Mapping of Subsites of Monoclonal, Anti-Carbohydrate Antibodies Using Deoxy and Deoxyfluoro Sugars

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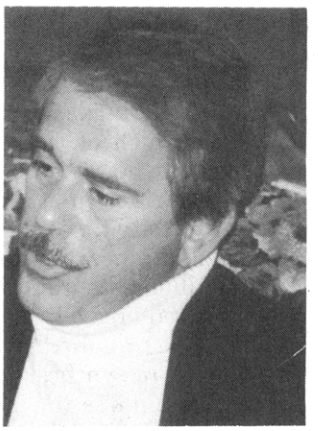

Cornelis P. J. Glaudemans obtained his B.Sc. at the Federal University in Utrecht, The Netherlands, and his Ph.D. degree from McGill University in Canada in 1958. Originally working on polysaccharides and synthetic organic chemistry, he became interested in bacterial- and immunochemistry in the middle 1960's, following a post-doctoral period with Professor Michael Heidelberger. Beginning when he was on the faculty at Yale University, he has worked on microbial and fungal capsules and—more extensively—on the molecular interaction of monoclonal antibodies with carbohydrate, and other, antigens. He learned much from his post-doctoral associates, but particularly from his colleague M. Potter, M.D., to whom this review is dedicated. Since 1975, Dr. Glaudemans has been Chief of the Section on Carbohydrates in the National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD.

I. Introduction

There are many classes of proteins whose biological significance results from their capability to bind carbohydrates. These include receptors,[1] lectins,[2] immunoglobulins,[3] and enzymes.[4] The number, and refinement, of studies of carbohydrate–protein interactions has dramatically increased lately and includes examination of hydrogen bonding between carbohydrate determinants and protein epitopes, using deoxy and deoxyfluoro sugars as probes. In early work, involving concanavalin A[5,6] and D-galactose oxidase,[7] some use was made of this. In such an approach the rationale is that, if in a hapten a hydroxyl group is involved in any hydrogen bonding with the antibody, then replacement of the hydroxyl group in question by hydrogen negates such an interaction. Substitution by fluorine would only prevent hydrogen donation by the ligand, since fluorine can receive a hydrogen bond,[8] and there is even evidence that increased interactions may occur when fluorine is involved.[9] The first studies of subsite arrangements of monoclonal antibodies with carbohydrate specificity used many related carbohydrate mono- and oligosaccharidic ligands that had been altered by substituting hydrogen, or fluorine for hydroxyl groups, at specific positions.[10,11] Monitoring of the changed antibody affinities of haptens so derivatized, can reveal patterns of binding that in turn can point to certain subsite arrangements in the antibodies investigated. Spacial effects due to substitutions are not expected, as the bond length as well as the van der Waals radius of the C–F bond (1.39 and 1.37 Å, respectively) are shorter than the corresponding values for the C–O (in OH) bond (1.43 and 1.40 Å, respectively).[12]

II. Binding Studies

The injection into mammals of even simple immunogens (substances foreign to the recipient, and thus potentially capable of invoking an immune response) activates many lymphocytes to differentiate, leading to antibody-secreting plasma cells. Since each lymphocyte is capable of producing a single molecular species of immunoglobulin (same as antibody), molecular mixtures of antibodies are obtained in a normal antibody response. The differentiation of a single lymphocyte and its expansion into a single clone of plasma cells (monoclonal) will produce a single molecular species of expressed antibody. Monoclonality can occur spontaneously (myeloma), or can be achieved following immunization by a given immunogen, followed by the in vitro clonal expansion of spleen cells obtained after their fusion with unrelated plasmacytoma cells, the so called "hybridoma" technique. The early work on mouse myeloma monoclonal immunoglobulins[13,14] furnished the groundwork for the discovery of hybridomas[15] and the subsequent, unlimited access to monoclonal antibodies of any, predetermined specificity. Antibody proteins are made up of two heavy (H) and two light (L) chains. The N-terminal ends of one H and one L chain together make up one of two identical antigen-combining areas the molecule has. Early work on antigen/antibody-binding of monoclonal immunoglobulins involved those with specificity for phosphorylcholine,[16] inulin,[17] levan,[18] cryptococcal,[19] and pneumococcal polysaccharides,[20] as well as galactan and dextran. The work on the latter two systems was expanded to include probing with deoxy and deoxyfluoro sugars, and that will be discussed below.

Galactan-Binding Monoclonal Immunoglobulins

The first systematic evaluation of binding patterns between carbohydrate haptens and monoclonal immunoglobulins using substrate derivatization was done on β-(1→6)-D-galactan-binding IgAs J539 and X24.[21,22] These immunoglobulins bind sequences of several β-(1→6)-D-galactopyranosyl residues, and the combining area appears on the surface of the antibody. It was suggested[23] that IgA J539 bound to internal tetragalactosyl sequences of a β-(1→6)-linked, fungal galactan, and subsequent binding studies confirmed the ability of antibodies of this family to bind to the internal tetrasaccharidic epitope.[24] The elucidation of the *sub*sites patterns began by studies on methyl 2-deoxy-2-fluoro-β-D-galactopyranoside and the corresponding 4-deoxy-4-fluoro compound. The former ligand did not bind whereas the latter one did with an affinity comparable to that of methyl β-D-galactopyranoside.[25] It was initially not clear which of the four galactosyl-binding protein subsites showed dominant binding. Since the monosaccharide (methyl β-D-galactopyranoside) must of necessity first fill the subsite with the highest affinity, and since the 2-deoxy-2-fluoro derivative did not exhibit measurable binding, it appeared that that subsite required a hydrogen bond from OH-2 to the protein. The proposed strategy for mapping of subsites was that of synthesizing many oligosaccharidic ligands wherein one (or more) of the galactosyl residues would contain a deoxy-fluoro group, thus forcing that (those) residue(s) away from the highest affinity subsite (introduction of the fluorine does not affect the conformation, as was shown by NMR spectroscopy). These oligosaccharidic ligands would thus "frame-shift" in order to optimize binding. From comparison of their affinities the arrangement of subsites can then be deduced. However, the synthetic ramifications of using the 2-deoxy-2-fluoro-galactosyl residue forecast difficulties. This, because the antibodies are specific for β-(1→6)-linked galactopyranosyl residues, and the β-linkage in galactose means a 1,2-trans arrangement for each sugar residue. That, in turn, means that in the synthesis of oligosaccharidic ligands it is necessary to have a temporary, equatorial *O*-acyl group at the 2-position of the galactosyl chloride intermediate in order to insure anchimeric assistance in the formation of the desired *trans*-β-intersaccharidic linkage. The presence of a fluorine atom at C-2 would, of course, prevent that. Serendipitously, methyl 3-deoxy-3-fluoro-β-D-galactopyranoside was found not to bind either. Thus, the required hydrogen bond in the highest affinity subsite apparently involved both the 2- *and* 3-OH groups.[10,26] This allowed the preparation and study of oligosaccharides having certain galactosyl residues bearing a deoxyfluoro group at the C-3 position. In those, the acylated 2-position was available for use in anchimeric assistance during the condensation reaction leading to the correct anomeric configuration of the oligosaccharides formed. In addition many 3- and 4-deoxy derivatives of corresponding oligosaccharides were synthesized and their binding studied.

IgAs J539 and X24, investigated in the greatest detail, are myeloma immunoglobulins which precipitate with β-(1→6)-D-galactan from *Prototheca zopfii*.[23] The latter is an essentially linear galactan, except for the presence of single side units of (1→3)-linked galactofuranosyl

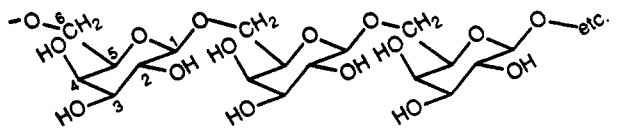

Figure 1. The sequence *O*-β-D-galactopyranosyl-(1→6)-*O*-β-D-galactopyranosyl-*O*-β-D-galactopyranosyl-(1→6)-..., the epitope defining the specificity of the anti-galactan monoclonal antibodies under discussion. One of the galactosyl residues shows the numbering of the carbon atoms.

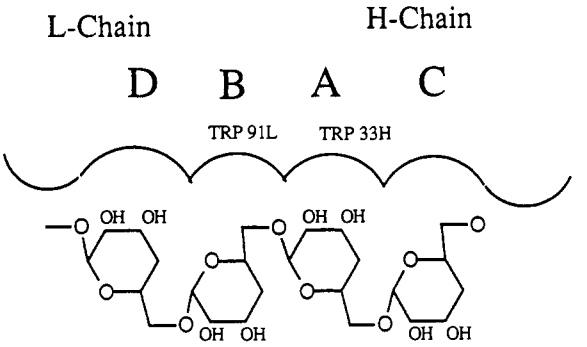

Figure 2. Schematic representation[10,11] of the binding mode of a β-(1→6)-D-galactopyranan segment to IgA J539. The view is looking down along the face of the combining area, and the polysaccharide fragment is shown with the reducing end oriented from the H chain toward the L chain. Protein subsites are labeled A, B, C, and D.

residues on every 10th galactopyranosyl residue in the main chain.[27] These antibodies belong to the X24 gene family[28] as do the hybridoma anti-galactan IgM's and IgG's, also extensively studied.[11] These were obtained by cell fusion with spleens of mice immunized[29] with phenyl β-D-galactopyranoside diazotized to BSA and gum ghatti, a predominantly β-(1→6)-linked D-galactan,[30] or gum ghatti alone. The sequence analysis of all these antibodies has been reported.[11,31,32]

In the galactan-binding immunoglobulins there are two solvent-exposed tryptophanyl (TRP) residues,[33] one at position 33 of the heavy (H), and one at position 91 of the light (L) chain. Both occur in the general combining area of the antibody. Since the monosaccharide methyl β-D-galactopyranoside, binding in the highest affinity subsite, showed ligand-induced (tryptophanyl) fluorescence change[34] (LIFC), that subsite is postulated to be near one of these tryptophan residues. The choice as to which one of the two would dictate the direction of the oligo- (or poly-) saccharidic chain (H → L or L → H) in the antibody combining area (Figure 2). Hybrid, recombinant immunoglobulins were prepared from the separated H and L chains of several IgAs in this group.[35] Some of these H_2L_2 chain-recombinant IgAs showed that methyl β-D-galactopyranoside bound to them with fluorescence characteristics more related to the H-chain donor than to the L-chain donor. The first subsite was therefore placed[10,35] near the TRP 33H. It was next observed that the disaccharide *O*-β-D-galactopyranosyl-(1→6)-β-D-galactopyranose, when binding to the IgA showed[22] double the LIFC, which was interpreted to mean involvement by perturbation of the second solvent-exposed tryptophanyl residue. Thus the antigenic chain would bind as in Figure 2.

From the data[36] listed in Table I, it can be computed that the subsite with highest affinity binds its galactosyl residue with some 52% of the total energy of binding exhibited by the maximally binding methyl β-(1→6)-

TABLE I. Binding Constants (K_a) and Free Energies of Binding for IgA J539 and Galactose Derivatives

	saccharide[a]	K_a, M^{-1}	$-\Delta G$, kcal
5	Gal→Me	1.0×10^3	4.14
7	Gal_2→Me	4.7×10^4	6.45
10	Gal_3→Me	4.8×10^5	7.85
15	Gal_4→Me	5.9×10^5	7.98
16	Gal_5→Me	5.9×10^5	7.98

[a] The names for the saccharides are abbreviated as follows: Gal→Me = methyl β-D-galactopyranoside; Gal_2→Me = methyl 6-*O*-β-D-galactopyranoside; Gal_3→Me = the corresponding trisaccharide, etc.

TABLE II. Binding Constants (K_a) and Percent Maximal Ligand Induced Fluorescence Change ($LIFC_{max}$) for IgA J539 Fab′ with Galactose Derivatives[10,11,40,80]

	ligand[a]	K_a, M^{-1}	$LIFC_{max}$
1	2FGal→Me	0	0
2	3FGal→Me	0	0
3	4FGal→Me	0.8×10^3	23
4	6FGal→Me	2.5×10^3	23
5	Gal→Me	1.0×10^3	20
6	6deoxyGal→Me	2.8×10^3	20
7	Gal_2→Me	4.4×10^4	36
8	Gal→3FGal→Me	3.7×10^4	45
9	3FGal→Gal→Me	0.8×10^4	21
10	Gal_3→Me	4.8×10^5	43
11	3FGal→Gal→Gal→Me	4.1×10^5	42
12	Gal→Gal→3FGal→Me	4.2×10^5	39
13	3FGal→Gal→3FGal→Me	3.6×10^5	40
14	Gal→3FGal→Gal→Me	4.2×10^4	41
15	Gal_4→Me	5.9×10^5	41
16	Gal_5→Me	5.9×10^5	42

[a] The names of the saccharides are abbreviated as in Table I. In addition, 2FGal→Me = methyl 2-deoxy-2-fluoro-β-D-galactopyranoside; Gal→3FGal→Me = methyl *O*-β-D-galactopyranosyl-(1→6)-β-D-3-deoxy-3-fluoro-galactopyranoside, etc.

linked β-galactopyranotetraoside. The next issue to be investigated, using the approach outlined above, was the order of affinity these four subsites have for their galactosyl residues: Neither methyl 2-deoxy-2-fluoro- nor methyl 3-deoxy-3-fluoro-β-D-galactopyranoside (2FGal→Me and 3FGal→Me, respectively) showed measurable binding, whereas the corresponding methyl 4-deoxy-4-fluoro- and the methyl 6-deoxy-6-fluoro-β-D-galactopyranoside (4FGal→Me and 6FGal→Me, respectively) bound well.[10] For the reasons of stereoselectivity[37] pointed out above, oligosaccharides, containing a nonbinding 3-deoxy-3-fluoro galactosyl residue, were prepared.[38] Their affinity constants (K_a) and those of related mono- and oligosaccharides with IgA J539 are listed in Table II. It can be seen that the monosaccharidic methyl glycosides[39] all bind with a K_a in the order of 10^3. Note also that the percentage change in antibody tryptophanyl fluorescence caused by the binding of the *mono*saccharides (ca. 20%) is nearly doubled (36%) by the binding of the disaccharide methyl *O*-β-D-galactopyranosyl-(1→6)-β-D-galactopyranoside (Gal_2→Me, **7**), indicative of involvement of a second tryptophanyl residue. Thus, the highest affinity subsite (A) and the next highest affinity subsite (B) together can bridge the distance between the two solvent exposed,[33] perturbable TRP residues. These are ca. 9.5 Å apart,[33] and the length of disaccharide **7** is approximately 10 Å in an extended conformation. Therefore, the two major affinity subsites were proposed as shown in Figure 2 (A and B). Since subsite A requires hydrogen bonding from the

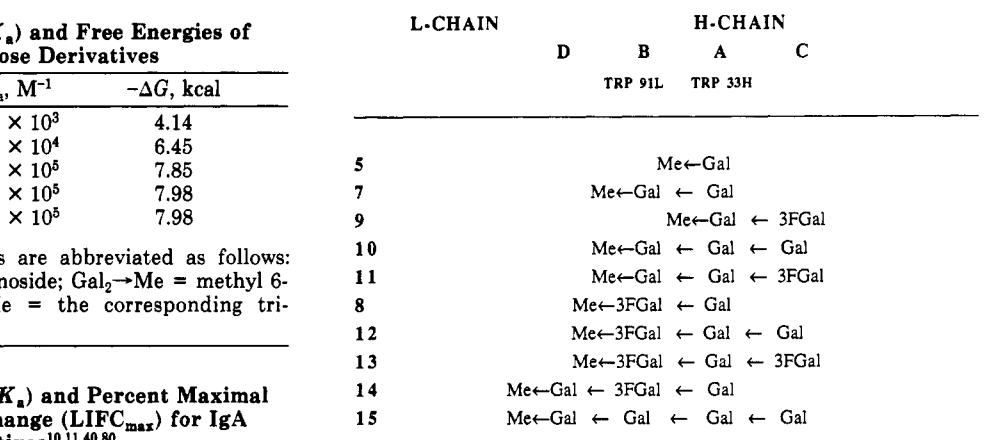

Figure 3. Schematic representation of the binding of various galactose derivatives to the subsites of IgA J539. Me←3FGal←Gal = methyl *O*-β-D-galactopyranosyl-(1→6)-β-D-3-deoxy-3-fluoro-galactopyranoside, etc.

3-OH group of the galactosyl residue it binds, **8** should and **9** should not be able to bind to both the subsites A and B (with the reducing end projecting toward the L chain (see also Figure 3). Table II shows that **8** does bind to A–B (K_a and LIFC similar to **7**), whereas **9** shows a lesser affinity, and has reverted to perturbing only one TRP residue. Thus the non-fluorine-bearing galactosyl residue of **9** binds to subsite A, and the remainder of the binding affinity results from the binding of the fluorine-bearing residue to subsite C, which must be located away from the TRP 91L, on the other side of A, toward the H-chain. Thus the trisaccharide **10** (Gal_3→Me) binds in the A–B–C sequence of subsites. If that is correct, trisaccharides **11**, **12**, and **13** should also be able to do so,[40] because in these three trisaccharides the deoxyfluorogalactosyl residue(s) are in either one, or both, of the flanking moieties of the ligand and can thus avoid the middle subsite A. Their K_a's and the LIFC are in fact essentially the same as those of **10**. In addition, it can be calculated from the K_a's of **7** and **10** that the affinity subsite C has for its galactosyl residue, should be ca. 11. By using that value, the K_a of the monofluorinated disaccharide **9** in subsites A–C is computed as 1.09×10^4, with an expected LIFC of ca. 20%, close to the values found for that disaccharide: 0.8×10^4 and 21%. To locate the fourth and last subsite D on the outside, either near B or C, the trisaccharide **14** was synthesized. In the former case compound **14** could bind to D–B–A, since in that mode it would have its central, fluorine-containing galactosyl residue avoid the subsite A. Thus, **14** would perturb both TRP 33H, and TRP 91L. In the other case, compound **14** would bind to A–C–D. In that case D would be away from TRP 91L, and **14** would then only perturb TRP 33H. The $LIFC_{max}$ of **14** reveals that it perturbs *both* TRP residues. Therefore, subsite D is next to B, and nearer to—or part of—the L chain. Could **14** possibly bind to A–C only? That appears not to be the case, since **9** binds to subsites A–C, and has a K_a of 0.8×10^4. Thus, the 5 times larger K_a of **14** clearly shows additional binding. Also, using the position of subsite D as adjacent to B, one can compute the K_a of **14**—using the values for compounds **8**, and **10** and **15**—to be 4.4×10^4, a value that agrees very well with the experimentally found value (Table II). From the affinity constants of **5**, **7**, **10**, and **15** it can be calculated that the various subsites have affinities for "their" ga-

TABLE III. Binding Constants (K_a) and Percent Maximal Ligand Induced Fluorescence Change ($LIFC_{max}$) for IgAs X24 and J539 (Fab') with Galactose Derivatives[42]

	immunoglobulin			
	X24		J539 Fab'	
ligand[a]	K_a, M^{-1}	$LIFC_{max}$	K_a, M^{-1}	$LIFC_{max}$
5 Gal→Me	0.6×10^3	18.2	1.0×10^3	18.8
17 2dGal→Me	0	0	0	0
18 3dGal→Me	0	0	0	0
19 4dGal→Me	0	0	0	0
7 Gal_2→Me	2.0×10^4	30	5.2×10^4	35
20 Gal→3dGal→Me	1.9×10^4	31	5.8×10^4	30
21 3dGal→Gal→Me	6.8×10^3	19	7.4×10^3	19
22 Gal→4dGal→Me	0.9×10^4	37	$1.3 \times 10^{4\,b}$	36
23 4dGal→Gal→Me	1.2×10^4	21	$1.3 \times 10^{4\,b}$	22
10 Gal_3→Me	3.2×10^5	41	4.8×10^5	43
24 3dGal→Gal→Gal→Me	1.9×10^5	34	4.8×10^5	37
25 Gal→3dGal→Gal→Me	2.6×10^4	31	5.2×10^4	36
26 Gal→Gal→3dGal→Me	3.5×10^5	37	4.4×10^5	38
27 4dGal→Gal→Gal→Me	2.3×10^5	41	$4.8 \times 10^{5\,b}$	44
28 Gal→4dGal→Gal→Me	2.2×10^4	37	4.7×10^4	35
29 Gal→Gal→4dGal→Me	1.6×10^5	30	1.8×10^5	38

[a] Names are abbreviated as in Tables I and II. In addition: Gal→3dGal→Me = methyl *O*-β-D-galactopyranosyl-(1→6)-3-deoxy-β-D-galactopyranoside; Gal→4dGal→Gal→Me = methyl *O*-β-D-galactopyranosyl-(1→6)-*O*-(4-deoxy-β-D-galactopyranosyl)-(1→6)-β-D-galactopyranoside, etc. [b] Measured on whole J539.

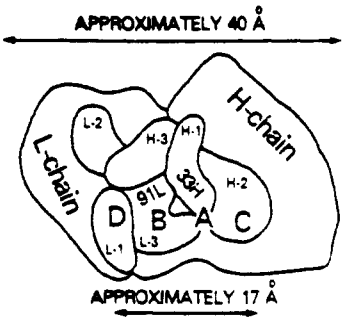

Figure 4. Schematic representation of the structure[33] of J539, with the H chain on the right and the L chain on the left, looking into the combining area. The three hypervariable regions for the H (H-1, H-2, and H-3) and the L (L-1, L-2, and L-3) chain are shown, and the general location of the solvent-exposed TRP 33H and TRP 91L are indicated. These are approximately 9.5 Å apart. A, B, C, and D denote the four galactosyl-binding subsites.

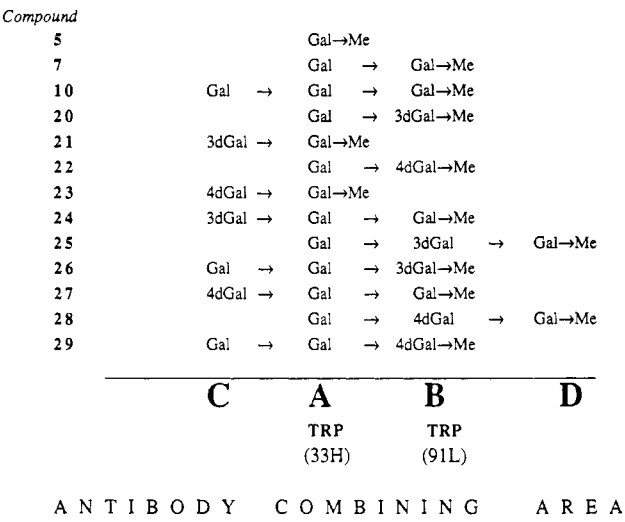

Figure 5. Schematic depiction of the immunoglobulin combining area with the four galactosyl-binding subsites. The general location of the two solvent-exposed tryptophan residues in subsites A and B is shown. Note that subsite A always accommodates an unaltered →6 galactopyranosyl 1→ residue.

lactosyl residues of 10^3 (A), 44 (B), 10.9 (C), and 1.2 (D). The "frame shifting" of the various oligosaccharides is schematically represented in Figure 3. It is the same for six other monoclonal antigalactan antibodies, two of which were hybridoma antibodies.[11] The proposed binding area on the surface of the immunoglobulin (see Figure 4) runs across the interface of the H and the L chain, under the general position of the TRP 33H. Both the H and L chain of any antibody contain short peptide segments that exhibit sequence hypervariability[41] when compared to the sequences of other antibodies (see Figure 4). These hypervariable regions define the individual specificity of the antibody in question, and frequently contain the antigen-contact amino acid residues. For the anti-galactans, some residues were proposed as possible contact residues[11] suggesting oligonucleotide-mediated alterations in the immunoglobulin that are currently being evaluated. In addition, measurements on these antibodies with a series of oligosaccharides possessing specific deoxy groups have completely confirmed[42] the binding mode in these subsites (Table III): It had earlier been found[25] that methyl 4-deoxy-4-fluoro-β-D-galactopyranoside (Table II, 3) bound with nearly undiminished affinity to the IgA, showing no hydrogen bond *donation* from that position. Since **19** (Table III) shows no binding, the highest-affinity subsite is proposed to hydrogen bond *to* the *O*-4 of its galactosyl residue. Therefore, disaccharide **23** finds it energetically more favorable to fit into the subsites C–A than in subsites A–B, as revealed by the perturbance of a single TRP residue (21%) and the fact that the observed K_a's with X24 and J539 are essentially equal to those calculated (10^4 and 1.1×10^4) from the value of the A (X24 = 600; J539 = 1000) and C (X24 = 16; J539 = 44) subsite. Note that **22** binds in the A–B subsites, as revealed by the value of the ligand induced fluorescence change, but that it does so at a somewhat reduced affinity. As for the trisaccharides **24**, **25**, and **26**, all three can use the subsites A–B, so they all show perturbation of both TRP residues. However, **24** binds in A–B–C, while **25** shifts to A–B–D, and **26** is again binding in A–B–C, as expected. This same pattern holds for the trisaccharides **27**–**29** (Figure 5).

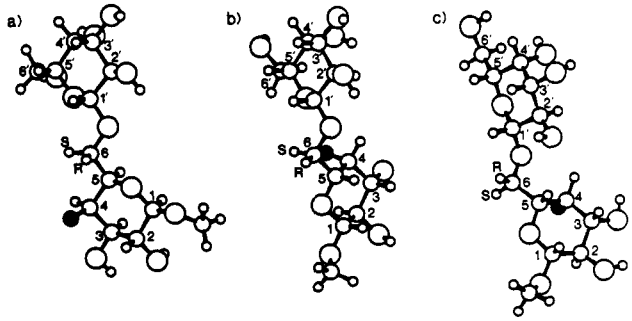

Figure 6. Comparison of the most populated conformer in free solution (a) and the two possible antibody-bound conformations (b,c) of disaccharide **7a**. The fluorine is indicated in black.

NMR Studies on Antibody–Hapten Interactions

The conformational flexibility of antigenic determinants, and the possible *selection* of one of these conformations as the one to be recognized by its antibody, has been the object of many studies.[43] For oligosaccharide epitopes, theoretical considerations have been advanced for the prediction of solution conformations.[44] Binding data obtained with oligosaccharides and proteins, including antibodies, together with measured or calculated conformations, have been used to arrive at specific binding interactions.[45,46] It is however quite possible that the free energy released upon binding of an epitope to an antibody can more than compensate for the energy required to *induce* changes in the existing solution conformations of that epitope. In the case of the antigalactans, nuclear magnetic resonance (NMR) spectroscopy using transferred nuclear Overhauser enhancement (TRNOE) spectroscopy,[48-50] was employed[47] to study the conformation of the bound hapten. The antibody used was IgA X24. This monoclonal immunoglubulin is representative for the entire antigalactan family.[28,51] The use of dissaccharide **7** and of its 2-deuterio-4-deoxy-4-fluoro derivative[52] (**7a**) with IgA X24 allowed unequivocal conclusions to be drawn. For a disaccharide linked 1→6, three torsion angles,[53] ϕ, ψ, and ω, are used to define these intersaccharidic angles: By sighting along the bond of interest, the heaviest atom near the observer is turned to eclipse the heaviest atom away from the observer. The degree of rotation gives the angle, while the direction determines the sign (clockwise is positive).

Extensive analysis of J couplings and NOE data showed[47] that, in free solution, no single conformation is present, but that the preponderant conformer has an extended conformation with $\phi = -120°$, $\psi = 180°$, and $\omega = 75°$. This predominant conformation of the free disaccharide (Figure 6) is close to that predicted by HSEA calculations.[38] No trace of an NOE interaction between H-2′ and H-4 could be observed in the solution spectrum. In the presence of antibody the NMR constraints were compatible with two conformations for the antibody-bound form of methyl *O*-β-D-galactopyranosyl-(1→6)-2-deuterio-4-deoxy-4-fluoro-β-D-galactopyranoside (**7a**), having interglycosidic torsion angles of $\phi = -152 \pm 9°$, $\psi = -128 \pm 7°$, and $\omega = -158 \pm 6°$; and a conformer with $\phi = -53 \pm 6°$, $\psi = 154 \pm 10°$, and $\omega = -173 \pm 6°$ (Figure 5). Since for the bound epitope the observed dihedral angles differ significantly from *any* of the observably populated solution conformations, this suggests that studies on conformation by theoretical calculations, or even by X-ray diffraction, of flexible epitopes may not always be relevant from a biological point of view. Finally, studies have been reported on the affinity labeling of antigalactan, monoclonal antibodies.[83] Two kinds of reactive ligands were prepared: Initially β-(1→6)-linked D-galacto-oligosaccharides, having an epoxypropyl group as the aglycone were synthesized.[54] Although that functionality has been successfully used in the affinity labeling of lysozyme,[55] it requires electrophilic or nucleophilic attack and is thus selective for certain amino acid residues. To avoid that restriction IgA X24 was labeled with more reactive groupings, such as diazerino aglycones,[56] capable of chain insertion.[57] The preparation of oligosaccharides bearing that aglycone led to a novel chemical method of glycosylation.[58] In addition, enzymatic transglycosylation has yielded *C*-diazerino galactosides, the β-(1→6)-linked one of which was, again, capable of labeling both the H and L chain of IgA X24.[59] This work is continuing with use of tetrasaccharidic labels of great complexity.[60]

Dextran-Binding Monoclonal Immunoglobulins

Many monoclonal immunoglubulins—such as the ones discussed above—have the capability to bind to either internal or terminal residues of a polysaccharide chain. There also are antibodies that can only bind to the terminus of a polysaccharidic antigenic determinant. Such an immunoglubulin is IgA W3129 having a combining area complementary only to the nonreducing terminal epitope of an α-(1→6)-D-glucopyranan (dextran).[61-64] The subsites of IgA W3129 have been mapped by an approach similar to the one discussed above for the anti-galactans.[10,11] Initially, the ligands studied were the methyl mono-deoxy-mono-fluoro-α-D-glucosides.[9,65] The IgA requires a critical hydrogen bond for its highest affinity subsite from the OH-6 and the OH-4 *to* the protein, with a possibility of a hydrogen bond from the protein to OH-3 of the glucoside (Table IV). That requirement for free OH-6 clearly indicates that that protein site is bound to the terminal nonreducing glucosyl group of the α-(1→6)-linked dextran, as that is the only glucosyl moiety in the entire antigenic epitope bearing a free OH group at position 6. The preparation of the required isomaltooligosaccharides and their specifically substituted deoxyfluoro derivatives was far more difficult in this case,[66-68] due to the intersaccharidic (cis) α-linkage required.[37] In order to again ensure the anomerically proper pyranoside of the terminus all ligands were synthesized as their methyl α-glucoside. The binding affinities of the isomaltooligosaccharides **39–44** (Table IV) show that the maximally binding dextran epitope consists of four sequential glucosyl residues, while glucosides **30–34** reveal the highest affinity subsite of the protein to involve the nonreducing terminus of that epitope, as pointed out above. From the association constants of **30** and **39–41** the affinities of the individual subsites for "their" glucosyl residue can again be computed, as free energies of binding of subsites are additive (see below), shown in Figure 7. Immunoglobulin W3129 lacks[69] the TRP at 91L, and the only solvent-exposed TRP is at position 33H. The data in Table IV suggest that TRP residue is located near to the highest-affinity subsite. This, because *all* the glucosyl ligands show LIFC. The op-

TABLE IV. Binding Constants (K_a) and Percent Maximal Ligand Induced Fluorescence Change ($LIFC_{max}$) for IgA W3129 with Glucose Derivatives[9]

ligand[a]	K_a, M^{-1}	$LIFC_{max}$
30 Glc→Me	1.8×10^3	−19
31 2FGlc→Me	1.8×10^3	−15
32 3FGlc→Me	8.7×10^3	−15
33 4FGlc→Me	0	0
34 6FGlc→Me	0	0
35 6FGlc→β-(1→2)Glc→Me	0	0
36 6FGlc-β-(1→3)Glc→Me	2.1×10^2	−18
37 6FGlc-α-(1→2)Glc→Me	0	0
38 6FGlc-α-(1→3)Glc→Me	0	0
39 Glc_2→Me	1.6×10^4	−16
40 Glc_3→Me	6.7×10^4	−16
41 Glc_4→Me	1.8×10^5	−13
42 Glc_5→Me	1.9×10^5	−14
43 Glc_6→Me	1.8×10^5	−15
44 Glc_7→Me	1.7×10^5	−14
45 6FGlc-Glc→Me	0.5×10^2	−12
46 6FGlc-Glc-Glc→Me	2.6×10^2	−16
47 6FGlc-Glc-Glc-Glc→Me	4.6×10^2	−19

[a] The names of the saccharides are abbreviated as follows: Glc→Me = methyl α-D-glucopyranoside; Glc_2→Me = methyl *O*-α-D-glucopyranosyl-(1→6)-α-D-glucopyranoside; Glc_3→Me = the corresponding trisaccharide (methyl α-isomaltotrioside), etc.; 6FGlc-Glc→Me = methyl *O*-(6-deoxy-6-fluoro-α-D-glucopyranosyl)-(1→6)-α-D-glucopyranoside; 6FGlc-β-(1→2)Glc→Me = methyl *O*-(6-deoxy-6-fluoro-α-D-glucopyranosyl)-(1→2)-α-D-glucopyranoside, etc.

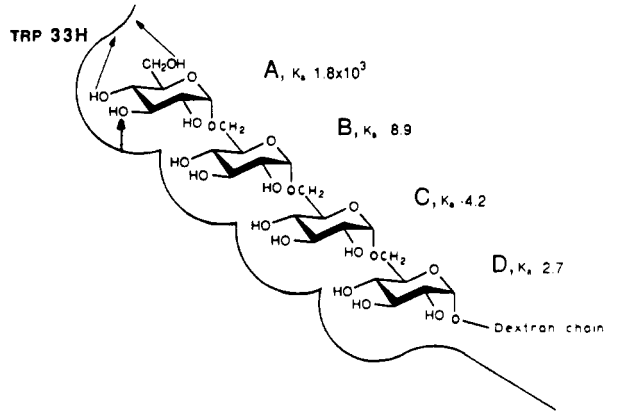

Figure 7. Schematic representation[9] of the subsites of IgA W3129, showing the tetrasaccharide epitope as well as the affinity constants each subsite possesses for its glucosyl residue. The approximate location of the TRP 33H is also indicated.

timally binding isomaltotetraose epitope has its terminal glucosyl group in that subsite, as do the other oligoisomaltosides. The amino acid sequence[70] of the variable part of the W3129 H chain (V_H) is similar to the corresponding part of the V_H of IgA J539, while the variable part of the W3129 L chain (V_L) is more similar to that of IgA McPC 603 than to the V_L of J539. A model of W3129 shows[70] a shallow cavity, primarily due to the combination of a long first hypervariable region of the L chain (hv_1-L) and the presence of smaller residues at positions 91 and 96 in the third hypervariable region of the L chain (hv_3-L). TRP 33 H lines this cavity, and it thus appears likely that said cavity is the one binding the terminal, nonreducing glucosyl group of the dextran, and that it is the subsite with the highest affinity. The 4-deoxy-4-fluoro or 6-deoxy-6-fluoro group causes cessation of measureable binding in methyl α-D-glucopyranoside (Table IV, cf. compounds **33** and **34**). From the affinities of **45**, **46**, and **47** an apparent K_a could be computed for the 6-deoxy-6-fluoro-glucosyl

TABLE V. Binding Constants (K_a) for IgA W3129 with Glucose Derivatives[71]

ligand[a]	K_a, M^{-1}
48 2dGluc→Me	2.9×10^2
49 3dGluc→Me	~30
50 4dGluc→Me	0
51 6dGluc→Me	0
52 Gal-β-(1→3)Gluc→Me	1.8×10^2
5 Gal→Me	0

[a] Abbreviations are as follows: 2dGluc→Me = methyl 2-deoxy-α-D-glucopyranoside, etc.; Gal-β-(1→3)Gluc→Me = methyl *O*-β-D-galactopyranosyl-(1→3)-α-D-glucopyranoside.

group in each of these ligands (by comparison with the association constants of **39**, **40**, and **41**, respectively). A remarkably consistent value of ca. 6 was obtained.[9] This observation confirmed that free energies of binding for each subsite are additive to give the value for the entire epitope. In addition, the data in Table IV show that methyl *O*-(6-deoxy-6-fluoro-β-D-glucopyranosyl)-(1→3)-α-D-glucopyranoside (**36**) binds reasonably well to the high affinity subsite through its un-fluorinated moiety, confirmed by the observation[71] that methyl *O*-β-D-galactopyranosyl-(1→3)-α-D-glucopyranoside (Table V, **52**) showed significant binding to W3129 (methyl β-D-galactopyranoside itself showed no binding).

Next, it was shown how the glucosyl group fits into that high-affinity subsite space, with use of data obtained[71] from binding measurement obtained in solution on a series of methyl α-deoxyglucosides with IgA W3129 (Table V). Whereas compounds **50** and **51** showed no binding by ligand-induced fluorescence change assay,[34] binding to the high-affinity subsite was moderate for **48** (K_a = ca. 3×10^2) and very weak for **49** (K_a nearly zero).[9,34] The nonbinding of **50** and **51** confirms the necessity of hydrogen bond donation for the binding of methyl α-D-glucopyranoside in the high-affinity subsite. The previously found[9] K_a for methyl 2-deoxy-2-fluoro-α-D-glucopyranoside (**31**, see Table IV) was identical with that of methyl α-D-glucopyranoside (**30**). The fact that **48** binds indicates that hydrogen-bond reception by the OH-2 of the glucoside is not critical. Apparently,[9] substitution of OH-2 in methyl α-D-glucopyranoside by F had yielded a hapten equally capable of receiving the hydrogen bond from the IgA. That methyl 3-deoxy-3-fluoro-α-D-glucopyranoside (**32**, see Table IV) binds 4.8 times stronger than the parent glycoside may be due to an inductive effect at position C-4, causing facilitated hydrogen-bond donation from OH-4 to the protein. Or, a positively charged amino acid side chain near O-3 of methyl α-D-glucopyranoside may be involved in hydrogen-bond donation, so that when that OH-3 is replaced by F, enhanced binding results. In W3129 there occurs a histidine which may be so situated.[71] Figure 8 shows a fit of methyl α-D-glucopyranoside (**30**) when bound to IgA W3129 (Fv). The conformation of **30** here used is that of the crystal structure of methyl α-D-glucopyranoside.[72] Glucoside **30** corresponds to the last glucosyl group in the antigenic α-D-(1→6)-dextran chain, and is postulated to bind near the solvent-exposed TRP 33H, since binding perturbs[9] the fluorescence of the IgA. In the mode,[71] this TRP is situated in a shallow cavity which appears to be the high-affinity subsite. Since the OH-4 and OH-6 of **30** show hydrogen-bond donation to the pro-

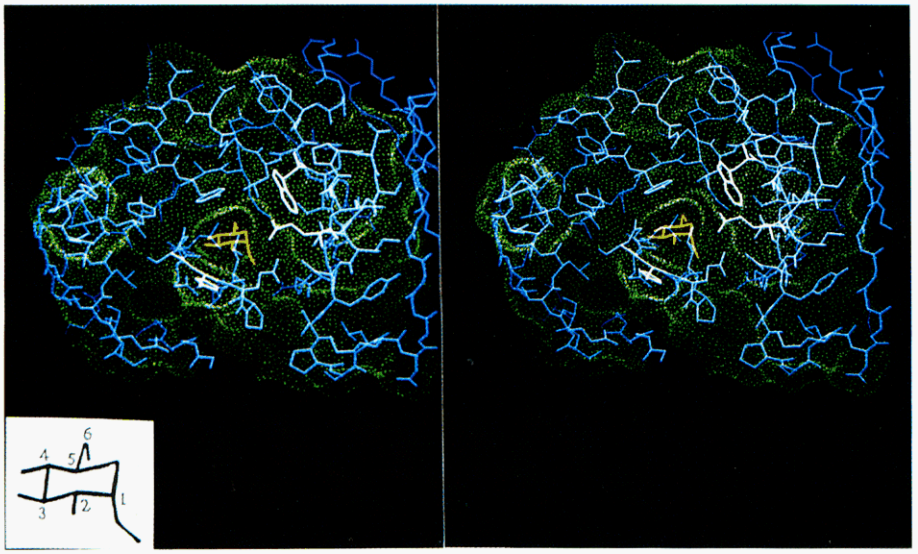

Figure 8. Stereo view[71] of the molecular surface[81] covering the CDR-residues of the H and L chain (the N-terminal ends of which together make up the general combining area of all immunoglobulins) of IgA W3129 according to Padlan and Kabat.[70] The view is with the first hypervariable (hv) region of the H chain in the foreground. The extended loop created by the five amino acid insert in the first hv region of the L-chain juts out to the left. The cavity located in the center shows methyl α-D-glucopyranoside bound in it, in red. Clearly visible in white are TRP 33H, GLU 50H, HIS 93L, and the carbonyl of SER 92L (for identification purposes the ligand is shown alone, with the carbon atoms in the glucose moiety numbered). Distances between the various oxygen atoms of the carbohydrate hapten and the nearest part of selected amino acid side chains are as follows: (O-4) serine 92L >C=O, 2.9 Å; (O-6) glutamic acid 50H $O_{\epsilon 1}$ ($-COO^-$), 3.0 Å; (O-3) histidine 93L $N_{\epsilon 2}$ (>N–), 3.3 Å; (O-5) tryptophan 33H $C_{\zeta 3}$ (>C=), 4.9 Å. The figures were generated with use of the molecular graphics program FRODO.[82]

tein, they must be placed near an appropriate amino acid receptor. The glycosidic $-O-CH_3$ (which represents the $-O-CH_2-$ of the next glucosyl residue in the dextran chain) should project out of the cavity to allow the continuation of the antigenic chain. The environment of the OH-3 should allow bulky substitution.[9] Thus, the OH-3 points out of the cavity into the solvent. In addition, OH-3 is likely to be near a positively charged amino acid residue. These prerequisites are fulfilled in Figure 8 (for distances in Å, see the legend to Figure 8): Glutamic Acid 50H can accept the OH-6 hydrogen bond, OH-4 is accepted by the carbonyl of serine 92L in the backbone, and histidine 93L can form a hydrogen bond to the O-3 and may be positively charged. The glucoside is near tryptophan 33H, and the OH-3 projects away from spacial restriction, into the solvent. The glycosidic oxygen also projects out of the cavity, providing space to continue the dextran chain. All-created hydrogen-bond distances are in the correct range.

Recently, a hybridoma anti-α-D-(1→6)-dextran IgA antibody (16.4.12E) has been produced.[73] It has a variable heavy- and light-chain (V_H and V_L) amino acid sequence derived from the same gene segments as is W3129 and also binds the nonreducing terminal epitope of the dextran chain. Affinity measurements on that antibody and all the same ligands used in the above studies were also performed (see Table VI).[71] Binding behavior is similar to that of W3129, and IgA 16.4.12E also shows a deep cavity. The 6-hydroxyl group of methyl α-D-glucopyranoside is again critical for binding of that determinant in the highest affinity subsite of IgA 16.4.12E, as methyl 6-deoxy- or methyl 6-deoxy-6-fluoro-α-D-glucopyranoside **51** and **34**, respectively) do not significantly bind, and it was concluded that the antibody also binds the nonreducing glucosyl terminus of the dextran in its pronounced cavity subsite. From the data (Table VI) the subsites were mapped. The observation that the methyl glycoside of β-D-Gal_p-(1→3)-α-D-Glc_p (**52**) did not bind showed that the terminus occupied the cavity with its C2–C3 face toward the wall instead of facing the solvent, as is the case in IgA W3129.

Protein–saccharide fittings can be used to select certain amino acids for changes by site-directed mutagenesis in order to experimentally evaluate their role in hapten contact. It is hoped that by fully elucidating the bonds that occur between saccharidic epitopes and their specific antibodies, an explanation can be proposed for the sometimes lower affinity found for carbohydrate antigens when compared to protein antigens with their immunoglobulins. The affinities of the former frequently are of the order of from 10^5 to 10^6 M^{-1}. Yet these antibodies are protective.[74] It has been shown that ligands with affinities of 10^5 or somewhat less, are capable of initating an immune response in mice.[75]

Anti-Blood Group Monoclonal Antibodies

Young et al.[76] prepared and characterized a set of murine monoclonal antibodies with specificity for the human Le^a blood group determinant β-D-Gal_p-(1→3)-[α-L-Fuc_p-(1→4)]-β-D-Glc_pNAc. In a later paper Lemieux et al.[77] studied the binding of a series of synthetically modified ligands (by inhibition techniques) in which hydroxyl groups in the trisaccharidic determinant had been replaced by hydrogen, one at the time. The deductions made by these workers was that one antibody (AH8-34) required the presence of the $2\text{-}OH_{galactose}$ and the $N—C(CH_3)=O_{N\text{-}Ac\ glucosamine}$ groups for binding, and that this antibody bound the trisaccharide determinant when its $4\text{-}OH_{galactose}$ was hydrogen bonded to the $3\text{-}O_{galactose}$. From the observation that the determinant shows improved binding upon replacement of the $4\text{-}OH_{galactose}$ by H, it appears to this reviewer that any hydrogen bonding proposed between these two

TABLE VI. Comparison of K_a and Maximum Ligand Induced Fluorescence Change (ΔF_{max}) for Glucose Derivatives with the IgAs 16.4.12E and W3129.

	16.4.12E		W3129	
ligand[a]	K_a, M^{-1}	ΔF_{max}, %	K_a, M^{-1}	ΔF_{max}, %
30 Glc→αMe	4.5×10^3	(+51)	1.8×10^3	(−19)
39 Glc_2→αMe	5.2×10^4	(+45)	1.6×10^4	(−16)
40 Glc_3→αMe	8.7×10^4	(+41)	6.7×10^4	(−16)
41 Glc_4→αMe	3.9×10^5	(+47)	1.8×10^5	(−13)
42 Glc_5-αMe	2.4×10^5	(+47)	1.9×10^5	(−14)
43 Glc_6→αMe	3.8×10^5	(+45)	1.8×10^5	(−15)
48 2dGlc→αMe	0.2×10^2 [b]	(+25)	2.9×10^2	(−12)
31 2FGlc→αMe	0.8×10^2	(+36)	1.8×10^3	(−15)
37 6FGlcα(1→2)Glc→αMe	0		0	
35 6FGlcβ(1→2)Glc→αMe	0		0	
49 3dGlc→αMe	0		0.3×10^2 [b]	(−47)
32 3FGlc→αMe	1.2×10^2	(+36)	7.7×10^3	(−15)
38 6FGlcα(1→3)Glc→αMe	0		0	
36 6FGlcβ(1→3)Glc→αMe	0		2.1×10^2	(−18)
52 Galβ(1→3)Glc-αMe	0		1.8×10^2	(−16)
50 4dGlc→αMe	0		0	
33 4FGlc→αMe	0		0	
51 6dGlc→αMe	0		0	
34 6FGlc→αMe	0.3×10^2	(+47)	0	
53 6-*O*-MeGlc→αMe	0.6×10^2	(+50)	0	
45 6FGlc→Glc→αMe	3.6×10^2	(+40)	0.5×10^2	(−12)
46 6FGlc→Glc_2→αMe	6.3×10^2	(+48)	2.6×10^2	(−16)
47 6FGlc→Glc_3→αMe	3.7×10^3	(+49)	4.6×10^2	(−19)
5 Gal→βMe	0		0	

[a] Abbreviations are as follows: Glc→αMe = methyl α-D-glucopyranoside; Glc_2→αMe = methyl *O*-(α-glucopyranosyl)-(1→6)-α-D-glucopyranoside, Glc_3→α = the corresponding trisaccharide glucoside, etc. [unless specifically noted, for instance as in **9**, all linkages are α-(1→6)], 2dGlc→αMe = methyl 2-deoxy-α-D-glucopyranoside, 2FGlc→αMe = the corresponding 2-deoxy-2-fluoroglycoside, etc. [b] Poor, if any, binding.

positions may actually have to go the other way: from the $3\text{-}OH_{galactose}$ to the $4\text{-}O_{galactose}$. The other murine anti-Le^a antibody discussed in this study (CF4–C4) reads the trisaccharidic epitope when it is in the identical conformation, according to these authors, but a different set of intramolecular hydrogen bonds apparently plays a role in mediating the affinity with the antibody site: Here a bond is proposed from $2\text{-}OH_{galactose}$ to the O=C of the acetamido group, as well as a hydrogen bond from the $4\text{-}OH_{fucose}$ to the $3\text{-}O_{fucose}$. No deoxy-fluoro derivatives of the various sugar moieties were prepared or tested to verify or refute these assignments. From the method of measurements in this work, it must be remembered that the $\Delta\Delta G°$ values presented are relative and not absolute. It was proposed that the association of antibodies and carbohydrate ligand, being enthalpy driven, are so due to a significant ordering of "conformationally labile protein" on binding. Some rearrangement of an initial, transient antibody–hapten complex has in fact in one case been observed (Vuk-Pavlovic et al., ref 43), but it is not obvious to this reviewer that antibody is conformationally labile in the absence of binding hapten.

Work on the interaction Le^b human blood group α-L-Fuc_p-(1→2)-β-D-Gal_p-(1→3)-[α-L-Fuc_p-(1→4)]-β-D-Glc_pNAc with a monoclonal antibody has also been reported.[78] In this report only the galactosyl residue was altered systematically. It was shown that the 3-OH was critical to binding, and that the 6-OH, when changed to H, F, or Cl, allowed more potent binding to the antibody. This was attributed to the presence of a hydrogen bond between the 4-OH and the 6-OH (presumably 4-OH to 6-O). This would mean that substitution of OH by highly electronegative F would enhance binding in what the authors refer to as a hydrophobic cleft. Intermolecular hydrogen bonding between antibody and hapten was not suggested. Finally, binding of the human blood group B determinant α-L-Fuc_p-(1→2)-[α-D-Gal_p-(1→3)]-β-D-Gal_p was studied with two murine monoclonal immunoglobulins.[79] Replacement of certain hydroxyl groups by hydrogen (position 2 in the α-galactosyl residue, and position 4 in the β-galactosyl residue) led to cessation of binding. It was observed that the two 6-deoxy derivatives (either galactose) bound the antibody with essentially the same affinity as did the original determinant. From that it was suggested that two intramolecular hydrogen bonds existed in the determinant, directed toward the respective ring oxygens.

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