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Surface Glycans of *Candida albicans* and Other Pathogenic Fungi: Physiological Roles, Clinical Uses, and Experimental Challenges

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INTRODUCTION

Infections by pathogenic fungi, particularly *Candida* species, are both widespread and increasing in frequency (68, 276). Oral colonization by *Candida albicans* has been reported at 17.7% in the healthy population (40). Among hospitalized patients, oral carriage of *Candida albicans* rises to 40.6%. Healthy, asymptomatic women demonstrate an incidence of vaginal colonization by *Candida* of 15 to 20%. This percentage rises to 20 to 40% in healthy pregnant women and to 40 to 60% in human immunodeficiency virus-infected pregnant women (26, 209, 254, 273). Hospitals in the United States participating in the National Nosocomial Infection Survey System reported a nosocomial fungal infection frequency of 3.8 per 1,000 discharges in 1990, an increase from 2.0 per 1,000 discharges in 1980 (17). *Candida* species accounted for 78.3% of all such nosocomial fungal infections, followed by *Torulopsis* (now *Candida*) *glabrata* and *Aspergillus* species. *C. albicans* was the most frequently isolated of all the *Candida* species (17, 219). Furthermore, a European study reported that fungal species in general accounted for 17.1% of intensive care unit-acquired infections (306).

Using the National Nosocomial Infection Survey (1980 to 1990) data, Jarvis and Martone (131) reported that *Candida* species accounted for 9.4% of nosocomial urinary tract infections, 10.1% of nosocomial infections in adult and pediatric intensive care units, and 7.8% of nosocomial bloodstream infections. In 1990, fungi as a whole accounted for 10% of all

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nosocomial bloodstream infections (17). Ten years later, the rates of bloodstream infections due to *Candida* are still rising, with half of these candidemias due to *C. albicans* (230). More recently, other *Candida* species—such as *C. glabrata*, *C. tropicalis*, and *C. dubliniensis*—have been isolated with increasing frequency (220). A study carried out by five university hospitals in the Netherlands also reported increased fungemia rates. Between 1987 and 1995, episodes of fungal bloodstream infections rose from 0.37 to 0.76 per 10,000 patient days. Of these infections, 93% were due to *Candida* species while 7% were due to *Cryptococcus* species (307).

In addition to the observed increases in incidence, fungal infections continue to be a serious clinical problem with respect to increased morbidity and mortality (194, 314; see also reference 219 and references therein). An analysis of records from the National Center of Health Statistics indicated that mycoses ranked 10th among the underlying causes of death in the United States in 1980 but that by 1997 this rank had risen to 7th (193). Increased morbidity due to nosocomial fungal infections also takes a heavy toll, resulting in longer hospital stays (49, 178, 314) and higher patient care costs (91, 234, 285).

The increasing incidence of fungal infections has, to a certain degree, coincided with the incredible medical advances of the last few years. Life-prolonging technologies, although welcomed by medicine and those it cares for, have unfortunately created numerous opportunities for emerging fungal infections. The rising number of immunocompromised patients, whether through immunosuppressive therapy, AIDS, or other ailments, the prophylactic use of antimicrobics, and the increasing number of indwelling catheters and prosthetic devices seen among patients all unwittingly subject patients to the threat of invasive disease (68). Patients with central intravenous IV catheters were over three times more likely to have bloodstream infections than patients without such catheters (17). Patients undergoing orthotopic liver transplantation demonstrated an incidence of invasive fungal infection of 5 to 42%, with *Candida* species being the most commonly isolated organism, followed by *Aspergillus* species (54). A study of heart transplant patients reported that while fungal infections accounted for only 7% of all infections, they were associated with the highest (36%) mortality (194). Taken together, these clinical observations strongly reinforce the necessity for understanding the relationship between fungus and host.

The interaction between fungus and patient occurs first at the level of the cell wall (35). The cell wall of most fungi is composed of glycoproteins embedded within a polysaccharide matrix or scaffolding. Additionally, some fungal species produce a polysaccharide capsule that surrounds the cell wall (e.g. the glucuronoxylomannan capsule produced by *Cryptococcus neoformans* [221]). It is therefore possible, and even likely, that carbohydrate, not DNA or RNA and perhaps not even protein, is the first component to contact host tissue. Thus, characterization of these exterior carbohydrate groups could lead to (i) a better understanding of fungal adhesion and mechanisms by which fungi avoid the host immune system, (ii) proposals for countering this avoidance so as to enhance the immune response, and (iii) the development of diagnostic tests based on identification of carbohydrate components. The utility of cell wall and cell surface carbohydrate groups has been shown in other areas of clinical microbiology (e.g., *Mycobacterium*) (23). Fungal cell surface carbohydrates also play an important role in industry (132, 135) and in plant and nonhuman animal fungal pathogenesis.

The purpose of this review is to bridge the areas of fungal pathogenesis and glycobiology. To this end, the manuscript is divided into three major sections. First, a brief introduction to the carbohydrate components of the fungal cell wall and how they are assembled is provided. Second, relevant studies where carbohydrates have been shown to play a role in infection, function in diagnostic assays, and act as therapeutic agents are discussed. The final section is a discussion of the practical issues and difficulties that arise when working with microbial carbohydrates.

This review is limited in scope in two ways. First, although there is an extensive body of literature describing the various structures and pathways by which microorganisms interact with host species, the focus of this review is fungal glycobiology. Therefore, discussion is limited to the polysaccharide components of the fungal cell wall and does not include more general fungal cell fractions, fungal proteins and lipids, or elements of the host cells. In addition, examination of the *C. albicans* literature illustrates the need for both careful identification of fraction components and strategies for demonstrating that carbohydrate, rather than protein or lipid, is responsible for the observed effect. For example, while *C. albicans* mannan (defined below) is found only as a glycoconjugate with protein or lipid, many pathogenesis studies use the term "mannan" to refer globally to mannoprotein or similar glycoconjugate fractions.

Second, *C. albicans* will serve as the example organism for relating glycobiology to clinical microbiology. Studies describing the role of carbohydrate groups in pathogenesis have been carried out mainly with *C. albicans*; therefore, the review likewise focuses on this organism. A review by Nelson et al. (202) focused on *Candida* mannan and issues of mannan chemistry, immune suppressive effects, and mechanisms of action. This review expands on their discussion to include glucans and relevant mannan results reported since the publication of the previous review. Although the focus is *C. albicans*, it should be remembered that carbohydrates, as polysaccharides or components of glycoproteins, play a role in the pathogenesis and diagnosis of other fungal species such as *Cryptococcus neoformans*, *Aspergillus fumigatus*, *Kluyveromyces lactis*, and *Paracoccidioides brasilienis.* Where appropriate, selected results from studies of these species are included in the discussion.

GLYCANS IN THE *CANDIDA* CELL WALL

Basic Components

Three types of monosaccharides form carbohydrate chains, or glycans, within the *Candida* and *Saccharomyces* cell wall: D-glucose (Glc), *N*-acetyl-D-glucosamine (GlcNAc), and D-mannose (Man) (Fig. 1). A comparison of the cell envelope glycans from some pathogenic fungi is presented in Table 1, and a more general discussion of fungal cell wall composition, structure, and assembly can be found in reference 242. Recently, there have been several reports of sialic acid as a constituent of the *Candida* cell wall, presumably as terminal residues of glycoprotein glycans (2, 140, 272, 308). These reports are remark-

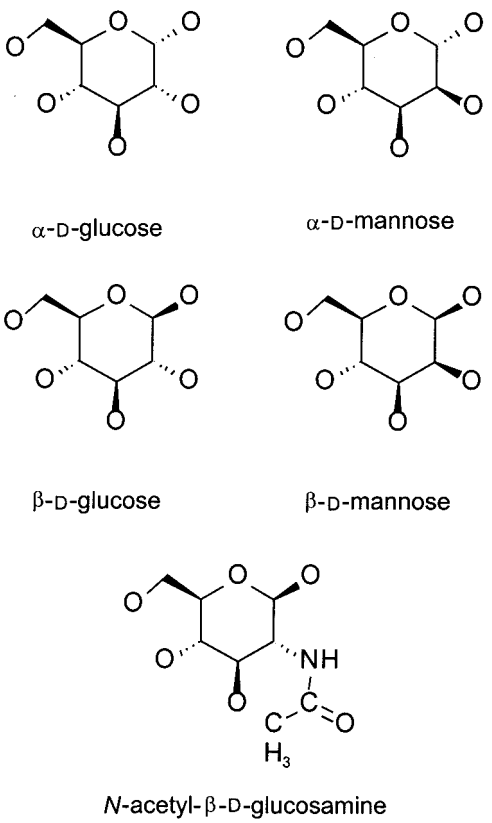

FIG. 1. Monosaccharides found in the *C. albicans* cell wall.

able in that the types of glycan normally containing sialic acids have not been described in *C. albicans.* The evidence for sialic acids will be described in greater detail below.

Glucan. Glycans composed of glucose homopolymers are generically referred to as glucans. The cell wall glucan of *Candida* and *Saccharomyces* is a highly branched polymer consisting of β-1,3 and β-1,6 linkages. Bishop et al. (21) determined the average degree of polymerization to be 30 ± 2, basing the β-anomericity of the glycosidic bond on its negative optical rotation value: $[\alpha]_D = -30°$. Chemical analyses allowed them to conclude that *C. albicans* glucan has a higher percentage of β-1,6-glucan cross-links than that seen in *Saccharomyces cerevisiae* (21). Rees and Scott (231), based on computational modeling, predicted that β-1,3-glucan forms wide helices, like a wire spring or a spiral staircase (designated type B), while β-1,6-glucan forms structures that are extended and flexible (type D). As discussed below, glucan forms the structural skeleton of the cell wall, although the exact molecular arrangement has not yet been determined (21).

Chitin. Linear polymers of β-1,4-D-GlcNAc, called chitin, provide cross-linking and strength to the glucan scaffolding. The amount of chitin found in the cell walls of *C. albicans* hyphae is three times that found in yeasts (50). In contrast, Kanetsuna et al. (149) found that the GlcNAc content of the mycelial forms of *Paracoccidioides brasiliensis* and *Blastomyces dermatitidis* was one-quarter to one-third that of the yeast form. Lipke and Ovalle, based on findings of Kapteyn et al., have suggested that chitin plays a role in a cellular integrity rescue mechanism (153, 181). That is, when the cell wall is weakened by decreased amounts or quality of glucan, the cells compensate by increasing chitin production. Bahmed et al. (12) noted an increase in cell wall chitin in *Kluyveromyces lactis* and *K. bulgaricus* strains resistant to amphotericin B. Their results indicate that the net increase in chitin is due to a decrease in chitinase activity rather than an increased chitin synthase activity. However, the specific link between antifungal resistance and chitin content remains to be identified. Chitin also forms the core of the septum during bud growth and separation (see reference 29 for a review of the process in *S. cerevisiae*). GlcNAc also appears in the fungal cell wall as a component of glycoproteins. A chitobiose [D-GlcNAcβ-

TABLE 1. Carbohydrate components of some of the organisms responsible for invasive mycoses

Species	Cell envelope glycan	Components[a]	Other sugars reported	Reference(s)
Aspergillus fumigatus	Nigeran (glucan)	α-1,3- and α-1,4-Glc		175, 242
	Galactomannan (glycoprotein)	α-1,2- and α-1,6-Man core with β-1,4-Gal side chains		
	Chitin	β-1,4-GlcNAc		
Blastomyces dermatitidis	Glucan	α-1,3-Glc (yeast), β-1,3-Glc (mycelia)		242
Candida albicans	Glucan	β-1,3- and β-1,6-Glc	Sialic acid	13, 21, 50, 272, 292
	Mannan (glycoprotein/glycolipid)	α-1,6/α-1,2/α-1,3/β-1,2-Man		
	Chitin	β-1,4-GlcNAc		
Coccidioides immitis	Glucan	Glc	Gal, Xyl	214, 242
	Glucomannan (glycoprotein)	Glc, Man		
Cryptococcus neoformans	Glucan (wall)	α-1,3-Glc β-1,6- and β-1,3-Glc	Sialic acid	52, 129, 237
	Glucuronoxylomannan (capsule)	Glucuronic acid, Man, Xyl		
	Galactoxylomannan (glycoprotein)	Gal (O acetylated), Man, Xyl		
	Mannoprotein (glycoprotein)	Glucuronic acid, Gal, Man, Xyl		
Paracoccidioides brasiliensis	Glucan (yeast)	α-1,3-Glc	Sialic acid	79, 148, 149, 270
	Glucan (mycelia)	β-1,3-Glc		
	Galactomannan (glycoprotein)	Gal, Man		
	Chitin	β-1,4-GlcNAc		
Pneumocystis carinii	Glucan	β-1,3-Glc Man, Gal, GlcNAc	Fucose, ribose, sialic acid	65, 66, 302

[a] Gal, galactose; Glc, glucose; GlcNAc, *N*-acetylglucosamine; Man, mannose; Xyl, xylose.

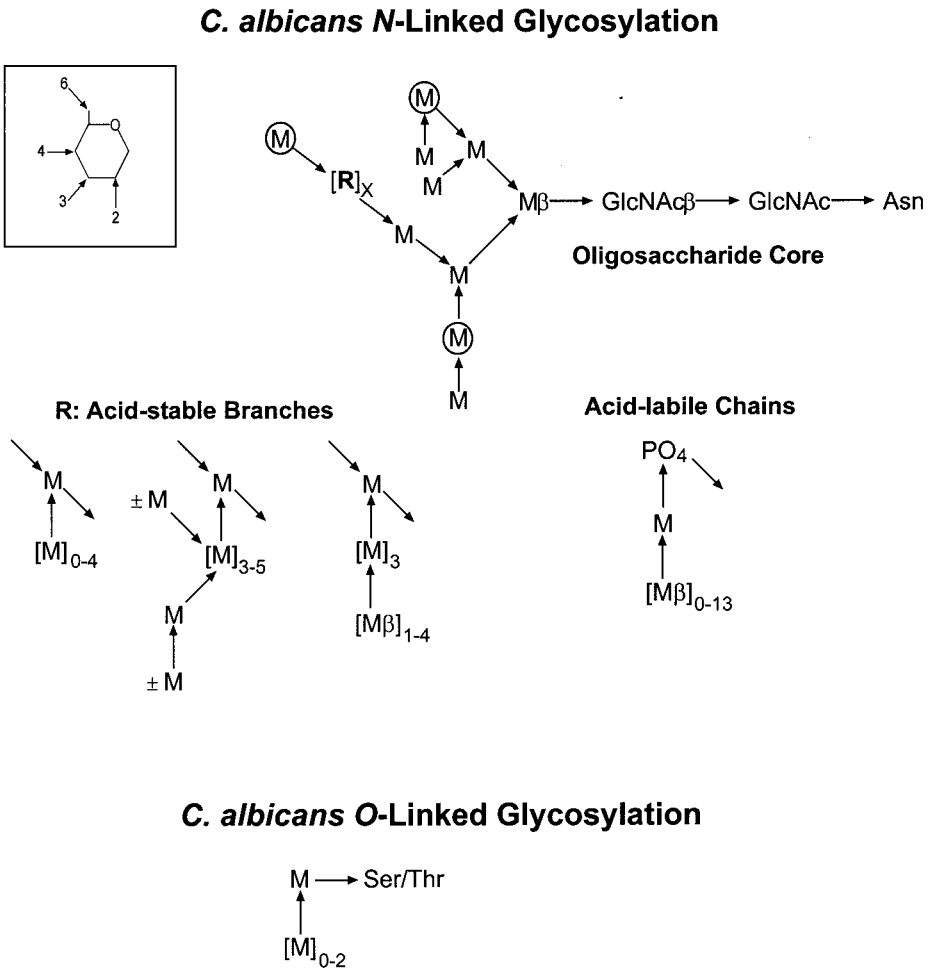

FIG. 2. Glycan structures in *C. albicans* glycoproteins. The processed oligosaccharide core is extended from the α-1,3-linked mannose at its C-6 position (R). R structures represent possible side branch structures that can be incorporated into this outer-chain elongation. X can be up to 60 to 70. The acid-labile phosphooligomannosides are added to a side branch mannotetraose. Circled mannose units are potentially phosphorylated. Inset: Map key for linkage points. All bonds are α-1,*n*, unless otherwise indicated. Structure scheme adapted from reference 88 with permission. Structural information based on work in references 13, 167 to 169, 211, 264, 265, and 293 and R. C. Montijn (1998), Ph.D. thesis, University of Amsterdam. GlcNAc, *N*-acetylglucosamine; M, mannose.

(1→4)–D-GlcNAc] forms the link between protein asparagine residue and the inner core of N-linked glycans (Fig. 2).

Mannan. Mannose polymers, or mannan, are found only as part of a glycoconjugate in *C. albicans* and *S. cerevisiae*—either glycoprotein (13) or glycolipid (292)—and not as an unconjugated oligosaccharide (13). Because of this, the terms "mannan" and "mannoprotein" have often been used interchangeably in the *Candida* literature. However, to maintain clarity, in this review "mannan" refers to the glycan itself while glycoconjugates are referred to in specific terms (mannoprotein, glycoprotein, glycolipid, etc.). The mannan preparations of Bishop et al. (21) gave an average degree of polymerization of 41 ± 2. Highly positive optical rotation values (+78° ± 2°) indicated that α-glycosidic bonds predominate. The authors concluded that yeast mannan comprised short α-1,2 linked oligomannosides joined by α-1,6 linkages (21). Subsequent work, mainly by Ballou and coworkers, resulted in a more complete structural characterization of mannan from *S. cerevisiae* and other species (reviewed in reference 13). Similarly, elegant experiments by Suzuki and colleagues (163–166, 261, 262, 264) described the structures of the N-linked glycans from *Candida* species (Fig. 2). These glycans are composed of an internal core, one branch of which is extended to form the outer chain consisting of an α-1,6-linked backbone from which side branches are linked. To some of these side branches, additional β-1,2-oligomannosides are attached through a phosphodiester. Because this phosphodiester bond can be cleaved by 10 mM HCl and heat, the β-1,2-mannan is referred to as acid labile while the remaining outer chain groups are described as being acid stable (Fig. 2).

As alluded to above, mannose can be linked through either α- or β-anomeric bonds. O-linked glycans and the outer chain side branches of N-linked glycans are composed mainly of α-1,2- or α-1,3-oligomannosides. Three types of β-1,2-mannose additions have been reported by Suzuki and coworkers (266). The first type forms the acid-labile mannan in strains of *C. albicans* (both serotype A and serotype B), *C. glabrata*, and *C. tropicalis* (164–167, 263) The second is found on the nonreducing terminal of α-1,2-oligomannoside outer chain side branches of serotype A *C. albicans* strains and of *C. glabrata*

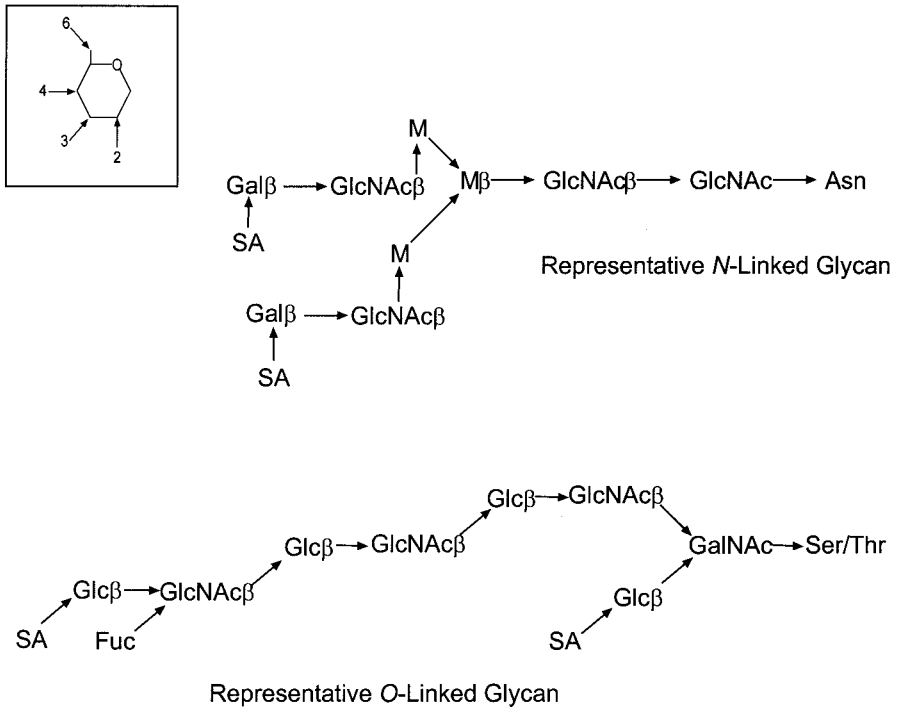

FIG. 3. Representative glycans from higher eukaryotic organisms. Inset: Key for linkage positions as in Fig. 2. Fuc, fucose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; Glc, glucose; M, mannose; SA, sialic acid.

and *C. tropicalis* strains (164–166, 263). The third type is connected to an α-1,3-linked mannose in the acid-stable mannan of *C. guilliermondii* and *C. saitoana* (261, 266). In addition, the first mannose in the N-linked glycan core is linked β-1,4 to the second GlcNAc (Fig. 2). The structural models of Rees and Scott (231) predicted that α-1,2-mannan forms type B helices like β-1,3-glucan, α-1,3-mannan forms extended ribbon chains (type A), and α-1,6-mannan forms type D chains.

β-1,2-Mannan was predicted to form crumpled or contorted chains (type C). Further, all 1,2-linked glycans, with the exception of α-1,2-mannan, were predicted to form these distorted chains (231). Because of steric clashes between nonadjacent residues, the authors expected such structures to be found only rarely in nature. This expectation is supported empirically by the determined structures of commonly occurring polysaccharides. For example, neither starch (α-1,4-D-Glc; type B), cellulose (β-1,4-D-Glc; type A), nor glycogen (α-1,4-D-Glc with α-1,6-D-Glc branches; types B and D) forms type C chains. Likewise, none of the commonly occurring fungal polysaccharides (Table 1) are predicted to form these crumpled, contorted chains. Nonetheless, examples can be found of 1,2-linked sugars, in addition to *C. albicans* mannan. Some these examples are *Cryptococcus neoformans* capsule glucuronoxylomannan (β-1,2-D-xylose and β-1,2-D-glucuronic acid), *C. neoformans* capsule galactoxylomannan (β-1,2-D-xylose), gum ghatti (β-1,2-D-glucuronic acid), and gum tragacanth (α-1,2-L-glucose) (9, 10, 22, 52, 157). In these cases, however, the 1,2-linked sugars occur either as a terminal group of a side chain or discontinuously as a component of a heteropolymer. In either case, the potential steric conflicts resulting from chain formation are avoided. The two instances of 1,2-linked sugars forming oligomers are a β-1,2-D-glucan from *Agrobacterium* species (94, 226) and the β-1,2-D-oligomannosides from *Candida* species.

Sialic acids. With only a few exceptions, sialic acids have been found only in higher eukaryotes (i.e., vertebrates and some invertebrates such as Echinoderms) (300). Besides these organisms, sialic acids have been found in *Drosophila* embryos and the capsule material of some bacteria species. Benhamou and Ouellette (19) also found sialic acid in the cell wall of a fungal plant pathogen, *Ascocalyx abietina*. Where they are typically found, sialic acids are components of O-linked glycans or complex-type N-linked glycans (Fig. 3). As mentioned above, several groups have presented evidence for the presence of sialic acids in the cell wall of *C. albicans* (2, 140, 272, 308). Until these reports, there had been no indication that yeast (*C. albicans* or *S. cerevisiae*) produce sialic acid in any capacity.

Alaei et al. (2) and Wadsworth et al. (308) provided indirect evidence for sialic acids in *C. albicans* glycans while characterizing the iC3b and CR2 binding proteins, respectively. Alaei et al. identified three proteins (66, 55, and 42 kDa) that bound to C3d-Sepharose. Endoglycosidase F (Endo F) was tested as a deglycosylation agent but had no effect as measured by electrophoretic mobility. On the other hand, treatment of the proteins with neuraminidase, which cleaves terminal sialic acids groups, apparently deglycosylated the 66- and 55-kDa proteins.

Several aspects of these results are puzzling. First, the *C. albicans* cells were disrupted by shearing with glass beads. The cell debris, containing the cell wall, was removed by centrifugation, and the supernatant fluid, containing mostly cytoplasmic proteins, was used as the source material for a C3 binding protein. Such a protein would be expected to be membrane or cell wall associated and would thus be more likely to be found

with the cell pellet. Second, the removal of terminal sialic acids by neuraminidase seems insufficient to account for a 20% decrease in apparent molecular mass, observed as an increase in relative electrophoretic mobility. Relative electrophoretic mobility is also unlikely to be affected by the presence or absence of negative charge from sialic acids because such charge effects are overwhelmed by the sodium dodecyl sulfate present during electrophoresis. Thus, any change in relative electrophoretic mobility must be due to changes in apparent molecular mass. The neuraminidase preparation used (Sigma; type V) reports potential protease contamination. The presence of proteases could be an alternative explanation for the observed decrease in molecular mass. However, because the 42-kDa protein was unaffected by neuraminidase treatment, the presence of generally acting proteases seems unlikely. Third, Endo F treatment did not deglycosylate the C3d binding proteins. This enzyme cleaves N-linked glycans between the two GlcNAc residues of the oligosaccharide core. It is effective against high-mannose or hybrid glycans but not against complex glycans. While this is consistent with the types of glycans typically containing sialic acids, it is not consistent with the types of glycans made by *C. albicans*.

Similar evidence was presented by Wadsworth et al. (308) in their characterization of a type 2 complement receptor (CR2). Proteins were extracted from the *C. albicans* cell wall with dithiothreitol. Glycans were labeled with biotin-hydrazide either before or after protein separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) and subsequent transfer to nitrocellulose. Sodium periodate oxidizes *cis*-diols (e.g., between C-2 and C-3 of mannose), cleaving the ring and producing two aldehydes. These aldehydes can then react with the hydrazide group, resulting in covalent linkage of the attached biotin to the glycan. Wadsworth et al. showed that a 50-kDa protein from *C. albicans* blastoconidia and a 60-kDa protein from hyphae were recognized by anti-CR2 antisera. Initial results indicated that both proteins were glycosylated by biotin-hydrazide labeling. A 50-kDa protein also bound concanavalin A (Con A) and, to a lesser extent, wheat germ agglutinin (which normally binds GlcNAc but has been reported to also bind sialic acids). However, treatment with *N*-glycanase (which cleaves between the first GlcNAc and the Asn) or Endo H (which acts in a similar manner to Endo F) did not significantly reduce lectin binding, as would be expected. Furthermore, *N*-glycanase, Endo F, or neuraminidase treatment had no effect in immunoblots using the anti-CR2 antisera as probes. Based on these results, and considering that the protein separations were one-dimensional PAGE, it seems more likely that the 50-kDa CR2 protein is the deglycosylated form and that the biotin-labeled protein was a comigrant.

The 60-kDa protein also appeared to bind Con A, although this binding also was unaffected by Endo H treatment. This protein gave a stronger signal with regard to wheat germ agglutinin binding, which was markedly reduced on digestion with N-linked glycanase. The remaining results, however, led to some conflicting conclusions. Neuraminidase treatment of the 60-kDa protein led to loss of signal in immunoblots, which might be expected if the epitope included the removed sialic acid. In addition, neuraminidase treatment, either before or after biotinylation, eliminated detection of the biotin signal. This result is consistent with the terminal sialic acid being linked to a galactose (Gal) residue, as is seen in complex-type N-linked glycans. Although galactose can be labeled with biotin-hydrazide, a preliminary reaction with galactose oxidase is required to generate the required hydrazide-reactive aldehydes (248). *N*-Glycanase and Endo F deglycosylated the 60-kDa protein, as indicated by a decrease in apparent molecular mass in immunoblots. *N*-Glycanase cleaves both complex and high-mannose N-linked glycans, but Endo F is effective only against high-mannose glycans. This result, in contrast to the neuraminidase result, suggests that the glycans on the 60-kDa protein are high mannose rather than complex.

Jones et al. (140) examined the electrostatic properties of the *C. albicans* cell surface by using a cationic fluorophore, 9-aminoacridone. The electronegative *C. albicans* surface attracts the fluorophore, leading to self-quenching and a decrease in detectable fluorescence. Treatment of the cells with neuraminidase resulted in a time-dependent decrease in fluorescence intensity, leading these authors to conclude that the *C. albicans* surface contained sialic acid groups. However, the neuraminidase used in this study was from the same source as that used by Alaei et al., so that protease contamination is a potential confounding factor for concluding that the observed loss of electronegativity was due specifically to loss of sialic acids.

More direct evidence was presented by Soares et al. (272), who used fluorophore-labeled lectins to detect sialic acids on the *C. albicans* cell surface. Fluorescein isothiocyanate-conjugated *Sambucus niger* agglutinin (SNA) strongly labeled the entire surface of blastoconidia but did not appear to label hyphae. This lectin is highly specific for the sialic acid *N*-acetylneuraminic acid linked α-2,6 to galactose or GalNAc. Labeling by SNA was completely eliminated by pretreatment of *C. albicans* cells with *Clostridium perfringens* sialidase. Fluorescein-isothiocyanate-conjugated agglutinins from *Limax flavus* and *Limulus polyphemus* also bound to the *C. albicans* cell surface. These lectins are not specific for sialic acid linkage position and do not require a specific subterminal sugar group. As with the SNA result, *L. flavus* and *L. polyphemus* agglutinin binding was significantly reduced by pretreatment of the *Candida* cells with sialidase. In addition, sialidase treatment allowed the binding of peanut agglutinin to occur. This lectin recognizes terminal β-galactose units, groups that would be exposed from complex-type N-linked glycans following removal of the terminal sialic acids (Fig. 3).

Using similar methods, sialic acids have been found to be also associated with the cell surface or exogenous glycoproteins of *Cryptococcus neoformans*, *Fonsecaea pedrosoi*, *Paracoccidioides brasiliensis*, *Pneumocystis carinii*, and *Sporothrix schenckii* (237, 270, 271; reviewed in reference 6). In addition, Rodrigues et al. (236) have recently shown the presence of sialyltransferase activity in *C. neoformans*. Taken together, these results not only demonstrate the presence of sialic acids on pathogenic fungi but also show that these sugars are probably the termini for complex-type N-linked glycans. This observation is significant because these glycans had not previously been found on these organisms. In mammals, sialylated glycans are often a component of glycoconjugates that modulate intercellular interactions, such as the sialyl Lewis x selectin ligand (155, 252).

A similar role for sialylated glycans has been demonstrated for *C. neoformans* and *S. schenckii*, where treatment of fungal

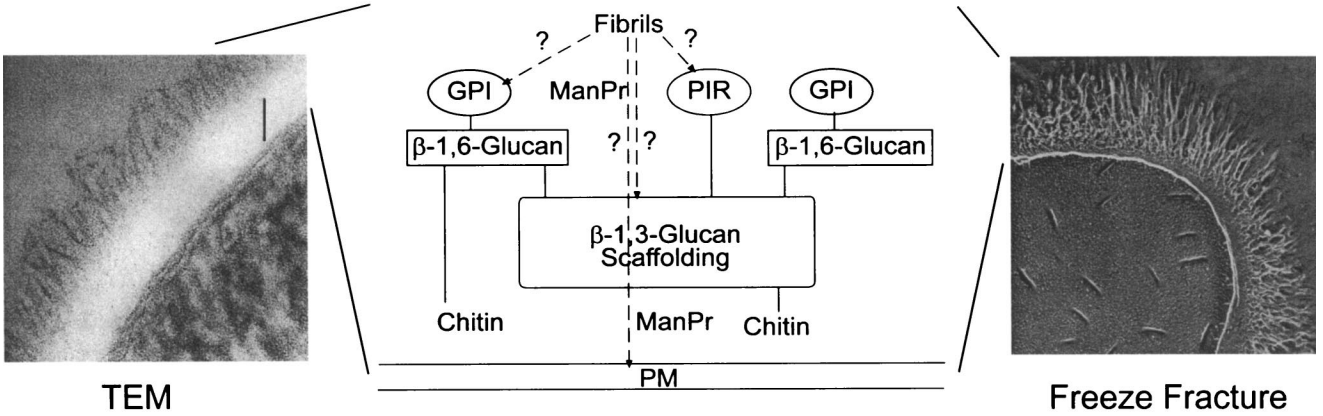

FIG. 4. Schematic of the *C. albicans* wall structure. The wall structural cartoon is based on information from references 150, 152, 213, and 269. Electron micrographs are reprinted from reference 108 with permission. GPI, glycosylphosphatidylinositol-linked glycoproteins; ManPr, mannoprotein; PIR, *PIR*-family glycoproteins; PM, plasma membrane.

cells with neuraminidase significantly increased phagocytosis by murine peritineal macrophages (5, 6, 237). *S. schenckii* or *C. neoformans* yeast cells were treated with neuraminidase from *Clostridium perfringens* or *Vibrio cholerae* for 1.5 to 2 h and then washed. Treated cells and untreated control cells were then added to adherent peritoneal macrophages. Neuraminidase treatment of *C. neoformans* cells led to a twofold increase in the phagocytic index (the percentage of macrophages phagocytizing at least one yeast multiplied by the average number of phagocytized yeast cells) (237). For *S. schenckii* cells, the increase was almost eightfold (207). In the studies above, neuraminidase was specifically chosen to complement the other work demonstrating the presence of sialic acids. It is unknown what effect, if any, treatment with other glycosidases, such as mannosidase, would have on phagocytosis. However, the neuramindase results are consistent with a model, proposed by Schauer and coworkers, that terminal sialic acid groups mask ligands for macrophage-bounds receptors (155, 156, 177). This raises the possibility that sialylated glycans on fungi and other microorganisms can assist in adaptation to life in the host by avoiding recognition or killing by the immune system.

Previous studies of mannoprotein fractions from the cell walls of *S. cerevisiae* or *C. albicans* gave no indication of the presence of sialic acids or complex-type N-linked glycans. There are two potential explanations for this (until recently) lack of evidence. First, the usual methods of mannoprotein preparation (hot water or citrate extraction followed by precipitation with Cetavlon or Fehling's reagent [see below]) may be harsh enough to degrade the sialic acid groups, which are known to be sensitive to chemical conditions (301). Second, the N-linked glycans on mannoproteins are strictly high mannose, as described in all previous reports, but the sialic acids are components of other glycoproteins as complex-type glycans. Such glycoproteins would not be expected to precipitate with Cetavlon because they lack the long mannan chains needed to interact with the Cetavlon (discussed in reference 191) and the mannose *cis*-diols needed to interact with borate (in the Cetavlon precipitation) or copper (in Fehling's reagent).

Combination To Form the Cell Wall

Building on previous studies of *S. cerevisiae* and *S. rouxii*, Bishop et al. (21) were the first to study the cell wall composition of *C. albicans* and were the first to isolate glucan and mannan from the cell wall as separate entities. They also determined that chitin was present by using X-ray diffraction (21). Earlier work by Kessler and Nickerson had reported glucomannan-protein and glucan-protein complexes (158).

Visualization of the cell wall structure began with electron microscopy, which showed layers of differing electrodensity (42, 43, 260). These electron micrographs, combined with biochemical data, led to the development of a cell wall schematic (Fig. 4) (35, 232, 260) that has been further refined as the cell wall components have been studied more closely (161, 213). Although these schematics are often depicted as having a layered structure, it has become increasingly apparent that the cell wall is actually a dynamic structure and that any given layer should be more properly referred to as a zone of enrichment (260).

Moving outward from the plasma membrane, mannoproteins are the major component of the first zone of enrichment, sometimes referred to as the periplasmic space (213). The next, more electron-dense zone is composed mainly of the β-1,3- and β-1,6-glucan "scaffolding." The proximal region of this zone is enriched for chitin (295), and there is evidence for a covalent link between chitin and glucan (278). The outermost zone is composed of mannoproteins (294, 325). Work by Kapteyn et al. indicated that at least some cell wall mannoproteins, as well as the chitin component, are covalently linked to the glucan chains (150–152). Some mannoproteins are linked through a glycosylphosophatidylinositol remnant structure to the β-1,6-glucan, which is subsequently linked to β-1,3-glucan or, in some cases, to chitin (150). Other proteins, coded for by members of the *PIR* (for "Proteins with Internal Repeats") gene family, are covalently linked directly to the β-1,3-glucan (150). A subset of the mannoproteins forms fibrillar structures that extend into the surrounding environment (42, 260). Using rapid-freezing techniques that better preserve cell surface ultrastructure, Tokunaga et al. (288) observed that this fibrillar

layer was brush-like and evenly spaced on the cell surface. Tokunaga's work was extended by Osumi to look at *S. cerevisiae* and regenerating *C. albicans* protoplasts (213).

The detailed structure of the fibrils is still unclear. One possibility is that the protein component is globular and lies close to the glucan scaffolding, with the observed fibril being entirely glycan. Alternatively, the fibril may contain a linear or tightly helical protein core decorated with glycan. Some cell wall proteins, which are anchored into the plasma membrane, possess a "lollipop on a stick" structure (134). A long stretch of polypeptide (the stick) extends from the membrane and passes through the wall. Short O-linked glycans are attached along the length of the stick, presumably to help maintain a linear structure. A globular domain (the lollipop) is then stuck out into the extracellular space. However, it is unknown if the fibrils are anchored in such a way into the membrane, covalently attached to glucan or chitin, or simply trapped within the glucan matrix. It is also not known if the fibril is composed of subunits, but some initial experiments suggest that a fibril subunit exists (319).

Some organisms produce extensive carbohydrate structures at the cell wall exterior. For example, *C. parapsilosis* has been reported to produce a slime layer similar to those produced by *Staphylococcus epidermidis* and other bacterial pathogens (186, 187). Although the composition of this slime layer has not been characterized in detail, Kuhn et al. (171) have shown that cell wall-like polysaccharides comprise the matrix. Their conclusions were based on staining with a fluorescent Con A conjugate, which binds to terminal mannose and glucose units, and staining with Calcofluor White, which binds to β-linked polysaccharides such as chitin and β-glucan. These authors also demonstrated that *C. albicans*, in addition to *C. parapsilosis*, was able to construct this slime layer biofilm. As mentioned above, *Cryptococcus neoformans* constructs a polysaccharide capsule composed mainly of glucuronoxylomannan (52, 297) on the cell wall exterior. Galactoxylomannan and mannoproteins are also elements comprising the capsule. The genetic and temporal regulation of capsule synthesis has recently been reviewed by Bose et al. (22).

FUNGAL GLYCANS IN PATHOGENESIS AND HOST RESPONSE

Protein Folding and Tertiary Structure

At the most basic level, the glycans present in the cell wall affect the nature of the proteins to which they are conjugated. N-linked glycans can increase protein stability, affect local secondary structure, affect protein packing at the cell surface, and prevent protein aggregation (119, 182). As mentioned above, O-linked glycans can provide structure and stability to proteins that are anchored in the cell membrane but must span the cell wall to present a domain at the surface (134, 182).

Adhesion

Attachment or adherence of pathogenic cells precedes infectious diseases (218, 305). Several reviews have discussed the adhesion of *C. albicans* in a more general fashion (33, 40, 115, 218). The discussion below is limited to how glycans specifically are involved in the adhesion process. Further, work has generally progressed from examination of the cell in its entirety to cell fractions, sequentially working toward refinement of experiments to allow discrimination between specific effects due to particular fractions. Thus, each of the following sections regarding the roles played by glycans in fungal pathogenesis begins with studies looking at glycans in a more global sense followed by those that focus on particular components.

Adhesion to host components. The presence of polysaccharide in the intercellular junction of *C. albicans* cells bound to epithelial or endothelial cells has been known for some time. Marrie and Costerton (188) described a ruthenium red-stainable matrix surrounding the *C. albicans* cell. Ruthenium red is a cationic dye with affinity for anionic polymers such as certain polysaccharides. The observed matrix was shown to exist between fungal cells and buccal tissue as well as between fungal cells and bacterial cells. Barnes et al. (16) demonstrated fibril connections between the yeast cell surface and the endothelium of renal peritubular and glomerular capillaries. Although the specific composition of the fibrils (i.e., carbohydrate versus protein) was not investigated, the authors speculated that they were the same as, or similar to, polysaccharide fibrils found on the *S. cerevisiae* cell surface as described by Johnston and Latta (136). Tronchin et al. (296) also reported that fibrillar structures and polysaccharide granules on the yeast cell surface appeared to mediate adhesion to epithelial cells. Some of this material was released from the cell surface into the surrounding medium under certain conditions (109, 183).

Other work focused on specific glycans that might be involved in adhesion events. The majority of evidence pointed to mannan as the active glycan. The studies discussed immediately below established the role of mannan in adhesion. Subsequent work extended these results to show which specific mannan components (β-1,2-oligomannosides, acid-stable side branches, or O-linked glycans) were the critical ones.

Maisch and Calderone (184) found that the mannoprotein fraction from *C. albicans*, when conjugated to sheep red blood cells (SRBC), was sufficient to cause the conjugated SRBCs to adhere to a fibrin-platelet matrix. Mannosidase treatment eliminated the adherence, indicating that the mannan was the adhering component. Subsequent results indicated that the matrix component involved in binding to mannan was fibronectin (253).

Sandin et al. (245) showed that pretreatment of yeast cells with Con A inhibited the binding to buccal epithelial cells (BEC), from 100 to 18% adherence. Con A pretreatment of the BECs also reduced binding. Con A is a lectin that binds α-linked terminal mannose or glucose residues of an oligosaccharide (93). Of the carbohydrates in the *Candida* cell wall (mannan, glucan, and chitin) only mannan is α-linked. Furthermore, Con A-mediated inhibition of binding was partially reversible with increasing amounts of α-methylmannoside added as a mannan competitor. Lectins that bind *N*-acetylgalactosamine (GalNAc), GlcNAc, fucose, or galactose did not significantly alter percent adhesion. Sandin et al. also reported that α-methylmannoside itself inhibited the binding to BEC while GlcNAc, ribose, α-methylglucoside, galactose, and xylose did not. This is in partial disagreement with the results of Sobel et al. (274), who reported that treatment of *C. albicans* with

fucose inhibited adhesion but treatment with mannose, α-methylmannoside, or galactose did not.

Centeno et al. (47) investigated the possible adhesion interactions between epithelial cells, bacteria, and fungi. Earlier observations had been made that bacterial binding to epithelial cells blocked similar binding by fungi. In addition, bacterial structures (e.g., pili) can bind to fungal cells, leading to fungal agglutination. Centeno et al. found that binding of yeast to BEC was enhanced by pretreatment of the BEC with piliated, but not nonpiliated, strains of *Klebsiella pneumoniae* or *E. coli*. The enhanced binding was reversed by the addition of mannose. Further, mannose, but not glucose, inhibited *C. albicans* binding to urothelial cells and BEC in the absence of bacterial cells. The authors hypothesized that the bacterial pili added mannose-recognizing lectin-like proteins that provided extra attachment sites for the fungal cells.

Sawyer et al. (250) examined a liver perfusion model for studying yeast adhesion. Mouse liver was isolated (in an ex vivo manner) into a perfusion system. *C. albicans* cells were then infused into the liver, and the system was switched back to perfusion medium. Clearance of cells from the perfusate exiting the system was due either to endothelial trapping or, to a lesser extent, to adherence to and subsequent phagocytosis and killing by Kupffer cells. Treatment of the yeast cells with α-mannosidase (from Jack Bean), subtilisin, α-chymotrypsin, and papain increased the hepatic killing of yeast cells but did not affect trapping. Mannoprotein, isolated from either *C. albicans* or *S. cerevisiae*, blocked fungal cell trapping in the liver and was also able to elute previously trapped cells. These results, combined with previous results from this group showing that mannose or α-methylmannoside (but no other sugar) was able to block liver trapping, suggest that it is the carbohydrate portion of the mannoprotein fraction that is involved in hepatic endothelium adhesion.

The use of monosaccharides (e.g., mannose or α-methylmannoside) to inhibit binding is commonly applied to demonstrate the recognition of glycans. In the case of competition for the Con A site, this practice seems reasonable since it is known that Con A binds to terminal saccharides. However, other lectins or receptors may recognize oligosaccharides or even oligosaccharides in the context of the polypeptide or lipid to which they are conjugated. Further, these binding interactions can be specific for not only chain length and saccharide composition but also anomericity and linkage. Some of these instances are mentioned in the discussion below. Thus, it seems that the use of bioses or longer oligosaccharides as competitors would be a more physiologically relevant choice. There are a couple of issues, however, that make this choice problematic. First, the specific configuration (i.e., saccharide, anomericity, linkage) needed for a competitor often cannot be known a priori. Therefore, several oligosaccharides may have to be tried before a suitable one is identified. Second, in cases such as the β-1,2-oligomannoside adhesin described below, the oligosaccharides are not commercially available and must be fractionated and purified in-house, which can be time-consuming. The problem of oligosaccharide availability is discussed in more detail in the last section of this review.

(i) β-1,2-Mannan. Miyakawa et al. (195) developed *C. albicans* mutants that lack a β-1,2-oligomannoside sequence found in the acid-stable mannan of serotype A strains. They observed a decrease in adhesion to BEC and to a mouth squamous carcinoma cell line by the mutant compared to the parent. Adherence of the mutant was comparable to that seen for wild-type serotype B cells that lack the β-1,2-oligomannosides in the acid-stable region. Pretreatment of the epithelial cells with mannoprotein from the parent strain decreased adhesion of parent cells, but mannoprotein from the mutant did not.

A series of papers from Cutler and coworkers identified specific mannan groups involved in *C. albicans* binding to macrophages in the splenic marginal zone. Kanbe et al. (147) first demonstrated this binding in an ex vivo assay. Subsequent studies (146, 179) investigated the possible cell wall groups that might be involved in the observed adhesion. *C. albicans* cells were extracted with β-mercaptoethanol, and the released material was fractionated using Con A affinity and ion-exchange chromatography (146). The crude β-mercaptoethanol extract inhibited yeast cell binding to the splenic marginal zone and lymph node epithelial cells in a dose-related manner, and this inhibition activity could be isolated to one particular fraction. Heat and protease treatment of the fraction did not affect inhibition, but periodate and mannosidase treatment did, indicating that the active moiety was carbohydrate. This conclusion was supported by experiments using a monoclonal antibody (10G) that recognizes a β-1,2-mannotetraose (179). The antibody was able to inhibit *C. albicans* attachment to murine splenic marginal-zone macrophages in the ex vivo assay. Recently, Fradin et al. (83) identified the receptor molecule on the macrophage for β-1,2-oligomannosides to be a 32-kDa protein with homology to galectin 3. Since the strain that was used for these studies, A9, is serotype B, the tetraose epitope must be part of the acid-labile fraction of N-linked glycans (Fig. 2).

In a recent study using an in vivo model, Dromer et al. (71) supported the importance of β-1,2-oligomannosides in adhesion. Intestinal colonization in mice was assayed by both survival and clearance (measuring CFU in fecal pellets). Expression of, or presence of, β-1,2-oligomannosides, as measured by agglutination with an anti-β-1,2-oligomannoside monoclonal antibody, positively correlated with virulence as assayed in mouse and rat systemic models. In the developed infant-mouse gastrointestinal (GI) colonization model, the investigators tested virulent (10261, high β-1,2) and avirulent (10231, low β-1,2) strains and measured the CFU/fecal pellet. The median score was always lower for 10231, and colonization was more protracted/prolonged with 10261 (which contained more β-1,2). Synthetic β-1,2-oligomannosides, but not α-1,2-oligomannosides, administered by gavage 1 h preinoculation, decreased or eliminated the CFU/fecal pellet (day 7 postinfection) in a dose-related manner. The authors therefore concluded that β-1,2-oligomannosides play a role in colonization, presumably at the adhesion step.

(ii) Acid-stable mannan. Other work by Cutler's group (144) showed that the acid-stable mannan inhibited *C. albicans* binding to splenic marginal zone epithelial cells, demonstrating that this mannan is also involved in attachment. Inhibition was not affected by treatment of mannoprotein with pronase, confirming the importance of the carbohydrate portion. Although the exact structure of the mannan group involved is not yet known, certain characteristics have been determined. Evidence suggests that both the α-1,2-linked side branches and the α-1,6-

linked backbone are involved (145). Mannoproteins from *S. cerevisiae MNN2* deletion mutants, which do not attach side branches to the outer chain backbone, did not inhibit binding, showing that the α-1,6-linked backbone alone is not sufficient. Acetolysis of the acid-stable mannan cleaves the α-1,6-linked backbone bonds and releases the side branches. Acetolysates of acid stable mannan also had no effect on binding, indicating that the side branches alone are not sufficient to produce the observed acid stable mannan-dependent adhesion. Partial sulfuric acid digestion, which sequentially releases the terminal sugar groups from the nonreducing ends, was carried out on acid-stable mannan. As the digestion progressed, producing shorter side branch chains, the inhibition of *C. albicans* binding decreased. Taken together, these results indicate that the acid-stable mannan mediating *C. albicans* binding to splenic marginal zone macrophages comprises two or more side branches, joined through the outer chain backbone and of a certain minimum length. Based on their results, Kanbe and Cutler (145) further concluded that chain length was more important than a specific side branch conformation. Other specifics of the macrophage mannose receptor and mannose-binding protein have been recently reviewed (84).

(iii) O-linked glycans. Buurman et al. (27) suggested that O-linked glycosylation plays a role in adhesion. Mutant strains with defects in O-linked glycosylation (leading to truncated glycans) showed decreased adhesion to human buccal epithelial cells in vitro and decreased adhesion to rat vaginal epithelial cells in vivo. They also observed that guinea pigs and mice had increased survival when challenged with the mutant strains. This increased survival was coincident with a decreased organ burden. These results, combined with the in vitro adhesion experiments, led the authors to conclude that decreased adhesion resulted in decreased organ burden and increased survival.

Herrero et al. (110) looked at the issue of glycosylation from the supply side. GDP-mannose is brought in from the cytosol to the Golgi lumen, where mannose is transferred to the growing mannan chain. The other product, GDP, is then hydrolyzed by a GDPase to give GMP and inorganic phosphate. GMP is then transported to the cytosol through an antiporter that brings in the GDP-mannose. Thus, a defect in the GDPase should have an effect on protein glycosylation. Previously, deletion of the *S. cerevisiae* GDPase gene (20) decreased glycosylation in the Golgi. The authors cloned the *C. albicans* GDPase gene, designated *CaGDA1*, and used the URA-blaster (80) procedure to knock out both alleles in *C. albicans.* Unexpectedly, they observed no change in N-linked glycans. However, a significant change in O-linked mannan was observed. There was a loss of the mannobiose and mannotriose groups and a corresponding increase in the monomer. This suggested a loss of ability to extend the O-linked chain. However, this loss of O-mannosylation did not affect measured adherence to HeLa cells.

These O-linked glycan results present a few possibilities. (i) The defect in the Herrero strain was not severe enough to alter adhesion. The authors themselves speculate that there might be backup GDPases in *C. albicans*. (ii) the residual O-linked M_1 glycans (from Buurman et al. [27]) or the residual M_4-M_5 O-linked chains observed by Herrero et al. (110) were sufficient to maintain whatever structural requirement was necessary for glycoprotein stability and adhesin function. (iii) Some of the results observed by Buurman et al. (e.g., increased survival, increased vaginal clearing, and decreased organ load) were due to processes other than adhesion per se, although this later case is harder to reconcile with the observed decrease in adhesion to BEC in vitro. (iv) If O-linked glycans play a structural role (as in the lollipop-on-a-stick model), the effect of disrupting O-linked glycosylation may have more to do with protein structure than the glycans acting directly as an adhesin.

There is some evidence for the involvement of glycans other than mannan in adhesion. Reversing the typical manner in which adhesion studies are done, Forsyth and Matthews (81) investigated carbohydrate inhibition of lymphocyte adhesion to *C. albicans* hyphal cells. While mannose and α-methlymannoside both inhibited interleukin-2 (IL-2)-activated mouse lymphocyte adhesion to *C. albicans* hyphal cells by approximately 40%, GlcNAc inhibited adhesion by activated mouse and human lymphocytes by 80%. No effect on mouse lymphocytes was observed when glucose or sucrose was used. As with the mouse lymphocytes, glucose had no effect on adhesion of IL-2-activated human lymphocytes, but small amounts of β-glucan (4 mg ml^{-1}, or approximately 40 μM) inhibited adhesion by 70%. From these results, the authors concluded that lymphocyte binding occurs through Mac-1, which has a lectin-like domain, known ligands for which include β-glucan and chitin. Placing their observations in context, Forsyth and Matthews concluded that interactions between fungal cells (*C. albicans*) and lymphocytes are multidimensional but involve lectin-like binding sites on the lymphocytes and glycans on the fungal cell surface.

The inhibition of adhesion by GlcNAc and the chitin binding domain of Mac-1 echoed an earlier result of Segal et al. (255), who found that, of the cell wall polymers, chitin (although not mannoprotein or glucan) inhibited the binding of *C. albicans* to vaginal epithelial cells. Chitin hydrolysate, GlcNAc, glucosamine, and mannosamine inhibited adhesion to a similar extent (255). On first inspection, these results would seem to be in conflict with the current model of the cell wall that has the chitin component in the inner layers of the cell wall, near the plasmalemma (Fig. 4). It has been shown, however, that the hyphal cell wall is thinner than that of the blastoconidia (43). Further, there is evidence for a small amount of chitin being present on the cell surface (295). These observations serve as illustrations of the dynamic nature of the cell wall and support the evidence by Forsyth and Matthews and Segal et al. for a role of chitin in adhesion, but they do not explain the ineffectiveness of mannose or mannoprotein as an adhesion inhibitor observed by Segal et al.

Another possible explanation is that the experimental conditions are playing a role. Researchers have used various strains, growth conditions, and substrates in adhesion experiments (Table 2). A comparison of additional experimental conditions used in adhesion studies has been done recently by Calderone and Gow (32). The multidimensional aspect of adhesion noted by Forsyth and Matthews may indicate that the relative importance of a specific adhesin depends on the combination of fungal strain and tissue substrate, as well as experimental design.

Adhesion to other members of the microflora. Cell surface carbohydrate has been implicated in the interaction between *C. albicans* and other members of the microflora, particularly

those from the oral cavity (Table 3). Bagg and Silverwood (11) first reported that coaggregation of *C. albicans* with *Fusobacterium nucleatum* and *Actinomyces viscosus* decreased significantly when the yeast cells were treated with periodate, which oxidizes and cleaves glycan chains. This treatment did not, however, affect the coaggregation of yeast with oral streptococci. These results suggest that carbohydrates on the yeast cell surface are involved in interactions between *C. albicans* and *F. nucleatum* or *A. viscosus* but not oral streptococci (e.g., *Streptococcus mutans*, *S. mitis*, and *S. salivarius*).

The latter conclusion was confirmed by Jenkinson et al. (133), who reported that *C. albicans* coaggregation with *S. sanguis*, *S. gordonii*, and other oral streptococcal isolates was not inhibited by the addition of 60 mM D-mannose, D-glucose, GlcNAc, or other monosaccharides. The authors state that their findings are in contrast with those of Bagg and Silverwood. However, the only possible point of contention is the *S. sanguis* result, and Bagg and Silverwood indicated that *S. sanguis* coaggregation was equivocal (11). In all other respects, the two sets of results correlate exactly. Jenkinson et al. measured coaggregation by using a radioactive assay in which bacterial cells were metabolically labeled with [methyl-^{3}H]thymidine before being mixed with yeast cells. Coaggregation was determined by measuring the amount of radioactivity not associated with the yeast cells after pelleting. This assay is different from the simpler visual assay used in the other three studies mentioned in this section, but how it may affect conclusions regarding the efficacy of inhibitors is unknown.

Hsu et al. (117) looked at coaggregation of *C. albicans* and oral bacteria isolated from bone marrow transplant patients. They reported that in contrast to previous results (11), sugars did not inhibit coaggregation of *C. albicans* and *A. viscosus.* Neither did the sugars inhibit coaggregation with *S. sanguis* or *S. epidermidis*, in agreement with the other results. Coaggregation of *C. albicans* with *S. mitis* and *Lactobacillus salivarius* was inhibited by α-methylglucoside. Glucosamine inhibited coaggregation with *S. salivarius*, while glucose inhibited coaggregation with *Bacteroides gingivalis.* Mannose, α-methylmannoside, glucose, and α-methylglucoside all inhibited coaggregation with *Lactobacillus amylovorus*. Coaggregation with *F. nucleatum* was inhibited by the addition of mannose or α-methylmannoside. More recently, Jabra-Rizk et al. (121) showed that mannose inhibited the coaggregation of *F. nucleatum* with *C. dubliniensis* as well as *C. albicans*.

The summary of these results seems to be that the strains of *A. viscosus* (one strain, tested twice) and *F. nucleatum* (three strains) tested were positive for coaggregation and that the evidence is that a glycan on the fungal cell surface is involved in that coaggregation. Because the fungal cells were cultured and prepared for assay under different conditions, it is difficult to predict how other fungal phenotypes are involved in coaggregation. For example, results by Jabra-Rizk et al. (121, 122) suggest that cell surface hydrophobicity plays a role in coaggregation of *C. albicans* and *C. dubliniensis* with *F. nucleatum*. This conclusion is supported, to a certain extent, by the observation of Jenkinson et al. (133) that *Streptococcus gordonii*, *S. sanguis*, and *S. anginosis* adhered best to *C. albicans* germ tubes, structures that are highly hydrophobic, especially at the distal end (106, 107). These authors, however, concluded that cell surface hydrophobicity was not involved since star-

TABLE 2. *C. albicans* glycans and adhesion

C. albicans strain [serotype]	Substrate[a] (source)[b]	Growth medium[c] (temp)	Assay format	Evidence for glycan[d]	Reference
Clone 4 [B]	Fibrin-platelet matrix	Phytone peptone + Glc, 1 mg ml^{-1}	Radiolabeled fungi	Inhibition by α-mannosidase	184
Clinical isolate	Urothelial cells (h)	Tryptose phosphate	Microscopy	Ruthenium red staining	188
CBS562 (ATCC 18804)	VEC (h)	Yeast extract (28 or 37°C)	Mix and filter	Inhibition by chitin, GlcNAc, ManN, GlcN	255
MSU-1	BEC (h)	TSB + 4% Glc; M199 (37°C)	Mix and filter	Inhibition by Con A (reversed by α-MM)	245
Clinical isolate	Urothelial cells, BEC (h)	SDA (37°C)	Mix and filter	Inhibition by Man	47
VW32 [A]	BEC (h)	SDA (37°C)	Mix and filter	Staining with Con A	296
MSU-1	BEC (h)	TSB + 4% Glc; M199 (37°C)	Mix and filter	Inhibition by Con A, extraction of carbohydrates	244
20A	Liver (m)	TSB + 0.5 M Gal, RPMI 1640 + Glc (24 or 37°C)	Perfusion and flow (elution)	Inhibition of trapping by Man, α-MM	250
M1012R AL mannan mutants, clinical isolates [A and B]	BEC (h)	SDB (27°C)	Mix and filter	Decreased adhesion in absence of β-1,2-oligomannosides	195
A9 [B]	Splenic and lymph node sections (m)	GYEP (37°C)	Microscopy	Inhibition by periodate oxidation or α-mannosidase	146
A9 [B], Strain 1 [A], 222 [B]	Splenic sections (m)	GYEP (37°C)	Microscopy	Inhibition by monoclonal antibody 10G or its antigen (β-1,2-Man4)	179
A9 [B], TCH32 [A], NUM1385 [A]	Splenic sections (m)	GYEP (37°C)	Microscopy	Periodate oxidation prevents binding activity	144
A9 [B], *S. cerevisiae* x2180, *S. cerevisiae MNN* mutants	Splenic sections (m)	GYEP (37°C)	Microscopy	Inhibition by α-mannosidase	145
SC5314 [A], CAI4, NGY21, NGY23, NGY24	Vaginitis (r), BEC (m or gp)	Specific medium not indicated	Colony counts	Decreased adhesion in absence of O-linked glycans	27
ATCC 10231, ATCC 10261	GI epithelium (m)	SDA (37°C)	GI colonization and pass through	Inhibition by β-1,2-Man4	71
SC5314 [A], CAI4, SS19-4, BAB1-4	HeLa cells	YEPD (GYEP), synthetic dextrose	Colony counts		110
ATCC 58716 [A]	IL-2-activated lymphocytes (m), NIH 3T3 cells (m)	SDA, RPMI 1640 (37°C)	Radioactivity (^{51}Cr-labeled mammalian cells)	Inhibition by Man, α-MM, GlcNAc, and glucan	81

[a] BEC, buccal epithelial cells; VEC, vaginal epithelial cells.
[b] gp, guinea pig; h, human; m, mouse; r, rat.
[c] Gal, galactose; Glc, glucose; GYEP, glucose/yeast extract/peptone; SDA, Sabouraud dextrose agar; TSB, Trypticase soy broth.
[d] GlcN, glucosamine; GlcNAc, *N*-acetylglucosamine; Man, mannose; ManN, mannosamine; α-MM, α-methylmannoside.

TABLE 3. Coaggregation of *C. albicans* with other members of the microflora

Bacterial species	Strain	Coaggregate	Periodate (10 mg ml^{-1})	Inhibition by[d]:						Reference(s)
				D-Man	α-MM	D-Glc	α-MG	D-GlcN	L-Fuc	
A. viscosus	ATCC 15987	Y (4 of 7)[a]/Y	Y	N	N	N	N	N	N	11, 117
B. gingivalis	CS213	Y (3 of 7)[a]		N	N	Y	N	N	N	117
B. intermedius	NCTC 9336	N								11
E. faecalis	JH2-2	10%[b]								133
E. coli	ATCC 25922	N								117
	NCTC 10418	Y								11
F. nucleatum	ATCC 49256	Y		Y						121
	NGB 15-73	Y	Y							11
	VPI 10197	Y		Y	Y	N	N	N	N	117
L. acidophilus	ATCC 521	N								117
L. amylovorus	JH05	Y		Y	Y	Y	Y	N	N	117
	JH06	Y		Y	Y	Y	Y	N	N	117
L. salivarius	ATCC 11741	Y (2 of 7)[a]		N	N	N	Y	N	N	117
S. epidermidis	ATCC 12228	Y (2 of 7)[a]		N	N	N	N	N	N	117
S. anginosus	ATCC 27335	69%[b]								133
	NCTC 10709	<10%[b]								133
	NCTC 10713	58% (CA2)[b]								133
S. gordonii	ATCC 10558	54%[b]								133
	DL1 (Challis)	81%[b]								133
S. mitis	JH05	Y (4 of 7)[a]		N	N	N	Y	N	N	133
	NCTC 10712	Y	N							11
S. mutans	ATCC 1600	N								117
	ATCC 25175	12%[b]								133
	NCTC 10832	Y								11
S. oralis	ATCC 10557	58%[b]								133
S. pyogenes	M4	35%[b]								133
S. salivarius	HB	28% (CA2)[b]								133
	ATCC 25975	Y (1 of 7)[a]		N	N	N	N	Y	N	117
	NCTC 8606	Y	Y/N							11
S. sanguis	ATCC 10556	Y (5 of 7)[a]		N	N	N	N	N	N	117
	ATCC 10557	Y		N	N	N	N	N	N	117
	ATCC 10558	Y	N							11
	ATCC 903	Y (5 of 7)[a]		N	N	N	N	N	N	117
	CN3410	86%[b]		N		N	N			133[c]
	CR311	80%[b]								133
	FW213	62% (CA2)[b]		N		N	N			133[c]
	NCTC 7869	90%[b]								133
	PSH1b	75%[b]								133

[a] *C. albicans* strains tested were JH01, UMCC, JH05, JH06, JH08, JH (blood), and ATCC 18804 (117).
[b] Results reported as percent input cpm bound to *C. albicans* cells. Low percentages (<20%) were taken to indicate no coaggregation. Primary test strain was 6406, but CA2 was used for the three indicated.
[c] Also tested (and negative) for D-fucose, D-galactose, lactose, raffinose, L-rhamnose, GlcNAc, and GalNAc.
[d] Man, mannose; α-MM, α-methlymannoside; Glc, glucose; α-MG, α-methylglucoside; GlcN, glucosamine; Fuc, fucose.

vation of fungal cells increased coaggregation but had no effect on cell surface hydrophobicity.

In contrast to *F. nucleatum* and *A. viscosus*, no strain of *Klebsiella pneumonia*, *Lactobacillus casei*, *Peptostreptococcus anaerobius*, *P. magnus*, *P. micros*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Pseudomonas maltophila*, *P. stutzeri* (2 strains), *P. aeruginosa*, or *Staphylococcus aureus* formed coaggregates. The *Streptococcus* species present more variable results that seem to be species and strain dependent. For example, *S. sanguis* appears to form some kind of coaggregate, but the evidence points to the relevant glycan being on the bacterial, not the fungal, cell surface.

Immune Cell Receptors and Interactions

The interactions between immune system components and *C. albicans* cells and cell fractions have been the subject of several recent reviews (36, 57, 58, 70, 189, 225, 282). A broader discussion of the interactions between the immune system and the fungal cell wall has been provided by Latgé and Boucias (174). *C. albicans* has been called a mosaic of immunoregulatory molecules (44) because of both the numbers of cell surface moieties implicated in regulation of immune system response and the types of regulatory effects observed. In some cases, particularly those involving mannan fractions, separate studies using similar cellular fractions report seemingly conflicting conclusions about whether the fraction is immunostimulatory or immunosuppressive (reviewed in reference 70). As discussed below, some of these apparent conflicts may be due to dose effects. In addition, most of the cellular fractions tested were a mixture of glucan, mannan, and mannoprotein, and different preparations resulted in different proportions of the several components. As these studies have progressed, the cellular fractions have been refined to the point where certain aspects of the interaction between the immune system and the fungal cell can be attributed to specific glycans (Table 4). For example, several types of immune cells have been reported to have receptors that bind fungal glycans that can then lead to modulation of cytokine production. In addition, antiglycan an-

TABLE 4. Glycan interaction with immune cells and immune system components

Glycan	Strain [serotype]	Growth medium[a] (temp)	Immune system component (source)[b]	Immune cell source[b]	Glycan effect	Reference
β-Glucan (particulate)	*S. cerevisiae*	Commercial (dried) yeast	Complement (gp, h, r)	DHC-BA (gp), Long-Evans hooded (r), commercial source (h)	Decreased complement activation	90
β-Glucan (particulate)	*S. cerevisiae*	Commercial glucan	Monocytes (h)	Isolation from healthy volunteers	Stimulate leukotriene production	60
β-Glucan (soluble, particulate)	*S. cerevisiae*	Commercial glucan	Monocytes (h)	Isolation from healthy volunteers	Particulate glucan stimulated released of *N*-acetylglucosaminidase	130
Mannoprotein O-linked mannan	*C. albicans* 2252 (ATCC 44806)	YNB/AMS/2% Glc (RT)	PBMC	Isolation from healthy volunteers	O-linked glycan inhibits proliferation in response to *Candida* antigen	224
β-Glucan (particulate)	*S. cerevisiae*, barley	Commercial glucan	Neutrophils (h)	Isolation from healthy volunteers	β-Glucan particles induce a superoxide burst	239
β-Glucan (soluble)	*S. cerevisiae*	Commercial glucan	Monocytes (h)	Isolation from healthy volunteers	Identified β-glucan receptor	61
β-Glucan (particulate)	*S. cerevisiae*	Commercial glucan	Monocytes (h)	Isolation from healthy volunteers	Stimulated production of TNF-α and IL-1β	1
β-Glucan (particulate)	*S. cerevisiae*	Commercial glucan	PBMC	Isolation from healthy volunteers	Binding of β-glucan upregulates expression of CR3	198
β-Glucan	*S. cerevisiae*	Commercial glucan	Macrophages (rb, alveolar)	New Zealand White (rb)	Glucan inhibits *C. albicans*-stimulated release of arachidonic acid; inhibits phagocytosis of *C. albicans*	45
β-1,2-Oligomannosides	*C. albicans*	YEPD	Macrophages (m, peritoneal exudate)	BALB/c mice 20–24 wk	β-1,2-Man4, β-1,2-Man5, and β-1,2-Man8 induce TNF-α production	143
β-1,2-Mannotetraose, β-glucan (soluble)	*C. albicans* VW32 [A]	SDA, YE/Glc/$MgSO_4$/NH_4OH (37°C)	Peritoneal epithelial cells (m), J774 (m, macrophage-like cell line)	Peritoneal cavity rinse (BALB/c mice 4–6 mo, female), commercial cell line	β-Mannan and β-glucan receptors on macrophages	82
β-Glucan (soluble)	*S. cerevisiae*, barley, mushroom	Commercial glucan	NK cells	Isolated from healthy volunteers	β-Glucan priming of NK cells allowed lysis of iC3b-opsonized SRBC	303
Chitin	*C. albicans* E-139	GYE agar, medium 199 (24°C)	Macrophages (m)	Swiss OF1 mice 6–8 wk, female	Chitin inoculation increased subsequent phagocytosis and candidacidal activity	233
β-Glucan (soluble)	*S. cerevisiae*	Commercial glucan	NK cells	Isolated from healthy volunteers	β-Glucan priming induced NK cell cytotoxicity toward mammary carcinoma cells	304
β-1,2-Mannan (from phospholipomannan)	*C. albicans* VW32 [A]	SDA (28°C)	J774 (m, macrophage-like cell line), L929 (m fibroblast cell line)	Commercial cell line	Identify receptor for β-1,2-mannan from glycolipid	141
Mannan (mannoprotein)	*C. albicans* VW32 [A]	SDB	IL-2 (h)	Commercial source of IL-2	IL-2 binds to *C. albicans* mannan	323
Mannan (mannoprotein)	*C. albicans* NIH A-207 [A]	SDB (27°C), RPMI 1640 (37°C)	RBC (h)	Isolation from healthy volunteers	Mannoprotein acts as hemolytic factor; activity removed by periodate	311
β-Glucan (soluble)	*S. cerevisiae*	Commercial glucan	Macrophages and NK cells (m), P388D1 (m, monocytoid line), Ptas64 (m, mammary tumor)	BALB/c mice 6–8 wk, female	Mouse leukocyte CR3 is soluble and particulate β-glucan receptor	315
β-1,2-Mannan	*C. albicans* VW32	SDA	J774 (m, macrophage-like cell line)	Commercial cell line	β-1,4-Man4 induces release of TNF-α	142
β-Glucan	*C. albicans* BP [A]; CA2	Winge medium (28°C), Lee's medium (28 or 37°C)	Monocytes (h)	Isolated from healthy volunteers	β-glucan induced MCP-1, MIP-1b, and IL-8 production	291
β-Glucan (soluble, CSBG)	*C. albicans*	C-limiting medium	DC (m)	C57BL/6 mice, 8 wk, female	CSBG augments DC maturation	159
β-Glucan (particulate)	*C. albicans* IFO 1385	SDA, C-limited medium (25°C)	PBMC (h), RAW 264.7 (m, macrophage cell line)	Isolated from healthy volunteers	Particulate glucan induced release of IL-8 (PBMC) and TNF-α (RAW264.7)	120
Glucan (soluble, curdlan)	*Alcaligenes faecalis*	Commercial glucan	RAW264.7, RAW-R12 (m, macrophage cell lines)	Commercial cell lines	Glucan increases activation of NF-κB, iNOS, and TNF-α	154
Mannan (mannose)	*C. albicans* UC 820	SDB (37°C)	DC (h)	Isolated from healthy volunteers	Pututive second mannan receptor on DC	39

[a] AMS, ammonium sulfate; Glc, glucose; GYE, glucose-yeast extract; SDA, Sabouraud dextrose agar; SDB, Sabouraud dextrose broth; YE, yeast extract; YNB, yeast nitrogen base. RT indicates room temperature.
[b] gp, guinea pig; h, human; m, mouse; r, rat; rb, rabbit.

tibodies have been described, some of which confer protection against subsequent *Candida* infection.

Glycan binding to and activation of immune cells. (i) Mannan. Various receptors on the surface of immune cells recognize one or more glycan moieties. For example, mannan receptors have been found on macrophages (144, 179, 298). Speert and Silverstein (275) observed that macrophages (human macrophages, mouse peritoneal macrophages, and a mouse macrophage line, J774) phagocytose zymosan in the absence of opsonins. Zymosan was originally identified as a yeast component that inactivated complement component C′3 (222). It is an insoluble residue of *S. cerevisiae* that is prepared by extraction of yeast cells with hot water and digestion with trypsin. The resulting product is then further extracted with ethanol and water. Di Carlo and Fiore (67) determined the composition of zymosan as being glucan (54.7%), mannan (18.8%), and protein (14.5%), with the remainder comprising fat, inorganic material, and chitin. They also described zymosan visualized with the Gram stain as being "ghost" cells. From this description, zymosan probably represents some remnant of the cell wall scaffolding with covalently linked chitin and mannoprotein.

Because zymosan phagocytosis was inhibited by a mannoprotein fraction, Speert and Silverstein (275) concluded that phagocytosis occurs after recognition by a mannose receptor. Similarly, Cross and Bancroft (56) reported that phagocytosis of an acapsular *Cryptococcus neoformans* mutant was inhibited by the addition of mannoprotein from *S. cerevisiae*. Scaringi et al. (251) reported that injection of mice with *C. albicans* mannoprotein stimulated the generation of natural killer cells and activated macrophages. Podzorski et al. (224) reported an increase in lymphoproliferation due to mannoprotein. The preceding results, however, lack the evidence necessary to prove that the effect was due to the carbohydrate portion of the mannoprotein fraction rather than the protein.

Several recent studies have linked glycan specifically to effects on the immune system. Podzorski et al. (224) refined their results mentioned above and examined the effect of O-linked mannan on lymphoproliferation. These oligosaccharides, separated from the mannoprotein fraction by beta elimination, inhibited lymphoproliferation in response to *Candida* antigen, tetanus toxoid, and herpes simplex virus type 1 (224). Fradin et al. (82) showed that pretreatment of macrophages (either peritoneal macrophages or the J774 macrophage cell line) with β-1,2-linked, acid-labile oligomannosides decreased the macrophage binding of both live and heat-killed *C. albicans* cells. The extent of inhibition was the same when a synthetic β-1,2-mannotetraose was used as the inhibitor. Another study from the same group showed that the β-1,2-oligomannosides can also be released from *C. albicans* cells during *C. albicans*-macrophage coculture as a component of a glycolipid fraction (141). A recent paper by Cambi et al. (39) reported that along with the mannose receptor, dendritic cells (DC) bind *C. albicans* cells through a DC-specific intercellular cell adhesion molecule (ICAM)-grabbing nonintegrin (DC-SIGN) protein. Binding of fungal cells was inhibited in the presence of mannose, but only by 40%. Even with the addition of an anti-DC-SIGN antibody, binding was still 20% of control levels. In addition, when DC-SIGN was transfected into K562 cells (a moderately undifferentiated leukemic derivative cell line), mannose had no effect on *C. albicans* binding. These results suggest that the interaction of *C. albicans* cells and DC-SIGN is not mediated solely by lectin-like activity.

Watanabe et al. demonstrated that the glycan component of *C. albicans* mannoprotein acts as a hemolytic factor (311). RBC were mixed 1:1 (vol/vol) with cell fraction samples, and hemolysis was monitored by measuring the absorbance of the suspension at 405 nm. Hemolysis was associated with a sugar-rich, high-molecular-weight chromatographic fraction characterized as having low protein and high sugar content. Treatment of this fraction with periodate, which cleaves the glycan chains, eliminated hemolytic activity, demonstrating that carbohydrate was the active group.

Cross and Bancroft (56) extended their inhibition experiment to show that mannose binding protein bound to the acapsular *Cryptococcus neoformans* mutant, but not to encapsulated wild-type cells. These results indicated that the glucuronoxylomannan capsule produced by *C. neoformans* could mask potential glycan ligands recognized by immune cell receptors. Subsequent work by Mansour et al. (185) showed that mannoprotein released by *C. neoformans* into the culture supernatant fluid inhibited, in a dose-related manner, the binding of the macrophage mannose receptor to one of its natural ligands, horseradish peroxidase type II. They further showed that deglycosylation drastically reduced the ability of *C. neoformans* mannoprotein to activate primary T cells. In addition, binding of *C. neoformans* to DC is mediated, at least in part, by a mannose receptor (283). There is also evidence that peritoneal macrophages bind and phagocytose *Paracoccidioides brasiliensis* cells via a mannose receptor. Phagocytosis of *P. brasiliensis* cells was significantly inhibited by the addition of mannose or, to a lesser extent, fucose (3). Mannose reduced the phagocytic index to between one-third and one-half that of the control. This result again raises the issue of using monosaccharides as substitutes for oligomers or polymers. An oligomannoside, used as a competitor, may have completely inhibited phagocytosis because of more complete blockage of the macrophage receptor.

(ii) Chitin. Suzuki et al. (279) first reported the immunological effects of chitin in a murine model of candidiasis. Mice receiving pretreatment with chitin (50 mg kg^{-1}) were better able to survive a lethal challenge with *C. albicans* than were mice receiving no treatment. In addition, polymorphonuclear leukocytes (PMN) from circulating blood and peritoneal exudate cells from treated animals showed a higher level of reactive oxygen species (O_2^- and H_2O_2) generation than did those isolated from control animals. However, when macrophages were separated from peritoneal exudates, candidacidal activity was not dependent on reactive oxygen species (280) but was due to a serine protease released by the chitin-treated macrophages. Candidacidal activity was present in macrophage culture supernatant fluid and was inhibited by actinomycin D and diisopropylfluorophosphate, which inhibit protein synthesis and serine protease activity, respectively. One potential confounding factor of these studies is that the authors imply that the control animals were simply untreated rather than receiving sham injections. Thus, it is possible that some of the response is due to the act of injection rather than to receiving chitin specifically. However, chitosan, a deacetylated chitin derivative, was also tested for immunological effects (279, 280),

and although chitosan-treated mice also had increased levels of PMN and macrophages, the distribution of these cells (circulation versus peritoneal exudate) was different. Thus, it seems less likely that the immunopotentiating effects of chitin were due entirely to the injection itself.

The effect of fungal chitin (as opposed to chitin extracted from crustacean shells) on immune system components is described in a single study. Rementería et al. (233) extended the earlier studies to examine chitin purified from the *C. albicans* cell wall. They reported that, as observed by Suzuki et al. (279), injection of mice with purified *C. albicans* chitin (30 mg kg^{-1}) increased the survival time in response to a *C. albicans* challenge of 10^5 cells. In these experiments, it was explicitly stated that control mice were given an injection of saline to control for the stress of the injection itself. As a further refinement, the authors quantified the chitin present in the organs of experimentally infected animals. Chitin treatments were then based on these measured values.

To begin to look at more specific reasons for this increased survival, peripheral macrophages were isolated over the course of a 20-day study period (233). Macrophages isolated from mice treated with chitin and macrophages isolated from mice treated and then infected with *C. albicans* both showed increases in phagocytosis and candidacidal activity over those for macrophages isolated from control animals. However, the peak of phagocytosis, measured as (number of macrophages ingesting at least one yeast cell/total number of macrophages) $\times$ 100, did not coincide with the peak of cytotoxicity, measured as percentage of killed yeast cells in samples − percentage of yeast cells killed in controls lacking macrophages. Nitric oxide production was increased only in macrophages from mice receiving both chitin treatment and *C. albicans* infection. Because peak nitric oxide production preceded peak cytotoxicity, the authors concluded, as did Suzuki et al. above (280), that macrophage cytotoxicity in these animals was not occurring through an oxidative burst. In their study, Rementería et al. suggested that a lower dose of chitin (10 mg kg^{-1}) also produced immunosuppressive effects. However, these data, while suggestive, are not as convincing as those from the experiment with the higher dose.

(iii) Glucan. Studies that examine the interaction between fungal glucan and immune cells can be separated into two groups: those that use soluble glucan and those that use insoluble or particulate glucan. Szabó et al. used soluble glucan (prepared by sonication of *S. cerevisiae* glucan particles) to characterize the ligand binding domain of a previously identified β-glucan receptor on mononuclear phagocytes (61, 130, 284). These receptors were shown to also bind *C. neoformans* glucan (56). In a study mentioned previously, Fradin et al. (82) found that laminarin, a polymer of β-1,3-linked glucose units with β-1,6-glucan branches, inhibited the binding of heat-killed, but not live, *C. albicans* cells to macrophage cells. The authors speculated that exposing the cells to heat released cell surface components and exposed the deeper glucan layers. This supports the evidence that macrophages contain proteins that bind glucan in addition to the mannose receptor. It further suggests that the mannose receptor is not sterically blocked when glucan is bound or that binding of mannan is able to displace bound glucan.

Tokunaka et al. (290) examined the immunopharmacologic activity of a soluble glucan preparation from the *C. albicans* cell wall (*Candida* soluble beta glucan [CSBG]). They observed that CSBG increased the hematopoietic response to cyclophosphamide-induced leukopenia, increased vascular permeability, and acted as an adjuvant for the purposes of antibody production. They also observed several effects with respect to cytokine production (discussed below). Kataoka et al. (154) looked at macrophage activation by several types of soluble β-1,3-glucans by using an NF-κB–luciferase reporter assay. Activation culminated in the induction of proinflammatory mediators, inducible nitric oxide synthase (iNOS), tumor necrosis factor alpha (TNF-α), and macrophage inflammatory protein 2 (MIP-2). The authors found that a linear β-1,3-glucan, such as curdlan, gave a stronger response than branched glucans, such as laminarin. They also reported that β-1,3-oligoglucosides were ineffective at macrophage activational, although a β-1,3-glucoheptaose was able to inhibit curdlan activation of macrophages. Kikuchi et al. (159), also using the CSBG preparation, showed that β-glucan augmented or induced maturation of DC. Xia et al. (315) demonstrated that, as with human leukocytes, soluble and particulate β-glucan binds to mouse leukocytes via CR3 (CD11b/CD18, $\alpha_M\beta_2$-integrin). Binding of β-glucan to natural killer (NK) cells, macrophages and the monocytic cell line $P388D_1$ was inhibited by other β-glucans but not yeast α-mannan. Further, glucan binding was inhibited by anti-CR3 antibody and did not occur on cells from CR3-deficient mice. Earlier work by this group demonstrated that binding of soluble glucan to CR3 enabled NK cells and neutrophils to lyse iC3b-opsonized SRBC (303). The authors proposed that binding of soluble β-glucan to these cells primed them for cytotoxicity against iC3b-opsonized cells, even when the opsonized cells are normally resistant to CR3-dependent killing.

Particulate glucan (e.g., in the form of zymosan or unopsonized yeast cells) binds to and is phagocytosed by neutrophils and monocytes in the absence of opsonins (60, 238). Ross and coworkers further demonstrated that binding of particulate, although not soluble, β-glucan induced superoxide bursts from neutrophils (239, 303). Binding of particulate β-glucan also led to upregulation of CR3 expression on the surface of NK and cytotoxic T cells (198). Czop and Austen (60) showed that binding of particulate β-glucan to monocytes induced leukotriene production. This action is inhibited by pretreatment of the monocytes with soluble β-glucan but not α-mannan. In an earlier paper, Glovsky et al. (90) reported that particulate glucan affected human serum complement activity, mainly by decreasing the C2 and C3 activity. Lesser effects on C1 and C5 activity were also seen. This coincided with a release of C3a from normal human serum. In vivo experiments showed that particulate glucan activates guinea pig complement via the alternative pathway. Ishibashi et al. (120) compared soluble and particulate forms of β-glucan having the same primary structure. The particulate glucan had a significantly greater effect than did the solubilized glucan on the activation of various cells, as measured by production of IL-8 by human peripheral blood mononuclear cells (PBMC), TNF-α production by a murine macrophage cell line (RAW 264.7), and peroxide production in ICR mouse peritoneal exudate. These results indicate that immune cell activation is a function of both sol-

ubility and particle size (termed degree of assembly) (120). Vassallo et al. (302), working with particulate β-glucan from *Pneumocystis carinii*, demonstrated that dose is also a factor in the immune cell response (see below).

Alkali-insoluble polysaccharide from *Paracoccidioides brasiliensis*, containing β-glucan, also activated PBMC isolated from healthy volunteer donors (7). Incubation of PBMC in the presence of this cell wall fraction (F1) induced production of peroxide similar to that induced by gamma interferon (IFN-γ). Incubation of PBMC with IFN-γ and F1 increased fungicidal activity and TNF-α production over the levels produced by either component alone.

Glycan influence on cytokine production. (i) Mannan. Several studies implicated mannan in the modulation of cytokines. Castro et al. (45) reported that mannoprotein from *C. albicans*, but not *S. cerevisiae*, inhibited arachidonic acid release by alveolar macrophages. In addition, pretreatment with mannoprotein inhibited the phagocytosis of *C. albicans* cells. Garner and Hudson (86) reported that injection of mice with mannoprotein induced TNF-α production. Wang et al. (310) injected mannoprotein into mice that were sacrificed over time to determine the mRNA levels of various cytokines. They observed no apparent induction of IL-2 but did find some increase in IL-4 and IFN-γ levels between 48 and 96 h. The IL-10 signal was strong throughout the assay period (2 to 96 h), while IL-12p40 levels were reduced in the first 12 h but returned to normal levels by 48 h.

All of these studies were carried out using preparations or extracts that release mannoproteins, not isolated polysaccharide. It is not clear, therefore, if the active portion is the glycan or the protein. More recent studies have identified specific glycan components that are involved in cytokine interaction and induction. Garner et al. (87) observed that mannan induced TNF-α production by mouse alveolar macrophages. They implicated the carbohydrate portion specifically based on two results. First, pretreatment of the mannoprotein with Con A (a lectin that binds terminal mannose, as well as glucose, units) inhibited TNF-α induction. Second, pretreatment of the macrophages with mannose or α-methlymannoside also inhibited induction. These two results indicated the presence of a mannose receptor or mannose binding protein on macrophages. They further showed that there was some species specificity involved with regard to the ability of mannoprotein to induce TNF-α secretion. Mannoprotein from *S. cerevisiae* had no effect on TNF-α secretion. Mannoprotein from *C. tropicalis* induced TNF-α secretion, but the effect was about one-fifth the effect produced by *C. albicans* mannoprotein. N-linked glycans from *C. tropicalis* mannoproteins contain β-1,2-oligomannosides both in the acid-stable portion and as acid-labile groups, although the latter do not get as long as those from *C. albicans* (164). The comparison of the three fungal species suggests that the active mannan is a β-1,2-oligomannoside because these groups are absent from *S. cerevisiae* glycans.

Jouault et al. (143) extended the results of Garner et al. and showed that individual oligosaccharides in the acid-labile N-linked glycan groups (specifically β-1,2-Man_4, Man_5, and Man_8) induced TNF-α production by murine alveolar macrophages. Furthermore, TNF-α production following macrophage stimulation by the octaose was nearly fivefold higher than that due to stimulation by the tetraose or pentaose. TNF-α production in response to the heptaose, although the heptaose differs from the octaose by only a single mannose, was virtually undetectable. As the authors note in their discussion, this suggests a structural specificity to mannan binding (143). The relative activity of the tetra- and pentaose also suggests that the β-1,2-oligomannoside has a repetitive characteristic. Four or five mannose units may form the base of the structure recognized by the macrophage receptor, but the additional four units, perhaps completing another helical turn, are needed to form the complete ligand.

A subsequent study showed that this stimulation had the effect of desensitizing the macrophages, causing an inhibition of TNF-α release after a second stimulation (142). The results from these two groups support, and are supported by, those from Cutler's group demonstrating that the *C. albicans* macrophage adhesin is a β-1,2-mannotetraose (see the discussion above). This does not yet, however, provide a mechanism for the observation of Garner et al. (87) that TNF-α induction by *Candida* mannan is not a simple on/off switch. One possibility is that cross-linking of the mannose receptors is required for full induction and that *C. tropicalis* glycosylation is not extensive enough to provide maximal cross-linking. Another possibility is that some other element required for full induction is present in *C. albicans* but absent in *C. tropicalis* (87).

Studies by Zanetta and coworkers (46, 323) demonstrated that IL-2 directly interacts with *C. albicans* mannoprotein in a lectin-like manner. Binding was inhibited by the Man_5 and Man_6 cores, but not the Man_9 glycan core (Fig. 5), leading the authors to speculate that the IL-2 carbohydrate recognition domain requires an unsubstituted Man(α1–6). Based on these and previous results, this group proposed a model that states that mannan on the *Candida* cell wall, or larger mannan pieces released from the cells into the surrounding-medium, binds IL-2 and prevents IL-2 signaling in the host. Additionally, IL-2 may thus act as a bridging unit, allowing *Candida* cells to bind T cells, possibly acting as the mannan binding receptor described by Durandy et al. (73).

(ii) Glucan. Castro et al. (45) reported that β-glucan from *S. cerevisiae* or *C. albicans* inhibited arachidonic acid release from rabbit alveolar macrophages by 36% at 1 mg ml^{-1}. Arachidonic acid initiates and mediates inflammation. A further result was that at 100 μg ml^{-1}, β-glucan completely inhibited phagocytosis of fungal cells by these macrophages. Torosantucci et al. (291) reported increased MIP-1b production by human monocytes in response to glucan, but not to mannan. The authors further concluded that β-1,6-glucan, not β-1,3-glucan, was the main chemokine inducer. This conclusion was supported by studies using glucan fractions and by using purified pustulan (a linear β-1,6-glucan) and laminarin (a β-1,3-glucan with β-1,6-glucan branches). *Pneumocystis carinii* β-glucan induced the release of MIP-2 from rat alveolar macrophages and epithelial cells (98, 302). Subsequent work by Lebron et al. (176) showed that macrophage activation in response to *P. carinii* β-glucan occurred through activation of NF-κB. They further showed that activation does not go through the classical lipopolysaccharide (LPS) receptor, TLR4. However, some Toll receptor is likely to be involved since depletion of the Toll adapter protein, MyD88, severely attenuated macrophage response.

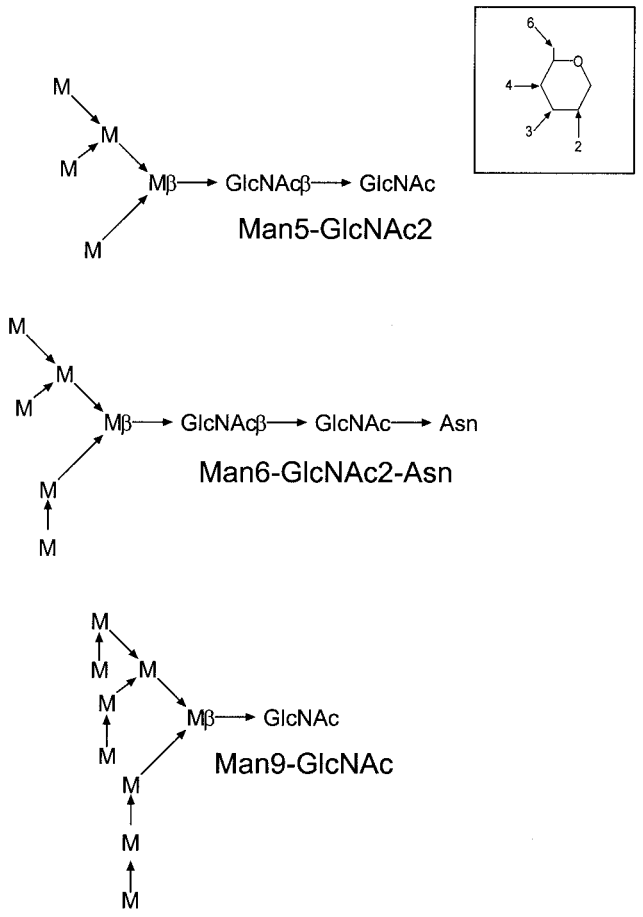

FIG. 5. N-linked glycan core structures used as inhibitors of IL-2 binding to *C. albicans* mannan (46, 323). Inset: Key for linkage positions as in Fig. 2.

Tokunaka et al. (290) published the results of immunological studies performed with a preparation of CSBG. Their results led to several conclusions: (i) β-Glucan, in a dose-related manner, augmented IL-6 synthesis in IFN-γ-stimulated peritoneal macrophages. (ii) CSBG competes with zymosan for sites on macrophages. This conclusion was based on the observation that addition of CSBG to the reaction decreased TNF-α production in response to zymosan. However, since CSBG did not itself stimulate TNF-α production, it is difficult to say if CSBG is truly acting as a competitive inhibitor of zymosan or acting through some other mechanism. (iii) LPS-induced TNF-α and nitric oxide production in RAW264.7 cells was enhanced by the presence of glucan. The authors conclude here that glucan was working thorough an additional or independent signal mechanism. However, again, since CSBG alone had no effect, it is difficult to see what CSBG-specific signal mechanism this could be.

Olson et al. (212) reported that while low and intermediate concentrations of glucan induce TNF-α production, higher concentrations suppress it. In a similar fashion, *P. carinii* β-glucan induced TNF-α release by rat alveolar macrophages. This induction peaked at 2.5×10^7 particles ml^{-1} (302). Higher concentrations of glucan resulted in diminished TNF-α release until the baseline was reached at 1×10^8 particles ml^{-1}. The paper by Olson et al. was an extension of an earlier paper by Hoffman et al. (111), who showed that β-glucan suppressed TNF-α release by rat alveolar macrophages stimulated with LPS. Olson et al. suggested that these observations might help explain some of the earlier, seemingly contradictory results reported in the literature where, under certain experimental conditions, *C. albicans* cell fractions produced a stimulatory effect in the immune system while others produced immune system suppression. This might also explain how the organism produces a seemingly self-harming interaction with the immune system. Another observation from this work is that TNF-α seems to bind glucan directly. This binding was inhibited by diacetylchitobiose (β-1,4-$GlcNAc_2$) and glucose but not cellobiose (β-1,4-Glc_2) or fucose. Olson et al. suggested that at low concentrations all the glucan was phagocytosed but at the higher concentrations glucan saturated the system, so that the remaining glucan could bind up circulating TNF-α, thus preventing its activity. TNF-α release from murine macrophages was also induced by β-glucan from *Paracoccidioides brasiliensis* but not by α-glucan, chitin, or galactomannan fractions (79). The level of induction did not reach that due to LPS but was at least fourfold higher than the control level. Czop and Austen (60) reported that particulate, as well as soluble, *C. albicans* β-glucan was phagocytosed by monocytes. They observed that this action stimulated oxidative generation of leukotrienes (LTB_4 and LTC_4). LTB_4 acts as a chemotactic factor, while LTC_4 is a permeability-enhancing factor.

Recognition by antibodies. The first separation of *C. albicans* strains into serotypes A and B was subsequently attributed to recognition by different antisera to specific mannosyl side chains on the mannoproteins (44, 105). Another illustration of mannan antigenicity is that an endotoxin-free *C. albicans* mannan preparation, when used to treat SRBC, significantly activates a primary antibody response to those SRBC (44). Faux et al. (78) described an enzyme-linked immunosorbent assay (ELISA) using specific antibodies (immunoglobulin G [IgG]) to *Candida* mannan. Although the epitope was not specified, the presumption by the authors is that a glycan group is recognized. The authors used this assay to determine the normal ranges of serum mannan in a population. Mannan/mannoprotein-specific IgG levels increased with age, and patients with primary antibody deficiency had uniformly low responses.

Anti-mannoprotein antibodies have been of particular interest in the field of atopic dermatitis. IgE-mediated hypersensitivity to mannoprotein (presumed to be due to the polysaccharide part) was thought to be associated with atopic dermatitis and respiratory allergies. Doekes et al. (69) reported that a large proportion of atopic dermatitis patients had IgE specific for a glycan epitope on *Pityrosporon ovale* (now a *Malassezia* species), another commensal yeast. They concluded that the epitope was a glycan because the IgE bound to cell extract fractions that were both high molecular weight by size exclusion chromatography and bound to the lectin Con A by affinity chromatography. Further, pretreatment of the extract with periodate eliminated its ability to prevent IgE from binding to extract adhered to the bottom of an ELISA well. Nermes et al. (203) looked at an ELISA for anti-mannoprotein IgA, IgG, and IgM, and compared it to a radioallergosorbant test for anti-mannoprotein IgE. Savolainen et al. (249) reported higher specific IgE responses to *C. albicans* mannoproteins and cell

lysate proteins in atopic dermatitis patients than in healthy controls. No difference existed between the two groups in IgG response, although IgE and IgG were measured using different protocols. They also reported that both mannoprotein and cell lysates from *Candida* strains isolated from atopic dermatitis patients produced higher levels of lymphoproliferation induction than did those from healthy controls. However, as noted with other studies, the "mannan" used in the last two studies was a mannoprotein cell wall preparation, with no indication of separation of glycan from protein.

Although glucan and chitin in the cell wall of *C. albicans* act as immunomodulators, they have low antigenicity (44, 189). Uchiyama et al. also recently noted that there have been few reports of anti-glucan monoclonal antibodies, though they themselves describe an antibody raised against the CSBG (299). The lack of anti-β-1,3-glucan antibodies was taken by Ballou to indicate that glucan is not exposed on the cell surface (13). Thus, immunological studies have tended to focus on the mannoprotein component.

DIAGNOSTICS AND THERAPEUTICS

Diagnostics

Mannan assays. As mentioned above, the first instance of mannan used as a diagnostic target for *C. albicans* was for the segregation of isolates into two serotypes. Hasenclever and Mitchell (105) attempted to determine whether *C. albicans* strains could be categorized by antigenic groups, as was done earlier by Evans and Kessel for *Cryptococcus* strains (76). Using adsorbed or unadsorbed antisera generated in rabbits, the strains they tested segregated into two serotypes, A and B. Serotype A has an antigen(s) not present in serotype B but otherwise contains all of the serotype B set. However, no further characterization of the antigen was done in this study. In 1964, Summers et al. (277) confirmed the differences between serotypes A and B and concluded that the serotype epitopes were glycans. They further concluded that mannan was the major or predominant antigen in crude cell wall extracts.

Subsequent analyses of mannan epitopes have led to production of more specific antisera. A collection of such antisera (recognizing epitopes or factors designated 1, 4, 5, 6, 8, 9, 11, 13, 13b, and 34) is commercially available as the Candida Check kit (Iatron), which allows species identification of clinical isolates based on the combination of positive agglutination reactions (268). Shibata et al. (262), while defining antigenic factors 5 and 6, identified the specific structural differences between serotypes A and B (reviewed in reference 243). They also provided the first evidence for β-1,2-linked mannose in the acid-stable portion of serotype A strain mannan. Lectins have been proposed as an alternative tool to these factor antisera for species identification. For example, Muñoz et al. (197) recently described using a panel of 14 commercially available lectins to produce agglutination profiles for 93 clinical *Candida* isolates. Only in the case of *C. glabrata* and *C. tropicalis* did all isolates (8 and 12, respectively) fall into a single pattern, although 90% of the *C. albicans* isolates gave the same profile. Further, 25% of the *C. parapsilosis* isolates gave the same profile as the *C. glabrata* isolates and another 37.5% produced the same pattern as the one most commonly produced by *C. albicans* isolates. Nevertheless, the results from this lectin panel suggest that it would be possible to discriminate *C. albicans*, *C. glabrata*, *C. tropicalis*, and *C. guilliermondii*. Thus, while this type of assay may not stand on its own, the use of lectins as agglutinating reagents can be useful as a confirmatory test or in conjunction with other types of diagnostic assays.

Diagnosis of disseminated candidiasis has proved particularly problematic because its clinical signs and symptoms are nonspecific. Also, blood culture isolation of the organism as an assay is low (50%) in specificity (208). Therefore, there has long been interest in a diagnostic assay for disseminated candidiasis. Many of the assays developed, especially early systems, were designed to detect *C. albicans* antigens in patient serum. In 1976, Weiner and Yount (313) developed a hemagglutination inhibition assay for *Candida* antigen detection (antigenemia), using mannan as the detected group. Antisera specific for mannan were first identified by passive hemagglutination of SRBC coated with a *C. albicans* mannoprotein fraction prepared from cell wall extracts. Because the protein level was low (3% [dry weight] protein), the authors concluded that the identified sera recognized polysaccharide. The presence of mannan in test serum was determined by inhibition of the agglutination reaction, as the serum mannan would compete with mannan coating the RBCs for antiserum binding sites. The authors note, however, that the success of this assay depends on the absence of any antimannan antibodies in the test serum. Weiner and Coats-Stephen (312) later developed a radioimmunoassay for mannan antigenemia detection, which they reported as being more sensitive. Serum mannan would compete with iodinated reagent mannan for antiserum binding sites, leading to lower counts on precipitation of the antigen-antibody complex. The method of iodination utilized chloramine-T, which incorporates ^{125}I into protein through a covalent linkage of the iodinated label to amine groups. Although both assays effectively detected *Candida*-specific antigen, it can be seen that recognition of a protein, rather than a glycan, epitope cannot be completely ruled out.

Since they were originally developed, improvements have been made to antigenemia detection assays to make them both standardized and highly specific. There remains, however, a lack of the sensitivity necessary for early detection of systemic candidiasis. Sendid et al. (257) have noted that this lack of sensitivity is probably due to rapid clearance of antigen as well as the latex agglutination format typically used. Detection of antimannan antibodies in patient sera has also been studied as a diagnostic strategy and, in fact, was proposed before the antigenemia assays. However, in a 1992 review, Buckley et al. (25) pointed out that an inherent deficiency of immunodiagnostics designed to detect antibodies is that these assays will be positive only if the patient/host recognizes the invader and mounts a response. In addition, the specificity of these tests is diminished because false-positive results are likely to arise in patients who are colonized but do not have clinical candidiasis (34, 208, 257).

Poulain and coworkers sought to overcome the individual problems of antigen- and antibody-based tests and to develop a diagnostic test based on the detection of both mannan antigen and patient-derived antimannan antibodies. The test that they developed used a monoclonal antibody that recognizes an

α-linked mannopentaose from the acid-stable mannan portion of N-linked glycans (PLATELIA *CANDIDA* Ag EIA; Bio-Rad) (128, 257, 318). The assay was subsequently tested for diagnosis of candidemia caused by species other than *C. albicans* (256). This strategy worked for *C. albicans*, *C. glabrata*, and *C. tropicalis* but did not work as well with *C. parapsilosis*, *C. kefyr*, or *C. krusei*. The differences in sensitivity were attributed to the particular antibody (EBCA1) used in the mannan detection test, which recognizes a mannose epitope present to a much lesser extent in mannan from the last three species (128). Because the incidence of invasive candidiasis due to non-*albicans Candida* species is increasing, Rimek et al. (235) recently tested the PLATELIA CANDIDA assay for cross-reactivity to antigens from 54 fungal species. They found that only *Candida* species gave positive results, although one strain of *Geotrichum candidum* and one strain of *Fusarium verticilliodes* gave borderline results. However, as was seen by Sendid et al. (256), *C. kefyr*, *C. krusei*, and *C. parapsilosis* tested negative in this assay.

Antigen detection has also been the focus of diagnostic tests for aspergillosis. The *Aspergillus* cell wall contains a neutral polysaccharide antigen, galactomannan (72). This polysaccharide comprises a branched core of α-1,2- and α-1,6-mannan with β-1,4-oligogalactosyl side chains. One characteristic worth noting is that the side chain termini, as well as some of the internal units, are in the galactofuranosyl (five-member ring) rather than the galactopyranosyl (six-member ring [Fig. 1]) form (175). A study of experimentally infected rabbits and patients diagnosed with invasive aspergillosis suggested that antigen detection in urine produces higher sensitivity than detection in serum (72).

A different approach to using *C. albicans* mannan as a diagnostic was presented by Goins and Cutler (92), who released acid-labile and O-linked oligomannosides from intact cells. The released oligomannosides were then labeled with a fluorophore and electrophoretically separated. This treatment of intact cells released oligomannosides other than those from purified mannan. The authors suggest that these groups may come from other sources in the cell wall rather than being part of mannan purified by Fehling's reagent (92). Nevertheless, electrophoretic profiles of oligosaccharides released by either acid-hydrolysis or beta-elimination demonstrated species and strain differences, suggesting that this might prove to be a useful approach to species and strain identification.

Glucan assays. *Candida* glucan is immunologically less active than mannan (44, 170, 189). Nevertheless, direct measurement of glucan has been described in an assay for candidemia. Miyazaki et al. (196) reported the development of a diagnostic test, called the G Test, which detects β-1,3-glucan. This test is based on the *Limulus* test originally developed for the detection of endotoxin (LPS). Comparison of this new G Test to other *Limulus*-based tests and mannan detection tests indicated that the G Test was more sensitive than the other assays tested. All rabbits infected in a systemic candidiasis model tested positive by day 2 after inoculation. A later study by Nakao et al. (201) determined that the amount of detected glucan was directly proportional to organism load. One complicating factor noted by Miyazaki et al. was that patients treated with anti-tumor polysaccharides could give false G Test positives. Thus, they recommend the combined use of the G Test and a mannan test for maximum sensitivity and specificity.

Therapeutics

A recent review by Tzianabos (298) discussed glycans as therapeutics. However, most of the studies cited in that review used mannoprotein preparations, and so the effect of the glycan specifically cannot be demonstrated. However, there are a few examples where glycan was used in a therapeutic treatment. Cassone et al. (41) reported protection from *Candida* vaginitis in a rat model involving active immunization with mannan. They suggested, based on their results, that antimannan antibodies were responsible.

Several papers, primarily from Cutler and coworkers, describe *Candida* oligomannosides or antibodies raised against them as being useful therapeutic agents. This group reported the production and testing of monoclonal antibodies against fractions of *C. albicans* mannoprotein. One in particular, an IgM designated B6.1, protected mice from disseminated candidiasis, while another IgM, B6, did not (100, 101). B6.1 recognizes a β-1,2-mannotriose (101). B6 recognizes a portion of the acid-stable mannan whose exact structure has not been completely characterized. B6.1, administered either intravaginally or intraperitoneally, also protected mice against vaginal candidiasis. This effect occurred whether the antibody was given prior to or after infection. B6 was protective, although to a lesser degree than B6.1, when administered before infection but had no protective effects when given after infection. The authors speculate that the more acidic environment in the vagina results in changes in glycan structure (162, 169) that allow greater exposure of the B6 epitope in the vaginal mucosa than in the bloodstream. B6.1, but not B6, was also protective against vaginal infection with *C. tropicalis.* Protection by B6.1 was expected because *C. tropicalis* N-linked glycans also contain β-1,2-mannotriose groups in both the acid-labile and acid-stable mannan fractions (164). However, B6 does react with *C. tropicalis*, and so the reason why this antibody was not protective against vaginal candidiasis remains to be discovered.

A paper by Caesar-TonThat and Cutler (30) suggested that the protective nature of B6.1 in the case of disseminated candidiasis was due to its enhancement of phagocytosis and cytotoxicity of PMN in the presence of complement. Subsequent studies (324) showed that mannoprotein adsorbed with normal human serum delayed the accumulation of C3. Since this effect could be reversed by B6 or B6.1, they concluded that the glycan portions of mannoprotein fractions were involved in enhancement of the classical complement pathway. Han et al. (102) later presented data showing that an IgG3, recognizing the same epitope as B6.1, was also a protective antibody. Antibody isotype, in addition to epitope specificity, was first shown to be important in the protective efficacy of anti-*Cryptococcus* antibodies by Sanford et al. (246). In other studies of experimental cryptococcosis, a nonprotective IgG3 antibody became protective after a switch to IgG1 (320, 321). The protective nature of the IgG3 antibody is, however, consistent with the earlier findings and hypothesis that complement activation is involved in resistance to disseminated candidiasis (102). Han et al. (103) further showed that mannan, conjugated to bovine serum albumin, was effective as a vaccine against subsequent *Candida*

infections. More recently, Bystricky et al. (28) reported similar results using a mannan-human serum albumin conjugate.

An extension of this work was presented by Glee et al. (89). They identified a series of peptides from a peptide display library that bound to IgM antibodies such as B6.1. It was thought that these peptides were acting as mimetics to the carbohydrate antigen. The results of this study, however, led the authors to conclude that these particular peptides were binding at a site other than the traditional antigen binding site. Each peptide was able to bind only IgM (no other class) but could bind all IgM antibodies tested, regardless of species or epitope. Thus, the binding site conformation and specificity must have been conserved among the antibodies. Nevertheless, the authors point out that these peptides may have therapeutic value as affinity matrices for IgM purification, as B cell activators, or to prevent unwanted or disadvantageous antigen-antibody interactions.

As described above, Suzuki et al. (279) demonstrated that mice treated with chitin from a commercial source (unspecified, but probably extracted from crustacean shells) had increased survival in response to a lethal challenge with *C. albicans* than did untreated mice. Rementería et al. (233) further showed that inoculation of mice with purified *C. albicans* chitin led to increased survival in response to a subsequent fungal challenge. Glucans extracted from mushrooms have been used as tumor immunotherapeutic agents for some time (see references in reference 303). Two recent papers discuss the use of *C. albicans* glucan as a therapeutic agent. Tokunaka et al. (289) demonstrated the use of glucan itself as an antitumor therapy. Soluble glucan (CSBG) inhibited the growth of P815 mastocytoma tumors and increased the survival of mice with implanted tumors. Ross and coworkers have investigated the mechanism by which these protective effects might occur. As mentioned above, glucan binds to CR3, which results in a magnesium- and protein tyrosine kinase-dependent conformation change in the receptor (303). This, then, without directly causing cellular activation, primes the cells to be cytotoxic toward C3-opsonized cells. Subsequent work showed that β-glucan-primed cells were effective against opsonized mammary carcinoma cell lines (304). Furthermore, primed cells showed 32 to 54% cytotoxicity against freshly excised mammary tumor cells, indicating that at least a subpopulation of tumor cells are opsonized in vivo but that β-glucan binding is required for NK cell recognition and killing of the opsonized cells (304). In the second study, Bromuro et al. (24) reported the presence of antiglucan antibodies. They also showed that some components of these sera aggravate infection while others inhibit infection. This led the authors to speculate that humoral protection requires not only the presence of the right antibodies but also the absence of other antibodies.

ISSUES IN CARBOHYDRATE CHEMISTRY

Clearly, glycans on the fungal cell surface play a role in the interaction between the fungus and its host and can be useful as both diagnostic and therapeutic agents. However, as mentioned in the previous paragraph, glycan epitopes can give rise to antagonistic as well as helpful responses. Thus, it will be important to identify and characterize specific glycan structures to discriminate between those that are helpful and those that are not. With regard to studying fungal surface glycans, several challenges exist that hinder such characterization. Generally, glycans are difficult to detect, they are often difficult to separate and purify, there is a high degree of complexity to glycan structure, and there is currently a lack of key tools needed for sequencing or compositional analysis.

Detection and Separations

These challenges, to a large extent, are fundamentally due to carbohydrate chemistry. Neutral sugars (mannose, glucose, etc.) which make up the majority of fungal glycans, are uncharged and thus will not move within an electric field, making electrophoretic separations problematic. Separations have been done in the past using chromatographic methods, such as liquid chromatography (particularly high-pH anion-exchange chromatography) and thin-layer chromatography, but even with these techniques, detection of the separated fractions was difficult because the sugars have limited UV absorbance (low extinction coefficients), are not colored, and do not fluoresce (112). Furthermore, UV detection is complicated by the presence of UV-absorbing contaminants in the sample (48). Nonetheless, despite these low extinction coefficients, UV absorption has been used to detect glycan fractions. Hofstetter-Kuhn et al. (112) added borate to aqueous solutions of carbohydrates to form a complex that had increased absorption at low UV wavelengths. This increase was due, in part, to shifting the carbohydrate conformation equilibrium toward the carbonyl form (112). Glycan UV absorbance is also increased by the presence of GlcNAc, GalNAc, or sialic acid (75). Other detection systems that have been used include indirect UV, refractive index, pulsed amperometric detection, light scattering (75), and isotopic labeling. However, with many of these techniques, sensitivity remains an issue. In some cases, lectin binding has been used to detect glycan species, but this technique is limited to structures for which a lectin exists.

One early report describing improvements to glycan separations was presented by Honda et al. (114), who took advantage of the ability of borate to form complexes with the *cis*-diols on sugars mentioned above. The resulting anionic borate complexes not only have increased UV absorbance but also have mobility in an electric field, allowing separation by capillary zone electrophoresis or other electrophoretic methods. Other complexes were later formed by direct conjugation of groups to the oligosaccharides (Table 5). These conjugation reactions are typically carried out using reductive amination, in which an amine group on the molecule to be added reacts with the aldehyde at the oligosaccharide reducing end (C-1). There are two main advantages to this approach. First, reductive amination reactions can be done under relatively mild conditions (e.g., dilute acetic or citric acid at 37°C for 18 h), minimizing potential hydrolysis or other unwanted reactions. Second, because each oligosaccharide contains only one reducing end, the labeling reaction is stoichiometric at one label per oligosaccharide. As a result, depending on the specific detecting instrument, labeled oligosaccharides can be detected quantitatively.

A general discussion of glycan labeling follows. However, a recent review by Lamari et al. (173) covers the physicochemical properties of the labeling compounds and some experimental considerations for carbohydrate derivatization in greater de-

TABLE 5. Selected reports of glycan labeling, detection and separation

Label[a]	Detection	Separation[a]	Yr of study	Reference
Perbenzoylation	UV (230 nm)	(RP)-HPLC	1981	63
O-benzoylation	UV (230 nm)	RP-HPLC	1987	62
PMP	UV (245 nm)	RP-HPLC	1989	113
2-AP	UV (240 nm)	CZE	1989	114
ANTS	Fluorescence	PAGE	1990	125
None	UV (195 nm)	CZE	1991	112
ANTS	Fluorescence	PAGE	1991	127
ANTS	UV (214 nm) Fluorescence	CZE	1993	53
2-AP	UV (200 and 230 nm)	HILIC	1994	4
AMAC	Fluorescence	PAGE	1994	126
AMAC and ANTS	Fluorescence	PAGE	1994	123 (R)[b]
AMAC and ANTS	Fluorescence	PAGE	1994	124 (R)
APTS	Fluorescence	CZE	1995	51
2-AP	UV (320 nm)	CE-HPLC	1995	77
APTS	Fluorescence	CGE	1995	97
Various	Various	Various	1996	75 (R)
2-AB	Fluorescence	HPLC (NP and RP)	1996	96
N-(4-Aminobenzoyl)-L-glutamic acid	UV (291 nm)	CZE	1997	223
2-AB and 2-aminobenzoic acid	Fluorescence PAD	HPAEC NP-HPLC	1998	8
AMAC	Fluorescence	UHV-MECC	2000	118
ANTS and APTS	Fluorescence	Electrophoresis	2000	228 (R)
O-(4-Nitrobenzyl)hydroxylamine	Fluorescence	RP-HPLC	2001	229
p-Aminobenzoic acid ethyl ester	UV (340 nm)	Cellulose chromatography RP-HPLC	2001	267
p-Sulfophenyl hydrazone	UV (200 nm)	CZE	2001	309

[a] Abbreviations: 2-AB, 2-aminobenzamide; 2-AP, 2-aminopyridine; AMAC, 2-aminoacridone; ANTS, 8-aminonaphthalene-1,3,6-trisulfonic acid; APTS, 1-aminopyrene-3,6,8-trisulfonic acid; CE, capillary electrophoresis; CGE, capillary gel electrophoresis; CZE, capillary zone electrophoresis; HILIC, hydrophilic interaction liquid chromatography; HPAEC, high-pH anion-exchange chromatography; MECC, micellar electrokinetic capillary chromatography; NP, normal phase; PAD, pulsed amperometric detection; RP, reversed phase; UHV, ultrahigh voltage.

[b] R indicates review article.

tail, with emphasis on preparing samples for analysis by chromatography, electrophoresis, and mass spectrometry. One of the first such derivatives incorporated 3-methyl-1-phenyl-2-pyrazolin-5-one (MPP, also referred to as PMP), which produced a complex that had strong UV absorbance and that could be detected electrochemically. Benzoyl groups have also been used to facilitate the detection of oligosaccharides (62, 223).

Detection sensitivity was increased severalfold by the introduction of fluorescent labels. Several compounds with the ability to add fluorescence have been used to conjugate oligosaccharides. These include 2-aminopyridine (2-AP) (4, 77), 2-aminobenzamide (223, 267), anthranilic acid (8), 2-aminoacridone (AMAC) (126), 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) (53, 125), and 1-aminopyrene-3,6,8-trisulfonic acid (APTS) (51, 97). ANTS, APTS, and similar compounds also contain strongly acidic functional groups that dissociate at low pH, thus intrinsically providing the capability of electrophoretic separation. One advantageous characteristic of APTS is that the unconjugated molecule has a much lower fluorescence than do APTS conjugates. While not affecting UV detection, the noise from the underivatized label is greatly reduced when using fluorescent detection (228).

In 1990, Jackson (125) was interested in finding a highly sensitive (subpicomolar range) alternative to radiolabeling. He labeled oligosaccharides with ANTS and carried out electrophoretic separations using high-percentage (20 to 40% gradient) polyacrylamide gels. This system is referred to as Fluorophore-Assisted Carbohydrate Electrophoresis (FACE). The method showed higher sensitivity than the paper chromatography, thin-layer chromatography, and noncapillary electrophoretic methods used previously. The basic FACE method was later combined with enzymatic digestions for more complete oligosaccharide characterization (127). The method was subsequently modified to use AMAC as the label. Since AMAC is not charged, only charged oligosaccharides maintain electrophoretic mobility in the absence of a borate buffer. This modification can be used as an additional tool for characterizing glycan structure, especially if there is evidence that some of the sugar residues are charged (e.g., sialic acids). The labeling reagents, precast gels, and gel buffers for carrying out FACE had, until recently, been commercially available. Gao and Lehrman (85) recently published a methods paper directly addressing this issue. Although perhaps less convenient, it is still possible to obtain all necessary reagents from other sources. Furthermore, FACE gels poured in-house are just as effective as precast gels, although more difficult technically to make than traditional protein PAGE gels.

Chiesa and Horváth (53) used capillary zone electrophoresis (CZE) for separation of ANTS-derivatized maltooligosaccharides (α-1,4-linked oligoglucosides). Detection was by either UV or laser-induced fluorescence. Alpert et al. (4), in a study of complex-type glycans, labeled them with 2-aminopyridine (2-AP) and carried out separations using hydrophilic interaction liquid chromatography (HILIC). These authors concluded that HILIC was the better chromatographic choice for combination with mass spectrometry (than, for example, high-pH anion-exchange chromatography) because volatile mobile phases can be used. They also showed that HILIC had better selectivity than reverse-phase high-performance liquid chromatography (HPLC). Chen and Evangelista (51) were able to resolve structural isomers by labeling with APTS and separating by capillary electrophoresis (CE). Based on their analyses,

they concluded that the mechanism of separation they observed was different from that for separation in borate or high-pH buffers and was based on apparent hydrodynamic volumes. Guttman and Pritchett (97) used capillary gel electrophoresis to separate APTS-labeled high-mannose glycans isolated from glycoproteins. This method is distinguished from CZE in that the capillary is filled with a solid-phase gel, much like a chromatography column.

Plocek and Novotny (223) addressed some practical aspects of glycan separations. They noted that UV-absorbing tags are less expensive than fluorescent tags and that CE instruments with UV detectors are readily available. This led them to develop a method for labeling oligosaccharides with aminobenzoyl-glutamic acid in preparation for separation by CE. Most recently, Shimizu et al. (267) described the derivatization of glycoprotein hydrazinolysates with *p*-aminobenzamide and fractionation by cellulose chromatography. Raju recently reviewed electrophoretic separations of carbohydrates in more detail (228). El Rassi and Mechref have reviewed specific capillary electrophoretic applications (75).

Glycan labeling overcomes the limited UV absorbance and lack of color inherent to carbohydrates and thus provides higher sensitivity for detection. The labeling methods described above have the further advantage of using mild reaction conditions and do not require high temperature or pressure. The choice of label can also provide insight into glycan structure. Labels containing ionizable groups (e.g., ANTS) are often used to provide charge for mobility during electrophoretic separations. However, the presence of a charged group (e.g., sialic acids) can be confirmed by maintaining mobility using a neutral label (e.g., AMAC and 2-AP).

Complexity and Characterization

The high complexity of glycan structure also has its roots in carbohydrate chemistry. Nucleic acids and proteins are synthesized as one-dimensional chains. However, while sugars are added to a growing complex through the carbon at position 1, this sugar can be attached at more than one position on the previous residue. For example, pyranoses (six-carbon sugars) can be linked to five of the six carbon atoms. In addition, the position 1 carbon bond shows anomericity, meaning that the new sugar can be added in an α or β conformation (Fig. 1 and Fig. 2 inset). This results in a much higher complexity per number of residues for oligosaccharides than for peptides or oligonucleotides (182, 258). A collection of four different nucleotides can combine to form 24 unique oligomers, but four different monosaccharides can combine to form 35,560 unique oligosaccharides (258).

Additional complexity arises when describing the glycosylation of proteins. As glycans are added and modified during procession through the endoplasmic reticulum and Golgi, the glycan chains are elongated and terminated based on a number of factors, including transferase accessibility to the growing oligosaccharide chain and competition for substrate (14, 64). As a result, a single glycoprotein is present as several, or many, glycoforms (182). Even proteins with a single glycosylation site, isolated from a homogeneous cell population, contain glycans with several structures (95, 287). This increases the amount of structural information available to the cell in terms of sequence variability and ligand specificity (e.g., for cell recognition sites) but complicates structural studies and protein identification.

Many tools are available for the characterization of glycans, beginning with separation of the glycans from glycoproteins by chemical and enzymatic means. N-linked glycans have been chemically released by using hydrazinolysis (215) and TFMS (trifluoromethanesulfonic acid) (74). Until recently, there was at least one instrument designed to automate the deglycosylation of protein fractions by hydrazinolysis (GlycoPrep), but it is no longer commercially available or supported. One disadvantage of hydrazinolysis is that the protein component is destroyed during the glycan release reaction because hydrazine cleaves peptide bonds. On the other hand, deglycosylation with TFMS maintains the integrity of the aglycone (deglycosylated glycoconjugate) but not the integrity of the glycan (18, 74). O-linked glycans are typically released by treatment with mild alkali (beta-elimination), incubating the glycoprotein in 0.05 M NaOH at 23°C for 18 h (92, 116).

Several endoglycosidases are also available that will release glycans from their protein backbones. N-linked glycans can be released with peptide-N^4-(*N*-acetylglucosaminyl)asparagine amidase (PNGase F), Endo H, or *N*-glycosidase F (Endo F), among others (see reference 286 for a review). These enzymes are commercially available from several suppliers and have the advantage of being able to isolate the aglycone after deglycosylation. Care needs to be taken when choosing an enzyme because of cleavage site specificity. For example, Endo F does not cleave oligosaccharides containing an α-1,3-fucose linked to the oligosaccharide core, such as are found in many plants. *N*-Glycosidase A removes glycans containing this group. Several endo-β-*N*-acetylglucosaminidases have been described which cleave between the two GlcNAc residues of the core oligosaccharide. However, their substrate specificities limit their usefulness in the study of hybrid-type or high-mannose-type (the type found in *Candida* and *Saccharomyces*) glycans. PNGase F cleaves the bond between the asparagine residue and the first GlcNAc and so is an amidase rather than an endoglycosidase per se. Nevertheless, because of this, it can act on a wide variety of glycan structures. O-linked glycans from higher eukaryotes can be released with *O*-glycanase. Unfortunately, this enzyme is not useful for the analysis of *Candida* or *S. cerevisiae* glycoproteins because the fungal O-linked glycans are composed solely of mannose and *O*-glycanase cleaves between the serine/threonine and the attached Galβ1 → 3GalNAc.

The glycans themselves (glucan and mannan/mannoprotein) can be extracted from cell walls by autoclaving the cell mass in citrate buffer (217) or even distilled water. Mannan, in the form of mannoprotein, can be separated from glucan by subsequent differential precipitation with detergent (Cetavlon) and alkaline borate (15, 210). Extracted and purified glycans can then be separated into fractions by chemical and enzymatic means.

One of the most common approaches to glycan fractionation is the use of acid hydrolysis. Mild-acid hydrolysis, with 0.1 N HCl in a boiling-water bath for 1 h, breaks the phosphodiester bond and releases the oligosaccharides linked through this mechanism (giving rise to the term "acid-labile," mentioned above). Stronger conditions can be used to hydrolyze carbohydrate chains down to their monosaccharide components. How-

ever, susceptibility and rates of cleavage of glycosidic bonds depend on position and anomeric configuration (216). Hence, the the conditions of hydrolysis for a given glycan must often be empirically determined. Another caveat to acid hydrolysis is the process of reversion, the acid-catalyzed polymerization of monosaccharides. This is particularly important when preparing polysaccharide acid hydrolysates for ladders or standards. Under the wrong conditions, the polysaccharide could hydrolyze but then revert, making unexpected oligosaccharides from monosaccharides or combinations of oligosaccharides and monosaccharides (157).

Another common chemical approach to glycan fractionation is acetolysis. This reaction has proven useful because under controlled conditions, the α-1,6-linkages are preferentially broken. As a result, the α-1,6-links between mannose residues in the outer-chain backbone can be cleaved, releasing the side branches intact for further structural studies (99, 281). The process of acetolysis begins with acetylation of all free alcohol groups to prevent any modification or derivatization. Sulfuric acid is then used to cleave the glycosidic bonds under conditions where the α-1,6-links are preferentially cleaved. The alcohol groups are then reverted to their previous form using alkaline sodium methoxide.

Glycans can also be fractionated by using a variety of commercially available exoglycosidases (e.g., glucosidases, mannosidases, and sialidases) with various substrate specificities and from a variety of natural sources (generally microbial or plant). However, several of the more commonly used enzymes are relatively inactive toward yeast mannan (138, 180). This difficulty prompted Ballou and colleagues to search for novel enzymes that were active on *S. cerevisiae* mannan. Several enzymes were isolated from soil bacteria, but they have not yet become commercially available (137–139, 199). One, however, an α-1,6-endo-D-mannanase from *Bacillus circulans*, has recently been cloned by Maruyama and Nakjima (190).

Exoglycosidases play an important role in strategies for determining oligosaccharide sequence (240, 241). In general, glycans are removed from their aglycones and then separated into fractions containing unique glycans. These fractions are then subjected to a battery or array of exoglycosidases for analysis. For example, if a particular exoglycosidase is capable of removing the end unit, then the treated glycan will be smaller than the parent. Other modifications or refinements to the basic scheme include in-gel deglycosylation of proteins separated by PAGE (172, 200), separation of glycans by capillary electrophoresis (322) or normal-phase HPLC (172), and separation and identification of glycans by mass spectrometry (104, 172, 247, 259, 322). In some cases, these analytical strategies require instruments that are expensive and not always available (for example, quadrupole ion trap mass spectrometry and nuclear magnetic resonance spectroscopy). However, Callewaert et al. (38) have recently described a method for profiling and sequencing glycans by using standard equipment already in use for sequencing DNA. Yanagida et al. (317) have presented a strategy for the automation of glycan removal and labeling. Further, Rudd et al. (240) note that robust and relatively rapid glycan analysis can be accomplished with an HPLC system (equipped with a fluorescence detector and normal-phase columns) and a collection of enzymes and standards. To this might be added equipment for running and imaging FACE gels.

Analysis of fungal glycans, particularly *Candida* glycans, has been problematic with regard to enzymes and standards. The ineffectiveness of some commercially available enzymes against yeast glycans has already been discussed. In addition, for *Candida* species specifically, no β-1,2-mannosidase (exo- or endo-) has been identified. An exo-β-1,2-mannosidase would be particularly useful for sequence and structure characterization of *Candida* glycans. Also missing have been oligosaccharide standards for fungal glycan side branches. Characterization of the side chains requires knowledge of both the monosaccharide sequence and the linkages in that sequence. Homopolymers of different sugars and different linkages all show different electrophoretic mobilities (J. Masuoka, B. Fraser-Reid, and K. C. Hazen, unpublished data). Thus, well-defined standards are required for glycan identification from electrophoretic separations. Because the β anomer is less energetically favored, synthesis of β-linked oligosaccharides has been particularly problematic. However, several groups have recently reported effective syntheses of β-oligomannosides (55, 160, 205, 206). These synthetic oligosaccharides are conjugated through their reducing end, complicating fluorophore labeling, or have protecting groups still attached to nonbonding alcohol groups. Fraser-Reid and coworkers have recently described a method for synthesis of unconjugated β-1,2-oligomannosides (192). Syntheses, in general, are increasing in their ability to produce oligosaccharides of increasing length and complexity of other fungal glycan groups. Ning and Kong (204) have published the synthesis of a nonasaccharide from the wall mannan of *C. albicans*, which represents a section of the outer-chain backbone with the beginnings of two side branches. More recently, Xing and Ning (316) have described the synthesis of the mannan antigenic determinant of *C. kefyr*.

CONCLUDING REMARKS

Fungal diseases are widespread and increasing in frequency, especially among immunocompromised patients. Fungal infections caused by *Candida* have been known since the fourth century B.C.E. (34) and are responsible for the majority of fungal infections currently diagnosed. There has been an increase in efforts over the last few years to identify antifungal strategies and compounds. The main difficulty, however, is that fungi, as eukaryotes, have cellular machinery very close to that of humans, making it extremely difficult to identify points that are lethal for the fungus but not the patient. Currently available antifungal agents either are toxic or act in a fungistatic manner, and resistance to these agents is already an emerging problem.

It seems clear that the pathogenesis of *Candida* is a multifactorial process with the participation of many cellular groups; and it is the interaction of these groups with host counterparts at certain times and under certain conditions that leads to disease (31, 37, 59). Cell wall glycans are exposed to the surrounding medium and are probably the first, spatially and temporally, to interact with components on the host cell surface. As discussed in this review, these glycans can, separately or in combination with protein or lipid components, affect various aspects of the host response to the invading fungal cells. Understanding glycan structure and function could thus

provide an important handle for the treatment of fungal diseases. More specifically, because of the involvement of glycans in host-pathogen interactions, it may be possible to use these interactions to provide fungus-specific protection if the interaction mechanisms were known.

Chronologically, host-fungus studies first began with the general: looking at what happens in an animal model during an infection with intact cells. From there, they progressed to looking in vitro at the action of fungal cells on specific immune system components, to breaking up the fungal cell to see which specific fractions elicited the observed effect, and now to breaking up the individual molecules into chemical fractions (e.g., glycan and protein) to see which specific molecular component of the fungal cell interacts with which receptor/molecular component of the host cell. With the advent of the methods and strategies of glycobiology (227) presented in this text, we are now at the threshold of being able to break apart the glycans and study how the component moieties influence pathogenesis, immune response, therapeutics, and diagnostics. Further, by breaking glycans down into individual, understandable pieces, we can revise our model of the fungal cell wall and use it to create a detailed map of the host-fungus interaction. From there, the road to better diagnostics and treatments should be much shorter.

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