Molecular recognition of the Lewis Y antigen by monoclonal antibodies

Magdalena Blaszczyk-Thurin¹, Ramachandran Murali², M.A. Julie Westerink³, Zenon Steplewski⁴, Man Sung Co⁵ and Thomas Kieber-Emmons⁶

¹The Wistar Institute of Anatomy and Biology, ²Department of Pathology and Laboratory Medicine, University of Pennsylvania, 36th and Hamilton Walk, Philadelphia, PA 19104-6082, ³Department of Medicine, Medical College of Ohio at Toledo, ⁴Department of Medicine, Thomas Jefferson University, ⁵Protein Design Labs, Inc., USA

To whom correspondence should be addressed

Introduction

Cell-surface carbohydrates play a role in tumor growth, progression and metastases (Hakomori, 1989, 1991). Among the carbohydrate types, the histo-blood group Lewis antigens are highly associated with a number of cancers including human breast, colon, lung and ovarian carcinomas (Fukushi et al., 1986; Itzkowitz et al., 1986; Kim et al., 1986; Blaszczyk-Thurin et al., 1987; Hoff et al., 1989; Cooper et al., 1991; Tsukazaki et al., 1991; Itzkowitz 1992; Miyake et al., 1992; Murata et al., 1992; Ogawa et al., 1992; Ichihara et al., 1993; Ilioopoulos et al., 1993; Kobayashi et al., 1993). The Lewis Y (Y) difucosylated type 2 lactoseries structure, expressed on both glycoproteins and glycolipids, is one tumor-associated carbohydrate structure being explored as a target for monoclonal antibody (Mab)-based imaging and therapy (Trail et al., 1993; Choe et al., 1994). It is postulated that the Y determinant is of key importance for tumor cell growth or maintenance since it mediates internalization and killing with Y-specific Mab (Hellstrom et al., 1990; Pai et al., 1991, 1992; Steplewski et al., 1991; Schreiber et al., 1992, Garrigues et al., 1993).

While a large number of anti-Y antibodies have been generated, their detailed specificity has been studied in only a few cases (Blaszczyk-Thurin et al., 1987; Hellstrom et al., 1990; Pastan et al., 1991; Kitamura et al., 1994). Reactivity patterns of antibodies with synthetic Y determinants might be different when compared with antibody tumor cell binding which depends on the presentation of Y on the tumor cell surface and also the number of hexose subunits defining the Y epitope. We previously described a monoclonal antibody, BR55-2, generated against a human gastric adenocarcinoma cell line, that specifically recognizes the Y determinant (Blaszczyk-Thurin et al., 1987; Steplewski et al., 1990, 1991, Scholz, 1991). Monoclonal BR55-2 (IgG3) and its isotype switch variants are found to mediate both antibody-dependent cell-mediated and complement-dependent cytoxicity and efficiently inhibit tumor growth in xenografted nude mice (Steplewski et al., 1990, 1991). Humanized or chimeric forms of anti-Y antibodies that share some of the properties of BR55-2 are being considered for passive therapy (Kaneko et al., 1993; Kitamura et al., 1994) and BR55-2 is in clinical trials. An increased understanding of the structural basis for antibody recognition of the Y antigen might be exploited to develop improved diagnostic agents and passive and active immunotherapeutics for Y expressing solid tumors.

NMR studies of synthetic Y and Lewis b (Leb) structures show that the major difference in topography between Leb and Y molecules is provided by the change of glycosidic linkage from β1→3 to β1→4 in type 1 and 2 chains, respectively (Lemieux et al., 1980; Thogersen et al., 1982; Lemieux and Bock, 1983; Hindsgaul et al., 1985; Rao and Bush, 1988, Cagas and Bush, 1990, 1992; Chai et al., 1992, Strecrer et al., 1992). The differences in the glycosidic linkages result in conformers in which the N-acetyl and hydroxymethyl groups of the GlcNAc moiety are projected on opposite sides of the type 1 and 2 structures (Figure 1a). Functional groups that are shared or define the common topography between the type 1 and type 2 structures account for their mutual recognition by GS4 (Sporr et al., 1985a). The immunodominant portion of the antigen detected by BR55-2 and other related MAb is specific for the type 2 Y determinant but not the type 1 isomeric determinant, Leb. This type 2 specific recognition is in contrast to the lectin IV protein of Griffonia simplicifolia (GS4), suggesting that BR55-2 and related MAb mimic only a portion of the salient recognition features of GS4 for Y.

The aim of the present study was to identify the molecular basis for BR55-2 Y specificity by characterizing the dimensions and similarities of BR55-2 with other anti-Y antibodies. BR55-2 was cloned and sequenced and molecular modeling was...
used to elucidate its three-dimensional structure. During the performance of this work the crystal structure of an anti-Y antibody, BR96 in complex with a Y epitope was reported (Jeffrey et al., 1995). Since the crystal coordinates for BR96 are not yet available, molecular modeling of BR55-2 was utilized to identify similarities in the recognition scheme for the Y tetrasaccharide core by BR55-2 based on the BR96-Y co-complex structural features. Potential BR55-2 interaction sites with the putative tetrasaccharide of the Y determinant were identified and further compared with related anti-Y antibodies, B3 (Brinkmann et al., 1991; Pastan et al., 1991; Pai et al., 1991, 1992; Choe et al., 1994), and H18A (Kaneko et al., 1993). The BR55-2 model emphasizes key polar and non-polar interactions contributing to the molecular recognition features for Y that are shared among the related anti-Y antibodies, consistent with results from examination of reactive profiles of lactoseries oligosaccharide probes with BR55-2. We observe that a major source of differential specificity for the type 1 and 2 difucosylated structures emanates from its interaction with the β-D-N-acetylglucosamine residue as compared with GS4, which most likely extends to other anti-Y antibodies.

Materials and methods
Cloning and sequencing of the heavy-chain and light-chain variable-domain cDNA for BR55-2

The variable-domain cDNA for the heavy-chain and light-chain of BR55-2 was cloned by the anchored PCR method (Co et al., 1992). First, a total RNA preparation was prepared using the hot phenol method. Briefly, $1 \times 10^7$ BR55-2 hybridoma cells were resuspended in 1.2 ml of RNA extraction buffer (50 mM sodium acetate, pH 5.2–1% SDS), vortexed and incubated at room temperature for 2 min. The cell lysates were then incubated with 0.6 ml of phenol, pH 5.2, at 65°C for 15 min, followed by another 15 min incubation on ice. The extract was spun in a microfuge; the aqueous phase was recovered and ethanol precipitated twice. The RNA pellet was resuspended in water and quantitated at OD$_{260}$. cDNA was synthesized from the total RNA using reverse transcriptase [5 μg total RNA, 40 ng dT$_{12-18}$ (Pharmacia), 200 units of M-MLV reverse transcriptase (BRL), 40 units of RNAsin (Promega), 50 mM Tris–HCl, pH 8.3, 75 mM KCl, 10 mM DTT, 3 mM MgCl$_2$ and 0.5 mM each dNTP in a 20 μl reaction volume]. The G-tailing was achieved with terminal deoxynucleotidyl transferase (TdT) [cDNA, 15 units TdT (BRL), 0.1 M potassium cacodylate, pH 7.2, 2 mM CoCl$_2$, 0.2 mM DTT and 1 mM dGTP in a 20 μl reaction volume]. Under the conditions described, tails generally contained about 20 bases. One half of the G-tailed product was then amplified to generate the V$_L$ gene and the other half amplified to generate the V$_H$ gene, using Taq polymerase. The V$_L$ gene was amplified with the primer TATATCTAGAATTCC-CCCCCCCCCCCCC that anneals to the G tail and a primer TATAGAGCTCAAGCTTGGATGGTGGGAAGATG-GATACGTTGGTGC that anneals to the C region of gamma chains. The sequences in parentheses indicate base degeneracies, which were introduced so the primer would be able to recognize all gamma chain isotypes. EcoRI and HindIII sites are included in the upstream and downstream primers for convenient subcloning into pUC18 vector. An alternative set of restriction sites (XbaI and SacI) are also included in the primers for the rare event that EcoRI and HindIII sites are present in the variable region genes.

The PCR reactions were performed in a programmable
heating block using 30 rounds of temperature cycling (92°C for 1 min, 50°C for 2 min and 72°C for 3 min). The reaction included the G-tailed product, 1 μg of each primer and 2.5 units of Taq polymerase (Perkin-Elmer Cetus) in a final volume of 100 μl, with the reaction buffer recommended by the manufacturer. The PCR product bands were excised from a low-melting agarose gel, digested with restriction enzymes and cloned into pUC18 vector for sequence determination.

**Modeling of BR55-2**

We and others have developed procedures for antibody modeling (Fine et al., 1986; Brucoleri and Karplus, 1987; Shenkin et al., 1987; Brucoleri et al., 1988; Novotny et al., 1990; Cheetham et al., 1991; Martin et al., 1991; Mas et al., 1992; Nell et al., 1992; Tomiyama et al., 1992; Lohman et al., 1993; Bajorath et al., 1994; Lin et al., 1994) based on the fact that the shapes of heavy and light chains are closely associated with the lengths of complementarity determining regions (CDRs) and that framework (FR) residues play an important role in influencing CDR conformations (Chothia et al., 1992; Tramontano and Lesk, 1992). The conformations of the variable domains of BR55-2 were deduced by comparison with known immunoglobulin crystal structures (Bernstein et al., 1977). Sequence comparisons were used to identify templates for the localized structural folds of the hypervariable or CDR1, CDR2 and CDR3 regions of the heavy and light chains. For modeling of the heavy chain CDR3 region in particular, we utilized both a knowledge-based approach (Blundell et al., 1987) to search for suitable structures that fit a geometry to the base residues of the CDR3 domain (Fine et al., 1986; Shenkin et al., 1987; Martin et al., 1991) and a conformational search procedure (Brucoleri et al., 1988; Novotny et al., 1990; Mas et al., 1992; Nell et al., 1992). The latter involved using the program AbM (Oxford Molecular).

To determine a base geometry for H-CDR3, the Ca positions of several antibodies of known crystal structure were superimposed to define invariant residue positions. These positions defines the amino-terminal beginning and the carboxyl-terminal end that are shared among the putative CDR3 domains of varying lengths (Tomiyama et al., 1992; Karp et al., 1993; Lohman et al., 1993; Lin et al., 1994). The systematic superpositioning of the CDR3 domain over short sequences defines a consensus region where the base geometry is conserved among the antibody templates. The description of invariant positions effectively reduces the length of the loop to search to those that are representative of a sufficient saturation of conformational space in the crystallographic database; usually six to seven residues in length. The crystallographic database was searched to identify loops of the same size as the CDR3 loop being examined using the program InsighII (version 3.5, Biosym Technologies). The spatially conserved Cartesian positions of the N- and C-terminal regions of CDR3 were held fixed in this search procedure. The 10 best matches were examined using the program InsighII and an appropriate choice was made based upon similarities in positions of side chains at the junctures of the CDR3 loop. As an alternative to this approach we also utilized the automated antibody modeling program AbM which incorporates a knowledge based search, followed by conformational mapping.

The CDRs and the FRs of the templates were mutated to those of the respective antibody heavy and light chains using InsightII or automatically assigned by AbM. For the InsightII-built structures, the side chain angles of the substituted residues were set according to angles identified in a database of side chains. Each CDR and framework region was changed individually, followed by 1000 cycles of energy minimization to eliminate close contacts between atoms. As in our previous studies, the program Discover (version 2.95, Biosym Technologies) was used for conformational calculations with the supplied consistent valence force field (CVFF) parameters. After model building, the respective structures were energy optimized to convergence. Molecular dynamics (MD) at 300 and 600 K was used to alleviate further any close contacts within the antibodies.

Initially a molecular dynamics simulation over 30 ps using the program Discover was performed. The structure was then energy minimized using conjugate gradients to convergence. Following this initial equilibration, the calculation was resumed for another 20 ps at 600 K at constant pressure and then cooled to 300 K over 30 ps. During the second dynamics procedure atoms lying further than 15 Å from all atoms of the CDR loops were held fixed. Non-hydrogen atoms of residues lying in the region 9–15 Å from all CDR loop atoms were harmonically restrained to their initial positions with a force constant of 30 kcal/mol/Å². These distance approximations result in fixing or restraining atoms of residues within the framework region of the antibodies. The backbone conformational torsion angles, φ (ψ) and ψ (φ), of non-CDR loop residues were restrained to their initial values with a force constant of 1600 kcal/mol/Å². In addition, a torsional restraint of 10 kcal/mol/rad² was employed around the peptide bond. A time step of 1 fs was used. The resulting structure for BR55-2 was energy minimized using conjugate gradients to convergence.

**Systematic conformational search of Lewis antigens**

Systematic conformational searches over torsional space on both Y and Le⁠³ were performed to relate their binding mode conformations. Utilizing a grid search approach each dihedral angle is stepped through a range of values, and resulting conformations are then steric free. Conformational search calculations were performed using the program Search and Compare (version 2.3.5, Biosym Technologies). The respective units in Y and Le⁠³ were searched over conformational space at 1 or 5° intervals either holding (φ,ψ) angles for fixed glycosidic linkages to their binding mode values or allowing all angles to move concomitantly. Glycosidic (φ,ψ) angle definitions follow those previously published (Lemieux and Bock, 1983; Mukhopadhyay and Bush, 1991). Angle (φ¹,ψ¹) defines rotations about H₁-C₁-O-C₆ (φ¹), and C₁-O-C₆-H₈ (ψ¹). Similarly, (φ²,ψ²) corresponds to the IUPAC convention in which φ² corresponds to O ring–C₁–O₁–C₆, and ψ² is defined as C₁–O₁–C₆–C₇–C₈–1.

Molecular dynamics calculations were also performed for both Le⁠³ and Y tetrasaccharide structures. The structures were first equilibrated at 300 K for 50 ps, followed by 100 ps of molecular dynamics at 300 K. A total of 100 000 conformations were sampled with instantaneous dynamics structures minimized at 1 ps intervals, reducing the number of structures to be examined to 100. The 100 structures were minimized to convergence using conjugate gradients. The differences in populations of the two molecules were analyzed using the Analysis option in InsighII. In vacuo calculations were performed with a dielectric of 1 or 80 to monitor electrostatic effects on final conformations.
Docking of Lewis Y to BR55-2

Individual subunits derived from crystal structures of carbohydrate fragments was used to model the putative Y tetrasaccharide core Fucα1→2Galβ1→4(Fucα1→3)GlcNAc structure. The modeled structure was first energy minimized and compared with published computational (Lemieux and Bock, 1983; Mukhopadhyay and Bush, 1991) and NMR (Cagas and Bush, 1990, 1992) results on Y and Leb determinants. Residues associated with the CDR loops were identified for possible interaction with the Y determinant based upon BR96 binding to Y (Jeffrey et al., 1995) and Lewis antigen recognition by GS4 (Delbaere et al., 1993). The approach taken in the placement of the Lewis Y core in the antibody combining site was that described previously by us (Lin et al., 1994). The binding surface on the modeled antibodies was first defined as sites accessible to probe spheres of varying radii (1.4–1.7 Å) to identify possible positions which could be occupied by the atoms of the Y structure. The probe spheres were rolled on the binding surface of the models, with the continuum of loci reduced to a set of discrete points by clustering neighboring spheres much like a set of site points (Lin et al., 1994). These site points are localized at atom positions accessible to the probe spheres on each residue on the surface of the antibody. The site points were then used as a guide in the placement of the Y structure. Hydrogen bonding restraints were applied to enhance potential contacts between the respective antibody combining site residues with sugar groups identified in the BR96/LeY structure (Jeffrey et al., 1995), allowing the glycosidic angles of the Y structure to change and the BR55-2 model to adjust to these restraints.

After minimization, a restrained molecular dynamics calculation over 100 ps using the program Discover was performed, preserving the hydrogen bonding constraints. The dynamics run was not intended to be a detailed study, but to alleviate further any close contacts within the antibody and between tetrasaccharide and the antibody. The calculation was initialized and equilibrated for 50 ps at 300 K at constant pressure and resumed for another 50 ps. The resulting structure was energy minimized using conjugate gradients to convergence. In the minimization and dynamics run, no constraints other than the retention of Y-antibody hydrogen bonds were placed on the antibody or binding site. Charges and non-bonded parameters for the Y structure were assigned from atom types from the CVFF parameter list supplied with Discover/InsightII.

Results

Mapping of recognition sites of Lewis binding proteins

Natural oligosaccharides and synthetic probes of Lewis antigens have been used in a variety of studies to map potential recognition sites on lectins and antibodies (Young et al., 1983; Lemieux et al., 1984, 1985; Hindsaul et al., 1985; Spoehr et al., 1985a,b; Blaszczyk-Thurin et al., 1987; Lemieux et al., 1988; Spoehr and Lemieux, 1988; Lemieux et al., 1990). Based upon analysis of the binding profiles of lectoseries isomeric structures by BR55-2, we previously identified epitope reactive groups that include the OH-4 and OH-3 groups of the β-D-galactose unit, the 6-CHα groups of the two fucose units and the N-acetyl group of the subterminal β-D-α-N-acetylgalactosamine (βDGlcNAc) (Blaszczyk-Thurin et al., 1987). A similar interaction pattern involving OH-3 and/or OH-4 of the terminal galactose as key polar groups is postulated for the interaction of six other Mabs (Leb, B blood group, Lea and I Ma specific antibodies) (Lemieux et al., 1984, 1985, 1988; Spoehr et al., 1985b) and lectins [Ulex europaeus (Hindsaul et al., 1985) and GS4 (Spoehr et al., 1985a)] (Table I).

Our interpretation of the reactivity patterns (Table I) indicates that OH-4, OH-3, OH-4 and OH-3 of the Y molecule might be involved in BR55-2 binding (Figure 1b). A similar hydroxyl group cluster which consists of OH-3, OH-4 and OH-4 is found to be a part of a topography recognized on Leb and Y determinants by GS4 (Spoehr et al., 1985a) and on the H-2 structure recognized by U.eurpaeus lectin I (Hindsaul et al., 1985). Adjacent to the hydroxyl clusters, a lipophilic surface formed by CHα and CHα as well as O-5 is postulated to interact with both BR55-2 and GS4 (Blaszczyk-Thurin et al., 1987). The OH-4 unit might also contribute to the amphiphilic binding surface since it is present at the edge of the proposed epitope for both BR55-2 and GS4. The involvement of the OH-2 group in α-2-fucose recognition of Leb with MAbs and GS4 is also observed. Since GS4 binds to the common surface in both Leb and Y, this functional group might also be involved in Y recognition by BR55-2.

The acetamido group of the βDGlcNAc residue, part of the binding epitope for Y of BR55-2, is also involved in binding of specific MAbs with Ma I (Lemieux et al., 1984) and Leb (Spoehr et al., 1985b). The specific recognition of the H-2 structure by the lectin U.eurpaeus does not significantly involve the N-acetamido group, but does involve the 6-hydroxyl group in the binding reaction (Hindsaul et al., 1985). On the other hand, MAbs specific for the type 1 Lea determinant recognize both sides of the β-D-N-acetylgalactosamine unit with the recognition of CHαOH being polar in character and that on the acetamido side non-polar (Lemieux et al., 1988).

The nature of the combining site for several anti-Y antibodies that include BR64, BR96 (Hellstrom et al., 1990), BR55-2 (Blaszczyk-Thurin et al., 1987), BR16-5A (Rodeck et al., 1987), B1 and B3 MAbs (Pastan et al., 1991) and AH-6 (Abe et al., 1986) appear to be similar in that they all map to the terminal portion of the Y oligosaccharide. Examination of cross-reactive patterns of the antibodies with synthetic Y probes suggest that they require α-2-fucose for binding and that α-3-fucose increases the binding but is not required. The B1 antibody displays slight cross-reactivity with the type-1 Leb structure and B-3 binds to di- and tri-fucosylated LeX structures as well as a trifucosyl Leb/LeX hybrid structure. A distinct group of antibodies recognize only extended Y structures (Kaiizu et al., 1986; Kim et al., 1986; Nudelman et al., 1986). These differences in reactivity clearly suggest that Y reactive antibodies recognize different epitopes. This differential specificity might also be extended to interaction with both tumor and normal cells. This difference in the length of the oligosaccharide side chains has important implications for the binding of MAbs to certain cells which have the ability to synthesize extended type 2 chain antigens, such as carcinoma cells.

Sequence and structural similarities between anti-Y antibodies

To define better the functional groups involved in molecular recognition of Y determinants, we cloned and sequenced BR55-2 and compared its sequence/structural properties with several other described anti-Y antibodies, B3 (Brinkmann et al., 1991), H18A (Kaneko et al., 1993) and BR96 (Bajorath, 1994; Jeffrey et al., 1995). The nucleotide sequence and the translated amino acid sequence of the light chain and heavy-
Fig. 2. Nucleic acid and amino acid sequence of (a) the heavy chain and (b) the light chain of BR55-2. The first amino acid of the mature protein is double underlined. CDRs are underlined.

Table 1. Summary of functional groups implicated in molecular recognition of Lewis structures with MAb and lectins

<table>
<thead>
<tr>
<th>MAb/lectin</th>
<th>Type 1</th>
<th>Type 2</th>
<th>Key polar interactions</th>
<th>Non-polar interactions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y</td>
<td>Lea</td>
<td>BR55-2</td>
<td>OH-4b</td>
<td>OH-4c</td>
</tr>
<tr>
<td>H-2, Ulex</td>
<td>Br55-2</td>
<td>OH-3b</td>
<td>OH-3c</td>
<td>OH-3c</td>
</tr>
<tr>
<td>europaeus</td>
<td></td>
<td>OH-3b</td>
<td>(OH-3c)</td>
<td>(OH-2c)</td>
</tr>
<tr>
<td>Lea</td>
<td></td>
<td></td>
<td>OH-2b</td>
<td>OH-2d</td>
</tr>
<tr>
<td>AH8-34</td>
<td></td>
<td></td>
<td>OH-3b</td>
<td>OH-3c</td>
</tr>
<tr>
<td>CF4-C4</td>
<td></td>
<td></td>
<td>OH-3b</td>
<td>OH-3c</td>
</tr>
<tr>
<td>Leb</td>
<td></td>
<td></td>
<td>OH-3b</td>
<td>OH-3c</td>
</tr>
<tr>
<td>96FR</td>
<td>Y/Leb</td>
<td></td>
<td>OH-3b</td>
<td>OH-2d</td>
</tr>
<tr>
<td>Griffonia</td>
<td></td>
<td></td>
<td>OH-4b</td>
<td>(OH-3c)</td>
</tr>
<tr>
<td>simplicifolia</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>B blood</td>
<td>Mb-1</td>
<td></td>
<td>OH-4b</td>
<td>OH-4c</td>
</tr>
<tr>
<td>terminal</td>
<td>Mb-2</td>
<td></td>
<td>OH-4b</td>
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</tbody>
</table>

Atom lower case scripts are those defined in Figure 1. The designation e* refers to the terminal Gal residue of Gal[1→4Galβ1→4GlcNAc B blood group structure. Parentheses indicate marginal interactions depending on the number of saccharide units associated with the antigen.

Ref: 1, Blaszczzyk-Thurin et al. (1987); 2, Hindsgaul et al. (1985); 3, Lemieux et al. (1988); 4, Spohr et al. (1985b); 5, Spohr et al. (1985a); 6, Delbaere et al. (1993); 7, Lemieux et al. (1985); 8, Lemieux et al. (1984).

Sequence analysis of BR55-2 shows that VH and V kappa genes are in the VH 7183 family and V kappa C1 family, respectively. Sequence alignment (Figure 3) indicates that the anti-Y antibodies are homologous with each other, except in their heavy-chain hypervariable or CDR regions.

Sequence comparison with known immunoglobulin crystal structures in the Brookhaven Protein database (Bernstein et al., 1977) provide a template for the variable regions of the antibodies (Figure 3). The primary structure of the light chain of the anti-cholera toxin antibody TE33 (1TET) and the autoantibody BV04-01 (1CBV) displayed 96% and 89% identity, respectively, with the BR55-2 light chain (Figure 3). Both of these antibodies have been elucidated by X-ray crystallography as a complex with their respective ligands. Superposition of these two light chains indicates that their CDR conformations are nearly the same except for CDR1 around the sequence tract 'S-N-G' of BV04-01. The conformational difference appears to be due to an induced conformation in TE33 upon the binding of the cholera toxin.
Fig. 3. Sequence alignment of (a) the light and (b) the heavy chains of BR55-2, B3, H18A and BR96, among themselves and with template crystal structures as identified by Blast search of the protein crystallographic database on the NCBI BLAST server. Dashes indicate identities with respect to BR55-2. Numbering corresponds to that of Kabat et al. (1997). Only a partial sequence has been presented for BR96 (Bajorath, 1994). The structure of the light chain of TE33 (Brookhaven code ITET (Bernstein et al., 1977)) was used as a template for the modeling of BR55-2 and B3 light chain. For the heavy chain the B13i2 structure (2IGF) was used as the template. This structure displayed equivalent length CDRs with respect to the anti-Y antibodies. The antibody 17/9 has a longer CDR3 region than the anti-Y antibodies.

peptide to TE33. Subsequently these structures appear to reflect conformational changes that might be evident upon ligand binding. In the modeling of BR55-2 we utilized the TE33 template for the light chain, but the CDR1 conformation of BV04-01.

For the heavy chain of BR55-2, we identified two immunoglobulin templates, 17/9 (1H1M) and B13i2 (2IGF) which are co-complexed with peptides derived from their respective antigens, displaying 78% and 69% identity, respectively, with BR55-2 up to CDR3. Superpositioning of these structures indicates that they display nearly the same conformations up to the CDR3 region (residues 1-95) with an r.m.s. of 0.47 Å, suggesting CDR1 and CDR2 conformations have undergone similar conformational transitions, if any. This aspect is of relevance in considering that the CDR2 of 17/9 and BR55-2 each display three glycines (Figure 3) which in principle can adopt unusual $\phi/\psi$ angles. In comparison with B13i2, which displays a 'G-G-S' sequence for residues 53-55, the canonical conformations are equivalent for 17/9 and B13i2.

To model the CDR3 region of the BR55-2 heavy chain, we first examined CDR3 folding patterns of several crystallographically known immunoglobulins. Analysis of immunoglobulin crystal structures indicates that CDR3 loop regions can adopt conformations that fall into two general classes which have been referred to as kinked and unkinked (Mas et al., 1992), despite their considerable variability in length, sequence
and conformation. Conformational search procedures applied to CDR3 clearly suggest that these are the only conformations available to the base of the loop (Mas et al., 1992). It has been suggested that packing of residues at loop base can be used to differentiate between these structures (Mas et al., 1992).

Based upon the sequence similarities found in B13i2 with the anti-Y antibodies, we utilized this kinked base geometry template and searched for additional loops in the database (Table II). In splicing each loop of seven residues identified from the search into the B13i2 template (Table II), and heating the system to 600 K and then cooling to 300 K, we found that the CDR3 loops approached the conformation of the B13i2 loop. In each of the starting conformations the replaced Gly residue at position 5 in the putative loop did not adopt the conformation analogous to the proline in the B13i2 template (Figure 4). A major difference in the two conformation types was centered on Gly H99 (Figure 4). In effect, the substituted Gly in the second conformer type, Gly at position 5 in the B13i2 template points downward, whereas the substituted Gly in the second conformer type points upward (Figure 4). Analysis of the low-energy form of the preferred AbM-generated structure displayed correspondence to the second conformer type, indicating that conformational searching leads to structure types identified by the molecular dynamics calculations.

We found that the B13i2 loop was energetically favorable on average by 4 kcal/mol. This finding is probably related to the starting bias in the choice of the B13i2 template. Analysis of VH:VL interfaces indicate that the structure of the CDR3 region and the VL:VH association are interdependent as residues at the C-terminal end of CDR3 form part of the VH:VL interface. However, if we take a different approach and consider the degree of sequence homology for the CDR3 loop of BV04.1 compared with BR55-2, the BV04.1 displays a conformation in which the Trp residue at position H100a is oriented differently with respect to the center of the antigen binding site relative to the calculated low-energy forms. This orientation is similar to that observed in the BR96 crystal structure (Jeffrey et al., 1995), suggesting that perhaps hydrophobic interactions involved in DNA and sugar interactions are similar. These results further suggest that sequence similarities are perhaps a more important consideration for modeling than energetics alone and that multiple starting geometries should still be considered. Subsequently we choose the BV04.1 loop for CDR3.

Docking of Y

Using site point information as a guide, we examined the respective CDR domains for residue types implicated in binding the difucosylated Y structure as determined by the co-complex of BR96. Previous studies have implicated tracts such as YYMY and YYGY as potential regions for carbohydrate interactions (Hoess et al., 1993). The YYMY sequence tract is found in CDR1 of the heavy chain in BR96, B3 and BR55-2 (Figure 3b) and is implicated in BR96 binding to Y. We positioned the Y fragment within the BR55-2 binding site, relying upon distance difference maps between the Y structure and the site points and also the reported contacts in the BR96 co-crystal structure.

In the placement of Y we performed a restrained molecular dynamics calculation using the reported contacts for BR96 with the cFuc functional group contacts. This model is shown in Figure 5a and summarized in Table III. In this model O-4a can potentially form a hydrogen bond with the backbone NH group of Tyr H33 as observed in BR96, while a bifurcated hydrogen bond is observed between His L31 and OH-3 and OH-4. Tyr H35 forms a hydrogen bond with OH-6. Unlike the crystal structure we observe a hydrogen bond between OH-2 and Asn H97. In the crystal structure Asp97 is interacting with the nonoate methyl ester group attached to βD-GlcNAc. Our Y structure does not account for this functional moiety (Figure 1b). We also observe hydrogen bonds with dFuc functional groups which are not reported for the crystal structure.

To evaluate the model further we examined alternative placements of Y based upon the GS4 lectin structure in complex with Leβ. In these models (Figure 5b–d) site points associated with residues displaying homology with GS4-Leβ contact residues were identified and used as a guide in the placement of the Y structure. We observed a range of possible polar and non-polar arrangements illustrated in Figure 5b–d. We identified several variations of a putative binding mode for BR55-2 in which many of the contacts were conserved in GS4 and the other anti-Y antibodies. In particular, we observed a potential bifurcated hydrogen bond between Asn L31b and OH-3β and OH-2β (Figure 5b and d) which mimics Asn L31 binding to a tetrasaccharide form of Leβ in GS4. Differences in the glycosidic angles of Y can change the bifurcated hydrogen bond to a single hydrogen bond with OH-2β (Figure 5c). The bifurcated hydrogen bonding by Asp89 in GS4 to OH-3β and OH-4β is potentially mimicked by Ser L33a in the antibodies (Figure 5b–d). In B3 we observe a Trp residue contacting OH-3β (Figure 5b), again mimicking that observed for Leβ binding in GS4, while in BR55-2 this contact involves a Tyr. In BR55-2, B3 and H18A Tyr H33 can bind to OH-4β (Figure 5b–d). Again, variations in the Y structure can introduce a hydrogen bond between Ser L31a and OH-2β in BR55-2 (Figure 5b and c). This residue is conserved in B3 and mutated to a Thr in H18A (Figure 3a). In BR96 this position is mutated to an Asn (Figure 3a), which presumably can still form a hydrogen bond with OH-2β.

An electrostatic interaction involving His L93 (assuming a physiological pH of 7.0) is
Val L94 potentially stabilizes the polar interactions with the available hydroxyls of cFuc. This interaction can approach hydrogen bonding distances (Figure 5b and c). Val L94 can also interact with cFuc via hydrophobic interactions (Figure 5c). As expected from experimental data (Blaszczyk-Thurin et al., 1987), we observe interactions with the GlcNac residue. Our alternative placement of Y results in a hydrogen bond between Ser H56 in BR55-2 with OH-6α (Figure 5b and c).
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Fig. 5. Summary of hydrogen bonding and hydrophobic interactions identified for Y binding to BR55-2. Hydrogen bonding distances identified by the program LIGPLOT (Wallace et al., 1995) reflect idealized heavy atom distances less than 3.3 Å. Interaction energies for the Y structure for BR55-2 range from -88 kcal/mol (a) to -64 kcal/mol (d). Model (a) corresponds to contacts observed in the BR96-Y crystal structure which are conserved between BR55-2 and BR96. Models (b)–(d) are those based upon a presumed common topography of Leb and Y binding to the anti-Y antibodies based upon the GS4-Leb crystal complex.

This residue is an Ile in H18A, providing a hydrophobic interaction with GlcNAc (Figure 5b). The acetamido group of GlcNAc is involved in either electrostatic or hydrophobic interactions with residue position H58 in the antibodies. Interactions with either the N-2 or O-7 is dependent on the final conformation of the acetamido group of the GlcNAc residue (Figure 5c and d).

Comparison of interaction energies for the various binding
minimum energy conformation represented by NMR results about 10° and are within 3—4 kcal/mol around a single
Le structures indicate that low-energy conformers fluctuate during MD calculations (Table IVa, columns 1 and 2) of unbound
TV). computational or NMR approaches (Table IV, column 3), HSEA (Table IVb, column 2) calculations indicate that final minimized models are dependent on the starting conformation, we used the HSEA conformer (Table IVb, column 2) as a starting geometry. Minimization of this geometry resulted in the same conformer (Table IVa, column 5, and Table IVb, column 3) as that starting from the binding mode conformation of Leb in GS4 (Table IVa, column 4, and Table IVb, column 1). A converged structure for Leb (Table IVa, column 5 and Table IVb, column 3), which started from an average of low-energy conformers identified from a grid search, was found to be within this range of angle fluctuations.

To determine the extent to which final minimized models are dependent on the starting conformation, we used the HSEA conformer (Table IVb, column 2) as a starting geometry. Minimization of this geometry resulted in the same conformer (Table IVa, column 5, and Table IVb, column 3) as that starting from the binding mode conformation of Leb in GS4 (Table IVa, column 4, and Table IVb, column 1). The HSEA-derived starting conformation in Table IVb (column 2) was found to be only 0.8 kcal/mol above the converged conformer (Table IVa, column 5, and Table IVb, column 3), with the Leb binding mode conformation 2.8 kcal/mol above the converged structure. These calculations confirm that the binding mode of Leb for GS4 and the HSEA-derived structure are local minima within the range of fluctuations for the glycosidic angles observed from MD calculations (Mukhopadhyay and Bush, 1991).

Performing similar calculations for the Y structure, it was found that averaged low-energy forms identified from a grid search, and also the HSEA starting conformation (Table V, column 2) as a starting geometry. The calculations confirm that the binding mode of Leb for GS4 and the HSEA-derived structure are local minima within the range of fluctuations for the glycosidic angles observed from MD calculations (Mukhopadhyay and Bush, 1991).
Table V. Comparison of \( \phi/\psi \) glycosidic torsion angles (°) in Lewis Y structures

<table>
<thead>
<tr>
<th>Linkage</th>
<th>( \phi^1,\psi^1 ) (HSEA)</th>
<th>( \phi^1,\psi^1 ) (CVFF)</th>
<th>( \phi^1,\psi^1 ) (BM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>bGal(1→3)GalNAc</td>
<td>55/10</td>
<td>49/12</td>
<td>46/14</td>
</tr>
<tr>
<td>eFuc(1→4)GalNAc</td>
<td>50/25</td>
<td>46/22</td>
<td>45/25</td>
</tr>
<tr>
<td>dFuc(1→2)bGal</td>
<td>50/10</td>
<td>48/17</td>
<td>50/19</td>
</tr>
</tbody>
</table>

*HSEA angle values are from hard-sphere exomeric–anomeric calculations (Lemieux and Bock, 1983). CVFF is a minimized structure from average conformation identified in grid search and is a low-energy structure starting from column 1. BM is BR55-2 binding mode conformation in Figure 5a.

column 1), led to a converged conformer (Table V, column 2) similar to that found for the Le\(^b\) structure (Table IVa, column 3) for the representative units. Minimization of the binding mode conformation (Table V, column 3) also led to the converged structure in column 2. The binding mode structure (Figure 5a) (Table V, column 3) was 1.5 kcal/mol above its optimized structure. In comparison, the HSEA conformation (Table V, column 1) was less stable, being 2.3 kcal/mol above its optimized structure shown in column 2. Minimization of binding mode structures in Figure 5b–d resulted in converged structures either representative of that in Table V (column 2) or higher energy conformers.

To examine further the Y specificity of BR55-2, we superimposed the corresponding structure for Le\(^b\) on the BR55-2 binding mode Y structure observed in Figure 5a. The binding mode conformation for Le\(^b\) in complex with GS4 is not very different from that based upon NMR and theoretical calculations. Minimization of the Le\(^b\)-BR55-2 complex resulted in a recognition scheme less stable (14 kcal/mol) than the Y-BR55-2 complex, with the majority of the contribution coming from loss of van der Waals interactions involving the N-acetamido group.

**Discussion**

Structures from lactoseries reactive antibodies (Table I) and crystal structures of BR96 and GS4 have defined potential sites on Lewis structures that might dictate specificity. Comparison of calculated and NMR-derived conformers of precursors of type 1 (Galβ1→3GalNAc) and type 2 (Galβ1→4GalNAc) chains show that they display very similar topographical features (Figure 1a). Spohr et al. (1985a) have shown that the binding of these structures by GS4 should occur at the termini of the molecule involving the common surface to both structures based on a large number of chemically modified structures related to Le\(^b\) and Y human blood group determinants. These interactions are to some extent retained by anti-Y antibodies but important differences are observed.

The major sugar moieties, (bGal, cFuc and dFuc) are stabilized by hydrogen bonds and hydrophobic interactions (Table III). Residue types that form hydrogen bonds with the hexose subunits are different in GS4 in binding to bGal functional groups, but a potential bifurcated hydrogen bond is observed in BR55-2 involving OH-3 and OH-4 as implicated in GS4. A hydrogen bond is observed involving OH-6 which is however absent in GS4 binding. For cFuc, backbone hydrogen bonds are observed to contribute to GS4 binding as observed in BR55-2 and BR96. For dFuc, our model for BR55-2 (Figure 5a) implicates both OH-2 and OH-4 as forming hydrogen bonds.

Based on the oligosaccharide specificity of lectins and antibodies that distinguish between the difucosylated type 1 and 2 lactoseries structures, it appears that a critical recognition feature for type 1 and 2 chains is an involvement of the 6-CH$_2$OH and/or acetamido group of the GlcNAc residue with the antibody combining site. The involvement of the β-d-N-acetylgalcosamine residue in the binding epitope for Y-specific MAbs was postulated previously (Błaszczzyk-Thurin et al., 1987). In the BR96 crystal structure and BR55-2 model (Figure 5a), this moiety forms a hydrogen bond involving the O-7 group, and provides an enhanced stability over its isosteric homolog Le\(^b\). These differences and those reflective of the nature of the structures extended at the reducing site of the fucosylated lactosamine probably translate into different affinities for Y by these antibodies. Mutation of Asp97 by Ala in BR96 indicates enhanced tumor cell binding (Jeffrey et al., 1995). This residue is native to B3 (Table III). An intermolecular interaction energy calculation of a substituted Ala at position 97 in the BR55-2 model indicates an enhanced hydrophobic interaction with the CH$_3$ group of the N-acetamido moiety (data not shown). Subsequently, the interaction of the βDGlcNAc residue with Y-specific antibodies is a driving mediator in their specificity as well as the number of hexose subunits comprising the Y structure that are recognized by the respective antibodies.

Anti-Y antibodies differ in their recognition of epitopes on the Y antigen. Based upon the crystal structure of the BR96–Y tetrasaccharide complex and the relative sequence similarities between the anti-Y antibodies examined here, it is apparent that the MAb binding groove of Y-specific MAbs is sufficiently large to bind four monosaccharide units of the Y determinant. Further binding studies to determine specificity and cross-reactivity, and also simple docking studies with other than Y conformers which are recognized by some MAbs (such as H$_2$, LeX and Le\(^b\)), will provide information on the different conformational epitopes presented to Y-specific MAbs, elucidating contact points that might participate in the formation of the carbohydrate–protein complexes. Although the specificity of either BR55-2 or BR96 MAbs was not tested against difucosylated extended Y or trifucosylated Y conformer structures, the analysis of the BR96–Y complex shows that trifucosylated structures extended at the reducing site with a fucosylated lactosamine unit are accommodated in the binding groove of some MAbs. Similarly, we conclude from our model that BR55-2 might bind to the additional trisaccharide unit, similar to the BR96 geometry. The antibody AH-6 was previously shown to bind equally well to Y hexaosylceramide, difucosylated Y octaosylceramide and trifucosylated nonaosylceramide (Nudelman et al., 1986), suggesting that the epitope is limited to the Y hexasaccharide or the extension at the reducing part of the oligosaccharide is incorporated into the binding groove without influencing antibody binding.

Structural studies on Lewis antigens have generally substantiated that conformations are determined mainly by steric repulsion brought about by changes in the glycosidic dihedral angles. Molecular dynamics calculations on Lewis antigen structure prototypes indicate the lack of spontaneous conformational transitions to other minima during the simulations, suggesting that these oligosaccharides maintain well defined conformations with relatively long lifetimes (Mukhopadhyay and Bush, 1991). These results further indicate that hard-sphere or rigid-geometry calculations, albeit in the absence of solvent, provide a good picture of the steric repulsion that modulates the conformational properties of the Lewis antigens.
Our minimizations, starting from binding mode geometries, were found to converge to such conformations. Subsequently, the binding mode conformation for BR55-2, and presumably also for BR96, is in excellent agreement with the expectations for the Y structure derived from consideration of both MD (Mukhopadhyay and Bush, 1991) and NMR (Cagas and Bush, 1990, 1992) studies.

Current procedures for predicting ligand–antibody interactions are limited, mainly owing to the conformational flexibility of ligands and antibodies and the role of solvent in mediating ligand recognition and binding. Comparison of the models in Figure 5a–d, which are reflective of different assumptions about the interaction of Y with the Y binding proteins, indicate that modeling and intermolecular energy calculations can discriminate differences in binding orientations. Intermolecular interaction calculations for the structures in Figure 5b–d were within 5 kcal/mol of each other, with the model in Figure 5a 22 kcal/mol more stable than the most stable alternative model (Figure 5d). While the general features of Y recognition is retained in the BR55-2 model (Figure 5a) in comparison with the BR96 crystal structure, the notion of induced fit upon Y binding as representative of the crystal structure of BR96 involving CDR3 of the heavy chain was not predictive within our calculations. This is an obvious limitation to predictive approaches in elucidating recognition of ligands by antibodies. In addition, the comparison of the various models indicates that BR55-2 and BR96 do not faithfully mimic Y recognition by the GS4 lectin, binding to a different topography on the Y antigen. The recognition of different functional groups on trifucosylated Y structures probably explains differences in the efficacy of anti-Y antibodies.

In summary, the sequence and structural analysis presented here indicates that BR55-2, BR96 and, to some extent, H18A and B3, share similarities in binding the putative Y tetrasaccharide core. An understanding of the three-dimensional basis for the molecular recognition of Y by these and related antibodies can be applied for future diagnosis in tumor progression and micrometastasis and also active immunotherapy. Further structural studies of Y antigen forms binding to anti-Y antibodies will provide information relevant to vaccine design strategies and improved immunotherapeutics.

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