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Candida Mannan: Chemistry, Suppression of Cell-Mediated Immunity, and Possible Mechanisms of Action

ROBERT D. NELSON,[1,2]* NOBUYUKI SHIBATA,[3] RAYMOND P. PODZORSKI,[4] AND MICHAEL J. HERRON[1]

Departments of Dermatology[1] and Microbiology,[2] University of Minnesota Medical School, Minneapolis, Minnesota 55455; The Second Department of Hygienic Chemistry, Tohoku College of Pharmacy, Sendai, Japan[3]; and Department of Immunology, Mayo Clinic-Foundation, Rochester, Minnesota 55905[4]

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INTRODUCTION

Clinical and scientific interest in *Candida* infections is high and increasing, as evidenced by many recent publications (6, 17, 32, 73, 76, 101, 106, 118, 120, 137, 141, 144, 152, 187). Increasing clinical interest in candidiasis is due to the rising frequency of infections involving this pathogen, attributable in part to improved detection of the pathogen and in part to a real increase in the incidence of *Candida* infections associated with immunosuppressive therapies and immunodeficiency associated with human immunodeficiency virus infection. Increasing scientific interest is related to needs for improved methods for earlier detection of infection and a safe and effective antibiotic to reduce the high probability of mortality (>35%) (120) associated with disseminated candidiasis. Scientific interest in *Candida* has also been influenced by the need for information about mechanisms that allow for persistence of the pathogen in tissue.

Studies conducted by several laboratories over the past

* Corresponding author.

decade have provided evidence that *Candida* cell wall mannan, a mannose glycoprotein, has an immunosuppressive property that could play an important role in fungal persistence. Other studies have been directed to identification of mannan species and oligosaccharide catabolites of mannan that possess this property and their mechanism(s) of action. With the progress made in these areas of investigation, it is appropriate to (i) consider the relationship of mannan to antigens and virulence factors of *Candida*; (ii) describe the chemistry of mannan, together with methods for preparation of mannan and mannan fragments for immunological studies; (iii) review the evidence for immunosuppression by *Candida* mannan and mechanisms proposed for this property; and (iv) speculate upon still other mechanisms by which mannan might influence immune function.

Efforts to define immunosuppressive effects of mannan further should produce clues to novel therapies for candidiasis that will enhance the efficacy of both available and future anti-*Candida* agents. They may also generate new clues to fundamental aspects of immune responses to polysaccharide antigens and provide novel reagents and techniques for dissecting these responses.

PATHOGEN-MEDIATED IMMUNOSUPPRESSION IN CANDIDIASIS

Two major questions can be raised about the significance of *Candida*-mediated immunosuppression in vivo. First, if systemic infections occur only when host immunity is compromised by drugs or primary disease, how can further immunosuppression by the pathogen occur? Second, if superficial forms of infection, especially chronic mucocutaneous candidiasis (CMC), are associated with diverse underlying clinical, genetic, and immunological features, how can immunosuppression by the pathogen provide a unifying explanation for the disease? These questions challenge the significance of *Candida*-mediated immunosuppression in vivo, but a role for immunosuppression perpetuated by the pathogen can be supported in both forms of candidiasis.

For systemic infections, noncandidal causes of immunosuppression may be eliminated, but immunosuppression will be perpetuated by the persisting pathogen. For CMC, there may be no unifying explanation for initiation of the infection; infection may begin as a consequence of a minor defect in any of several host defense mechanisms, but the established pathogen induces suppression of specific immune functions to exacerbate and sustain the infection (120). Whether *Candida*-mediated immunosuppression in all forms of infection is comparable in mechanism and consequence will be a debatable issue for the time being.

CANDIDA ANTIGENS AND VIRULENCE FACTORS

Although we emphasize the role of mannan as an immunosuppressant in *Candida* infections, mannan is not the only component of *Candida* known or suspected to have a role in the pathogenesis of candidiasis. A brief survey of known antigens and potential virulence factors of *Candida* is therefore described.

Antigens

The antigenic structure of *Candida* has been studied primarily for two purposes: to identify antigens useful for improved identification of *Candida* species or strains of *Candida albicans* and for diagnosis of infection. Antigens useful for serological applications are identified by agglutination with antisera raised against surface components of yeast cells. The antigens considered for diagnosis of deep-seated infections have been associated with cytoplasmic components of the organism because they may become available in amounts sufficient to evoke a humoral response only when fungal cells are destroyed by the host.

Cell wall antigens. Serological studies to identify *Candida* species (182) and studies to identify serotypes of *C. albicans* (62) have been based on agglutination reactions that use antisera to components of the cell wall. Because mannan is the major antigenic component of the *Candida* cell wall, these antisera must recognize subtle variations in the linkage or number of mannose residues in side chains of the molecule and the primary structure of the peptide component of mannan, all of which have yet to be fully detailed. The glucan polymers, which are in greater abundance than mannans in the *C. albicans* cell wall (21), are immunologically less active (134).

The cell wall of *C. albicans* is not antigenically constant (134). It is a dynamic structure, and its antigenic characteristics vary in response to growth conditions in vitro and in vivo. Variations in antigenicity related to strain, growth medium, and age of the cells cultured in vitro have been demonstrated with monoclonal antibodies recognizing epitopes of the outer cell wall (9, 10, 69). Antigenic variability of *C. albicans* serotypes has been related to mannan (135, 138), and antigenic differences between a virulent wild-type and an avirulent derived strain of *C. albicans* (14) could likewise be related to a structural change in mannan. It is tempting to speculate that such facultative control of cell wall mannan structure might similarly influence the pathogenicity of *C. albicans*. If this is so, then careful consideration must be given to the strain of the organism and growth conditions used to obtain mannan for studies of mannan-mediated immunosuppression.

Cytoplasmic antigens. The number of antigenic components in extracts of broken cells is considerable, and it is likely that the 168 identified by Manning and Mitchell (98) is an underestimate. These antigens have not yet been associated with specific cytoplasmic components of the cell; their metabolic or structural roles also remain unknown and, to our knowledge, none have been examined for an immunoregulatory influence.

Virulence Factors

To separate discussions of antigens and virulence factors of *Candida* implies that different fungal components contribute to antigenicity and virulence, but this is not true. Separate discussions of these components reflect the independent studies of these qualities of the pathogen. Antigenic components have been identified immunologically, without emphasis on biological functions of associated molecular species, and virulence factors have been characterized in terms of function, without consideration of antigenicity.

Toxins. A considerable effort has been made over the years to identify *Candida* toxins analogous to bacterial exotoxins and endotoxins, but toxins with biological properties equivalent to those of bacteria have not been found. Glycoprotein extracts of *Candida* cell walls, like bacterial endotoxins, are lethal and pyrogenic and induce anaphylactic shock in various animal models (27), but their potencies are not comparable. Failure to identify a potent candidal exotoxin or endotoxin may not be surprising, however, since a microorganism in frequent commensal contact with hu-

mans is unlikely to produce a potent toxin and retain its status as an opportunistic pathogen (120).

Enzymes. Fourteen hydrolytic enzyme activities have been described for *C. albicans* (120), but only two of these activities may have a role in pathogenesis. A phospholipase activity has been correlated with mouse lethality and adherence of fungal cells to buccal epithelium (5). An acidic carboxyl protease secreted by pathogenic species of *Candida* has been reported to be rare or absent in nonpathogenic species of the organism (96), and mutant strains of *C. albicans* that do not secrete this enzyme show significant reductions in lethality for mice (88, 97). This protease has also been reported to have a role in fungal attachment to mucosal cells (7). We speculate that it could also promote release of cell wall mannan by cleaving the peptide component of the glycoprotein.

Adhesins. The ability of different *Candida* species to adhere to host cells correlates with their virulence (37) and clearly plays a major role in the pathogenesis of candidiasis. Binding of *Candida* to epithelial and endothelial cells is controlled by adhesins on the surface of the fungus that interact with receptors on the host cells. Inhibition studies to characterize this interaction have suggested that the adhesin is a mannoprotein (36) and that both the polysaccharide and protein components of the mannoprotein have functional roles in adhesion (25, 26). The ligands on the host cell for fungal adhesin remain to be identified but, by analogy with receptors for other microorganisms on the surface of animal cells, carbohydrate components of membrane glycoproteins or glycolipids are likely candidates (72). Recent studies suggest that fibronectin (160) and laminin (8) may mediate attachment of the pathogen to human cells and basement membranes.

Complement receptors. A quality recently described for *C. albicans* that might influence its virulence is an ability to bind complement-derived opsonins. Receptors for C3d and iC3b, CR2 and CR3, respectively, were first described (49) as surface molecules of phagocytic leukocytes that mediate endocytosis of complement-opsonized microorganisms. One or more molecules reacting with anti-CR2 and anti-CR3 antibodies have now also been found on the surface of *C. albicans* yeast cells (42, 57) and pseudohyphae (93). A role for these binding functions in the pathogenesis of candidiasis is suggested by the observation that, except for *C. stellatoidea*, the other less pathogenic *Candida* spp. lack an ability to bind these complement fragments (63, 122). These complement receptors may bind and mask opsonic ligands to reduce uptake and killing of the pathogen by phagocytes. Alternatively, the *Candida* iC3b receptor may mediate adhesion of the pathogen to mammalian cells (58, 60a). This receptor has recently been identified as a member of the integrin receptor superfamily because it reacts with Mo-1 and BU-15 antibodies recognizing human leukocyte integrins (68, 69a).

Phenotype switching. Phenotype switching denotes the ability of organisms of a single strain to switch reversibly and at high frequency among different colony phenotypes. Although phenotype switching was first noted for *C. albicans* in vitro (161), subsequent studies have linked switching in vitro with virulence in vivo (164). Mechanisms to explain the relationship of colony switching to pathogenicity of *C. albicans* are not yet known, but several clues are available. A role in adherence phenomena is suggested by the correspondence of phenotypic differences in colonial morphology to different propensities for coadherence and attachment to epithelial cells (74). A role in avoiding elimination is suggested by differences in sensitivity of the white and opaque yeast phenotypes to the candidacidal activity of neutrophils (85). Definition of the specific biochemical differences responsible for these phenotypic variations also remains for the future.

Cell Wall Mannan

Among the antigens and potential virulence factors of *Candida*, mannan has special significance. It provides the antigenic variability most useful for species identification and subtyping, it may be the antigen most useful for rapid and early serodiagnosis of infection, and it has been the component chosen most often for studies of effects of *Candida* on immune function. Mannan can stimulate or suppress cell-mediated and humoral immune functions, and oligosaccharide fragments of mannan appear to be potent inhibitors of cell-mediated immunity; it can therefore be considered both antigen and virulence factor. Thus, while multiple components of *Candida* appear to have a role in establishing an infection, mannan may play the major role in immunosuppression of the host leading to persistence of the infection.

CHEMISTRY OF MANNAN

Location of Mannan in the Cell Wall

The cell wall of *C. albicans* makes up approximately 30% of the dry weight of the cell (170). Purified walls from yeast cells and germ tube cells are composed predominantly of carbohydrate (77 to 85%, wt/wt), with small amounts of protein (3 to 6%) and lipid (2%). The carbohydrates are represented by beta-glucan, a glycoprotein containing glucose in β-1,3 or β-1,6 linkages (48 to 60%, wt/wt); mannan, a glycoprotein containing mannose in a variety of α and β linkage arrangements (20 to 23%); and chitin, containing *N*-acetylglucosamine in β-1,4 linkage (0.6 to 2.7%). The glucan and mannan content of the cell wall is invariable throughout cell growth and germ tube formation (170), but mannan composition (148, 159) and features of the cell wall identifiable by electron microscopy (16) do vary in association with these events.

By transmission electron microscopy it is possible to identify at least five layers in the cell wall of *C. albicans* (151). The striated appearance of the cell wall in electron micrographs suggests that each layer has a qualitatively unique chemical composition, but it is more likely that differences in electron density are related to quantitative differences in chemical compositions of the layers (119). The architecture of the cell wall proposed by Shepherd (151) includes a fibrillar external layer followed, in order, by zones enriched in mannan, glucan, glucan-chitin, and mannan. The mannan composition of the outermost layer of the cell surface has been confirmed by studies of lectin (18) and antibody (188) binding and cytochemical staining (135). This architecture of the carbohydrate components of the cell wall is also supported by studies of the sequence of resynthesis of cell wall carbohydrate components by *C. albicans* protoplasts. During wall regeneration chitin fibrils are synthesized first; they are then overlaid with glucan, and mannoproteins are added last (45, 46, 112).

Chemical Structure of Mannan

Chemical analysis has shown that mannan from *C. albicans* is structurally similar to that of *Saccharomyces cere-*

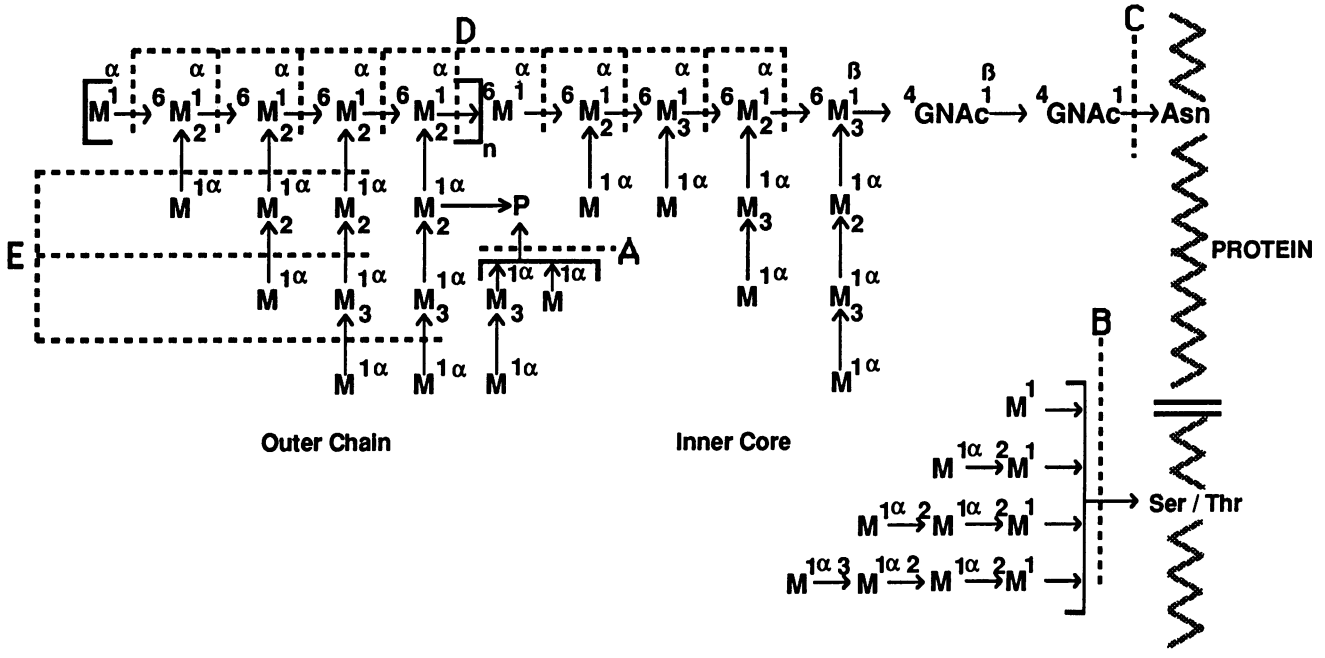

FIG. 1. Schematic representation of structure of mannan from *S. cerevisiae* X2180 (114) that serves as a model for the mannan of *Candida* species (137). *C. albicans* mannan shares the same structural features of inner core, outer chain, and base-labile oligomannosides, but is more highly phosphorylated, has longer side chains, and contains β-linked mannose units. The dashed lines associated with letters A through E denote sites at which the molecule can be cleaved by chemical or enzymatic methods to generate fragments for further structural analysis and studies of mannan-mediated immunosuppression. M, Mannose; P, phosphate, GNAc, *N*-acetyl-D-glucosamine; Asn, asparagine; Ser, serine; Thr, threonine.

visiae (173). The structure of mannan prepared from *S. cerevisiae* X2180 is illustrated in Fig. 1. Mannan is a complex glycoprotein composed of mannose polymers and oligomers attached in different ways to a peptide that accounts for approximately 10% of the molecular weight of the mannan molecule (3). The complex, branched mannose polymers are attached to asparagine residues through an *N*-acetyl-D-glucosamine dimer bridge ("N linked"). These N-linked polymers are long, linear chains of mannose units, joined through α-1,6 linkages, which provide a "backbone" for attachment of either single mannose residues or oligomers containing a variable number of mannose residues joined through α-1,2 and occasional terminal α-1,3 linkages. "Outer chain" and "inner core" are terms used to differentiate portions of the polymeric backbone which contain or do not contain phosphate esterified to the oligomeric side chains, respectively. Different transferase enzymes may also be involved in addition of the side chain mannose residues to the outer chain and inner core aspects of the polymeric backbone (3). Mannose or unbranched mannose oligomers are attached directly to the peptide through the hydroxyl group of serine or threonine residues ("O linked"). These O-linked oligomers range in length from two to six mannose units joined through α-1,2 and occasional terminal α-1,3 linkages. The dashed lines in Fig. 1 have been included to indicate how mannan can be cleaved by chemical and enzymatic methods to generate fragments for further structural analysis.

Methods for selective fragmentation of mannan have recently been applied to determine the chemical basis of antigenic differences between the A and B serotypes of *C. albicans* (62). Figures 2 and 3 illustrate the structures of the outer chain segments of mannans isolated from serotype A and B strains of *C. albicans* as they are currently known. A family of β-1,2-linked mannose oligosaccharides is present in phosphodiester form in both serotype A and B mannans and can be removed by treatment with weak acid (158); serotype A mannan contains additional β-linked residues in branching, acid-stable forms (157). That the acid-labile β-1,2-linked oligosaccharides are the major antigenic feature of serotype B mannan is supported by observations that their removal decreases precipitation by anti-serotype B antibody and that three-fourths of the monoclonal antibodies prepared against serotype B mannan are specific for these oligosaccharide moieties (179).

A more definitive description of mannan structure is not yet available. One cannot expect, however, that mannan produced by even one strain of *C. albicans* has a singular, constant structure. The 14 kinds of structurally different branches described for mannan isolated from *C. albicans* J-1012 (81) illustrate the possibilities for variation among mannan molecules from a single source. The heterogeneity of mannan observed in terms of size by electrophoresis (44) and in terms of charge by ion-exchange chromatography (35) also illustrate this principle. Mannan must therefore be thought of as a family of glycoproteins structured on a common theme, as a heterogeneous mixture of polymers of varying sizes and compositions.

It is not surprising that mannan composition has been found to vary between *Candida* species (95), strains of *C. albicans* (95, 147), and hyphal and yeast forms of the same strain (148). Strain-related variation appears to involve the number of O-linked oligosaccharides and the number of α-1,6 linkages and phosphate-linked side chains (147). Variation related to hyphal transformation was related to a decrease in the average chain length of mannooligosaccha-

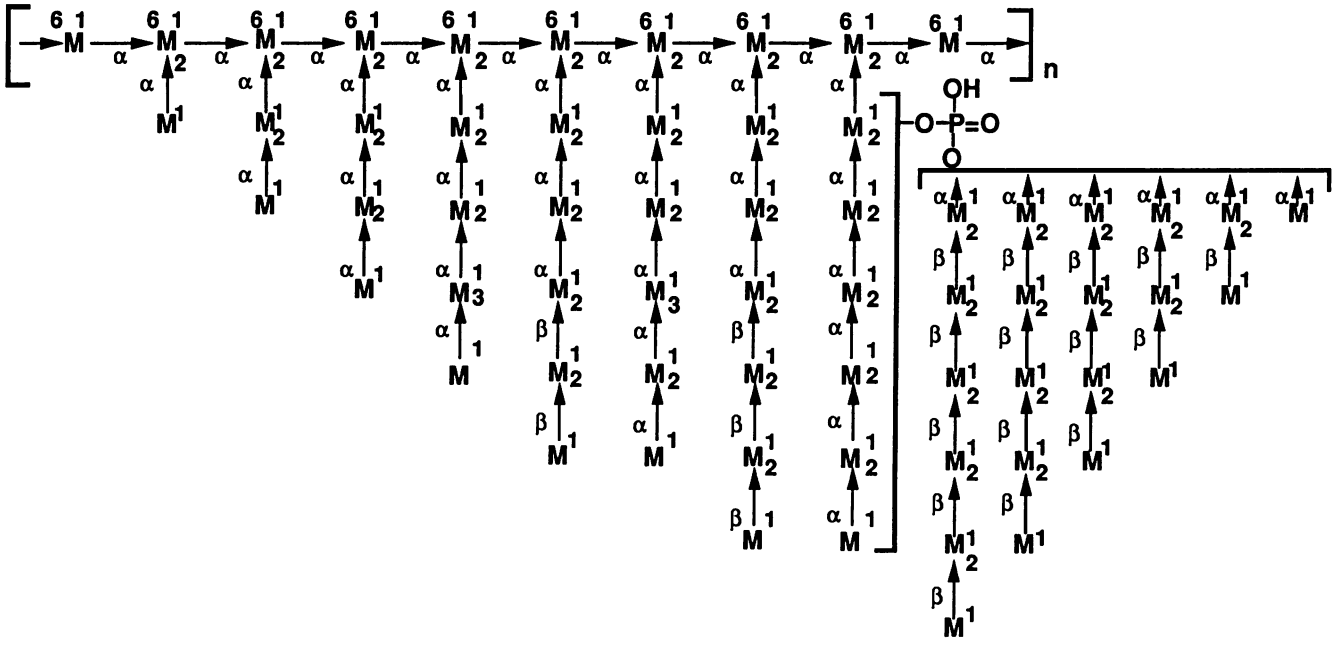

FIG. 2. Schematic representation of structure of the outer chain component of mannan from serotype A *C. albicans* NIH A-207 adapted from *Carbohydrate Research* (157) with permission of the publisher. Note the presence of β-1,2 linkages between mannose units in both the oligosaccharides esterified to phosphate and the oligosaccharides attached directly to the backbone polysaccharide.

rides (148). The composition of mannan can also be affected by methods used for its extraction and isolation.

Methods of Mannan Preparation

In Table 1 we describe four methods used to extract mannan together with the characteristics of each product. The most popular methods for preparing larger amounts of mannan include extraction with alkali, citrate buffer, or water at temperatures of 100 to 140°C, but these methods have limitations. For example, when mannan is extracted with alkali (53, 60, 174), mannose-serine and mannose-threonine linkages, phosphodiester linkages, and some peptide bonds are cleaved at the basic pH. The loss of the O-linked and β-linked oligosaccharides attached through phosphate leaves a grossly modified mannan product that is greatly altered in its antigenicity and biological effects. Extraction of mannan with neutral citrate buffer (114, 124) or water (121, 124, 158) preserves its carbohydrate component, but the temperature of extraction may denature the protein component. Treatment of cells with Zymolyase (Kirin Brewery Co., Tokyo, Japan), a mixture of beta-glucanase and proteinase, with a trace amount of mannanase, optimally preserves mannan structure (157, 159). The mannoprotein product released by Zymolyase treatment has a high molecular weight, but the yield is not high, and the cost of enzyme and scale of treatment present unique problems to this method of extraction.

No extraction method is selective for mannan. Separation of mannan from other carbohydrate and protein components of the cell wall and cytoplasm must therefore be achieved with another step. Two methods (Table 2) are available for the selective precipitation of mannan from crude extracts. The Fehling method (53, 67, 68), which uses Fehling's solution, exploits the ability of mannan to chelate and be precipitated by copper. A disadvantage of this method is that the high pH of Fehling's solution modifies the structure of the mannan product by cleaving the mannose-serine and mannose-threonine linkages and some peptide bonds. Another disadvantage is that considerable copper remains bound to the mannan, even after the product is washed and reprecipitated several times with methanol-acetic acid (116). While not a problem for studies of mannan structure, the mannan-copper product can negatively influence antigen-stimulated lymphocyte proliferation in vitro (116). It is therefore essential that contaminating copper be removed with a chelating resin (116) before copper-precipitated mannan is used in immunological studies.

The Cetavlon method for separation of mannan from other extraction products uses the reagent cetyltrimethylammonium bromide (CTAB) (94, 114, 158). In this method, selective precipitation of mannan involves formation of a boric acid complex with mannan, giving a negative charge to the molecule, which then forms an insoluble complex with CTAB. Because of the weaker alkaline condition with this method, linkages sensitive to cleavage in Fehling's solution are preserved. This difference may explain in part the higher molecular weight and protein content of mannan precipitated with CTAB, although some of this protein could represent contamination.

Because the mannans prepared from *C. albicans* by these precipitation methods have different compositions, we have compared their abilities to stimulate the in vitro proliferation of lymphocytes isolated from *Candida* antigen-sensitive donors (132). A consistently greater stimulatory activity of mannan precipitated by CTAB has confirmed that the method used to selectively precipite mannan has a significant influence on the outcome of studies to evaluate its immunomodulatory properties. Because the mannose-serine and mannose-threonine linkages are preserved, the CTAB-precipitated product is also a better source of O-linked oligosaccharides for studies of effects of mannan catabolites on immune functions.

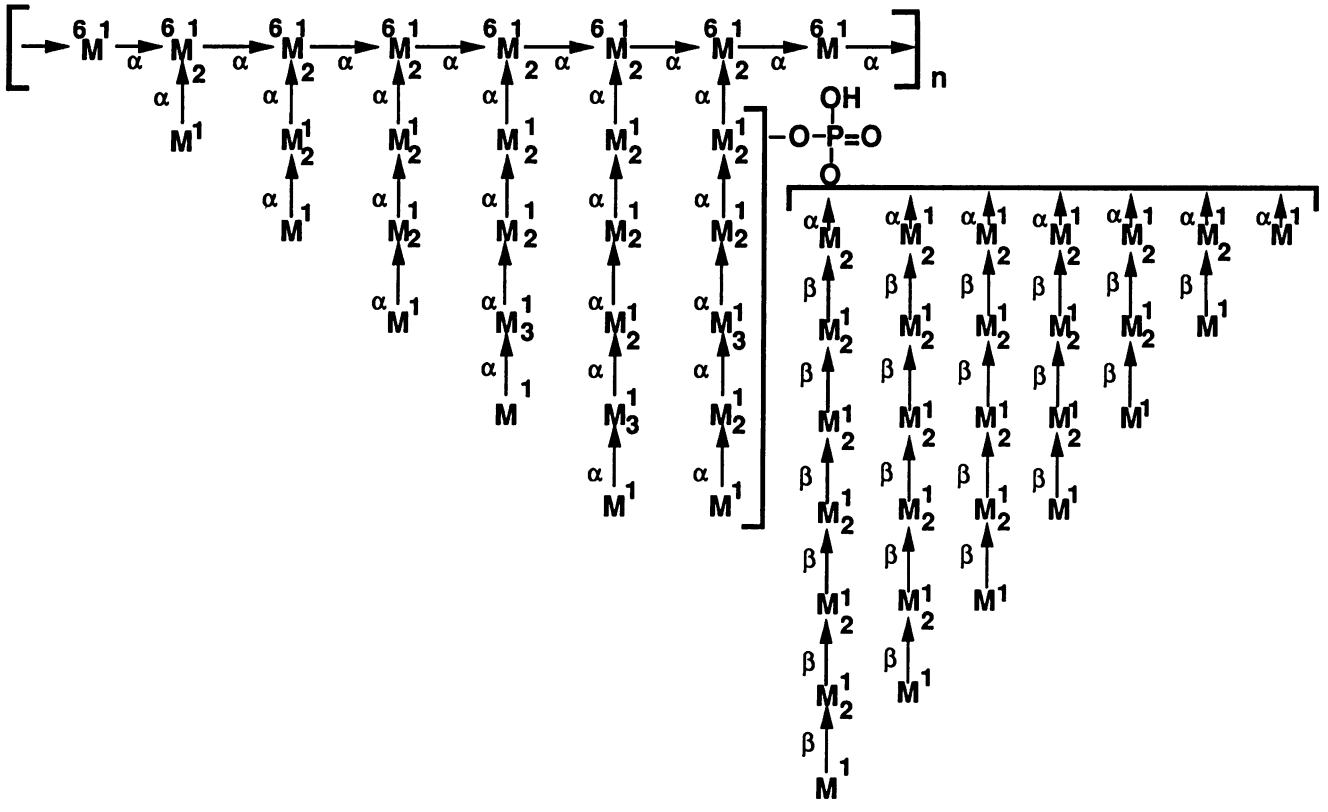

FIG. 3. Schematic representation of structure of the outer chain component of mannan from serotype B *C. albicans* NIH B-792 adapted from the *Archives of Biochemistry and Biophysics* (159) with permission of the publisher. Note the presence of β-1,2 linkages between mannose residues of the oligosaccharides esterified to phosphate and the absence of β linkages between mannose residues of oligosaccharides attached directly to the backbone polysaccharide.

Because of the complex structure of mannan and the influence of various extraction and precipitation methods, no "mannan" can be considered a homogeneous product. While isolation of individual mannan species is essentially impossible, mannan can be further fractionated by ion-exchange chromatography (35). This procedure has been used to provide five charge classes of mannan but, because the salt gradient used to elute the column is discontinuous, even these mannan fractions cannot be considered homogeneous.

Other *C. albicans* polysaccharide preparations used for immunological studies include a "Mangion purified" polysaccharide (127), glycoproteins extracted with ethylenediamine or alkali (33, 43), a mannoprotein purified by concanavalin A affinity chromatography (43, 149, 180), and peptidoglucomannan extracted from defatted purified cell walls (33, 140). These studies are cited because they call attention to the variety of procedures used to prepare cell wall polysaccharides from *C. albicans* and variations in the sugar and protein components of the products obtained. Information on chemical and physical characteristics of several of these polysaccharide preparations is available in references 21, 75, 86, and 95.

Methods of Chemical and Enzymatic Degradation of Mannan

In Fig. 1 information useful for understanding methods available to prepare mannan fragments for immunological testing is shown. Dashed lines identified by uppercase letters

TABLE 1. Methods for extraction of cell wall mannan

Method	Conditions	Limitations	References
Hot alkali extraction	2% KOH at 100°C for 2 h	Glycosyl-serine and -threonine linkages, phosphodiester linkages, and some peptide bonds are cleaved	53, 60, 174
Citrate buffer extraction	20 mM citrate buffer (pH 7.0) at 120°C for 90 min	Protein is denatured	114, 124
Hot water extraction	Distilled water at 140°C for 2 h	Protein is denatured	121, 124, 158
Enzyme treatment	Phosphate buffer (pH 7.5) at 28°C for 1 to 3 h		157, 159

TABLE 2. Methods for separating mannan from cell wall extract

Separation method	pH	Limitations	Mol wt (10^3)[a]	Protein content (%)[a]	References
Fehling method	>14.0	Glycosyl-serine and threonine linkages and some peptide linkages cleaved	40	6.0	53, 116, 124, 174
Cetavlon method	8.8	None	133	8.3	94, 114, 157–159

[a] Values are those of mannans from *S. cerevisiae* (114).

A through E denote linkages within the mannan molecule which can be selectively cleaved by chemical or enzymatic treatments. Additional technical information related to carbohydrate analysis is available in reference 20 and in literature available from companies developing reagents and analysis systems for glycobiology, i.e., BioCarb Chemicals (Lund, Sweden), Boehringer Mannheim (Mannheim, Federal Republic of Germany), Dionex (Sunnyvale, Calif.), Oxford GlycoSystems (Rosedale, N.Y.), and TaKaRa Biochemical (Berkeley, Calif.). Note that these same fragmentation methods, in combination with ^{1}H- and ^{13}C-nuclear magnetic resonance analyses, are also used in the structural characterization of yeast phosphomannans (82).

Phosphodiester linkage (A). The phosphodiester linkage, through which oligosaccharides are attached to side chains of the outer chain polymer, can be cleaved by exposure to weak acid (10 mM HCl) at 100°C for 30 to 60 min (136). The oligosaccharides released by this treatment are the β-1,2-linked oligomers that provide the major antigenic structures of *C. albicans* serotype B mannan (159). Subsequent separation and isolation of these oligosaccharides can be achieved by sizing chromatography, using Bio-Gel P-2 (Bio-Rad Laboratories, Richmond, Calif.) (115). Bio-Gel is the matrix of choice for this application because it is polyacrylamide, which does not support bacterial growth or leach carbohydrate, and has a limited residual charge, which allows for use of water as eluant.

Mannose-serine, mannose-threonine linkage (B). The oligosaccharides linked to serine and threonine can be released by the process of beta-elimination, using 100 mM NaOH at 25°C for 18 h (61). Subsequent separation and isolation of members of this family of oligosaccharides can also be achieved by chromatography with Bio-Gel P-2. Oligosaccharides in each size class from this source are not homogeneous because the trimers and larger oligomers may contain either an α-1,3- or an α-1,2-mannose unit at the nonreducing terminus. Physical separation of oligomers differing in this respect has not been reported, but could be achieved by affinity chromatography with antibody recognizing one of the linkage arrangements (83). Under the alkaline condition of beta-elimination, still further heterogeneity of these oligomers occurs due to epimerization of mannose to glucose or fructose at reducing termini (131).

***N*-Acetylglucosamine-asparagine linkage (C).** The polysaccharides linked to asparagine can be removed with their side chains intact by treatment with 1 M $NaBH_4$–1 M NaOH at 100°C for 5 h (3) or with hydrazine at 100°C for 12 h (105) or *N*-glycosidase F (176). The potential for structural heterogeneity among these polymers is obvious, making further fractionation essential for a rigorous analysis of their immunomodulatory effects. Some degree of separation by size may be achieved by column chromatography with sizing gels, but there is no known method to provide for their ultimate physical separation.

α-1,6 linkage (D). The oligosaccharides branching from the long polymers linked to asparagine can be released by controlled partial acetolysis, but different acetolysis conditions yield different oligosaccharides. Fragmentation depends on the increased acid lability of the α-1,6 linkages in the mannan backbone. The classical procedure for fragmentation of mannan by acetolysis involves treatment with the reagent composed of acetic anhydride-acetic acid-sulfuric acid (10:10:1, vol/vol) at 40°C for 12 h (84). With such treatment, both α-1,6 linkages of the backbone polymer and β-1,2 linkages within the side chains are cleaved, thereby preventing recovery of the side chains containing mixed α-1,2 and β-1,2 linkages. The β-1,2 linkages can be preserved, however, by decreasing the concentration of sulfuric acid to 50:50:1 (40°C for 12 h) (157) or to 100:100:1 (40°C for 36 h) (83). Separation of individual side chain species can be achieved by a combination of sizing and affinity chromatographic methods.

α-1,2 and α-1,3 linkages (E). For removal of α-linked mannose residues from oligosaccharides, the exo-α-mannosidase prepared from *Arthrobacter* strain GJM-1 is effective (139). The enzyme cleaves mannose residues from the nonreducing termini of oligomannoside chains having α-1,2 and α-1,3 linkages, but the α-1,6-mannan backbone is not attacked. This enzyme is not commercially available.

IMMUNOSUPPRESSIVE EFFECTS OF MANNAN

Immunosuppressive effects of mannan have been identified primarily through studies to define the mechanism of anergy associated with the superficial forms of *Candida* infection: CMC and vaginal candidiasis. Early experiments showed that patient serum inhibits the proliferative response of lymphocytes from healthy donors to *Candida* antigen in vitro, thus stimulating attempts to isolate and describe a circulating inhibitor. Neutralization of the serum inhibitor by anti-*Candida* antibody provided evidence that the inhibitor was a pathogen-derived antigen. Immunosuppressive effects of cell wall mannan on antigen-stimulated lymphoproliferation in vitro were then described, and the inhibitor in patient serum was identified as mannan. The clinical and immunological features of CMC and their potential relationship to mannan are described.

Clinical Features of CMC

Superficial candidiasis most commonly affects the mouth or vagina; infection remains limited to these surface body sites and can be treated successfully. A much less frequent form of superficial candidiasis, CMC, involves infections at multiple sites, including the mouth, skin, and fingernails. CMC is refractory to currently available therapy and persists for years.

CMC is not a single disease. Patients with CMC share only the persistent, superficial lesions caused by *Candida*, which may be few and mild to extensive and severe. Schemes to

classify these patients have been based primarily on clinical features (90), clinical features and familial history of disease (196, 198), or responses to in vivo and in vitro tests of cell-mediated immune function (185), but none of these schemes has been accepted as satisfactory. Although the heterogeneity of CMC may defy strict classification, such efforts have continued. The most recent scheme (120) is based on a review by Odds of 283 cases reported from the period 1966 to 1986. He has organized the primary features of CMC into six categories as follows: (i) CMC with endocrinopathy, with no evidence of inheritance; (ii) familial CMC with endocrinopathy; (iii) familial CMC with no endocrinopathy; (iv) idiopathic CMC with onset before 20 years of age; (v) idiopathic CMC with onset after 20 years of age; and (vi) CMC with thymoma.

Immunological Features of CMC

In CMC, as in other superficial forms of candidiasis, *C. albicans* penetrates only as far as the stratum corneum. Histologically, affected tissues also contain infiltrations of monocytes, lymphocytes, and polymorphonuclear neutrophils. Accumulation of inflammatory cells at infected sites suggests that the host is able to mobilize cellular defenses against *Candida*, but unable to eliminate the pathogen totally. Failure of the host to eradicate the fungus appears to be related in some way to defective cell-mediated immunity commonly associated with CMC. In vivo and in vitro tests used to evaluate patient immune and nonspecific defense functions have included the following: (i) skin test reactivity to *Candida* and other antigens; (ii) in vitro lymphocyte proliferation in response to *Candida* antigen and mitogens; (iii) in vitro effect of patient serum on antigen-stimulated proliferation of lymphocytes from healthy donors; (iv) in vitro production of migration inhibitory factor (MIF) by lymphocytes in response to *Candida* antigen; (v) neutrophil killing of *Candida* and migration in response to a chemoattractant; (vi) level of serum antibodies to *Candida* antigens; and (vii) level of autoantibodies.

No delayed-type cutaneous hypersensitivity to *Candida* antigen has been observed in more than 80% of the CMC patients tested (120). The frequency of negative skin tests across all six CMC subtypes ranged from 72 to 88%. Some 50 to 60% of the patients tested also exhibited negative skin test responses to other microbial antigens. Therefore, defective cell-mediated immunity to *Candida* antigen is a dominant feature of all CMC clinical subtypes and often extends to other microbial antigens.

Reviewing in vitro tests of patient cell-mediated immune function, Odds observed that, among all clinical subtypes, 57 to 78% of the patients tested exhibited deficient lymphocyte proliferation in response to *Candida* antigen (120). Failure to respond normally to mitogens was much less frequent, ranging from 12 to 30% among the patient subtypes. This differential ability of patient lymphocytes to proliferate in response to antigen and mitogen is helpful in defining the mechanism of failure to respond to antigen. A normal response to mitogens suggests that the defect in immune function in CMC must be unrelated to a basic ability of patient lymphocytes to produce and respond to cytokines that stimulate their proliferation.

Testing for production of macrophage MIF by patient lymphocytes has been done much less often, but negative or subnormal production of MIF in response to *Candida* antigen is also a common finding in CMC patients. Abnormal production of MIF has been observed for 72 to 86% of the patients tested in three of the CMC clinical categories (120).

The high frequency of abnormal results in individual tests of patient immune functions suggests that CMC patients may share a common immune defect and mechanism of immune dysfunction. These results also suggest that the tests used are relevant to studies of the defect in cell-mediated immunity associated with CMC. When results of multiple tests of immune function of individual patients are considered, however, the frequent lack of concordance of test results brings these conclusions into question.

To account for discrepancies in tests of cell-mediated immunity, the proposal has been made that different patients may have defects in different aspects of their immune response (68, 78). The observation that proliferation and production of MIF may be separate functions of different lymphocyte subpopulations (142) supports this possibility. Negative and positive skin test results may reflect systemic or local distribution of a pathogen-derived inhibitor related to the level of infection. However, neither skin testing at sites proximal and distant to foci of infection nor quantitation of organism growth have been done to evaluate this possibility. We speculate that variable outcomes of in vitro tests of cell-mediated immune function may also be attributable to aspects of the tests themselves. For example, we have observed that the lymphoproliferative response of patient leukocytes to *Candida* antigen can become positive when the cells are cultured at higher numbers or densities, changes that did not cause leukocytes from healthy donors to convert from negative to positive responses. Culture conditions might similarly affect the rate and level of MIF production by patient cells.

Relative to the MIF assay, it is also important to note that there is another important limitation to its usefulness as a measure of immune function. Migration inhibitory activity was originally attributed to a single molecular species, but it is now known that gamma interferon (178) and interleukin-4 (IL-4) (102), in addition to a cloned human macrophage MIF (194), can inhibit macrophage migration. Further, MIF was assumed to have both migration inhibition (28) and macrophage activation (129) properties, but cytokines (193) and a number of noncytokine agents (165, 171) possess distinct MIF or macrophage activation factor activities. Finally, migration inhibition and cell activation can involve separate agonists in a two-stage process of priming and triggering (65). The results of early tests for MIF activity described for CMC patients (120) therefore cannot be interpreted in contemporary terms.

The traditional MIF assay therefore seems inappropriate for current studies of CMC immunology. More relevant to this purpose would be assays that directly measure cytokines known to stimulate the microbicidal activity of phagocytic leukocytes, control the proliferation of lymphocytes, and stimulate proliferation of epidermal cells. Cytokines known to stimulate the fungicidal activity of macrophages and neutrophils include gamma interferon (125), tumor necrosis factor alpha (31), and colony stimulating factors (190). Cytokines known to influence T-lymphocyte proliferation include gamma interferon, and IL-1, and IL-2 (30). Cytokines influencing epidermal growth include growth factors and lymphokines that regulate growth factor production (189). The potential importance of cytokine-stimulated epidermal growth in elimination of *Candida* is suggested by experiments involving a guinea pig model of cutaneous candidiasis (163). Only animals with an intact cutaneous

delayed-type hypersensitivity to *Candida* could control fungal growth by scaling and sloughing infected skin.

Early Studies Identifying an Immunoinhibitor in CMC

During the early 1960s, when experimental interest in CMC began to build, in vitro tests of cell-mediated immune function were not routinely available. Tests were limited to measurements of cutaneous delayed-type hypersensitivity, effects of patient serum or plasma on the clumping, viability, or transformation of *Candida* yeast cells, and secreted and circulating anti-*Candida* immunoglobulins. The lymphocyte "transformation" assay, used as an analog of cell-mediated immunity, was described in 1960 (117). Over the remainder of the decade, many investigators exploited the in vitro lymphoproliferation assay to study immune function in CMC and reported mostly normal (14, 22, 99) or defective (15, 58, 64, 186) responses of patient lymphocytes to *Candida* antigen. Chilgren et al. (22) noted that lymphocytes isolated from three patients proliferated normally in vitro in response to *Candida* antigen and phytohemagglutinin, in spite of the inability of these patients to manifest skin test positivity to *Candida* antigen or to be actively sensitized with 2,4-dinitrofluorobenzene. This result suggested to them that host factors prevented antigen processing and/or cellular expression of immunity only in vivo.

Marmor and Barnett (99) proposed that the pathogen might contribute to the immunosuppression associated with CMC, citing the possible role of "antigen load" by analogy with other infectious diseases (93, 146). More direct evidence for pathogen-induced immunosuppression in CMC was provided by Buckley et al. (13), who described clinical improvement in a patient receiving a bone marrow graft following amphotericin B therapy. They considered that immunological reconstitution was achieved because preliminary clearing of skin and oral lesions by antifungal therapy had reduced pathogen-derived antigen responsible for inducing immune paralysis.

The first direct evidence of a circulating inhibitor of immune function in CMC was reported in 1969 (15). Patient serum inhibited the proliferative response of control lymphocytes in vitro to *Candida* and mumps antigens and to allogeneic cells. This serum immunoinhibitory property was subsequently confirmed by others (91, 123, 185), and evidence that the inhibitor derived from the pathogen was provided in two of these studies. Paterson et al. (123) and Twomey et al. (183) observed that the inhibitor became undetectable in serum following rigorous treatment of the patient with amphotericin B or plasmaphoresis. Loss of inhibitor was concurrent with clearing of infectious lesions and return of in vivo and in vitro immunoreactivity after initiation of therapy. Reappearance of the inhibitor on return of disease provided evidence that it might be a circulating component of the pathogen.

A mechanism to account for the immunoinhibitory effect of suspected circulating fungal antigen was proposed by two investigators in 1970. Rocklin (143) suggested that patient lymphocytes were being desensitized by constant exposure to high levels of circulating antigen based on the short remission period of a CMC patient receiving transfer factor therapy. At that time, transfer factor was thought to be released from antigen-sensitive lymphocytes either on exposure to an antigen or by lymphocyte lysis and to be capable of conferring delayed-type hypersensitivity to that antigen (89). Kirkpatrick et al. (77) considered that CMC patients may become desensitized by chronic exposure to (*Candida*) antigen such as occurs with prolonged exposure to poison ivy. They subsequently reported that treatment of a CMC patient with transfusions of functional leukocytes (transfer factor?) could similarly produce a temporary clearing of infection and restoration of skin test reactivity and MIF production by isolated patient leukocytes (79).

All CMC sera tested for immunoinhibitory activity have not, however, contained the inhibitor. Of 37 CMC patients tested for serum suppression of lymphocyte proliferation, only 17 possessed this activity (120). This inconsistent presence of inhibitory activity could be interpreted to indicate that the same "serum inhibitory factor" may not occur in all patients with CMC. The difference in sensitivity to freezing between inhibitors studied by Canales et al. (15) and Twomey et al. (183) would support this possibility. Alternatively, immune dysfunction in some patients may not be mediated by a circulating inhibitor, the inhibitor may be present intermittently, or it may be neutralized to a variable degree by antibody or other serum component. Despite these discrepant observations, investigators have continued to pursue "the immunoinhibitor" in CMC.

Studies Identifying Mannan as an Immunoinhibitor in CMC

In 1978, Fischer and his colleagues described efforts to characterize the physicochemical properties of the inhibitory material in patient serum and to establish whether a correlation between presence of the inhibitor and the immunological disorders was associated with infection (50). For 6 of 23 patients studied, the serum inhibitory activity was present during active disease in association with *Candida*-specific defects in cell-mediated immunity and was absent during periods of remission induced by antifungal therapy. The inhibitor was nondialyzable, thermostable, and nonprecipitable with ammonium sulfate; it could also be removed from patient serum by affinity chromatography by using agarose conjugated with anti-*Candida* antibodies or concanavalin A, and active inhibitor could be eluted from the column. When normal serum or noninhibitory patient serum was passed through the column, no inhibitory activity was retained. These characteristics and evidence that the eluted inhibitor had a high carbohydrate/protein ratio supported the idea that the inhibitor is pathogen derived and a mannose-containing glycoprotein. The identity of the serum inhibitor as mannan was further supported by the abilities of a component of *Candida* metabolic antigen (Institut Pasteur, Paris, France) isolated by lectin affinity chromatography as well as mannan prepared from the pathogen to reproduce the in vitro inhibitory effect of the serum factor.

The observations of Fischer et al. (50) are compatible with, but do not prove, the identity of mannan as the inhibitor of cell-mediated immunity in CMC. No formal criteria exist for this purpose, but criteria analogous to three of four of Koch's postulates to establish that a microorganism caused a specific disease could be applied here as a guideline. Accordingly, evidence is needed of an association of serum inhibitor and serum mannan with disease and immune dysfunction, recovery of the inhibitor as mannan from the patient, and reproduction of the same immune deficit in vivo on administration of serum mannan or purified *Candida* mannan to a healthy host.

Except for the absence of a precise physicochemical description of the mannan isolated from patient serum, the first two criteria seem currently satisfied by data available (50). The observation that "mannan" purified from patient serum and mannan prepared from laboratory-grown *C. albi-*

cans suppress the proliferative response of control lymphocytes to *Candida* antigen in vitro (50) partially satisfies the third criterion needed to establish the identity of the inhibitor as mannan. Additional satisfaction of the third criterion is provided by the report that *Candida* mannan can significantly suppress delayed hypersensitivity reactions when transfused into a healthy murine host (34), though one can argue that this phenomenon is unrelated to immune dysfunction in CMC. Reconstitution of patient immunoreactivity by selective removal or neutralization of plasma mannan then becomes the ultimate test of the immunoinhibitory role of mannan in CMC. Also remaining to be established is the form or forms of immunoinhibitory mannan circulating in vivo.

Inhibitors of cell-mediated immunity unrelated to cell wall mannan may also occur in CMC. The lymphocyte-inhibitory factor isolated from the serum of a patient with thymoma and late-onset mucocutaneous candidiasis would support this possibility (54). The heat-stable 50- to 65-kDa substance was thought to be derived from the thymus rather than the pathogen because it disappeared following tumor irradiation. Because this patient's inhibitor likely predated the infection, its presence may have predisposed the patient to candidiasis as well as compromised elimination of the pathogen. Although this patient may be unusual, the identification of mannan as a serum inhibitory factor does not eliminate the possibility that other inhibitors, including nonmannan components of the pathogen and host-derived agents, might also contribute to immunosuppression associated with CMC.

Immunostimulatory and Immunoinhibitory Forms of Mannan

Mannan isolated from patient serum or laboratory-grown organisms is not a homogeneous compound. Different molecular species making up such mannans therefore may not possess the same abilities to influence immune function. Indeed, considerable evidence is available to illustrate the influence of mannan composition, size, and charge on its immunomodulatory effects.

We have obtained evidence that the structure of mannan, determined in part by method of preparation, significantly influences its ability to stimulate or inhibit human lymphocyte proliferation response to antigen in vitro (116). We initially observed that mannan isolated from *Saccharomyces cerevisiae* grossly suppressed lymphoproliferation in response to six antigens, including *Candida*, tetanus toxoid mumps, streptokinase-streptodornase, cytomegalovirus, and herpes simplex virus, and slightly inhibited proliferation in response to three mitogens. Although the mannans of *S. cerevisiae* and *C. albicans* are structurally similar, the nonspecific immunoinhibitory quality noted for the bakers' yeast product made us suspect that the mechanisms of immunosuppression by mannans from these sources might not be comparable due to subtle differences in their structures.

The nonspecific immunoinhibitory activity of bakers' yeast mannan was subsequently found to be related to copper remaining complexed with the mannan prepared by the Fehling method (116). The contribution of bound copper to the nonspecific inhibitory influence of this mannan was demonstrated by removing the contaminating copper and comparing the inhibitory activities of the copper-deficient and copper-sufficient mannans: the mannan from which copper had been removed selectively suppressed the proliferative response of lymphocytes to *Candida* antigen. Although the copper-deficient *S. cerevisiae* mannan produced the same specificity of immunosuppression as the *C. albicans* mannan prepared by Fischer et al. (50), still other compositional features of mannan may influence its immunomodulatory function.

Recognizing that the method of extraction and precipitation of mannan can affect mannan structure, we have more recently compared the immunomodulatory properties of *C. albicans* mannans precipitated with copper or CTAB (132). Mannan prepared by both precipitation methods was observed to stimulate lymphoproliferation, but the CTAB-mannan consistently produced a peak response two to three times higher than that obtained with Fehling mannan, and the CTAB-mannan was more stimulatory at lower doses. Removal of copper from the Fehling mannan increased its ability to stimulate lymphoproliferation, but the stimulatory influence of the copper-deficient Fehling mannan was never comparable to that of the CTAB mannan. Exposure of the CTAB mannan to copper grossly reduced its ability to stimulate lymphoproliferation. Neither the CTAB nor the copper-deficient Fehling mannan inhibited the proliferative response of lymphocytes to other antigens, including commercial *Candida* antigen. These results demonstrate that *C. albicans* mannan isolated by conditions preserving its composition can be a potent stimulatory of lymphoproliferation in vitro, in apparent contradiction to other reports.

Other investigators who have evaluated *C. albicans* mannan as an antigen have reported that it has minimal or no immunostimulatory activity in vitro (2, 34, 56). We must therefore explain either the unexpected stimulatory activities of our mannan preparations or the inability of mannans prepared by others to stimulate lymphocyte proliferation. Mannans prepared from a virulent and a nonvirulent derived strain of *C. albicans* have different antigenic properties (14); therefore, mannans made by some laboratory strains of *C. albicans* may not stimulate lymphoproliferation in vitro. In addition, the changes in cell surface composition of *C. albicans* grown in different media (100) may involve mannan and, therefore, its ability to stimulate lymphoproliferation. Alternatively, noting that all other investigators have used Fehling mannan exclusively in their studies, we can hypothesize that either structural alterations caused by exposure to alkali or failure to remove all bound copper may have been responsible for the common failure of mannans prepared by others to stimulate lymphoproliferation. Our observation that removal of the Fehling-sensitive oligosaccharide side chains nearly abrogates the ability of CTAB mannan to stimulate lymphoproliferation in vitro (132) would seem to support this possibility. Because the mannosyl-hydroxyamino acid O-glycosidic bond has no known counterpart in glycoproteins of humans and other animals (168), either these O-linked oligosaccharides or the O linkage itself may be the primary epitope recognized by mannan-sensitive T lymphocytes. Observations that Fehling mannan catalyzes the dismutation of superoxide to hydrogen peroxide (116), which is known to suppress lymphocyte proliferation (104), and that mannan species obtained by ion-exchange chromatography differ in their abilities to stimulate and suppress immune function in vitro (35) also illustrate a relationship between mannan composition and function.

The inconsistent immunomodulatory properties of mannans prepared by different investigators could compromise theories about the role of mannan in immune dysfunction associated with CMC. Alternatively, these discrepancies could be interpreted to suggest that mannan exhibiting immunoinhibitory properties is not a native molecular spe-

cies. We propose that the circulating inhibitor in CMC is not a native form of mannan, but some altered form or forms, and suggest that an effort should be made to better define the inhibitory mannan in patient serum, as well as the immunomodulatory effects of mannan fragmented by various chemical and enzymatic methods. This proposal is based on our recent discovery that oligosaccharides derived from *C. albicans* CTAB mannan by hydrolysis with weak alkali (the O-linked oligosaccharides) are potent inhibitors of antigen-stimulated lymphoproliferation in vitro (132). These purified oligomers, containing two to six α-1,2-linked sugar residues and used at doses in the micromolar range, all inhibited proliferation of human lymphocytes in response to multiple antigens in vitro, but did not inhibit proliferation in response to mitogen. The alkali-modified fragment (deficient in O-linked oligosaccharides) was minimally stimulatory and did not inhibit antigen-stimulated lymphoproliferation. These results establish that the catabolism of mannan may enhance, rather than neutralize, its immunoinhibitory property and that inhibitory fragments can be as simple as a dimer.

In more recent studies we have determined that oligosaccharides prepared by acetolysis of a mutant *S. cerevisiae* (Mnn2) mannan, which contain α-1,6-linked mannose residues, may be more potent inhibitors of antigen-stimulated lymphoproliferation than the α-1,2-linked oligomers and that oligosaccharides prepared by weak acid hydrolysis of *C. albicans* mannan (which contain β-1,2-linked mannose residues) have no inhibitory influence (unpublished observations). These observations establish that mannan catabolism can yield very inhibitory carbohydrate fragments and demonstrate that the inhibitory property of oligosaccharide catabolites of mannan is partly linkage dependent. They also corroborate an earlier report by Muchmore et al. (109) that Man(α,1-6)Man isolated from human urine during pregnancy and its counterpart prepared from *C. albicans* mannan suppress antigen-stimulated lymphocyte proliferation in vitro. It therefore seems probable that mannan sloughed by *C. albicans* in vivo may be catabolized by fungal enzymes and/or enzymes in leukocytic or reticuloendothelial phagocytes to yield multiple oligosaccharide species of various sizes and linkage composition which make up the inhibitory mannan of CMC serum.

Mechanisms of Mannan-Mediated Immunosuppression

If mannan mediates immunosuppression in CMC, then what is its target cell, how does it influence cell function, and what cell functions are compromised? Historically, two experimental approaches have been followed to obtain answers to these questions. The approach common to early studies of CMC involved identification of the defective leukocyte in patients with CMC. The contemporary approach has been to identify effects of purified mannan on leukocytes isolated from healthy subjects. Each of these approaches has provided clues to leukocyte deficiencies associated with defective cell-mediated immunity in CMC, but neither is sufficient for all of the answers. Given that all patients with CMC do not share a common leukocyte defect and that an immunosuppressive influence of mannan could be secondary to an inherent leukocyte dysfunction, all observations deriving from in vitro applications of mannan must ultimately be confirmed with cells from multiple CMC patients. This section describes available clues to the defective leukocyte in CMC and the mechanism involved.

Defective lymphocyte. Early studies of cell-mediated immunity in CMC identified the lymphocyte as the defective leukocyte. Canales et al. (15) found that lymphocytes from their patient did not incorporate thymidine in response to *Candida* antigen in vitro and suggested that the lymphocytes either failed to recognize the antigen or were incapable of responding to it. Alternatively, Chilgren et al. (22) proposed that the immunological defect in CMC might reflect defective mediator production by lymphocytes, because lymphocytes from their patients proliferated in response to mitogen or *Candida* antigen. Subsequently, Valdimarsson et al. (186) confirmed the latter possibility and identified the mediator as MIF. Lymphocytes from a patient studied by Goldberg et al. (58) failed to proliferate in response to *Candida* antigen, but did produce MIF. This patient's cutaneous anergy could not be explained by failure of the lymphocyte to either recognize or respond to *Candida* antigen. Our unpublished observation that patient lymphocytes can proliferate in vitro under modified culture conditions also argues against a lymphocyte defect in CMC. Later observations (183, 197) that coculture of monocytes from healthy donors with lymphocytes from CMC patients allows lymphoproliferation to occur in response to *Candida* antigen would seem to further establish that some patients' lymphocytes recognize and respond to *Candida* antigen, even though the mechanism of reconstitution of immune reactivity by immunologically incompatible monocytes in these experiments remains unknown.

While there is no current basis for believing that the lymphocyte is defective in CMC, an observation by Durandy et al. that T lymphocytes bind *C. albicans* mannan specifically makes the T lymphocyte a potential candidate for a direct inhibitory influence of mannan (38). Binding was assayed by measuring binding of tritiated mannan to mannan-specific T lymphoblasts. The ligand bound only to the mannan-specific T cells, and binding was reversible and saturable, indicating that the binding was receptor mediated. A monoclonal anti-T3 antibody inhibited mannan binding to the T lymphoblasts, suggesting identity of the mannan receptor with the antigen receptor. The level of mannan binding to the T lymphoblasts (3.8×10^4 molecules per cell) approximated the number of antigen receptors on human T lymphocytes (200), providing additional evidence for identity of the mannan and antigen receptors. Whether binding of mannan to antigen receptor produced a direct effect on lymphoblast function or whether binding of soluble mannan interfered with the presentation of stimulatory mannan analog was not addressed in this report. Therefore, the functional relevance of this ligand-receptor interaction is not known at this time.

Defective monocyte-macrophage. Recognizing that the cooperative interaction of monocytes and lymphocytes is necessary for lymphoproliferation (145), failures of patient lymphocytes to respond to antigen have also been considered to reflect a monocyte defect. The earliest experiment implicating the monocyte as the cell responsible for immune dysfunction in CMC was described by Twomey et al. (185). They observed that patient monocytes cultured with either autologous lymphocytes or purified lymphocytes from healthy donors failed to mediate proliferation in response to *Candida* antigen; in contrast, monocytes from healthy donors mediated proliferation in response to antigen when cultured with purified patient lymphocytes.

Fischer et al. (52) next suggested a monocyte defect in CMC. Their discovery of mannan in patient serum led them to consider why mannan persisted only in some patients' sera and to identify the mechanism of inhibition of antigen-stimulated lymphoproliferation by mannan. They found that

monocytes from two of three patients with serum inhibitory activity took up mannan normally, but released mannan catabolites at a very slow rate. Such defective mannan catabolism occurred in both active and remission phases of infection. They also found that normal monocytes preincubated with *Candida* antigen in the presence of mannan failed to promote lymphoproliferation when recombined with autologous T lymphocytes. Together, these observations were taken to indicate that mannan may accumulate in patient plasma because it is slowly catabolized and that mannan is immunoinhibitory because it interferes with the presentation of *Candida* antigen to T lymphocytes.

Additional evidence for a monocyte defect in mucocutaneous candidiasis is available in two reports by Witkin et al. describing studies of women with recurrent *Candida* vaginitis (197, 198). Noting that patient mononuclear leukocytes inhibited the proliferative response of control leukocytes to *Candida* antigen, they attempted to identify the responsible cell by recombining purified monocytes and lymphocytes from control and patient donors. Finding that patient lymphocytes responded to *Candida* antigen in the presence of control monocytes and that patient monocytes inhibited the response of control lymphocytes to the antigen, they concluded that the monocyte was the defective cell associated with recurrent *Candida* vaginitis (197). Although this interpretation of their results appears acceptable, several issues can be raised to challenge it.

One issue is related to the lymphocyte proliferation that occurs on coculture of leukocytes from unrelated donors. It is possible that proliferation stimulated by allogeneic monocytes (197) in some way allowed the patients' lymphocytes to respond to antigen; the control monocytes may not have simply replaced the defective patient monocytes as antigen-presenting cells (APC) (184). We, too, have noted the ability of control monocytes to reconstitute the response of patient mononuclear leukocytes to *Candida* antigen (115a) and were unable to distinguish the contribution of the background proliferation to this phenomenon.

The second issue critical to interpretation of leukocyte coculture experiments is how monocytes and lymphocytes not matched for histocompatibility can interact to allow antigen-stimulated lymphoproliferation to occur. It is dogma in immunology that proliferation of T lymphocytes in response to antigen is controlled by molecules of the major histocompatibility complex (MHC) on the surface of the accessory cell or APC. The T lymphocyte can productively bind protein antigens only when presented in association with self-MHC class II molecules on the surface of the APC (184), and it is possible that mannan must be presented as antigen in the same manner (39). Without compromising this principle, one must then speculate that the normal monocytes in the experiments described by Witkin et al. (197) may have processed the *Candida* antigen for presentation by autologous B-lymphocytes (1) and provided a source of IL-1 to potentiate T-lymphocyte proliferation (55). Alternatively, the normal monocytes may have provided a missing second signal essential to stimulation of lymphoproliferation (191).

In additional experiments to determine the mechanism of the immunosuppression by patient monocytes, Witkin et al. (197) added ibuprofen or indomethacin to cultures of patient monocytes and patient lymphocytes or patient monocytes and control lymphocytes. They observed that these reagents enhanced antigen-stimulated lymphoproliferation and concluded that the defect in patient monocytes was related to synthesis of prostaglandin E_2 (PGE_2), known to inhibit the production of IL-2 (48).

PGE_2 production. Establishing that prostaglandin production by monocytes is a component of immune dysfunction in CMC would have tremendous significance. This mechanism would (i) give the patient monocyte the role of suppressor cell (4), (ii) provide for the possibility that a suppressor T lymphocyte might also be involved (192), and (iii) allow classification of CMC with other chronic infections in which macrophages and T lymphocytes have a significant immunoregulatory role (19, 24, 41, 70, 71, 87, 103, 113, 126, 130, 177, 181).

Recent findings in our laboratory have shown that the inhibition of antigen-stimulated lymphoproliferation induced by the mannan-derived oligosaccharides described previously is attributable to suppression of IL-2 production by PGE_2 (132a). Culture medium from human mononuclear leukocytes incubated for 2 days with antigen and oligomer was devoid of IL-2 bioactivity and contained an amount of PGE_2 in excess of that known to inhibit lymphoproliferation in vitro (59). We have also determined that excessive production of PGE_2 in the presence of oligomer may be an indirect consequence of oligomer-stimulated production of IL-1. Although IL-1 normally functions to augment the production of IL-2 by helper T lymphocytes (55), this cytokine has also been shown to operate in autocrine fashion to stimulate the monocyte to produce PGE_2 (23, 29). In our experiments PGE_2 production may also have been enhanced by oxygen metabolites (104) and other cytokines, and suppression of IL-2 production may have occurred through PGE_2-stimulated activation of suppressor T lymphocytes.

Induction of suppressor T lymphocytes by PGE_2 was observed initially in a murine system (192) and soon after shown to apply to human leukocytes as well. Fischer et al. demonstrated that human monocyte suppressive activity in vitro is mediated predominantly through release of PGE_2 (51). PGE_2, in turn, activates short-lived suppressor T lymphocytes that negatively influence mitogen- or allogeneic leukocyte-induced lymphocyte proliferation and mitogen-induced B-lymphocyte maturation.

A second observation made by Fischer et al. (51) was that two events were essential for optimal activation of suppressor T lymphocytes. They found that concanavalin A provides a first signal that primes the suppressor lymphocytes to become more sensitive to PGE_2. This finding, together with another by Koster et al. (87), suggests that lymphocytes can also become sensitized to PGE_2 in vivo by conditions that affect the number and/or affinity of membrane receptors for this agonist. Koster et al. observed that lymphocytes from different individuals differ in their sensitivity to inhibitory effects of low doses of PGE_2 when stimulated with a suboptimal dose of phytohemagglutinin. The individuals most sensitive to PGE_2 were Q-fever patients exhibiting *Coxiella* antigen-induced, prostaglandin-mediated suppression of lymphocyte proliferation in vitro. This phenomenon could explain different abilities of lymphocytes from patients with CMC to respond to *Candida* antigen.

Suppressor T lymphocytes. Evidence for the existence of suppressor T lymphocytes in CMC has been provided by Durandy et al., using two experimental approaches (40). In tests of the ability of patients' leukocytes to produce antibody to mannan in vitro, they observed that leukocytes isolated from CMC patients during active disease did not produce anti-mannan antibody and that this phenomenon was the result of suppressor T-lymphocyte activity. This suppressor activity was also demonstrated by the ability of patient lymphocytes identified by rosetting with sheep erythrocytes to interfere with the production of anti-mannan

antibody by control leukocytes in vitro. In other experiments they observed that incubation of control lymphocytes with autologous monocytes and a high concentration of mannan in vitro also activated suppressor T lymphocytes, that the mannan-activated suppressor cells did not have to be histocompatible to exert their inhibitory effect, and that irradiation of the mononuclear leukocytes before addition of mannan prevented generation of the suppressor lymphocytes. The patient-derived suppressor cells and those induced by mannan in vitro differed only in terms of the ability of the patient cells to inhibit both lymphocyte proliferation and helper activity. Piccolella et al. (128) and Domer et al. (35) have also reported that suppressor cells are generated in response to *C. albicans* polysaccharide or components of bulk mannan, but the mechanism(s) involved in these phenomena was not further defined.

From other studies unrelated to candidiasis, it becomes possible to speculate about additional characteristics of a suppressor lymphocyte-monocyte circuit in patients with CMC. Extrapolating from the report by Chouaib et al. (23), we can guess that the radiosensitive suppressor cell precursors become radioresistant after activation by PGE_2 and that the suppressor precursors segregate predominantly with the suppressor-cytotoxic (CD8) cell population and only with this subset after differentiation. Considering the report by Koster et al. (87), we can identify features of antigen-specific or nonspecific immunosuppression. Suppression can be elicited by an antigen-specific mechanism when the response to antigen activates a CD8 cell to produce a lymphokine that stimulates monocytes to produce PGE_2; suppression can be expressed in an antigen-nonspecific manner through PGE_2-mediated inhibition of IL-2 production. Mannan and its antigenic catabolites might therefore elicit specific immunosuppression limited to local sites of infection as long as lymphokine or PGE_2 does not escape to the systemic circulation. Nonantigenic mannan catabolites, acting locally or escaping to the systemic circulation, may produce nonspecific immunosuppression by directly stimulating or potentiating PGE_2 production.

Interaction with cytokines. Another mechanism to explain inhibitory effects of mannan and its catabolites on cell-mediated immunity in CMC can be found in additional literature unrelated to the immunology of candidiasis. In a series of reports, Muchmore et al. describe immunosuppressive effects of mannose, a mannose dimer, and a urinary glycoprotein, uromodulin. Studies to identify effects of mannose on immune function were based on an earlier observation that simple sugars could block cytotoxic effects of human monocytes on xenogeneic erythrocytes by interfering with binding of the effector cell to target (110). These investigators therefore considered that sugars might also interfere with the physical interaction of monocytes and lymphocytes essential to antigen-stimulated lymphoproliferation (109). Of 29 sugars tested, mannose was one of six sugars found to inhibit lymphoproliferation in response to tetanus toxoid in vitro. Although these sugars were immunoinhibitory only at a nonphysiological concentration of 25 mM, limitation of this activity to only six of them suggests that this phenomenon might have biological relevance. In other experiments, mannose was observed to inhibit lymphoproliferative responses to *Candida* antigen, diphtheria toxoid, and streptokinase-streptodornase as well and to be inhibitory only when added during the first 48 h of the culture period. These results were interpreted to reflect the competitive interference of sugars with carbohydrate receptors mediating the interaction of monocytes with lymphocytes.

An α-1,6-linked mannose dimer, obtained by Muchmore et al. from human pregnancy urine and *C. albicans* mannan, is 100 times more potent than mannose as an inhibitor of antigen-stimulated T-lymphocyte proliferation in vitro (111). Preincubation of mononuclear leukocytes with dimer for 20 h, followed by washing and resuspension of the cells in fresh medium, resulted in suppression of antigen-stimulated lymphoproliferation; preincubation of monocytes or lymphocytes separately with dimer, however, did not produce a similar inhibitory effect. Whether this mannose dimer circulates during pregnancy to perform an immunosuppressive function is not known, but it is tempting to speculate that it might and that *C. albicans*-derived mannose dimers and larger oligosaccharides may exploit an existing mechanism to control cell-mediated immunity during chronic candidiasis.

In other studies to characterize additional immunoregulatory factors in pregnancy urine, Muchmore and Decker discovered an 85-kDa mannose-containing glycoprotein that also selectively suppressed antigen-specific T-lymphocyte proliferation in vitro (107). It was named uromodulin to reflect its source and biological effect. The polypeptide portion of uromodulin is identical to Tamm-Horsfall glycoprotein in urine, but uromodulin is significantly more immunosuppressive than the glycoprotein (66), likely due to differences in glycosylation. Tamm-Horsfall glycoprotein is produced by epithelial cells in the ascending limbs of Henle's loop and thought to play a role in transport of macromolecules between the tubular lumen and peritubular capillaries (175). Although uromodulin likely has no direct role in immunosuppression associated with CMC, features of its mechanism of action as an immunoinhibitor provide an important clue to mechanisms of immunoinhibitory effects of mannan and mannose oligomers in CMC.

The immunosuppressive effect of uromodulin occurs over a dose range of 10^{-9} to 10^{-11} M and is not attributable to a cytotoxic influence or inhibition of cell division (107). Rather, uromodulin is a high-affinity ligand for IL-1 (112) and inhibits the bioactivity of IL-1 (11). A role for N-linked glycosylation of uromodulin in its interaction with IL-1 has been suggested (108), and clear evidence of the lectin-like nature of this interaction has been provided by the demonstration that uromodulin also binds to tumor necrosis factor α (155) and IL-2 (156). The binding of uromodulin to each cytokine could be effectively blocked by saccharides including *N*-acetylglucosamine dimer, diacetyl-chitobiose; a mannose trimer, Man(α-1-6)(Man(α-1-3))-Man-*O*-ethyl; and high-mannose glycopeptides (Man_{5-6}GlcNac2-*R*) thought to mimic core N-linked oligosaccharides of the uromodulin glycoprotein. Still further characterization of the mannose-binding activity of IL-2 was provided by observations that IL-2 binds to ovalbumin (a high-mannose glycoprotein) and yeast mannan and that IL-2 contains a peptide sequence in its N-terminal region that shares 27% homology with a 33-residue sequence of the carbohydrate-binding domain of human mannose-binding protein (47, 156).

The accumulated data describing interactions of IL-1, tumor necrosis factor α, and IL-2 with mannoproteins and mannose-containing saccharides tempt speculation that *C. albicans* mannan and/or its catabolites might influence the activities of these cytokines in chronic candidiasis, thus contributing to immune dysfunction associated with CMC. These pathogen-derived ligands might bind to the cytokines directly to inhibit their bioactivities, or they might competitively inhibit the interaction of uromodulin with these cytokines to compromise their detoxification and renal clear-

ance. The observations that uromodulin inhibits IL-1 activity (11) and effectively protects mice from lethal challenge with endotoxin (155), an event known to involve cytokine toxicity, support this possibility. The observations that uromodulin does not interfere with binding of IL-2 to cell surface receptors (156) and that binding of these cytokines to uromodulin and other ligands is not comparable in solid and soluble phase assays detract from our proposal, but we suggest that conditions may ultimately be defined under which mannan and/or its catabolites might influence the bioactivities of these cytokines in some significant way.

Interference with costimulation activities. The APC provides both specific and nonspecific signals to T cells in initiation of an immune response. The specific signal is the complex of antigen fragment bound to self-MHC molecules displayed on the APC surface for recognition by the T-cell receptor complex on T cells sensitive to the antigen. Recognition of the MHC-antigen complex alone, however, may not be sufficient to fully activate most T cells.

The additional signaling events occurring through other receptors are known collectively as costimulatory activities (reviewed in reference 150). In some cases, costimulation is provided by secreted ligands such as IL-1; in other cases cell-cell interactions are required (reviewed in reference 191). Interactions of lymphocyte function-associated antigen-3 with the T-cell receptor (12) and lymphocyte function-associated antigen-1 with intercellular adhesion molecule-1 (166) are examples of these costimulatory mechanisms involving physical interactions of the presenting cell with T cell. We have already noted the ability of mannose glycoproteins to interfere with IL-1 bioactivity (11), but there is no evidence that mannose glycoproteins might also interfere with cell-cell interactions involved in costimulation. Nevertheless, it is appealing to consider the latter possibility because of known consequences of antigen presentation in the absence of costimulator: T-cell recognition of MHC plus antigen in the absence of costimulator can lead to failure of clonal expansion as well as long-term refractoriness, or "tolerance" (150, 191).

Interference with leukocyte homing. Recruitment of lymphocytes, monocytes, and neutrophils to extravascular sites of infection is based on their ability to recognize and adhere to specialized venular endothelial cells adjacent to sites of inflammation. In mice, the adhesion receptors thought to mediate this binding interaction are known as MEL-14 (reviewed in reference 199); the human homolog of MEL-14 has recently been identified, using a battery of "DREG" monoclonal antibodies (80). Although the endothelial cell surface ligand recognized by these adhesion molecules is not yet known, available evidence suggests that they may interact with carbohydrate determinants on endothelial cells (194). The DREG-56 antibody inhibits lymphocyte binding of the phosphomannan monoester core prepared from the yeast *Hansenula hostii* (80), an activity associated with human and murine lymphocyte recognition of lymph node venules (reviewed in reference 169). The *Hansenula* product, mannose-6-phosphate, fructose-1-phosphate, and the fucose sulfate-rich polysaccharide fucoidin also efficiently block attachment of lymphocytes to lymph node venules (reviewed in reference 199). It is therefore not unreasonable to expect that *C. albicans* mannan or fragments thereof might also bind to DREG receptors and compromise recruitment of leukocytes to sites of fungal infection.

Leukocyte receptors for mannan and mannan fragments. The antigenic activity of *Candida* mannan may involve intracellular degradation, or processing, by monocytes or macrophages for presentation of the "antigen" to sensitive lymphocytes. The immunoinhibitory activity of mannan catabolites must involve interaction of these fragments with monocytes or macrophages in a way that stimulates cytokine production and/or prostaglandin production. These activities of mannan and its catabolites imply that monocytes and macrophages possess surface receptors for such ligands, but only a few clues to their identity exist.

Mannose/fucose receptors found on mammalian macrophages bind glycoproteins terminating in mannose, fucose, galactose, or *N*-acetylglucosamine, a mannan (Mnn2) synthesized by a mutant strain of *S. cerevisiae* (167), and zymosan (172). The mannose/fucose receptors on human alveolar macrophages have been characterized in terms of their relative affinities for glycosylated derivatives of bovine serum albumin (BSA): fucose-BSA $\geq$ mannose-BSA > *N*-acetylglucosamine–BSA >> galactose-BSA (153). Mannose-glycoprotein therefore is a preferred ligand for mannose/fucose receptors, but these receptors are not expressed on monocytes freshly isolated from human blood (153); they are expressed on human monocytes only after 2 to 3 days of maturation in culture. This timing of receptor upregulation would seem to be too late to effectively mediate either uptake of mannan as antigen or interaction with immunoinhibitory mannan fragments.

Receptors recognizing mannose-6-phosphate might also mediate binding of mannan and selected mannan fragments to leukocytes. Mannose-6-phosphate receptors with an apparent molecular weight of 215,000 have been isolated from human fibroblasts (162) and rabbit alveolar macrophages (154). Receptors with this ligand specificity, but a lower molecular weight of 46,000, have also been isolated from murine macrophages (67, 133). Both receptor populations function in the intracellular transport of newly synthesized lysosomal enzymes to lysosomes, but the larger receptors also mediate binding and endocytosis of exogenous lysosomal enzymes. Because mannose-6-phosphate receptors have not been identified on human monocytes and mannan fragments may not contain phosphate, it seems unlikely that these receptors have a role in the effects of mannan on immune function. More likely, the receptors involved in mediating uptake of mannan as antigen, and mannan fragments as immunoinhibitory agents, remain to be identified.

FUTURE PROSPECTS

During the 1960s and early 1970s, CMC provided an exciting challenge for many clinician-scientists with an interest in the emerging field of immunology. That was a time when the deficit in delayed hypersensitivity in CMC was recognized and when rapid strides were made in understanding and measuring functions of leukocytes that differentiate cellular and humoral immune responses. In this environment, new opportunity was available for further defining the immune deficit in CMC and applying novel therapies to restore host immune function. As the treatments applied were found to be effective only over the short term, and knowledge and experimental techniques available had been fully exploited, academic interest in CMC moderated. Most research efforts became directed instead toward characterization of the biological, antigenic, and pathogenic properties of *C. albicans* and clinical interest in *Candida* infection was associated with the much more frequent problem of disseminated, deep-seated candidiasis occurring in other patient populations.

Today, new opportunity exists to define the immune deficit in CMC and to identify treatments aimed at successfully restoring immune function in patients with CMC. A definition of the cell-mediated immune deficits associated with CMC is available from early studies, and evidence that immune dysfunction in CMC may be primarily an acquired problem is provided by repeated observations that temporal recovery of immune function occurs when patients are treated aggressively to reduce infection. The presence of mannanlike material in patient plasma demonstrates that immune dysfunction is likely caused by a component of the pathogen. Immunosuppressive properties observed for isolated *Candida* mannan and its catabolites in vivo and in vitro provide additional evidence that fungal mannan is responsible for patient immune dysfunction. A rapidly growing understanding of the cellular interactions and soluble mediators involved in elicitation and control of an immune response suggest new experiments to better define the immune deficit in chronic candidiasis and to explain suppressive effects of fungal mannan. New techniques to prepare, separate, and characterize carbohydrates will permit a more detailed characterization of the immunosuppressive mannan in patient plasma and catabolites of mannan generated by chemical or cellular degradation in the laboratory. The knowledge and techniques necessary to acquire the final details to explain the mechanism of immune dysfunction in chronic candidiasis and successfully treat this disease now seem to be in place.

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