

The Glycosylation of Human Serum IgD and IgE and the Accessibility of Identified Oligomannose Structures for Interaction with Mannan-Binding Lectin¹

James N. Arnold,^{2*} Catherine M. Radcliffe,[†] Mark R. Wormald,[†] Louise Royle,[†] David J. Harvey,[†] Max Crispin,[†] Raymond A. Dwek,[†] Robert B. Sim,^{*} and Pauline M. Rudd[†]

Analysis of the glycosylation of human serum IgD and IgE indicated that oligomannose structures are present on both Igs. The relative proportion of the oligomannose glycans is consistent with the occupation of one *N*-linked site on each heavy chain. We evaluated the accessibility of the oligomannose glycans on serum IgD and IgE to mannan-binding lectin (MBL). MBL is a member of the collectin family of proteins, which binds to oligomannose sugars. It has already been established that MBL binds to other members of the Ig family, such as agalactosylated glycoforms of IgG and polymeric IgA. Despite the presence of potential ligands, MBL does not bind to immobilized IgD and IgE. Molecular modeling of glycosylated human IgD Fc suggests that the oligomannose glycans located at Asn³⁵⁴ are inaccessible because the complex glycans at Asn⁴⁴⁵ block access to the site. On IgE, the additional C_H2 hinge domain blocks access to the oligomannose glycans at Asn³⁹⁴ on one H chain by adopting an asymmetrically bent conformation. IgE contains 8.3% Man₅GlcNAc₂ glycans, which are the trimmed products of the Glc₃Man₉GlcNAc₂ oligomannose precursor. The presence of these structures suggests that the C_H2 domain flips between two bent quaternary conformations so that the oligomannose glycans on each chain become accessible for limited trimming to Man₅GlcNAc₂ during glycan biosynthesis. This is the first study of the glycosylation of human serum IgD and IgE from nonmyeloma proteins. *The Journal of Immunology*, 2004, 173: 6831–6840.

There are five classes of Ig in humans, IgG, IgM, IgA, IgD, and IgE. All are glycoproteins and therefore their populations may contain glycoforms which can be recognized by the lectin-like recognition proteins of the innate immune system, such as mannan-binding lectin (MBL),³ macrophage mannose receptor (mMR), and the surfactant proteins SP-A and SP-D. Much is already known about the glycosylation of IgG (1) and IgA (2, 3). IgG contains a conserved *N*-glycosylation site in the C_H2 domain (Fig. 1) at Asn²⁹⁷ in the Fc region. The biantennary complex glycans at this site have been shown to be variable (4). One set of glycoforms, referred to as IgG-G2, contains glycans at the Asn²⁹⁷ site terminating in a galactose residue on both arms. In IgG-G1 glycoforms, a terminal galactose residue is missing from one arm, exposing a GlcNAc residue. In IgG-G0 glycoforms, neither arm is galactosylated so that a GlcNAc residue is exposed at

the terminals of each arm. In normal human serum, ~20% of IgG glycans at this glycosylation site terminate in GlcNAc on both arms. The IgG-G0 glycoforms have been shown to bind MBL (5) and also to interact with the mMR (6). MBL has been shown to bind to polymeric forms of serum IgA (7). The interaction of MBL with human polymeric IgM is uncertain. It has been shown that mouse IgM and to a lesser extent, human serum IgM can be purified by affinity chromatography using immobilized rabbit MBL (8), but immobilized polyclonal human IgM does not bind MBL (9). The L chains of the Igs contain no conserved *N*- or *O*-linked glycosylation sites.

MBL, also known as mannan/mannose-binding protein, is a member of the collectin family (10). MBL binds in a calcium-dependent manner to sugar arrays on the surfaces of microorganisms, including bacteria, viruses, and fungi (11). MBL is an important component of the innate immune system and has a structure and function very similar to that of C1q, which is the recognition protein that initiates the classical pathway of complement activation. MBL has two important roles; opsonization (12) and activation of the lectin pathway of the complement system (reviewed in Ref. 13). MBL circulates bound to MBL-associated serine proteases (MASPs) (14), of which three have been identified to date; MASP-1, MASP-2 (15, 16), and MASP-3 (17). MASP-2 has been shown to cleave the complement proteins C4 and C2, resulting in the formation of a C3 convertase C4b2a. The biological roles of MASP-1 and MASP-3 are currently unknown. MBL circulates in the serum at an average concentration of ~1.2 μg/ml. Concentration varies widely between individuals (18) from <50 ng/ml to above 10 μg/ml. MBL binds to sugars that have hydroxyl groups orientated on the carbon-3 and carbon-4 in the equatorial plane of the pyranose ring (19). This gives MBL affinity for mannose, fucose, and GlcNAc (18), but not galactose or sialic acid that

*Medical Research Council (MRC) Immunochemistry Unit and Oxford Glycobiology Institute, [†]Department of Biochemistry, University of Oxford, Oxford, United Kingdom

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² Address correspondence and reprint requests to Dr. James N. Arnold, Department of Biochemistry, MRC Immunochemistry Unit, University of Oxford, Oxford OX1 3QU, U.K. E-mail address: james.arnold@bioch.ox.ac.uk

³ Abbreviations used in this paper: MBL, mannan-binding lectin; mMR, macrophage mannose receptor; MASP, MBL-associated serine protease; CRD, carbohydrate recognition domain; PEG, polyethylene glycol; RT, room temperature; 2AB, 2-amino-benzamide; NP-HPLC, normal phase HPLC; GU, glucose unit; ABS, *Arthobacter ureafaciens* sialidase; BTG, bovine testis β-galactosidase; GuH, β-*N*-acetyl glucosaminidase; Glc, glucose; GlcII, glucosidase II; ER, endoplasmic reticulum; GlcNAc, *N*-acetyl glucosamine; BKF, bovine kidney fucosidase; LC-ESI-MS, liquid chromatography/electrospray ionization mass spectrometry; NAN1, *Streptococcus pneumoniae* sialidase; SP, surfactant protein.

are the terminal sugars on most human glycoprotein glycans. Glycoprotein glycans contain a variety of terminal sugars and linkages with different binding affinity for MBL and other lectins (20, 21). The affinity of a single carbohydrate recognition domain (CRD) of MBL for carbohydrate is very weak (10^{-3} M) (22). There is increased avidity of binding when multiple CRDs of MBL interact with a carbohydrate array.

An earlier report on glycosylation of a myeloma IgD indicated that the Ig contains oligomannose glycans (23), that are potential binding sites for MBL. The ability of MBL to bind IgD and IgE has not previously been examined. Normal, polyclonal IgD and IgE are difficult to purify from human plasma due to their low abundance (~ 30 $\mu\text{g/ml}$ and <1 $\mu\text{g/ml}$, respectively). The biological role of IgD in blood is uncertain. In 1972, IgD was found to be membrane-bound as part of the BCR (24) on immature B cells. IgD is secreted into the serum as part of the primary Ab response upon B cell activation. The serum half-life of IgD is short at 2.8 days (25) perhaps because of an extended hinge region between the Fc and Fab which renders IgD susceptible to proteolytic degradation (26). IgD has three *N*-linked glycosylation sites (Fig. 1) in the Fc region at positions Asn³⁵⁴, Asn⁴⁴⁵, Asn⁴⁹⁶ (27). Studies of a myeloma IgD protein (23) found that Asn³⁵⁴ in the C_H2 domain was occupied by oligomannose structures. These if exposed, could provide a binding site for MBL or macrophage mannose receptor. The other sites contained sugars terminating in galactose and sialic acid (complex glycans). The oligomannose sugars of Asn³⁵⁴ have been shown to play a structural role, as mutagenesis of this site resulted in nonsecretion of the H chain (28). The hinge region of IgD contains multiple *O*-linked glycosylation sites (Fig. 1), though it has been found that complete inhibition of *O*-linked glycosylation using benzyl 2-acetamido-2-deoxy- α -D-galactopyranosidase did not affect assembly and secretion (28).

IgE is the least abundant serum Ab. IgE directed toward allergens leads to the symptoms of allergy through binding to the Fc ϵ RI found on tissue mast cells and basophils (reviewed by Refs. 29 and 30). This binding leads to the release of proinflammatory mediators such as histamine, causing the symptoms of allergy. There are seven *N*-linked glycosylation sites in the ϵ -chain at Asn¹⁴⁰, Asn¹⁶⁸, Asn²¹⁸, Asn²⁶⁵, Asn³⁷¹, Asn³⁸³, and Asn³⁹⁴ in the constant region of IgE (Fig. 1) (31). Quantitative site analysis of glycans from a myeloma IgE protein identified Asn³⁸³ as an unoccupied site and Asn³⁹⁴ as containing oligomannose structures (31).

We report here the first study of serum (nonmyeloma) IgD and IgE glycosylation. This study confirms the presence of oligomannose glycans and evaluates the accessibility of these *N*-linked glycans for MBL binding using a combination of molecular modeling and ELISA-style binding assays. It has previously been demonstrated that MBL binds to the IgG-G0 glycoforms and polymeric forms of serum IgA. Serum IgD and IgE have not previously been assessed as targets of MBL binding.

Materials and Methods

Abs and antisera

Purified human IgD from pooled normal serum was supplied by Kent Laboratories (cat. no. 12770; Bellingham, WA) and human IgE from single donor hyperimmune serum was supplied by Biotest International (A10164H; Saco, ME). IgG known to contain 34% IgG-G0 (data not shown) was obtained from the Glycobiology Institute (Oxford, U.K.). The samples were shown to be pure by SDS-PAGE and direct ELISA using goat anti-human IgD (δ -chain specific) alkaline phosphatase conjugate (A6406), goat anti-human IgE (ϵ -chain specific) alkaline phosphatase conjugate (A3525), and goat anti-human IgG (γ -chain specific) alkaline phosphatase conjugate (A3187) obtained from Sigma-Aldrich (Poole, U.K.). Rabbit anti-MBL polyclonal antiserum was raised by Charles River Laboratories (Romans, France) against the recombinant head and neck region of MBL (N. K. Lim, MRC Immunochemistry Unit; unpublished work).

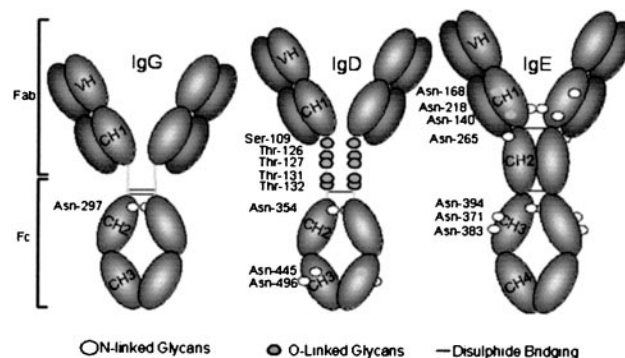


FIGURE 1. Diagrammatic structure of IgG, IgD, and IgE. Diagrammatic representation of IgG, IgD, and IgE showing their variable H chain (V_H), constant H chain (C_H) domains, and their two L chain domains. The diagram also shows the split into Fab and Fc domains. The structures of IgD and IgE are hypothetical as no crystal structures exist, although the Fc region of IgE has been crystallized (43). The asparagines to which *N*-linked glycans are attached are marked in sites predicted by molecular modeling. The *O*-linked glycosylation sites of IgD, which are found in the extended hinge region of the C_H1 and C_H2 domains are shown. It is uncertain whether both Thr¹³¹ and Thr¹³² or only one are glycosylated (27). Asn³⁸³ on IgE is a potential *N*-link glycosylation site, but was shown not to be glycosylated in a myeloma IgE (31).

The antiserum was depleted of anti-mannan Abs by passage on a mannan-agarose resin (M9917; Sigma-Aldrich) run in Dulbecco's PBS (8.2 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 139 mM NaCl, 3 mM KCl, pH 7.4) obtained from Oxoid (Hampshire, U.K.) made 0.5 mM EDTA.

MBL purification

The purification was based on the method of Tan et al. (32). One liter of citrated human plasma (HDS Supplies, High Wycombe, U.K.) was made 20 mM CaCl₂ and left overnight at 4°C to clot. The clot was filtered through muslin, and the serum made 7% w/v polyethylene glycol (PEG; m.w. 3350, P-3640; Sigma-Aldrich) and left stirring for 2 h at 4°C for a precipitate to form. The precipitate was spun down at 11,000 × *g* for 30 min at 4°C. The supernatant was discarded and the pellet was resuspended and washed in 400 ml of TBS-TCA-PEG (50 mM Tris, 140 mM NaCl, 0.05% Tween 20, 20 mM CaCl₂, 7% PEG, pH 7.8). The pellet was redissolved in 50 ml of HS-TBS-TCA (50 mM Tris, 1M NaCl, 0.05% Tween 20, 20 mM CaCl₂, pH 7.8). These dissolved proteins were then incubated for 2 h at 4°C with 20 ml of mannan-agarose resin (M-9917; Sigma-Aldrich) equilibrated with HS-TBS-TCA, rotating slowly. The resin was then washed with 500 ml of HS-TBS-TCA at 4°C and packed into a 1.5-cm diameter column. The MBL/MASPs were eluted with HS-TBS-TEDTA (50 mM Tris, 1M NaCl, 0.05% Tween 20, 10 mM EDTA, pH 7.8) and fractions collected and pooled. The MBL concentration was calculated using a standardized MBL detection ELISA (as described below). This partially purified MBL, which was used for the binding studies, contained contaminants of MASPs, IgG, and IgM and traces of other proteins. Highly pure MBL was obtained by dialyzing the preparation into gel filtration running buffer (0.1 M sodium acetate, 0.2 M NaCl, 5 mM EDTA, pH 5.0) and running on a Superose 6 gel filtration column. The fractions (1 ml), judged to contain MBL, were analyzed by SDS-PAGE and pooled. This material, which still contained some IgM contamination, was dialyzed into PBS 0.5 mM EDTA and run on a 5-ml anti-human IgM (μ -chain specific) agarose resin (A9935; Sigma-Aldrich). The resulting MBL was pure and quantified by amino acid analysis.

MBL quantification ELISA

ELISA plates (Nunc-Maxisorp; Roskilde, Denmark) were coated with 100 μl of 50 $\mu\text{g/ml}$ mannan (M-7504; Sigma-Aldrich) in 0.1 M NaHCO₃, pH 9.5. After each step the wells were washed three times at room temperature (RT) with 200 μl of wash buffer (10 mM HEPES, 5 mM CaCl₂, 1 M NaCl, 0.1% Tween 20, pH 7.4). The wells were blocked with 400 μl of PBS, 0.1% Tween 20 (PBST) for 2 h at RT and then washed and incubated for 1 h at RT with 1/100–1/10,000 (v/v) dilutions of the purified MBL in 10 mM HEPES, 1 M NaCl, 5 mM CaCl₂, pH 7.4. Negative controls were diluted in 10 mM HEPES, 1 M NaCl, 5 mM EDTA, pH 7.4, to prevent MBL binding. The ELISA was standardized using the highly purified

MBL. The wells were washed and then incubated for 1 h at RT with 100 μ l of a 1/250 (v/v) dilution of rabbit anti-MBL antiserum, washed and incubated with 100 μ l of a 1/700 (v/v) dilution of goat anti-rabbit IgG (whole molecule) Ab alkaline phosphatase conjugate (A-3812; Sigma-Aldrich). After another washing, 100 μ l of 1 mg/ml *p*-nitrophenyl phosphate substrate in 0.2 M Tris buffer (N-2770; Sigma-Aldrich) was added. The absorbances at 405 nm were recorded after 10 min.

Denaturation of Igs

A total of 20 μ g of IgE (in 100 mM Tris, 200 mM NaCl, 0.1% sodium azide, pH 7.5) or IgD (in 10 mM Tris, 200 mM NaCl, 0.05% sodium azide, pH 8) was denatured by mixing 1:1 (v/v) with 0.2 M Tris, 40 mM DTT, 8 M guanidine-HCl, pH 8.2, and incubated for 2 h at 37°C. The samples were then made 42 mM iodoacetamide and incubated at 37°C for a further 15 min. The guanidine was then removed by microdialysis for 20 h against PBS at 4°C.

Assay for MBL binding to targets

ELISA plate (Nunc-Maxisorp) wells were coated with 100 μ l of 10 μ g/ml IgD, IgE, or IgG, the last of which was used as a positive binding control. BSA (A3912; Sigma-Aldrich) was used as a negative binding control and all were diluted in 0.1 M NaHCO₃, pH 9.5. The wells were incubated at 4°C overnight, then blocked with 400 μ l of PBST for 2 h at RT, washed three times with 200 μ l of wash buffer (10 mM HEPES, 1 M NaCl, 5 mM CaCl₂, 0.1% Tween 20, pH 7.4), and incubated in triplicate for 1 h at 37°C with 50 ng/well of the purified MBL diluted in 10 mM HEPES, 1 M NaCl, 5 mM CaCl₂, pH 7.4. Other wells were incubated with MBL diluted with 10 mM HEPES, 1 M NaCl, 5 mM EDTA, pH 7.4, as controls for non-calcium-dependent binding. The wells were washed three times with the wash buffer and incubated for 1 h at RT with 100 μ l of 1/250 (v/v) anti-MBL polyclonal antiserum in wash buffer. The wells were washed and incubated with 100 μ l of a 1/2000 (v/v) dilution of monoclonal anti-rabbit IgG (γ -chain specific: clone RG-96 alkaline phosphatase conjugate; A-2556, Sigma-Aldrich) in wash buffer, washed again, and 100 μ l of an AmpliQ DakoCytomation (Cambridgeshire, U.K.) amplification kit reagent mixture was added and the OD was read at 492 nm after 30 min.

Removal of N-linked glycans for analysis

SDS-PAGE gels were prepared and run according to Küster et al. (33) and in-gel N-linked glycan release was performed as described by Radcliffe et al. (34). IgE (10 μ g in 100 mM Tris, 200 mM NaCl, 0.1% sodium azide, pH 7.5) or IgD (10 μ g in 10 mM Tris, 200 mM NaCl, 0.05% sodium azide, pH 8) was reduced with 50 mM DTT and incubated for 10 min at 70°C then alkylated with 10 mM iodoacetamide. The sample was then incubated at RT for 30 min in the dark before being loaded onto the gel, and run at 500 V, 25 mA for 1 h. The separated glycoproteins were visualized with Coomassie blue stain and the relevant bands were cut into small pieces and dried using vacuum centrifugation. Three units of peptide N-glycanase F (PNGase, EC 3.5.1.52, 1000 U/ml) diluted in 27 μ l of 20 mM NaHCO₃ was added per 10–15 mm² of gel and incubated for 16 h at 37°C. N-linked glycans were extracted from the gel pieces by collecting the supernatants of sequential gel incubations with 3 $\times$ 200 μ l of water, then 200 μ l of acetonitrile, then 200 μ l of water, and finally 200 μ l of acetonitrile in a sonicating water bath for 30 min at RT. The collected supernatants were concentrated in the vacuum centrifuge to ~500 μ l, then depleted of any ions using 50 μ l of an AG-50 X12 (H⁺ activated) ion-exchange resin, which was removed by filtering through a 0.45- μ m LH Millipore filter using a syringe. The N-linked glycans were dried for 2-aminobenzamide (2AB) labeling.

Release of O-glycans by hydrazinolysis

The O-linked glycans were released chemically with anhydrous hydrazine as described by Royle et al. (35).

2AB labeling

Released glycans were labeled by reductive amination with the fluorophore 2AB according to Bigge et al. (36), using a Ludger Tag 2AB glycan labeling kit (Ludger, Oxford, U.K.). Excess 2AB reagent was removed by ascending chromatography on Whatman 3MM paper (Clifton, NJ) in acetonitrile.

Normal phase-HPLC (NP-HPLC) and anion exchange-HPLC

Labeled glycans were separated on NP-HPLC as described by Guile et al. (37). NP-HPLC used a 4.6 $\times$ 250-mm TSK amide-80 column (Anachem, Luton, U.K.) with a linear gradient of 20–58% solvent A (50 mM formic

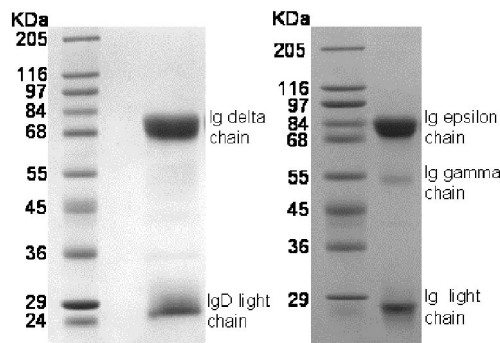


FIGURE 2. SDS-PAGE analysis of reduced and alkylated IgD and IgE. The gels were 10% acrylamide, the molecular mass marker (molecular mass 6,500–205,000) was supplied by Sigma-Aldrich (M4038). The presence of IgD was confirmed using goat anti-human IgD (δ -chain specific) alkaline phosphatase conjugate. The presence of IgE was confirmed using goat anti-human IgE (ϵ -chain specific) alkaline phosphatase conjugate. The H chains appeared as doublets. Bands were excised and analyzed separately but as the glycan profiles were identical the doublets were pooled. A trace of IgG is present in the IgE preparation, identified as IgG H chain from its molecular mass and by ELISA using a goat anti-human IgG (γ -chain specific) alkaline phosphatase conjugate supplied by Sigma-Aldrich (A3187).

acid adjusted to pH 4.4 with ammonium hydroxide) with solvent B (acetonitrile). Fluorescence was measured at 420 nm with excitation at 330 nm. Weak anion exchange HPLC was conducted as described by Zamze et al.

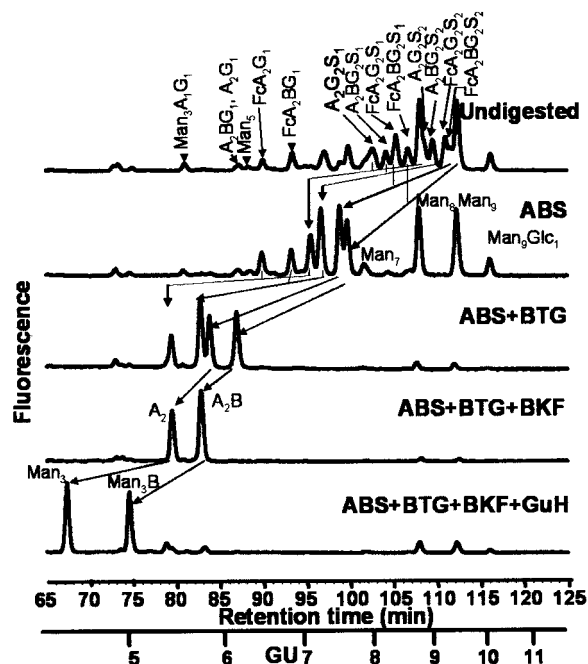


FIGURE 3. NP-HPLC exoglycosidase digestion profile of glycan pool from IgD. 2AB-labeled N-linked glycans were digested by exoglycosidases and analyzed by NP-HPLC. Structure abbreviations: all N-glycans have two core N-acetyl-glucosamines (GlcNAcs): Fc, core fucose linked α 1–6 to the inner GlcNAc; Man χ , number χ of mannose residues on core GlcNAcs; A, number of antennae on the trimannosyl core; A2, biantennary; B, bisecting GlcNAc linked β 1–4 to inner mannose; G χ , number χ of galactose residues on antennae; S χ , number χ of sialic acids on antennae. The profile shows most notably the presence of oligomannose structures. Percentage areas and GU values are shown in Table I. The exoglycosidases used were: ABS (removes sialic acid), BTG (removes galactose), BKF (removes core fucose), GuH (removes terminal GlcNAc).

(38) using a Vydac 301VHP575 7.5 × 50-mm weak anion exchange column (Hichrom, Berkshire, U.K.). Glycan profiles from NP-HPLC were calibrated against a dextran ladder prepared from hydrolyzed and 2AB-labeled glucose oligomers (37). Glycans were assigned glucose unit (GU) values and glycan structure/composition was predicted by reference to a glycan database using the program PeakTime (E. Hart, R. A. Dwek, P. M. Rudd (Glycobiology Institute), unpublished).

Exoglycosidase digestions

Exoglycosidases were used to confirm the structures of glycans present in the preparations, in conjunction with HPLC (34). Enzymes were used at manufacturers' recommended concentrations and digests were conducted using 50 mM sodium acetate buffer, pH 5.5, for 16 h at 37°C. Enzymes were supplied by Glyko (Upper Heyford, U.K.); 1–2 U/ml *Arthrobacter ureafaciens* sialidase (ABS; EC3.2.1.18); 3 mU/ml almond meal α -fucosidase (AMF; EC 3.2.1.111); 1 U/ml bovine testis β -galactosidase (BTG; EC 3.2.1.23); 100 mU/ml jack bean α -mannosidase (JBM; EC 3.2.1.24); 120 U/ml *Streptococcus pneumoniae* β -hexosaminidase (SPH; EC 3.2.1.30); 40 U/ml β -N-acetylglucosaminidase (GuH) (cloned from *S. pneumoniae*, expressed in *Escherichia coli*, EC 3.2.1.30) (Europa Bioproducts, Cambridge, U.K.); 1 U/ml *S. pneumoniae* sialidase recombinant from *Escherichia coli* (NAN1, EC 3.2.1.18); 100 U/ml bovine kidney fucosidase (BKF; EC 3.2.1.51); glucosidase II (GlcII) was prepared in the Glycobiology Institute (Oxford, U.K.).

Mass spectrometry

Non-2AB-labeled N-linked glycans were analyzed by MALDI-MS and O-linked glycans were analyzed by liquid chromatography/electrospray ionization mass spectrometry (LC-ESI-MS)/MS as described earlier (35).

Molecular modeling data

Sequence alignment was performed using Align (39) on the equivalent domains of IgG, IgD, and IgE (Swiss-Prot: P01857, P01880, and P01854, respectively). Molecular modeling was performed on a Silicon Graphics

Fuel workstation using InsightII and Discover software (Accelrys, San Diego, CA). Figures were produced using the program Molscript (40). Crystal structures used as the basis for modeling were obtained from the Protein Data Bank (41). Molecular models for the Fc domains of IgG and IgD were based on the crystal structure of IgG Fc (42) and the molecular model for IgE was based on the crystal structure of IgE hinge and Fc domains (43). N- and O-glycan structures were generated using the database of glycosidic linkage conformations (44, 45) and in vacuo energy minimization to relieve unfavorable steric interactions. The Asn-GlcNAc linkage conformations were based on the observed range of crystallographic values (46).

Glycosylated Asn side-chains can adopt three distinct conformations (46), with χ_1/χ_2 values of 60°/180°, 180°/180°, and 300°/180° ($\chi_1 = \text{N-C}^\alpha - \text{C}^\beta - \text{C}^\gamma$ and $\chi_2 = \text{C}^\alpha - \text{C}^\beta - \text{C}^\gamma - \text{N}^\delta$), each conformation being reasonably flexible and with easy interconversion between them. In IgD, Asn³⁵⁴ and Asn⁴⁹⁶ can only adopt one of these conformations, whereas two of these conformations are sterically allowed for Asn⁴⁴⁵ (180°/180° and 300°/180°). Thus, IgD was modeled with Asn⁴⁴⁵ on one H chain in the 180°/180° conformation and on the other H chain in the 300°/180° conformation. In IgE, Asn³⁸³ and Asn³⁹⁴ can only adopt one of the side-chain conformations. All three distinct side-chain conformations are sterically allowed for Asn³⁷¹, and so IgE was modeled with Asn³⁷¹ on one H chain in the 180°/180° conformation and on the other H chain in the 300°/180° conformation. For Asn²⁶⁵, none of the three possible side-chain conformations is sterically disallowed due to interactions with the C_H2 domain (the domain to which it is attached). However, for the Asn²⁶⁵ on the H chain closer to the C_H4 domains, the -60°/180° conformation leads to fewer steric interactions between the glycan outer arms and the C_H3/C_H4 domains and so this conformation was used.

Results

N-linked glycans of IgD

2AB-labeled glycans released from the δ -chain (Fig. 2) were assigned structures from GU values, shifts with the enzyme digest arrays and MALDI-MS (Fig. 3 and Table I). Sialylated glycan

Table I. Pooled normal IgD δ chain N-linked glycans

Structure ^a	GU	Molecular Mass ^b	Composition ^c				% Glycan Pool
			Hex	HexNAc	Fuc	Neu5Ac	
A ₂	ND	1339.3	3	4	0	0	0.0
Man ₃ A ₁ G ₁	5.60	1298.4	4	3	0	0	1.5
FcA ₂	5.79	1485.5	3	4	1	0	0.2
FcMan ₃ A ₁ G ₁	5.79	1444.5	4	3	1	0	0.2
Man ₅	6.29	1257.3	5	2	0	0	1.4
A ₂ G ₁	6.41	1501.5	4	4	0	0	0.5
A ₂ BG ₁	{6.59}	1704.6	4	5	0	0	{1.8}
FcA ₂ G ₁		1647.6	4	4	1	0	
FcA ₂ BG ₁	6.97	1850.7	4	5	1	0	3.7
Man ₆	7.15	1419.4	6	2	0	0	0.8
A ₂ G ₂	7.23	1663.6	5	4	0	0	0.8
A ₂ BG ₂	{7.42}	1866.7	5	5	0	0	{5.4}
A ₂ BG ₁ S ₁		DS	4	5	0	1	
FcA ₂ G ₂	7.65	1809.7	5	4	1	0	1.3
FcA ₂ BG ₁ S ₁	{7.77}	DS	4	5	1	1	{5.0}
FcA ₂ BG ₂		2012.8	5	5	1	0	
Man ₇	8.05	1581.5	7	2	0	0	1.3
A ₂ G ₂ S ₁	8.14	DS	5	4	0	1	6.1
A ₂ BG ₂ S ₁	8.35	DS	5	5	0	1	3.0
FcA ₂ G ₂ S ₁	8.52	DS	5	4	1	1	7.3
FcA ₂ BG ₂ S ₁	8.72	DS	5	5	0	1	4.7
Man ₈	8.92	1743.6	8	2	0	0	14.4
A ₂ G ₂ S ₂	8.99	DS	5	4	0	2	4.4
A ₂ BG ₂ S ₂	9.16	DS	5	5	0	2	6.5
FcA ₂ G ₂ S ₂	9.38	DS	5	4	1	2	7.6
FcA ₂ BG ₂ S ₂	9.48	DS	5	5	1	2	3.0
Man ₉	{9.61}	1905.6	9	2	0	0	13.5
Man ₈ Glc ₁		1905.6	9	2	0	0	
Man ₉ Glc ₁	10.26	2067.7	10	2	0	0	3.3

^a Structures are explained in Fig. 3.

^b Molecular mass of unlabeled glycans, detected as [M + Na]⁺ by MALDI-MS. All masses were within 0.2 mass units of calculated values. DS, desialylated mass detected. Sialylated structures during MALDI lose much sialic acid. ND, not detected by NP-HPLC.

^c Compositions deduced from mass values.

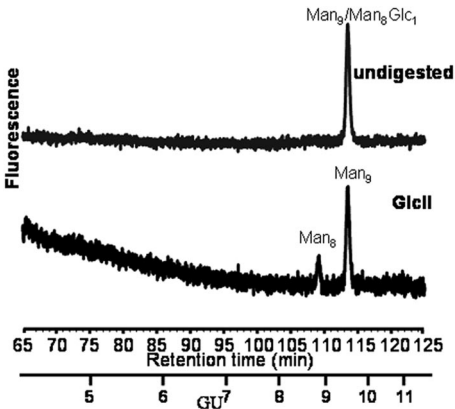


FIGURE 4. Man₈Glc₁ glycan identified under the Man₉ peak from IgD. The α-GlcII (removes glucose) digestion of the GU 9.61 peak fraction analyzed by NP-HPLC results in a Man₈ peak, showing that a Man₈Glc₁ species is present in addition to Man₉. Man₈Glc₁ makes up 15% of the GU 9.61 peak and 2.4% of the overall glycan pool

peaks digested with ABS but not with NAN1 indicating that all sialic acid residues were 2,6- (and not 2,3-) linked to galactose. Weak anion exchange HPLC and digests showed the presence of neutral, mono-, and disialylated structures. Oligomannose sugars Man₅–Man₉ (for oligomannose structures see Table IV) were identified as previously described on an IgD myeloma WAH (23). In addition, there was a Man₉Glc₁ structure (Table I) that accounted for ~3.3% of the glycan pool. A GlcII digest of Man₈ and Man₉ collected from NP-HPLC revealed the presence of a Man₈Glc₁ structure which accounted for 15% of the GU 9.61 peak (Fig. 4). The sum of the oligomannose structures comprised 37% of the glycan pool, which is compatible with the occupation of one of the three *N*-linkage glycosylation sites and in agreement with data from the work of Mellis and Baenziger (23), who showed that in a myeloma IgD, oligomannose glycans occupy the Asn³⁵⁴ site. The remaining 63% of glycan structures terminated in galactose or sialic acid. No glycans were detected on the L chain of IgD by NP-HPLC (data not shown).

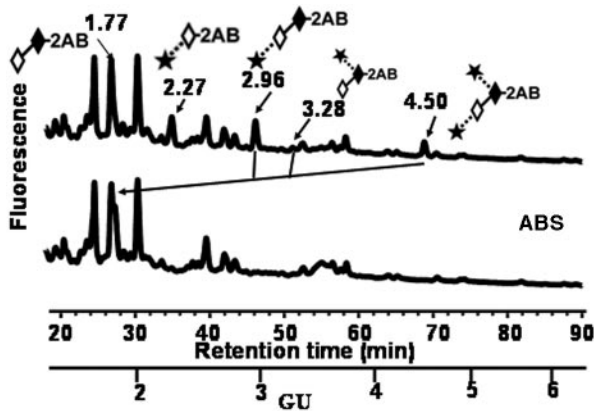


FIGURE 5. NP-HPLC analysis of *O*-linked glycans from IgD. NP-HPLC of the *O*-linked glycans of IgD before and after sialidase digestion. Numbers are GU values for the glycan peaks. All other peaks are not glycans. *O*-linked glycans identified were neutral, mono-, and disialylated Core I structures as shown. The sialylated peak at GU 2.27 is a product of peeling where Core I glycans have broken down in the release procedure and the galactose attached to a sialic acid is 2AB labeled. The structures are represented by: GlcNAc, ■; galactose, ◇; fucose, ◇ with a dot inside; sialic acid, ★; β linkage, solid line; α linkage, dotted line; 1–6 linkage, \; 1–4 linkage, —; 1–2 linkage, /.

Table II. Pooled normal IgD δ chain *O*-linked glycans

Structure ¹	GU	Molecular Mass ²	Composition ³		
			Hex	HexNAc	Neu5Ac
 2AB	1.77	504.36	1	1	0
 2AB	2.27	592.42	1	0	1
 2AB	2.96	795.55	1	1	1
 2AB	3.28	795.55	1	1	1
 2AB	4.50	1086.74	1	1	2

^a Structures are explained Fig. 5.
^b Molecular mass of 2AB-labeled glycan detected as ESI [M + H]⁺ by LC-ESI-MS. All masses were within 0.3 mass units of calculated values.
^c Composition deduced from mass values.

O-linked glycans of IgD

The *O*-linked glycans were removed chemically by hydrazinolysis. The 2AB-labeled glycans were run on NP-HPLC before and after digestion with ABS, and ABS + BTG. Peaks were distinguished from background noise and structural assignments were made based on GU values. The profile (Fig. 5) shows the presence of neutral, mono-, and disialylated Core I structures. The HPLC assignments were consistent with results from LC-ESI-MS/MS (Table II).

N-linked glycans of IgE

The structures of 2AB-labeled glycans from the ε-chain (Fig. 2) were assigned from GU values, shifts with enzyme digestion arrays, and MALDI-MS (Fig. 6 and Table III). Weak anion exchange chromatography separated the neutral, mono-, di-, and confirmed the absence of trisialylated structures. A NAN1 digestion showed no 2,3-linked sialic acid, indicating that all sialic acids were 2,6-linked to galactose as subsequently confirmed by digestion with ABS. Oligomannose structures Man₄–Man₈ comprised ~14.2% of the glycan pool, which is compatible with the occupation of one of the potential seven *N*-linkage sites. The presence of Man₄ is caused by an incomplete digestion during glycan processing of Man₅ to Man₃ by α-mannosidase II or α-mannosidase III in the golgi. Man₅ was the most abundant oligomannose structure that accounted for 8.3% of the glycan pool. Dorrington and Bennich (31) established that Asn³⁹⁴ was occupied by oligomannose structures in an IgE myeloma. The remaining 85.8% of glycans terminated in galactose or sialic acid. No glycans were detected on the L chain of IgE by NP-HPLC (data not shown).

Comparison of the N-linked glycan pools

The *N*-linked glycans of IgD and IgE contain many of the same glycan structures (Table I and III). IgD and IgE both contain oligomannose ladders (Man₅–Man₉ and Man₄–Man₈, respectively). Comparing the overall glycan profiles, 97% of the structures present in IgE are also present on IgD. IgD, however, contains a larger number of glycan structures, and only 65% of the structures found in IgD were also present in IgE.

MBL-binding studies

The binding of MBL to the Igs was assessed using ELISA plates coated with IgD, IgE, and IgD and IgE that had been denatured to expose any inaccessible oligomannose glycans present (Fig. 7). IgG was used as a positive binding control and BSA was used as a negative binding control. Assays were done in triplicate with

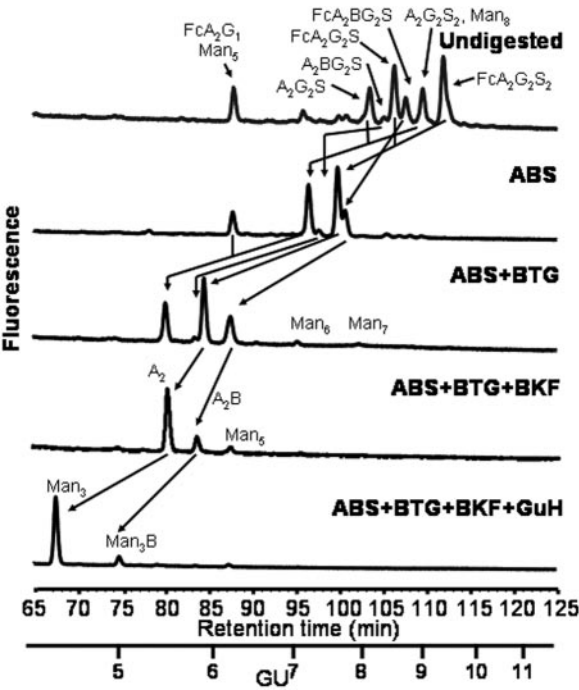


FIGURE 6. NP-HPLC exoglycosidase digestion profile of glycan pool from IgE. NP-HPLC profiles of *N*-linked IgE glycans, and results of digestion arrays of exoglycosidases (see legend of Fig. 4 for details).

additional EDTA negative controls. There was no significant binding of MBL to native IgD, however, unfolding of the IgD resulted in significant binding to MBL, indicating that the oligomannose structures became exposed upon unfolding. MBL showed low levels of binding to IgE which increased after unfolding, again indicating exposure of oligomannose structures after denaturing.

Discussion

Normal pooled serum IgD glycosylation

IgD contained a variety of *N*-linked glycans terminating in galactose and sialic acid, as well as oligomannose structures (Table I).

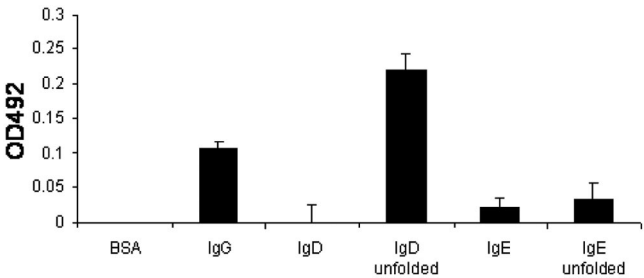


FIGURE 7. MBL binding to native and unfolded Igs. Microtiter plate wells were coated with 1 μ g/well of each Ig, and incubated with 50 ng/well MBL. Wells were then incubated with the anti-MBL antiserum and then the monoclonal anti-rabbit IgG (γ -chain specific) alkaline phosphatase conjugate, as described in *Materials and Methods*. The bars show the mean of the triplicate MBL binding assay $\pm$ 1 SD unit, read at 492 nm. EDTA and BSA negative control readings were subtracted from the results. Results are shown for both native IgD and IgE and the denatured Abs. A positive control of native IgG (determined to contain 34% IgG-G0 glycoform) was used. The absorbances were read after 30 min.

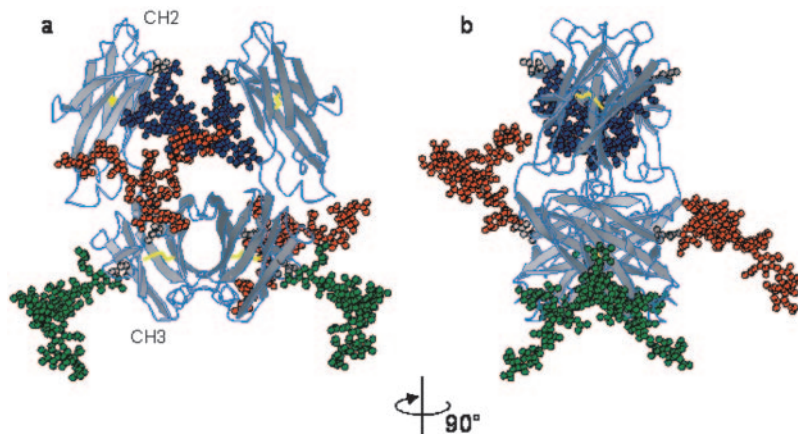
The oligomannose glycans comprised 37.1% of the *N*-linked glycan pool, consistent with occupation of one of the three *N*-linked glycosylation sites. Previous studies on a human myeloma IgD have shown that the Asn³⁵⁴ *N*-linkage site contains exclusively oligomannose glycans (Man₅-Man₉), including the glucosylated Man₉Glc₁, Man₈Glc₁, and Man₇Glc₁ (23). Serum IgD shows the same range of oligomannose glycans and glucosylated oligomannose glycans as the myeloma, with the exception of Man₇Glc₁. Man₉Glc₁ is an endoplasmic reticulum (ER) retention signal for the glycoprotein to which it is attached, to ensure proper quality control and correct folding (47). A Man₉Glc₃ structure becomes attached to the Asn of the *N*-link site cotranslationally in the ER. Man₉Glc₃ is digested to Man₉Glc₁ by ER glucosidase I and II. Mono-glucosylated glycans on secreted proteins are discussed in more detail by Crispin et al. (48).

The complex glycans on myeloma IgD previously identified by Mellis and Baenziger (23) are very similar to those that have been identified in this study of normal serum IgD. The biggest difference was in the quantity of sialylated structures. In normal serum

Table III. IgE ϵ chain *N*-linked glycans (see Table I for details)

Structure	GU	Molecular Mass	Composition				% Glycan Pool
			Hex	HexNAc	Fuc	Neu5Ac	
Man ₃ A ₁	4.99	1136.4	3	3	0	0	0.5
Man ₄	5.61	1095.3	4	2	0	0	0.7
FcMan ₃ A ₁	5.73	1282.5	3	3	1	0	0.4
Man ₃ A ₁ G ₁	5.90	1298.5	4	3	0	0	0.3
FcMan ₃ A ₁ G ₁	6.19	1444.6	4	3	1	0	1.3
Man ₅	6.19	1257.4	5	2	0	0	8.3
Man ₄ A ₁ G ₁	6.33	1460.5	5	3	0	0	0.5
A ₂ G ₁	6.67	1501.5	4	4	0	0	0.7
FcA ₂ G ₁	6.94	1647.6	4	4	1	0	1.0
Man ₆	7.07	1419.6	6	2	0	0	3.7
A ₂ G ₂	7.14	1663.6	5	4	0	0	1.2
A ₂ BG ₂	7.30	1866.7	5	5	0	0	1.1
FcA ₂ G ₂	7.57	1809.6	5	4	1	0	1.6
FcA ₂ BG ₂	7.68	2012.7	5	5	1	0	2.2
Man ₇	7.95	1581.6	7	2	0	0	0.8
A ₂ G ₂ S ₁	8.04	DS	5	4	0	1	11.3
A ₂ BG ₂ S ₁	8.27	DS	5	5	0	1	1.4
FcA ₂ G ₂ S ₁	8.44	DS	5	4	1	1	18.1
FcA ₂ BG ₂ S ₁	8.62	DS	5	5	0	1	8.2
Man ₈	8.92	1743.6	8	2	0	0	0.7
A ₂ G ₂ S ₂	8.92	DS	5	4	0	2	11.0
FcA ₂ G ₂ S ₂	9.28	DS	5	4	1	2	25.1

FIGURE 8. Molecular model of IgD Fc. A model of IgD Fc (C_H2 and C_H3 domains) based on the crystal structure of IgG Fc (see *Materials and Methods* for details) showing (a) front view and (b) side view (rotated by 90° clockwise about the y-axis relative to a). The peptide backbone and glycosylated Asn residues are shown in gray. An oligomannose glycan (Man_6) is attached to Asn³⁵⁴ on both H chains (blue). Complex biantennary glycans, with core fucose, bisecting GlcNAc and sialic acid ($FcA_2BG_2S_2$) are attached to Asn⁴⁴⁵ (red) and Asn⁴⁹⁶ (green) on both H chains. The Asn⁴⁴⁵ side chains on the two H chains have been modeled in the 180°/180° (left side of b) and the 300°/180° (right side of a) conformations. The Asn⁴⁴⁵ side chains are expected to be flexible so the glycans attached to these residues will move between the two positions.



IgD, 45% of the total glycan pool was made up of sialylated structures, both mono- (24%) and disialylated (21%). This is a higher overall sialylation level than reported for the myeloma IgD where Mellis and Baenziger (23) reported that sialylated structures were present at Asn⁴⁴⁵, and at this site only 50% of the glycans were monosialylated (of the overall glycan pool ~16% of the glycans were sialylated).

The *O*-linked glycans released from IgD are present in the hinge region (Fig. 1) (49). Neutral, mono-, and disialylated Core I structures were identified (Table II). This finding is consistent with those of Mellis and Baenziger (49) who report the same Core I glycans present on a myeloma IgD. Our study also identified the product of peeling which is a sialylated galactose from the breakdown of Core I structures from the release and extraction procedure (Fig. 5) that becomes 2AB labeled. The Core I structures attached to IgD all terminate in galactose or sialic acid and these sugars are not potential ligands for MBL.

Serum IgE glycosylation

Analysis of serum IgE *N*-linked glycans revealed the presence of oligomannose sugars Man_4 – Man_8 that composed 14.2% of the glycan pool (Table III). Of the seven potential *N*-linked glycosylation sites per H chain of IgE, only Asn³⁹⁴ contained solely oligomannose glycans in a myeloma IgE (50, 31). We report here that oligomannose glycans account for one-seventh of the glycan pool. It has been previously found by Dorrington and Bennich (31) that Asn³⁸³ in the C_H3 domain is an unoccupied site in a myeloma IgE. Modeling to identify the positioning of this site showed that Asn³⁸³ is on the surface of the quaternary structure and therefore completely accessible for glycan processing. As the oligomannose

structures identified accounted for one-seventh of the glycan pool and not one-sixth, these findings suggest that all seven *N*-linked sites are occupied in the normal serum IgE. Modeling of glycans onto this site also showed that glycosylation would not hinder or block the docking of the FcεRIα from the crystal structure of Garman et al. (51).

Both mono- and disialylated glycans have been shown to be present on a myeloma IgE (50), and were assigned to the *N*-linkage sites Asn²¹⁸ and Asn²⁶⁵ (31). Here, we report the presence of 39% of monosialylated and 36% disialylated glycans on serum IgE, indicating that other *N*-linked sites must also be occupied by sialylated glycans. No *O*-linked glycans have been described in the conserved regions on IgE.

The conserved Ig Fc glycosylation site

IgG has a single glycosylation site at Asn²⁹⁷ in the C_H2 region of the Fc domain. This glycosylation site is localized on a β -turn at the top of the Fc domain, near the hinge and the glycans are situated in the space between the two C_H2 domains (42). Complete removal of this glycan alters both the stability of the Fc domain and its ability to bind FcγRs (reviewed by Ref. 52). The sequence of the glycan at Asn²⁹⁷ has also been shown to modulate receptor binding (53).

Sequence alignment between IgG, IgD, and IgE indicates that the IgG Asn²⁹⁷ site is completely conserved in all three, corresponding to site Asn³⁵⁴ in IgD and Asn³⁹⁴ in IgE. None of the other glycosylation sites are conserved between IgD and IgE suggesting that this Asn-common glycan may have a conserved role in the folding, assembly, or function of Ig Fc domains.

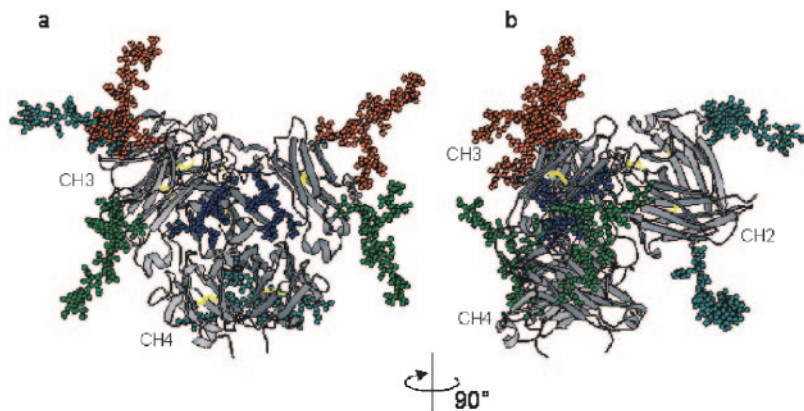


FIGURE 9. Molecular model of IgE Fc and hinge domain. A model of IgE Fc (C_H2 , C_H3 , and C_H4 domains) based on the crystal structure of IgE Fc (see *Materials and Methods* for details) showing (a) front view, with the C_H2 domains behind the C_H3 domains, and (b) side view (rotated by 90° clockwise about the y-axis relative to a), with the C_H2 domains to the right. The peptide backbone and glycosylated Asn residues are shown in gray. An oligomannose glycan (Man_6) is attached to Asn³⁹⁴ on both H chains (blue). Complex biantennary glycans, with core fucose, bisecting GlcNAc and sialic acid ($FcA_2BG_2S_2$) are attached to Asn²⁶⁵ (light blue), Asn³⁷¹ (red) and Asn³⁸³ (green) on both H chains. The Asn³⁷¹ side chains on the two H chains have been modeled in the 300°/180° (left of a) and the 180°/180° (right side of a) conformations. The Asn²⁶⁵ and Asn³⁷¹ side chains are expected to be flexible.

Glycan processing at the conserved Ig Fc glycosylation site

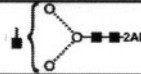
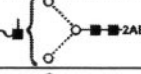
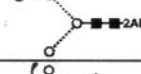
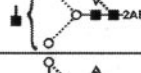
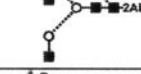
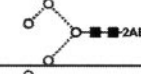
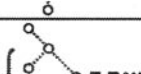
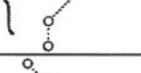
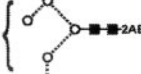
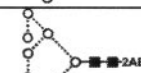
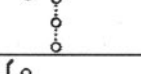
In IgG, the Asn-common glycans are complex and thus accessible to ER and golgi glycan-processing enzymes (54, 55). It has been shown that the glycans IgG-G1 and -G2 that collectively make up ~80% of normal human serum IgG glycoforms have restricted motion because the terminal galactose residues interact with the peptide surface, holding them in place. The IgG-G0 glycans are free to move as their glycans are missing the terminal galactose residues. As a result the glycans are more accessible at this stage for processing (5), this explains why most glycans get terminal galactoses attached to the 6-arm GlcNAc. IgD has the same domain structure as IgG; however the glycans found at this site are virtually unprocessed oligomannose or glucosylated oligomannose glycans, indicating low accessibility to ER and golgi glycan processing enzymes. Modeling of IgD (Fig. 8) showed that access to glycans at Asn³⁵⁴ may be blocked by the glycans attached to Asn⁴⁴⁵. This steric hindrance to access by the ER and golgi glycosidases and by glycosyltransferases to the glycans of Asn³⁵⁴, may thus prevent further processing of the Asn³⁵⁴ glycans once the protein quaternary structure has formed. Precedents within the glycosylation of CD4 have also shown the environment around a glycosylation site and the attached glycan to be important to site-specific processing (56).

IgE has a different domain structure to both IgG and IgD. The hinge peptides are replaced by Ig domains which form a rigid dimer. The IgE Asn-common glycans are also oligomannose, but more processed than in IgD indicating that the ER and Golgi glycan processing enzymes have access to at least the outer-arm residues. Modeling of IgE from the crystal structure (43) (Fig. 9) shows an asymmetrically bent quaternary structure with the C_H2 hinge domain completely blocking access to the oligomannose glycans at Asn³⁹⁴ on one H chain. There is no obvious steric blocking of access to the oligomannose glycan on the other H chain, assuming that it has sufficient mobility to move out of the central cavity in the Fc quaternary structure. The high abundance of Man₅ glycans (58.5% of the oligomannose structures identified). Table III indicates that the mannosidase access to the outer arm residues but not the core 5 mannose residues of the Asn³⁹⁴ glycans on both H chains. This suggests that the C_H2 hinge domains must be mobile, possibly flipping between two bent quaternary conformations with the C_H2 domains on either side of the Fc domain, in order to allow access to both Asn³⁹⁴ sites and so provide less steric blocking than the additional glycans on IgD.

The accessibility of the oligomannose glycans to lectin binding

The pattern of MBL binding to folded proteins (Fig. 7) mirrors almost exactly the accessibility of the glycans to processing. Although IgD and IgE have glycans to which MBL binds (Table IV), native IgD shows no interaction with MBL and native IgE only weak binding, ruling out these as potential binding targets and inducers of the activation of the lectin pathway of complement. There are previous reports of glycoproteins that contain oligomannose glycans that are inaccessible for MBL binding. Solis et al. (57) reported that the oligomannose structures present on complement component C3 and RNase B were negative for MBL binding in the native proteins, although the isolated oligomannose structures converted to neoglycolipids (20) did bind MBL. It should be noted that both IgD and IgE both contain <1% of glycans that terminate in GlcNAc. These glycans are potential targets for MBL binding and must occupy a site other than the Asn-common. Although these glycans are likely to be accessible, at the quantities they appear (~2% IgD and 1% IgE) they would have no physiological effect. Only one IgD and IgE of eight would carry such a

Table IV. IgE and IgD N-linked glycans to which MBL could bind^a

Glycan	Structure	IgD % Arca	IgE % Arca
Man ₃ A ₁		—	0.5
Man ₃ A ₁ G ₁		1.5	—
Man ₄		—	0.7
FcMan ₃ A ₁		—	0.4
FcA ₂		0.2	—
FcMan ₃ A ₁ G ₁		0.2	—
Man ₅		1.4	8.4
Man ₆		0.8	3.7
Man ₇		1.3	0.8
Man ₈		14.4	0.7
Man ₉		13.5	—
Man ₈ Glc ₁		2.4	—
Man ₉ Glc ₁		3.3	—

^a Summary of the glycans identified from the glycan profiles of IgD and IgE that could be potential binding targets for MBL, and the relative percent of the total profile. Both IgD and IgE have been shown to contain oligomannose ladders, Man₃–Man₉ in IgD and Man₄–Man₈ in IgE. Glycan structures are explained in Fig. 5.

glycan. At these concentrations the abundance would be too low throughout the Ag bound IgD and IgE for MBL to bind with high avidity.

These findings lead to a more general hypothesis that, for serum proteins, the presence of oligomannose glycans on the mature protein indicates a lack of access of the glycan-processing enzymes

which will be accompanied by a lack of recognition by MBL. Glycans that are accessible to MBL will also be accessible to the glycan processing enzymes and so be converted to complex glycans in the Golgi. This ensures that self proteins are in general not recognized by lectins such as MBL. This hypothesis holds true for the MBL binding to IgG-G0, where the glycans attached at the Asn-common site are accessible to ER glycan-processing enzymes and as a result are complex glycans. It is only at the point when the terminal galactose is attached to the 6-arm that the glycans become inaccessible as the galactose interacts with the peptide surface (5). It is when these galactoses are missing in the IgG-G0 glycoform that the glycan-protein linkage becomes flexible, and the terminal GlcNAcs are targets for MBL binding.

Acknowledgments

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