

Structural and functional characterization of glycosylation in an immunoglobulin G1 to *Cryptococcus neoformans* glucuronoxylomannan

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Abstract

Analysis of the N-linked oligosaccharides of the murine IgG1 monoclonal antibody (mAb) to *Cryptococcus neoformans* by LC/MS revealed five different core fucosylated, biantennary complex-type oligosaccharides at Asn-293, with the major species being a mono-galactosylated oligosaccharide with the glycosyl composition of Hex₄HexNAc₄Fuc (39% of the total glycan pool). The primary sequence predicted from nucleic acid sequencing differed from that measured by mass spectrometry at position 33 (ASN to ASP), a finding that may represent post-translational modification caused by spontaneous ASP deamination. Analysis of mAb 18B7 from three hybridoma clones revealed the same heterogenous N-glycan pattern, indicating that diversity in oligosaccharide structures originated from individual cells. The binding of native and de-glycosylated mAb 18B7 to cryptococcal Ag was comparable but the de-glycosylated 18B7 had shorter serum half-life and did not activate complement (C). De-glycosylated mAb 18B7 was opsonic for *C. neoformans* with murine macrophages through a mechanism that involved C-independent ingestion through the C receptor. Passive administration of de-glycosylated mAb 18B7 mediated comparable protective efficacy to the native mAb in mice with lethal infection. The results imply that the contribution of N-glycan structure to immunoglobulin function varies depending on the Ag–Ab system.

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1. Introduction

Immunoglobulin G (IgG) is the predominant Ab class in serum and its presence in convalescent sera is associated with immunity for many infectious diseases. The majority of immunoglobulins in therapeutic use are IgG. Each IgG molecule is composed of two heavy and light chains that

are linked by disulfide bridges. IgG has a single glycosylation site at CH₂ domain of Fc region. IgG glycosylation is important for the interaction of immunoglobulin with the Fc receptor (Radaev and Sun, 2001), immunoglobulin stability (Ghirlando et al., 1999), metabolism (Wright et al., 2000), half-life (Wawrzynczak et al., 1992), and complement (C) activation (Wright and Morrison, 1994, 1998). The IgG is a dimer with two glycan molecules that occupy the space between the two Fc chains and their presence results in an open conformation (Krapp et al., 2003). The interaction between the IgG Fc domain and the Fc receptor is dependent

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on IgG glycosylation, even though crystallographic analysis has shown that the carbohydrate structures are outside the interface between the immunoglobulin and its receptor. Thermodynamic studies suggest that carbohydrate chains function to stabilize the immunoglobulin Fc structure and the interaction between the Ab molecule and its receptor (Mimura et al., 2001; Radaev and Sun, 2001).

The IgG1 murine mAb 18B7 binds glucuronoxylomannan (GXM) and is protective against *Cryptococcus neoformans* (Casadevall et al., 1998). MAb 18B7 is currently in clinical testing for the treatment of human cryptococcosis (Larsen et al., 2005). Since IgG glycosylation depends on the cell line that the immunoglobulin is expressed, and differences in glycosylation can translate into differences in Ab-dependent cellular cytotoxicity (Lifely et al., 1995), we have carried out a detailed study of mAb 18B7 glycan structure and function. The results indicate a complex *N*-glycan pattern whereby five core fucosylated, biantennary complex-type oligosaccharides are attached to Asn-293. In contrast to other IgGs where glycans are essential for proper interaction of the Ig molecule with the Fc receptor, de-glycosylation of mAb 18B7 did not abolish its opsonic properties because Ab-mediated phagocytosis of *C. neoformans* can occur through the C receptor (CR). Our result show that the properties conferred by *N*-glycans to immunoglobulin function can vary depending on the biological system.

2. Materials and methods

2.1. Chemicals and reagents

Sequanal grade guanidine hydrochloride was obtained from Pierce (Rockford, IL). Analytical grade dithiothreitol, iodoacetamide, and 2-mercaptoethanol were obtained from Sigma (St. Louis, MO). Sequencing grade-modified trypsin was obtained from Promega (Madison, WI). Sequencing-grade peptide-*N*-glycosidase F (PNGaseF), Glyko *N*-glycanase, sialidase A, β -galactosidase (β -GALase, Jack Bean), and β -*N*-acetylhexosaminidase (Hexase, Jack Bean) were obtained from Prozyme (San Leandro, CA).

2.2. Yeast strains and culture conditions

C. neoformans (CN) strains 24067 and H99 were used in our experiments. The cells were grown overnight in Sabouraud's dextrose broth (Difco, Sparks, MD, USA) at 30 °C with agitation (150–180 rpm). The cells were then collected at 3000 rpm at 4 °C, washed with PBS buffer and counted with a hemacytometer. To induce capsule size, the cells were grown in Sabouraud medium at 30 °C with moderate shaking, collected in the logarithmic phase of growth, washed with PBS, and placed in Sabouraud (10%) diluted with MOPS buffer (50 mM, pH 7.3) at a cell density of 10^7 ml⁻¹ and incubated with moderate shaking at 30 °C overnight (Zaragoza et al., 2003).

2.3. Preparation of mAb 18B7

MAb 18B7 was prepared at the Massachusetts Public Health Biologic Laboratories (Jamaica Plain, MA) and is from a lot of purified bulk material generated while synthesizing material for the clinical trial. Briefly, hybridoma 18B7 cells were adapted to and grown in HyQ-CCM-1 (Hyclone Laboratories) in a hollow fiber bioreactor and mAb 18B7 protein was purified by protein A affinity chromatography, passed over a Q-sepharose column and equilibrated in PBS, pH 7.4. For studies of *N*-glycan heterogeneity within the 18B7 hybridoma cell line, clones were recovered by cloning in soft agar, the cells were expanded, supernatant was collected, concentrated and mAb 18B7 was purified by protein G affinity chromatography.

2.4. Preparation of de-*N*-glycosylated mAb 18B7

A volume of 200 μ l of 400 mU PNGase F in 20 mM Tris-HCl buffer, pH 7.5, containing 50 mM NaCl, and 50% glycerol was added to 350 μ l of 1.94 mg/ml mAb 18B7 and the resulting solution was incubated for 24 h at 37 °C. The positive control was prepared without PNGaseF and the negative control without mAb 18B7. For the circular dichroism study, de-glycosylated 18B7 was prepared using 400 mU Glyko *N*-glycanase in 20 mM Tris-HCl buffer, pH 7.5, containing 50 mM NaCl and 1 mM EDTA instead of 400 mU PNGase F. The de-glycosylated mAb 18B7 was confirmed by trypsin digestion and LC/MS/MS analysis.

2.5. Characterizing *N*-linked oligosaccharides of mAb 18B7 by LC/MS and LC/MS/MS

MAb 18B7 was reduced and alkylated using previous described method for IgM (Wang et al., 2003). The 18B7 heavy chain was separated from the light chain by HPLC. The fractions were collected and centrifuged by SpeedVac Concentrator to evaporate organic solvents. Trypsin digestion of the heavy chain and light chain were performed at 37 °C for overnight, respectively. The resulting digests were analyzed by HPLC coupled with mass spectrometry (LC/MS). A volume of 20 μ l of the digest was loaded onto a 1 mm \times 250 mm Vydac C₁₈ column. A HP 1100 HPLC equipped with a degasser and a binary pump was used to degas the solvents and generate acetonitrile gradients at the flow rate of 30 μ l/min. Solvent A was deionized water containing 0.1% trifluoroacetic acid or formic acid and solvent B was 80% (v/v) acetonitrile containing 0.1% trifluoroacetic acid or formic acid. The sample was desalted at 1%B for 30 min and separated by a 5 min 1%B–18%B gradient followed by a 0.3%B min⁻¹ gradient. After 20 min of commencing the acetonitrile gradient, the column effluent was delivered directly to a mass spectrometer. The monoisotopic masses of the tryptic peptides were measured with the QSTAR triple quadrupole time of flight mass spectrometer (Applied Biosystems, Foster City, CA), which was tuned to

provide a mass resolving power of ca. 8000 (full width half maximum, FWHM). MS/MS analysis of tryptic peptides was performed with the LCQ quadrupole ion trap mass spectrometer (Thermo, San Jose, CA), which was operated in the data dependent mode in concert with dynamic exclusion, detecting the intensity of the ions in the m/z range of 400–2000 and switching to the collision-induced dissociation (CID) mode to acquire a MS/MS spectrum when certain criteria were met. The mass isolation window for CID mode was set as 3 mass units and dynamic exclusion time for each ion was set as 1 min after 3 scans were acquired. The relative collision energy was set arbitrarily at 30%. The glycopeptides were located during HPLC run by in-source CID with the ion trap mass spectrometer as described previously (Wang et al., 2003). The mass spectrometer was set to cycle between two different scan functions. The mass spectra were recorded in the m/z range of 600–2000 in the first scan and in the second scan, the mass spectrometer detected the daughter ions produced during ion source CID at the collision energy of 25 V in the m/z range of 150–450.

2.6. Exoglycosidase digestion of glycopeptides

The heavy chain tryptic digest was treated with several exoglycosidase combinations. Exoglycosidases (3 μ l each) were added to each sample at 0 and 12 h of incubation time. The exoglycosidases used were: sialiasase which releases non-reducing terminal sialic acid residues such as *N*-glycolylneuraminic acid (NeuGc) and *N*-acetylneuraminic acid, β -GALase which cleaves β (1–4), β (1–6) and β (1–3)-linked, non-reducing terminal galactose, and HEXase which releases all non-reducing terminal β -linked *N*-acetylglucosamine and *N*-acetylgalactosamine residues. Exoglycosidase combination were: reaction 1, no enzyme; reaction 2, sialiasase A; reaction 3, sialiasase, β -GALase; 4, sialiasase, β -GALase, HEXase. The samples were incubated at 37 °C for 24 h. The exoglycosidase digests were then subjected to LC/MS/MS analysis.

2.7. Circular dichroism spectroscopy

Circular dichroism (CD) spectra were recorded using a Jasco Model J-720 spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan), at mAb 18B7 concentration of 0.4 mg/ml, using quartz cells with a 0.5 mm path length. CD spectra between 200 and 250 nm were obtained using a scanning speed of 50 nm/min, a time response of 2 s, a bandwidth of 1 nm, and an average of five scans. The measured ellipticity data were converted into mean residue ellipticity, $[\theta]$, which is defined as $[\theta] = \theta/10nCl$, where θ is the measured ellipticity in millidegrees, l the path length of the cell in centimeters, C the concentration (moles per liter) of the Ab, and n the number of its residues. The CD data were plotted as mean residue ellipticity $[\theta]$ (in degrees centimeter squared per decimole) versus wavelength, in 0.2 nm steps.

2.8. Immunofluorescence and ELISA binding studies

MAB 18B7 (10 μ g/ml) was added to a suspension of 10^6 *C. neoformans* cells and incubated at 37 °C for 30 min. The cells were then washed three times with blocking solution (1% BSA, 0.5% horse serum), incubated with 10 μ g/ml of fluorescein isothiocyanate-labeled goat anti-mouse IgG1 (Southern Biotechnology, Birmingham, AL) for 30 min at 37 °C. After three washes, cells were suspended in mounting medium (0.1 M of *n*-propyl gallate). The slides were viewed in an AX70 microscope (Olympus, Melville, NY). MAb binding to GXM was studied by ELISA. Briefly, polystyrene plates were coated with 1 μ g/ml of GXM and blocked with 1% BSA. MAb binding was detected by using alkaline phosphatase-conjugated goat anti-mouse IgG1 followed by addition of *p*-nitrophenyl phosphatase substrate.

2.9. Complement binding studies

A suspension of 2×10^6 CN cells with induced capsule was used for each sample. Fresh mouse serum samples (50 μ l) were prepared containing 100 μ g/ml of the native and deglycosylated 18B7. Negative controls consisting of cells without Ab to the capsule were carried out in parallel to observe C localization in untreated cells. When indicated, EGTA 10 mM was also included as an inhibitor of the classical C pathway. The cells were incubated for several time intervals (1.5, 5, and 15 min) at 37 °C, and C and Ab binding was stopped by diluting the serum solution with 1 ml of blocking solution (0.5% fetal bovine serum, 1% BSA in PBS) and immediately collecting the cells by centrifugation. After three washes, the cells were suspended in 100 μ l of blocking solution. To detect capsule by immunofluorescence in the samples not treated with Ab, mAb 18B7 (10 μ g/ml) was added for 30 min at 37 °C. After the cells were washed, mAb to GXM and C localized in the capsule were detected by immunofluorescence with a fluorescein-isothiocyanate (FITC) conjugated goat Ab to mouse C (5 μ g/ml, Cappel) and goat anti-mouse IgG or IgM conjugated to tetramethyl-rhodamine-isothiocyanate (TRITC) (Southern Biotechnology Associates, Inc, Birmingham, AL). To detect the cell wall by immunofluorescence, calcofluor white (50 μ g/ml) was added in this step. The cells were then incubated for 30 min at 37 °C and washed with blocking solution. The cells were finally washed again with blocking solution, and suspended in mounting medium (50% glycerol and 50 mM *N*-propyl gallate in PBS). Cells were viewed in an AX70 microscope (Olympus, Melville, NY) and pictures were taken with a QImaging Retiga 1300 digital camera using the QCapture Suite V2.46 software (QImaging, Burnaby BC, Canada), and processed with Adobe Photoshop 7.0 for windows (San Jose, CA).

2.10. Murine macrophage-like cells

Murine macrophage-like cell line J774.16 (Ralph et al., 1975) was used in the phagocytosis assays. J774.16 cells

express Fc and CR3 receptors and behave like primary murine peritoneal macrophages with regards to their phagocytic efficacy (Taborda and Casadevall, 2002). These macrophages were grown at 37 °C, in an atmosphere containing 10% CO₂ in feeding medium containing DMEM, 10% NCTC-109 medium (Gibco), 10% heat-inactivated (56 °C for 30 min) FCS (Gemini Bio-products, Woodland, CA, USA), and 1% non-essential amino acids (Mediatech Cellgro, Washington, DC, USA).

2.11. Phagocytosis assays

Phagocytosis assays were performed as described previously (Zaragoza et al., 2003). Briefly, 5×10^4 J774.16 cells were plated and incubated overnight in each well of 96-well tissue culture plates. *C. neoformans* strain 24067 was added at a ratio of 1:1, the cells were then incubated for 2 h, fixed, stained and the phagocytosis index was determined for each well. Phagocytosis via the C receptors (CR) was blocked by adding 10 µg/ml Ab to CD18, CD11b, and CD11c (Pharmin-gen, San Diego, CA) for 1 h at 4 °C. The phagocytosis index was defined as the number of macrophages with ingested CN out of 100 counted macrophages in total. Counting was carried out in triplicate.

2.12. Pharmacokinetic studies

The serum half-life of native and de-glycosylated mAb 18B7 was determined in Balb/c mice. An amount of 50 µg of immunoglobulin was injected intravenously and blood samples were obtained at various time intervals. The amount of mAb 18B7 protein in serum was calculated by measuring serum Ab binding activity for GXM relative to mAb 18B7 standards of known concentration. Immunoglobulin serum half-life was calculated using the GraphPad Prism software (GraphPad Software, San Diego, CA).

2.13. Mouse protection studies

The protective efficacy of native and de-glycosylated mAb 18B7 was evaluated in 6–8 week old female A/J mice obtained from the National Cancer Institute (Bethesda, MD). Ab (100 µg/mouse) was administered i.p. 24 h before i.v. infection with 1×10^5 *C. neoformans* cells. Mice were observed daily and the survival of the various groups was analyzed by log–rank statistic.

3. Results

3.1. Glycosylation of mAb 18B7

The tryptic peptides derived from the light chain were recovered by both monoisotopic mass measurement (the difference between the experimental mass and the theoretical mass below 50 ppm) and MS/MS except for two peptides. The experimental masses of 2929.2 and 2557.8 Da did not match to any tryptic peptides derived from the deduced amino acid sequence of the light chain of mAb 18B7. MS/MS spectra of these two ions were identified as tryptic peptides T1 and T2 with the substitution of one amino acid residue (data not shown). The proteomic analysis revealed two discrepancies from the predicted DNA sequence that presumably reflect sequencing errors. Ala7 from the nucleic acid sequence should be threonine and the Asn33 should be aspartic acid. The calculated monoisotopic masses of these two tryptic peptides after correction were matched to the experimental masses with the error below 0.12 Da, as listed in Table 1. The protein-derived light chain V region sequence is deposited in GenBank under accession number AJ309277. MAb 18B7 has a kappa light chain and the light chain constant region sequence for kappa light chain is used. Ninety two percent of the amino acid sequence of the heavy chain were recovered by both monoisotopic mass measurement with the error within 50 ppm and MS/MS. The protein-derived heavy chain V region sequence is deposited in GenBank under accession number AJ309276. The heavy chain constant region sequence of mouse hybridoma Ab 4F8, deposited in GenBank under accession number M60432, was used as the reference heavy chain constant region sequence for analyzing the mAb 18B7 MS results. The tryptic peptide E288-R297 containing the one potential *N*-glycosylation site at position Asn-293 was glycosylated. Five oligosaccharides were attached to Asn-293. MS/MS was performed on each of glycopeptide ions and the carbohydrate compositions were unambiguously assigned and their corresponding structures were proposed (Table 2).

The proposed oligosaccharide structures were further verified using exoglycosidase cleavage and LC/MS/MS analysis. Five doubly charged glycopeptide ions were present in the heavy chain tryptic digest (Fig. 1A). Two glycopeptide ions with *m/z* values at 1536.9 and 1618.0 disappeared after sialidase digestion (Fig. 1B), respectively. Thus, these two

Table 1
Monoisotopic mass obtained for tryptic peptides T1 and T2 from IgG 18B7 light chain

Tryptic peptide	Amino acid sequence	Calculated monoisotopic mass	Experimental monoisotopic mass	Δ_{mass} (Da)
Peptide 1–24 (predicted)	DVVMTQAPLALPVSLGDQASISCR	2527.277	2557.187	29.91
Peptide 1–24 (MS/MS)	DVVMTQTPLALPVSLGDQASISCR	2557.288	2557.187	–0.10
Peptide 25–50 (predicted)	SSQSLAHSNGNTYLHWYLQKPGQSPK	2927.431	2928.294	0.86
Peptide 25–50 (MS/MS)	SSQSLAHS D GNTYLHWYLQKPGQSPK	2928.416	2928.294	–0.12

Table 2
Oligosaccharides attached to Asn-293 of glycopeptides (E289–R297)

Monoisotopic mass of glycopeptide (Da) ^a	Theoretical monoisotopic mass of glycopeptide (Da)	Mass difference (Da)	Carbohydrate composition ^b	Proposed oligosaccharide structure ^c	Oligosaccharide type	Relative abundance (%) ^d
3232.102	3232.245	−0.14	Hex ₅ HexNAc ₄ FucNeuGc		Hybrid	7
3070.051	3070.192	−0.14	Hex ₄ HexNAc ₄ FucNeuGc		Complex	7
2925.010	2925.155	−0.15	Hex ₅ HexNAc ₄ Fuc		Hybrid	14
2762.973	2763.102	−0.13	Hex ₄ HexNAc ₄ Fuc		Complex	44
2600.916	2601.049	−0.13	Hex ₃ HexNAc ₄ Fuc		Complex	29

^a Monoisotopic masses of glycopeptides were obtained by LC coupled with Qstar QqTOF mass spectrometer.

^b Carbohydrate composition was assigned on the masses of glycans and MS/MS.

^c The structures of *N*-glycans were proposed based on the fragment ions of glycopeptides obtained by MS/MS. (▣) *N*-acetylglucosamine, (⊗) core mannose, (○) mannose, (▽) fucose, (●) galactose and (◇) NeuGC.

^d The relative abundance were calculated based on the relative intensities of glycopeptide ions obtained by LC/MS.

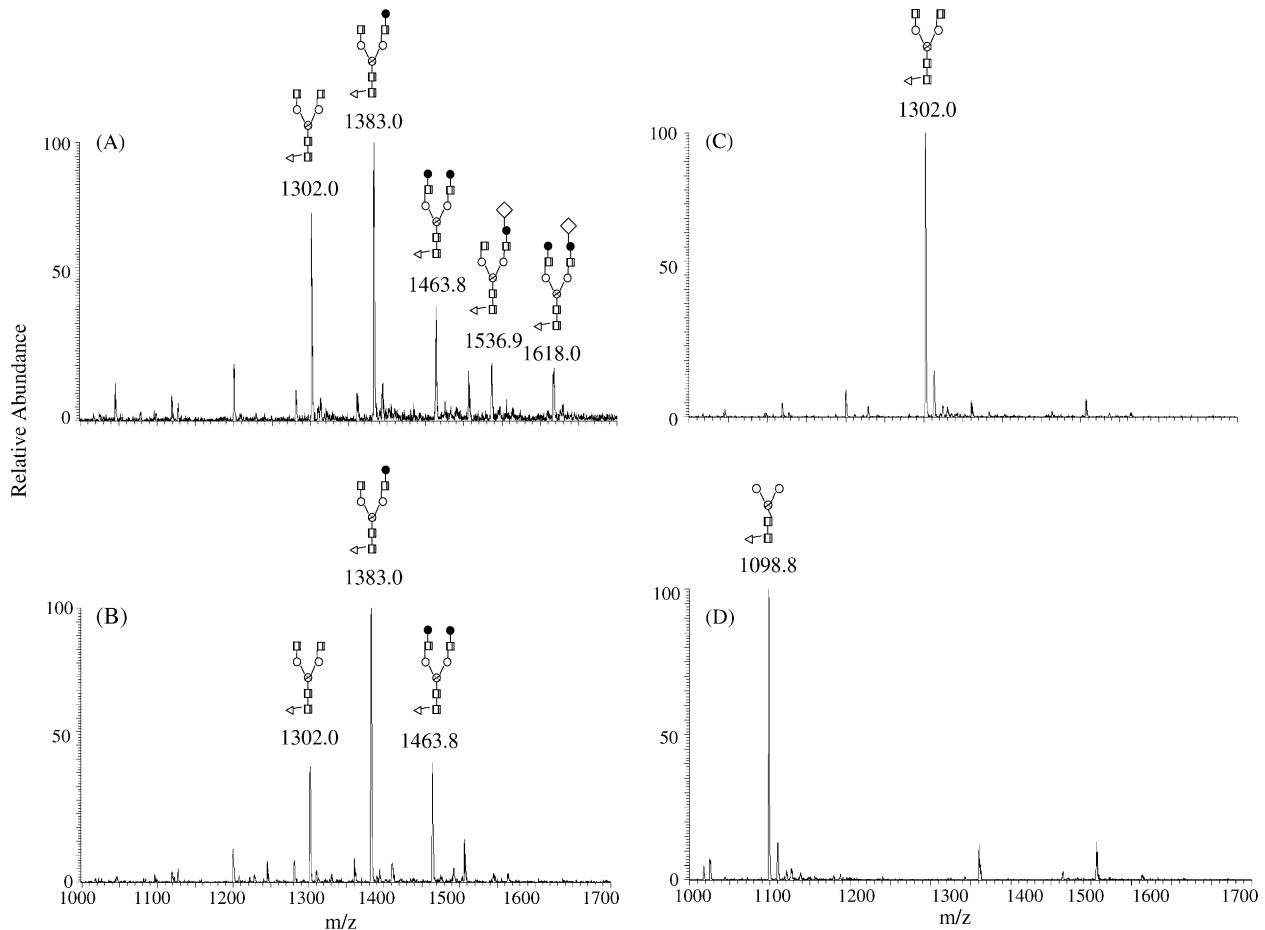


Fig. 1. The mass spectra of glycopeptide ions after exoglycosidase treatment. (A) no reaction; (B) sialidase treatment; (C) sialidase and β -GALase treatment; (D) sialidase, β -GALase and HEXase treatment. (\square), *N*-acetylglucosamine; (\circ), mannose; (∇), fucose; (\bullet), galactose; (\diamond), *N*-glycolylneuraminic acid.

peptides contained terminal sialic acid. After treating the glycopeptides with sialidase and β -GALase, only one glycopeptide ion appeared at m/z 1302.0 (Fig. 1C), due to the release of both NeuGc and galactose. Two *N*-acetylglucosamines were released after digestion with sialidase, β -GALase and HEXase, and a glycopeptide ion with m/z value at 1098.8 was observed (Fig. 1D).

Earlier studies had shown that the relative intensities in the electrospray ionization mass spectrometry data correlated closely with the relative quantities of each glycoform for glycopeptides with the same peptide sequence (Settineri and Burlingame, 1996). The mono-galactosylated, core fucosylated, biantennary complex-type oligosaccharide with the glycosyl composition of Hex₄HexNAc₄Fuc was the major glycan species, which comprised approximately 39% of the total glycan pool (Table 2). Two oligosaccharides had terminal *N*-glycolylneuraminic acid (NeuGc) residues, and each of which comprised approximately 8% of glycan pool. Analysis of the glycosylation pattern of three 18B7 subclones recovered from the parent 18B7 hybridoma by soft agar cloning in our laboratory revealed that each had the same glycosylal-

tion pattern as the original mAb 18B7 prepared for clinical use.

3.2. Circular dichroism spectra of native 18B7 and de-glycosylated 18B7

Circular dichroism spectroscopy is a sensitive method for probing the conformational changes in the secondary structure of proteins. The CD spectra of native 18B7 and de-glycosylated 18B7 were essentially similar (Fig. 2).

3.3. Binding of native and de-glycosylated mAb 18B7 to GXM and *C. neoformans*

Native and de-glycosylated mAb 18B7 demonstrated similar binding to GXM by ELISA (Fig. 3). Similarly, the indirect immunofluorescence pattern of native and de-glycosylated binding to encapsulated *C. neoformans* cells was indistinguishable. Hence, removal of glycans did not have a significant effect on the binding properties of mAb 18B7 for GXM by ELISA or on yeast cells.

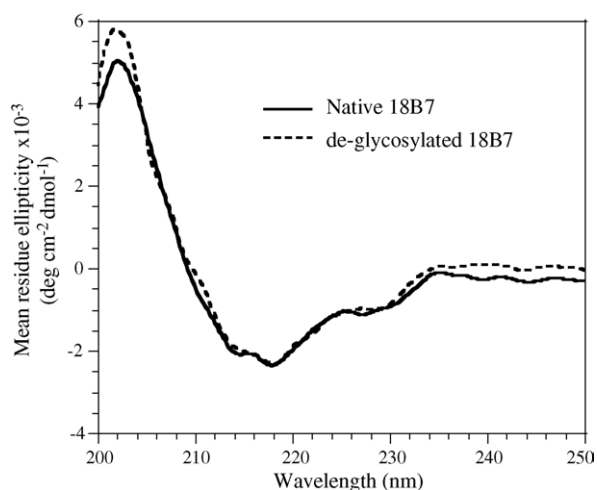


Fig. 2. Circular dichroism spectra of native (solid line) and de-glycosylated-18B7 (dashed line). The spectra were recorded at room temperature ($\sim 21^\circ\text{C}$).

3.4. Pharmacokinetics of native and de-glycosylated mAb 18B7

De-glycosylated mAb 18B7 was cleared from serum faster than native mAb 18B7 with a half-life of 110 ± 4.4 and 382 ± 9.0 h, respectively.

3.5. Activation of the C pathway by the native and de-glycosylated 18B7

Ab–Ag complexes can activate C through the classical pathway. *C. neoformans* is unusual among encapsulated microbes in that its capsule is a potent activator of the alternative C pathway although this phenomenon is much slower than Ab-mediated classical pathway activation (Kozel, 1996;

Kozel et al., 1998). Since Ab glycosylation is important for C1q binding and classical pathway activation we performed a kinetic study to infer the function of the native and de-glycosylated 18B7 in C activation in this system. As shown in Fig. 4, addition of native mAb 18B7 to *C. neoformans* cells produced fast deposition of C in the cryptococcal capsule with C3 being detected by immunofluorescence after 1.5 min of incubation. This C activation occurred through the classical pathway, since it could be inhibited by EGTA (Fine et al., 1972). In the same conditions, de-glycosylated 18B7 coated cells did not have any C bound, consistent with a requirement for glycosylation in classical C activation pathway. Longer incubations (15 min) resulted in C deposition in the cryptococcal capsule in all the cases, even in EGTA- or de-glycosylated 18B7-treated cells, indicating that C3 deposition in the cryptococcal capsule was a result of alternative pathway activation.

3.6. Opsonic and protective efficacy of native and de-glycosylated 18B7

Removal of mAb 18B7 N-glycans with PNGase F reduced, but did not abrogate, the opsonic efficacy of the IgG1 for *C. neoformans* with J774.16 macrophage-like cells (Fig. 5). The ability of de-glycosylated 18B7 to promote phagocytosis was unexpected given that Fc glycans are essential for IgG interaction with the Fc receptor. Since antibodies to *C. neoformans* capsular polysaccharide are capable of promoting opsonization by inducing changes in the polysaccharide capsule that allow C-independent ingestion by the CR we investigated the effect of blocking CR with antibodies to CD11b and CD18. In the presence of antibodies to CR there was a marked reduction in the opsonic efficacy of de-glycosylated 18B7 whereas only a modest reduction was observed for the native mAb 18B7. Passive transfer experiments revealed that native and

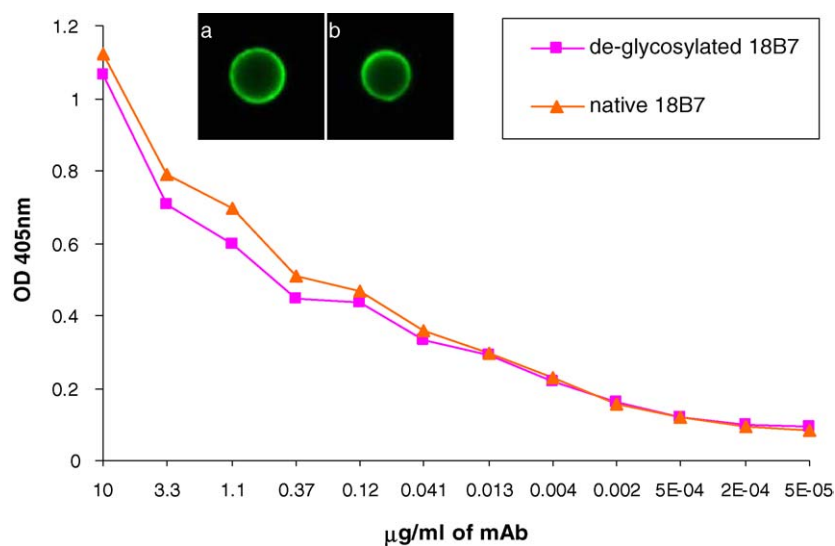


Fig. 3. Binding of native and de-glycosylated 18B7 to GXM by ELISA. Inset: the indirect immunofluorescence patterns of native 18B7 (a) and de-glycosylated 18B7 (b) on binding to encapsulated *C. neoformans* cells.

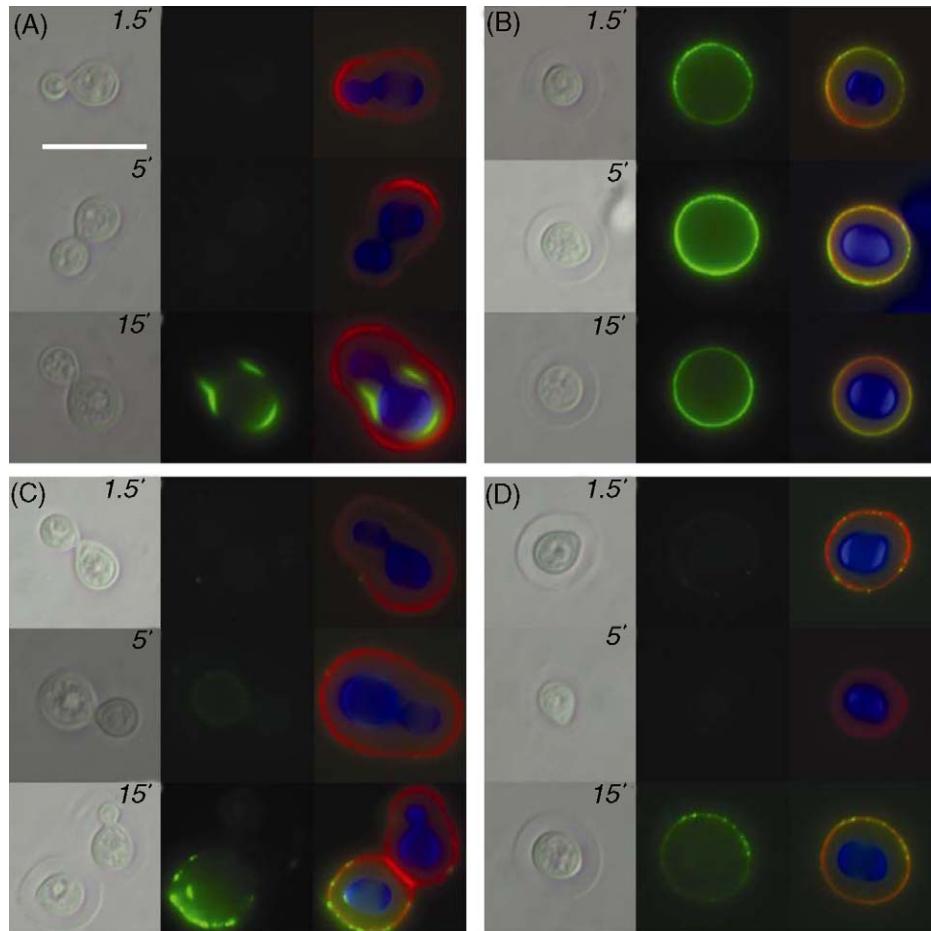


Fig. 4. Activation of C by native and de-glycosylated 18B7. *C. neoformans* H99 cells were grown in Sabouraud and incubated in 10% Sabouraud in MOPS 50 mM pH 7.3 to induce capsule size as described in material and methods. Approximately 2×10^6 cells were incubated in serum (A), serum + 100 $\mu\text{g/ml}$ native 18B7 (B), serum + 100 $\mu\text{g/ml}$ native 18B7 + 10 mM EGTA (C) and serum + 100 $\mu\text{g/ml}$ of de-glycosylated 18B7 (D). The cells were incubated at 37 °C for 1.5 (upper row), 5 (middle row) or 15 min (lower row). In (A), the cells were incubated with 10 $\mu\text{g/ml}$ of native 18B7 after the serum incubation to delimit capsule edge (see Section 2). The capsule edge and location of C3 binding were detected with GAM-IgG1-TRITC (red) and GAM-C3-FITC (green) respectively. In this experiment, calcofluor white (blue) was added to localize the cell wall. Left column, light microscopy; middle column, C3 (fluorescein); right column, merge of the fluorescence due to the capsule (rhodamine), C (fluorescein) and cell wall (calcofluor). Bar in first panel denotes 10 μm and applies to all the pictures. A parallel experiment with *C. neoformans* and serum + 100 $\mu\text{g/ml}$ of de-glycosylated 18B7 + 10 mM EGTA produced the same results as those shown in the absence of EGTA (not shown).

de-glycosylated mAb 18B7 had comparable protective efficacy (Fig. 6).

4. Discussion

We report the peptide and glycan structure of a murine mAb that is in clinical trial for the treatment of human cryptococcosis and the effect of de-glycosylation on various functional properties that can affect its therapeutic efficacy. The protein sequence for mAb 18B7 deduced from mRNA and cDNA sequencing (Casadevall et al., 1998) was confirmed by peptide mass mapping and tandem mass spectrometry. However, two differences were observed for V_L at positions 7 and 33 where the protein sequence yielded threonine and aspartic acid, respectively, instead of the alanine and asparagine predicted by nucleic acid sequence data obtained

by DNA sequence of amplified V_L mRNA (McLean et al., 2002) and/or direct V_L mRNA sequencing (Mukherjee et al., 1993). A reappraisal of sequence data shows that the difference at position 7 is almost certainly an error arising during DNA sequencing of the amplified V_L mRNA since the direct mRNA sequence data also predicted a threonine residue. However, both direct mRNA sequencing (Mukherjee et al., 1993) and DNA sequencing of amplified V_L mRNA (McLean et al., 2002) carried out a decade apart predicted an asparagine residue at position 33. Hence, it is difficult to attribute the N33D difference to sequencing errors. The N33D change could be the result of spontaneous hydrolysis of asparagine to aspartic acid, a phenomenon that has been demonstrated in certain proteins (Carter and McFadden, 1994a,b). Asn \rightarrow Asp hydrolysis may have occurred during affinity chromatography when mAb 18B7 was eluted by the use of an acidic solution since spontaneous deamination of

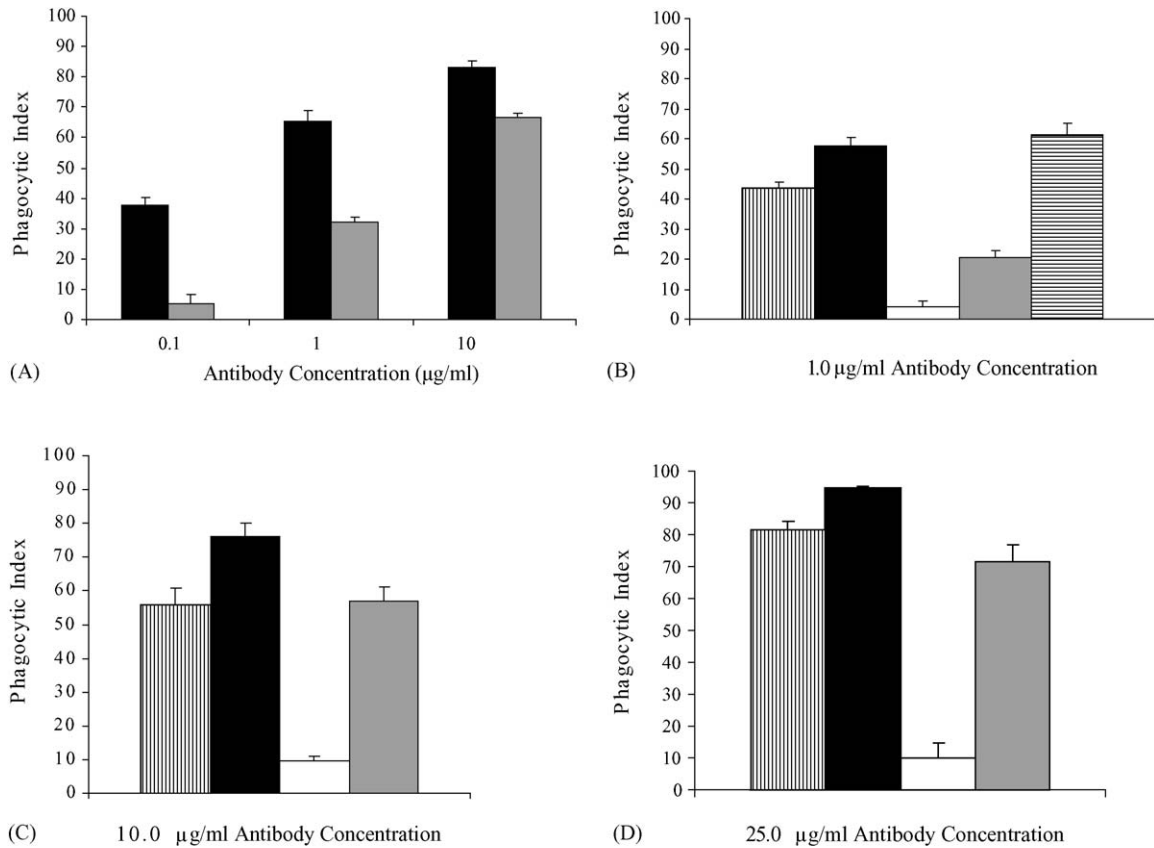


Fig. 5. (A) Phagocytic index of macrophages was determined for different doses (0.1, 1.0, and 10.0 µg/ml) of native 18B7 (■) and deglycosylated 18B7 (▨). (B–D) Effect of complement receptor blockage on phagocytosis using 1 (B), 10 (C), and 25 µg/ml (D) of native and de-glycosylated mAb 18B7. Since de-glycosylated mAb 18B7 had reduced opsonic efficacy we considered the possibility of toxicity to macrophage-like cells but were able to rule it out by showing no reduction in opsonic efficacy when native and de-glycosylated mAb 18B7 preparations were mixed and added to cells (column with horizontal lines on panel B). (▨) Native 18B7 with anti-CR3/CR4 Ab. (▨) De-glycosylated 18B7 with anti-CR3/CR4. Open and grey bars denote native and de-glycosylated mAb 18B7, respectively, as indicated in (A).

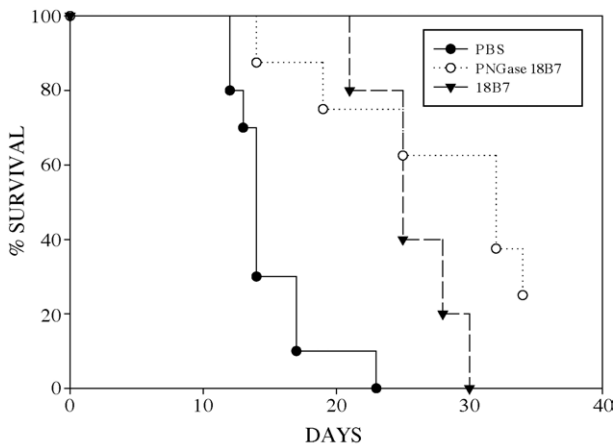


Fig. 6. Survival of mice treated with PBS, native mAb 18B7, or de-glycosylated mAb 18B7 and then lethally infected with *C. neoformans*. Mice given either mAb 18B7 or de-glycosylated mAb 18B7 lived significantly longer than mice given PBS ($p=0.013$ and 0.005 relative to PBS group, respectively).

asparagine in proteins can be facilitated by low pH (Maeda and Kuromizu, 1977). Alternatively, it may be associated with this particular protein. We can only speculate as to why this phenomenon occurred only for N33 and not for other asparagine residues. In this mAb, the local electronic environment resulting from neighboring interactions may facilitate deamination of asparagine. To our knowledge this is the first example of an apparent spontaneous asparagine deamination event in an immunoglobulin, which raises the tantalizing possibility that this effect is yet another mechanism by which sequence diversity can be generated in Ag combining regions.

Mass spectrometric analysis of mAb 18B7 tryptic peptides revealed that glycosylation was limited to the peptide E288-R297, which contains one potential *N*-glycosylation site at Asn-293. No evidence for *O*-glycosylation was detected as has been described for some murine IgG2 mAbs (Kim et al., 1994). This combined with the observation that peptide-*N*-glycosidase F released Ab-associated glycans implies that this mAb has a conventional IgG glycosylation pattern with a single *N*-linked glycosylation site at position Asn-293.

The mAb 18B7 glycans manifested considerably less diverse structural diversity than reported for other murine IgG mAbs (Saba et al., 2002). For example, a murine IgG_{1K} mAb was reported to have 10 N-linked oligosaccharides, of which the majority are core-fucosyl, asialyl biantennary chains with varying galactosylation and a minority are afusoyl, bisected, and mono-sialyl oligosaccharides (Saba et al., 2002). In contrast, mAb 18B7 had five core fucosylated, biantennary complex-type oligosaccharides, of which four were galactosylated, comprising 71% of the total oligosaccharide pool and two were monosialylated by *N*-glycolyneuraminic acid, comprising 16% of the total oligosaccharide pool.

CD spectral analyses of native and de-glycosylated mAb 18B7 were essentially identical, indicating no major change in secondary structure as a result of *N*-glycan removal. Since the Ag recognized by mAb 18B7 is a large polysaccharide that is presumably multivalent, Fc glycosylation could conceivably affect the binding properties by affecting Fc–Fc interactions that influence avidity and/or the quaternary structure of Ag–Ab complexes. Comparison of native and deglycosylated mAb 18B7 by ELISA revealed comparable binding curves. Similarly, no differences in immunofluorescence pattern were observed for the binding of native and deglycosylated mAb 18B7 to the *C. neoformans* capsule. Remarkably, we found that deglycosylated mAb 18B7 was opsonic for *C. neoformans* with only a small reduction in opsonic efficacy relative to native mAb 18B7 being evident at higher Ab concentrations. Since IgG Fc region glycosylation is essential for the interaction with the Fc receptor (Wright and Morrison, 1997), we hypothesized that this effect was due to C-independent phagocytosis through the CR, as has been described for certain mAbs to *C. neoformans* (Taborda and Casadevall, 2002). This mechanism is postulated to involve the occurrence of Ab-mediated structural change in the *C. neoformans* capsule such that the capsular polysaccharide can then interact directly with the CR (Taborda and Casadevall, 2002). The inhibition of opsonic efficacy by Ab to the CR components CD11b and CD18c for deglycosylated mAb 18B7, but not native 18B7, indicates that the deglycosylated IgG1 promotes complement-independent phagocytosis through CR. Furthermore, native and de-glycosylated mAb 18B7 demonstrated comparable protective efficacy in passive transfer studies. Hence, for microbes where Ab binding can trigger phagocytosis through an alternative receptor or by modifying microbial surfaces, glycosylation may not be an absolute necessity for IgG opsonic efficacy.

Fc N-linked glycosylation has been implicated in the serum half-life of immunoglobulins but the effect observed has varied with the IgG studied and the host used to measure clearance. For a murine IgG2b, deletion of the CH₂ N-linked glycosylation site reduced serum half-life from 7.4 to 4.8 days, an effect attributed to increased catabolism in extravascular tissues (Wawrzynczak et al., 1992). In addition to the quantitative defects described in these studies, qualitative differences in glycosylation structure are important for Ab pharmacokinetics. In this regard, mouse–human

chimeric IgG1 mAbs expressed in cells unable to process high-mannose intermediates through the terminal glycosylation products had significantly shorter serum half-life in mice (Wright and Morrison, 1994). The serum half-life of deglycosylated mAb 18B7 was significantly shorter than native mAb 18B7 in mice. This result is consistent with studies indicating a role for glycosylation in IgG serum half-life and that the serum clearance of glycoproteins can reflect interactions with mannose and/or asialoglycoprotein receptor in liver cells and that this effect has been implicated in serum half-life differences TNFR–IgG (Raju et al., 2001).

IgG Fc region glycosylation is essential for C activation. The IgG N-linked glycosylation site in CH₂ is located near the binding site for C1q, the first component of the classical C pathway. *C. neoformans* has the unusual characteristic that its capsule is a potent activator of the alternative C pathway (Kozel, 1996). Consequently, incubation of *C. neoformans* cells in serum results in C deposition in the capsule without the need for specific Ab although this process is slower than Ab-mediated classical C activation (Kozel, 1996). Since de-glycosylated mAb could conceivably alter C3 deposition even if could not promote classical C activation by changing the capsule properties and/or blocking C activation sites, we compared C3 deposition on *C. neoformans* cells coated with native and de-glycosylated mAb 18B7. Incubation of *C. neoformans* cells coated with native mAb 18B7 in serum resulted in rapid binding of C3 to the rim of the capsule, consistent with classical C activation. In contrast, incubation of *C. neoformans* cells coated with de-glycosylated mAb 18B7 in serum led to slow deposition of C3 on the capsule surface. Since de-glycosylated IgG does not bind C1q and consequently cannot activate the classical C pathway this result implies C3 deposition in the capsule resulting from polysaccharide-mediated activation of the alternative C pathway. Hence, the eventual outcome of C3 deposition in the presence and absence of native and de-glycosylated IgG1 is qualitatively similar in that C3 is deposited in the *C. neoformans* surface, although the process is much slower with de-glycosylated IgG.

To investigate the mechanism of *N*-glycan heterogeneity hybridoma 18B7 cells were cloned in soft agar to recover fresh clones originating from single cells, mAb was prepared from three different clones and protein analyzed for *N*-glycan content. The glycan pattern in each of the three clones was identical and corresponded to that in the mAb 18B7 generated from the parent cell line. This observation indicates that the variation in the glycosylation pattern of mAb 18B7 arises from individual cells, each of which produces hybridoma protein in multiple glycoforms. Hence, it is unlikely that one can generate a more homogenous Ab preparation by subcloning this hybridoma cell line.

In summary, we have characterized the glycan structures associated with mAb 18B7 and compared the Ag binding characteristics, opsonizing properties, serum pharmacokinetics, and C-activating activities of the native and deglycosylated forms of this therapeutic Ab. With regards to the

functional studies, Ag binding was not significantly affected by removal of *N*-glycans. Consistent with prior studies, *N*-linked glycosylation in mAb 18B7 was essential for Fc γ R-mediated phagocytosis and classical C activation. However, de-glycosylated mAb 18B7 remained opsonic by promoting C-independent phagocytosis through CR3 and serum incubation of *C. neoformans* coated with de-glycosylated mAb 18B7 resulted in capsular deposition of C3 through activation of the alternative C pathway. These results illustrate how the effect of IgG glycosylation on immunoglobulin function can vary depending on the biological characteristics of the system being studied. In this system mAb 18B7 de-glycosylation did not abrogate phagocytosis or C deposition on the capsule because certain characteristics of the *C. neoformans* capsule allow the polysaccharide to interact directly with cellular receptors when Ab binding alters capsule structure and to activate the alternative C system. These results suggest that IgG glycosylation is dispensable for some biological effects of Ab function against *C. neoformans* and imply that it may be possible to use de-glycosylated mAbs in therapy. For example, de-glycosylated mAbs may have advantages in delivery of microbicidal radiation (Dadachova et al., 2003), since they would not trigger Fc γ R-mediated phagocytosis and thereby spare certain host cells radiation damage. Hence, *N*-linked glycosylation function should be considered in the context of the system being studied rather than being a global attribute conferring singular effector properties to immunoglobulin molecules. In fact, IgG glycosylation can be viewed as another facet of immunoglobulin architecture amenable to engineering that can be modified to improve the therapeutic usefulness of certain mAbs.

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Glossary

- Ag*: antigen.
- C*: complement.
- CD*: circular dichroism.
- CID*: collision-induced dissociation.
- CN*: *Cryptococcus neoformans*.
- CR*: complement receptor.
- Fuc*: fucose.
- GlcNAc*: *N*-acetylglucosamine.
- GXM*: glucuronoxylomannan.
- Hexase*: β-*N*-acetylhexosaminidase.
- HexNAc*: *N*-acetylhexosamine.
- Hex*: hexose.
- IgG*: immunoglobulin G.
- LC/MS*: HPLC coupled with mass spectrometry.
- LC/MS/MS*: HPLC coupled with tandem mass spectrometry.
- mAb*: monoclonal antibody.
- MS*: mass spectrometry.
- MS/MS*: tandem mass spectrometry.
- NeuGc*: *N*-glycolylneuraminic acid.
- PNGase F*: peptide-*N*-glycosidase F.
- β-*GALase*: β-galactosidase.