

## Chemical structure, biosynthesis and genetic regulation of carbohydrate antigens: retrospect and prospect

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**Abstract** - The carbohydrate antigens associated with the human ABO and Lewis blood group systems are prime examples of allogenic diversity. Elucidation of their structures, pathways of biosynthesis and genetic regulation has provided a model for understanding the genetic and enzymic basis of changes in expression of cell surface carbohydrates that occur in normal differentiation and malignancy.

### INTRODUCTION

Interest in the carbohydrate moieties of glycoconjugates has greatly increased in the last few years since molecular biologists and protein chemists have been forced to acknowledge the vital role played by oligosaccharides as receptors on cell surfaces and as structural components essential for the correct conformation and biological functioning of soluble glycoproteins. However, in a few laboratories throughout the world detailed research on the oligosaccharide structures of glycoproteins and glycolipids has been in progress for the last 40 years or more and most of the general principles concerning structural patterns of the sugars, their method of attachment to protein or ceramide, the existence of heterogeneity of the oligosaccharide chains and their mechanisms of biosynthesis have been long established. As in all fields exciting new observations have arisen in the pursuance of other more immediate objectives, such as the discovery of a new type of O-glycosidic linkage involved in the glycosylation of nuclear and cytoplasmic components (ref.1) and the finding that certain proteins in mammalian cells are anchored in the membrane via glycosyl-phosphatidylinositol structures (ref.2). However, much of the progress today consists in the definition of the biological function of the glycoconjugates and the introduction of refined methodologies that enable ever smaller quantities of materials to be subjected to structural analysis. These advances supply details that permit more accurate models to be constructed and realistic attempts made to determine the nature of the molecular interactions between carbohydrate and protein (ref.3), or carbohydrate and carbohydrate (ref.4). Gradually more knowledge is being gained concerning the enzymes, the glycosyltransferases, involved in the biosynthesis of the oligosaccharide chains and this is enabling approaches to be made to isolate the genes encoding these enzymes and to use these probes to gain further understanding of the genetic regulation of oligosaccharide synthesis (ref.5). This aspect is obviously one that has to be understood if we are to have the ability to ensure the correct glycosylation patterns of recombinant glycoprotein products and to interpret the changes that occur in cell surface carbohydrates in differentiation and malignancy.

One of the first group of glycoproteins to receive detailed study were those associated with the water-soluble blood-group substances (refs.6,7). The carbohydrate antigens defining the human ABO blood-group system, namely the A and B antigens, and the precursor H structure, are examples of inherited characteristics which occur not only on erythrocytes but also on cell surfaces of many tissues and in soluble form in body fluids and secretions. Insofar as erythrocytes are concerned, with very rare exceptions, the A or B alleles are expressed whenever an individual inherits one or both of these forms of the gene (ref.8). The polymorphism at the ABO locus results in the inheritance of alleles that give rise to immunologically and chemically distinct antigens in different individuals belonging to the same species; they thus provide a classic example of allogenic diversity (ref.9). In epithelial tissues, however, expression of A and B is dependent on inheritance of another gene, Se, which controls the formation of the precursor H substance (ref.10). Also, in the course of normal development an A, B or H antigen may, in certain organs, be expressed early in embryonic life only to disappear later (ref.11). The reappearance of the foetal antigens in some malignant conditions leads them to be considered as onco-developmental antigens (refs.12,13). Since an A or a B gene is subjected to regulatory processes that modulate its expression in different tissues and at different developmental stages, the distribution of the antigens provides excellent examples of epigenetic diversity (ref.9). Information concerning the chemistry, biosynthesis and genetic regulation of the blood group antigens therefore furnishes a valuable model for the

understanding of the expression and control of other cell-surface and soluble glycoconjugates.

### EARLY BIOCHEMICAL STUDIES ON A AND B ANTIGENS

After a few rather unsuccessful attempts to determine the chemical nature of the A and B antigens on erythrocytes the earlier biochemists turned their attention to the water-soluble sources of blood group-active materials, such as urine, saliva and gastric juice (see ref.6). However, the richest supply of material for chemical study was found to be the pathological fluid from ovarian cysts (ref.7). Active substances isolated from this source were characterised as richly glycosylated, macromolecular, mucin-type materials containing some 85-90% carbohydrate and 10-15% protein. The standard methods of structural carbohydrate analysis available in the early 1950s had relatively little to offer for the elucidation of the structure of such large, highly complex, molecules. As the sugar composition turned out to be almost identical irrespective of the blood group of the individual from whom the cyst was obtained (ref.7) it became evident that an unconventional approach was needed if the differences underlying the blood-group specificities were to be elucidated. In the event, results coming from three different approaches combined to demonstrate that one specific sugar in each substance, namely N-acetyl-D-galactosamine in A, D-galactose in B and L-fucose in H, played an important role in determining the individual blood-group specificities of the macromolecules. Moreover the evidence allowed the conclusion that each of these sugars was bound to the remainder of the molecules in  $\alpha$ -anomeric linkage. The methods used in our laboratory at the Lister Institute, London were 1) inhibition of specific erythrocyte agglutination by simple sugars 2) examination of the products of degradation of soluble blood-group substances by specific glycosidases and 3) inhibition by simple sugars of the enzymic decomposition of the blood-group substances by glycosidases (ref.6,15). In Elvin Kabat's laboratory in New York inhibition of specific immunoprecipitation and enzymic degradation featured as the main analytical tools in the early stages (ref.6). Although inhibition of haemagglutination or specific precipitation with sugars followed a principle established by Landsteiner (ref.16), namely, that a simple substance with a structure closely related to the immunological determinant group of an antigen can combine with the antibody and thereby inhibit the reaction between antigen and antibody, such a technique had not previously been applied to determine the nature of the determinant structure in a naturally occurring antigen. Our task at this stage was greatly helped by the recent finding of an endogenous lectin in eel serum (ref.17), and of certain plant lectins (ref.18,19), which emulated the human antibodies in their capacity to specifically agglutinate erythrocytes of certain blood-groups. The extremely narrow and exclusively carbohydrate specificity of these lectins had not been realised until it was revealed by these simple agglutination inhibition tests (ref.20). Interestingly, experiments with monosaccharides differing from L-fucose only by substitutions of OH groups at one or two positions enabled the conclusion to be drawn in 1952 (ref.21) that the configurations of OH-3 and OH-4 in the fucose ring were essential for combination with the anti-H eel agglutinin; a conclusion very similar to that reached some 30 years later concerning the importance of OH-3 and OH-4 of L-fucose in the binding of lectin 1 of Ulex europeaeus to the H determinant (ref.22).

In the 1930s a number of enzyme preparations of microbial or mollusc origin were described that destroyed the serological activity of water-soluble blood-group substances (reviewed in ref.7,15). Examination of the products of enzyme action usually revealed the release of all the component sugars in the blood-group-active molecules. However, by applying the principle that many enzymes are inhibited by the products of their own activity it was possible in certain enzymic mixtures to identify a single, specific glycosidase that accounted for the loss of blood group activity (ref.23). The idea that the so-called blood group destroying enzymes were, in fact, exo-glycosidases was at that time novel but, by pursuing this finding in the direction of purifying the enzymes and determining their action on both synthetic substrates and the macromolecular blood group substances, valuable tools were provided for subsequent studies. The use of these glycosidases confirmed the presence of N-acetyl-D-galactosamine, D-galactose and L-fucose as terminal non-reducing sugars in A, B and H substances, respectively. Importantly for further understanding of the determinant structures they also revealed the sequential pattern of the sugars and the exposure of new immunological specificities upon the release of single, terminal sugars from the oligosaccharide chains (ref.24). Although the use of the enzyme inhibition method does not appear to have been widely applied to this type of problem since this time the sequential enzymic degradation of oligosaccharides has now become an established method of structural analysis of glycoconjugates (ref.25).

The sequential glycosidase digestion of A and B blood group substances made clear the precursor-product relationship of H to A and B and provided an explanation for an earlier important observation of Iseki & Masaki (ref.26) that treatment of A substance with a culture filtrate from *Clostridium tertium* "transformed" it into O(H) substance. Any lingering doubts that the antigen detected on the red cells and in the secretions of group O persons was the product of an allele at the ABO locus were dispelled by these observations and it became clear that the O allele is silent and the antigen detected by so-called anti-O reagents is in fact unconverted precursor H substance. All three early indirect approaches to finding the chemical basis of blood-group specificity gave evidence that the immunodominant sugars in the ABH determinants on the red cells are the same as those on the glycoproteins in secretions.

The 1950s saw the publication of the double helix structure of DNA and the unravelling of the genetic code (ref.27). Until this time, because of the apparently straightforward manner of their inheritance, it had been widely thought that the A and B blood group antigens were the direct products of the blood group genes. However, since the major tenet of the new ideas was that proteins were the only directly translated products of the genes, it became evident that the carbohydrate ABH antigens must be secondary gene products. The exposure of new specificities by the sequential removal of sugars (ref.24) suggested that biosynthesis proceeds essentially by a reverse process of degradation, namely that individual sugar units are added in a stepwise manner to the non-reducing ends of the carbohydrate chains in a precursor molecule (ref.28). It was proposed that the blood group genes were encoding glycosyltransferases, a class of enzymes whose existence had been recently discovered at that time (reviewed in ref.29), and that these products in each instance catalysed the addition of the monosaccharide that completed the determinant structure. Thus the A gene encoded enzyme was envisaged as an  $\alpha$ -N-acetyl-D-galactosaminyltransferase, the B gene encoded enzyme as an  $\alpha$ -D-galactosyltransferase and the H gene encoded enzyme as an  $\alpha$ -L-fucosyltransferase (Table 1). From this stage on work proceeded in two directions; chemical studies were carried out to isolate blood-group-active oligo-saccharide fragments from the ovarian cyst glycoproteins and a search was begun to find the enzymes proposed as the primary protein products of the genes.

### STRUCTURAL STUDIES ON A, B, H AND LEWIS ANTIGENS

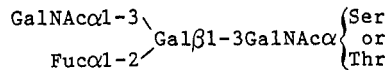
Fragments were isolated first from mild acid hydrolysis products and subsequently from mild alkaline degradation products of the glycoproteins (ref.30). Separation was achieved by the charcoal-Celite chromatography method of Whistler and the structures were determined by compositional analysis, periodate oxidation, partial acid hydrolysis, exo-glycosidase digestion, optical rotation and paper chromatographic mobility. The chromatograms were developed with various reagents such as benzidine and p-dimethylamino-benzaldehyde and considerable emphasis was placed on the colour reactions observed (ref.31). Although such evidence would probably be considered rather flimsy and insecure today most of the structures that were characterised by these methods have withstood the test of time. By 1967 the minimal structures for the A, B and H antigens, and the phenotypically closely related Le<sup>a</sup> and Le<sup>b</sup> antigens, had been established (Table 1)(ref.32). Although the major aim was to identify the blood-group-specific groupings, in the course of the work much was learnt about the glycoprotein molecules that carried these active structures. An early observation was that two types of chain endings, Type 1 (Gal $\beta$ 1-3GlcNAc) and Type 2 (Gal $\beta$ 1-4GlcNAc), could form the basis of the blood-group-active structures (Ref.33). Subsequently many larger oligosaccharide fragments were isolated and characterised from ovarian cyst glycoproteins and the presence of branched structures was established (ref.34).

With the introduction of new methods for the isolation of membrane components work was undertaken first in laboratories in Japan (ref.35) and subsequently in Poland (ref.36) and the United States (ref.37) to investigate the blood group antigens on the erythrocyte. The characterisation of, at first short chain, and then larger and more complex, glycosphingolipids bearing A, B and H activity at first appeared to uphold the belief that the blood-group activity of these cells was entirely associated with lipid-bound materials. Subsequently, around 1980 a number of different laboratories reported that a large part, possibly up to 70%, of the determinants on erythrocytes are carried on glycoprotein molecules (reviewed in ref.38). The oligosaccharide chains in the secreted blood-group active glycoproteins are O-glycosidically linked to the protein moiety but on erythrocytes the determinants are carried on N-linked chains. Most of the ABH structures are located on the anion transport protein (Band 3), and the glucose transport protein (Band 4.5). Each of these proteins carries a single highly branched poly-N-acetylactosamine

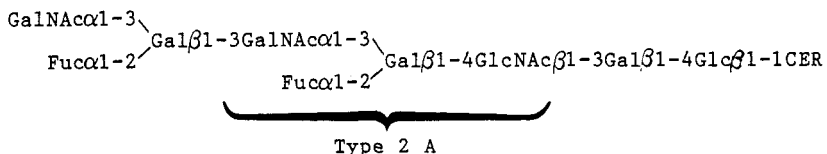
Table 1. Structures of the H, A, B, Le<sup>a</sup>, Le<sup>b</sup>, X and Y determinants.

Blood group specificity	Enzymic products of blood group genes	Structure of determinant
H	$\alpha$ -2-L-Fucosyl transferase ( <u>H</u> ( <u>Se</u> )gene)	Fuc $\alpha$ 1-2Gal $\beta$ 1-R
A	$\alpha$ -3-N-Acetyl-D-galactosaminyl transferase ( <u>A</u> gene)	GalNAc $\alpha$ 1-3 Fuc $\alpha$ 1-2 } Gal $\beta$ 1-R
B	$\alpha$ -3-D-Galactosyl-transferase ( <u>B</u> gene)	Gal $\alpha$ 1-3 Fuc $\alpha$ 1-2 } Gal $\beta$ 1-R
Le <sup>a</sup>	$\alpha$ -4-L-Fucosyl-transferase ( <u>Le</u> gene)	Gal $\beta$ 1-3 Fuc $\alpha$ 1-4 } GlcNAc $\beta$ 1-R
Le <sup>b</sup>	$\alpha$ -2-L-Fucosyl- and $\alpha$ -4-L-fucosyl transferases	Fuc $\alpha$ 1-2Gal $\beta$ 1-3 Fuc $\alpha$ 1-4 } GlcNAc $\beta$ 1-R
X	$\alpha$ -3-L-Fucosyl-transferase ( <u>X</u> gene)	Gal $\beta$ 1-4 Fuc $\alpha$ 1-3 } GlcNAc $\beta$ 1-R
Y	$\alpha$ -2-L-Fucosyl- and $\alpha$ -3-L-fucosyl transferases	Fuc. $\alpha$ 1-2Gal $\beta$ 1-4 Fuc $\alpha$ 1-3 } GlcNAc $\beta$ 1-R

(Type 2) chain. The blood-group-specific ABH determinants are therefore examples of terminal structures that may be carried on both O- and N-linked oligosaccharide chains in glycoproteins and on both short chain and complex glycosphingolipids. The diversity of chain ending that can carry the determinants has also been shown to be greater than earlier thought. Two types of structures based on the disaccharide Gal $\beta$ 1-3GalNAc have been identified. In Type 3 structures the GalNAc residue is  $\alpha$ -linked: it may occur in glycoproteins as a short chain attached to serine or threonine (ref.39)):

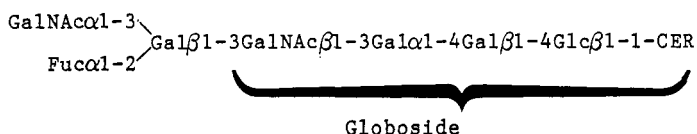


or as part of a repetitive A structure in glycolipids (ref.40)



This remarkable structure, in which a Type 2 A determinant is extended to form another A-active grouping, is now believed to account for a very significant part of the total glycosphingolipid bearing A determinants on the surface of group A<sub>1</sub> erythrocytes (ref.41). The occurrence of this structure is of considerable interest because it confounds previous ideas that the terminal sugars in the completed blood-group determinants are unable to function as substrates for any other glycosyltransferases. The repetitive A structure does not occur to any extent on A<sub>2</sub> erythrocytes (ref.41) but extended precursor H structures have been identified (ref.41) indicating the presence of cryptic A structures on A<sub>2</sub> cells. The repetitive A structure has not so far been reported to occur in glycoproteins and it is therefore still controversial as to the extent to which it can be said to account for the chemical basis of the A<sub>1</sub>-A<sub>2</sub> subgroups.

Extension of a different kind occurs in the Type 4 (Globo-series) structures. In the Type 4 A determinant the second GalNAc residue is  $\beta$ -linked to the next sugar and indeed forms the the terminal residue of globoside (ref.41):





structure or by an  $\alpha$ -4-L-fucosyltransferase to make an  $Le^a$  structure. An  $Le^a$  determinant is not a substrate for an  $\alpha$ -2-L-fucosyltransferase or for the A or B transferases and hence in a cell where a strong  $\alpha$ -4-L-fucosyltransferase is expressed much of the available Type 1 substrate may be converted to  $Le^a$  at the expense of a less active  $\alpha$ -2-L-fucosyltransferase. H structures once formed, however, may be converted either to A and B structures or to  $Le^b$  determinants (reviewed in ref.10). Although the difucosyl  $Le^b$  structure is not a substrate for the A and B transferases, the monofucosyl Type 1 A and B determinants are substrates for the Le transferase and hence may be further converted into  $ALe^b$  and  $BLE^b$  structures. Just as the H and  $Le^a$  specificities are masked in the  $Le^b$  determinant, so A and B specificities are masked in the  $ALe^b$  and  $BLE^b$  structures and monoclonal antibodies have been raised that may be used to establish the cellular localisation of these determinants (ref. 41,43). A similar series of interactions may occur between Type 2 H, A and B determinants and the X gene associated  $\alpha$ -3-L-fucosyltransferase. As a result of these interactions, in any particular cell the antigens detectable on the cell surface will depend not only on the genes expressed but also on the relative activities of the various enzymes competing for the acceptor substrates. Although the A and B genes, for example, are expressed co-dominantly at the enzyme level they may appear to assume dominance over each other at the antigen level on group AB erythrocytes since in individuals with a highly active B transferase an  $A_1$  gene may be expressed as if it was the product of one of the much weaker A alleles, or even be suppressed completely (ref.46). The apparent loss of an antigen in development may therefore result from the addition of a sugar that masks a previously detected specificity and the reappearance of an antigen in malignancy from the loss of a glycosyltransferase that normally is expressed in that tissue. Equally an increase in the level of activity of a glycosyltransferase may prevent the expression of another enzyme that cannot compete effectively for the substrate.

One tenet derived from earlier studies on glycosyltransferases was that each glycosidic linkage is formed by the action of a single highly specific enzyme and this led to the concept of "one enzyme-one linkage" and by extension to one gene being responsible for one glycosidic linkage (ref.47). Although this is largely true as a generalisation there are exceptions to this rule. Some ten years ago now we found that the B gene associated  $\alpha$ -3-D-galactosyltransferase had some capacity in *in vitro* experiments to transfer N-acetyl-D-galactosamine to H-active structures to make A determinants (ref.48) and later showed that the A gene associated  $\alpha$ -3-N-acetylgalactosaminyltransferase had the capacity to transfer D-galactose to make B determinants (ref.49). This overlapping specificity was not entirely unexpected since the two substrates are structurally very closely related and bovine galactosyltransferase has been shown to utilise UDP-glucose to a limited extent as a substrate (ref.50). Serologically, however, A and B blood group characters have always been considered to be quite distinct and we assumed that this overlapping function of the two glycosyltransferases was not manifested *in vivo*. Recently, however, it was reported that a very powerful monoclonal anti-A reagent could detect weak A activity on the red cells of certain group B individuals (reviewed in ref.51). The possibility that the antibody was recognising conformations common to both A and B determinants was shown not to be correct because the reactivity could be inhibited by group A saliva and not by B saliva and was destroyed by a specific  $\alpha$ -N-acetylgalactosaminidase and not by an  $\alpha$ -galactosidase. Glycosyltransferase tests on the serum from the group B persons whose erythrocytes showed A activity disclosed high levels of  $\alpha$ -3-D-galactosyltransferase that could be up to five times greater than those found in the sera of group B individuals not reacting with the monoclonal anti-A reagent. Since *in vitro* the ability to transfer N-acetylgalactosamine was directly related to the strength of the  $\alpha$ -3-D-galactosyltransferase it appears probable that these highly active B enzymes are responsible *in vivo* for the transfer of small amounts of N-acetylgalactosamine to H precursor structures to form a few A determinants that are detectable by the highly active monoclonal anti-A reagents.

#### MOLECULAR BIOLOGY OF THE ABO LOCUS

The ABO locus is still to my knowledge the only mammalian gene locus described so far which has alleles encoding enzymes with qualitatively different specificities. This fact in the past caused some geneticists to doubt whether A and B were really alternative forms at a single genetic locus but evidence has accumulated over the years to support their allelic status. Antibodies raised in rabbits to partially purified preparations of the human A transferase (ref.52,53,54) and monoclonal antibodies directed to this enzyme (ref.55) have been shown to immunoprecipitate the B transferase, thus giving

evidence of structural homology. The overlapping functions of the A and B transferases, discussed above, are also consistent with behaviour to be expected of allelic gene products. The proof of allelic status must come, however, from molecular genetic studies. Many methods are now available for gene cloning but for the biochemist the most direct approach is to purify the enzymes to homogeneity, and either to raise antibodies to the purified protein or to obtain a partial amino acid sequence that can be used to prepare a corresponding oligonucleotide probe. The antibodies or the oligonucleotide can then be used to screen the appropriate cDNA libraries. In the case of the A and B transferases the preliminary step of purifying the enzymes in sufficient quantities for amino acid sequence analysis, or antibody production, has been rendered difficult by the very low abundance of these glycosyltransferases and the problems of obtaining sufficient quantities of human tissues. Recently, however, the A transferase has been purified to apparent homogeneity from human lung (ref.56) human intestinal mucosa (ref.57) and human plasma (ref.58). All three enzymes were found to have molecular weights of about 40,000 and specific activities in the range of 6-16 mol/min/mg.

Determination of a partial amino acid sequence of the lung enzyme enabled a corresponding oligonucleotide probe to be prepared which was used to isolate complementary DNA encoding the A transferase (ref.59). In common with the six other glycosyltransferases for which cDNAs have been obtained (reviewed in ref.5) the A transferase consists of three domains: a short N-terminal, a hydrophobic transmembrane, and a long C-terminal domain. This latter domain most likely contains the catalytic site since the purified soluble form of the enzyme lacks the N-terminal and hydrophobic domains. Despite the similarity in general domain structure there is no major sequence homology between the various glycosyltransferases with known primary structures. The cloned DNA encoding the A transferase has subsequently been used as a probe to determine the sequence differences between ABO genes (ref.60). The results are still preliminary but suggest that the A and B genes differ in a few single-base substitutions and that changing four amino acid residues may be responsible for differences in A and B transferase specificity, that is, for the differences in their primary structure that result in their ability to transfer D-galactose or N-acetyl-D-galactosamine to H acceptor structures. Now that the nucleotide sequence is known the way is open to investigate the many variant forms at the ABO locus that lead to aberrant expression of ABO groups on erythrocytes.

Of considerable interest is the finding that a critical single-base deletion was found in the O gene near the N-terminal region which results in a frame-shift in the DNA (ref.60) with the result that this allele encodes an entirely different protein with no activity towards the H acceptor substrate. If this protein is actually translated *in vivo* one can ask whether antibodies to this protein occur in homozygous AA, BB or heterozygous AB individuals, especially those who have received blood or organs from group O donors, and whether such antibodies have any role to play in blood transfusion or organ transplantation.

Significant progress has also been made by Lowe and his colleagues towards cloning the genes involved in the expression of H and Lewis structures. They have used an alternative approach, the transfection method, to isolate human genomic DNA determining the expression of  $\alpha$ -2- and  $\alpha$ -3/4-L-fucosyltransferases associated with the H and Lewis genes (ref.61, 62). When these genes have been fully sequenced valuable tools will be available to probe the relationships between the family of fucosyltransferases and the products of the genes at the ABO locus.

## CONCLUSIONS

Diversity of the carbohydrate blood group antigenic structures in different individuals and in different tissues in the same individual is dependent on 1) the polymorphism at the ABO, H(Se), Le and X gene loci, 2) the specificity requirements of the glycosyltransferases encoded by these genes, 3) The competition or cooperation of glycosyltransferases encoded by the genes at the same or independent loci and 4) the expression of these genes in some tissues and not in others and the apparent switching on and off of the genes at different stages of development and in tissues that undergo malignant change. Although the structures of the antigens and their pathways of biosynthesis are now well understood we know little about the factors regulating the expression of the glycosyltransferases in different cellular types or about their rates of turnover. The phase of the work that is now beginning, involving the isolation of the genes, should provide probes that will enable answers to be obtained to many of these unanswered questions.

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