Human Follicular Lymphoma Cells Contain Oligomannose Glycans in the Antigen-binding Site of the B-cell Receptor

Catherine M. Radcliffe, James N. Arnold, David M. Suter, Mark R. Wormald, David J. Harvey, Louise Royle, Yusuke Mimura, Yoshinobu Kimura, Robert B. Sim, Susana Inoges, Natalia Zabalegui, Ascensión López-Díaz de Cerio, Kathleen N. Potter, C. Ian Mockridge, Raymond A. Dwek, Maurizio Bendandi, Pauline M. Rudd, and Freda K. Stevenson

Expression of surface immunoglobulin appears critical for the growth and survival of B-cell lymphomas. In follicular lymphoma, we found previously that the Ig variable (V) regions in the B-cell receptor express a strikingly high incidence of N-glycosylation sequons, NX(S/T). These potential glycosylation sites are introduced by somatic mutation and are lymphoma-specific, pointing to their involvement in tumor pathogenesis. Analysis of the V region sugars from lymphoma-derived IgG/IgM reveals that they are mostly oligomannose and, remarkably, are located in the antigen-binding site, possibly precluding conventional antigen binding. The Fc region contains complex glycans, confirming that the normal glycan processing pathway is intact. Binding studies indicate that the oligomannose glycans occupying the V regions are accessible to mannose-binding lectins. These findings suggest a potential contribution to lymphoma pathogenesis involving antigen-independent interaction of surface immunoglobulin of the B-cell receptor with mannose-binding molecules of innate immunity in the germinal center.

Expression of slg() is critical for the survival of normal B-cells in the periphery, even in the absence of antigen (1), indicating that a stimulatory surface Ig-mediated signal, independent of antigen, is required (2). The majority of malignancies derived from mature human B-cells may also require such a signal, since slg-negative tumors are rare. In FL, slg retention is remarkable, since in most tumors, one Ig allele is compromised by the characteristic t(14;18) chromosomal translocation. (3, 4) In terms of pathogenesis, the translocation that up-regulates expression of the BCL-2 oncoprotein appears necessary, but not sufficient, for tumor development, since it can also occur in B-cells of healthy individuals (5). Other factors must be required for survival in the hostile germinal center (GC), where normal B-cells that fail to be selected by antigen usually die.

FL occurs in the GC of lymph nodes, where tumor cells maintain many features of normal GC B-cells. A nodular or nodular/diffuse growth pattern is characteristic, with conservation of the microenvironment of follicular dendritic cells and CD4+ T cells. In normal B-cells, the Ig V region genes undergo somatic mutation in the GC. B-cells expressing Ig sequences that can bind antigen are rescued from the default death pathway, allowing further differentiation and subsequent exit as plasma cells or memory B-cells (6, 7). Somatic mutation is also activated in FL cells, with evidence of ongoing mutational activity in tumor clones (8). Although Ig expression is retained, it has been difficult to envisage a role for multiple potential antigens in supporting the growth of neoplastic B-cells. Interestingly, we observed a striking difference in the B-cell receptor of lymphoma cells as compared with normal B-cells, which might provide an alternative stimulatory pathway. In normal B-cells, N-glycosylation is mainly confined to conserved sites in the Ig constant regions, although a few germ line encoded V regions do carry potential N-glycosylation sites. In FLIg, the number of potential sites increases dramatically during the somatic mutation process. By analyzing V(14) sequences, we previously found that 55 of 70 (79%) cases of FL contained these sequons. Sites were also present in V(1). This high incidence has been confirmed in 24 of 24 cases (10). Since glycosylation sites do not
accumulate significantly in somatically mutated normal B-cells, the positive selection of B-cells in FL that express B-cell receptor containing N-linked glycans suggested a potential role for the oligosaccharides in tumorigenesis.

Sites generated by somatic mutation are frequent and possibly mandatory in FL, but they also exist in other GC-associated lymphomas (11). The incidence of potential N-glycosylation sites in endemic Burkitt’s lymphoma (BL) is high (82%), although sporadic BL and diffuse large cell lymphoma have lower levels, 43 and 41%, respectively (9), possibly reflecting the known heterogeneity of these tumors. In contrast to GC-associated B-cell malignancies, sites are found at insignificant levels in chronic lymphocytic leukemia (CLL) and multiple myeloma (9).

The process of N-linked glycosylation is initiated in the ER by the transfer of the dolichol phosphate oligosaccharide precursor, N-acetylglucosaminel-mannose3-glucose2, to suitable asparagine residues in the glycosylation sequons of nascent proteins (12). Following the removal of glucose and mannose residues, the fully folded protein is transported to the Golgi, where enzymes further process the glycans to hybrid and complex-type. The exact processing of the glycans depends on factors such as the cell in which the glycoprotein is expressed and the three-dimensional structure of the protein around the glycosylation site (13, 14). When enzyme access is restricted, oligomannose sugars may not be fully processed.

We have now characterized the glycosylation of the Fab region of FLIgGs that results from somatic mutation and probed the accessibility of the glycans to C-type lectins. We have analyzed V region-associated sugars in tumor-specific Ig derived from six cases of FL. We focused first on IgG-expressing cases, since there are no conserved sites in the IgG Fab constant regions, analyzing heavy chain (HC), light chain (LC), and Fab fragments. Interestingly, the Fab glycans are mostly unprocessed oligomannose. FLIgM HC, which has a conserved glycosylation site in the constant region at Asn171 occupied by complex glycans, was also analyzed and showed a significant increase in oligomannose sugars when compared with normal human serum IgM (15, 16).

Studies with MBL, a C-type lectin, with both immobilized FLIgG and slg, which is part of the B-cell receptor, have revealed that the terminal mannose residues on the Fab glycans are accessible for binding. Molecular modeling based on amino acid substitution of the Fab region has been used to ascertain the location of N-linked glycosylation sites.

**EXPERIMENTAL PROCEDURES**

**Idiotypic Ig Production and Identification of Tumor-derived Gene Sequences**—Six patients with Stage IVA FL had surgical biopsy at first relapse following chemotherapy; five expressed slgG, and one expressed slgM. The control patient had IgG+ CLL, which expressed IgG without a potential V region N-glycosylation site. Ig protein was generated from tumor cells isolated from blood by producing heterohybridomas with the K6H6/B5 cell line (ATCC: CRL1823) as previously described (17–19). Resulting hybridomas were screened by enzyme-linked immunosorbent assay for the production of Ig matching the isotype of the tumor. The identities of fusions and tumor were determined by comparing Ig V_{H} CDR3 sequences. Ig was purified from culture supernatants by affinity chromatography (IgG, Protein A (Amersham Biosciences); IgM, anti-IgM antibody columns). Protein purity was determined by SDS-PAGE electrophoresis and in each case was >95%. All Ig proteins were adjusted to a final concentration of 1 mg/ml. V_{H} and V_{L} gene sequences were determined as previously described (9).

**N-linked Glycan Analysis**—15 μg of five FLIgG samples (FL2, -4, -11, -31, and -32), one FLIgM (FL3), CLL1, normal human serum NIgG, and NIgM were run on 10% SDS-polyacrylamide gels (20, 21). HC and LC protein bands migrating with an apparent molecular mass of 53–58 and 24–30 kDa, respectively, for IgG and 75–90 and 29 kDa, respectively, for IgM were excised, cut into ~1 mm², frozen for ~2 h at −20 °C, and washed with alternating 1 ml of acetonitrile and 1 ml of 20 mM NaHCO₃, pH 7 (five washes, 30 min each). N-Linked glycans were released in situ with peptide-N-glycanase F (PNGase F; Roche Applied Science) (20). The extracted glycans were labeled with the fluorophore 2-aminobenzamide (2AB; Ludger Ltd., Oxford, UK) (22) and processed through normal phase (NP) HPLC (23). Neutral, monosialylated, and disialylated fractions were also collected from weak anion exchange HPLC and processed by NP-HPLC for further analysis and confirmation of NIgG, FL2, NIgM, and FL3 peak assignments.

Exoglycosidase digestions were carried out on 2AB-labeled glycan pools of N-linked glycans (23). Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectra of unlabeled glycans were recorded as described previously (20).

**Papain Digestion of IgG and Fab/Fc Separation**—Digestion of IgG samples FL2, FL4, FL31, and NIgG (100 μg) was performed with 1 μg of papain (Sigma) in 250 μl of 0.1 mM phosphate buffer containing 2 mM EDTA, 12 mM cysteine (pH 7) for 16 h. To test the completion of the digestion, aliquots (4 μl) were treated with 50 mM iodoacetamide at 4 °C for 30 min to inactivate papain, added to nonreducing SDS-sample buffer preheated to 100 °C, and heated at 100 °C for 3 min. The samples were analyzed by 8.5% SDS-PAGE. The papain digests were dialyzed against 0.1 mM phosphate buffer (pH 8) overnight and applied to DEAE-cellulose (Whatman, Kent, UK) that was equilibrated with the same buffer and packed into microcolumns (~100–150-μl bed volume). Undigested IgG, Fab, and papain were eluted in flow-through fractions. Fc was eluted with phosphate-buffered saline, pH 7.4. The separation of Fab and Fc was confirmed by Western blotting using horseradish peroxidase-conjugated versions of goat anti-human κ chains, goat anti-human λ chains, and mouse anti-human IgG-Fc (Serotec). Glycans from Fab and Fc fractions were released from in-gel bands and analyzed.

**PNGase F Digestion in Solution of FL2 Fab**—8 μl of FL2 Fab were treated as above to inactivate the papain. 4 μl were incubated with PNGase F in solution for 36 h (24). The nonreduced sample was run on 8.5% SDS-PAGE together with an undigested aliquot.

**MBL Purification and Binding Assay**—Rabbit anti-MBL polyclonal antiserum was depleted of any anti-mannan antibodies on a mannan-agarose resin (catalog number M9917; Sigma) and then pre-equilibrated in phosphate-buffered saline,
TABLE 1
HC and LC gene segment usage and glycosylation sites

| Case | Ig   | VH  | VH Percentage homology | VL  | VL Percentage homology | No. of sites/region | Sequence |
|------|------|-----|------------------------|-----|------------------------|---------------------|----------|
| FL2  | IgG  | 3–21| 6                      | 90.2| 1e                     | 2/CDRH2            | INGSN    |
| FL4  | IgG  | 1–18| 6                      | 90.5| 1b                     | 1/CDRH3            | STPS     |
| FL11 | IgG  | 3–72| 6                      | 86.4| 1b                     | 3/CDRH3            | RNCS     |
| FL31 | IgG  | 3–30| 6                      | 90.5| 1b                     | 1/CDRH3            | RNCS     |
| FL32 | IgG  | 4–39| 5                      | 88.8| 1e                     | 2/CDRH3            | KNEITW   |
| FL3  | IgM  | 4–59| 2                      | 85.6| 2e                     | 2/CDRH2            | MNITSN   |
| CLL1 | IgG  | 3–73|                        | 93.3| A19                    | 1/FR4              | NLTIFV   |

For each assay, 1 × 10⁶ cells (L3055 or BL-2) were pelleted in 5-ml fluorescence-activated cell sorting tubes (1500 rpm, 5 min, 4 °C). Cells were resuspended in 500 μl of either HBSS (Sigma) supplemented with 5 mM CaCl₂ or 5 mM EDTA to demonstrate Ca²⁺-dependent binding. The cells were centrifuged as before and resuspended in 250 μl of appropriate buffer and incubated with 5 μg (20 μg/ml) of FITC-conjugated MBL or 5 μg of goat anti-human IgM (μ chain-specific) for 30 min on ice prior to analysis on a BD Biosciences FACSCalibur. The effect of apoptosis on MBL binding to L3055 cells was investigated by dual staining with MBL-FITC followed by Annexin V-PE (BD Biosciences) according to the manufacturer’s protocol.

L3055 and BL-2 cells were labeled with mouse anti-human Igλ FITC (BD Biosciences) and analyzed by fluorescence-activated cell sorting to determine surface immunoglobulin expression. To mediate endocytosis of slg, L3055 cells were incubated overnight at 37 °C with goat F(ab)₂ anti-IgM (μ chain-specific) (Southern Biotech) at 10 μg/ml. Loss of slg was monitored by staining with anti-human Igλ FITC, and the cells were then tested for MBL binding.

Molecular Modeling—Molecular modeling was performed as described previously (25). Briefly, sequence alignment was performed using Align (29) on the equivalent domains of IgG (Swiss-Prot: P01857) together with appropriate amino acid substitutions. Crystal structures used as the basis of the modeling were obtained from the Protein Data Bank (30). The structure of FL2 was based on the crystal structure of murine NIG9 antibody (31). The models of FL3, FL4, and FL31 were based on the crystal structure of the Fab domain of the monoclonal antibody against HIV-1 GP41 (32). N-Glycans were generated using the data base of glycosidic linkage conformations and in vacuo (33, 34) energy minimization to relieve unfavorable steric interactions.

RESULTS

V Gene Analysis—Analysis of gene segment sequences encoding V regions of FL cases (Table 1) show that a range of VH and VL genes were used, all of which were somatically mutated. In all cases, potential N-glycosylation sites NX(S/T) were identified. All contained sites in VH; FL32 (IgG⁺) and FL3 (IgM⁻) each had an additional site in VL. The position of sites varied; FL2 contained two in CDRH2, at Asn⁵¹ and Asn⁵⁵, and
Mannose Sugars in Antigen-binding Site of FL slg

Complete NP-HPLC glycan profiles were obtained for the 2AB-labeled HC glycans of all patient-derived samples and controls (Fig. 1, c and d). The system was calibrated using an external standard of hydrolyzed and 2AB-labeled glucose oligomers to create a standard curve. The retention times for the individual glycans were converted to glucose unit (GU) values using this curve. Preliminary peak assignments were made by comparing the GU values with the Oxford Glycobiology data base of experimental values, and the assignments were confirmed by exoglycosidase array digestions (23). These were carried out to remove the monosaccharides sequentially to elucidate the structures. Weak anion exchange HPLC data were used to facilitate the assignments of neutral and sialylated peaks of FL2 that coeluted (data not shown). All of the FLIgG samples contained the characteristic profile of complex glycans seen in NlgG (peaks 3, 11, 12, 20; Table 2 and supplemental Table S1), but there was unexpectedly also an oligomannose (M) series, M5–M9 (peaks 5, 15, 24, 31, and 36), with both types of glycan in varying proportions (Fig. 1, Table 2, and supplemental Table S1). The complex glycans were consistent with previous analyses of serum IgG Fc (35, 36), confirming that the normal glycan processing pathway was intact. CLL1 showed a characteristic IgG HC glycan NP-HPLC profile with 30% fucosylated (F; for assignment nomenclature, see Tables 2 and 3), agalactosylated, and monogalactosylated (G1) glycans with only 1% sialylation (S) but lacking glycans with bisecting (B) GlcNAc seen in human NlgG, marked with an asterisk in Fig. 1c. Structural representations of the biantennary (A2) glycan FA2G2Ga1S2 (1%) either absent in the other samples and controls or present in very small quantities (~1%) (Fig. 1c, Table 2, and supplemental Table S1). The production of more processed one in CDRH3 at Asn95. In FL2, both CDRH2 sites resulted from codon insertions, with a Gly residue inserted between Asn and Ser and an Asn residue inserted prior to TS. The other four patient IgG samples each had a single site in VH: FL4 at Asn95, FL11 at Asn96, FL31 at Asn95, all in CDRH3, and FL32 at Asn50 in CDRH2. FL32 also had a potential site in VL, within CDRH2 at Asn50. FL3 had two potential sites at Asn21 in framework region H1 and Asn50 in CDRH2 and a potential site in VL within framework region L4 at Asn103. The Ig derived from CLL1 as a hybridoma control had none.

N-linked Glycan Analysis—lg secreted by tumor-derived heterohybridoma cells was run on 10% SDS-polyacrylamide gels under reducing conditions. An increase in molecular mass of FL HC bands is evident when compared with controls, which is indicative of glycosylation. c, profiles of FLIgG HC and two controls. Glycans contained in the two controls, CLL1 and NlgG, are evident in the FLIgG samples, interspersed with oligomannose, M5–M9. d, profiles of FLIgM (FL3) together with NlgM. Major peaks are named or numbered (Tables 2 and 3). * peak containing bisected structure, FA2BG1, present in human IgG but not in mouse glycans. e, structural representations of M6 and FA2BG1. Open circle, mannose; filled square, GlcNAc; open diamond, galactose; open diamond with dot, fucose; M, mannose; F, α1-6 core fucose; A, antennary; B, bisected; G, galactose. Linkage is indicated by the angle linking adjacent residues. ↓, 1–2; ↓–, 1–3; →, 1–4; ↓, 1–6; dotted line, α linkage; solid line, β linkage; −, unknown linkage. The NP-HPLC system was calibrated using an external standard of hydrolyzed and 2AB-labeled glucose oligomers to provide a dextran ladder from which the retention times for the individual glycans were converted to GU. These were compared with the Glycobiology Institute Database of experimental values to obtain preliminary assignments for the glycans that were confirmed by digestions with arrays of exoglycosidases and mass spectrometry.
glycans is consistent with the more usual pattern of glycosylation that occurs in the ~10% of NgG Fab where there is an N-glycosylation site due to somatic mutation that is fully accessible (38, 39).

FL3 has a potential N-glycosylation site on the LC, but no glycans were recovered. Molecular modeling showed that the site is on an exposed loop close to the V_H/V_L interface. Glycosylation could interfere with the assembly of the IgG, leading to the secretion of nonglycosylated isoforms only. None of the other IgG^+ LCs had potential glycosylation sites.

The glycans of NgM (Table 3 and supplemental Table S2) were consistent with previous analyses (16). NgM has five glycosylation sites on the HC (Asn^171, Asn^332, Asn^395, Asn^402, and Asn^563). Oligomannose glycans occupy conserved sites at Asn^402 and Asn^563 (15, 40). In this study, ~31% of the glycans from NgM were oligomannose. This is consistent with 100% occupancy of Asn^402, which is homologous with Asn^297 of IgG and is always fully occupied, and ~50% occupancy of Asn^563. It has been shown previously (16) that the oligomannose glycans in these conserved sites are not accessible for MBL binding. Two further NgM samples had 28 and 30% oligomannose glycans (data not shown). FL3 (Fig. 1d, Table 3, and supplemental Table S2) showed an increase to ~49% oligomannose glycans, which is consistent with the occupancy of one of the two additional N-glycosylation sites in the Fab with oligomannose. The NP-HPLC profile shows that 9% of the glycans had a molecular mass higher than those of NgM, including triantennary (A3) trisialylated (S3) structures (Table 3 and supplemental Table S2, peaks 42–46). The potential site on the LC contained oligomannose glycans, ~80% M6 and ~20% M5 (data not shown). Structural representations of all of the glycans from Tables 2 and 3 are shown in supplemental Table S3.

**Jack Bean α-Mannosidase Digestions**—The glycan pool of FL31 HC (Fig. 2a, Table 2, and supplemental Table S1) is representative of three other samples (FL4, -11, and -32). Jack bean α-mannosidase digestion of FL31 and FL2 glycans (Fig. 2, b and d) confirmed the presence of oligomannose glycans, and the profile remaining after removal of the mannose glycans was very similar to that of Cll1 (Fig. 1c). There are additional glycans (~3%) in the FL2 profile (Fig. 2d) that are hybrid structures (Peaks 17 and 22 and included in peaks 5, 15, and 24; Fig. 2, b and d) which are consistent with the occupancy of one of the two additional N-glycosylation sites in the Fab with oligomannose.
Mannose Sugars in Antigen-binding Site of FL sIg

TABLE 3
Peak assignments and percentage abundance of glycans of NIgM and FLIgM

| Peak number | Assignmenta | NIgMb | FL3c |
|-------------|-------------|-------|------|
| 1           | A2          | <1    |      |
| 2           | A2B         | <1    |      |
| 3           | FA2         | <1    |      |
| 4           | A1G1        | <1    |      |
| 5           | M5          | 24    | 7    |
| 6           | FA1G1       | <1    |      |
| 7           | M4A1G1      | 2     | 2    |
| 8           | FA2BG1      | 4     |      |
| 9           | FA2         | 4     |      |
| 10          | M6          | 4     | 26   |
| 11          | A2G2        | <1    |      |
| 12          | FA1G1S1(6)  | 2     | <1   |
| 13          | FA2G2       | 4     | 2    |
| 14          | A3G2        | 4     |      |
| 15          | A2G1S1(6)   | 4     |      |
| 16          | FA3G2/Fa2BG2| 7     | <1   |
| 17          | M5A1G1      |       |      |
| 18          | M4A1G1S1(6) |       |      |
| 19          | FA2G1S1(6)  |       |      |
| 20          | M7          | 3     | 9    |
| 21          | A2G2S1(6)   | 3     | 4    |
| 22          | A2BG2S1     | 3     | 2    |
| 23          | FA2G2S1(6)  | <1    |      |
| 24          | FA2BG2S1(6) | 17    | 9    |
| 25          | FA2BG2      | 21    |      |
| 26          | M8          | 5     |      |
| 27          | A2G2Ga1S1(6)| 1     |      |
| 28          | FA2G2S1(6)  | <1    |      |
| 29          | FA2G2a1S1(6)| 1     |      |
| 30          | M9          | 2     |      |
| 31          | FA3G2S1(6)  | <1    |      |
| 32          | FA2G2S1(6)  | 4     | 9    |
| 33          | FA2BG2S1(6)| 4     | 9    |
| 34          | FA2BG2S1(3,6)| 5     |      |
| 35          | FA2BG2S1(6)| 5     |      |
| 36          | FA3G3a1S1(6)| 2     |      |
| 37          | A3G2S1(6)   | 2     |      |
| 38          | FA3G2S2(3,6)| 2     |      |
| 39          | FA3G2S2(6,6)| 2     |      |
| 40          | FA3G3a1S1(6)| 2     |      |
| 41          | FA3G2S2(3,6)| 2     |      |
| 42          | FA3G3a1S1(6)| 2     |      |
| 43          | FA3G2S2(3,6)| 2     |      |
| 44          | FA3G3a1S1(6)| 2     |      |
| 45          | FA3G3S3(3,6)| 1     |      |
| 46          | FA3G3S3(6,6)| 3     |      |
| 47          | FA3G3S3(6,6)| 1     |      |

a A, antennary (followed by number of N-acetylglucosamine residues); A2, biantennary; B, bisect; F, α 1–6-linked core fucose; G, β-galactose; Ga, α -galactose; M, mannosae; S, sialic acid; (3) or (6), α2-3- or α2-6-linked sialic acid.
b Percentage determined from NP-HPLC areas.

d (Fig. 2e) was also confirmed. Again, there are additional structures from digested hybrid glycans (Fig. 2f). Details of GU values, peak areas, and mass spectrometry of both FLIgM and NLgM are shown in Table 3 and supplemental Table S2.

Papain Digestion of IgG—In order to confirm the location of the oligomannose glycans, the Fab and Fc regions were cleaved by papain digestion. The HC of NLgG was also digested by papain. Fractions containing Fab and Fc were confirmed by Western blotting (Fig. 3, a, b, c, and d). Fab and Fc fractions of NLgG, FL2, and FL4 were separated by DEAE. Separation of the corresponding fractions of FL31 was achieved by SDS-PAGE alone (Fig. 3c). FL2 Fab, which migrated as three bands (Fig. 3d), was digested by PNGase F in solution. The three bands collapsed into two, x and y (Fig. 3e). Glycan analysis of these bands showed that the protein in the major band y was not glycosylated, and the minor band x contained only M9 caused by incomplete digestion (data not shown). Variable occupancy of the three N-linked glycosylation sites was therefore confirmed.

**FIGURE 2.** NP-HPLC profiles of FLIg with jack bean α-mannosidase digestions. FL31 (a), FL2 (c), and FL3 (e) are undigested glycan pools together with b, d, and f, which are jack bean α-mannosidase digestions. Oligomannose peaks are labeled. The removal of these peaks confirms the presence of oligomannose glycans and reveals the presence of complex glycans and profiles that correlate with normal controls. In the profiles of both FL2 and FL3, there is also evidence of hybrid structures in which mannosae residues, on the 6' arm of the trimannosyl core that were not processed by glycosylation pathway enzymes, have been digested. There are structural representations of M5 and M7–M9 (as described in the legend to Fig. 1).

**FIGURE 3.** Glycoprotein staining and Western blot analysis of Fab and Fc fractions (a–d). 8.5% SDS-polyacrylamide gels and Western blots of Fab and Fc fractions of NLgG, FL4, FL3, and FL2. Fab and Fc fractions were obtained after DEAE chromatography of NLgG, FL4, and FL2 following papain digestion of FLIgG samples. Lanes a, flow-through fractions; lanes b, eluate. Separation of FL31 (c) was achieved by gel electrophoresis alone. FL4 and FL31 contained single bands of Fab, whereas FL2 (d) contained three, labeled 1–3, e. FL2 Fab was treated with PNGase F in solution. The gel shows two bands. Following glycan analysis, x was shown to contain M9 due to incomplete deglycosylation, whereas y was completely deglycosylated.
glycans consisted of triantennary and biantennary structures, whereas FL31 appeared to contain only oligomannose glycans (Fig. 4, a). The mixture of oligomannose and complex glycans on Fab2 and -3 indicates that there may not have been complete separation of the two bands by SDS-PAGE.

FIGURE 4. NP-HPLC glycan profiles of HC, Fab, and Fc fractions. FL4 (a) and FL31 (b) show HC glycosylation together with Fc and Fab, demonstrating the presence of oligomannose on the Fab fragments only. c and d show the chromatograms of FL2 glycans with the Fc without oligomannose plus three glycosylated Fab bands. The combination of complex and oligomannose glycans in Fab2 and -3 indicates that there may not have been complete separation of the two bands by SDS-PAGE.

process complex glycans in the Fc regions. Although FL4 and FL31 appeared to contain only oligomannose glycans (Fig. 4, a and b), the three bands of FL2 Fab contained both oligomannose and complex glycans (Fig. 4d). The high proportion (~69%) of oligomannose glycans from the fully occupied Fab1 indicates that mannosidase I and GlcNAc transferase I have restricted access to two of the sites. Half of the ~31% complex glycans consisted of triantennary and biantennary structures with the addition of α-galactose plus mono- and disialylation. This compares with no triantennary structures and 5% disialylation of the Fc, confirming that the third glycosylation site is more accessible than the Fc to the glycosylation pathway enzymes. The mixture of oligomannose and complex glycans on Fab2 and -3 bands, ~59% ~71% oligomannose, respectively, indicates that there may not have been complete separation of the two bands by SDS-PAGE. The ~59% of oligomannose of Fab2 suggests that the two sites are occupied by one oligomannose and one complex/hybrid-type glycan (38/3%, respectively). About 10% of the Fab of NLgG HC contained complex structures, including glycans of molecular mass higher than those on the Fc, as has been shown previously (data not shown) (39). The two other papain-digested samples, FL4 and FL31, had ~73% (63% M6) and 88% (56% M6) oligomannose glycans, respectively, with a small percentage of complex glycans. With FL4, these could be from small quantities of Fc that were not completely retained by DEAE-cellulose chromatography. With FL31, complete separation was achieved by SDS-PAGE, indicating that ~10% of the glycans were processed to complex glycans. These data are consistent with one glycosylation site in the Fab region. Analysis of the Fc regions (Fig. 4, a–c) also revealed some differences between the samples. FL2 and FL4 have similar quantities of asialylated, galactosylated (14%), and monogalactosylated (30%) structures, whereas FL31 contains 27 and 45% respectively. A reduction in galactosylation is known to be related to age and to some diseases, in particular rheumatoid arthritis and Crohn disease (41, 42). FL2 has 32% monosialylated structures with and without core fucosylation, whereas FL4 and FL31 have 21 and 11%, respectively. This demonstrates some differences in the activities of galactosyl and sialyl transferases of the individual tumor-derived heterohybridoma cell lines.

IgG Fab has N-glycosylation sites acquired only through somatic mutation, whereas IgM Fab has a conserved N-glycosylation site at Asn^{171}, occupied by complex glycans (43), in the C_{H1} region as well as the possibility of sites acquired through somatic mutation. The glycan pool released from FLIgM Fab would therefore include glycans from both regions. The contribution from glycosylation resulting from somatic mutation in FLIgM was elucidated by comparing the percentage changes of the total glycan pool with those from NLgM.

FIGURE 5. MBL binding to three FLIgG samples, FL2, FL11, and FL31. MBL binding to FLIgG immobilized on microtiter plates was assessed. Binding to FL2 is approximately twice that of the other two samples. FL2 has two N-linked glycosylation sites in the antigen-binding region; FL11 and FL31 each have a single N-linked glycosylation site. CLL1 is a negative control. The bars show the mean binding ± 1 S.D. Mannan was used as a positive control. The mean binding less the EDTA control was 3.783 ± 0.094 S.D. (data not shown).

MBL Binding Assay—MBL bound to all three FLIgGs tested, confirming that the terminal mannosyl residues were accessible to the lectin. Mannan and CLL1 were used as positive and negative binding controls, respectively. Protein concentrations for the Igs were normalized to CLL1 to account for MBL binding to any terminal GlcNAc present in the Fc (44). The graph in Fig. 5 shows that MBL binds most strongly to FL2, which has three glycosylation sites, two of which are occupied by oligomannose glycans. The binding is approximately twice that of the other two samples, which correlates with the single mannosylated site in the Fab of FL11 and FL31.

Binding of MBL to slgM—To assess specific binding of MBL to cell surface Ig, two Burkitt’s lymphoma cell lines were used. Motifs similar to those in FL have been found in these GC-associated B-cell tumors. FL cell lines were not used, since they are not as well characterized, and there would be no negative control. Two parallel Epstein-Barr virus-negative cell lines, each derived from sporadic BL and expressing comparable levels of slgM, were analyzed (Fig. 6, a and b). In both cases, V_{H}4 gene segments were used to encode Ig V_{H}, but L3055 carries an N-linked glycosylation site in CDR2, whereas BL-2 has no motif (27). No sites were found in either of the V_{L} sequences. L3055...
Mannose Sugars in Antigen-binding Site of FL sIg

cells were able to bind to biotinylated MBL preconjugated with streptavidin-FITC, and this was ablated by removal of Ca\(^{2+}\) (Fig. 6a). In contrast, BL-2 cells showed only marginal binding of MBL, not significantly affected by removal of Ca\(^{2+}\) (Fig. 6b). This confirms that the oligomannose glycans in the conserved sites were not accessible for binding. Although viability was >90% and only viable cells were analyzed, L3055 cells tend to undergo apoptosis upon removal from HK cells (28). It was important, therefore, to assess any effects of early apoptosis on binding of MBL. Annexin V-positive and annexin V-negative L3055 cells showed similar binding to MBL, indicating no significant influence of apoptosis on binding (data not shown).

To correlate binding of MBL by L3055 cells with expression of slgM, cells were treated with goat F(ab')\(_2\) anti-human IgM (\(\mu\) chain-specific) overnight in order to reduce surface expression by endocytosis. On gated live cells, there was a reduction in the expression of slgM following endocytosis (Fig. 6c) as compared with the control (Fig. 6a). This was mirrored by a significant loss of ability to bind MBL, which became comparable with the low level of binding in the absence of Ca\(^{2+}\) (Fig. 6c). These data locate the binding of MBL to slgM and strongly indicate that the glycosylation site in the Fab is critical for binding.

**DISCUSSION**

Tumor cells in the majority (~85%) of FL cases carry N-glycosylation sites in the CDRs (9, 10), and these cells are positively selected and functional (9). We have established the location and structures of the oligosaccharides attached to the V region of FLIg and their binding to the C-type lectin MBL and discuss their possible role in B-cell proliferation. Although most of the data in this paper are derived from protein secreted by hetero-hybridomas established from lymphoma cells, the evidence from the unmanipulated BL cell line points to a comparable expression of surface IgM with characteristics similar to those of FLIg.

V region glycans of slgG and slgM from FL tumor cells have been shown to be mostly oligomannose; in contrast, the Fc regions of the same molecules contain processed complex glycans, confirming that the normal glycan processing pathway is intact. In all of the FL cases, there is a high proportion of oligomannose, indicating an inaccessibility of these glycans to mannosidase I, which trims back the \(a1-2\)-linked mannose sugars, and to GlcNAc transferase I, which puts on the first GlcNAc.
prior to the processing of complex structures. This allows the glycans to retain a composition not generally found at the cell surface. The Fab of both FL2 and FL3, which have three and two potential N-glycosylation sites, respectively, in the V regions, contained complex glycans in addition to the oligomannose, suggesting that sites are differentially glycosylated according to location. It is generally accepted that more exposed sites contain more highly processed glycans; however, amino acid sequence is also important, and the local three-dimensional protein structure is a major factor in regulating the degree of processing. For example, a study of the glycosylation of the influenza A virus hemagglutinin (45) showed that the glycan processing is site-specific and that one site that is buried in the hemagglutinin trimer contained only oligomannose glycans. The investigation of Thy-1 of rat brain and thymus (13) demonstrated that there is both tissue- and site-specific glycosylation and that the processing of oligosaccharides at one site is influenced by the glycosylation at other sites. A restriction in the processing of complex sugars was observed previously in the V regions of an artificially generated antibody expressed by a mouse hybridoma. The natural antibody had an NYT motif introduced at Asn in CDR2 of V, following somatic mutation at position 60 (Asn to Thr). This site was glycosylated with complex sugars. However, site-directed mutagenesis of Lys to Thr resulted in the introduction of a new glycosylation site at Asn, which contained oligomannose glycans although the site was on the exposed loop of CDR2 (46, 47).

Molecular modeling of the Fab of FL1gG and FL1gM has revealed that, surprisingly, one or more glycosylation sites are located within the antigen-binding region, which would be expected to restrict the access of the processing enzymes. Glycan analysis has shown that the proportion of oligomannose glycans in the HC is consistent with complete occupancy of the antigen-binding regions in most cases. Where there is more than one potential glycosylation site in the V region, these occur both in the antigen-binding region and in more exposed positions. In FL2, when all the three potential sites are glycosylated, the proportions of oligomannose and complex glycans are consistent with two of the sites being occupied by oligomannose glycans and the third site by complex glycans.

The GC is a site where antigen-stimulated B-cells are selected or die, the decision being based mainly on the strength of antigen binding (48). Since slg-negative tumors are rare, tumors located in the GC may retain a requirement for the engagement of slg. FL1g bound to MBL, demonstrating the exposure of diequatorial hydroxyl groups at C3 and C4 on the terminal mannose (49) and indicating that C-type lectin receptors (CLR) could be involved in FL. Indeed, MBL is present on the surface of immature dendritic cells (DCs) (50). Other cell surface candidates for binding the oligosaccharides include mannose receptor, a CLR expressed on macrophages, DCs, and endothelium, and also endo 180, DEC-205, and DC-SIGN (51–53). CLRs are highly expressed on immature DCs, and DC-SIGN is expressed by DCs in lymphoid tissue and lymph nodes. A DC subset has been found that localizes within B-cell follicles (54). Interactions between the BL cell line L3055, which has sugars located in the CDR, and a follicular DC line have been shown to lead to the proliferation of L3055 cells (28). The normal function of the receptors of the innate immune system is to bind to pathogens, but they can also bind endogenous and self-ligands (53), suggesting a mechanism for the interaction of the oligomannose glycans with cell surface lectins that provides a substitute for antigen clustering.

The survival of patients with FL correlates with genes expressed by nonmalignant immune GC cells that infiltrate the tumor. Environmental signals from the GC cells, which

![Molecular models of FL31 Fab domains showing V, glycosylation.](image)

*FIGURE 7. Molecular models of FL31 Fab domains showing V, glycosylation. The molecule is shown in two orientations, side on (above) and looking down on the antigen-binding site (below). Light blue, LC; dark blue, HC; yellow, cysteines; orange, oligomannose glycans.*

*MARCH 9, 2007 • VOLUME 282 • NUMBER 10 • JOURNAL OF BIOLOGICAL CHEMISTRY*
Mannose Sugars in Antigen-binding Site of FL sIg

include follicular dendritic cells and macrophages, appear to promote survival or proliferation of malignant cells (55). It cannot be ruled out that the infiltrating cells may also interact with the oligomannose glycans on the surface of FL B-cells. It is also possible that the new glycosylation sites may alter local folding and domain structure, such that new peptide epitopes may become accessible and contribute to antigen-independent survival of FL B-cells.

The role of the oligomannose glycans is likely to be important in the early stage of tumor growth and is an example of the processes by which tumor cells adapt to and exploit hostile environments. At later stages, further chromosomal transformations would be expected to allow wider dissemination of tumor cells (8). In terms of therapy, the targeting of small molecules or antibodies to block the putative CLR interaction may be effective at the early stage of disease. It is possible that the highly effective treatment of lymphoma with anti-idiotypic antibody directed at sIg could be acting in this way (17, 56, 57). The unexpected finding that oligomannose sugars are covalently bound within the antigen-binding region of FLsIgs opens up the possibility of novel therapeutic approaches to GC-associated lymphomas.

Acknowledgments—We thank Dr. Luisa Martinez-Pomares for helpful discussions and Dr. D. A. Mitchell for assistance with the biotinylation of MBL.

REFERENCES

1. Lam, K. P., Kuhn, R., and Rajewsky, K. (1997) Cell 90, 1073–1083
2. Smith, S. H., and Reth, M. (2004) Mol. Cell 14, 696–697
3. Tsujimoto, Y., Cossmann, J., Jaffe, E., and Croce, C. M. (1985) Science 228, 1440–1443
4. Yunis, J. J., Frazzera, G., Oken, M. M., McKenna, J., Theologides, A., and Arnesen, M. (1987) N. Engl. J. Med. 316, 79–84
5. Limpens, J., Stad, R., Bos, C., de Vlaam, C., de Jong, D., van Ommen, G. J., Schuuruing, E., and Kluin, P. M. (1995) Blood 85, 2528–2536
6. Liu, Y. J., and Arpin, C. (1997) Immunol. Rev. 156, 111–126
7. Manser, T. (2004) J. Immunol. 172, 3369–3375
8. Zelenetz, A. D., Chen, T. T., and Levy, R. (1992) J. Immunol. 149, 1137–1148
9. Zhu, D., McCarthy, H., Ottensmeier, C. H., Johnson, P., Harbin, T. J., and Stevenson, F. K. (2002) Blood 99, 2562–2568
10. Zabalegui, N., de Cerio, A. L., Inoges, S., Rodriguez-Calvillo, M., Perez-Calvo, J., Hernandez, M., Garcia-Foncillas, J., Martin-Algarra, S., Rocha, E., and Bendandi, M. (2003) Haematologica 88, 1438–1440
11. Rodriguez-Calvillo, M., Inoges, S., Lopez-Diaz de Cerio, A., Zabalegui, N., Villanueva, H., and Bendandi, M. (2004) Crit. Rev. Oncol. Hematol. 52, 1–7
12. Rudd, P. M., and Dwek, R. A. (2002) J. Biol. Chem. 277, 4615–4623
13. Zabalegui, N., de Cerio, A. L., Inoges, S., Rodriguez-Calvillo, M., Perez-Calvo, J., Panizo, C., Fernandez, M., Cuesta, B., Rocha, E., and Bendandi, M. (1996) Haematologica 88, 1438–1440
14. Rudd, P. M., and Dwek, R. A. (1997) Blood 85, 2528–2536
15. Bigge, J. C., Patel, T. P., Bruce, J. A., Goulping, P. N., Charles, S. M., and Parekh, R. B. (1995) Anal. Biochem. 230, 229–238
16. Wang, L. D., and Clark, M. R. (2003) Immunochemistry 41, 411–420
17. Morris, L. S., and Morrison, S. L. (1989) J. Immunol. 142, 2011–2018
18. Wang, L. D., and Clark, M. R. (2003) Immunochemistry 41, 411–420
19. Weis, W. I., Taylor, M. E., and Drickamer, K. (1998) Immunochemistry 35, 19–34
20. Downing, I., Koch, C., and Kilpatrick, D. C. (2003) Immunology 109, 360–364
21. Kuster, B., Wheeler, S. F., Hunter, A. T., Dwek, R. A., and Harvey, D. J. (1997) Anal. Biochem. 258, 80–101
22. Parekh, R., Bopp, P. M., Wing, D. R., Prime, S. B., and Dwek, R. A. (1996) Anal. Biochem. 240, 210–226
23. Kane, S. M., Chung, M. C., Kon, O. L., Thiell, S., Lee, S. H., and Lu, J. (1996) Biochemistry 35, 329–332
24. Chang, S.-C. (1993) J. Biol. Chem. 268, 360–364
25. Zhu, D., Ottensmeier, C. H., Du, M. Q., McCarthy, H., and Stevenson, F. K. (2002) J. Biol. Chem. 277, 4615–4623
26. Tandai, M., Endo, T., Sasaki, S., Masuho, Y., Kochibe, N., and Kobata, A. (1995) Nature 371–375
27. Takahashi, K., Nakayama, T., Shima, I., Arata, Y., and Satow, Y. (1995) J. Biol. Chem. 268, 368–372
28. Petrescu, A. J., Petrescu, S. M., Dwek, R. A., and Wormald, M. R. (1999) Glycobiology 9, 343–352
29. Zabalegui, N., de Cerio, A. L., Inoges, S., Rodriguez-Calvillo, M., Perez-Calvo, J., Hernandez, S., Garcia-Foncillas, J., Martin-Algarra, S., Rocha, E., and Bendandi, M. (1996) Haematologica 88, 1438–1440
Mannose Sugars in Antigen-binding Site of FL sIg

51. Cambi, A., and Figdor, C. G. (2003) *Curr. Opin. Cell Biol.* **15**, 539–546
52. Geijtenbeek, T. B., van Vliet, S. J., Engering, A., ’t Hart, B. A., and van Kooyk, Y. (2004) *Annu. Rev. Immunol.* **22**, 33–54
53. McGreal, E. P., Martinez-Pomares, L., and Gordon, S. (2004) *Mol. Immunol.* **41**, 1109–1121
54. Berney, C., Herren, S., Power, C. A., Gordon, S., Martinez-Pomares, L., and Kosco-Vilbois, M. H. (1999) *J. Exp. Med.* **190**, 851–860
55. Dave, S. S., Wright, G., Tan, B., Rosenwald, A., Gascoyne, R. D., Chan, W. C., Fisher, R. I., Braziel, R. M., Rimsza, L. M., Grogan, T. M., Miller, T. P., LeBlanc, M., Greiner, T. C., Weisenburger, D. D., Lynch, J. C., Vose, J., Armitage, J. O., Smeland, E. B., Kvaloy, S., Holte, H., Delabie, J., Connors, J. M., Lansdorp, P. M., Ouyang, Q., Lister, T. A., Davies, A. I., Norton, A. J., Muller-Hermelink, H. K., Ott, G., Campo, E., Montserrat, E., Wilson, W. H., Jaffe, E. S., Simon, R., Yang, L., Powell, J., Zhao, H., Goldschmidt, N., Chiorazzi, M., and Staudt, L. M. (2004) *N. Engl. J. Med.* **351**, 2159–2169
56. Bendandi, M., Gocke, C. D., Kobrin, C. B., Benko, F. A., Sternas, L. A., Pennington, R., Watson, T. M., Reynolds, C. W., Gause, B. L., Duffey, P. L., Jaffe, E. S., Creekmore, S. P., Longo, D. L., and Kwak, L. W. (1999) *Nat. Med.* **5**, 1171–1177
57. Timmerman, J. M., Czerwinski, D. K., Davis, T. A., Hsu, F. J., Benike, C., Hao, Z. M., Taidi, B., Rajapaksa, R., Caspar, C. B., Okada, C. Y., van Beverloo, A., Liles, T. M., Engleman, E. G., and Levy, R. (2002) *Blood* **99**, 1517–1526