Glycobiology: An Expanding Research Area in Carbohydrate Chemistry

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Nucleic acids, proteins, and sugar chains are biopolymers widely distributed in living organisms. Although all of these polymers are composed of covalently linked units A–B–C..., sugar chains have a characteristic feature not found in nucleic acids and proteins. Let us consider the smallest possible chain: A–B. In the case of protein, only one chemical structure is made when, for example, alanine and leucine are assigned to A and B, respectively. In the case of nucleic acid also, only one chemical structure is formed by assigning, for example, adenylic acid to A and guanylic acid to B. The situation of sugar chains, however, is quite different. Suppose that galactose is taken for A and mannose for B. As shown in Figure 1, galactose can be linked to a mannose residue at four different positions: C-2, C-3, C-4, and C-6. Therefore, four isomeric structures can be formed. Because a galactose residue can take two anomeric configurations, the number of possible isomeric structures becomes eight. Furthermore, a galactose residue can occur in the furanose form as well as the pyranose form shown in Figure 1. Taking this evidence into account, 16 isomeric structures are possible for the disaccharide Gal→Man. When the number of units increases to three, four, etc., only one structure with a particular sequence can be formed in the case of proteins and nucleic acids, because they are linear constructs. In contrast, the number of isomeric sugar chains increases by geometrical progression, because branches can be formed in sugar chains larger than disaccharides. This means that sugar chains, but not nucleic acids and proteins, have a characteristic feature that they can form many possible structures with a small number of units.

Recent advances in molecular biology have elucidated roles of nucleic acids and proteins as molecules containing biological information. The information in nucleic acids is constructed by the linear arrangement of nucleotide bases. On the other hand, the biological information of proteins is expressed not only by the linear arrangement of amino acids but also by the tertiary structure formed by amino acid residues remote in the polypeptide chains. Gene technology and protein engineering, which have developed from knowledge obtained by studies of nucleic acids and proteins, enable us to utilize living organisms more effectively for the fermentation industry by directing them to produce a particular protein. Thanks to these biotechnologies, we can now obtain substantial amounts of bioactive proteins, which are useful but occur in very minute amounts in animals. However, many proteins produced by animal cells occur as glycoproteins. Because bacteria such as *Escherichia coli* lack glycosylation machinery, recombinant proteins produced by them lack sugar chains. Since many of these nonglycosylated proteins do not express the expected biological activities, functional roles of the sugar chains have recently been attracting the interest of molecular biologists. On the basis of the idea of elucidating the biological information contained in the sugar chains of glycoconjugates and using them for the understanding of biology, the scientific field called "glycobiology" was recently established. This Account will introduce this new field.

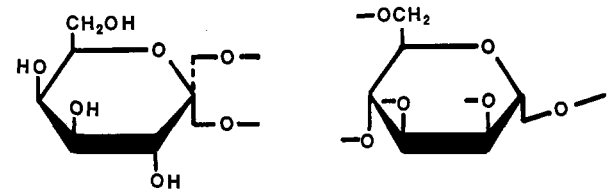

Figure 1. Possibilities for construction of sugar chains.

Rules Included in the Sugar Chain Structures of Glycoproteins

As described above, even a trisaccharide containing three different monosaccharide units can have several hundred different isomeric structures. Since the sugar chains found in glycoproteins usually contain more than 10 units, the structural multiplicity possible for sugar chains of such a size is theoretically enormous. It might be impossible to elucidate the biological information of sugar chains, if we had to consider such a large number of isomers. Fortunately, studies of the sugar chain structures of various glycoproteins reveal that a series of structural rules can describe them and variable regions occur in a limited part of their structures. With such rules in mind, elucidation of the sugar chain structures related to a particular biological function comes within the range of laboratory investigation.

The sugar chains of glycoproteins can be classified into two groups. Those that are called mucin type or

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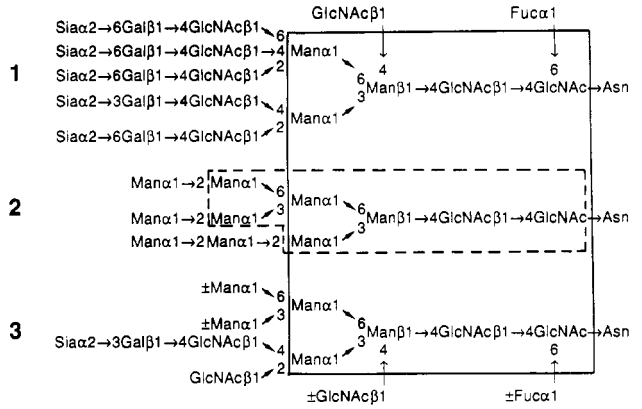

Figure 2. Three subgroups of N-linked sugar chains: (1) complex type sugar chain; (2) high mannose type sugar chain; (3) hybrid type sugar chain. The structure within the solid line is the trimannosyl core common to all N-linked sugar chains. The structure enclosed with a dotted line is the common heptasaccharide core of high mannose type sugar chains. Structures outside these lines can vary by sugar chains.

O-linked sugar chains contain an *N*-acetylgalactosamine residue at their reducing termini. This *N*-acetylgalactosamine is linked to the hydroxyl group of either a serine or a threonine residue of a polypeptide. The sugar chains that belong to another group called asparagine-linked or N-linked sugar chains contain an *N*-acetylglucosamine residue at their reducing termini and are linked to the amide group of an asparagine residue of a polypeptide. Studies on the functional aspects of sugar chains reveal that they play two major roles. One is to confer particular physicochemical properties on proteins. The other is to act as signals of cell surface recognition phenomena, which are important in multicellular organisms. Generally speaking, O-linked sugar chains mainly work for the former function and N-linked sugar chains for the latter function. Because the signal roles of sugar chains are the central topics of glycobiology and also because N-linked sugar chains are more readily described by structural rules, I will limit the remainder of this Account to N-linked sugar chains.

All N-linked sugar chains contain the pentasaccharide Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc as a common core, which will be called the "trimannosyl core". On the basis of the structure and the location of sugar residues added to the trimannosyl core, N-linked sugar chains are further classified into three subgroups as shown in Figure 2.[1] Sugar chains of the *complex type* contain no mannose residues other than those in the trimannosyl core. Outer chains with an *N*-acetylglucosamine residue at their reducing termini are linked to the two α-mannosyl residues of the trimannosyl core. The presence or absence of the α-fucosyl residue linked to the C-6 position of the proximal *N*-acetylglucosamine residue and the β-*N*-acetylglucosamine residue linked to the C-4 position of the β-mannosyl residue of the trimannosyl core (bisecting GlcNAc) contributes to the structural variation of complex type sugar chains. *High mannose type* sugar chains contain only α-mannosyl residues in addition to the trimannosyl core. A heptasaccharide with a two-branch structure, Manα1→6(Manα1→3)Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc, is commonly included in this type of sugar chain as shown by the dotted line in Figure 2.[2] The third group is called *hybrid type*, because the sugar chains have the characteristic features of both complex type and high mannose type sugar chains.[3,4] One or two α-mannosyl residues are linked to the Manα1→6 arm of the trimannosyl core as in the case of high mannose type, and the outer chains found in complex type sugar chains are linked to the Manα1→3 arm of the core. The presence or absence of the α-fucosyl residue and the bisecting GlcNAc linked to the trimannosyl core also produce structural variations of the sugar chains of this subgroup.

Among the three subgroups of N-linked sugar chains, the complex type has the largest structural variation. This variation is caused mainly by two factors. As shown in Figure 3A, from one to five outer chains are linked to the trimannosyl core by different linkages, resulting in the formation of mono-, bi-, tri-, tetra-, and pentaantennary sugar chains.[5] Two isomeric triantennary sugar chains containing either the GlcNAcβ1→4(GlcNAcβ1→2)Manα1→3 group or the GlcNAcβ1→6(GlcNAcβ1→2)Manα1→6 group are found. These isomeric triantennary sugar chains are called 2,4-branched and 2,6-branched triantennary sugar chains, respectively. Various structures are found in the outer chain moieties of complex type sugar chains as shown in Figure 3B. The combination of different antennary structures and various outer chains forms a large number of different complex type sugar chains.

Receptors That Specifically Bind to Particular N-Linked Sugar Chains

If sugar chains are working as signals of biological recognitions, there must be receptors for the signals. Such a receptor was found for the first time in 1970 by Ashwell.[6] In collaboration with Morell, he studied the functional role of the sugar chains of ceruloplasmin, a serum glycoprotein responsible for the transport of copper, by investigating the effect of trimming of its sugar chains by sequential exoglycosidase digestion. Although removal of sugar chains did not alter the capacity of ceruloplasmin to bind to copper, its clearance from the blood stream was strongly accelerated by the removal of sialic acid residues. By investigating the mechanism of this interesting phenomenon, Ashwell found a glycoprotein, which specifically binds to the β-galactosyl residues, in the plasma membrane of liver parenchymal cells. In the beginning, this glycoprotein was thought to bind any β-galactosyl residues. Therefore, it seemed strange that asialo-transferrin is not effectively cleared by the liver receptor despite its having two β-galactose terminated biantennary complex type sugar chains in one molecule. In order to find the

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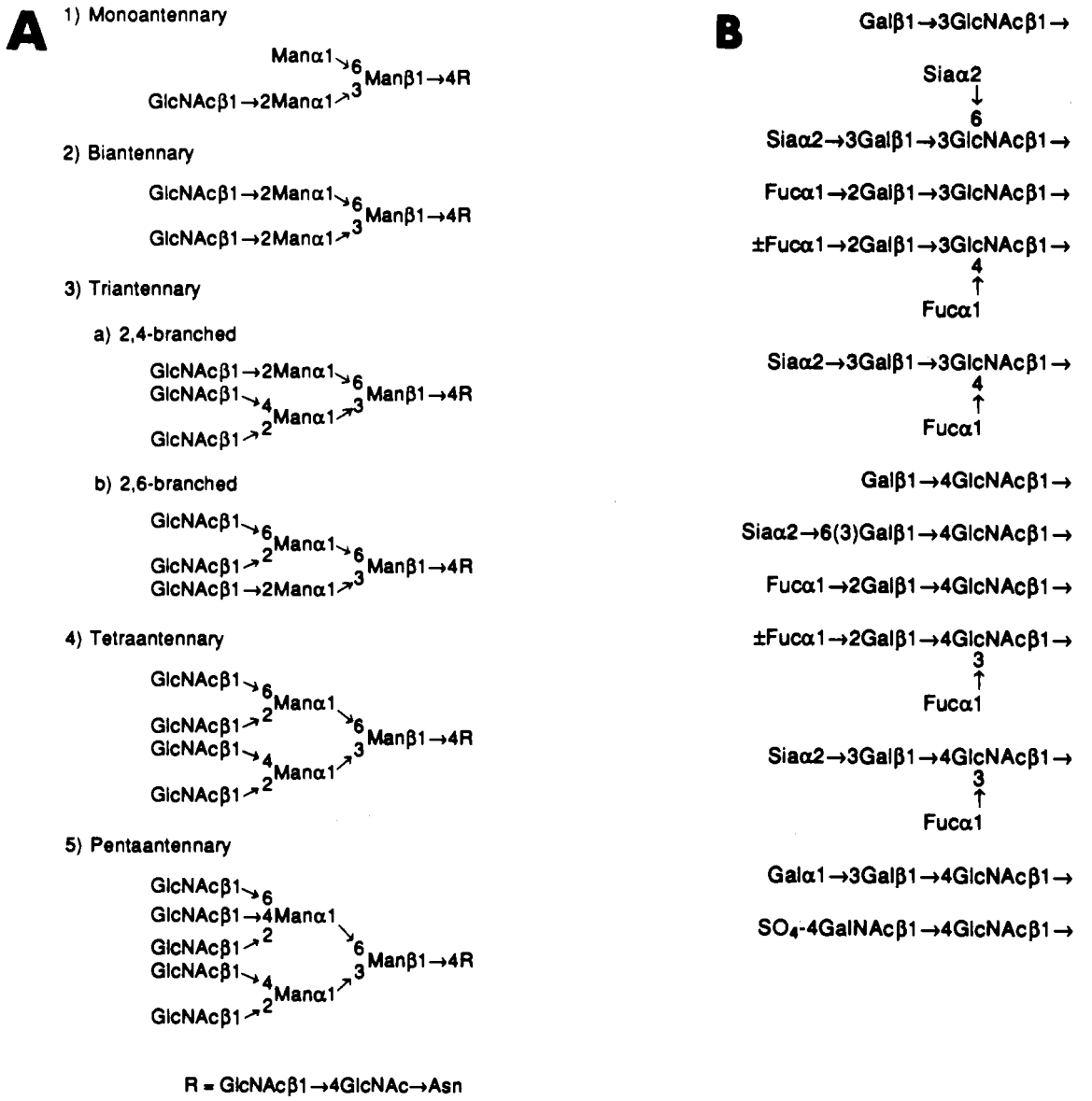

Figure 3. Two major elements that form the various structures of complex type sugar chains: (A) branching of complex type sugar chains; (B) various outer chain structures found in complex type sugar chains.

background of this inconsistency, Yamashita et al.[7] comparatively studied the sugar chain structures of ceruloplasmin and transferrin and found that the former glycoprotein contains a 2,4-branched triantennary sugar chain (I in Figure 4) in addition to two biantennary sugar chains, while the latter contains only biantennary sugar chains. On the basis of this evidence, they suggested that the triantennary sugar chain might be the true ligand for the liver receptor. This speculation was substantiated later by Lee et al.,[8] who found that the branched structure of oligosaccharide I enclosed by the dotted line in Figure 4 binds most strongly by investigating the binding constants of various oligosaccharides to the purified hepatic receptor.

A second sugar-binding protein of animal origin was found by investigating the transport mechanism of lysosomal hydrolases.[9] Many acidic hydrolases in lysosome contain mannose-6-phosphate residues in their sugar chain moieties, and the residues serve as ligands for binding to the Golgi membrane receptor. By this mechanism, the acid hydrolases are sorted from secretory glycoproteins and packed in clathrin-coated vesicles to be transported to prelysosomes.[10]

Two distinct mannose-6-phosphate receptors with M_r of 275 000 and 46 000 were found. The latter requires divalent cations for optimal ligand binding. That the larger receptor is identical to the insulin-like growth factor II receptor was found in 1987, and the interesting possibility that the receptor functions in two distinct biological processes was reviewed by Kornfeld.[11]

The finding of these receptors that recognize particular sugar chain structures revealed a new receptor family, animal lectins, that recognizes sugar chain ligands. Many animal lectins have since been found,[12] and the functional role of sugar chains as signals of recognition is now widely accepted to occur in multicellular organisms.

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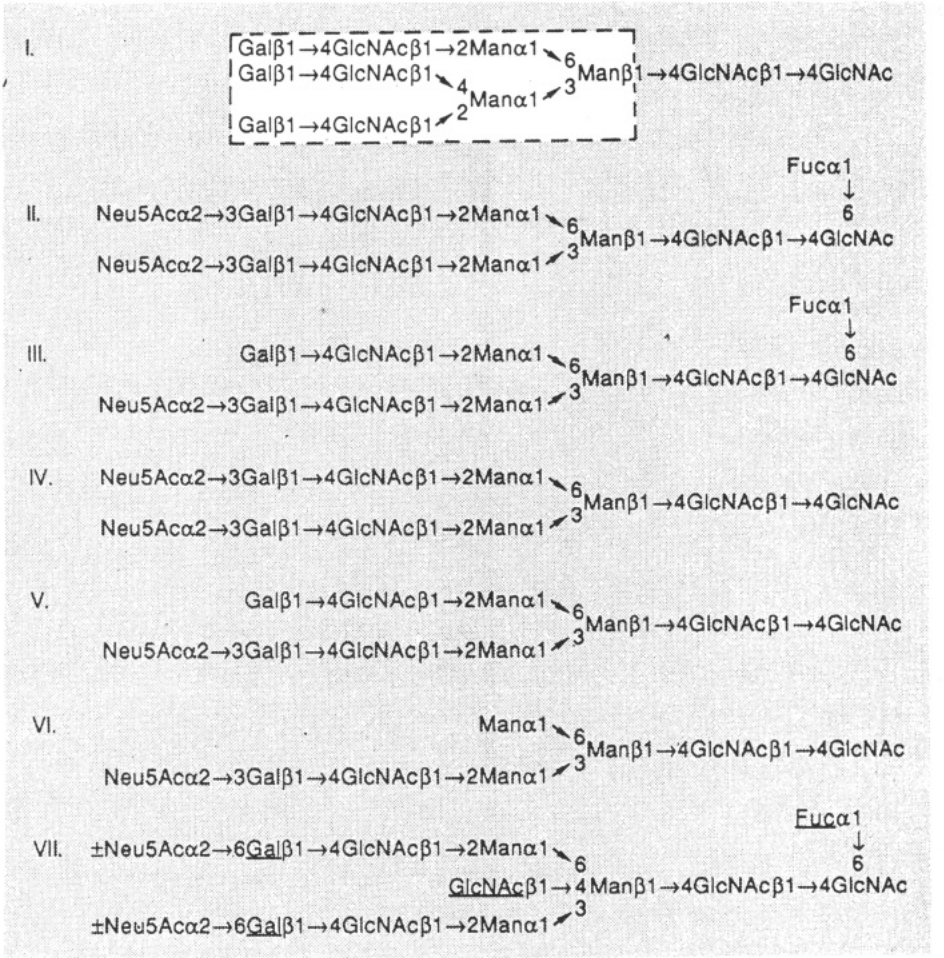

Figure 4. Structures of the N-linked sugar chains of human ceruloplasmin (I), hCG (II–VI), and human serum IgG (VII).

Functional Roles of N-Linked Sugar Chains

Accumulated information about structural characteristics of the sugar chains of glycoproteins enabled us to consider their functional roles in molecular terms. Because of space limitations, two topics will be introduced here to help readers consider this exciting research area.

Function of the Sugar Chains of Glycoprotein Hormones. Four glycohormones have been found in a variety of mammals. Three of these are produced in the same organ: luteinizing hormone and follicle-stimulating hormone are produced by gonadotrophs,[13,14] while thyroid-stimulating hormone is synthesized by thyrotrophs in the anterior pituitary.[15] Only chorionic gonadotropin is produced by trophoblasts of placenta.[16]

All of these hormones are composed of two noncovalently linked subunits of different sizes, designated α and β. Since the α subunits of all four glycohormones have identical amino acid sequences within an animal species,[17] it has been believed without proof that the specificity of each hormone to bind to its target cells resides in its β subunit. However, recent investigation of the sugar chain structures of the four hormones revealed that this well-accepted concept is not correct.

Structural information on the sugar moiety was first obtained from human chorionic gonadotropin (hCG). The two subunits of this hormone contain two N-linked sugar chains.[18,19] Structural studies of the N-linked sugar chains of whole hCG revealed that it contains five acidic oligosaccharides (II–VI in Figure 4).[20] Among these oligosaccharides, III–VI could be incomplete biosynthetic products of oligosaccharide II. However, comparative study of the N-linked sugar chains of α and β subunits revealed that this is not true.[21] It was found that the α subunit contains IV + V and VI in approximately a 1:1 molar ratio and no II and III. In contrast, the β subunit contains II + III and IV + V in a 1:1 molar ratio but no VI. These data indicate that the two N-linked sugar chains of the α subunit are never fucosylated, and one of them remains at the level of monoantennary sugar chains. Both N-linked sugar chains of β subunit are converted to biantennary sugar chains, but one of them is never fucosylated, while the other is completely fucosylated.

Around the mid 1980s, several groups investigated the effect on hCG hormonal activity of removing sugar

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chains by enzymatic or chemical means.[22-25] An important result of these studies is that deglycosylated hCGs bind more strongly to target cells than natural hCG, but express no hormonal activity. The functional role of the sugar moiety of hCG was more precisely pointed out by the report of Calvo and Ryan.[26] The adenylyl cyclase of rat corpora luteal membrane is increased in linear fashion in response to the addition of hCG. They found that addition of glycopeptides, obtained from hCG by exhaustive pronase digestion, to this reaction mixture inhibited the adenylyl cyclase activation by hCG in a dose-dependent manner. This result indicates that a membrane lectin, which binds to the sugar chains of hCG, may be involved in the regulation of luteal cell hCG-stimulated adenylyl cyclase. Although all sialic acid residues of the N-linked sugar chains of hCG occur only as the Neu5Acα2→3Gal group, the linkage is not included in the lectin recognition because artificially made isomeric hCG, in which sialic acid residues occur only as the Neu5Acα2→6Gal group, shows the same level of hormonal activity as natural hCG.[27]

Recently, structures of the N-linked sugar chains of the other three glycohormones were elucidated.[28-30] The data indicate that the sugar chains of the four glycohormones are quite different. Therefore, the α subunits of the four glycohormones should no longer be considered the same. They may also play important roles when the hormones interact with the surface of target cells.

Function of the Sugar Chains in Immunology. Recent studies in immunology revealed that both cellular and humoral immunological systems are controlled by complicated networks connecting the interactions of immunocompetent cells. In response to soluble immunological and inflammatory factors, leukocytes adhere to each other and to other types of cells such as platelets and vascular endothelial cells. These interactions are characteristic in that they form a strong contact between cells within a relatively short period compared to the permanent cell adhesion observed in tissue-forming cells. Therefore, such interaction should be mediated by special adhesive molecules that appear on the surface of activated cells.

Actually, a membrane-integrated glycoprotein that mediates binding of monocytes and neutrophils is found on the surface of vascular endothelial cells.[31] This glycoprotein, called ELAM-1, is not constitutively expressed on the endothelial cells but is rapidly induced on the cells activated by the action of cytokines such as interleukin 1 and tumor necrosis factor.[31] Accordingly, the glycoprotein plays an important role in accumulating monocytes and neutrophils at the site of inflammation within an animal body. Adhesion of the cells to ELAM-1 correlated with the presence of X determinant, Galβ1→4(Fucα1→3)GlcNAcβ1→..., on the cell surface and was eliminated by sialidase treatment of the cells, suggesting that the sialylated form of X determinant may be a ligand.[32] This estimation was evidenced by the recent finding that the Neu5Acα2→3Galβ1→3 or 4(Fucα1→4 or 3)GlcNAc group works as the ligand of ELAM-1.[33-36]

Mature lymphocytes repeatedly migrate from or to the blood circulation to or from the lymphatic circulation. This phenomenon, called lymphocyte homing or lymphocyte recirculation, is specific to lymphocytes and is not found for other blood cells. When lymphocytes migrate from the bloodstream to the lymphatic organs, they initially bind to the high endothelial (HE) cells of peripheral lymph node high endothelial venules (HEVs) and pass through the HE cells by an endocytotic mechanism. Since the lymphocytes pretreated with MEL-14 monoclonal antibody failed to bind to the HE cells, this antibody was considered to recognize a homing receptor of lymphocytes. Purification of a MEL-14 antigen (gp 90 MEL), followed by cloning of the gene from cDNA library of mouse splenic T cells, revealed that the protein contains the domain homologous to the C type animal lectin in its amino terminus.[37] The binding of lymphocytes on the cryo section of lymph node containing HEVs was inhibited haptenically by the addition of mannose, mannose-6-phosphate, fucose-4-sulfate, fucoidan, sulfatides, and related compounds including sialic acid.[38,39] Accordingly, the combination of a negatively charged group such as phosphate, sulfate, or sialic acid with mannose or fucose should be involved in the ligand of gp 90 MEL.

Mutual interactions of immunocompetent cells are mediated by cell surface glycoproteins encoded by genes within the major histocompatibility complex (MHC). The mixed lymphocyte reaction has been considered as a useful model for thymus-derived lymphocyte recognition of MHC-encoded determinants involved in mediating the cell–cell interaction.[40-42] Pretreatment of allogenic stimulator cells with tunicamycin, which specifically inhibits the biosynthesis of N-linked sugar chains, prevents their induction of blastogenic response by thymic lymphocytes.[43] This result indicates that the N-linked sugar chains of the surface stimulator cells play an essential role in the cell–cell interaction involved in the regulation of immune responses.

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Not only the direct interaction of immunocompetent cells but also several soluble mediators were found to connect them. Although all these mediators are glycoproteins, no evidence is available to indicate the functional role of their sugar chains. However, recent reports that interleukins 1 and 2 have lectin activities suggested that sugar–receptor interaction may also be included in their actions.[44-45]

Immunoglobulin G (IgG) plays a major role in humoral immunity. It is unique among serum glycoproteins in that it contains sugar chains of extremely high microheterogeneity produced by the presence or absence of the two galactoses, the bisecting *N*-acetylglucosamine and the fucose residue underlined in sugar chain VII in Figure 4.[46] Interestingly, the galactose residues are extensively deleted in the serum IgG of patients with rheumatoid arthritis. The degalactosylated IgG binds less effectively to the subcomponent C1q of the first component complement and to the Fc receptor.[46] The functional role of the sugar chains of IgG and their pathology are described in a recent review.[47]

The evidence currently available indicates that information about sugar chains is essential for elucidating the mechanisms of many aspects of immunology.

Concluding Remarks

As introduced in this Account, addition of the biological information included in the sugar chains of glycoproteins extensively alters our way of looking at physiological phenomena which have up to now been considered for proteins only. Although only two examples of the functional roles of sugar chains were introduced here, the topics are related to many other biological recognition phenomena ranging from fertilization[48] through all the life processes of multicellular organisms to aging.

Because the biosynthetic machinery of sugar chains does not include a template, the structures of sugar chains are not so restricted as those of proteins and nucleic acids. This means that the structures of sugar chains can be altered by the physiological condition of cells. In addition to the galactose deletion phenomenon found in the serum IgG obtained from patients with rheumatoid arthritis, such alteration is well documented by the comparative study of the sugar chains of glycoproteins produced by normal and malignant cell.[49-51] Accumulation of more such information will contribute to the development of another sugar chain-related research field: glycopathology.

The mechanisms of signal transduction, which are now being elucidated for the study of hormonal action of hCG, will be extended to the field of soluble mediators such as interleukins.[44,45]

Illustration of a glycoprotein molecule by computer graphics shows that a sugar chain occupies quite a large space (Figure 5), indicating the importance of the sugar chains in considering the function of glycoproteins. For example, recent studies on the sugar chains of recombinant human erythropoietin[52] revealed that an understanding of sugar chains is essential for the sound development of gene technology and protein engineering. Therefore, the knowledge obtained from glycobiology research will effectively be used for the development of biotechnology in the future.

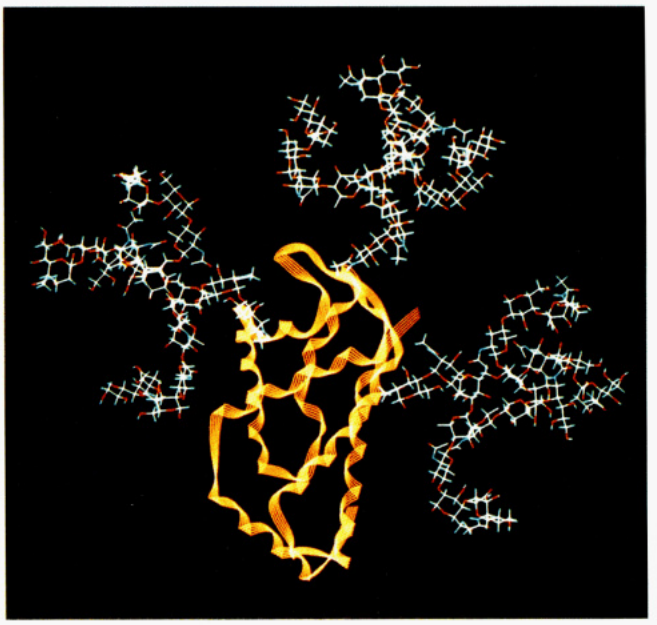

Figure 5. Computer graphic size illustration of a glycoprotein. The figure was made by arranging the three major N-linked sugar chains of human erythropoietin on human hemoglobin β, which has a molecular size similar to that of human erythropoietin. Note that the sugar chains cover most of the polypeptide portion (figure provided by courtesy of Dr. H. Iijima, Kirin Brewery Co., Ltd., Central Laboratories for Key Technology).

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