ABSRACT

THE EPITOPE SPECIFICITY OF THE HUMAN ANTI-CANDIDA ALBICANS ANTIBODY M1G1

By

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Mannan is one of the major cell wall components in *Candida albicans*. Previously, we generated a human recombinant IgG1 antimannan antibody known as M1g1 and showed a protective role in host resistance to systemic candidiasis in mice. The purpose of this study was to characterize the epitope of M1g1. Oligomannosides were isolated by thin layer chromatography, and identity was determined by ^{1}H-NMR. Purified oligomannosides were used either as inhibitors of M1g1 binding to mannan in a competition ELISA or as neoglycolipid-conjugates in ELISA for direct binding by M1g1. Furthermore, molecular recognition of oligomannosides by M1g1 was analyzed by 3D modeling and by modification of interacting amino acids with site-directed mutagenesis. Reactivity of serum natural antimannan antibody from normal adults for neoglycolipid-conjugated oligomannosides was determined by ELISA. β-1,2-linked mannotriose is the specific epitope for the protective human recombinant antimannan antibody M1g1 and is reactive with natural antimannan antibody found in normal individuals, suggesting the presence of naturally occurring protective antimannan antibody.

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CHAPTER 1

INTRODUCTION

Candida albicans and Disseminated Candidiasis

C. albicans is a dimorphic fungus that often commensally colonizes the skin, oral cavity, urogenital and gastrointestinal tracts without causing disease. However, *C. albicans* can cause opportunistic infections when the normal host defense mechanisms are suppressed or impaired as seen in patients who are HIV-infected, undergoing chemotherapy or organ transplantation. Disseminated candidiasis, a systemic *Candida* infection, is a life-threatening disease. It has an incidence of between 1.1 and 24 cases per 100,000 individuals and is associated with a mortality rate greater than 30% (Wisplinghoff et al., 2004). *C. albicans* and several other *Candida* species including *C. albicans*, *C. glabrata*, *C. tropicalis* and *C. parapsolosisi* are now the fourth most common cause of bloodstream infections in hospitalized patients (Edmond et al., 1999); *C. albicans* is the most frequently isolated of all the *Candida* species. Currently, treatment options for disseminated candidiasis are limited to a few antifungal drugs such as amphotericin B, triazole, and fluconazole. Due to drug toxicity, limited availability of antifungal drugs, and emergence of drug-resistant *C. albicans* strains, it is of interest to pursue new therapeutic approaches such as combining chemotherapy with immunotherapy (Stevens, 1998).

Candida Cell Wall Mannan

Common to all *Candida* species is the presence of mannan on the cell surface. Mannan is covalently associated with cell wall proteins (glycoproteins) and lipids (glycolipids); therefore, mannan has also been referred to phospsphomannoprotein or phosphopeptidemannan (Martinez et al., 1998). The structure of mannan has been extensively investigated. Chemical analysis by Shibata and his colleagues (Shibata et al., 1992a, 1992b, 1995, 2007) has shown that mannan contains homopolymers of D-mannose as the main component. Mannose polymers attached to asparagine residues of cell wall proteins are called N-linked oligomannosides. These are long, linear chains of mannose units joined by α-1,6-linkages and they are attached to the side chains of mannose units joined mainly through either α-1,2, α-1,3, or β-1,2-linkages. Because the backbones and side chains are stable in the presence of boiling 10 mM HCl, this part is called the acid-stable region. Additional oligomannosides with β-1,2-linkages may be attached to the acid stable side chains via phosphodiester-linkages. Mildly acidic treatment with 10 mM HCl causes hydrolysis of the phosphodiester bond, releasing β-1,2-linked oligomannosides; thus, this region is called the acid labile region. In addition, there are O-linked oligomannosides in which mannose or un-branched oligomannosides are attached to the peptide through the hydroxyl groups of serine or threonine residues of mannoprotein. These O-linked residues range in length from one to three mannose units joined through α-1,2 or occasional α-1,3-linkages (Figure 1).

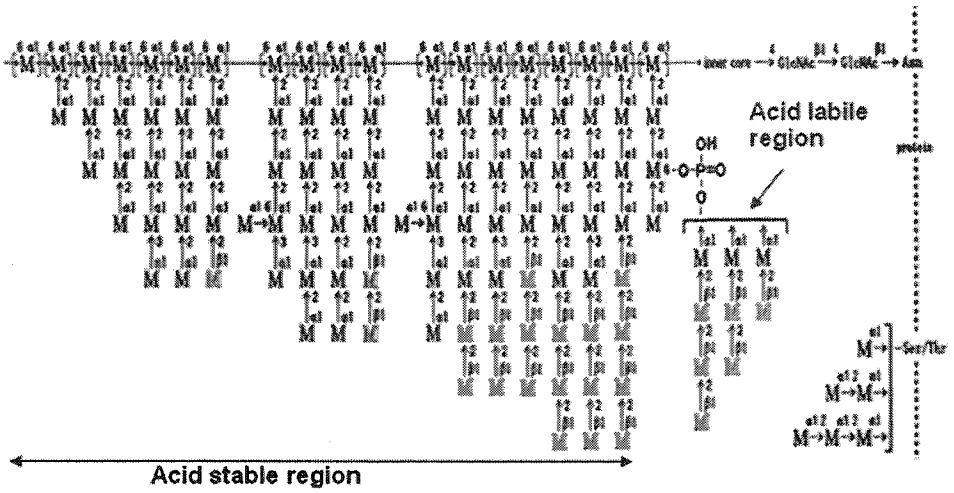

FIGURE 1. Proposed structure of *C. albicans* serotype A mannan (Shibata et al., 2007). "M" denotes D-mannose residue. Carbohydrate sugar linkages, acid stable and acid labile regions of mannan are indicated.

There are two serotypes of *C. albicans*: A and B, which are determined by the subtle variations in carbohydrate composition of cell wall mannan. In this context, Kobayashi et al. (1989, 1990) discovered that the acid labile region of both serotypes exclusively consist of β-1,2-linked oligomannosides. The only feature that distinguishes the two serotypes is within the acid stable region of mannan in which the oligomannosyl side chains of serotype A contain additional β-1,2-linked oligomannosides in the acid-stable region, while serotype B lacks such oligomannoside linkages.

Antimannan Antibodies

Mannan is an important antigenic component and a potential virulence factor for all *Candida* species. Early immunochemical studies of *Candida* mannan have

identified ten potential antigenic factors by using rabbit polyclonal antisera which can recognize subtle variations in the primary structure of mannan (Suzuki, 1997). Furthermore, both mannoproteins and polysaccharide components of mannan have functional roles in adhesion of *Candida* cells to host cells, which plays a major role in the pathogenesis of candidiasis (Critchley and Douglas, 1987a, 1987b). *C. albicans* mannan is immunogenic. It has been shown that there are naturally occurring antibodies in human individuals reactive with specific *Candida* mannan epitopes. (Zhang et al., 1998; Kozel et al., 2004; Lehmann and Reiss, 1980). These antibodies in sera are primarily immunoglobulin G (IgG) isotype with much lower levels of IgA, IgM and IgE (Chiani et al., 2009).

Many studies have provided additional evidence to support the hypothesis that antimannan antibodies may have a protective role in host resistance to systemic candidiasis. Two murine antimannan antibodies, IgM B6 and B6.1, generated by Cutler and his colleagues (Han et al., 1997) have been shown to react with the *C. albicans* cell surface mannan epitopes. However, only mAb B6.1 showed protection against candidiasis in mice. The epitope of the protective antibody B6.1 is β-linked oligomannoside (β-1,2-linked mannotriose) in the acid-labile region of mannan, whereas the non-protective B6 is specific for α-linked oligomannosides in the acid-stable region of mannan (Han et al., 1997). Furthermore, protection by mAb B6.1was found to require mouse neutrophil candidacidal activity and the host complement system (Han et al., 2001). It has been previously reported that antimannan IgG is required for complement opsonization of *Candida* yeast cells through either the

classical or alternative complement pathways (Zhang et al., 1997, 1998; Kozel et al., 2004). Our lab generated a human recombinant antimannan antibody, M1g1, and showed a protective role in host resistance to systemic candidiasis in mice. M1g1 was found to mediate phagocytosis and killing of *Candida* yeast cells by mouse macrophages and to activate the mouse complement system (Zhang et al., 2006).

Methods for Determination of Carbohydrate Epitopes

Due to the growing impact of fungal diseases and treatment challenges in using antifungal drugs, there has been a growing interest in developing immunological therapeutics utilizing protective antibodies against fungal infection. To achieve such a goal, it is essential to investigate the specific epitope of protective antibodies, because the structural characteristics of epitopes determine the production of protective or non-protective antibodies (Han et al., 1997; Cutler, 2005). Monoclonal antibody M1g1 is the first reported human recombinant antimannan antibody that has a protective role against candidiasis. However, the specific epitope of M1g1 has not yet been determined. Therefore, identification of the epitope of M1g1 may provide additional evidence for the effect of epitope specificity on antimannan antibodies-mediated protection against fungal infection. Such study will also be important for the development of anti-*Candida* antibodies as preventive and therapeutic agents.

Recognition of complex polysaccharides on the cell surface by antibody is an important part of the immune response to microbial infection. Interactions of antibodies with carbohydrates may be investigated in detail by immunochemical assays or even crystallography methods. However, detailed analysis of carbohydrate-

protein interactions have been challenging due to the complexity of polysaccharide structures, the limited amounts that can be isolated, and the low affinities of the interactions (Liu et al., 2007). Moreover, due to the difficulties in performing crystallography experiments, only a few carbohydrate-antibody crystal structures have been determined. According to RBSC protein data bank (Bernstein et al., 1977), to date, there are only 11 carbohydrate-antibody crystal structures in a total of roughly 250 published antibody crystal structures as of 2009. Out of these 11 structures, 6 antibodies are against anti-bacterial polysaccharides. Therefore, it will be of great interest to study how M1g1 recognizes mannan epitopes.

There are several methods available for analysis of carbohydrates epitope specificity. One of the most common methods is the competitive enzyme-linked immunosorbent assay (ELISA), in which *C. albicans* mannan is coated onto ELISA microwells as the solid phase. After various concentrations of mannose oligosaccharides are added with antibody of interest to the ELISA microwells, the inhibitory effects of each oligosaccharide can be assessed to determine the epitope specificity of the antibody. The second method to determine the molecular basis of recognition of carbohydrate epitopes is the neoglycolipid immunoassay developed by (Chai et al., 2003). With this method, oligomannosides are coupled to a lipid carrier DHPE (1, 2-O-dihexadecyl-sn-glycero-3-phosphoethanolamine), for binding to conventional substrates for immunoanalysis in a mole-mole carbohydrate/lipid conjugation. The hybrid molecules, called neoglycolipids (NGLs), exhibit both immunogenicity and antigenicity, which allows the direct analysis of antibodies

binding to immobilized NGLs in an ELISA format. Many studies have successfully used this method to identify the epitopes of monoclonal antibodies reactive with polysaccharide moieties of *C. albicans* mannoprotein (Trinel et al., 1992), and to analyze the human antibody response to *C. albicans*-derived oligomannosides (Faille et al., 1990, 1992b; Hayette et al., 1992). Moreover, computer molecular modeling and docking can also be used to predict antigen-antibody interactions (Sotriffer et al., 2000). Although binding sites are best defined by crystallography, computer simulated modeling incorporating site-directed mutagenesis has been used successfully to study the binding sites important for antigen-antibody interactions (Paula et al., 2005; Sivasubramanian et al., 2008). Since antibodies have highly conserved structures and are one of the best studied families of proteins using X-ray crystallography, the generation of computer simulated models and molecular docking provide methods to identify interacting amino acids unique to each antigen-antibody interaction. The information gathered from models is broadly applicable in antibody engineering, antigen docking and structurally-based drug design (Sotriffer et al., 2000).

Objectives and Major Findings

The goal of this study was to determine the epitope of the human recombinant antibody M1g1 with the following objectives: (i) to use preparative thin layer chromatography for isolation of oligosaccharides from *C. albicans* mannan, (ii) to identify mannan fractions that are reactive with M1g1, (iii) to identify antibody-antigen interacting amino acids of M1g1 with site-directed mutagenesis and 3D

modeling, and finally (iv) to determine the presence of M1g1-like antibodies in normal human adults.

The major findings of this study are that (i) preparative TLC can be used to isolate mannose oligosaccharides from *C. albicans* mannan, (ii) the specific epitope of M1g1 is β-1,2-linked mannotriose, (iii) molecular modeling and docking simulation predict recognition of β-1,2-linked oligomannosides by M1g1 (iv) natural antimannan antibodies that recognize β-1,2-linked oligomannosides are present in sera of normal human adults.

CHAPTER 2

MATERIALS AND METHODS

Antibody and Antibody Production

The recombinant human antimannan IgG1 antibody M1g1 used in this study was expressed in a Chinese hamster ovary cell line (ATCC CHO-K1) and purified as previously described (Zhang et al., 2006).

Yeast and Yeast Mannan Preparation

Yeast cells of *C. albicans* serotype A (strain 3153A) and serotype B (strain CA-1) were maintained and grown as described previously (Kozel et al., 1996). Briefly, yeasts cells were grown in 3 ml of GYEP (2% glucose, 1% peptone, 0.3% yeast extract) at 37°C, passaged daily 3 times, and then used to initiate a large broth culture. Each large culture was shaken overnight at 37°C, 250 rpm in an incubator. Yeast cells were inactivated by 1h treatment with 1% formaldehyde at room temperature, harvested by centrifugation, washed, resuspended in phosphate-buffered saline (PBS, pH 7.0). Purification of *C. albicans* mannan followed standard procedures (Kocourek and Ballou, 1969) with some minor changes to fit the scale of the production. Briefly, yeast cells from the large culture were treated with acetone to remove lipids and then were heated in deionized H_2O for 4 h at 121°C. Water-soluble mannan was precipitated by using Fehlings solution, and mannan in the precipitate was released by using Amberlite IR-120 (H+) resins (Acros Organics, Fair Lawn, NJ).

The mannan solution was neutralized, dialyzed against deionized H_2O at 4°C, and lyophilized.

Fractionation of Mannan

The phosphodiester-linked acid-labile oligomannosides (Figure 1) were released by the method of Okubo et al., (1981). Purified mannan (10 mg/ml) was heated in 10 mM HCl at 100°C for 60 min, and the solution was neutralized with 100 mM NaOH. The acid-stable mannan portion was precipitated overnight at −20°C with three volumes of ice-cold absolute ethanol. Precipitates were removed by centrifugation at 10,000 × *g* for 10 min. The acid-labile mannan in the supernatant was obtained by lyophilization, dissolved in distilled H_2O, and stored at −20°C until use.

Purification of Mannose Oligosaccharides by Thin Layer Chromatography

Thin-layer chromatography was done following the method of Faille et al. (1990). Briefly, isolated oligosaccharides were separated on silica gel 60-precoated TLC plastic plates (EMD Chemicals, Gibbstown, NJ) with n-butanol: acetic acid: methanol: water (20:10:20:10, v/v/v/v) as the solvent. Mannose oligosaccharides were purified by Preparative TLC (PTLC) (Sherma and Fried, 2003). The edges of the TLC plates were stained with orcinol (2 mg/ml; Acros Organics, Geel, Belgium) in 20% sulfuric acid at 105°C for 10 min to locate individual mannose oligosaccharide bands. Silica gel in the non-stained portions of the bands was scraped off and collected and then suspended in 10 ml of 80% ethanol with thorough mixing to extract mannose oligosaccharides. Insoluble silica gel was removed by centrifugation at 5,000 rpm for

15 min. Oligosaccharides were precipitated by lyophilization, dissolved in distilled H_2O, and stored at -20°C until use.

Quantification of Mannose Oligosaccharides

The phenol-sulfuric acid method of Dubois et al. (1956) was used with minor modifications to estimate the concentration of purified carbohydrates. Briefly, 5 µl of oligomannosides in distilled H_2O was serially diluted with 5 µl H_2O in an untreated 96-well ELISA plate (Thermo, Milford, MA) and incubated with 20 µl of 5% phenol and 200 µl of 80% sulfuric acid for 10 min at room temperature in a fume hood for color development. Absorbance of each well was read at 490 nm. Concentrations of oligosaccharides were estimated by a mannose standard curve.

Analysis of M1g1 Binding Specificity by Competition ELISA

Competition ELISA was performed following the method of Duro et al. (2003) with minor modifications. Briefly, *C. albicans* 3153A mannan (400 ng/well) in carbonate buffer (30 mM Na_2CO_3, 70 mM $NaHCO_3$, pH 9.5) was coated overnight onto 96-well ELISA plates (Thermo, Milford, MA) at room temperature. Mannan-coated plates were washed three times with PBS-Tween 20 (0.05%) and blocked with PBS-Tween 20 for 2 h at room temperature. M1g1 in the presence of increasing amounts of a specific oligosaccharide in PBS-Tween 20 was added to each well. The competing oligosaccharides include cell wall mannan derived mannose, β-1,2-linked mannobiose, β-1,2-linked mannotriose, β-1,2-linked mannotetrose and commercially prepared β-1,4-linked oligomannosides ranging from mannobiose to mannohexose (Megazyme, Wicklow, Ireland) and α-1,2-linked mannobiose, α-1,3-1,6-linked mannotriose, and α-1,3-1,3-1,6-linked mannopentose (V-Labs, Covington, LA). The

plate was incubated at 37°C for 1 h and washed 3 times with PBS-Tween 20. Goat anti-human IgG antibody conjugated with horseradish peroxidase (1:25,000; SouthernBiotech, Birmingham, AL) in PBS-Tween 20 was added to detect mannan-bound M1g1 and incubated for 1 h at 37°C. The plate was again washed 3 times with PBS-Tween 20, and 100 µl of a peroxidase substrate (SureBlue TMB 1-Component; KPL, Gaithersburg, Maryland) was added. After a 30 min-incubation at room temperature, the reaction was stopped by addition of 100 µl of 1M H_3PO_4. Absorbance was read at 450 nm, and the difference in absorbance between wells with or without competing oligosaccharides was used to calculate percent inhibition. IC50 values (concentration at which M1g1 was 50% inhibited) were calculated to determine the inhibitory effects of competing oligosaccharides.

Synthesis of Neoglycolipids (NGLs)

Neoglycolipids were synthesized from oligosaccharides and DHPE (1, 2-O-dihexadecyl-sn-glycero-3-phosphoethanolamine; Fluka Chemicals, Dorset, UK) by reductive amination as described in Figure 2 (Chai et al., 2003). Briefly, each oligosaccharide (200 nmol) was mixed in a sealed glass vial with 100 µl of DHPE [8 nmol/µl chloroform-methanol (1:1, v/v)] and 20 µl of 20 µg/µl freshly prepared sodium cyanoborohydride in methanol (Sigma-Aldrich, St. Louis, MO). The reaction was carried out at 60°C for 16 h. The purity and structure of each neoglycolipid were confirmed with TLC. One µl of each neoglycolipid was spotted onto a silica gel 60-precoated HPTLC aluminum plate (EMD Chemicals, Gibbstown, NJ), and the plate was developed in chloroform-methanol-water (60:35:8, v/v/v). Fractionated NGLs were stained with a 1:100 dilution of premulin stock solution (1mg/ml in 1:9 v/v

acetone-water; Sigma-Aldrich, St. Louis, MO) with acetone-water (4:1, v/v) and viewed under a UV lamp (Chai et al., 2003). NGLs derived from commercial synthetic oligosaccharides were used as size standards.

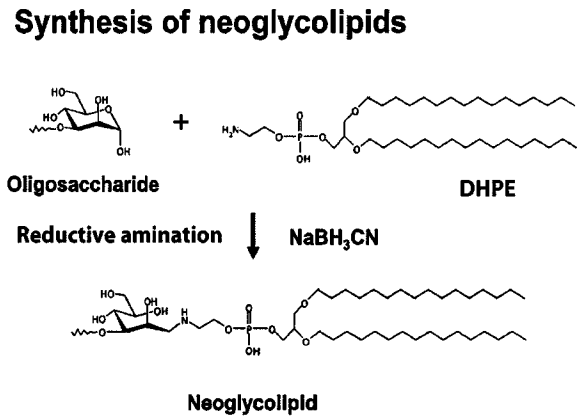

FIGURE 2. Schematic of synthesis of neoglycolipids (NGLs). Oligosaccharides were conjugated to DHPE by reductive amination as described in the methods.

Analysis of Direct M1g1 Binding to NGLs

M1g1 binding to lipid-linked oligosaccharides immobilized on ELISA plates was detected essentially as described (Chai et al., 2003; Lopez and Schnaar, 2006; Freudenberg et al., 1989) with minor modifications. In brief, NGLs in the presence of carrier lipid cholesterol (4 μg/ml) were serially diluted in an ELISA plate with chloroform-ethanol (1:9, v/v), and the solvent was allowed to evaporate overnight at 37°C. The amount of oligosaccharides in each NGL complex coated on the ELISA plate was determined using the phenol-sulfuric acid method as described above. Adjustments were made to ensure that equivalent amounts of oligosaccharides were

used to coat ELISA microwells. The wells were washed 3 times followed by a 2 h blocking with TBS-1% casein (150 mM NaCl, 50 mM Tris -HCl, pH 7.5). The same solution was used in the subsequent washing steps. M1g1 at 80 ng in TBS-0.1% casein was added to each well and allowed to incubate with immobilized NGLs for 1 h at 37°C. M1g1 binding to NGL complexes was detected with goat anti-human IgG HRP (1:10,000) and quantified with a peroxidase substrate at 450 nm as described above. Binding activity of M1g1 for a specific NGL complex was determined after subtracting the background binding in control wells that were coated with DHPE.

^{1}H-NMR Analysis of M1g1 Epitope

TLC fractionated β-1,2-linked mannotriose (0.4 mg) was exchanged twice with 99.85% D_2O (Acros Organics, Geel, Belgium) with intermediate lyophilizations. The sample was dissolved in 0.5 ml of 99.85% D_2O and injected into a 5-mm nuclear magnetic resonance (NMR) tube (Wilmad LabGlass, Vineland, NJ). The NMR experiment was recorded at 20°C on an Apollo 400 MHz spectrometer (Techmag, Houston, TX). A proton spectrum was obtained following 6,500 scans, and the width was 5,000 Hz. Chemical shifts were assigned relative to D_2O ($\delta = 4.87$). MestReNova software (Mestrelab Research, Escondido, CA) was used to analyze ^{1}H NMR data.

Molecular Modeling of M1 Antibody Variable Region and Oligosaccharides

Molecular modeling of the three-dimensional structure of the Fv region of mAb M1g1 was performed using the online modeling software RosettaAntibody (Sivasubramanian et al., 2009; Sircar et al., 2009). Briefly, amino acid sequences of the light and heavy chains of the Fv region were submitted to the server. In the initial stage, the server identified the most sequence homologous template structures for the

light and heavy framework regions and each of the complementarity determining regions (CDRs). An optimized crude model was generated based on the most homologous templates. In the second stage, a full protocol was executed to generate a high-resolution model. The server additionally modeled the hyper-variable CDR H3 loop, and relieved steric clashes by optimizing the CDR backbone torsion angles. RosettaAntibody generated 2,000 independent structures, and the top ten scoring high-resolution structures in rank order by energy were displayed as the output. The final M1 model used in this study was selected from the top ten models.

Molecular modeling of mannose and β-1,2-linked oligomannoses (from mannobiose to mannohexose) was performed using the modeling software W3-SWEET, which is a carbohydrate modeling server available online at www.dkfz-heidelberg.de/spec (Bohne et al., 1998). The primary sequence and the glycosidic linkages between sugar residues were defined by the user. The program converted the input information directly into a preliminary but reliable 3D model, with consideration of the range of flexibility allowed for each dihedral angle by calculating the optimum Van der Waals interactions between sugar residues. Glycosidic Phi and Psi torsion angles of disaccharides constructed in this program were similar to the values reported in the crystal structures of disaccharides available in the Cambridge Structure Database (Allen and Kennard, 1993).

Molecular Docking of M1 and its Epitopes

Computer docking studies were performed with the program Autodock Vina (Trott and Olson, 2010) which is a newly improved version of Autodock 4.0 (Morris et al., 2009). Autodock Vina achieves an approximately 2 orders of magnitude speed-

up compared with Autodock 4.0, and also significantly improves the accuracy of binding mode predictions (Trott et al., 2010). Briefly, mannose was docked onto the M1 model. Before docking, M1 Fv 3D model and mannose were prepared using the program Autodock 4.0 MGL tools in which M1 Fv was set as a rigid molecule, while mannose was set to be flexible with rotational bonds. The binding site of M1 was defined and calculated using a grid map of 20 x 20 x 20 dimensions surrounding all the CDR loops. After docking was performed by Autodock Vina, 100 docking conformations were generated as output with ranking in the order of binding energy. The binding mode between M1 and mannose with the highest binding energy was selected as the best binding model. The M1 epitope β-1,2-linked mannotriose was docked onto the M1 model by superposing the ring of the terminal mannose residue on the ring of the mannose molecule bound to the same binding site predicted by Autodock Vina. All docking results were visualized and analyzed using PyMOL (Delano et al., 2002). Hydrogen bonds interactions were analyzed by Swiss-Pdb Viewer (Guex and Peitsch, 1997).

Generation of M1 Fab Variants

The gene construct of wide-type M1 Fab contains the DNA sequences for the light chain (V_L-C_L) and the heavy chain (V_H-C_H1) of M1g1. Therefore, M1 Fab and M1g1 are expected to have the same epitope specificity but only M1 Fab lacks the Fc region. M1 Fab variants were engineered with the QuickChange mutagensis kit (Stratagene, La Jolla, CA) according to the manufacturer's directions with pComb3H vector DNA, encoding wide-type M1 Fab (Zhang et al., 2006). The sequences of the

M1 wide-type and variant clones were verified by DNA sequencing within the variable regions.

Crude Fab supernatants were prepared as previously described (Zwick et al., 2003). Briefly, the variant clones, and M1 wild type were transformed separately into *E. coli* XL-Gold cells (Stratagene, La Jolla, CA), and single colonies were used to inoculate 2 ml cultures in SB medium containing 50 μg/ml carbenicillin. The cultures were shaken at 300 rpm overnight at 37°C. The same cultures were scaled-up by pouring them into 20 ml SB medium and were shaken at 300 rpm at 37°C for 6-8 h, then induced with 1 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside), and incubated overnight at 30°C with shaking. The next day, the cultures were centrifuged at 3,000 rpm for 15 min at 4°C, and the pellets were resuspended in 1 ml of PBS (pH 7.0), and the bacterial suspensions were subjected to 10 rounds of freeze-thawing. The debris was pelleted at 13,000 rpm in microcentrifuge tubes, and the supernatants were supplemented with bovine serum albumin (1% BSA) and 0.025% Tween 20. These Fab supernatants were quantified by goat-anti human Kappa capturing ELISA with goat-anti human Kappa HRP (SouthernBiotech, Birmingham, AL) as the detecting antibody, and M1 wild-type Fab was used as the standard to estimate the concentrations. After quantification, Fab supernatants were used directly for ELISA assays described below.

Analysis of Binding of M1 Fab and its Variants to Mannan by ELISA

Ninety six-well plates were coated with 400 ng/well of purified *C. albicans* 3153A mannan overnight at 25°C. The wells were washed twice with PBS/0.05% Tween-20 and blocked with the same solution for 2 h. One hundred μl of bacterial

supernatants containing variant or wild-type Fab in equivalent amounts were serially diluted in PBS-T. The plates were incubated for 1 h at 37°C and washed 3 times. Goat anti human kappa-HRP (1:8,000; SouthernBiotech, Birmingham, AL) in PBS-T was added to each well, and the plates were incubated for 45 min at 37°C. After washing the plate 3 times, 100 µl TMB peroxidase substrate was added and incubated for 30 min at room temperature, and the reaction was stopped by adding 100 µl 1M H_3PO_4. Absorbance was read at 450 nm. A two-fold dilution ELISA binding curve was generated for each Fab variant alongside with M1 wide-type. Apparent affinities were calculated as the antibody amount at the half-maximal binding. Apparent affinities as a percentage of that of wild-type Fab M1 were calculated with the formula [(apparent affinity of the wild type)/ (apparent affinity of the variant)] x 100. All samples were tested at least twice, and the mean was taken as the final report value.

Detection of Naturally Occurring Antimannan Antibody for β-1,2-linked Mannotriose

Comparison of pooled and 30 individual serum IgG titers against mannan serotype A (strain 3153A), serotype B (strain CA-1) and specific epitope β-1,2 linked mannotriose were determined by an ELISA as described above using mannan or NGLs in the solid phase. Binding of human IgG antibodies was assessed by use of goat anti-human IgG HRP (1:10,000). Antibody titers were calculated by application of a sigmoid plot of optical density at 450 nm versus serum dilution by use of SigmaPlot (SPSS Inc., Chicago, Ill.). An end point optical density of 0.5 after 30 min of incubation with a substrate was used for calculation of antibody titers.

Statistical Analysis

Correlation coefficients were calculated as *r*, the Pearson product moment coefficient. All plots and statistical analyses were done with the assistance of SigmaStat and SigmaPlot (SPSS Inc., Chicago, Ill.).

CHAPTER 3

RESULTS

Purification of Acid-labile Oligosaccharides by Thin Layer Chromatography

Previous studies have shown that M1g1 epitope is broadly distributed among *Candida* species. M1g1 was found to bind to yeast cells of serotype A and B of *C. albicans*, as well as strains of *C. tropicalis, C. parapsilosis, C. glabrata,* and *C. guilliermondii,* but not to yeast cells of *C. krusei*, *C. kefyr*, and *Saccharomyces cerevisiae* (Zhang et al., 2006). However, the specific mannan epitope recognized by M1g1 has not been characterized. Chemical methods for selective fragmentation of mannan have been used to study the structure of mannan and determine the chemical basis of antigenic factors in different serotypes of *C. albicans* (Nelson et al., 1991). In this study, *C. albicans* serotype A strain 3153A was used. A TLC analysis of oligosaccharides released by mild acid hydrolysis of mannan revealed four major oligosaccharide fractions. Individual oligosaccharide fractions were purified by the Preparative TLC (PTLC) method (Sherma and Fried, 2003) which resulted in isolation of individual fractions containing one to four mannose residues (Figure 3). The quantitatively major components were mannose and mannobiose. The chemical nature of these mild acid released residues was determined to be a family of β-1,2 linked oligomannosides (Shibata et al., 1985). Therefore, based on the mannose

standard, the four residues collected from PTLC were mannose, β-1,2 linked mannobiose, β-1,2 linked mannotriose and β-1,2 linked mannotetrose.

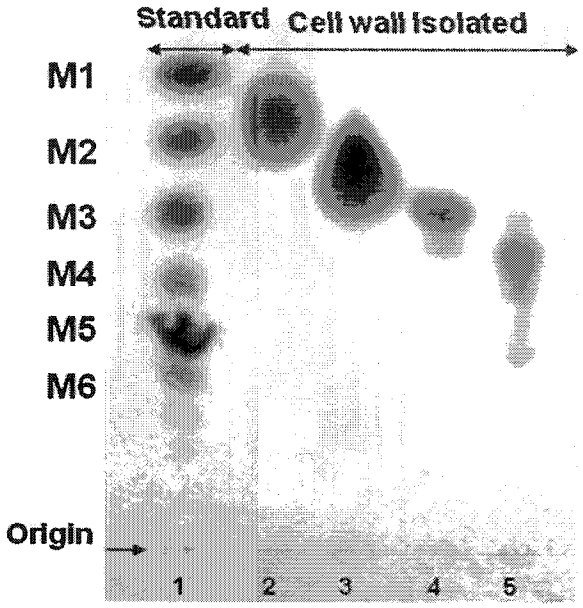

FIGURE 3. Purification of acid-labile oligosaccharides released by mild acid hydrolysis of *C. albicans* mannan by thin-layer chromatography. Lane 1 shows the sugar control; M1 – M6 represent commercial β-1,4-linked oligomannosides containing 1 to 6 mannose units. Lanes 2 – 5 show isolated oligomannosides.

Determination of M1g1 Epitope by Competitive ELISA

Competitive ELISA assays were performed to determine the binding specificity of M1g1. PTLC-purified mannose and β-1,2-linked oligomannosides and commercial α- and β-linked oligomannosides were evaluated for their abilities to inhibit the interaction between M1g1 and mannan A in a solution-phase competition ELISA. As shown in Figure 4, binding of M1g1 to mannan A was greatly affected in the presence of *C. albicans* cell wall isolated β-1,2-linked oligomannosides, but much

less affected by α-linked oligomannosides such as α-1,2-linked mannobiose, α-1,3-1,6-linked mannotriose, and α-1,3-1,3-1,6-1,6-linked mannopentose. To further define the specific epitope of M1, structurally similar oligomannosides containing β-linkages (from β-1,4-linked mannobiose to mannohexose) were also tested for their abilities to inhibit M1g1 binding to mannan A. The results indicated that β-1,4-linked oligomannosides did not affect the M1g1 binding activity. As summarized in Table 1, the IC50 values (concentration by which M1g1 was 50% inhibited) indicated that β-1, 2 linked mannotriose was the most potent inhibitor (IC50 = 0.26 μM), followed by β-1,2-linked mannobiose (IC50 = 0.86 μM) and β-1,2-linked mannotetrose (IC50 = 0.90 μM). Mannose (IC50 = 56 μM) alone showed a moderate inhibitory effect on M1g1 binding to mannan.

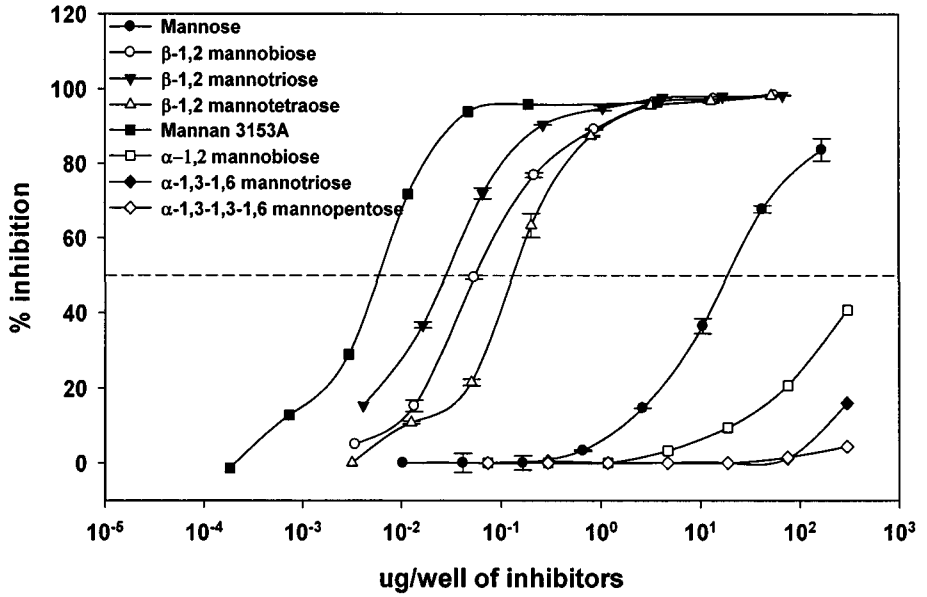

FIGURE 4. Competition ELISA assay for binding of M1g1 to immobilized mannan in the presence of soluble α- and β- linked oligomannosides. Dash line indicates the amounts of oligomannosides required for 50% inhibition. The data are means from two independent experiments ± the standard error of the means.

TABLE 1. Average Inhibition Titer (ug) to Cause IC50 (Concentration Which Causes 50% Inhibition).

Oligomannoside Inhibitors	**Average +/- SEM**	**μM**	**Relative Potency**
Mannose	18.66 +/- 1.46	558	0.05%
β-1,2-linked mannobiose	0.0613 +/- 0.0023	0.862	29.81%
β-1,2-linked mannotriose	0.03 +/- 0.00085	0.257	100.00%
β-1,2-linked mannotetrose	0.115 +/- 0.005	0.900	28.56%
Mannan 3153A	0.00555 +/- 0	NA[a]	NA[a]
α-1,2-linked mannobiose	NI[b]		
α-1,3-1,6 linked-mannotriose	NI		
α-1,3-1,3-1,6 mannopentose	NI		
β-1,4-linked mannobiose	NI		
β-1,4-linked mannotriose	NI		
β-1,4-linked mannotetrose	NI		
β-1,4-linked mannopentose	NI		
β-1,4-linked mannohexose	NI		

Note: Table shows inhibition by isolated oligosaccharides and mannan of the binding of monoclonal antibody M1g1 to *C. albicans* 3153A mannan antigen. SEM is the standard error of mean from two experiments.

[a] The molecular weight of *Candida* 3153A mannan is unknown. Its molality was not available.

[b] NI indicates non-inhibitory.

Determination of M1g1 Binding to Immobilized Neoglycolipids Containing β-1,2 linked Oligomannosides

To carry out a more detailed characterization of the epitope specificity of M1g1, solid-phase ELISA using neoglycolipid technology (Chai et al., 2003) was used. All the oligomannosides used in competition ELISA assays were conjugated to a lipid component, DHPE, to generate a set of neoglycolipids (NGLs). Because of their amphipathic nature, NGL complexes containing different oligosaccharides are spontaneously and stably adsorbed to the hydrophobic surface of ELISA microwells. This method enabled direct M1g1 binding studies to be performed with

oligosaccharides in the solid phase. The products of NGLs were analyzed by thin-layer chromatography. Individual components were visualized either under UV light, according to their lipid component, or after orcinol staining according to their carbohydrate components (data not shown). Binding activities of M1g1 to different NGLs adsorbed onto ELISA plates were tested. The absorbance of M1g1 binding at 450 nm was expressed as binding activity. The binding activity of M1g1 was the highest for β-1,2-linked mannotriose, followed by β-linked mannobiose and mannose (Figure 5). In contrast, M1g1 did not show measurable binding activities for other α- and β-linked oligomannosides (data not shown). All data shown here are consistent with the competition ELISA data, suggesting that the epitope of M1g1 is restricted to β-1,2-linked oligomannosides, specifically β-1,2-linked mannotriose.

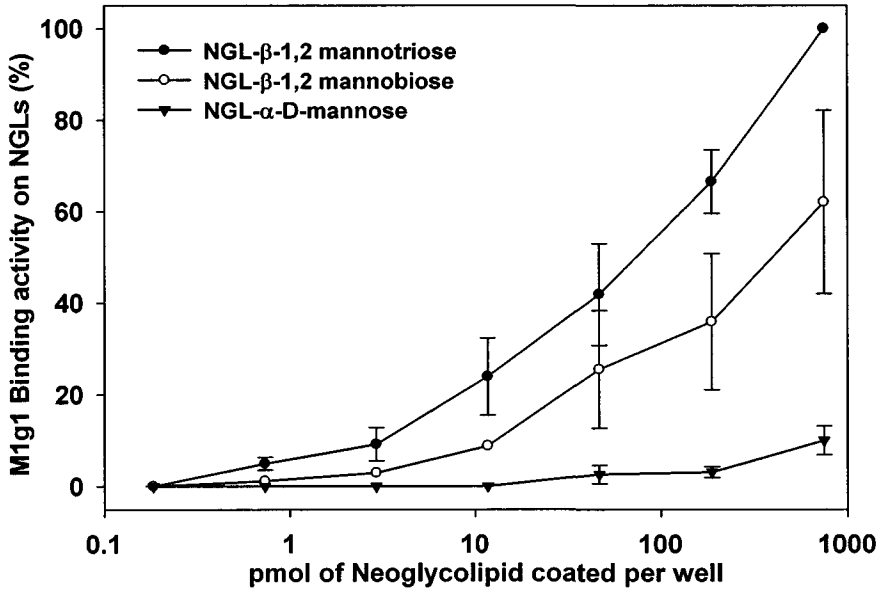

FIGURE 5. Comparison of M1g1 binding activity for different neoglycolipids in the solid-phase of ELISA. Error bars represents three independent experiments. Binding activity of M1g1 to β-1,2 linked mannotriose was considered as 100%.

The data presented above indicate that the epitope of the human recombinant antimannan mAb M1g1 is β-1,2-linked mannotriose. In order to obtain structural information and verify the identity of the M1g1 epitope as β-1,2-linked mannotriose, NMR spectroscopy was used. In carbohydrate NMR, typical ^{1}H NMR chemical shifts of carbohydrate ring protons are 3-6 ppm. Most of the resonances are clustered between 3-4 ppm which mainly contains non-anomeric protons (Bubb, 2003). As expected (Figure 6), the NMR spectrum from 3.3-4.0 ppm had chemical shift overlaps which made it difficult to assign individual proton signals. However, the ^{1}H NMR spectrum of β-1,2-linked mannotriose contained some well-resolved signals from anomeric protons which were the most diagnostic component of the spectrum. Table 2 and Figure 6 show that the signals from the anomeric ^{1}H of all three mannose residues were detected.

Two sets of three chemical shifts were detected in the anomeric region of the spectrum: the first set represents the mannotriose with anomeric protons having the α configuration at its reducing terminus, (III)Man-β-1,2- (II)Man-β-1,2-(I)Man-α; the other set represented the mannotriose with anomeric protons having the β configuration at its reducing terminus instead, (III)Man-β-1,2-(II)Man-β-1,2-(I)Man-β. It was also clear from the spectrum that the two forms of the mannotriose were present in the sample in equilibrium, but the majority was of α anomer. Comparing the measured chemical shifts of proton 1 and 2 of the three mannose residues from mannotriose to those published data, the results confirmed that the M1g1 epitope was identical to the β-1,2-linked mannotriose described by (Han et al., 1997), (Faille et al., 1990, 1992a), and (Shibata et al., 1992a, 1992b,1993).

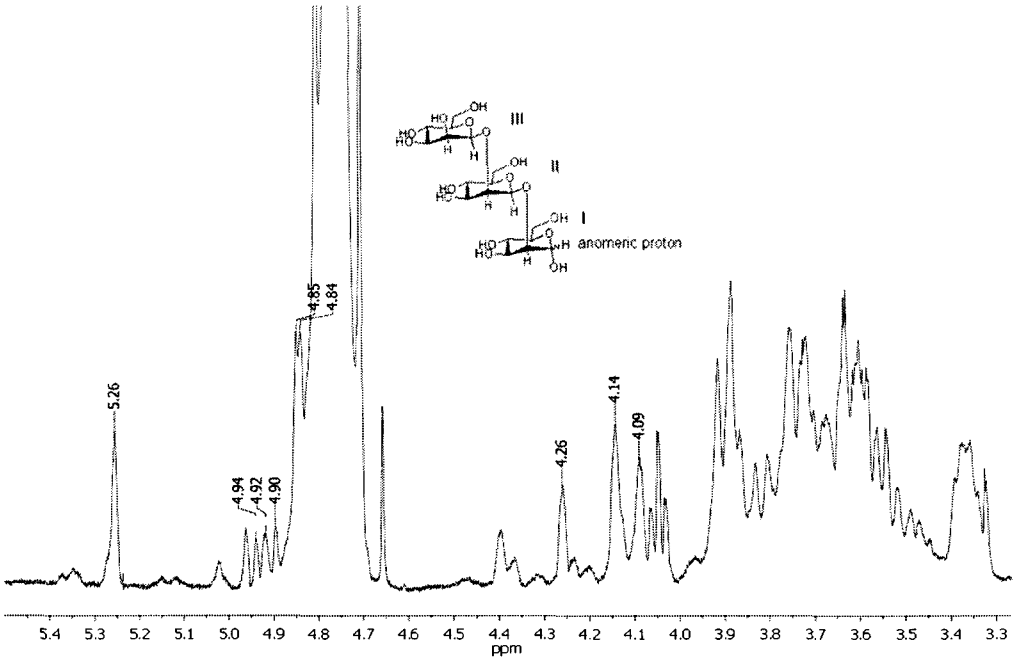

FIGURE 6. 400- MHz partial ^{1}H NMR one dimensional spectroscopy spectrum of β-1, 2 linked mannotriose with chemical shifts labeled. The cartoon represents the drawing of β-1, 2 linked mannotriose, and roman numerals refer to the Man I, Man II, and Man III residues.

TABLE 2. Experimental ^{1}H NMR Chemical Shifts and Literature's Data for β-1, 2-linked Mannotriose Isolated from *C. albicans* Serotype A (strain 3153A)

β-1,2-linked mannotriose	Proton 1						Proton 2		
	α anomer			β anomer					
	This work	Ref 1	Ref 2	This work	Ref 1	Ref 3	This work	Ref 4	Ref 3
Man I	5.26	5.27	5.28	4.94	4.97	4.98	4.09	4.10	4.16
Man II	4.84	4.84	4.86	4.90	4.90	4.91	4.26	4.27	4.41
Man III	4.85	4.85	4.87	4.92	4.94	4.95	4.14	4.15	4.15

Ref 1: Shibata et al., (1992 a,b); Ref 2: Faille et al., (1990); Ref 3: Faille et al., (1992 a); Ref 4: Shibata., et al (1993).

Three-Dimensional Homology Model of the Variable Region of mAb M1g1

Analysis of genomic DNA of mAb M1g1 Fv region identified the heavy chain sequence as VH3 family gene 74*02 and the light chain sequence as Vk2 family gene 28*01. The RosettaAntibody Server identified the heavy-chain residues 26-35 (H1), 50-66 (H2), 100-108 (H3) and light-chain residue 24-40 (L1), 56-69 (L2), 105-113 (L3) as the CDR-forming amino acids (Table 3). Molecular modeling of the Fv region of mAb M1g1 revealed structures typically found in an immunoglobulin fold (Novotny et al., 1983), two β sheets that pack closely to form a cylindrical barrel (Figure 7). In the center of the antibody, a large and deep cavity is present, which is a possible antigen binding site and is formed primarily by residues of CDRs. The 3D model of mAb M1g1 antigen binding site (Figure 7), obtained from the RosettaAntibody Server, passed standard quality tests for protein structures. These tests included RAMPAGE for a geometric evaluation of Phi and Psi backbone torsion angles (Lovell et al., 2000) and PROBITY tool (Davis et al., 2004) for a comparison of dihedral angles of side chains to rotamers frequently found in high-resolution protein structures (data not shown). Therefore, this M1g1 model was subsequently used for ligand docking simulations.

Molecular Modeling of Mannose and β-1,2-linked Oligomannosides

Mannan epitopes consisting of mannose or β-1,2-linked oligomannosides (from mannobiose to mannohexose) were modeled using the software W3-SWEET (Figure 8). Compared to the NMR and molecular dynamics determined solution structure of β-1,2-linked oligomannosides (Nitz et al., 2002), the W3-SWEET models also displayed a helical character for this type of oligomannoside chain. This type of

β-1,2-linked oligomannoside conformation was also confirmed by the crystal structure of a synthetic tetrasaccharide by Crich and his colleagues (Crich et al., 2001). Therefore, theses computer generated oligomannoside models can be treated as ligands in docking studies.

TABLE 3. The Experimentally Determined Amino Acid Sequence of the Fv Region of the Heavy and Light Chains of mAb M1g1.

H1 Framework (1-25)	**CDR H1 (26-35)**	**H2 Framework (36-49)**	**CDR H2 (50-66)**	**H3 Framework (67-99)**	**CDR H3 (100-108)**	**H4 Framework (109-118)**
VQLLEESGG GLIQPGGSL RLSCEAS	GFAFS RYWM H	WVRQVPG KGLVWVS	RTNED GSWTN YADSV RG	RFTISRDN AKNTLYL QMNSLRV EDTGVYY CAR	DVCGA DDQ	WGRGTLV TVS
L1 Framework (1-23)	**CDR L1 (24-40)**	**L2 Framework (41-55)**	**CDR L2 (56-69)**	**L3 Framework (70-104)**	**CDR L3 (105-113)**	**L4 Framework (114-121)**
ELVLTQSPL SLSVTPGEP ASISC	RSSQSL LHSNG DHWLA	WYLQKPG QSPQVLIY	LGTNR AS	GVPDRFS GSGSGTD FTLKISRV EAEDVGV YYC	MQGLQ TSYT	FGQGTKLE

Note: Framework and CDR residues were determined by RosettaAntibody server. H and L represent heavy and light chains of the antibody. The brackets indicate amino acid numbering.

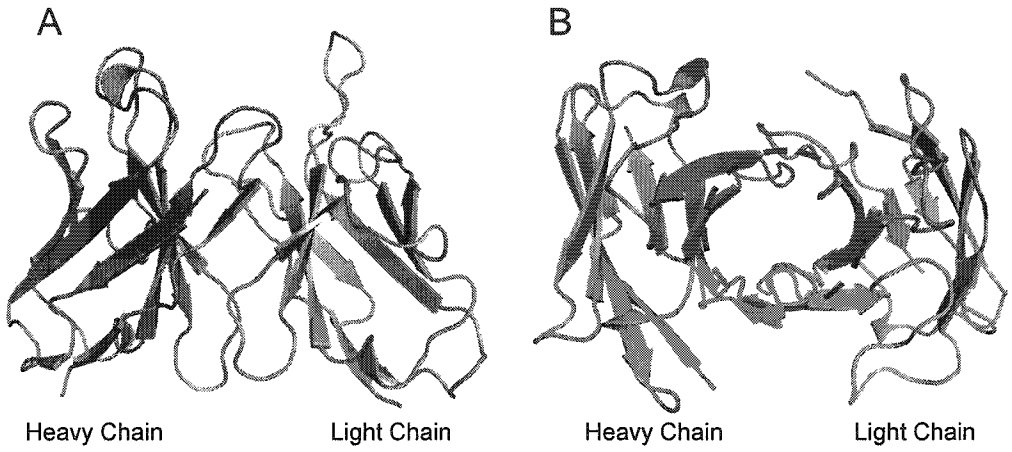

FIGURE 7. A 3D homology model of the Fv region of mAb M1g1 in ribbon format. (A) A side view of the heavy chain in green and the light chain in blue. (B) A perpendicular view of the Fv shown in (A). The putative antigen binding site is circled.

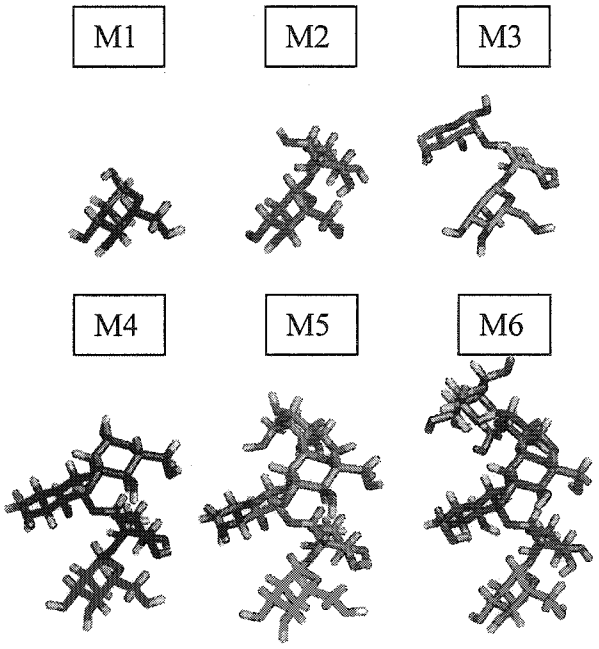

FIGURE 8. Structures of mannan epitopes modeled by W-3 SWEET program. M1 represents mannose and M2-M6 represent β-1,2-linked mannosides from biose to hexose.

Molecular Docking of M1g1 and its Epitopes

The binding mode of mannan epitope and interactions with mAb M1g1 were studied by using Autodock Vina (Trott et al., 2010). Docking of mannose to M1g1 revealed the possible binding mode of the terminal mannose by M1g1. The docking result (Figure 9: A, B and C) shows that the mannose was positioned in the cavity formed by CDRs of heavy and light chains, with the majority of contacting residues from the H3 CDR loops. The binding affinity was the result of two basic contributions arising from antibody-carbohydrate hydrogen-bond interactions with the hydroxyl group and the van der Waals interactions with the hexose carbon ring (Figure 9D). The mannose appears to be stabilized by seven direct hydrogen bonds formed between all hydroxyl groups within the CDR H1, H3 and CDR L3 loops. Four of these bonds are from residues Asp100, Gly103, Ala104 of CDR H3 loops, the other CDR H3 residues Val101 and Cys102 seem to be involved in the van der Waals interactions with the mannose. The only residue from CDR L3 is Tyr112; it seems to have an important role in binding as it provides a protonated imidazole ring for formation of a hydrogen bond with O1 of mannose. Thus, the terminal mannose is well situated in a hydrogen bonding network formed by M1 CDR loops (Figure 9). The other important feature is that the anomeric C-1 carbon of the bound mannose (which is linked to downstream mannose units in the mannan cell wall) extends away from the antibody binding cavity. In consequence, the 2^{nd} or 3^{rd} mannose unit in the M1-disaccharide and trisaccharide complex is likely positioned at the exterior of the Ab binding cavity and makes fewer contacts with the Ab.

When β-1,2-linked mannotriose was docked into the binding site of M1g1, it was apparent that mannotriose can be nicely fitted into the binding site. The deep cavity formed by CDRs is able to accommodate not only the terminal mannose, but also the 2nd and 3rd mannose residues. One important feature that needs to be emphasized is that the helical nature of β-1,2-linked mannotriose enabled the three residues of mannose to be orientated to be packed into the binding site. Furthermore, the 2nd and 3rd mannose residues also participate in forming hydrogen bonds and van der Waals interactions with M1g1 CDRs amino acids. Figure 10 (D) shows that the second mannose unit forms three H-bonds with Trp33 of CDR H1, Arg50 of CDR H2, and Thr94 of CDR L3. For the third mannose subunit, Asp53 forms two H-bonds with hydroxyl OH3 and OH4 (Table 4). When longer oligomannosides range from β-1,2-linked mannotetrose to mannohexose were docked into the binding site of M1g1, the 4th mannose residues made very few contacts with CDR amino acids, while the 5th and 6th residues had no direct contact with M1g1 and were exposed to the solvent (data not shown). Therefore, it is apparent that the binding site of M1g1 is capable of accommodating oligomannoside with the specific size of a triose.

Using Fab Variants to Evaluate the Importance of Interacting Residues in Antigen Binding

The molecular docking of β-1,2-linked mannotriose to M1g1 identified a number of amino acids from the CDR regions of both heavy and light chains crucial for antibody-antigen binding. In this study, extensive mutational analysis of the antigen binding site of M1 Fab was carried out to investigate the validity of the model and the binding specificity of M1g1 for β-1,2-linked mannotriose. The candidates for

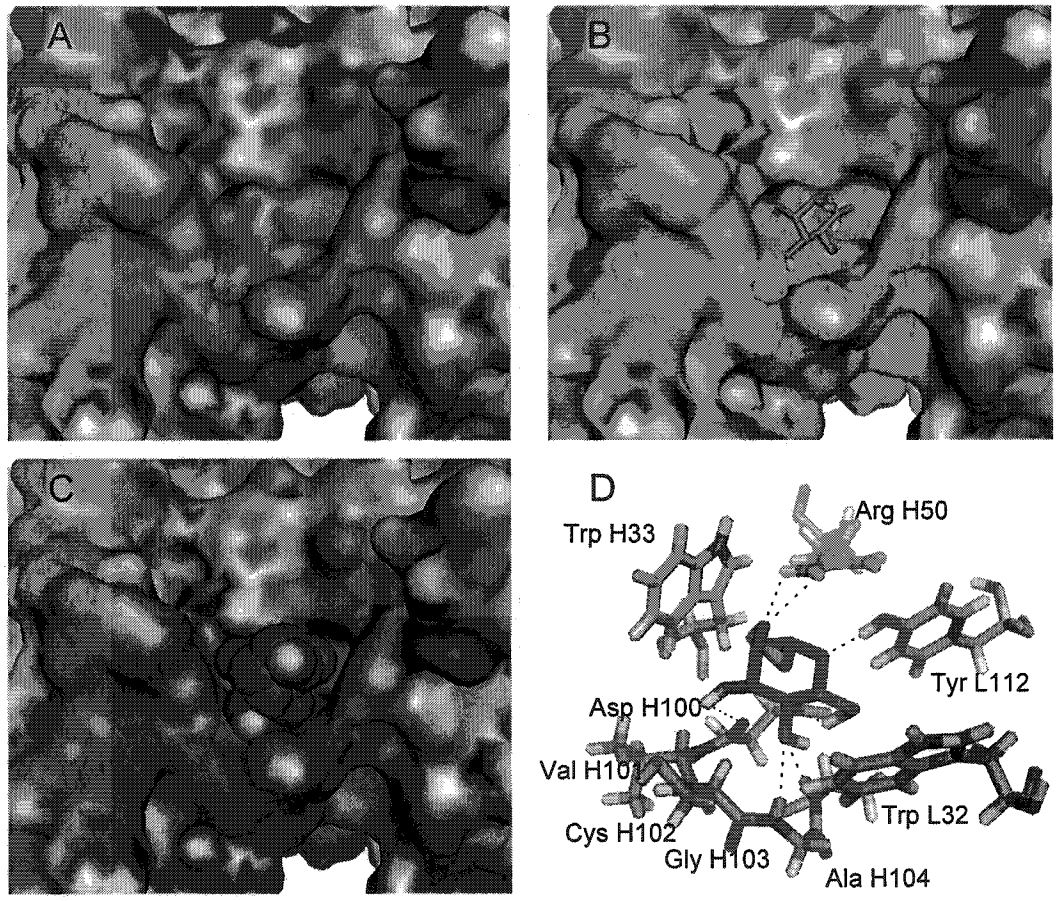

FIGURE 9. Molecular surface images of the modeled mAb M1g1 Fv structure (A) without the ligand mannose, (B) with the docked ligand in a stick representation and, (C) with the docked ligand in a spherical representation. (D) Amino acids from CDRs interacting with the terminal mannose. Dash lines indicate possible hydrogen bonds.

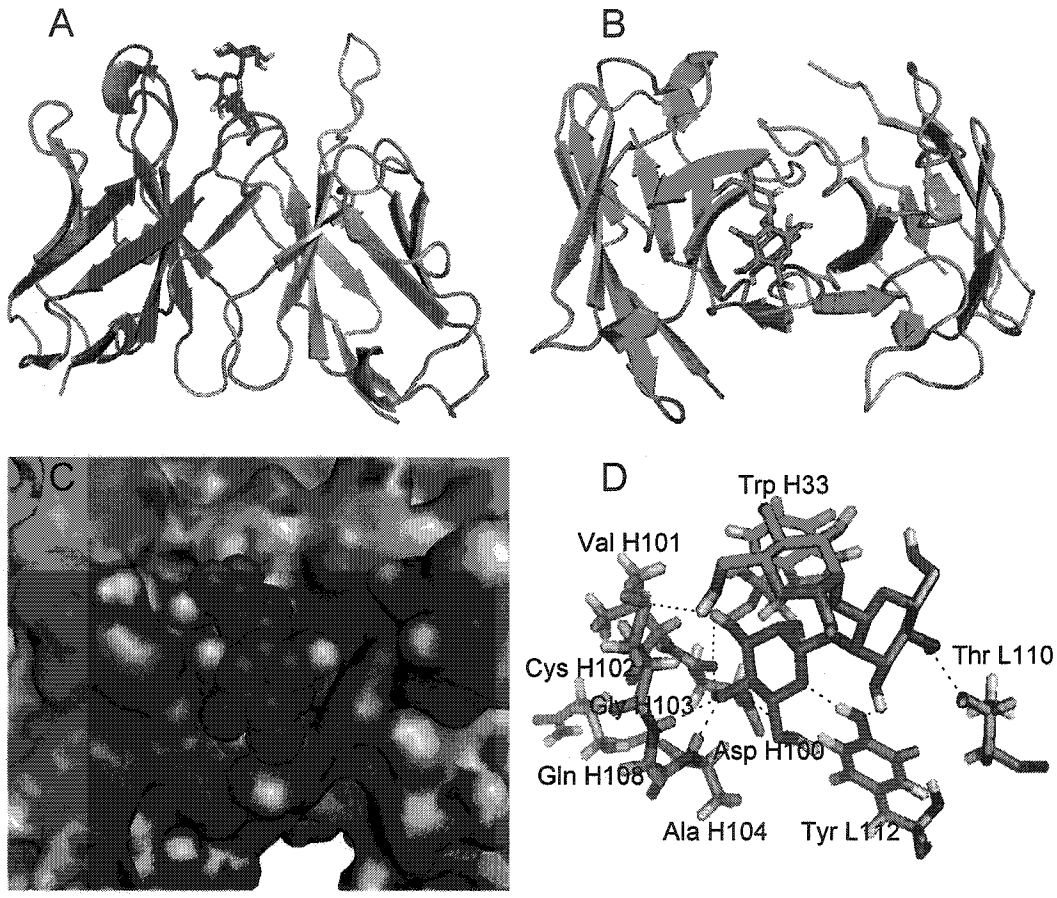

FIGURE 10. Molecular ribbon and surface images of the modeled mAb M1g1 Fv structure with a docked β-1,2-linked mannotriose. (A) A side view, (B) a top view, (C) a spherical representation of the molecular surface of M1, and (D) amino acids from CDRs that interact with β-1,2-linked mannotriose (dash lines indicate possible hydrogen bonds).

TABLE 4. Summary of Hydrogen Bond Interactions between M1g1 and Oligomannosides Residues

SUGAR ATOM	RESIDUES	ATOM	DISTANCE ( )
Terminal Mannose			
O-5	Tyr L112	OH	2.22
O-2	Arg H50	NH	2.72
O-2	Arg H50	N	2.86
O-3	Asp H100	O	3.29
O-4	Gly H103	NH	2.92
O-4	Ala H104	NH	1.94
O-6	Asp H100	O	2.47
Second Mannose			
O-5	Arg H50	NH	2.79
O-4	Thr L110	OH	2.66
O-6	Arg H50	NH	2.88
Third Mannose			
O-3	Asp H54	O	2.84
O-4	Asp H54	O	3.78

mutagenesis were selected from the interacting amino acids from docking studies. Nucleotide sequence analysis of the mutated Fab fragments revealed no changes in amino acid sequence of the variable regions other than the intended ones. All mutant Fabs were expressed in *E. coli* cells and produced in a soluble form in the culture supernatant, facilitated the rapid analysis of a large number of mutants.

M1 Fab variants were compared to the wild type in apparent affinity by ELISA (Figure 11). CDR-H3 D100A mutation resulted in a complete loss of binding of M1 Fab to mannan. Glycine at H103 of CDR was examined next to determine its role in epitope interaction when it was mutated to alanine, serine, and proline. G103A retained about 70% binding, while G103S and G103P resulted in significant loss of binding affinity, suggesting that G103 has a significant role in epitope binding. A104G mutant affected binding affinity by about 50%. Mutants of CDR L3 both showed reduced apparent affinities, especially, Y112A mutants, which completely lost binding affinity for mannan. T110S and T110A mutants retain binding affinity, roughly 40% and 20% of the wild-type, respectively. The mutant Q108L, which involved an amino acid that has no direct contact with antigen based on docking studies, showed no apparent effects on affinity and retained the same binding affinity as the wild-type. Therefore, site-directed mutagenesis of interacting residues identified through docking studies confirmed the requirement of these amino acids for recognition of β-1,2-linked mannotriose.

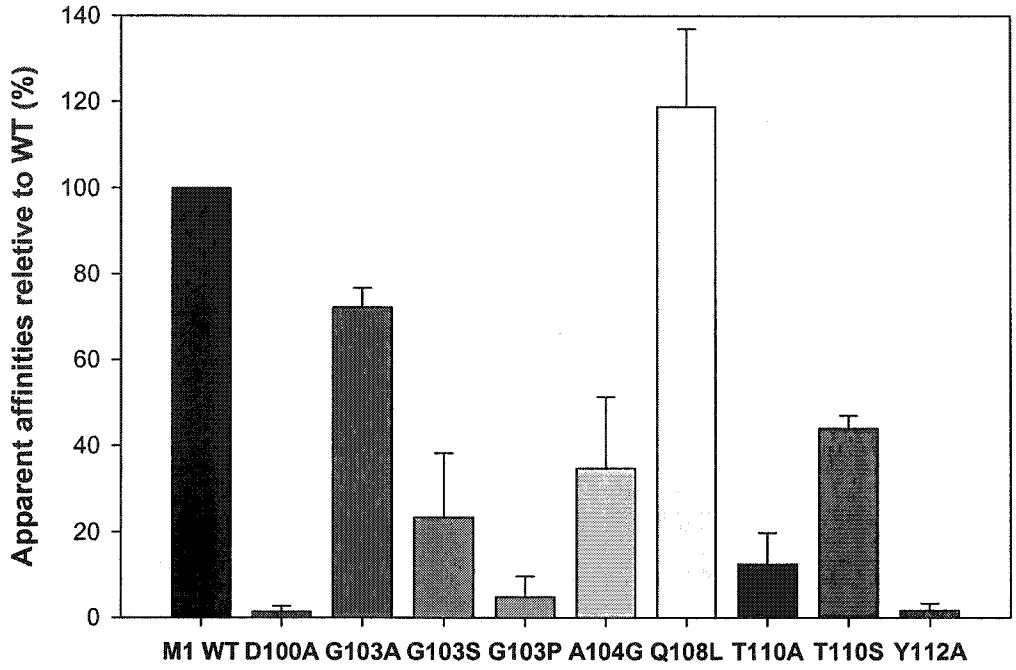

FIGURE 11. Binding of M1 Fab variants to *C. albicans* mannan. Selected amino acids of the wild type M1 Fab were replaced through site-directed mutagenesis. Bars indicate the apparent affinities of Fab mutants relative to that of wild-type (WT) M1 Fab for mannan. The data are means from two independent experiments ± the standard error of the means.

Detection of Naturally Occurring M1g1-like Antibodies in Normal Human Adults

In order to detect and characterize naturally occurring antibodies that share the same binding specificity as M1g1, *C. albicans* mannan serotypes A (strain 3153A) and B (strain CA-1) and mannan-derived β-1,2-linked mannotriose were selected for screening using ELISA. Sera from 30 normal, healthy adults were examined to determine the distribution of antimannan IgG for serotype A, B and β-1,2-linked mannotriose. The results (Figure 12) show a median IgG titer of 1/936 (range from 1/6 to 1/66,000) for antimannan serotype A, 1/62 for antimannan serotype B (range

from 1/13 to 1/1,300), and 1/652 for anti β-1, 2 linked mannotriose (range from 1/27 to 1/9,000). Overall, the titers were normally distributed around the medians, with antimannan serotype A IgG having the highest titers, followed by anti β-1,2-linked mannotriose titers and antimannan serotype B.

A correlation coefficient was used to assess the relationships between antibodies recognizing antimannan serotypes A, B and β-1,2-linked mannotriose. There was a significant correlation ($P < 0.001$) among titers of antimannan IgG of serotype A, B and β-1,2-linked mannotriose (Figure 13). For all three titers, the general pattern was that individual sera with a high IgG titer for mannan serotypes A, and B usually corresponded to a high titer for β-1, 2 linked mannotriose, and was true vice versa. However, a few serum samples did not follow such a pattern. The levels of antimannan IgG against *C. albicans* serotype A and serotype B mannan were compared based on titers. The results (Figure 13) showed a high degree of correlation between mannan A and B ($P < 0.001$). However, titers of serotype A antibody were much higher than that of serotype B antibody (about 10 fold). This result is consistent with the previous observation by Kozel et al., (2004). The above result presented evidence for the presence of human naturally occurring antibody that has epitope specificity for *C. albicans* mannan, more specifically β-1,2-linked mannotriose located in the mannan structure.

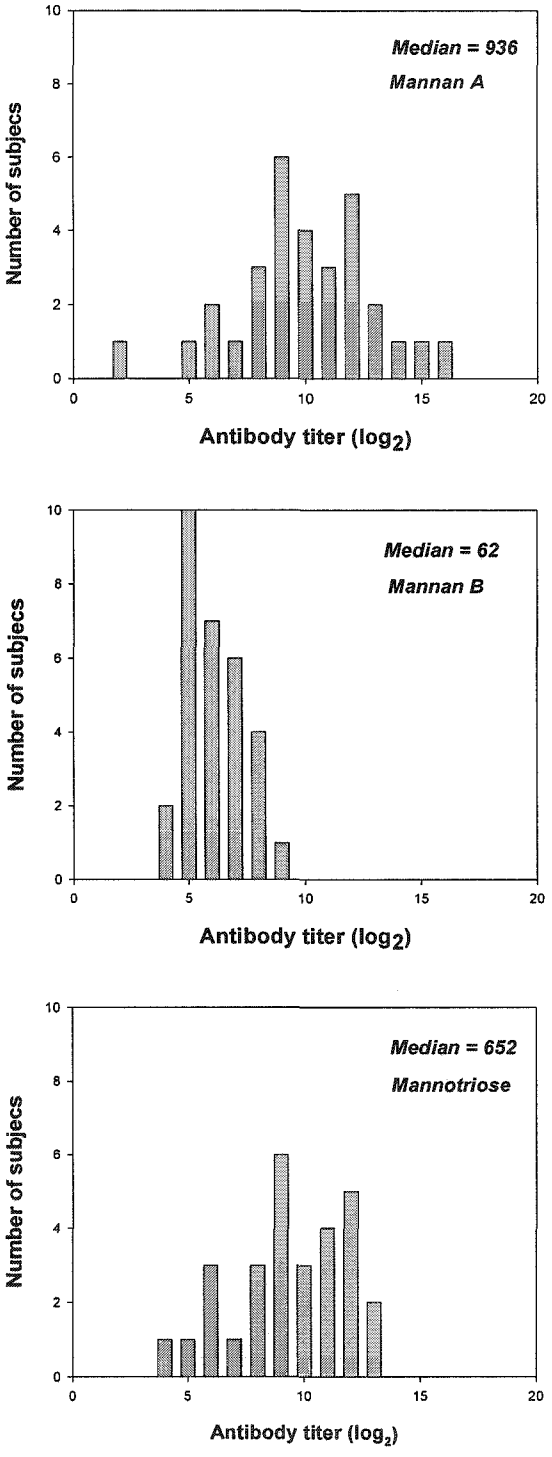

FIGURE 12. Distribution of antimannan antibody titers in 30 normal adults. Levels of antimannan antibody for serotype A (strain 3153A), serotype B (strain CA-1) or β-1,2-linked mannotriose were determined by ELISA.

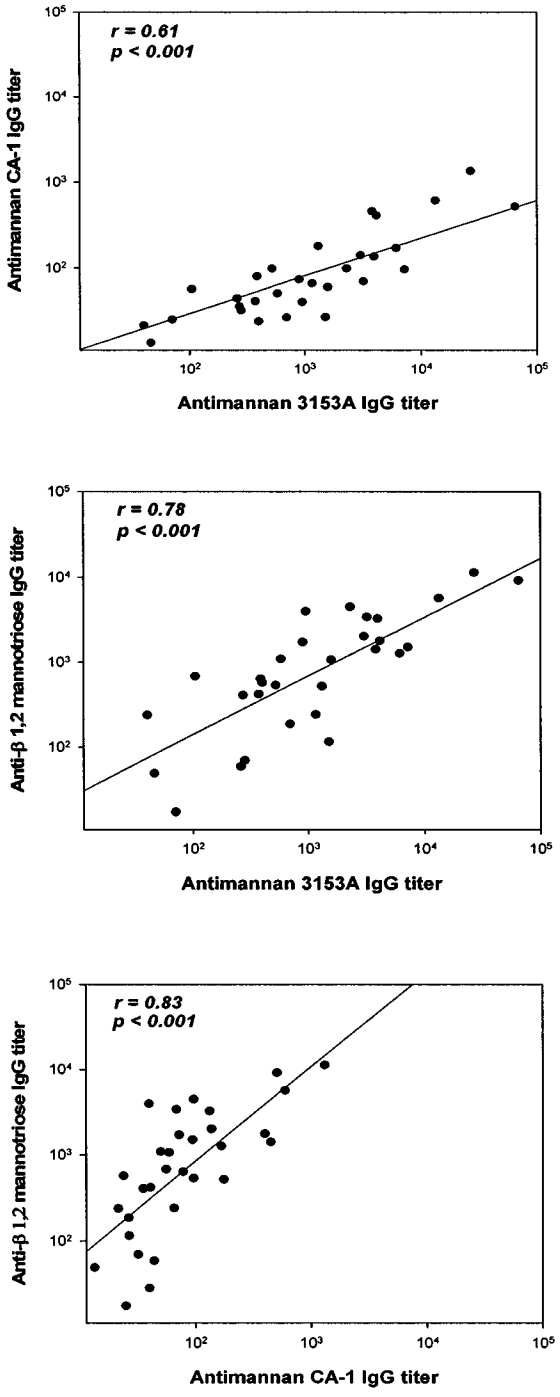

FIGURE 13. Correlation between antimannan antibody titers for serotype A (strain 3153A), serotype B (strain CA-1), or β-1,2-linked mannotriose.

CHAPTER 4

DISCUSSION

The first human recombinant antimannan antibody M1g1 is protective against systemic candidiasis in a murine model, but its specific epitope has not yet been determined. The approach to this question was to use immunochemical assays, molecular modeling and docking to identify the specific epitope of M1g1. In this study, competitive ELISA and NGL binding ELISA data both demonstrated that M1g1 recognizes β-1,2-linked oligomannosides derived from *C. albicans* cell wall mannan (Figures 4 and 5). Molecular modeling and docking incorporating site-directed mutagenesis also predicted the interactions between M1g1 and its mannan epitope (Figures 10 and 11). To my knowledge, it is the first model predicting the binding mode of human antimannan antibody with its mannan epitope. Lastly, NGL binding ELISA using normal human sera identified the presence of M1g1-like antibodies (Figures 12 and 13). These observations demonstrated that the specific epitope for the protective antimannan antibody M1g1 is β-1,2-linked mannotriose.

Interestingly, the M1g1 epitope β-1,2-linked mannotriose determined here is the same as that of a protective murine antimannan mAb B6.1 (Han et al., 1997). NMR spectroscopy of the mannotriose produced chemical shifts in the 1D proton spectrum that matched the NMR data for the β-1,2-linked mannotriose characterized by (Han et al., 1997), (Faille et al., 1990, 1992b), and (Shibata et al., 1992 a, 1992b.,1993). The

data presented here indicated that all three mannose residues of M1g1 epitope are also covalently linked through β-1,2-linkages. Although these two antimannan antibodies are from different species, both antibodies are reactive with β-1,2-linked oligomannosides uniformly distributed on the cell surface mannan of *C. albicans* serotype A and B. Importantly, both have protective roles in the mouse model of candidiasis.

According to the latest mannan structure proposed by (Shibata et al., 2007), β-1,2-linked oligomannoside is found in both acid labile and acid stable parts of *C. albicans* serotype A mannan. However, such linkage is only present in the acid labile part of *C. albicans* serotype B mannan. More importantly, it has been reported that the M1g1 epitope is a stable component of *Candida* mannan and has a broad distribution among *Candida* species (Zhang et al., 2006). Among the ten mannan antigenic factors identified for the rabbit immune system, factors 5, 6 and 9 all contain β-1,2-linked oligomannosides. In *C. albicans*, serotype A contains two of the antigenic factors (5 and 9) found in serotype B as well as an additional antigenic factor not present in serotype B. This additional antigenic determinant is factor 6 located in the acid-stable part of mannan, which consists of internal α-1,2-linked oligomannosides with up to three β-1,2-linked mannose residues at the non-reducing ends (Suzuki, 1997). In other *Candida* species, factor 5 is found in two *Candida* species (*C. albicans* and *C. tropicalis*), factor 6 is found in three *Candida* species (*C. albicans*, *C. tropicalis*, and *C. glabrata*), and factor 9 is found in *C. guilliermondii* (Suzuki, 1997). Apparently, M1g1 is able to recognize factor 5, 6 and 9 which are all common to a total of four *Candida* species. Thus, the fact that M1g1 can recognize

one of the most common *Candida* mannan epitopes can have great importance, because a broadly binding specificity for M1g1 is an attractive feature when considering it to be a potential prophylactic or therapeutic application of passive immunization against candidiasis.

C. albicans mannan is immunogenic. There are naturally occurring antibodies in normal human adults reactive with specific mannan epitope (Zhang et al, 2006; Kozel et al, 2004; Lehmann et al, 1980). In this study, experiments were carried out to detect anti- β-1,2-linked mannotriose antibodies in 30 normal human adults. Results show that anti β-1,2-linked mannotriose (M1g1-liked) antibody is present (IgG isotype) in human serum. The anti-β-1,2-linked mannotriose antibody titer correlated well with antimannan serotypes A and B antibodies. However, the titer of such antibodies is in between the titers of antimannan A and B antibodies. Such observations are not surprising, because it is reasonable to suspect that among many naturally occurring antibodies recognizing cell wall mannan in human serum, only some portion of them are specific for β-1,2-linked oligomannoside, while others are specific for other mannan epitopes such as α-linked oligomannosides (Casadevall and Pirofski, 2007). Furthermore, from common clinical observations in human candidiasis patients, the natural antimannan antibody repertoire in human serum does not confer anti-*Candida* protection, suggesting that inadequate amount of protective antibodies and/or the presence of both protective and nonprotective antibodies in human serum to play a significant role in natural protection against candidiasis (Casadevall and Pirofski, 2007).

Currently, the crystal structures of antimannan antibodies have not been elucidated, limiting the understanding of structural aspects of antimannan antibodies. However, crystallography studies of anti-bacterial polysaccharides antibodies provide important structural insights for anti-carbohydrate antibodies. For example, antibody Se155-4 recognizing the *Salmonella* serotype B O-antigen with an octasaccharide (Cygler et al., 1991), mAb S-20-4 whose specific epitope is a diasaccharide from LPS of *Vibrio cholera* (Villeneuve et al., 2000), mAb SYA/J6 recognizing *S. fleneri* Y cell wall lipopolysaccharide (Vyas et al., 2002), mAb F22-4 against hexasacharride from LPS cell wall of *Shigella flexneri* (Vulliez-Le Normand et al., 2008) and the latest mAb C.S-35 which is specific for lipoarabinomannan (LBM) of mycobacteria cell wall (Murase et al., 2009). These studies revealed important features of anti-carbohydrate antibodies, the size and nature of the epitope, and their relationships to the binding-site topology. Based on available structures of anti-bacterial polysaccharides antibodies, Cygler et al. (1994) summarized the features of binding sites of anti-carbohydrate antibodies including: the presence of aromatic residues such as tyrosine and histidine in the complementarity-determining-regions (CDR). Tyr and Trp side chains in the CDR frequently provide interacting surfaces for the antigens, while asparagines are involved in the intramolecular interactions and hydrogen bonds to the backbone atoms or other side chains. About 35 years ago, Kabat and coworkers proposed that antibodies recognizing linear polysaccharide chains through their terminal or non-terminal sugar residues could display either groove or cavity shape binding sites (Cisar et al., 1975). The carbohydrate epitope could vary from one to seven residues. It was later suggested that small epitopes (one to three residues)

generally including the non-reducing terminal sugar residue, bind to a cavity type antigen binding sites; whereas larger epitopes (four to seven residues) which are located along the length of the polysaccharide chain, are recognized by a groove-type antigen binding sites (Padlan et al., 1988).

Molecular modeling and docking have been used to study the recognition of mannan epitope by M1g1. Molecular modeling of M1g1 Fv region and its mannan epitopes using the RosettaAntibody Server and the W3-SWEET program, resulted in reasonable models for computational docking. The 3D modeling revealed that the M1g1 binding site is a cavity shape and can accommodate small-size mannan epitopes. This feature is in agreement with previous structural observations. After computational docking of β-1,2-linked mannotriose to the M1g1 binding site with Autodock Vina, the result revealed the possible binding mode and interactions between M1g1 and its epitopes. Important interacting amino acids were also identified near the CDR loops of M1g1, and these residues included tyrosine, tryptophan, asparagines and arginine which are commonly present in the binding sites of anti-carbohydrate antibodies as reported by Cygler et al., (1994).

In order to validate the molecular modeling and docking results, site-directed mutagenesis of these identified interacting amino acids were carried out to assess their importance in antigen recognition. Mutations in CDR H3 residues were examined. D100A mutation resulted in a complete loss of binding to mannan. According to the 3D model, D100 is the first residue of the CDR H3 and its side chain is partially exposed to the solvent; most importantly, its side chain forms the base for the binding cavity to accommodate the terminal mannose of the epitope. Mutation of D100 to Ala

residue likely caused the collapse of the binding cavity; it also may be possible that the exposure of hydrophobic Ala to the solvent causes partial unfolding of the Fab. Since M1 recognizes β-1,2-linked mannotriose as its epitope, upon binding the terminal mannose is completely fitted into the binding cavity. If the cavity is disrupted, M1 would likely lost affinity to the epitope, thus loss the ability to bind mannan.

Mutation G103 was examined next to determine its role in epitope interaction. The docking model indicates that Gly103 is located on the apex of the CDR H3 turn and the amide group of Gly103 backbone forms only one hydrogen bond with hydroxyl # 4 of terminal mannose. The G103A substitution which retains the backbone NH involved in hydrogen bonding has about 70% binding to mannan compared to wild-type, which was not a significant loss considering the mutation is on CDR H3. It is possible that G103 at this position is an important residue in forming the correct binding cavity, and it provides a hydrophobic environment for accommodation of the mannose ring. Furthermore, G103 is important in forming the correct beta hairpin in the CDR H3 region due to its intrinsic flexibility (Kuroda et al., 2008). When G103 was substituted by Ala, which contains a hydrophobic methyl group, the hydrophobic environment was still preserved, but its cavity shape or size may be altered by the addition of a methyl group. This would be possible explanation for the small loss of affinity to mannan. To further confirm this hypothesis, G103 was mutated to Ser which contains a bulkier polar site chain. Indeed, the G103S mutant bound about 10 fold less to the mannan than did the wild-type M1 Fab (Figure 9). When a G103P mutation was introduced, the mutant Fab failed to bind to mannan. Because of the intrinsic bend of Pro residues, its substitution in CDR H3 possibly

impaired the correct formation of the beta-turn. What's more, introduction of Pro in this position eliminated a hydrogen bond due to the missing peptidyl amide group of Pro. G102P greatly affected the binding cavity. Another interesting point is that Pro residue at this position is present in the closest germline sequence of M1. Therefore, it is reasonable to speculate that during the M1 antibody maturation process, Gly was selected preferably to have an effect in forming the correct binding site to fit the epitope.

In the binding model, A104 is located at the base of the CDR H3 C-terminal end and forms one hydrogen bond with the terminal mannose residue. Its role in epitope recognition is to use the methyl side chain to create a non-polar environment for the terminal mannose hexose ring. When this methyl side chain is abolished by Gly substitution, epitope recognition was retained, but the binding affinity dropped about five fold. The last CDR H3 mutation Q108L did not cause any impairment in binding to mannan, which indicated that Q108 was not involved in antigen-antibody interaction. It was clearly indicated in the docking model that Q108 is at the end of CDRH3 and has no contact with the terminal mannose.

Mutations in CDR L3 residues were also studied. In the docking model of β-1,2-linked mannotriose and M1 Fab, a majority of antigen-antibody interactions come from a hydrogen bonding network formed by CDR H3 residues. There are several important residues which could play roles in binding to the epitope. Residues T110 and Y112 were chosen for substitution to Ala. Both mutations resulted in significant impairments in mannan binding in which binding affinities were decreased more than 10 fold for T110A, and no binding was observed for Y112A (Figure 9). The docking

model showed that hydroxyl group of T110 forms an additional hydrogen bond with the second mannose unit in β-1,2-linked mannotriose. In order to confirm this, T110 was mutated to Ser, having a similar side chain which also contains a hydroxyl group. As predicted, the T110S mutant showed a similar (~ 80%) affinity to mannan compared to that of wild-type M1 Fab. Y112 is an important residue in forming the binding cavity of M1, and its hydroxyl group also forms a hydrogen bond with the O-5 of the terminal mannose ring. Binding to mannan would be greatly affected if this residue was mutated. As expected, the Y112A mutant had no observable binding to mannan. Overall, the modeling and docking results showed some important features for the specificity of M1g1 to β-1,2-linked mannotriose, in which the amino acids in the CDRs of the M1g1 binding site form an organized hydrogen bonding network, plus the favorable van der Waals and hydrophobic interactions specific for the accommodation of β-1,2-linked mannotriose.

The structure of β-1,2-linked mannotriose modeled by W3-SWEET was very similar to the modeled structure of synthetic β-1,2-linked mannopyranan oligosaccharides (Nitz et al., 2002). Both models displayed a helical character in this oligosaccharide chain (Figure 6), in which approximately every three mannose residues represent one repeating unit. It is of interest to consider the implications of oligomannosides adapting such a conformation. The helix hides the hydrophobic ring structures from exposure to solvent, except for the terminal mannose residues, which are available for the binding of oligomannosides by antibodies, where hydrophobic interactions can be important for antibody-antigen interaction. The molecular docking of mannose to M1g1 in this study demonstrated such a possibility. About four

decades ago, a paradigm of anti-carbohydrate antibodies first reported by Kabat E.A., proposed that in antibodies raised against the polymeric dextran antigens, the inhibitory power of oligosaccharides increased as the size of the inhibitor increased and reached a plateau at about the size of a hexamer (Kabat, 1966). Contrary to this findings, M1g1 exhibits significantly higher affinity for β-1,2-linked mannotriose, but not its longer or shorter counterparts . There are no patterns in inhibitory power observed when considering the length of oligomannosides. The same contradiction was also reported for murine antimannan mAb B6.1 (Nitz et al., 2002). The likely explanation for these observations mainly depends on the diversities of antibody binding sites and binding modes between antibody and carbohydrate epitope. It has been reported that there are two general types of antibody binding site: groove and cavity, which are predicted for carbohydrate epitopes (Lee et al., 2006; Padlan et al., 1991). The groove type binding site can recognize linear polysaccharide chains though either terminal or non-terminal sugar residues and might be expected to have a binding affinity that steadily increases with epitope length as proposed by Kabat, E.A. (Villeneuve et al., 2000). Indeed, there are such examples studied by crystallography (Vyas et al., 2002; Bundle et al., 1994; Vulliez-Le Normand et al., 2008). As predicted by computer modeling, the M1g1 binding site exhibits a cavity that can accommodate mannotriose specifically and therefore its affinity does not correspond to the epitope length. Cavity-type antibody binding sites were also been studied by crystallography (Villeneuve et al., 2000; Ramsland et al., 2004; Calarese et al., 2005).

Therefore, based on these findings, the following binding mode of M1g1 is proposed here: protective human recombinant antibody M1g1 recognizes short

oligosaccharide sequences (β-1,2-linked mannotriose) which are terminal mannose residues of larger oligomannosides present in the outer layer of the mannan cell wall of *C. albicans*, because given the conformation of the β-1,2-linked oligomannosides, it may be difficult for M1g1 to recognize the internal mannose residues in the mannan. Subsequently, it is not surprising to suspect that the expression of significant amounts of low molecular weight antigen may account for the induction of protective antimannan antibodies (such as M1g1, and mAb 6.1) recognizing β-1,2-linked oligomannosides in the immune system. In fact, it has been demonstrated that there is a high incidence of di- and trisaccharide length epitopes in the acid labile part of *C. albicans* mannan (Goins and Cutler, 2000).

The observations presented in this study indicate that human recombinant antimannan antibody, M1g1, recognizes a protective epitope β-1,2-linked mannotriose, which is one of the most common *Candida* mannan epitopes. This feature demonstrates the promise for antibody-based therapeutics against candidiasis.

REFERENCES

REFERENCES

Allen, F. H., Kennard O. 1993. 3D search and research using the Cambridge Structural Database. Chemical Design Automation News. 8, 31-37.

Bernstein, F. C., T. F. Koetzle, G. J. Williams, E. F. Meyer Jr, M. D. Brice, J. R. Rodgers, O. Kennard, T. Shimanouchi, and M. Tasumi. 1977. The Protein Data Bank: a computer-based archival file for macromolecular structures. J. Mol. Biol. 112:535-542.

Bohne, A., Lang, E., der Lieth, C.W., 1998. W3-SWEET: Carbohydrate modeling by Internet. Journal of Molecular Modeling 4, 33-43.

Bubb, W.A., 2003. NMR spectroscopy in the study of carbohydrates: Characterizing the structural complexity. Concepts in Magnetic Resonance Part A 19A, 1-19.

Bundle, D.R., Eichler, E., Gidney, M.A., Meldal, M., Ragauskas,A., Sigurskjold,B.W., Sinnott,B., Watson,D.C., Yaguchi,M., Young,N.M., 1994. Molecular recognition of a Salmonella trisaccharide epitope by monoclonal antibody Se155-4. Biochemistry 33, 5172-5182.

Calarese, D.A., Lee, H.K., Huang, C.Y., Best, M.D., Astronomo, R.D., Stanfield, R.L., Katinger, H., Burton, D.R., Wong, C.H., Wilson, I.A., 2005. Dissection of the carbohydrate specificity of the broadly neutralizing anti-HIV-1 antibody 2G12. Proc. Natl. Acad. Sci. U. S. A 102, 13372-13377.

Casadevall, A., Pirofski, L.A., 2007. Antibody-mediated protection through cross-reactivity introduces a fungal heresy into immunological dogma. Infect. Immun. 75, 5074-5078..

Chai, W., Stoll, M.S., Galustian, C., Lawson, A.M., Feizi, T., 2003. Neoglycolipid technology: deciphering information content of glycome. Methods Enzymol. 362, 160-195.

Chiani, P., Bromuro, C., Cassone, A., Torosantucci, A., 2009. Anti-[beta]-glucan antibodies in healthy human subjects. Vaccine 27, 513-519.

Cisar, J., Kabat, E.A., Dorner, M.M., Liao, J., 1975. Binding properties of immunoglobulin combining sites specific for terminal or nonterminal antigenic determinants in dextran. J Exp. Med. 142, 435-459.

Crich, D., Li, H., Yao, Q., Wink, D.J., Sommer, R.D., Rheingold, A.L., 2001. Direct synthesis of beta-mannans. A hexameric [-->3)-beta-D-Man-(1](3) subunit of the antigenic polysaccharides from Leptospira biflexa and the octameric (1-->2)-linked beta-D-mannan of the Candida albicans phospholipomannan. X-ray crystal structure of a protected tetramer. J. Am. Chem. Soc. 123, 5826-5828.

Critchley, I.A., Douglas, L.J., 1987 a. Role of glycosides as epithelial cell receptors for *Candida albicans*. J Gen Microbiol 133, 637-643.

Critchley, I.A., Douglas, L.J., 1987 b. Isolation and partial characterization of an adhesin from *Candida albicans*. J Gen Microbiol 133, 629-636.

Cutler,J.E., 2005. Defining criteria for anti-mannan antibodies to protect against candidiasis. Curr. Mol. Med. 5, 383-392.

Cygler,M., Rose, D.R., Bundle, D.R., 1991. Recognition of a cell-surface oligosaccharide of pathogenic *Salmonella* by an antibody Fab fragment. Science 253, 442-445.

Cygler, M., 1994. Recognition of carbohydrates by antibodies. Res Immunol 145, 36-40.

Davis, I.W., Murray,L.W., Richardson,J.S., Richardson,D.C., 2004. MOLPROBITY: structure validation and all-atom contact analysis for nucleic acids and their complexes. Nucl. Acids Res. 32, W615-W619.

DeLano, W.L. The PyMOL Molecular Graphics System. 2002. DeLano Scientific, Palo Alto, CA, USA. http://www.pymol.org.

DuBois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Colorimetric method for determination of sugars and related Substances. Analytical Chemistry 28, 350-356.

Duro, R.M., Netski, D., Thorkildson, P., Kozel, T.R., 2003. Contribution of epitope specificity to the binding of monoclonal antibodies to the capsule of *Cryptococcus neoformans* and the soluble form of its major polysaccharide, glucuronoxylomannan. Clin. Diagn. Lab. Immunol. 10, 252-258.

Edmond, M. B., S. E. Wallace, D. K. McClish, M. A. Pfaller, R. N. Jones, and R. P. Wenzel. 1999. Nosocomial bloodstream infections in United States hospitals: a three-year analysis. Clin. Infect. Dis. 29:239-244.

Faille, C., Michalski, J.C., Strecker, G., Mackenzie, D.W., Camus, D., Poulain, D., 1990. Immunoreactivity of neoglycolipids constructed from oligomannosidic residues of the *Candida albicans* cell wall. Infect. Immun. 58, 3537-3544.

Faille, C., Wieruszeski, J.M., Michalski, J.C., Poulain, D., Strecker,G., 1992 a. Complete 1H- and 13C-resonance assignments for D-mannooligosaccharides of the beta-D-(1-->2)-linked series released from the phosphopeptidomannan of *Candida albicans* VW.32 (serotype A). Carbohydr. Res. 236, 17-27.

Faille, C., Mackenzie, D.W., Michalski, J.C., Poulain, D., 1992 b. Evaluation of an enzyme immunoassay using neoglycolipids constructed from Candida albicans oligomannosides to define the specificity of anti-mannan antibodies. Eur. J. Clin. Microbiol. Infect. Dis. 11, 438-446.

Freudenberg, M.A., Fomsgaard, A., Mitov, I., Galanos, C., 1989. ELISA for antibodies to lipid A, lipopolysaccharides and other hydrophobic antigens. Infection 17, 322-328.

Goins, T.L., Cutler, J.E., 2000. Relative abundance of oligosaccharides in *Candida* species as determined by fluorophore-assisted carbohydrate electrophoresis. Journal of Clinical Microbiology 38, 2862-2869.

Guex, N., Peitsch, M.C., 1997. SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. Electrophoresis 18, 2714-2723.

Han, Y., Kanbe, T., Cherniak, R., Cutler, J.E., 1997. Biochemical characterization of *Candida albicans* epitopes that can elicit protective and nonprotective antibodies. Infection and Immunity 65, 4100-4107.

Han, Y., Kozel, T.R., Zhang, M.X., MacGill, R.S., Carroll, M.C., Cutler, J.E., 2001. Complement is essential for protection by an IgM and an IgG3 monoclonal antibody against experimental, hematogenously disseminated candidiasis. J. Immunol. 167, 1550-1557.

Hayette, M. P., G. Strecker, C. Faille, D. Dive, D. Camus, D. W. Mackenzie, and D. Poulain. 1992. Presence of human antibodies reacting with *Candida albicans* O-linked oligomannosides revealed by using an enzyme-linked immunosorbent assay and neoglycolipids. J. Clin. Microbiol. 30:411-417.

Kabat, E.A., 1966. The nature of an antigenic determinant. J Immunol 97, 1-11.

Kobayashi, H., N. Shibata, H. Mitobe, Y. Ohkubo, and S. Suzuki. 1989. Structural study of phosphomannan of yeast-form cells of *Candida albicans* J-1012 strain with special reference to application of mild acetolysis. Arch. Biochem. Biophys. 272:364-375.

Kobayashi, H., N. Shibata, M. Nakada, S. Chaki, K. Mizugami, Y. Ohkubo, and S. Suzuki. 1990. Structural study of cell wall phosphomannan of *Candida albicans* NIH B-792 (serotype B) strain, with special reference to 1H and 13C

NMR analyses of acid-labile oligomannosyl residues. Arch. Biochem. Biophys. 278:195-204.

Kocourek, J., Ballou, C.E., 1969. Method for Fingerprinting Yeast Cell Wall Mannans. J. Bacteriol. 100, 1175-1181.

Kozel, T.R., Weinhold, L.C., Lupan, D.M., 1996. Distinct characteristics of initiation of the classical and alternative complement pathways by Candida albicans. Infect. Immun. 64, 3360-3368.

Kozel, T.R., MacGill, R.S., Percival,A., Zhou,Q., 2004. Biological activities of naturally occurring antibodies reactive with *Candida albicans* mannan. Infect. Immun. 72, 209-218.

Kuroda, D., Shirai, H., Kobori, M., Nakamura, H., 2008. Structural classification of CDR-H3 revisited: a lesson in antibody modeling. Proteins 73, 608-620.

Lee,M., Lloyd, P., Zhang, X., Schallhorn, J.M., Sugimoto, K., Leach, A.G., Sapiro,G., Houk,K.N., 2006. Shapes of antibody binding Sites: qualitative and quantitative analyses based on a geomorphic classification scheme. The Journal of Organic Chemistry 71, 5082-5092.

Lehmann, P.F., Reiss, E., 1980. Comparison by ELISA of serum anti-*Candida albicans* mannan IgG levels of a normal population and in diseased patients. Mycopathologia 70, 89-93.

Liu, Y., T. Feizi, M. A. Campanero-Rhodes, R. A. Childs, Y. Zhang, B. Mulloy, P. G. Evans, H. M. Osborn, D. Otto, P. R. Crocker, and W. Chai. 2007. Neoglycolipid probes prepared via oxime ligation for microarray analysis of oligosaccharide-protein interactions. Chem. Biol. 14:847-859.

Lopez, P.H.H., Schnaar, R.L., 2006. Determination of glycolipid-protein interaction specificity. Functional Glycomics 417, 205-220.

Lovell, S.C., Word, J.M., Richardson,J.S., Richardson,D.C., 2000. The penultimate rotamer library. Proteins 40, 389-408.

Martinez, J. P., M. L. Gil, J. L. Lopez-Ribot, and W. L. Chaffin. 1998. Serologic response to cell wall mannoproteins and proteins of *Candida albicans*. Clin. Microbiol. Rev. 11:121-141.

Morris, G.M., Huey, R., Lindstrom, W., Sanner, M.F., Belew, R.K., Goodsell, D.S., Olson, A.J., 2009. AutoDock4 and AutoDockTools4: Automated docking with selective receptor flexibility. J. Comput. Chem.

Murase, T., Zheng, R.B., Joe, M., Bai, Y., Marcus, S.L., Lowary, T.L., Ng,K.K.S., 2009. Structural insights into antibody recognition of mycobacterial polysaccharides. Journal of Molecular Biology 392, 381-392.

Nelson, R.D., Shibata, N., Podzorski, R.P., Herron, M.J., 1991. Candida mannan: chemistry, suppression of cell-mediated immunity, and possible mechanisms of action. Clinical Microbiology Reviews 4, 1-19.

Nitz,M., Ling,C.C., Otter,A., Cutler,J.E., Bundle,D.R., 2002. The unique solution structure and immunochemistry of the *Candida albicans* beta -1,2-mannopyranan cell wall antigens. J. Biol. Chem. 277, 3440-3446.

Novotny, J., Bruccoleri, R., Newell, J., Murphy, D., Haber, E., Karplus, M., 1983. Molecular anatomy of the antibody binding site. J. Biol. Chem. 258, 14433-14437.

Okubo, Y., Shibata, N., Ichikawa, T., Chaki, S., Suzuki, S., 1981. Immunochemical study on bakers' yeast mannan prepared by fractional precipitation with cetyltrimethylammonium bromide. Archives of Biochemistry and Biophysics 212, 204-215.

Padlan, E. A., and E. A. Kabat. 1988. Model-building study of the combining sites of two antibodies to alpha (1--6) dextran. Proc. Natl. Acad. Sci. 85:6885-6889.

Padlan E A, Kabat E A. Modeling of antibody combining sites. 1991. Methods in Enzymology. 203:3–21

Paula, S., Monson,N., Ball,W.J., Jr., 2005. Molecular modeling of cardiac glycoside binding by the human sequence monoclonal antibody 1B3. Proteins 60, 382-391.

Ramsland, P.A., Farrugia, W., Bradford, T.M., Mark, H.P., Scott, A.M., 2004. Structural convergence of antibody binding of carbohydrate determinants in Lewis Y tumor antigens. J. Mol. Biol. 340, 809-818.

Sherma, J., Fried, B., 2003. Handbook of thin-layer chromatography. London: Taylor & Francis, Inc.

Shibata, N., Ichikawa, T., Tojo, M., Takahashi, M., Ito, N., Okubo, Y., Suzuki, S., 1985. Immunochemical study on the mannans of *Candida albicans* NIH A-207, NIH B-792, and J-1012 strains prepared by fractional precipitation with cetyltrimethylammonium bromide. Arch. Biochem. Biophys. 243, 338-348.

Shibata, N., Arai, M., Haga, E., Kikuchi, T., Najima, M., Satoh, T., Kobayashi, H., Suzuki,S., 1992 a. Structural identification of an epitope of antigenic factor 5

in mannans of *Candida albicans* NIH B-792 (serotype B) and J-1012 (serotype A) as beta-1,2-linked oligomannosyl residues. Infect. Immun. 60, 4100-4110.

Shibata, N., Hisamichi, K., Kikuchi, T., Kobayashi, H., Okawa, Y., Suzuki, S., 1992 b. Sequential nuclear magnetic resonance assignment of beta-1,2-linked mannooligosaccharides isolated from the phosphomannan of the pathogenic yeast *Candida albicans* NIH B-792 strain. Biochemistry 31, 5680-5686.

Shibata, N., Hisamichi, K., Kobayashi, H., Suzuki, S., 1993. Complete assignment of 1H and 13C nuclear magnetic resonance chemical shifts of beta-1,2-linked mannooligosaccharides isolated from the phosphomannan of the pathogenic yeast *Candida albicans* NIH B-792 strain. Arch. Biochem. Biophys. 302, 113-117.

Shibata,N., Ikuta,K., Imai,T., Satoh,Y., Satoh,R., Suzuki,A., Kojima,C., Kobayashi,H., Hisamichi,K., Suzuki,S., 1995. Existence of branched side chains in the cell wall mannan of pathogenic yeast, *Candida albicans*. Structure-antigenicity relationship between the cell wall mannans of *Candida albicans* and *Candida parapsilosis*. J. Biol. Chem. 270, 1113-1122.

Shibata, N., Suzuki, A., Kobayashi, H., Okawa, Y., 2007. Chemical structure of the cell wall mannan of *Candida albicans* serotype A and its difference in yeast and hyphal forms. Biochem. J.

Sircar, A., Kim, E.T., Gray, J.J., 2009. RosettaAntibody: antibody variable region homology modeling server. Nucl. Acids Res. 37, W474-W479.

Sivasubramanian, A., Maynard, J.A., Gray, J.J., 2008. Modeling the structure of mAb 14B7 bound to the anthrax protective antigen. Proteins 70, 218-230.

Sivasubramanian, A., Sircar, A., Chaudhury, S., Gray, J.J., 2009. Toward high-resolution homology modeling of antibody Fv regions and application to antibody-antigen docking. Proteins 74, 497-514.

Sotriffer, C.A., Flader, W., Winger, R.H., Rode, B.M., Liedl, K.R., Varga, J.M., 2000. Automated docking of ligands to antibodies: methods and applications. Methods 20, 280-291.

Stevens, D.A., 1998. Combination immunotherapy and antifungal chemotherapy. Clin. Infect. Dis. 26, 1266-1269.

Suzuki, S., 1997. Immunochemical study on mannans of genus Candida. I. Structural investigation of antigenic factors 1, 4, 5, 6, 8, 9, 11, 13, 13b and 34. Curr. Top. Med. Mycol. 8, 57-70.

Trinel, P. A., C. Faille, P. M. Jacquinot, J. C. Cailliez, and D. Poulain. 1992. Mapping of *Candida albicans* oligomannosidic epitopes by using monoclonal antibodies. Infect. Immun. 60:3845-3851.

Trott, O., Olson, A.J., 2010. AutoDock Vina: Improving the speed and accuracy of docking with a new scoring function, efficient optimization, and multithreading. J Comput. Chem. 31, 455-461.

Villeneuve, S., Souchon, H., Riottot, M.M., Mazie, J.C., Lei, P., Glaudemans, C.P., Kovac, P., Fournier, J.M., Alzari, P.M., 2000. Crystal structure of an anti-carbohydrate antibody directed against *Vibrio cholerae* O1 in complex with antigen: molecular basis for serotype specificity. Proc. Natl. Acad. Sci. U. S. A 97, 8433-8438.

Vulliez-Le Normand, B., Saul, F.A., Phalipon, A., Belot, F., Guerreiro, C., Mulard, L.A., Bentley, G.A., 2008. Structures of synthetic O-antigen fragments from serotype 2a *Shigella flexneri* in complex with a protective monoclonal antibody. Proc. Natl. Acad. Sci. 105, 9976-9981.

Vyas, N.K., Vyas, M.N., Chervenak, M.C., Johnson, M.A., Pinto, B.M., Bundle, D.R., Quiocho, F.A., 2002. Molecular recognition of oligosaccharide epitopes by a monoclonal Fab specific for *Shigella flexneri* Y lipopolysaccharide: X-ray structures and thermodynamics. Biochemistry 41, 13575-13586.

Wisplinghoff, H., Bischoff, T., Tallent, S.M., Seifert, H., Wenzel, R.P., Edmond,M.B., 2004. Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. Clin. Infect. Dis. 39, 309-317.

Zhang, M.X., Lupan, D.M., Kozel, T.R., 1997. Mannan-specific immunoglobulin G antibodies in normal human serum mediate classical pathway initiation of C3 binding to *Candida albicans*. Infect. Immun. 65, 3822-3827.

Zhang, M.X., Kozel, T.R., 1998. Mannan-specific immunoglobulin G antibodies in normal human serum accelerate binding of C3 to *Candida albicans* via the alternative complement pathway. Infect. Immun. 66, 4845-4850.

Zhang, M.X., Bohlman, M.C., Itatani, C., Burton, D.R., Parren, P.W.H.I., St.Jeor, S.C., Kozel, T.R., 2006. Human recombinant antimannan immunoglobulin G1 antibody confers resistance to hematogenously disseminated candidiasis in Mice. Infection and Immunity 74, 362-369.

Zwick, M.B., Parren, P.W.H.I., Saphire, E.O., Church, S., Wang, M., Scott, J.K., Dawson,P.E., Wilson,I.A., Burton,D.R., 2003. Molecular features of the broadly neutralizing immunoglobulin G1 b12 required for recognition of human immunodeficiency virus type 1 gp120. J. Virol. 77, 5863-5876.