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The chemistry and immunochemistry of blood group A, B, H, and Lewis antigens: Past, present and future

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This article traces reseach on the chemistry and immunochemistry of blood group A, B, H, and Lewis antigens from early work on the identification of soluble sources of these antigens, through the elucidation of the structures of the carbohydrate epitopes responsible for these specificities, to recent work on exploring their possible use as cancer vaccines. The various approaches used in the isolation of oligosaccharides from mucins for use in structural studies are discussed, as are recent efforts in the chemical systhesis of blood group-active oligosaccharides.

Keywords: **blood group antigens, oligosaccharides, mucins, glycolipids, epitopes, alkaline elimination, oligosaccharide synthesis, tumor antigens**

Introduction

The A, B, H(O) and Lewis antigens form a family of genetically and biochemically interrelated specificities originally detected on red cells but now known to be widely expressed in other tissues and secretions also. Their study has provided many challenges for the scientists who have been involved in the elucidation of their structure, genetics, enzymology and function. This review, which is partly autobiographical, will concentrate on the structural and immunochemical analysis of blood group antigens; other aspects of this field are discussed by other authors in this volume and in recent reviews [1–3].

Sources of blood group A, B, H and Lewis antigens

Although red cells are the eponymous source of blood group antigens, early studies in the field used the more readily available, water-soluble forms of the antigens found in secretions of the body as the material on which to carry out structural studies. As originally reported by Yosida in 1928 [4], fluids from benign ovarian cysts afforded a rich source of blood group antigens. Stomach secretions were also used in some studies. As their biochemical nature was poorly understood these materials were originally termed "blood group substances" [5] and only later did it become clear that they were mucin-type molecules. Blood group substances were purified by fractionation procedures involving precipitation with ethanol from 90% phenol (with or without prior protease digestion), a method that capitalizes on the high carbohydrate content of the antigens. These preparations were polydisperse with molecular weights ranging from 2×10^5 to several millions. Carbohydrates comprised about 85% of the weight of the samples with amino acids making up the remainder. All preparations contained the same four sugars (L-fucose, D-galactose, *N*-acetyl-D-glucosamine and *N*-acetyl-D-galactosamine) and a peptide moiety that was rich in serine, threonine and proline. This composition is now known to be characteristic of mucins.

First hints of specificity

Compositional analyses of blood group substances isolated from individual patients with different blood types showed that the basis for ABH specificities did not result simply from the presence or absence of a single sugar or amino acid since all preparations contained the same components (albeit in slightly different proportions). The first indications that the antigenic specificities were carried by the sugar moiety came from inhibition experiments with simple sugars. These experiments implicated L-fucose in H(O) specificity, *N*-acetyl-D-galactosamine in A specificity and D-galactose in B specificity [6]. Inhibition studies with more complicated, fucosylated oligosaccharides from milk provided the first information on Le^a and Le^b specificities [7,8].

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Isolation of oligosaccharides from mucins

Although *N*-acetylgalactosamine, galactose and fucose became recognized as the "immunodominant" sugars in determining ABH specificities, it was clear that the complete antigenic determinants comprised more complicated oligosaccharide structures. To analyze these specificities methods were devised to fragment the mucin glycan chains into small oligosaccharides whose structure and specificities could be studied.

(i) Partial acid hydrolysis methods

One of the first methods to be used for this purpose was partial acid hydrolysis under mild conditions (e.g. 0.1 N HCl at 100° for 30 min). Because of the acid lability of fucosyl linkages most of the oligosaccharides isolated were non-fucosylated di-, tri- and tetrasaccharides and were therefore partial determinants. Nevertheless, these studies enabled Winifred Watkins and Walter Morgan and coworkers [9] to make the fundamental observation that most A, B, and H determinants are based on two different oligosaccharide sequences, termed Type 1 (Galβ1 → 3GlcNAc) and Type 2 (Galβ1 → 4GlcNAc) structures. For example, two A active and two B active structures were isolated:

	Type I	*Type 2*
A	GalNAcα1 → 3Galβ1 → 3GlcNAc	GalNAcα1 → 3Galβ1 → 4GlcNAc
B	Galα1 → 3Galβ1 → 3GlcNAc	Galα1 → 3Galβ1 → 4GlcNAc

Although A, B, and H specificities are carried by both Type 1 and Type 2 structures, later studies showed that only Type 1 sequences, and not Type 2 sequences, were responsible for Le^a and Le^b epitopes.

(ii) Alkaline degradation under "peeling" conditions

The need to isolate fucose-containing oligosaccharides led to the development of other methods for the fragmentation of blood group mucins. The most productive method turned out to be the use of alkaline reagents, originally $BaCO_3$ and later tertiary amines or NaOH (with or without $NaBH_4$). My own involvement in this field began in 1963 in the laboratory of Elvin Kabat at Columbia University in New York utilizing this approach. Having been trained in carbohydrate chemistry with Stanley Peat and Peter Lloyd in Bangor, Wales and with John Sowden at Washington University in St. Louis I was able to make rapid progress on this problem. We applied a procedure utilizing 0.2 M NaOH with 0.25 M $NaBH_4$ at room temperature for 1 week for the production of reduced oligosaccharides from a series of A, B, H, and Lewis-active ovarian cyst fluid mucins. Using extremely laborious and time-consuming oligosaccharide fractionation procedures, involving mainly charcoal column, gel filtration and paper chromatographic methods, we isolated a large series of reduced oligosaccharides representing partial and the complete determinants of the A, B, H, $Lewis^a$ and $Lewis^b$ specificities [10–14]. Simultaneous studies on this problem in the Watkins and Morgan laboratory in London provided a competitive, but friendly, background to these studies. Fractionation techniques and particularly structural methodologies were rather limited at that time but this did not prevent the eventual correct elucidation of these structures. The introduction of gas chromatographic methods for the identification of *O*-methyl sugars (for methylation analysis) was a considerable advance, although the miniaturization of the procedure that we developed to study 2–3 mg of oligosaccharide sample was a considerable feat.

The combined efforts of these two groups resulted in the elucidation of the structures of the A, B, H type 1, H type 2, $Lewis^a$ and Le^b epitopes by 1966 (Figure 1). Our work in New York also yielded two new fucosylated structures based on Type 2 chains that did not correspond to any known blood group specificity [14]. We termed these structures "new gene interaction products"; they were later designated X (or $Lewis^x$) and Y (or $Lewis^y$) by Sen Hakomori. They are structural isomers of Le^a and Le^b, respectively (Figure 1). Although numerous mouse monoclonal antibodies detecting these structures have been developed, they do not represent true blood group specificities that are involved in blood typing. The expression of Le^x and Le^y is confined to epithelial cells, their secretions and a few other tissues.

Although the choice of alkaline conditions for the degradation of blood group substances was made empirically, it became clear that the mechanism for the formation of these oligosaccharides was the cleavage of carbohydrate-protein linkages and subsequent degradation of the carbohydrate chains by a step-wise β-elimination process known as "peeling". In the presence of sodium borohydride the peeling proceeded from the reducing end of the released carbohydrate chain until terminated by reduction of the terminal reducing sugar to its alcohol. Using this procedure we isolated a series of reduced oligosaccharides terminating in either D-galactitol or 2-acetamido-2-deoxy-D-galactitol (*N*-acetylgalactosaminitol). Surprisingly, a number of other oligosaccharides were isolated that did not seem to be terminated by any identifiable sugar alcohol. Although now of only passing interest, it was at the time an event of considerable excitement for me to find, using a simple decolorization of permanganate test, that these mysterious compounds were unsaturated alcohols [11]. Further work [15] demonstrated that these compounds were terminated by *erythro* and *threo* isomers of 1, 2, 5, 6-tetrahydroxy hex-3-ene (**3**). To account for their formation we postulated that they were derived from a branched galactose residue by a series of β-elimination steps as shown in Figure 2.

These studies also yielded two other structures of considerable importance in understanding the structure of glycan chains in mucins. The first (**1**) was a 3,6 branched oligosaccharide containing with Type 1 and Type 2 chains, that partially accounts for the formation of the hexene residue by alkaline elimination (Figure 2).

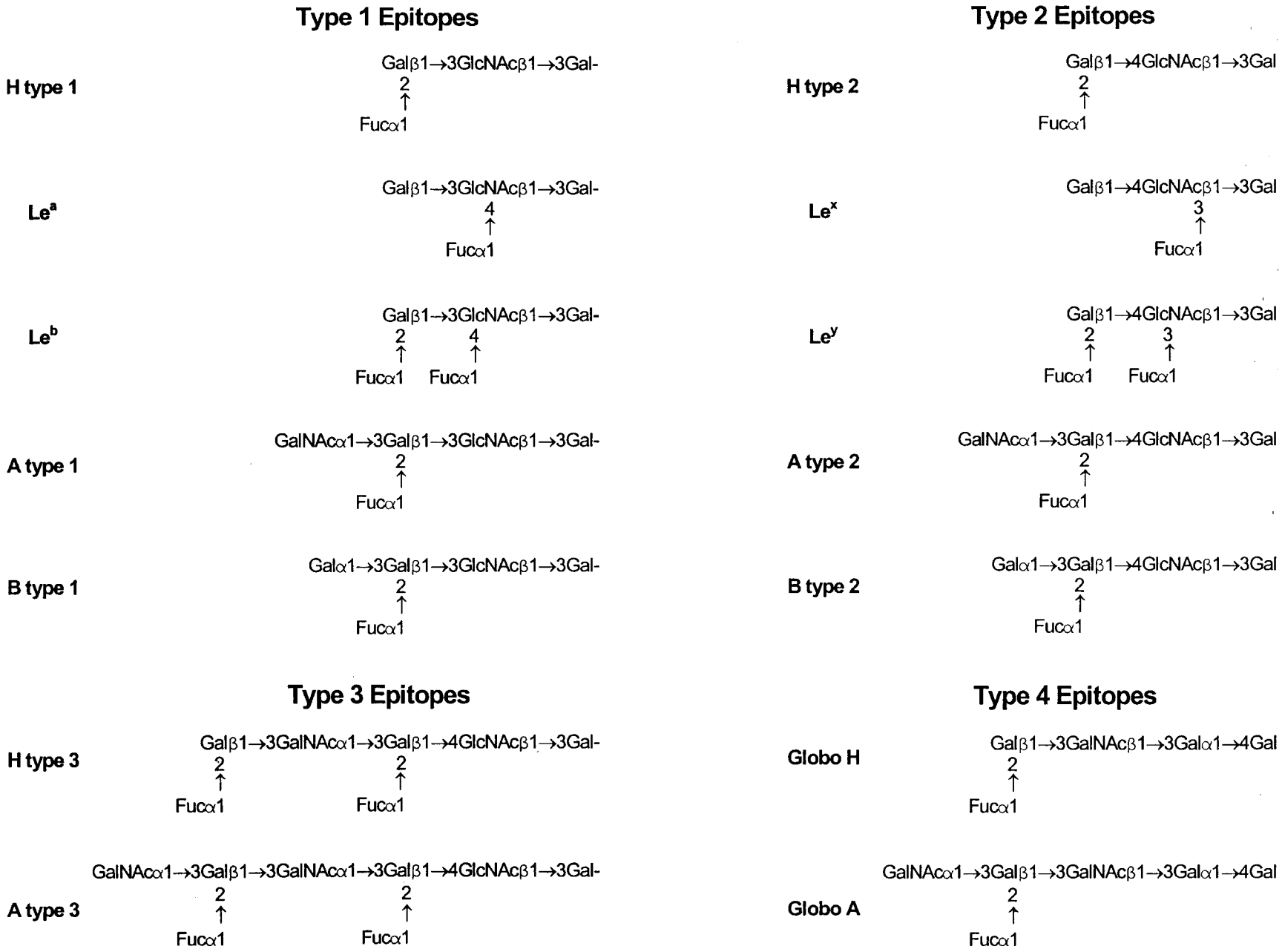


Figure 1. Structures of blood group A, B, H and Lewis epitopes. Not shown are difucosylated A and B structures, e.g. Ale^b; these may, in fact, be more common than the monofucosylated structures.

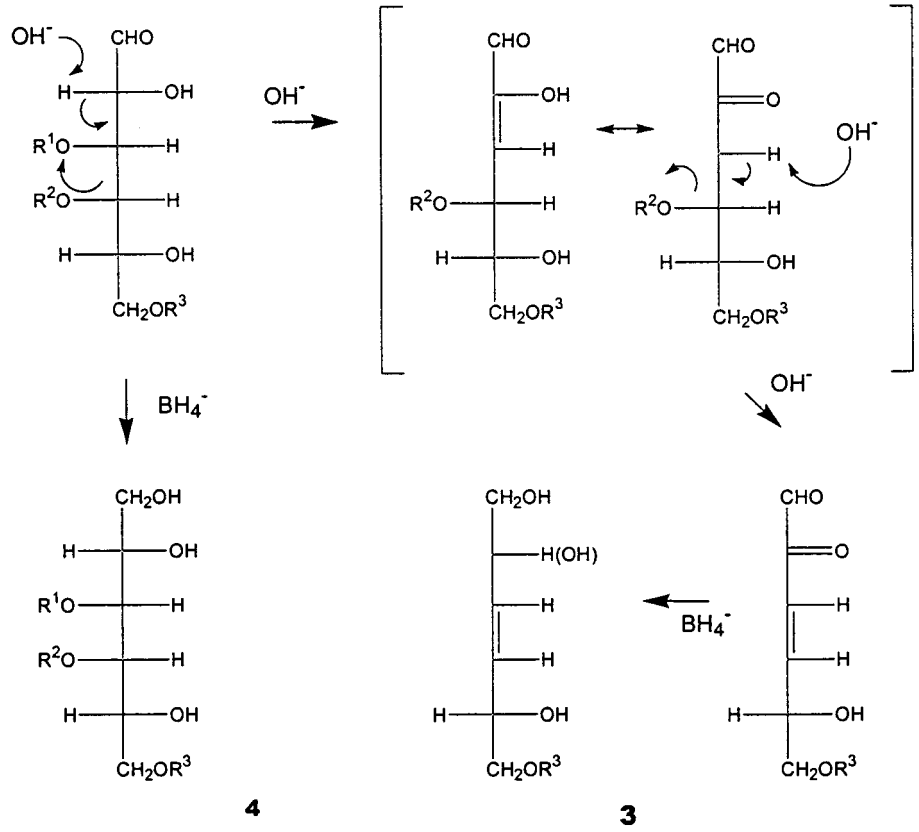
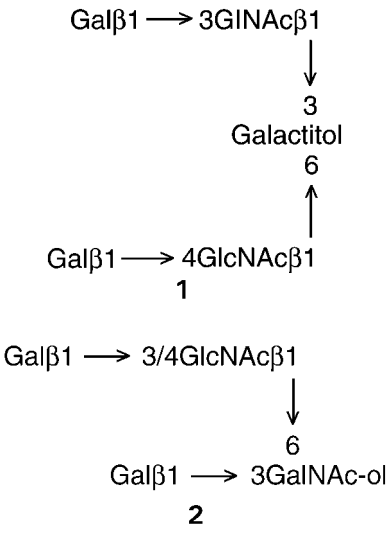


Figure 2. Postulated mechanism for the formation of 1, 2, 3, 4 tetrahydroxy-hex-3-ene-terminated oligosaccharides from a branched galactose residue during the alkaline degradation of blood group-active mucins. The process involves competition between β-elimination to form the hexene derivative (**3**) and reduction to form galactitol (**4**). Modified from Figure 10 in Ref. [13].



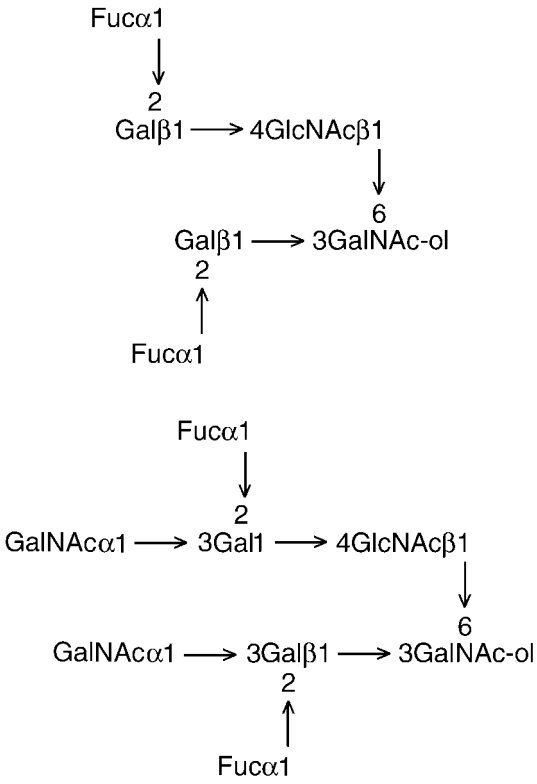
The second (**2**) was a tetrasaccharide containing a 3,6 branched *N*-acetylgalactosaminitol residue. This was the first demonstration of the so-called "Core 2" structure now known to be characteristic of many mucins. Recent work has shown that the importance of the Core 2 branch in modulating glycan structure during cell differentiation [16] and malignancy [17].

During the course of these studies we isolated a disaccharide (GlcNAcα1 $\rightarrow$ 3/4 galactitol) from a hog gastric mucin that had not been identified in human mucins [18]. The terminal α-linked *N*-acetylglucosamine residue was shown to be responsible for the unusual reactivity of gastric mucins with concanavalin A. Recently, Nakayama *et al.* [19] cloned the gene for the glycosyltransferase responsible for the synthesis of this structure and confirmed its selective expression in gastric glands.

(iii) "Non-peeling" alkaline degradation methods

A limitation of the earlier alkaline degradation method was that although it provided oligosaccharides of a size that could be fractionated and analyzed, it did not produce many oligosaccharides derived from internal and core regions of the glycan. The demonstration by Don Carlson [20] that changing the concentration of the reagents to 0.05 M NaOH and 1.0 M $NaBH_4$ allowed release of the glycan chains with minimal degradation by peeling was an important advance in the field and permitted the isolation of many new structures. Analysis of these oligosaccharides by other investigators in the Kabat laboratory (particularly Lucina Rovis and Albert Wu) resulted in elucidation of internal sequences and helped us to

understand the structure of the entire glycan chains in blood group mucins [21,22]. Similar studies were carried out by Allen Bush and coworkers [23]. These studies provided support for the composite structure proposed by Lloyd and Kabat (see below) and also generated new information on the structure of the glycan chains. In particular, they demonstrated that blood group epitopes are present on short structures directly linked to the GalNAc core in contrast to the long chains in the proposed composite structure. Two such structures were:



Composite structure of the glycan chains of A, B, H and Lewis-active mucins

Even before the large, reduced oligosaccharides produced under the Carlson conditions were analyzed Kabat and I bravely postulated a composite structure for the complete glycan chains assembled from the various oligosaccharides isolated in earlier studies (Figure 3). Subsequent work has shown that this structure is essentially correct although an oligosaccharide representing the whole structure has yet to be isolated. This is because techniques for the separation of oligosaccharides of this size are still not available. It should be emphasized that the proportion of chains consisting of this long, most complex structure, is probably quite small; most of the structure consists of heterogeneous mixtures of portions of this sequence. Moreover, Kochetkov *et al.* [24] and Rovis *et al.* [21] isolated oligosaccharides in which the branched galactose residue (**2**) is linked directly to the core GalNAc.

We attempted to estimate the lengths of the carbohydrate chains and their degree of heterogeneity by applying a sequential series of Smith degradation reactions to an H-active mucin sample [14]. In this procedure sugars having unsubstituted vicinal hydroxyl groups are oxidized by sodium periodate, the product is reduced to its polyol by sodium borohydride and the hemiacetal linkage to the next sugar is hydrolyzed with mild acid treatment. This procedure results in the shortening of the glycan chain by one or more residue at a time. Reiterative application of this procedure permitted us to estimate the lengths and heterogeneity of the glycan chains in this sample. Another feature of this proposed structure is that it proposes an elongated chain on the 3-*O*-branch of the core GalNAc consisting entirely of β1,3 linkages. This feature explains the formation of short blood group-active oligosaccharides under alkaline conditions as 3-*O*-substitution would allow peeling of the chain by β-elimination from the sugar aldehyde group at each step. Recent work, however, has shown that chain elongation can

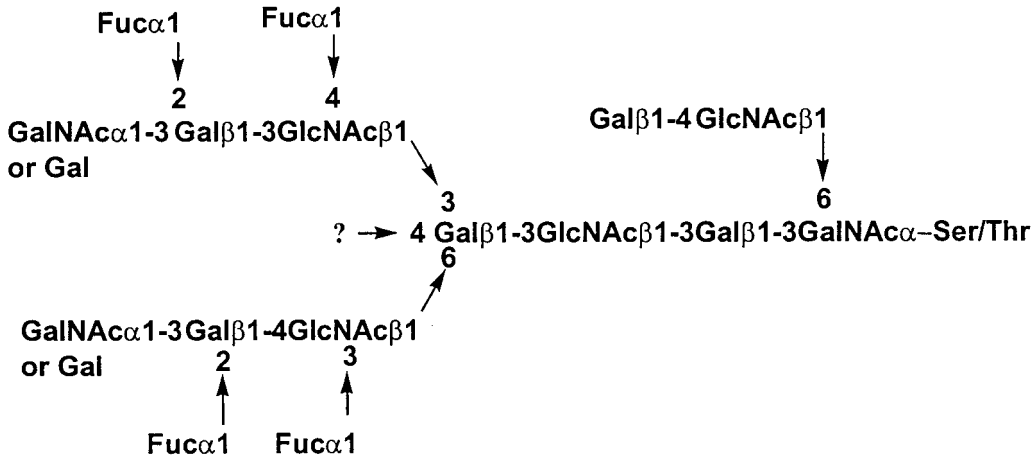


Figure 3. Composite structure of glycan chains of ovarian cyst mucin assembled from oligosaccharides produced by acidic and alkaline degradation of mucins. The nature of the substituent (?) on the C-4 of the branched galactose residue is uncertain. Modified from Figure 1 in Ref. [14]. Recent work has shown the presence of blood group epitopes on the GlcNAc$\beta \rightarrow$6GalNAc (core 2) branch also.

also occur on the GlcNAcβ1 → 6GalNAc branch of the core also; in contrast to the 3-*O*-branch these chains consist of alternate β1,4 and β1,3 inked (polylactosamine) structures.

Peptide cores of blood group mucins

As mentioned above, the amino acid composition of blood substances is characteristic of mucins, i.e. serine, threonine and proline constitute 40–60% of the total amino acid content. Analysis of ovarian cyst mucins before and after alkaline treatment demonstrated that the majority of the serine and threonine residues in the peptide core are substituted with glycan chains [25,26]. Recent work by investigators in the mucin field has shown that the peptide core of mucins invariably consist of tandem repeats of serine, and threonine-rich sequences, often flanked by non-repetitive or cysteine-rich regions. To date twelve different mucin sequences have been identified in mucins from a variety of sources, mainly using cDNA cloning approaches. These mucin sequences are designated MUC-1, MUC-2, MUC-3, MUC-4, MUC-5Ac, MUC-5B, MUC-6, MUC-7, MUC-8, MUC-9, MUC-11 and MUC-12 (reviewed in [27,28]). Some mucin species are membrane-bound (MUC-1, -3 and -4) whereas others are found only in secretions. Although one mucin species may predominate in a particular organ, most organs seem to synthesize more than one mucin. It is still unclear as to whether or not a particular mucin species is characterized by a unique carbohydrate content and blood group specificity. In a recent study we could identify only one mucin sequence (MUC-6) from a single Le^y/Le^b-active ovarian cyst mucin by chemical sequencing a deglycosylated mucin sample, although we suspect that other mucin species, particularly MUC-1, may have been present in the sample [29]. Other organs probably express blood group structures on different mucins; for example, salivary mucins, which are also blood group active, consist largely of MUC-7 [30].

Blood group structures on glycolipids

Although, as discussed above, most of the early work on the identification of blood group specificities was carried out on water-soluble materials later work, mainly by Sen Hakomori and coworkers, demonstrated the presence of identical determinants in the glycolipids of red cells also (reviewed in [1,31]). A major difference between the two sources was that the erythrocyte structures were based solely on Type 2 sequences. The small amounts of Type 1-containing species (Le^a or Le^b) found in red cells apparently originate from serum [32]. Erythrocytes also carry blood group determinants on glycoproteins, e.g. band 3 and band 4 [31].

These studies on erythrocyte antigens in the 1970–80 period also shed light on the structural basis for the blood group i and I antigens as well as demonstrating an important structural difference between A_1 and A_2 blood types. In a collaboration with Hakomori, Ten Feizi showed that unbranched poly-*N*-lactosamine chains are responsible for i antigen specificity, whereas as branched poly-*N*-lactosamine structures carry I specificity [33]. Although A_1 and A_2 erythrocytes differ in the quantity of A antigen expressed [34], they also differ in the structure of their A-active glycolipids. Specifically, A_1 cells expressed A type 3 and A type 4 chains (Figure 1) whereas A_2 cells lack these species and synthesize Type 3H and Type 4H (globo H) compounds instead [1,31].

Chemical synthesis of blood group-active oligosaccharides

The synthesis of oligosaccharides poses challenges that are far greater than those posed by the synthesis of oligonucleotides and peptides. This is mainly because of the multiple, nearly equivalent, hydroxyl groups present in each sugar ring which must be protected and/or deprotected during the synthetic process. Another problem is the potential creation of two anomeric forms of the product at each step of the synthesis. Considerable effort has been devoted by numerous groups to solving these problems but the synthesis of complex carbohydrates remains a difficult and laborious process. This section will not attempt to review the entire field but will briefly describe a collaboration with the laboratory of Samuel Danishefsky to synthesize a number of blood group-related structures of medical interest.

The general approach used by this group is termed "glycal assembly". The key feature of this method is the use of glycals as both donors and acceptors in a reiterative process (reviewed in [35,36]). As glycal linkages at the potential reducing end of even large oligosaccharides can function as donors it is possible to assemble quite elaborate structures relatively easily using this procedure. The approach also simplifies the process of achieving differential hydroxyl protection and presentation on the intermediate sugars. The use of this method is well illustrated by the synthesis of a Le^y structure for use in our vaccine studies discussed below [37]. Figure 4 shows the multi-step procedure used to synthesize a Le^y-active pentasaccharide starting from the readily available disaccharide lactal. In the final step, before deprotection, the pentasaccharide glycal functioned as a donor to allyl alcohol as acceptor to provide the allyl glycoside of the oligosaccharide. For use as an immunogen this glycoside was coupled to proteins (bovine serum albumin or keyhole limpet hemocyanin) by reductive amination after cleaving the double bond by ozonolysis.

In a recent extension of this technology we recently synthesized some very complex glycopeptides carrying blood group determinants. Particularly notable is a glycolipopeptide bearing three Le^y hexasaccharides in a clustered configuration (Figure 5) that is intended to mimic the natural form of Le^y epitopes as they occur on mucins [38]. The synthesis of this compound utilized an *N*-acetylgalactosamine group with an identified acceptor site, stereospecifically α-*O*-linked to a protected and activated serine or threonine

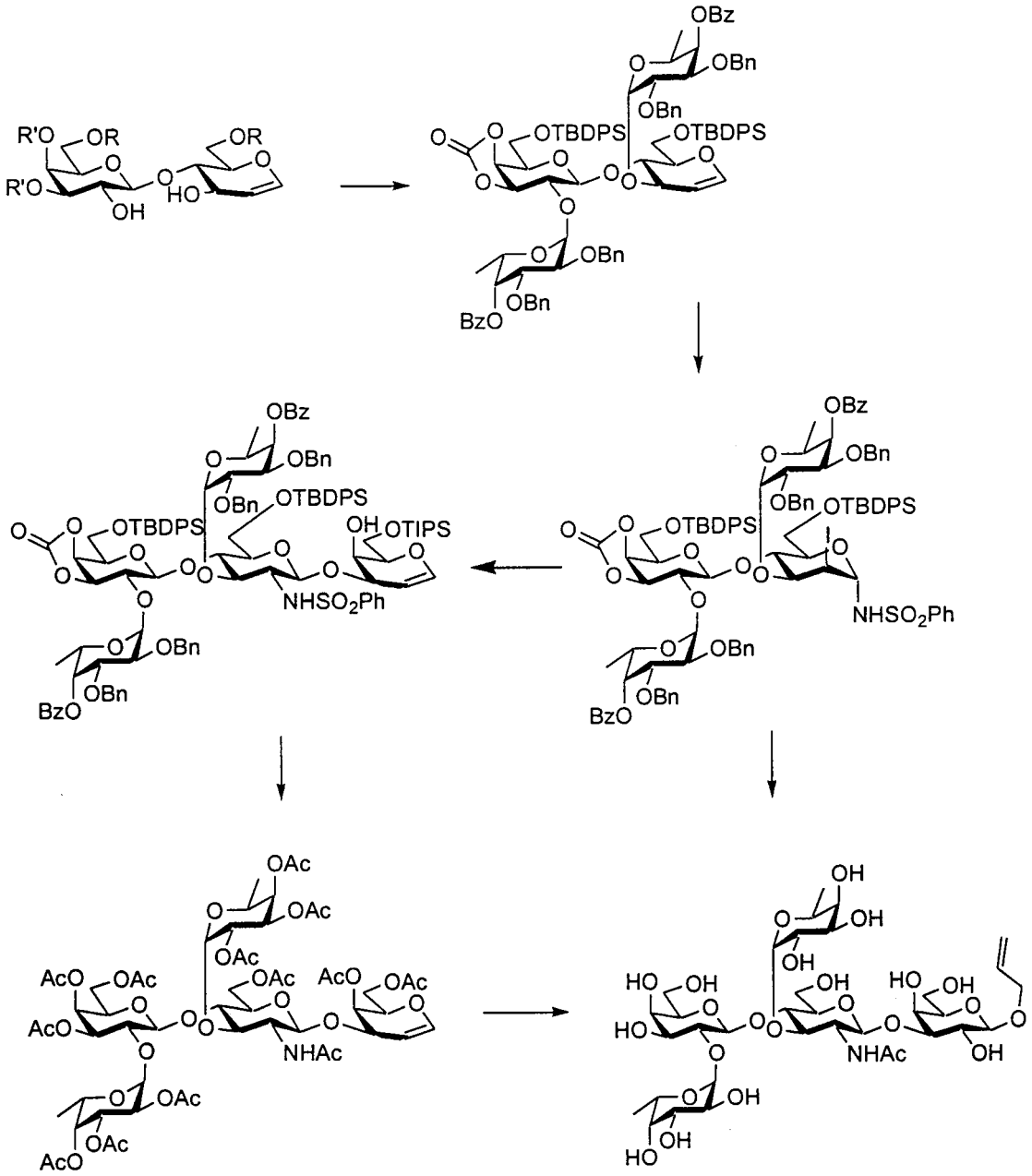


Figure 4. Scheme for the chemical synthesis of an allyl glycoside of a Le^y-active pentasaccharide using a glycal assembly approach. See Ref. [37] for details of the reagents involved.

residue. This acceptor cassette was joined to a Le^y oligosaccharide (assembled using the glycal method) via a reactive thioethyl or pentenyl group. This compound was then used in standard peptide synthesis chemistry to assemble the glycopeptide shown in Figure 5. This product was finally coupled to either Pam_3Cys (a B cell stimulating moiety) or to keyhole limpet hemocyanin for immunological experiments. Immunization of mice showed that this complex carbohydrate does indeed induce antibodies capable of reacting with natural forms of Le^y and with Le^y-positive tumor cells. Clinical studies with this construct are planned for the near future.

Blood groups in cancer

Although many earlier studies had suggested that cancer cells differ from normal cells in the expression of certain carbohydrate structures, it was with the application of monoclonal antibody technology in the 1980's that this situation became fully appreciated. Having joined the Sloan-Kettering Institute

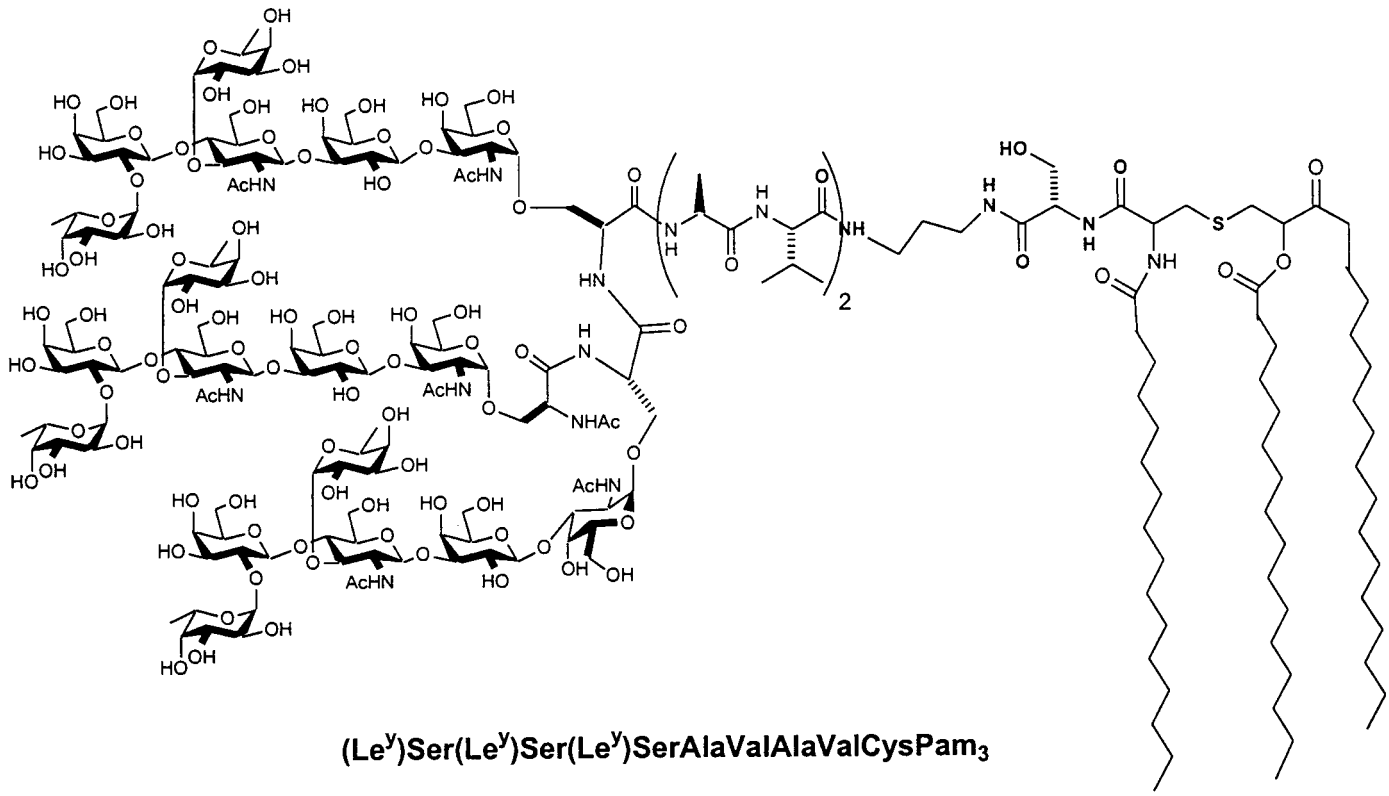


Figure 5. Structure of a glycolipopeptide bearing clustered Le^y epitopes synthesized as a possible cancer vaccine [38].

for Cancer Research in 1976, I was in a good position to capitalize on these findings by applying carbohydrate structural analysis to the elucidation of the specificity of anti-tumor monoclonal antibodies. Gangliosides were found to be characteristic antigens of most neural crest-derived tumors, such as melanoma and neuroblastoma (reviewed in [39]), and blood group-related structures were found to be overexpressed in most epithelial tumors (carcinomas).

Our own studies pinpointed previously known structures, such as Le^b and Le^y, as being important antigens in a number of types of epithelial cancer [40,41]. We also showed the importance of blood group secretor status in determining the degree of expression of these antigens in normal and malignant tissue [40,42]. Notably we found that certain secretor specificities are anomolously expressed in the cancers of non-secretors. Other investigators demonstrated that some anti-tumor monoclonal antibodies recognized novel structures, i.e. sialyl-Le^a and sialyl-Le^x [43,44]. These structures received increasing attention when it was later demonstrated that they were involved in cellular adhesion as receptors for selectins [45]. Following on the pioneering studies of Georg Springer [46], it was also shown that another family of blood group specificities (Thompson-Friedenreich, Tn and sialyl-Tn) were also selectively expressed in carcinomas [47,48].

Two different approaches for the application of these findings to the therapy of human cancers were immediately apparent. The first used monoclonal antibodies directed to blood group-related structures for the direct killing of tumor cells or as carriers for the delivery of radioisotopes, toxins or drugs to tumors. Although a number of non-carbohydrate directed antibodies have reached the clinical trial stage and some have obtained FDA approval, only a few anti-carbohydrate antibodies (e.g. anti-Le^y) have reached the clinical trial stage of investigation. A Doxorubicin-coupled anti-Le^y antibody showed potent anti-tumor effects in mouse models [49], however its clinical use was limited by anti-normal tissue toxicity [50], although other anti-Le^y antibodies did not have this problem. A humanized version of a different anti-Le^y antibody [51] will shortly be studied at our Institution by Lloyd Old, Sydney Welt and Chaitan Divgi in an imaging trial in ovarian cancer patients.

As a second approach we are studying the direct immunotherapy of human cancer by active immunization with a variety of carbohydrate antigens. The rationale for these studies is partly based on the work by Philip Livingston and coworkers [52] and Tadashi Tai, Reiki Irie and coworkers [53] showing that melanoma patients with circulating anti-GM2 ganglioside antibodies, either naturally-occurring or resulting from vaccination, show increased disease-free interval and survival. Three studies involving vaccination with blood group-related structures are presently underway at our Institution, i.e. Le^y in ovarian cancer [54], globo H in pancreas cancer [55], and sTn in breast cancer [56]. All three studies

utilize glycoconjugates in which the carbohydrate moiety is linked to a protein carrier (keyhole limpet hemocyanin), together with an immunological adjuvant (QS21), as the immunogen. In Phase 1 clinical studies it was shown that these conjugates elicit antibodies capable of reacting with the corresponding antigen on tumor cells. Importantly, given the normal expression of these antigens, it was also demonstrated that few side-effects resulted from the use of these vaccines. Randomized trials using polyvalent vaccines, planned for the future, are needed to demonstrate the clinical effectiveness of these carbohydrate-based vaccines.

Future perspectives

The study of blood group antigens has now entered a mature stage. The structure of all the major specificities has been known for some time. Moreover, with recent advances in gene cloning and enzymology the basis for such previously obscure phenomenon as secretor status, A_1/A_2 differences and the absence of an active gene product in O blood type individuals is now known. Future advances in the field will come from a better understanding of the biological functions of blood group specificities [2]. The retention of A, B and O (H) antigens, which have no apparent function, throughout human evolution has long been a puzzle. The expression of A, B and H antigens seems to be associated with susceptibility to infectious agents and this may provide one clue about their function. Various disease states, such as gastric ulcers and heart disease, are also associated with particular blood types. While provocative, these findings have been difficult to associate with actual function of the structures at the molecular level. Our understanding of the function of Lewis-related structures is on somewhat firmer ground. Numerous lines of evidence show that they serve as recognition units for molecules involved in cell–cell and cell–substrate interactions. The best evidence comes from the demonstration that the sialylated or sulfated derivatives of Le^a and Le^x are recognized by proteins, selectins, involved in leukocyte and endothelial cell interactions [44].

The function, if any, of unmodified Le^a, Le^x, Le^b and Le^y structures still remains uncertain. Le^x has been implicated in morulation in mouse embryogenesis, again suggesting a role in adhesion [57]. A recent study showing that Le^y and/or H expression is enhanced in cytokine-stimulated endothelial cells suggests a role for these structures in the inflammatory process [58]. The strong expression of some blood group specificities on cancer cells strongly implicates the involvement of these specificities in their biological behavior, e.g. in adhesion and metastasis, but supporting data are as yet largely lacking. Finally, whether or not altered blood group expression can form the basis for developing effective anti-cancer strategies also remains as a question for the future.

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