectin-ligand interactions, which may facilitate interaction with cellular proteins used as receptors of entry, and viral attachment proteins such as O-linked RSV-G protein.

C-linked modifications representing the third type of glycosylation are much less common and novel. C-linked modifications result in the attachment of an α-mannopyranose to tryptophan via a carbon [4]. Until 2007, the only described glycosylation of virus proteins was of the N or O-linked varieties. Falzarano *et al*. demonstrated C-linked modification of Ebola soluble glycoprotein representing the first description of a C-mannosylation of a viral protein [5]. The role of this modification in eukaryotic systems has not been fully clarified; however, it was first described in human RNase-2 and interleukin-12, the modification does also exist in several innate complement components (C6, C7, C8, and C9) and in the complement regulator properdin suggesting a role in innate defense [6].

The final modification is S-glycosylation which is a new and exceedingly rare post-translational modification found in glycopeptide bacteriocins where sulfur in the amino acid cysteine is modified by N-acetylhexosmine [3]. The biological role for this modification is still under investigation but may prove to be unique to bacteriocins.

Given the scope and breadth of protein modifications and the role that these modifications play in the physiology of the cell, it is not surprising that potential pathogens subvert these processes to promote their replication and survival.

2. Glycobiology of Infection

2.1 Bacterial Glycosylation

With respect to bacteria, the body of knowledge regarding glycosylation is small. There is an extensive repertoire of glycoconjugates on the bacterial cell surface that includes lipopolysaccharide, capsular polysaccharides, glycoproteins, and exopolysaccharides to name a few. These molecules are found on Gram-negative bacteria, but a group of surface layer (S-layer) glycoproteins in Gram-positive bacteria also exist [7]. Protein glycosylation was initially described in the 1930s and it was long believed that eukaryotic cells were singularly capable of glycosylation where more than two-thirds of the proteins are predicted to be modified by glycosylation [8].

It is now clear that prokaryotes are capable and may exhibit greater diversity of glycan composition and structure than eukaryotes [9-11]. The continued difficulty with understanding prokaryotic glycosylation lies in our inability for proper analysis and characterization. In general, the sugars that are employed for the modification of eukaryotic proteins are few and highly conserved. In contrast, the sugars that are utilized to produce glycoproteins in prokaryotic cells are more unusual and the structures may be more difficult to analyze with technologies developed to examine proteins. Characterization of carbohydrate modification also should include an analysis of amino acid consensus

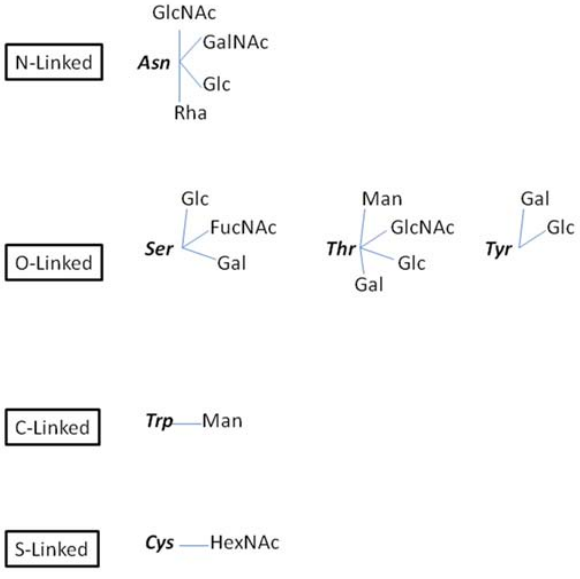

Figure 1. Schematic representation of the carbohydrate linkages known to occur in pathogens. The four described carbohydrate linkages display diversity in the possible carbohydrates that can be affixed to the amino acids. N-linkages employ the amide nitrogen on asparagine when associated with the consensus sequences N-x-S/T where x can be any amino acid except proline. The O-linkage utilizes the hydroxyl oxygen of serine, threonine or tyrosine. The C-linkage results from a carbon-carbon attachment of α-mannopyranose to a tryptophan within the consensus sequence of WxxW where the first tryptophan becomes mannosylated. S-linkage represents the most recently described and results from the addition of HexNAc linked to the sulfur on terminal cysteine. Abbreviations: Gal, Galactose; GlcNAc, N-acetylglucosamine; GalNAc, N-acetylgalactosamine; HexNAc, N-acetylhexosamine; FucNAc, N-acetylfucosamine; Man, Mannose; Rha, Rhamnose; Glc, glucose; Gal, Galactose; Fuc, Fucose.

sequences where carbohydrate addition may occur. In eukaryotic N-linked glycosylation a consensus sequon of Asn-X-Ser/Thr results in attachment carbohydrate to the peptide backbone. Thus far, a consensus sequon has not been clearly identified for O-linked addition outside of the requirement for a Ser/Thr residue.

Forty years after the discovery of eukaryotic glycosylation, bacterial S-layers were shown to be glycosylated in *Clostridium* species [12,13]. The glycans found on bacterial S-layer are often of the O-linked variety associated with serine, threonine or tyrosine. Over the past 10-15 years there has been an increase in the understanding of both N- and O-linked protein glycosylation systems. Currently, *Campylobacter jejuni* is the only bacterium that has been described as having a well characterized N-glycosylation pathway where up to 40 soluble and membrane proteins are glycosylated [14,15]. Among the glycoconjugates that are found on bacteria, there are examples in the archeal and bacterial S-layers [12,16], flagella [17] and pili [18] representing carbohydrate additions of glucose, galactose, heptose, pseudaminic acid, high mannose, N-acetylhexosamine and N-acetylglucosamine.

Carbohydrate modification is found in both commensal and pathogenic bacteria. The intestinal symbiont *Bacteroides fragilis* was described to have a system of O-glycosylation that is important to the physiology of the organism [19]. These organisms have developed an exquisite adaption for the gastrointestinal tract comprising one of the most abundant species in the human colon. Bacteroides have a complex system of mechanisms devoted to acquisition of and the metabolism of carbohydrates allowing the organism to rapidly adapt to shifts in food. Bacteroides also have mechanisms that allow for the capture of fucose from the host mucosal surfaces and a rare bacterial pathway for the incorporation of exogenous fucose into the bacterial capsule and into glycoproteins [20]. Pathogenic bacteria have also been demonstrated to employ glycosylation. From the standpoint of health and disease, the characterization of glycans on pathogenic bacteria is important to our understanding of pathobiology and eventual treatment. The pathogenic bacterium *Neisseria* was one of the first examples of O-glycosylated glycoprotein in a human pathogen. Several studies have demonstrated the genes important for the generation of pilin glycan and the mechanisms involved [21-23]. Gram-negative bacteria such as *Neisseria* produce a diverse array of pili that mediate microbe-microbe and host-pathogen interactions important in the development of disease. Both *Neisseria meningitides* [22] and *Neisseria gonorrhoeae* [24] make use of unusual sugars such as the trisaccharide β-Gal-(1-4)-α-GAL-(1-3)[2,4-diacetamido-2,4,6-trideoxyhexose] in O-linkage to serine [22]. In addition to the *Neisseria* species there is also evidence to support the addition of the O-linked glycan pseudaminic acid to serine residues, an analog of sialic acid to *Pseudomonas aeruginosa* pili [25] and to the flagellin of pathogens such as *Helicobacter pylori* [26], and *Campylobacter coli* [27]. In the case of *Campylobacter,* there have been at least 19 serine/threonine residues in the surface exposed flagella filaments that are modified by pseudaminic acid [28]. Glycosylation of pilin and flagellin in many cases is necessary for the assembly of pili and flagella and becomes important in the pathogenesis of infection because of the role that these proteins play in mobility, colonization and virulence. In addition to the aforementioned bacterial species, there has also been the description of carbohydrate addition to proteins in *Borrelia burdorferi* [29], *Chlamydia trachomatis* [30-32], *Escherichia coli* [33,34] and *Mycobacterium tuberculosis* [35] that contribute to attachment and infectivity (Table 1). The scientific community can now begin to appreciate bacterial glycosylation and the broad and unique range of sugars that are found in prokaryotic glycoproteins. The pathways and mechanisms in prokaryotic glycosylation are now also being described. With continued efforts, a clearer understanding of the biology of prokaryotic glycosylation will lead to enhanced antibacterial agents. Presently, the role of glycosylation in bacterial proteins is two-fold. First, many glycosylation events result in changes to protein function that affect assembly of flagellin/ pili and increase bacterial adherence. Secondly, the addition of carbohydrate to proteins can also dramatically affect interaction with the host immune system. The study of bacterial glycosylation has been made difficult by a lack of technologies and the complexity of bacterial carbohydrate modifications. Further developments in analytical capacity coupled with more intensive study and description of enzymatic pathways will bring greater understating of bacterial glycan and development of novel therapeutics.

2.2 Virus Glycosylation

Protein glycosylation is vitally important at various stages of the virus life cycle, from the initial attachment to the terminal stages of virus release. From this standpoint, the virus has been used as a model system to better understand the general mechanisms of protein glycosylation. Most viruses have minimal levels of glycosylation. However, the variation among viruses can be quite substantial. For example, the E protein of dengue has two putative sites [36] while other viruses such as human immunodeficiency virus

Organism	Carbohydrate	Protein	Reference
Borrelia burgdorferi	GlcNAc	Minor protein	[136]
Campylobacter	Pseudaminic acid	Flagellin	[137]
Mycobacterium tuberculosis	A-(1-2) mannose and fucose	Surface	[138]
Escherchia coli	Heptose	TibA	[17]
Neisseria	α-Gal-(1-3)GlcNAc	Pilin	[22]
Pseudomonas aeruginosa	Pseudaminic acid	Pilin/ flagellin	[25]
Clostridium	HexNAc	Slime layer	[139]
Helicobacter pylori	Pseudaminic acid, high mannose, sialic acids	Flagellin	[26]
Chlamydia trachomatis	High Manose	Minor protein	[32]
Influenza	High mannose	HA	[140]
Lymphocytic choriomeningitis virus	High mannose	GP1 and GP2	[141]
Filovirus	GlcNAc, high mannose	GP	[142]
RSV	Glucosaminoglycans	G	[143]
HIV-1	High mannose and fucose	Gp120	[144]

Table 1. Examples of glycosylated proteins in pathogens.

(HIV) are heavily glycosylated with an average of 25 sites [37]. In the case of HIV, the gp120 glycoprotein is the most highly glycosylated viral protein. The high level of N-linked glycosylation benefits the virus by serving as a glycan shield protecting the virus from the host immune system [38]. During evolution, sugars are added and deleted continuously bringing diversity and complexity to viral glycoproteins (Table 1). Alteration or abrogation of sites for glycosylation can have dramatic impact to survival and transmission of many viruses. Small alterations in glycosylation can alter folding and conformation that in turn affects the entire molecule [39-41]. Additionally, changes in the level of glycosylation can affect interaction with host receptors. Changes in glycosylation level can also cause a virus to be more readily recognized by innate host immune cells thus impacting viral replication and infectivity. Many viruses containing glycosylated proteins cause human and animal disease, however; this review for illustrative purposes will focus only upon two medically important virus families.

2.3 Orthomyxovirus and Paramyxovirus

Over the last 100 years influenza A virus (*Orthomyxoviridae*) is perhaps one of the most well studied viruses with respect to level and functional aspects of glycosylation. Several influenza proteins have been clearly demonstrated or predicted to have carbohydrate modifications. The surface proteins hemagglutinin (HA) and neuraminidase (NA) both use glycosylation for a variety of important functions including receptor binding, infectivity, virus release, and neurovirulence [42-48]. HA is the major antigenic surface glycoprotein of influenza and mediates attachment and entry into target cells. Traditionally it was believed that sialic acid was solely responsible for mediating entry events but recently, studies have shown that N-linked glycosylation can mediate sialic acid independent attachment and entry into the cells containing surface resident carbohydrate recognition molecules [49]. During infection, HA undergoes post-translational glycosylation that is indispensable to the proper folding and trafficking of the molecule during infection [50]. The number of sites has been described to range between 4 and 12 sites. Sites within the stalk region of the molecule remain relatively invariant since they are vital to the proper folding and processing of the molecule.

It is clear that influenza has had a monumental effect on global health and remains a persistent threat. The three pandemics that occurred during the 20th century (1918-1919, 1957-1958, and 1968) accumulated an estimated 40 million, 2 million and 1 million deaths respectively. The predicted level of glycosylation for these pandemic strains were 4 sites in 1918, 5 sites in 1957 and 7 sites in 1968. Presently, the commonly circulating H3N2 and H1N1 strains result in approximately 70,000 deaths each year, mostly within immunocompromized and elderly populations. Over the last 40 years there has been a gradual increase in sites for potential glycosylation and an increase in actual glycosylation [51]. Abe *et al*. introduced sites

for glycosylation by site directed mutagenesis into the pandemic Hong Kong 1968 strain, which resulted in the addition of carbohydrate [47]. Anecdotal evidence and recent studies would also indicate that the accumulation of carbohydrate leads to attenuation of disease. In a mouse model of infection, studies showed that as glycosylation was increased, disease was attenuated in part due to improved recognition and clearance by lung surfactant associated protein–D (SP-D) [52], illustrating that the addition of carbohydrate can have both positive and detrimental effects to the virus. The more recent swine 2009 H1N1 is predicted to have fewer sites for glycosylation than contemporary H1N1 strains and has been shown experimentally to have a single sequon for N-linked glycosylation on the globular head compared to seasonal H1N1 which carry 3-4 sequons [53]. Interestingly, that same study suggested that the loss of N-glycosylation led to resistance of SP-D and mannose binding lectin (MBL) activity.

Location and positioning of glycosylation can critically affect the activity of HA in either a deleterious or advantageous manner. For example, cleavage of HA by host proteases is vital to virus entry and can therefore affect and determine tissue tropism and viral virulence. Carbohydrate that is positioned in proximity to the HA cleavage site can prevent protease access and limit virus entry when this site is unoccupied by carbohydrate [54,55]. Alternatively, Klenk *et al*. illustrated that carbohydrate positioned near the receptor binding site is necessary to efficient replication and release of the virus [42]. Carbohydrate positioned around the globular head can mask antigenic sites from immune recognition and is believed to be the mechanism for antigenic drift in H3N2 viruses. Recently, Das *et al*. examined the role of glycosylation in antigenic drift within H1 globular domains [56]. They showed that the number of glycosylation sites in the globular head did not influence the overall variation in antigenic regions but rather variation occurred in regions that were unshielded by glycosylation. They concluded that glycosylation generally shields the virus from antibody-mediated recognition. Evidence for this view has came from earlier studies where successively adding additional Asn-x-Ser/Thr sites for potential glycosylation by site-directed mutagenesis revealed that virus could evade the host response without negatively impacting survival and biological activity [47]. Further, the addition of increased carbohydrate onto the globular head of the HA molecule of H3N2 viruses results in decreased receptor binding but had no effect on fusion activity, suggesting no negative impact to function. Conversely, the addition of glycan to the globular head of H2N2 HA does indeed affect both receptor binding and fusion activity [45,46]. The effect of glycan addition was especially important when looking at the interaction of HA and NA by showing that a balance is needed between receptor binding activity and virus release [52]. Overall HA depends on a balance of glycosylation to promote proper protein folding, facilitate interaction of virus with receptor, and elicit efficient particle release.

Although many virus glycoproteins contain N-linked glycan, some are extensively modified by O-linked glycosylation. One such example is respiratory syncytial virus attachment (RSV) (G) protein [57,58]. RSV (*Paramyxoviridae*) is perhaps the most important cause of viral lower respiratory tract infections in infants and children worldwide. RSV also causes severe disease and death among older persons and persons of all ages with compromised respiratory, cardiac, or immune systems and can exacerbate chronic cardiac and pulmonary conditions.

Membrane proteins from new and emerging viruses such as Hendra, Nipah, metapneumovirus and Sars-Cov have also been shown to be modified by glycan and play vital roles in viral infectivity, protein folding, tropism, protein processing and immune evasion [59-64]. Interestingly, in the case of Nipah virus, glycosylation plays a dual role: glycosylation leads to enhanced resistance to antibody neutralization yet causes a reduction in membrane fusion and viral entry [59,65].

2.4 Retrovirus

HIV-1 is a double stranded RNA virus with high mutagenic capacity. Virus from the *Retroviridae* family of viruses contains multiple subtypes or clades that are distinguished by wide intra-clade variation. The envelope protein (gp120) of HIV-1 is one of nature's most heavily glycosylated proteins [66,67]. N-linked glycan addition is vital to the proper functioning of the envelope glycoprotein. The envelope gp120 occurs as a trimeric complex with each monomer in association with the transmembrane viral envelope anchor, gp41. HIV-1 gp120 contains an average of 25 N-linked sequons (NxS/T) that can be extensively glycosylated. It has now been shown that the envelope glycans of immunodeficiency virus is almost entirely composed of oligomannose [68] creating an impressive glycan shield with implications to the development of vaccine design. Early studies suggested that the loss of glycan diminishes the binding, but does not abrogate the interaction of the virus with CD4 [69,70]. As a consequence of the reduced interaction with CD4, cell infectivity and cytopathic effect was reduced with little consequence to replication so it is clear that the structural changes resulting from glycosylation of gp120 plays a significant role in HIV-1 pathogenesis [71,72].

Neutralizing antibodies are one of the main adaptive components of our immune response to pathogens.

The role of neutralizing antibody in the response to HIV-1 is not clear. Recent studies suggest enhanced resistance of the virus to antibody neutralization over time that is accompanied by changes to the variable loop and to changes in potential glycosylation [73,74]. The contribution of N-glycan to protection is a question that remains open since many glycans are highly conserved components of gp120. For example, the neutralizing human monoclonal antibody 2G12 targets an epitope of gp120 that contains high oligomannose [75]. Analysis of this region by Calarese *et al*. [76,77] illustrates the importance of oligomannose to 2G12 neutralizing antibody activity. Conservation of this epitope in gp120 suggests a functional role in infection that can be speculated to be related to the mannose-dependent attachment of HIV-1 to innate surface molecules such as mannose receptor (MMR), dendritic cell-specific ICAM3 grabbing non-integrin (DC-SIGN) or other lectins that could facilitate entry into host cells [78]. It has been suggested that escape virus often contained addition of N-linked glycan to gp120 at positions that were not traditional neutralization epitopes. These findings suggest that glycan additions provide a mechanism of antibody evasion to provide protection from neutralizing antibody; however, multiple mutations were required. Frost *et al*. [79] suggests that viral escape from neutralizing antibody is promoted by accumulation of multiple amino acid within individuals. The evolving glycan shield that is produced represents one mechanism for viral persistence despite an increasing antibody repertoire.

2.5 Emerging viruses

Emerging viruses represent one of the current concerns as zoonotic infections become more prevalent. Recently there has been a strong push to explore the biology of several emerging viruses of importance to human disease. Typically, these are viruses that reside in a wild animal reservoir and are transmitted to humans via exposure to blood, excrement or bites. The *Arenaviridae* are enveloped single strand RNA viruses that typically are spread to humans by rodents. Infection from this family of viruses cause a spectrum of mild to severe, life-threatening systemic and neurological infections. Arenaviruses are classified based on their antigenic properties into two groups: Old World such as Lassa virus and lymphocytic choriomeningitis virus (LCMV) and New World such as Junin, Machupo, and several South American hemorrhagic fever viruses. Considerable focus has been directed to a better understanding of the role of glycosylation in arenaviruses. The prototype arenavirus LCMV contains 9 potential sites for glycan addition between the GP1 and GP2 glycoproteins. Bonhomme *et al*. demonstrated that there were 6 sites occupied on the GP1 and 2 of 3 sites occupied on the GP2. In this study, mutagenesis of sites was performed to examine the effect to viral fusion, infectivity and protein processing [80]. Results suggest that there is a high level of conservation in the sites for N-glycosylation and that the presence of glycan was vitally important to protein expression, processing, viral infectivity and cell fusion. Mutagenesis of several sites abrogated the function of GP and altered processing and overall infectivity.

Filoviruses, such as ebola virus, are a cause of severe hemorrhagic fevers in humans and in non-human primates. Filoviruses have a negative strand, non-segmented RNA genome that encodes seven proteins. The ebola surface glycoprotein GP is highly glycosylated with more than half of the molecular weight attributed to N- and O- linked glycans [81]. Entry of ebola requires GP to initiate attachment and fusion with host membrane. Earlier studies on the glycoprotein suggested that these proteins were involved in adherence and down-regulation of surface receptors such as major histocompatability complex-I (MHC-I), suggesting that innate responses to virus infection would be blunted [82]. Dowling *et al*. studied the antigenicity and immunogenicity of the ebola virus GP by generating mutations in sites for N-linked glycosylation [83]. This study performed mutation on eight sites for N-linked glycosylation with only one having an effect on antigenicity. Additionally, the results demonstrated clear differences to antibody responses among the mutations. The value of glycosylation in ebola has been shown to involve GP2 glycosylation and antigenicity. The study as a whole suggests that it may be possible to enhance immunity to ebola via specific changes to GP glycosylation.

Hantaviruses are members of the *Bunyavirdae* family of arthropod-borne and animal infecting viruses. Most viruses in this family are isolated or transmitted from arthropods; however, hantaviruses are an exception and are rodent borne and transmitted to humans by exposure to aerosolized urine or feces. Two membrane glycoproteins, Gn and Gc of Hantavirus, are modified by glycosylation. Collectively, these proteins contain six potential sites for the attachment of N-linked glycan. Shi and Elliott have demonstrated that five of the six sites are utilized to provide for proper folding, processing and intracellular trafficking of the glycoproteins [84]. More recently, studies have shown a role for N-linked glycosylation in cell fusion [85]. Extending work performed by Shi and Elliott, additional mutagenesis was performed to make single and multiple mutations in sites for glycosylation. Results indicate that one specific site on the G2 was found to be crucial for cell fusion

under low pH condition indicating that the G2 is likely to be the fusion protein for hantavirus.

3. Innate Immune Recognition

Research of late has clearly demonstrated that glycosylation is critically important to the pathobiology of bacterial and viral infection. Equally important, is the role that innate immune recognition molecules contribute to clearance of pathogens. Clearance in many cases is dependent on the level and type of protein glycosylation. The innate immune system of most organisms contain cell resident and soluble carbohydrate binding proteins with the capability of binding non-self carbohydrate structures. Within the immune system, several classes of receptors exist to recognize and target glycan structures displayed on pathogenic organisms. These receptors can be either secreted into the microenvironment or are maintained on the surface of immune cells. Most extensively studied, is a large family of calcium-dependent molecules called C-type lectin receptors (CLR). The C-type lectins are specific for proteins containing terminal mannose, fucose or galactose residues. Many of these CLR molecules are restricted to antigen presenting cells such as dendritic cells, macrophages and B-cells, although they have also been found on natural killer cells and endothelial cells [86,87]. Molecules such as the soluble and cell-associated macrophage mannose receptor (MMR), the macrophage associated macrophage associated receptor of collagen origin (MARCO), and the dendritic cell-specific ICAM-3 grabbing non-integrin (DC-SIGN) have been demonstrated to participate in the recognition and clearance of glycosylated pathogens. These molecules represent important sentinels within the respiratory, reproductive and gastrointestinal tracts where they play vital roles in the sensing and recognition of glycans from divergent organisms such as bacteria, yeast, viruses and helminths [88,89]. These molecules serve as a first line of defense and initiator of the adaptive immune response by their participation in the clearance and processing of pathogens. Recognition of glycan structures results in the internalization and processing of pathogens and packaging of antigen into MHC-I and II. Therefore, CLR molecules function as pathogen recognition receptors to induce antigen presentation and T-cell responses (Figure 2) [90]. In addition to the capacity to recognize and clear pathogen invaders, these molecules participate in cell signaling to relay information about the specific pathogen to the immune system by activating cytokine expression programs [91,92].

Although the role of the CLR molecule is to recognize and clear pathogens, several pathogens utilize these molecules as vehicles of binding and entry and target them in an effort to modulate immune responsiveness. For example, two recent studies suggest that the MMR represents an important receptor for virus entry of influenza and HIV [78,93]. Typically, one would suppose that viruses that encounter these molecules would be removed from the mucosal environment and destroyed; however, Influenza A virus and HIV have been reported to use cell resident carbohydrate recognition molecules as receptors for entry [94-96]. *Mycobacterium tuberculosis* contains high mannose residues as a cell wall component that targets both the MMR and DC-SIGN and elicits the production of anti-inflammatory cytokines such as interleukin-10 [90,97]. Additionally, *Helicobacter pylori* produces glycans to target CLR molecules and alter dendritic cells responses and activate T-helper type 2 cells with the net effect being the suppression of immune activation [98]. These findings are important since engagement of receptors that participate in endocytic or phagocytic processes may provide a direct route for the pathogen entry and provide the added advantage of evasion from innate recognition and clearance. Several immune cell surface receptors have been demonstrated to be important to the entry of HCV into host cells [99]. In particular, HCV envelope proteins have been found to associate with DC-SIGN and the related liver lectin L-SIGN through oligomannose N-glycans suggesting a mechanism for liver and dendritic cell tropism by illustrating two novel HCV binding receptors [100]. Glycosylation on west nile virus (WNV) and dengue prM and E proteins is sufficient to promote interaction and uptake by DC-SIGN and DC-SIGN receptor (DC-SIGNR) [101,102]. Davis *et al*. showed that WNV proteins modified by glycan can bind to DC-SIGN or DC-SIGNR but demonstrate a preference for DC-SIGNR [36,103,104]. Lin *et al.* have shown that Ebola virus glycoproteins with oligomannose interact with DC-SIGNR; whereas, those glycoproteins with complex glycan do not [105]. MMR and DC-SIGN have both been shown to bind and transmit HIV to T-cells via interaction with the envelope glycoprotein [95,96,106,107]. The Sars-Cov S protein and filovirus glycoproteins interact with the liver and lymph node sinusoidal endothelial cell C-type lectin (LSECtin) to enhance infection [108,109]. LSECtin is a cell-associated lectin that maps to the same chromosomal locus as DC-SIGN and is co-expressed in the liver and lymph nodes. Recent studies with LSECtin have also shown that this molecule interacts with DC-SIGN to promote HCV binding [110].

The utilization of CLR molecules such as MMR, DC-SIGN, DC-SIGNR and LSECtin for recognition, clearance and activation of the immune system is an important component of our defensive strategy from

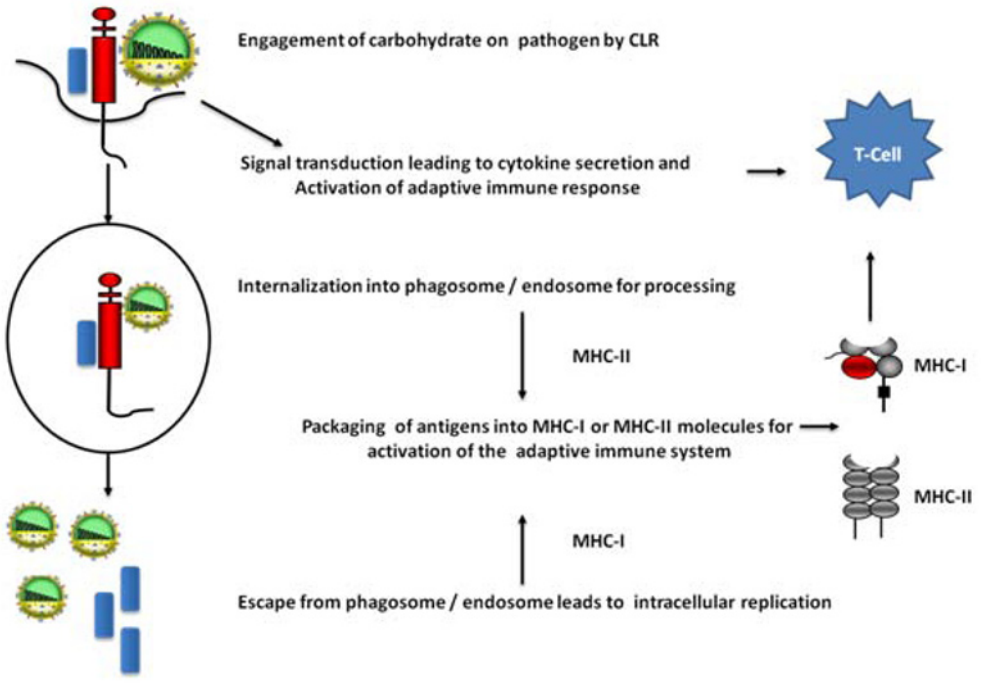

Figure 2. Schematic representation of pathogen associated carbohydrate interacting with CRL molecules. Bacterial (blue rectangle) and viral pathogens (yellow sphere) contain carbohydrate residues that interact with carbohydrate recognition domains on cell resident CRL molecules (red rectangle). Upon engagement with the CRL several biological processes occur to stimulate the immune system. First, signal transduction pathways are activated to secrete regulatory cytokines that further activate the adaptive immune response (represented as blue star). Second, engagement of carbohydrate on pathogen surfaces results in internalization of the pathogen into processing compartments that in turn package antigen into MHC-II molecules. Third, in cases where the CRL is a receptor of entry for the pathogen as is seen with HIV-1, influenza, Salmonella and Mycobacterium the pathogen can escape the degradative compartment and utilize the cell as a host for replication. During the process of replication pathogen proteins are captured and presented in MHC-I molecules. Packaging of pathogen components into the binding pocket of MHC-I or MHC-II activates the adaptive immune response for clearance.

infection. It is equally clear that many recent studies strongly suggest that pathogens take advantage of these molecules for attachment, entry and modification of the immune response. The role of glycosylation in pathobiology of infection and in defense from infection is complex and warrants continued investigation.

4. Potential for Therapy

The importance of glycosylation to pathogen protein function is clear. In the last several years the role of glycosylation in a variety of processes and diseases has expanded. As a consequence of this important role in pathobiology, many investigators have been prompted to explore plant, bacterial and algal derived lectins as potential therapeutics. This avenue of research is especially important given the scarcity of effective antiviral compounds to treat infections. The medical establishment has many viable therapeutic agents for the treatment of bacterial infection. Although more are needed in a growing age of antibiotic resistance, the development of effective agents against bacterial pathogens may lag until tools are available to better characterize bacterial glycans.

Aside from numerous HIV-1 antivirals, there exist very few antiviral compounds for the remainder of viral infections. Given the conservation of glycosylation among viruses and the important role of glycan in the life cycle, it is clear that this is a fruitful area to investigate. Several compounds have been classified as carbohydrate binding agents (CBAs) that show promise in the inhibition of virus activity [111] including the cyanobacterial cyanovirin-N (CV-N), plant lectins *Urtica dioica* agglutinin, *Galanthus nivalia*, concanavalin A, and Pradimycin A. All of which can interfere with attachment and neutralization of HIV-1, HCV and influenza virus *in vitro* [112-116]. Bertaux *et al*. showed that several of these agents can selectively inhibit the binding and entry of HCV and HIV but were unable to affect herpes simplex, RSV or parainfluenza-3 viruses [117]. Recently, a protein from the red algae *Griffithsia* was shown to bind oligomannose N-linked glycans on the surface of HIV with extremely high affinity preventing the entry of primary isolates and laboratory strains of T- and M-tropic

HIV-1 [118]. The above examples are just a few of the avenues for treatment currently being explored to utilize natural products to treat viral infection.

Several groups have successfully used the antimalarial drugs chloroquine and hydroxychloroquine in combination therapy to reduce the viral load of HIV in patients [119-122]. Both of these drugs have limited use in the management of malaria but because of the mechanism of action they can be repurposed to treat other diseases or infections. Naarding *et al*. performed studies illustrating the use of chloroquine to reduce the transfer of HIV-1 virus from DC-SIGN to $CD4^+$ T lymphocytes. Data from this study strongly suggested a viable means to limit virus transmission and replication *in vivo* [123]. Further *in vitro* studies with Sars-Cov and influenza [124-128], in conjunction with the field success of the drug in HIV, suggest that this is an approach that can be applied to other glycosylated viruses [129] since the action of chloroquine interferes with the viral life cycle at several stages.

Chloroquine may be important in the future therapy of viral infections for several reasons. First, the drug accumulates in the endosome preventing acidification which is a critical process for many viruses leading to uncoating and release into the host cell cytoplasm [130]. Second, chloroquine can interfere with the activity of glycosyltransferases in the ER and Golgi which can result in improper carbohydrate addition and lack of association with the chaperone proteins calnexin and calreticulin. Third, the pharmacology of this drug is well described since it has been in use as an antimalarial for more than 70 years. Recent studies suggest that chloroquine is an effective treatment for influenza *in vitro* [128,131,132] although it was not effective in the *in vivo* ferret model of infection. The ineffectiveness *in vivo* may be attributed to the level of drug present in the lung following oral administration. It is possible that the *in vivo* antiviral activity can be improved by changing the route of administration to a nebulized inhalation delivering the drug directly to the mucosa of the lung. Based on the field success of chloroquine in HIV infection, the low cost and the general safety of the drug, chloroquine could be a reasonable prophylactic therapy in a variety of acute viral infections where the virus relies on glycosylation for virus assembly and protein function.

5. Future Perspective

The study of glycosylation in disease remains a relatively new area of investigation. A growing body of knowledge is describing the role of carbohydrate in cancer, infectious disease, diabetes and chronic liver disease. The future of therapeutics is several fold: First, an expanding group of carbohydrate binding agents is showing the utility of these compounds in the development of effective drugs against enveloped viruses. New antiviral therapeutics against a broad range of enveloped viruses with oligosaccharide rich membrane glycoproteins will come from the field of pharmacognosy. Pharmacognosy is the science of the physical, chemical, biochemical and biological properties of compounds derived from natural origins. Drugs of natural origin could very well become the future treatment of infectious diseases. Second, the development of imino sugars that can interfere with glycosylation enzymes have shown promise in viral infections [133-135]. The difficulty with these compounds is that although these molecules have broad antiviral activity, their development has been limited by issues with efficacy and/or selectivity. Further work is needed to modify these compounds to better target pathogens. In the future these compounds have the potential to be powerful tools in the management of disease. Finally, an important consideration for the future is a better understanding of the interaction between glycosylated pathogen proteins and lectins of the innate immune system. Aside from jawed vertebrates, most of the animal kingdom relies on innate immune defenses using carbohydrate recognizing molecules such as MBL, MMR and DC-SIGN to defend from infections. As the scientific literature continues to describe glycosylation of pathogens, the importance of innate molecules will play a greater role in our understanding of infection and potential therapies.

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