Human Serum IgM Glycosylation
IDENTIFICATION OF GLYCOFORMS THAT CAN BIND TO MANNAN-BINDING LECTIN*

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The glycoprotein IgM is the major antibody produced in the primary immune response to antigens, circulating in the serum both as a pentamer and a hexamer. Pentameric IgM has a single J chain, which is absent in the hexamer. The \( \mu \) (heavy) chain of IgM has five N-linked glycosylation sites. Asn-171, Asn-332, and Asn-395 are occupied by complex glycans, whereas Asn-402 and Asn-563 are occupied by oligomannose glycans. The glycosylation of human polyclonal IgM from serum has been analyzed. IgM was found to contain 23.4% oligomannose glycans GlcNAc2Man5–9, consistent with 100% occupancy of Asn-402 and 17% occupancy of the variably occupied site at Asn-563. Mannan-binding lectin (MBL) is a member of the collectin family of proteins, which bind to oligomannose and GlcNAc-terminating structures. A commercial affinity chromatography resin containing immobilized MBL has been reported to be useful for partial purification of mouse and also human IgM. Human IgM glycoforms that bind to immobilized MBL were isolated; these accounted for only 20% of total serum IgM. Compared with total serum IgM, the MBL-binding glycoforms contained 97% more GlcNAc-terminating structures and 8% more oligomannose structures. A glycosylated model of pentameric IgM was constructed, and from this model, it became evident that IgM has two distinct faces, only one of which can bind to antigen, as the J chain projects from the non-antigen-binding face. Antigen-bound IgM does not bind to MBL, as the target glycans appear to become inaccessible once IgM has bound antigen. Antigen-bound IgM pentamers therefore do not activate complement via the lectin pathway, but MBL might have a role in the clearance of aggregated IgM.

The glycoprotein IgM is the major antibody produced in the primary immune response to foreign antigens. It exists both as part of the B cell antigen receptor on the surface of B cells and as a secreted glycoprotein. Human secreted IgM has a multimeric molecule consisting of five (pentameric) (Fig. 1) or six (hexameric) 190-kDa subunits, each of which consists of two heavy (\( \mu \)) and two light chains. Pentameric IgM has also a single 20-kDa J chain, which is absent in hexameric IgM (1).

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Electron microscopy studies have shown that a considerable conformational change occurs in the IgM pentamer when it has bound to antigen. This might allow IgM to expose its oligomannose glycans upon antigen binding, providing a potential mechanism of clearance of IgM after its initial recognition via an MBL-dependent pathway (3). IgM interacts efficiently with the complement system and constitutes a first line of defense against infection. However, hexameric IgM is 20 times more efficient at activating complement compared with pentameric IgM (1, 29, 30). The complement activation by IgM is attributed to C1q binding. The charged residues Asp-417, Glu-418, and His-420 in the Cε3 domain of IgM have been proposed as the binding site for C1q on IgM (31).

Here, we present the first comprehensive report of normal human serum IgM glycosylation and identify glycoforms of human serum IgM that do bind to MBL. Using molecular modeling, we have constructed a complete structure of glycosylated pentameric IgM and have assessed the accessibility of the identified oligomannose structures as binding targets for MBL.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Antisera**—Rabbit antiserum against recombinant MBL was adsorbed to remove anti-mannan antibodies and rheumatoid factors as described previously (32).

**Protein Concentration**—Protein samples (25 μg in any volume) were concentrated for general SDS-PAGE analysis by incubation with slow rotation at room temperature for 30 min with a 5-μl packed volume of StrataClean™ resin (Stratagene, Zuidoost, The Netherlands). The samples were then spun at 13,000 rpm for 1 min, and the supernatant was discarded. The resin was resuspended in 20 μl of SDS-PAGE sample buffer (200 mM Tris, 8 M urea, 2% SDS, and 1 mM EDTA, pH 8) and loaded onto the gel.

IgM was concentrated by chloroform/methanol precipitation adapted from the procedure described previously (33). To 100 μl of IgM sample (in phosphate-buffered saline (PBS) and 0.5 mM EDTA) was added 400 μl of methanol. This was vortexed and spun at 13,000 rpm for 30 s, and 100 μl of chloroform was added, vortexed, and spun again. To this was added 300 μl of water, followed by vortexing and spinning. There was a phase separation; the upper phase was removed and discarded; and 300 μl of methanol was added and vortexed. The precipitated IgM was pelleted by spinning at 13,000 rpm for 3 min. The supernatant was removed and discarded, and the pellet was dried in a vacuum centrifuge (Savant SpeedVac®, Thermo LifeSciences, Basingstoke, UK) for 30 min. This pellet was dissolved in 30 μl of sample buffer for SDS-PAGE analysis.

**Large-scale IgM Purification from Human Serum**—The purification method was adapted from that of Johnstone and Thorpe (34). A liter of citrated pooled normal human plasma (HDS Supplies, High Wycombe, UK) was made 20 mM CaCl₂ and left overnight at 4 °C to clot. The clot was filtered through muslin. IgM was precipitated from the serum at low salt by dialysis against 3 × 10 liters of 2 mM sodium phosphate, pH 6.0. The sample was centrifuged at 11,000 × g for 30 min at 4 °C; the supernatant was discarded; and the pellet was washed twice by resuspending in 100 ml of 2 mM sodium phosphate, pH 6.0, at 4 °C. The pellet was redissolved in 100 ml of Tris-buffered saline (10 mM Tris-HCl, 150 mM NaCl, and 0.5 mM EDTA, pH 7.3) at room temperature and centrifuged at 11,000 × g for 20 min at room temperature to remove undissolved material. A 50-ml sample was then applied to a Sepharose 4B gel filtration column (2.6 × 100 cm; Amersham Biosciences AB, Uppsala, Sweden) pre-equilibrated with Tris-buffered saline, pH 7.4, at 4 °C, and the fractions judged to contain IgM by SDS-PAGE analysis were pooled. The sample was run through 215 ml of Matrex Blue B resin (3.2 × 30 cm; Amicon Inc., Beverly, MA) pre-equilibrated with Tris-buffered saline, pH 7.4. The flow-through fractions containing IgM were collected and pooled. IgM still contained small amounts of IgG and IgA identified by SDS-PAGE analysis and direct enzyme-linked immunosorbent assay (ELISA) using alkaline phosphatase-conjugated goat anti-human IgG (γ chain-specific; A2187, Sigma, Poole, UK) and alkaline phosphatase-conjugated goat anti-human IgA (α chain-specific; Sigma). IgG was removed using a 5-ml HisTrap-protein G column (Amersham Biosciences AB), and IgA was removed by passage through an anti-IgA (α chain-specific)-Sepharose column (A2691, Sigma). The final IgM was judged by SDS-PAGE analysis to be >95% pure and was dialyzed against

**FIG. 1. Diagrammatic structure of pentameric IgM and IgG1.** The diagrammatic representation of pentameric IgM and IgG1 shows the variable and constant heavy chain (VH and CH, respectively) and light chain (VL and CL, respectively) domains. The diagram also shows the Fab and Fc domains. The locations of the asparagines to which N-linked glycans are attached are marked. The J chain is also shown on the IgM structure.
IgM Glycosylation and Structure

Dubelco's PBS (8.2 mM Na2HPO4, 1.5 mM KH2PO4, 139 mM NaCl, and 3 mM KCl, pH 7.4; Oxoid Ltd., Hampshire, UK) containing 0.5 mM EDTA.

Small-scale IgM Purification from Human Serum Using an ImmunoPure® IgM Purification Kit—The ImmunoPure® IgM purification kit was purchased from Pierce. The column was rabbit MBL-conjugated to a 4% bead agaroase support (5 ml), sold as a kit for purification of mouse IgM from ascites fluid. The column was run using human serum according to the directions of the manufacturer. The column was pre-washed with 5 ml of ImmunoPure® MBP (mannan-binding protein) column preparation buffer (Tris, sodium chloride, sodium azide, and EDTA, pH 7.4) at room temperature and then equilibrated with 20 ml of ImmunoPure® IgM binding buffer (Tris, sodium chloride, calcium chloride, and sodium azide, pH 7.4) at 4 °C. Human serum (500 μl) from citrated plasma as described above diluted 1:1 with ImmunoPure® IgM binding buffer was applied to the column at 4 °C and allowed to enter the resin completely. An additional 500 μl of ImmunoPure® IgM binding buffer was added and also allowed to enter the resin. The column was then incubated at 4 °C for 30 min and washed with 42 ml of ImmunoPure® IgM elution buffer (Tris, sodium chloride, sodium azide, and EDTA, pH 7.4).

The bound proteins were analyzed by SDS-PAGE and found to be a complex mixture containing >95% pure by SDS-PAGE analysis.

Preparation of 2,4,6-Trinitrophenyl (TNP)-conjugated Bovine Serum Albumin (BSA) and TNP-BSA-Sepharose—TNP-BSA was prepared by mixing 10 ml of 1% BSA in PBS and 0.5 ml EDTA with 2 ml of 5% (w/v) 3,3',4,4',5-pentachloroaniline (PP297, Sigma) and then mixing in a room temperature with 8 ml of ImmunoPure® IgM elution buffer (Tris, sodium chloride, sodium azide, and EDTA, pH 7.4).

Normal Phase (NP) HPLC and Weak Anion Exchange HPLC—Labeled glycosides were separated by NP-HPLC (37). Enzymes were used at the concentrations recommended by the manufacturer, and digestions were carried out using 50 mM sodium acetate buffer, pH 5.5, for 16 h at 37 °C. The following enzymes were supplied by Glyko Inc. (Upper Heyford, UK): ABS (1–2 units/ml), aldose-1fucosidase (3 milliunits/ml; EC 2.3.1.12), BTG (1 unit/ml), jack bean α-mannosidase (100 milliunits/ml; EC 3.2.1.24), Streptococcus pneumoniae β-N-acetyllactosaminidase (120 units/ml; EC 3.2.1.52), and bovine kidney fucosidase (100 units/ml; EC 3.1.1.51). Europa Bioproducts (Cambridge, UK) supplied S. penumoniae recombinant sialidase expressed in E. coli (1 unit/ml; Nanl; EC 3.2.1.18). The sialidase II was prepared in the Glycobiology Institute.

Mass Spectrometry—Non-2-AB-labeled N-linked glycosides were analyzed by MALDI-TOF mass spectrometry from 2,5-dihydroxybenzoic acid matrix.

Calculations of the Number of GlcNAc-terminating and Hybrid Glycans/IgM Pentamer—The average number of GlcNAc-terminating and hybrid structures/IgM pentamer was calculated to identify the number of potential MBL ligands present on each IgM pentamer. For example, taking values from Table III, pooled human IgM had a total of 7.5% structures that could potentially act as ligands for MBL occupying Asn-17, 38.3% for Asn-32, and Asn-395, with a probability of 0.075 at each site. There are 10 μ chains/IgM pentamer, each with the three N-linked glycosylation sites (Asn-17, Asn-32, and Asn-395) bearing complex sugars; therefore, 2.3 (i.e. 30 × 0.075) GlcNAc-terminating/hybrid glycans are present, on average, on each IgM pentamer.

2 A. Hart, R. A. Dwek, and P. M. Rudd, unpublished data.
MBL Purification—The purification procedure has been described previously (32, 42). Trace contaminants (IgG and IgM) were identified by SDS-PAGE analysis and direct ELISA using alkaline phosphatase-conjugated goat anti-human IgG (μ chain-specific) and alkaline phosphatase-conjugated goat anti-human IgM (μ chain-specific). The preparation was passed through a 1-ml HitTrap-protein G column and then 10 ml of anti-human IgM-agarose. The protein was dialyzed against 10 mM Hepes, 149 mM NaCl, and 5 mM CaCl\(_2\), pH 7.4, concentrated by binding to 500 μl of mannan-agarose (M6400, Sigma); and eluted with 10 mM Hepes, 140 mM NaCl, and 5 mM EDTA, pH 7.4. The sample was judged to be >95% pure by SDS-PAGE analysis. The concentration of MBL was calculated using a mannan capture MBL ELISA (32).

Assay for MBL Binding to IgM Preparations—The procedure for ELISA was that described by Arnold et al. (32). The wells of an ELISA plate were coated with 100 μl of 50 μg/ml TNP-BSA and blocked by the same method described for the IgM ELISA. After blocking, the wells were incubated with 100 μl of ABS- and BTG-treated anti-TNP-BSA IgM (120 ng/well) or IgM purified from the MBL-conjugated resin (120 ng/well), or IgM from the large-scale bulk-purified serum in PBS (previously shown by ELISA to saturate the TNP-BSA (data not shown)). In other wells, ABS- and BTG-treated anti-TNP-BSA IgM (120 ng/well), IgM from the large-scale bulk-purified serum IgM (120 ng/well), or IgM purified from the MBL-conjugated resin (120 ng/well) was coated directly onto the wells. Mannan (M7504, Sigma) and BSA were used for coating as positive and negative controls, respectively, at 50 μg/ml. An additional negative control of the enzymatic digestion mixture of ABS and BTG was bound directly to the well without anti-TNP-BSA IgM. MBL binding was assessed using a 1:700 dilution of rabbit anti-human polyclonal MBL antiserum (A2556, Sigma) as described by Arnold et al. (32) and developed with the substrate 1 mM 4-methylumbelliferyl phosphate (M413), Calbiochem) in 5 mM CHES and 1 mM MgCl\(_2\), pH 9.8, and the fluorescence was measured at λ\(_{em}\) = 355 nm and λ\(_{ex}\) = 460 nm after 1 h.

Molecular Modeling—Sequence alignment was performed using Align (43) on the equivalent domains of IgG and IgM (Swiss-Prot accession numbers P01857 and P01854, respectively). Molecular modeling was performed on a Silicon Graphics Fuel workstation using Insight II and Discover software (Accelrys Software Inc., San Diego, CA). Crystal structures used as the basis for modeling were obtained from the Protein Data Bank (44). The monomeric IgM molecular models for the Fc and hinge domains were based on the crystal structure of the IgE Fc domain (45), and the Fab domains were based on the crystal structure of IgG (46). The J chain was based on the J chain from the molecular model of secretory IgA (47). N-Glycan structures were generated using the data base of glycosidic linkage conformations (48, 49) and in vacuum energy minimization to relieve unfavorable steric interactions. The Asn-GlcNac linkage conformations were based on the observed range of crystallographic values (50). The details of the construction of the IgM pentamer from the monomer units will be published elsewhere.3

RESULTS

IgM Quantification from Purified IgM Preparations—The pooled serum for the IgM purifications contained 1774 μg of IgM/ml of serum. Large-scale IgM bulk-purified from 1 liter of pooled serum (Fig. 2a) gave a final yield of 78 μg of IgM/ml of serum (4% of total IgM), which was quantified by reading the absorbance at 280 nm.

The MBL column from the ImmunoPure® IgM purification kit had a binding capacity of ~180 μg of human IgM. Repeated passage of 0.5 ml of serum over the column indicated that ~200 μg of IgM could be extracted from 0.5 ml of serum (~22% of total IgM). Bound IgM that eluted with EDTA was impure, representing ~5% of the eluted proteins. When further purified using a protein G resin to remove IgG and an anti-IgM resin to select IgM (Fig. 2b), the final yield of IgM was 68 μg from 0.5 ml of serum (i.e. 8% of total IgM). Anti-TNP-BSA IgM was derived from 1 liter of pooled human serum (Fig. 2c) with a yield of 0.042 μg of anti-TNP-BSA IgM/ml of serum.

N-Linked Glycans of Pooled Human Serum IgM—Glycans were released from the μ chain of IgM prepared by large-scale purification (Fig. 2a). The released glycans were analyzed by MALDI-TOF mass spectrometry and NP-HPLC of the 2-AB-labeled material (Fig. 3). Structures of the glycans were assigned based on their glucose units, shifts in glucose units with enzyme digest arrays, and molecular masses (Table I). Sialylated glycans could be digested with ABS, but not with Nan1, indicating that all sialic acid residues are α2,6 (not α2,3)-linked to galactose. Weak anion exchange HPLC and enzyme digestions showed the presence of neutral and mono- and disialylated glycans as well as oligomannose glycans.

IgM Glycosylation and Structure

N-Linked Glycosylation of Asn-402 and Asn-563—Normal human serum IgM contains GlcNAc\(_2\)Man\(_n\) structures (Table I), with two predominant oligomannose structures, GlcNAc\(_2\)Man\(_n\) and GlcNAc\(_2\)Man\(_n\), which account for 9.5 and 4.1% of the total glycan pool, respectively. This is consistent with previous myeloma IgM glycan analysis (3, 11–12). Assuming full occupancy of all five glycosylation sites and that Asn-402 and Asn-563 contain only oligomannose glycans, then oligomannose glycans should compose 40% of the total glycan pool. In fact, oligomannose glycans composed only 23.4% of the N-linked glycan pool in this pooled normal human serum IgM. The Asn-402 site in the IgM Fc domain is homologous to the Asn-linked glycosylation site in IgG, Asn-354 in IgD, and Asn-394 in IgE, which are all fully occupied (32). Asn-563 has been shown to have a 75% occupancy in human-mouse hybridoma human IgM (51) and a 44% occupancy in murine IgM (52). Assuming 100% occupancy of Asn-402, 23.4% oligomannose glycans would account for 17% occupancy at Asn-563.

IgE and IgM possess a CH2 hinge domain, replacing the flexible hinge region in IgG, IgD, and IgA. The crystal structure of IgE (45) showed that the CH2 hinge domain adopts an asymmetrically bent conformation relative to the Fc domain. The relatively unprocessed glycans on the IgE Fc domain Asn-354 and IgD, and IgA. The crystal structure of IgE (45) showed that the CH2 hinge domain adopts an asymmetrically bent conformation relative to the Fc domain. The relatively unprocessed glycans on the IgE Fc domain Asn-354 and IgD, and IgA. The crystal structure of IgE (45) showed that the CH2 hinge domain adopts an asymmetrically bent conformation relative to the Fc domain.
domain (32). The extent of processing at the Asn-402 site in IgM is similar to that at Asn-394 in IgE. The CH2 hinge domain in IgM may also adopt this asymmetrically bent conformation and thus lead to similar processing of the glycans forming and thus lead to similar processing of the glycans terminating in mannose, occurring at Asn-171, and account for 42.5% of the glycans at that site. However, the human serum IgM studied here contained a significantly lower percentage of hybrid structures (9.9% of GlcNAc2Man4G1S1 and 2.5% of GlcNAc2Man5G1S1) (Fig. 3).

**N-Linked Glycosylation of the Pooled IgM J Chain**—The J chain of IgM has a single N-linked glycosylation site. The glycans of the J chain of human serum IgM, comparable with the results for both myelomas IgM-3 and IgM-12 (53).

In heterohybridoma human IgM (54), hybrid structures GlcNAc2ManG1, and GlcNAc2ManG2S1 (see the legend to Fig. 3 for explanation of glycan nomenclature) have one arm terminating in mannose, occur at Asn-171, and account for 15, 17, and 8% of these three structures, respectively, human-mouse hybridoma IgM3-4 has significantly fewer bisected glycans in transformed B cell line human IgM (3.7, 8.7, and 15.3% at each site, respectively); however, human-mouse hybridoma IgM3-4 identified a comparable content of FcGlcNAc2ManG2S1 structures, accounting for 44% of the glycans at that site. However, the human serum IgM studied here contained a significantly lower percentage of hybrid structures (9.9% of GlcNAc2Man4G1S1 and 2.5% of GlcNAc2Man5G1S1) (Fig. 3).

**Comparison of the Glycans in Pooled Serum IgM and Those in IgM Eluted from the MBL Resin**—The N-linked glycans were released from the purified IgM \( \mu \) chain eluted from the MBL-conjugated resin (Fig. 2b) and subjected to NP-HPLC, and...
glycan structures were assigned to the peaks. The same glycan structures were identified in both IgM preparations (Fig. 5), but there was a 97.3% increase in GlcNAc-terminating and hybrid structures (Table III, part A) in IgM eluted from the MBL resin. These structures occupied Asn-171, Asn-332, and Asn-563. The glycosylation sites Asn-171, Asn-332, and Asn-563 have been suggested to make Asn-563 a poor substrate for oligomannose glycans (47). The presence of these structures identified in IgM, indicating that secretory IgA and IgM may have similar glycan structures identified in IgM, that occupy the single N-linked glycosylation site. The glycans that occupy this site are predominantly galactose or sialic acid, indicating that the J chain is fully exposed for glycan processing (Table II). The J chain N-linked glycans of secretory IgA have been identified by Royle et al. (47) and are occupied by similar glycan structures identified in IgM, indicating that secretory IgA and IgM follow the same pathway of polymerization and J chain attachment.

Based on the proportion of oligomannose identified, we conclude that Asn-402 is fully occupied and that Asn-563 has an occupancy of only 17% in this pooled normal human serum IgM sample. The proximity of the Asn-563 site to the CH4 domain has been suggested to make Asn-563 a poor substrate for oligosaccharide transferase, the enzyme that attaches the GlcNAc2Man9Glc3 structure to Asn of the N-linked glycosylation of Normal Human Serum IgM. The N-linked glycans of the IgM J chain were analyzed by NP-HPLC before and after sialidase digestion. Percentage areas and glucose units (GU) are shown in Table II. All unlabeled peaks are not glycans.

### Table II

| Structure                   | GU | Glycan pool |
|-----------------------------|----|-------------|
| GlcNAc2A2G2                 | 7.12 | 12.5 |
| FeGlcNAc2A2G2               | 7.54 | 2.7 |
| GlcNAc2A2G2S1               | 7.95 | 44 |
| FeGlcNAc2A2G2S1             | 8.34 | 15 |
| GlcNAc2A2G2S2               | 8.78 | 17 |
| FeGlcNAc2A2G2S2             | 9.14 | 8 |

**DISCUSSION**

**N-Linked Glycosylation of Normal Human Serum IgM**—IgM contains a range of N-linked glycans (Table I) terminating predominantly in sialic acid and galactose, but also in mannose and GlcNAc. By reference to previous reports (3, 6, 12), we conclude that oligomannose glycans are confined to Asn-402 and Asn-563. The glycosylation sites Asn-171, Asn-332, and Asn-395 are likely to be occupied by complex glycans. The glycans at these sites terminated predominantly in galactose or sialic acid (95.1%), and a small population (4.9%) terminated in galactose or sialic acid, indicating that the J chain is fully exposed for glycan processing (Table II). The J chain N-linked glycans of secretory IgA have been identified by Royle et al. (47) and are occupied by similar glycan structures identified in IgM, indicating that secretory IgA and IgM follow the same pathway of polymerization and J chain attachment.

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| FeGlcNAc2A2G2S2             | 9.14 | 8 |

**MBL Binding Studies**—The binding of MBL to the purified IgM preparations was assessed using microtiter plates that had been coated with antigen-bound IgM or directly immobilized IgM (Fig. 6). Assays were carried out in triplicate or over 6 wells with additional negative EDTA controls. Only slight binding to antigen-bound IgM (ABS- and BTG-treated anti-TNP-BSA IgM) and bulk serum IgM was observed. However, directly immobilized IgM eluted from the MBL resin, and directly immobilized ABS- and BTG-treated anti-TNP-BSA IgM showed a much higher level of MBL binding. The ABS and BTG enzyme mixture alone showed no MBL binding.

**Fluorescence**

FIG. 4. NP-HPLC exoglycosidase digestion of glycan pool of pooled normal IgM J chain. The N-linked glycans of the IgM J chain were analyzed by NP-HPLC before and after sialidase digestion. Percentage areas and glucose units (GU) are shown in Table II. All unlabeled peaks are not glycans.

**Fluorescence**

FIG. 5. Comparison of the NP-HPLC glycan profiles of normal human IgM and the glycans of IgM eluted from the MBL resin. The two undigested NP-HPLC profiles are similar. There is a significant enrichment (highlighted) of N-linked glycans that terminate in sugars that could potentially act as ligands for MBL (either mannose or GlcNAc). No glycans were detected on the light chain of IgM by this method.

**Fluorescence**

FIG. 6. MBL binding to IgM preparations. Microtiter plate wells were coated with TNP-BSA (50 μg/well), blocked, and subsequently saturated with ABS- and BTG-treated anti-TNP-BSA IgM (120 ng/well). The ABS and BTG treatment cleaves all complex glycans to a terminal GlcNAc residue to maximize the number of MBL-binding glycan targets. ABS- and BTG-treated anti-TNP-BSA IgM, large-scale bulk-purified IgM, and purified IgM that eluted from the MBL resin were also immobilized directly at 120 ng/well. MBL was incubated at 20 ng/well for 1 h and then sequentially with anti-MBL antiserum and alkaline phosphatase-conjugated anti-rabbit monoclonal IgG (γ chain-specific) as described by Arnold et al. (32). The assay was developed with the sensitive 4-methylumbelliferyl phosphate substrate, and the fluorescence was measured at λ_em = 355 nm and λ_ex = 460 nm after 1 h.

**Fluorescence**

The bars show mean MBL binding ± S.D. EDTA was used in negative controls, and the EDTA readings were subtracted from the results.

GlcNAc. No glycans were detected on the light chain of IgM by NP-HPLC (data not shown).

This is the first study of the IgM J chain N-linked glycans that occupy the single N-linked glycosylation site. The glycans identified all terminated in galactose or sialic acid, indicating that the J chain is fully exposed for glycan processing (Table II). The J chain N-linked glycans of secretory IgA have been identified by Royle et al. (47) and are occupied by similar glycan structures identified in IgM, indicating that secretory IgA and IgM follow the same pathway of polymerization and J chain attachment.
FIG. 7. Molecular model of the glycosylated IgM pentamer with a glycosylated J chain. The model of pentameric IgM (see “Experimental Procedures”) shows the non-antigen-binding face (a) and a 90° rotation (b). The heavy chains are shown in blue; the light chains are shown in dark purple; and the J chain is shown in light purple. The complex glycans (FeGlcNAc2A2BG2S2 attached to Asn-171, Asn-332, and Asn-395) are shown in red. The oligomannose glycans (GlcNAcMan9, attached to Asn-402 and GlcNAcMan8, attached to Asn-563 at ~17% occupancy) are shown in green.

The antigen-binding face of pentameric IgM presents a large surface of complex glycans (Fig. 7b). IgM may bind to microorganisms via these glycan epitopes as an alternative route of antigen binding (other than through the Fab V regions). Influenza viruses initiate infection by binding the viral hemagglutinin to sialic acid on the cell surface (56, 57). IgM has 80.7% of its complex glycan pool terminating in sialic acid, which could allow IgM to bind to hemagglutinin, neutralizing the virus. Glycans with one or two terminal galactose residues account for 83.7% of the complex glycan pool and are a potential ligand for the asialoglycoprotein receptor, which binds to terminal galactose residues on serum glycoproteins. Upon binding, the asialoglycoprotein receptor conveys the ligand for intracellular degradation and is proposed to be involved in the clearance of IgA from the blood (58). Once pentameric IgM has bound antigen, these complex glycans on the antigen-binding face (Fig. 7b) appear to become inaccessible (Fig. 6). This is consistent both with the molecular model and with experimental data as discussed below. This may slow the clearance of IgM via receptors such as the asialoglycoprotein receptor and promote antigen-bound IgM to activate complement, as with the previously characterized C1q interaction (31).

MBL Binding to IgM—The glycans from normal total human serum IgM and those from IgM that eluted from an MBL-conjugated resin have been compared (Fig. 5 and Table III, parts A and B). The oligomannose structures that occupy normal serum IgM Asn-402 are similar in structure to those that occupy the homologous site in serum IgE (Asn-394) (32). MBL does not bind the oligomannose structures in serum IgE, as the additional CH2 hinge domain blocks access to these glycans (32).

This study has suggested that the occupancy of the normal human IgM Asn-563 site may be as little as 17%. Therefore, human serum IgM contains, on average, only one or two oligomannose sugars at the Asn-563 sites in each pentameric IgM molecule. The low number of potential ligands at this site does not present a binding surface to which MBL can bind with high avidity (Fig. 6). Oligomannose structures identified from pooled normal human serum IgM (23.4% of the glycan pool) were shown to be increased slightly to 25.3% in IgM eluted from the MBL resin. There were 43.7% more GlcNAc2Man8 structures occupying IgM eluted from the MBL-conjugated resin compared with normal serum IgM. GlcNAc2Man8 predominantly occupies the tail piece Asn-563 (3, 12). IgM that bound to the MBL resin had 26.4% of the Asn-563 sites occupied (two or three oligomannose structures at Asn-563 in each pentamer). We conclude that pooled normal human serum IgM does not have enough oligomannose occupying Asn-563 for MBL binding (which needs multiple glycan ligands) to give rise to high avidity binding. The occupancy of the Asn-563 site may be higher in lower vertebrates, as MBL has been shown to bind murine IgM (27). Glycerol analysis of murine myeloma IgM has shown Asn-563 to have an occupancy of 44% (four to five accessible oligomannoses/pentamer at the antigen-binding face) (31) appear to become inaccessible (Fig. 6). This is consistent both with the molecular model and with experimental data as discussed below. This may slow the clearance of IgM via receptors such as the asialoglycoprotein receptor and promote antigen-bound IgM to activate complement, as with the previously characterized C1q interaction (31).

MBL Binding to IgM—The glycans from normal total human serum IgM and those from IgM that eluted from an MBL-conjugated resin have been compared (Fig. 5 and Table III, parts A and B). The oligomannose structures that occupy normal serum IgM Asn-402 are similar in structure to those that occupy the homologous site in serum IgE (Asn-394) (32). MBL does not bind the oligomannose structures in serum IgE, as the additional CH2 hinge domain blocks access to these glycans (32).

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| Occupying Glycan | Pooled IgM | IgM eluted from MBL resin | Increase |
|------------------|------------|---------------------------|----------|
| A. Asn-171, Asn-332, and/or Asn-395 | GlcNAc2A1 | 1.0 | 3.7 | 208.3 |
| | GlcNAc2A0 | 0.5 | 1.0 | 100.0 |
| | GlcNAc2A1B | 1.6 | 4.0 | 150.0 |
| | FeGlcNAc2A2 | 0.3 | 0.3 | 0.0 |
| | GlcNAc2A3 | 0.3 | 0.3 | 0.0 |
| | FeGlcNAc2A3B | 0.3 | 0.4 | 33.3 |
| | GlcNAc2Man8S2G1 | 1.9 | 1.9 | 111.1 |
| | GlcNAc2Man8S2G1S1 | 2.4 | 3.2 | 33.3 |
| Total | 7.5 | 14.8 | 97.3 |
| B. Asn-402 and/or Asn-563 | GlcNAc2Man9 | 1.4 | 14.3 | 2.1 |
| | GlcNAc2Man9B | 23.8 | 21.0 | 11.8 |
| | GlcNAc2Man9B | 8.0 | 10.8 | 35.0 |
| | GlcNAc2Man9 | 10.3 | 14.8 | 43.7 |
| | GlcNAc2Man9B | 2.5 | 2.3 | 8.0 |
| Total | 58.6 | 63.2 | 7.8 |
IgM Glycosylation and Structure

14.8% at the complex glycan-occupied sites Asn-171, Asn-332, and Asn-395. MBL is able to interact with GnCNN-terminating structures in a similar manner to the interaction that is seen upon IgG-G0 binding. A small population of hybrid structures GlcNAc2A1Man4G1 and GlcNAc2A1Man4G1S1 was also identified; these structures were increased in IgM eluted from the MBL resin by 111.1 and 33.3%, respectively. Hybrid-type structures have been shown to occupy only Asn-171 in human-mouse heterohybridoma human IgM, accounting for 42.5% occupancy of the site (54). On average, there are four to five hybrid structures/IgM pentamer.

Pentameric IgM has 10 \( \mu \) chains. From the glycan analysis, each normal human serum IgM contains, on average, 2.3 GlcNAc-terminating/hybrid structures on each pentamer (Table III). As expected, directly immobilized bulk serum IgM showed poor MBL binding (Fig. 6). MBL is likely to require more than two ligand glycan structures to bind with a high enough avidity to be detected in the assays used or to trigger biological function. Directly bound IgM isolated from the MBL resin bound MBL much better (Fig. 6). IgM that eluted from the MBL resin had, on average, 4.4 GlcNAc-terminating/hybrid structures on each pentamer. The subset of IgM to which MBL bound comprised 20% of total serum IgM.

To establish whether these identified oligomannose and GlcNAc-terminating glycans are “presented” or “hidden” upon antigen binding by IgM, antigen-specific IgM was isolated. Anti-TNP-BSA IgM was tested both by binding it to the plate via antigen and by binding it directly (Fig. 6). The IgM preparation was digested with the exoglycosidases ABS and BTG to maximize the number of GlcNAc-terminating structures. The molecular model of pentameric IgM suggests that IgM has two distinct faces, only one of which can bind antigen. The complex glycans (including the GlcNAc-terminating structures) that cover the antigen-binding face of IgM (Fig. 7b) became inaccessible once IgM had bound antigen (Fig. 6). As expected, directly bound anti-TNP-BSA IgM bound MBL, but the antigen-bound form had very low MBL binding. This confirms, by experimentation, that, upon antigen binding, potential MBL-binding glycans become inaccessible. Antigen-bound IgM pentamers therefore do not activate complement via the lectin pathway, but MBL might have a role in the clearance of aggregated IgM.

Acknowledgments—We thank Dr. T. Butters for supplying glucosidase enzymes GlcNAc2A1Man4G1 and GlcNAc2A1Man4G1S1 was also seen upon IgG-G0 binding. A small population of hybrid structures/IgM pentamer.

REFERENCES

1. Randall, T. D., King, L. B., and Corley, R. B. (1990) Eur. J. Immunol. 20, 1971–1979
2. Wiersma, E. J., and Shulman, M. J. (1995) J. Biol. Chem. 270, 5565–5572
3. Mestecky, J., and Schrohenloher, R. E. (1974) Biochemistry 13, 131–139
4. Baker, M. D., Wu, G. E., Toone, W. M., Murialdo, H., Davis, A. C., and"