Brazilian Journal of Microbiology (2008) 39:195-208
ISSN 1517-8382

GLYCOCONJUGATES AND POLYSACCHARIDES OF FUNGAL CELL WALL AND ACTIVATION OF IMMUNE SYSTEM

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Submitted: September 11, 2007; Approved: February 24, 2008.

ABSTRACT

Glycoproteins, glycosphingolipids and polysaccharides exposed at the most external layers of the wall are involved in several types of interactions of fungal cells with the exocellular environment. These molecules are fundamental building blocks of organisms, contributing to the structure, integrity, cell growth, differentiation and signaling. Several of them are immunologically active compounds with potential as regulators of pathogenesis and the immune response of the host. Some of these structures can be specifically recognized by antibodies from patients' sera, suggesting that they can be also useful in the diagnosis of fungal infections.

Key-words: Glycoproteins, polysaccharides, glycosphingolipids, immune system, fungi

INTRODUCTION

The cell wall is a vital structure for all fungi, controlling shape and protecting the organism from the environment. It contains molecules involved in morphogenesis, reproduction, cell-cell and cell-matrix interactions. Although the fungal cell wall is a rigid structure, it must be dynamic in order to allow budding, growth and adaptation to environmental stress (43) This structure is composed of a number of unique interconnected polysaccharides, including chitin and a variety of glucans that are not found in mammalian cells, therefore it defines a prime target for drug development. However, the composition of the cell wall can differ substantially among fungal species. In general, they present a very similar polysaccharide structure but differ significantly in their protein composition which underscores the importance of cell wall proteins for pathogenesis.

In *Saccharomyces cerevisae*, chitin is found at the cell budding sites representing 1-2% of the cell mass. β 1-6 glucan chains are directly attached to β 1-3 glucan and both glucan (50%) can be linked to chitin. In *Aspergillus fumigatus* branching of β 1-3 glucan results in an increase of acceptor sites for chitin, galactomannan and a linear β 1-3 /1-4 – glucan which substitutes the β 1-6 glucan commonly expressed in other fungi. Glucans can also covalently bind to cell wall proteins (CWP). There are two major types of glycosyl modifications of proteins. *N*-linked glycans are attached to specific asparagine residues within a protein sequence and *O*-linked glycans to either serine or threonine residues. Many of these CWPs are attached to a glycosylphosphatidylinositol (GPI) anchor during their transport to the cell wall. At the cell surface, GPI-containing CWP are found linked to the other components through a remnant of their GPI anchor (see review 77).

Polysaccharides and peptidopolysaccharides are fundamental building blocks of organisms, contributing to the structure, integrity, and function of prokaryotic and eukaryotic cells. These molecules are especially relevant for the architecture of the cell wall, but several of them are immunologically active compounds with great potential as regulators of pathogenesis and the immune response of the host. In addition, some of these molecules can be specifically recognized by antibodies from patients' sera, suggesting that they can be also useful in the diagnosis of fungal infections.

Proteins and glycoproteins exposed at the most external layers of the wall structure are involved in several types of

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interactions of fungal cells with the exocellular environment. Thus, coating of fungal cells with host antibodies has the potential to strongly influence the host-parasite interaction by affecting antibody-mediated functions such as opsonin-enhanced phagocytosis and blocking the binding activity of fungal adhesins to host ligands (57).

It is known that host defence mechanisms influence the manifestation and severity of fungal infections, such that the clinical forms of the disease depend on a patient's immune response.

Glycoproteins have long been known to influence T cell immune responses to a wide variety of antigens. Carbohydrate-binding receptors (CBR are part of the larger pattern-recognition receptor (PRR) family and are highly expressed on front-line immune cells, particularly macrophages and dendritic cells. For fungi, the polysaccharide-rich cell wall is a major source of pathogen-associated molecular patterns (PAMPs), and it comprises the initial structure recognized by cells of the immune system. Identified PRRs for the detection of fungal surface components include Toll-like receptors TLR2 and TLR4, collectins SP-A and SP-D, pentraxin-3, CR3 integrin, and C-type lectins, all of which appear to detect fungal-associated carbohydrates (10). Recognition of fungal-surface polysaccharides initiates immediate responses such as phagocytosis, production of antimicrobial compounds, and induction of proinflammatory cytokines that activate and recruit other immune effector cells. Two of the most physiologically studied receptors are the macrophage mannose receptor (MMR) and the dendritic cell (DC)-specific ICAM-3-grabbing nonintegrin (DC-SIGN). Similar to other members in their class, MMR and DC-SIGN exist as single transmembrane chains and require Ca^{2+} for their carbohydrate-binding properties, and thus are termed C-type lectins (95,28,109,72). Recent work on the nonclassical C-type lectin, *dectin-1*, has defined its substrate to be oligomers of β-(1,3)-glucan (81) a constituent of the cell wall of all fungi and a potent immunostimulatory molecule that induces TNFα production by macrophages.

CBR affinity for sugars is diverse, but mannose is the most common monosaccharide recognized by this receptor (reviewed in Refs 109, 23).

Another important glycoconjugate class are glycosphingolipids (GSLs), which are the glycosides of either ceramide or myo-inositol-(1-O)-phosphoryl-(O-1)-ceramide. It is a structurally and functionally diverse sphingolipid subclass; GSLs are ubiquitously distributed among all eukaryotic species and are found in some bacteria (52). These molecules have been implicated in many fundamentals cellular processes including growth, differentiation, morphogenesis and contribute to host immune response. GSLs may also modulate cell signaling by controlling the assembly and specific activities of plasma membrane proteins (33,38). Phosphorylinositol-containing sphingolipids, which are absent in animals, have been reported in many plants, fungi, and protozoan (50). GSLs are present in fungi of the most primitive class of Phycomycetes (132) as well as in the most complex Basidiomycetes (6). Neutral and acidic GSLs have been characterized from fungal cells.

Polysaccharides and glycoproteins

Oportunistic yeasts:

Cryptococcus neoformans

The incidence of infections caused by *Cryptococcus neoformans* greatly increased in individuals with compromised T-cell-mediated immune systems and cryptococcosis has emerged as the second most common cause of death in persons with AIDS. The cryptococcal infection follows the inhalation of poorly encapsulated yeasts, which are deposited into the alveolar space and then reach the lung interstitium. The infection is normally limited to the lung, but can disseminate to other tissues (55).

Earlier studies demonstrated that protective T cell responses to the pathogenic yeast *Cryptococcus neoformans* are dependent on heavily mannosylated antigens termed mannoproteins. Extensive *O*-mannosylation, which occurs at the serine/threonine region, facilitates recognition by mannose receptors on antigen-presenting cells, particularly dendritic cells. This results in an efficient antigen uptake, processing and presentation to T cells. Inhibition of mannose receptors or deglycosylation of mannoproteins profoundly inhibits T-cell responses, demonstrating the crucial contribution of mannosylation to immunogenicity (55,61). Human and murine dendritic cells (DC) are able to capture fluorescent-labeled mannoprotein by a mannose receptor-mediated process. By confocal microscopy, intracellular mannoprotein trafficked to an endo-lysosomal compartment in DC, and at later time points extended into tubules in a similar fashion to the degradation marker DQ-OVA. Mannoprotein colocalized intracellularly with CD206 and CD209. These data suggest that DC provide the crucial link between innate and adaptive immune responses to *C. neoformans* via a process that is dependent upon the efficient uptake of mannoprotein by mannose receptors (60).

In addition, incubation of human peripheral blood mononuclear cells with cryptococcal mannoprotein leads to the secretion of interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), IL-1β, IL-6, IL-8 and IL-10 (131).

Other studies have demonstrated that secreted cryptococcal antigens were separated by concanavalin A affinity chromatography into adherent (mannoprotein [MP]) and nonadherent (flowthrough [FT]) fractions, and the fractions were tested in murine models of disseminated cryptococcosis. Mice that received two inoculations of MP and FT exhibited prolonged survival and reduced brain and kidney fungal loads following intravenous challenge with *C. neoformans* and MP-immunized animals had increased brain levels of tumor necrosis

factor alpha, gamma interferon, and interleukin-2.In this context, FT and MP immunization protected B-cell-deficient, but not T-cell-deficient mice, suggesting that protection was T-cell mediated (62).

During *C. neoformans* infection, mannoprotein reinforced IL-12 and IFN-γ secretion that coincided with enhanced antifungal activity of natural effector cells, early resolution of the inflammatory process, and clearance of fungal load from the brain. These studies show that MP is a key inflammatory mediator that induces a protective immune response against *C.neoformans* infection (85).

Glucuronoxylomannan (GXM), the major polysaccharide component of *Cryptococcus neoformans*, is found bound to the fungal cell in the form of a capsule or shed in soluble form as an exopolysaccharide during growth *in vivo* and in culture. GXM is a (1→3)-linked, linear α-D-mannopyranan with *β*-D-xylopyranosyl (Xyl*p*), *β*-D-glucupyranosyluronic acid (Glc*p*A), and 6-*O*-acetyl constituents (14) Variation in the structure of GXM results in antigenic differences that permit classification of *C. neoformans* strains into five serotypes known as A, B, C, D and AD. This molecule is associated with a variety of immunomodulatory effects. It inhibits the production of proinflammatory cytokines (125) induces inhibitory factors such as IL-10 (97), inhibits activation and maturation of dendritic cells (124), suppresses T cell proliferation in the presence of APC (98,111), dampens Th1 response and delayed-type hypersensitivity response (96), limits MHC class II expression on APC (98), reduces killing (70, 123) and chemotactic activity of natural effector cells (68), and induces apoptosis in splenic mononuclear cells from normal rats (15). These effects are believed to contribute to the pathogenesis of *C. neoformans* infections. Glucuronoxylomannan induces expression of Fas ligand in monocytes/macrophages resulting in apoptosis of T cells expressing Fas. The induction of FasL occurs in part through GXM-TLR4 interaction.(30). IgM, IgG1, and IgA mAbs to the capsule of *C. neoformans* are protective in murine models of cryptococcosis. Taborda and coworkers (2002) (112) reported that IgM and IgA to the *Cryptococcus neoformans* capsular glucuronoxylomannan (GXM) promote complement-independent phagocytosis by macrophages with efficiency comparable to that of IgG1. IgM- and IgA-mediated phagocytosis of *C. neoformans* was proportional to CR3 expression, inhibited by Abs to CR3 (CD11b/CD18) and CR4 (CD11c/CD18), and dramatically reduced with macrophages of CD18-deficient mice.

Pericollini and coworkers (2006) (82) investigated the effect of purified soluble GalXM on human T lymphocytes. Results indicate that, GalXM (i) can affect selected immune responses; (ii) causes significant impairment of T cell proliferation and increases interferon-γ and interleukin-10 production; and (iii) induces apoptosis of T lymphocytes through activation of caspase-8 that terminates with fragmentation of DNA.

Candida albicans

Fungal opportunistic infections, in particular, those caused by *Candida* species, have gained considerable significance as a cause of morbidity and mortality. Commensalism can easily turn into mucosal candidiasis in immunocompromised subjects, such as AIDS patients or those affected by idiopathic $CD4^+$ T lymphocytopenia (17); moreover, deep-seated candidiasis predominantly occurs in neutropenic bone-marrow transplant patients. Finally, a large incidence of vaginal infection by *Candida* is recorded in otherwise healthy women of premenopausal age (25,83,79).

Candida albicans has a multilayered cell wall composed of an outer layer of proteins glycosylated with *N*- or *O*-linked mannosyl residues and an inner skeletal layer of α-glucans and chitin. The glycans are in the form of phosphomannoprotein complexes and are important in fungal-host interactions, as they make first contact with the immune system. The beta-oligomannosides, which make up the acid-labile part of the phosphomannan complex, and alpha-oligomannosides, which make up the acid-stable part of the complex, serve as adhesins in the attachment of *C. albicans* yeast cells to host splenic and lymph node macrophages. The β-oligomannosides can induce release of tumour necrosis factor (TNF)-alpha, and antibodies specific to certain beta-oligomannosides enhance host resistance to various forms of candidiasis (18). Several Mabs generated against *C. albicans* have been shown to react to β-1,2-oligomannosides (35,36,57) In addition, Han and collaborators (37) demonstrated that a mannan-based vaccine formulation elicited antibodies that protected against disseminated systemic and vaginal candidiasis and that a monoclonal agglutinating IgM (MAb 6.1) specific for a *C. albicans* cell surface β-1,2-mannotriose was also protective in both types of infection (36)

The cytokine production by human mononuclear cells or murine macrophages is markedly reduced when stimulated by *C. albicans* mutants defective in mannosylation. The recognition of mannosyl residues is mediated by mannose receptor binding to *N*-linked mannosyl residues and by TLR4 binding to *O*-linked mannosyl residues. Residual cytokine production is mediated by recognition of β-glucan by the dectin-1/ TLR2 receptor complex. *C. albicans* mutants with a cell wall defective in mannosyl residues were less virulent in experimental disseminated candidiasis and elicited reduced cytokine production *in vivo* (75). A 65-kDa mannoprotein (MP65) has long been studied as a major, immunodominant antigen of the human opportunistic pathogen *Candida albicans*. Pietrella and coworkers (2006) (84) demonstrated that MP65 stimulates dendritic cells (DC) and induces the release of TNF-alpha, IL-6 and the activation of IL-12 gene. MP65 induces DC maturation by increasing costimulatory molecules and decreasing CD14 and Fc gammaR molecule expression. The latter effect is partly mediated by Toll-like receptor 2 (TLR2) and TLR4, and the

MyD88-dependent pathway is involved in the process. MP65 enables DC to activate T cell response, its protein core is essential for induction of T cell activation, while its glycosylated portion primarily promotes cytokine production. (84) MAbC7, a monoclonal antibody directed against a *Candida albicans* cell wall mannoprotein exerts three anti-*C. albicans* activities, i.e., inhibition of adherence to HEp2, inhibition of germination, and direct candidacidal activity.(71). The candidacidal activity of macrophage was strongly enhanced when *C. albicans* was opsonized by C7 (108).

The 58-kDa surface mannoprotein of *Candida albicans* (mp58) elicits strong antibody responses during infection. A monoclonal antibody directed towards the C-terminal epitope conferred protection in serum therapy experiments in a murine model of hematogenously disseminated candidiasis (130).

Lectins play a critical role in host protection against infection. The galectin family of lectins recognizes saccharide ligands on a variety of microbial pathogens. Galectin-3, a galectin expressed by macrophages, dendritic cells, and epithelial cells, bind to *Candida albicans* species that bear beta-1,2-linked oligomannans on the cell surface, but did not bind to *Saccharomyces cerevisiae* that lacks beta-1,2-linked oligomannans.This fact, induced death of *Candida* species containing specific beta-1,2-linked oligomannosides. Unlike other lectins of the innate immune system that promote opsonization and phagocytosis, galectin-3 has direct fungicidal activity against opportunistic fungal pathogens (45).

Filamentous fungi:

Aspergillus sp

Species of the genus *Aspergillus* are among the most common causal agents of deep mycoses in the developed world. They have been implicated as etiological agents of several lung diseases including allergic asthma, allergic bronchopulmonary aspergilllosis (ABPA), aspergilloma**,** and invasive aspergillosis (IA). Over 90% of cases of *Aspergillus*-related diseases are caused by *A. fumigatus* (47,41). Cell-wall polysaccharides and glycoproteins have been characterized in *Aspergillus* spp. and galactomannans are an important structural component of the *Aspergillus* cell-wall, being widely distributed among most *Aspergillus* species.(49, 31) A galactomannan was isolated from a culture filtrate (48) or extracted from the fungal cell wall and it consisted of a main chain of (1→6)-linked α-D-mannopyranosyl residues substituted at O-2 by 1 to 3 consecutive α-D-mannopyranosyl units that were (1→2)-linked, and β-D-Galactofuranosyl-containiing side-chains, with (1→5)-links. Such b-D-Gal*f*-bearing chains are regarded as immunodominant epitopes, especially when they are (1→5)-linked. Antibodies directed against this type of polysaccharide have been detected in patients with aspergilloma and in experimentally infected animals, or in rabbits or mice hyperimmunized with total extracts of *A fumigatus* (106). Gal*f*-containing molecules have been described to be important antigens among several human fungal pathogens, such as *Paracocciodioides brasiliensis* (1) and are not present in the human host and could help it recognize the fungus as non-self and induce cytokine synthesis to activate cellular immunity.

Monoclonal antibodies have been raised against these structures and are used with some success for detecting circulating antigens (110). Periodate treatment, partial acid hydrolysis, and alkaline, reductive β-elimination of peptidogalactomannans (pGM) removed most of the antibody-binding capacity (31).

An immunodominant 35 kDa antigen containing 70% carbohydrate and 30% protein, isolated from a strain of *Aspergillus flavus*, involved in invasive mold sinusitis, was fractionated by ConA-Sepharose chromatography. The role of the carbohydrate moiety in sera recognition has been demonstrated (3).

Leitão *et al*. (2003) (49) have demostrated that *O*-glycosidically terminated oligosaccharide may account for a significant part of the *A. fumigatus* peptidogalactomannan antigenicity, because de-*O*-glycosylation decreased by 50% its activity. The immunodominant epitopes were present in the tetra- and hexasaccharides, which contain β-Gal*f*-(1→5)- β-Gal terminal groups. These haptens are potent inhibitors (90%) of the recognition between the sera of patients and pGM.

Mature *A. fumigatus* conidia and germ tubes stimulate NF-kappabeta, secretion of proinflammatory cytokines and production of reactive oxygen by human monocyte-derived macrophages and murine macrophages from multiple anatomical sites. These responses are in part mediated by dectin-1, which binds cell wall beta-glucan that is not present on the surface of dormant conidia, but is present after cellular swelling and loss of the hydrophobic proteinaceous cell wall (29).

Binding and internalization of *A. fumigatus* conidia correlates with DC-SIGN cell surface expression levels and is abolished in the presence of *A. fumigatus*-derived cell wall galactomannans. The clinical relevance of this interaction is emphasized by the presence of DC-SIGN in lung DC and alveolar macrophages, and further illustrated by the DC-SIGN-dependent attachment of *A. fumigatus* conidia to the cell membrane of IL-4-treated monocyte-derived macrophages. These dates suggest the involvement of DC-SIGN in the initial stages of pulmonary infection as well as in fungal spreading during invasive aspergillosis (107).

C-type lectins represent a family of receptors, which recognize pathogen-specific carbohydrates. One of them is β1-3 glucan, a major component of the fungal cell wall. Luther and coworkers (2006) (58) provide evidence that β1-3 glucan plays an important role for the elimination of *A. fumigatus* conidia. Laminarin, a soluble beta1-3 glucan and antibodies to dectin-1, a well known beta1-3 glucan receptor, significantly inhibited conidial phagocytosis. Additionally, TLR2 and the adaptor protein MyD88 are required for efficient conidial phagocytosis,

suggesting a link between the TLR2-mediated recognition of *A. fumigatus* and the phagocytic response. TLRs as well as the TLR-associated adaptor molecule MyD88 have been implicated in the recognition of the fungal pathogens *Candida albicans*, *Aspergillus fumigatus*, *Cryptococcus neoformans* and *Pneumocystis carinii*. *Saccharomyces cerevisiae* and *C. albicans*-derived mannan seems to be detected by TLR4. Phospholipomannan, present in the cell surface of *C. albicans* has been shown to be recognized by TLR2, while TLR4 mainly interacts with glucuronoxylomannan, the major capsular polysaccharide of *C. neoformans*. MyD88 has been implicated in TLR signalling of linear (1 → 3)-β-D-glucan, and of β-glucan from *P. carinii*. These data point towards the ability of the innate immune system to utilize TLRs that are specific to different types and components of pathogenic fungi (102).

Emerging filamentous fungus*: Pseudallescheria boydi*

Several types of pathogenicity have been associated with *P. boydii*. It is known to cause human white-grain mycetoma (99) and it has recently emerged as an agent of systemic and disseminated mycoses. *P. boydii* resembles other fungal species in tissue specimens and is not easily distinguished from *Aspergillus* species (7).

In the search for structures that could be helpful in the diagnosis of pseudallescheriasis, much attention has been paid to the study of *P. boydii* cell-wall antigens. Polysaccharides and peptidopolysaccharides have been isolated from its mycelial form and characterized by our group using chemical and immunological methods. The chemical structure of a peptidoglycan of *P. boydii* was investigated. Chemical analysis showed it to contain α-Rha*p*-(1→3)-α-Rha*p*- side-chain epitopes linked (1→3) to a (1→6)-linked α-Man*p* core. (88) PRM reacted poorly with an antiserum raised against whole cells of *S. schenckii* and strongly with one against *P. boydii* hyphae. These characteristics and immunological differences suggest that this major rhamnose-containing antigen of *P. boydii* may be useful for diagnostic purposes, mainly in mixed allergic bronchopulmonary fungal disease due to *P. boydii* and *Aspergillus*. Nonreducing, *O*-linked oligosaccharides were obtained from PRM by alkaline β-elimination under reducing conditions. Three oligosaccharide fractions were obtained and the major oligosaccharide (oligo 1) was characterized. Oligo 1 was a branched structure, with a main chain of α-Rha*p*-(1→3)-α-Rha*p*-(1→3)-α-Man*p*-(1→2)-Man-ol substituted at O-6 of mannitol with an α-Glc*p*-(1→4)-β-Gal*p* group.Oligo 2, was a substructure of Oligo 1, lacking a hexose from Glc-Gal branch. Both oligo 1 and 2 blocked the reaction between PRM and rabbit anti-*P. boydii* mycelium hyperimmune serum by 75% (87).

Bittencourt *et al* (2006) (9) isolated and characterized the structure of an alpha-glucan from *P. boydii* cell wall and evaluated its role in the induction of innate immune response. These analyses indicated that α-glucan of *P. boydii* is a glycogen-like polysaccharide consisting of linear 4-linked α-D-Glc*p* residues substituted at position 6 with α D-Glc*p* branches. Soluble α-glucan, but not β-glucan, led to a dose-dependent inhibition of conidia phagocytosis. Furthermore, a significant decrease in the phagocytic index occurred when α-glucan from conidial surface was removed by enzymatic treatment with alpha-amyloglucosidase, thus indicating an essential role of α-glucan in *P. boydii* internalization by macrophages. α-glucan stimulates the secretion of inflammatory cytokines by macrophages and dendritic cells; again this effect is abolished by treatment with alpha-amyloglucosidase. Finally, alpha-glucan induces cytokine secretion by cells of the innate immune system in a mechanism involving toll-like receptor 2, CD14, and MyD88. These results might have relevance in the context of infections with *P. boydii* and other fungi, and α-glucan could be a target for intervention during fungal infections (9).

Dimorphic fungi

Histoplasma capsulatum

There are two varieties of *Histoplasma capsulatum* that are pathogenic to humans, *H. capsulatum* var. *capsulatum* and *H. capsulatum* var. *duboisii*, and a third variety that is an equine pathogen, *H. capsulatum* var. *farciminosum* (39), *H. capsulatum,* a dimorphic fungus, exists as a mold in the environment and two types of conidia (macroconidia or tuberculate and microconidia) are formed. At 37ºC *in vitro* and in tissues, the organism converts into the yeast phase that is composed of tiny oval budding yeasts found both inside and outside macrophages. Infection with *Histoplasma capsulatum* var. *capsulatum* occurs commonly in areas in the Midwestern United States and Central America, but symptomatic disease requiring medical care is manifest in very few patients. The extent of disease depends on the number of conidia inhaled and the function of the host's cellular immune system (40).

The major diagnostic antigens of *H. capsulatum* var. *capsulatum* are the H and M antigens, pluripotent glycoproteins that elicit both humoral and T-cell-mediated immune responses. The H antigen from *Histoplasma capsulatum*, isolated from histoplasmin, have carbohydrate-to-protein ratios of 0.78 and contains galactose, glucose, mannose, and hexosamine, with higher concentrations of galactose, mannose, glycine, and alanine. The amino acid sequence of this glycoprotein showed homology with β-glucosidases (20,21) Recombinant H antigen is able to stimulated splenocytes from mice immunized with viable yeast cells or with antigen suspended in adjuvant. Mice inoculated with H antigen were not protected against either a sublethal or a lethal inoculum of yeast cells. Thus, H antigen stimulates a cell-mediated immune response in BALB/c mice but does not induce a protective response to *H. capsulatum* (20).

One such yeast phase-specific component is α-(1,3)-glucan, a homopolymer of glucose with α-glycosidic linkages, which has been linked to fungal virulence. The cell walls of most

medically important fungi contain α-(1,3)-glucan. Reduction in α-(1,3)-glucan, in *Histoplasma* (94) has no effect on *in vitro* growth but severely attenuates virulence in murine respiratory infection models. Rappleye and collaborators (2007) (93) present evidence showing that *Histoplasma* cell wall α-(1,3)-glucan blocks host PRR recognition of the fungal pathogen-associated molecular patterns (PAMP) β-glucan, enabling *Histoplasma* yeast to avoid detection as a fungal invader. However, the role of this polysaccharide during infection, its organization within the cell wall, and its synthesis and regulation remain poorly understood. Organisms that present α-(1,3)-glucan as the most external cell wall layer may thus effectively mask their β-glucan signature and avoid alerting the host immune system and the production of proinflammatory TNFα by phagocytes. The generation of these cytokines necessary for a protective immune response to both primary and secondary histoplasmosis, was suppressed either by the presence of the α-(1,3)-glucan layer on yeast cells or by RNA interference-based depletion of the host β-glucan receptor *dectin-1*. Consistent with this hypothesis, the parasitic forms of the dimorphic fungal pathogens each possess α-(1,3)-glucan and can cause disease even in the face of normal host immune function.

Paracoccidioides brasiliensis

Paracoccidioidomycosis (PCM) is a human systemic granulomatous disease, prevalent in South America, which is caused by a dimorphic fungus, *Paracoccidioides brasiliensis* (Pb). This fungus grows in the mycelial phase at room temperature and in the yeast phase at 37ºC as well as in infected tissues. This disease is one of the most prevalent human systemic mycoses in Latin America (11). Inhalation is probably the common route of introduction of air-borne conidia into the human host, with the lung being the primary organ infected. Transformation of conidia into yeast forms starts the infectious process (67,105). Once these cells are installed, characteristic granulomatous lesions are formed in the lungs and, if not contained, yeast cells invade the lymphatic system (acute form). Granulomas are rich in viable fungi which, in immunologically compromised hosts, can disseminate to virtually all organs and tissues (74) It is suggested that cell-mediated immunity is the most important host defense mechanism against this fungus, although specific antibodies may also have a protective role (66). The gp43, first described by Puccia *et al*. (1986) (91), is the major diagnostic antigen of *P. brasiliensis* in a variety of serological tests (12,121). The 43 kDa glycoprotein (gp) was the predominant IgG reactive antigen, recognized by 100% of the patient's sera (13). Monoclonal antibodies to gp43 modulated the laminin-mediated fungal adhesion to epithelial cells and moderated pathogenesis of yeast cells in a hamster testicle infection model (126). The gp43, a high -mannose glycoprotein, was also immunodominant in crude antigenic preparation for eliciting delayed hypersensitivity reactions in guinea pigs (100).

The gp43 gene was cloned and completely sequenced by Cisalpino *et al.* (1996) (16). It encodes a polypeptide of 416 amino acids with a leader peptide of 35 residues: the mature protein has a single high mannose *N*-glycosylation site. It contains a neutral high-mannose core (Man7GlcNAc2) to which a (1→6)-linked alpha-D-Man*p* chain of variable length, substituted at the 2-O positions by single alpha-D-Man*p* residues, is attached. A terminal unit of beta-D-Galactofuranose is (1→6)-linked to one of the (1→2)-linked mannosyl residues, either in the C or in the A arm of the oligosaccharide. The heterogeneity of the oligosaccharide is determined by the different sizes of the A arm and the sites of insertion of the beta-Galactofuranosyl unit. (Almeida *et al.* 1996) (1). It bears peptide sequences homologous to those of beta-1,3-glucanases from *Candida albicans* and *Saccharomyces cerevisiae*. The gp43 is, however, devoid of hydrolase activity and does not cross-react immunologically with fungal glucanases.

Specific conformational peptide epitopes are recognized by the human antibodies as determined by antigen deglycosylation (120).

Apart from eliciting high antibody titers, gp43 is also immunodominant in delayed-type hypersensitivity reactions in infected animals and humans. The cellular immune response in mice to gp43 administered in complete Freund's adjuvant involves CD4+ Th-1 lymphocytes, secreting gamma interferon (IFN-gamma) and interleukin 2 (IL-2) but not IL-4 and IL-10. The T-cell epitope of this antigen was mapped to a 15-amino-acid peptide (P10) based on lymphoproliferations with primed cells from three different haplotypes. The HTLAIR inner core of P10 is the essential domain of the epitope, with various flanking regions possible. Immunization of mice with both gp43 and P10 led to vigorous protection against intratracheal challenge by virulent *P. brasiliensis*, with a >200-fold decrease in lung CFU and halting of dissemination to the spleen and liver. The protective effect of P10 is mainly attributed to an IFN-gamma-mediated cellular immune response. Unlike gp43, which induces an antibody response (IgG1, IgG2a, IgE and IgG2b subclasses) compatible with both Th-1 and Th-2 activation in infected BALB/c mice, P10 does not induce a humoral response. Protection by gp43 and P10 was characterized by a few well-demarcated lung granulomas with numerous nonviable yeast forms or resolved lesions with no detectable fungal cells (113,119). The treatment combined with peptide P10 and chemotherapy showed an additive protective effect when administered at 48 h or 30 days after intratracheal challenge in BALB/c mice (63).

In resistant mice (A/Sn), purified gp43 seems to have been preferentially presented by macrophages and stimulated Th1 lymphokine production. On the other hand, in susceptible animals (B10) gp43 was distinguishably presented by B cells, which led to stronger activation of Th2 subsets. Moreover, T cells from resistant mice responded as those from susceptible animals when stimulated by gp43 presented by APCs from

susceptible mice and vice versa, indicating that there are no significant differences in the T cell repertoires from resistant and susceptible mice (2).

Addition of different concentrations of gp43 to the culture medium inhibited, in a dose-dependent pattern, phagocytosis of live or heat-killed Pb by peritoneal macrophages from both B10.A and A/Sn mice. Gp43 also inhibits phagocytosis of zymosan particles but did not interfere with the uptake of opsonized sheep red blood cells. It was also shown that both gp43 and heat-killed Pb have an inhibitory effect on the release of NO by zymosan - stimulated macrophages. Moreover, gp43 inhibits the fungicidal ability of macrophages from both lineages (90).

Antigen presentation is an essential stage in the development of immune response to a specific antigen. This response can lead to the production of antibodies and/or effector T lymphocyte activation. Macrophages, dendritic cells and B-lymphocytes, among others, act as antigen presenting cells. B-1a and B1-b cells represent a small population in the adult spleen and are abundant in the peritoneal and pleural cavities. Previous studies demonstrated that B1-b cells express constitutively high levels of class II MHC and costimulatory molecules inducing an efficient proliferation of gp43 sensitized T lymphocytes. (128) Granulomas were observed either when total adherent peritoneal cells or when isolated B-1 cells were added to macrophage cultures. The data strongly suggest that an interaction of B-1 cells and macrophages plays an important role in granuloma-like formation in this experimental model and that the presence of gp43 strongly stimulates this response (127).

Purified gp43 lead to down-regulation of MHC-II and adhesion properties of immature DCs and in LPS-induced DCs maturation. It was also shown that purified gp43 from *P. brasiliensis* has the same inhibitory effect on IL-12 release. Mice infected with *P. brasiliensis* that received DCs plus gp43 plus LPS had a significant increase of the lung colony forming units when compared with control (not immunized) or those that received only DCs plus LPS. These data suggest that gp43 affects many functions of the host cells, indicating that these alterations might be used by *P. brasiliensis* to reduce the effectiveness of the immune response thus facilitating the establishment and fate of primary infection in susceptible host (24).

Another expressed glycoprotein, gp70, is recognized by 96% of sera from PCM patients and is able to induce lymphoproliferation. Using anti-gp70 MAbs, it was observed by confocal microscopy that gp70 is located mainly in the intracellular compartment of the fungus, although it was also detected in the culture supernatant. Purified gp70 was able to inhibit the activity of macrophages through the mannose receptors and also through the Fc receptors and inhibits NO and H(2)O(2) liberation by peritoneal macrophages *in vitro*. Passive immunization of mice during intratracheal infection with *P. brasiliensis* using anti-gp70 MAbs almost completely abolished granuloma formation in the lungs, suggesting that this glycoprotein may facilitate fungal establishment and progression of lesions in the primary fungal infection (65).

Glycosphingolipids

Glycosphingolipids (GSLs) are amphipathic molecules consisting of a ceramide (N-acylsphingosine) lipid moiety to a glycan chain of variable length and structure. Glycoinositolphosphoryl ceramides (GIPCs) belong to a class of glycosphingolipids that are distinguished from the classical GSL already described in that they contain their sugar portion linked to ceramide via an inositol phosphate bridge. The ceramide monohexosides (CMHs or cerebrosides) gluco- and galactosylceramides have been reported as membrane and cell wall constituents of both pathogenic and nonpathogenic fungi. They are structurally different from their mammalian counterparts in that the latter contain saturated, non-hydroxylated fatty acids bound to a sphingobase lacking branches, consequently, fungal CMHs can be selectively recognized by antifungal agents. Several studies revealed that fungal CMHs are the cellular targets for the action of human, rabbit and mouse antibodies with antimicrobial activity. The side effects and drug resistance are commonly observed during treatment of deep mycoses, which motivates the search for new antifungal drugs.

Structural aspects

The fungal CMHs are composed of a sugar unit, usually glucose or galactose, bound to a hydrophobic ceramide, containing the conserved C_{19} sphingoid base with a C-9 methyl branch group and two unsaturated linkages (Δ^4, Δ^8) in amidic linkage to 2-hydroxyoctadecanoic or 2-hydroxyhexadecanoic acids. These molecules are conserved structures, in which modifications include different sites of unsaturation as well as the varying length of fatty acids residues in the ceramide moiety. In plants the monosaccharide is normaly glucose and the sphingoid usually phytosphingosine. Galactose, sphingosine or dihydrosphingosine are the main components of animal glycolipids. The gangliosides contain at least one sialic acid residue. CMHs differ from globosides in that these glycolipids contain multiple sugar moieties and also from gangliosides that contain at least one sialic acid residue. CMHs have been widely detected in fungal cells and the current literature indicates that cerebrosides seem to be present in almost all the fungal species studied so far, with *Saccharomyces* cerevisae, *Torulaspora delbrueckii*, *Candida glabrata*,, *Kluyveromyces polysporus*, and *K. yarrowii* representing exceptions (103) Cerebrosides from *Saccharomyces kluyveri* have a rare trihydroxy sphingoid base as a unique feature (115).

The major CMH species from *F. pedrosoi* produced by conidial and mycelial forms display the same structure, an N-2'-hydroxyhexadecanoyl-1-beta-d-glucopyranosyl-9-methyl-4,8-sphingadienine. However, the major cerebroside species purified

from sclerotic cells carries an additional hydroxyl group, bound to its long-chain base (78).

Using several chromatographic approaches, mass spectrometry, and nuclear magnetic resonance, Maciel and coworkers (2002) (59) identified ceramide mono- and dihexosides (CDH) in purified lipid extracts from *Magnaporthe. grisea* cells. As described by other authors, CMH consists of a ceramide moiety containing 9-methyl-4,8-sphingadienine in amidic linkage to 2-hydroxyoctadecenoic or 2-hydroxyhexadecenoic acids and a carbohydrate segment consisting of one residue of glucose. CDHs, however, contain beta-galactose (1→4)-linked to beta-glucose as sugar units and phytosphingosine as the long-chain base, bound to a C24 alpha-hydroxylated fatty acid. This is the first report on the occurrence of CDH in a fungal species and illustrates the existence of an alternative path of ceramide glycosylation in fungal cells.

The long chain base 9-methyl-4-8-sphingadiene was first described in monohexosylceramides from *Aspergillus. oryzae* (27) and was subsequently isolated from *Schizophyllum commune* (42), *Fusicoccum amygdale* (4), *Clitocybe geotrope* and *C. nebularis* (26) CMHs were further characterized in lipid extracts from the fungal species *Aspergillus fumigatus* (129,116), *A. niger* (54), *A. versicolor* (131), *Acremonium chrysogenum* (104), *Candida albicans* (64), *Colletotrichum gloeosporioides* (19), *Cryptococcus neoformans* (101), *Fonsecaea pedrosoi* (78), *Fusarium sp* (22), *Hansenula anomala* (76), *Histoplasma capsulatum* (118), *Kluyveromyces waltii* (115), *Magnaporthe grisea* (44,122), *Paracoccidioides brasiliensis* (114), *Pichia pastoris* (104), *Pseudallescheria boydii* (89), *Saccharomyces klyuyveri* (115), *Sporothrix schenckii* (117), *Termitomyces albuminosus* (92).

Several studies (reviewed in reference Lester and Dickson 1993) (50) allowed the identification of three classes of GIPCs in *S. cerevisae*. These classes consist of inositolphosphoceramides (IPCs), mannosylinositol phosphoceramides (MIPCs) and the major sphingolipid, $M(IP)_2C$ which contains two inositolphosphates with a mannose unit attached to one of the inositols. Based on NMR analysis, $M(IP)_2C$ has been shown to have a structure consisting of Ins-1-P-6-man*p* α1→2Ins-1-P-1-Cer. Diversity in the long-chain base, degree of hydroxylation and chain length of the fatty acid from the ceramide portion, give rise to several members of these classes (133). In addition, non-glycosylated IPCs have been isolated from the fungus *Phytophtora* spp (56) and *Neurospora crassa* (134). The GIPCs structures from pathogenic fungi are shown in Table 2.

Structural determination of CMHs is greatly dependent on the use of mass spectrometry techniques, including analytical variations as fast atom bombardment mass spectrometry (FAB/MS), electrospray ionization (ESI-MS), and low energy collision-induced dissociation mass spectrometry (ESI-MS/CID-MS). ^{1}H and ^{13}C NMR have also been used successfully in cerebroside structural analysis. The combination of these techniques is usually sufficient for a complete structural elucidation.

Biological functions

The understanding of the functions of CMHs in fungal cells is only beginning to emerge. The old concept that cerebrosides and other glycosphingolipids are membrane structural components with exclusive "filling gap" (46) roles is simplistic, since it is now clear that such molecules are involved in cell growth, differentiation and signaling (32). Fungal cerebrosides induce cell differentiation in *S. commune* with formation of fruiting body (42). Seemingly, the 8E-double bond and the methyl group at C-9 in the sphingoid base are essential for differentiation. In fungal cells, CMHs have been characterized as bioactive molecules. For instance, the phytopathogen *Magnaporthe grisea* produces active elicitors of the hypersensitive response in rice (44,122) that were identified as monohexosylceramides. These molecules induced the accumulation of antimicrobial compounds, cell death, expression of pathogenesis-related proteins in rice leaves, and protected rice plants against fungal infection.

CMHs were characterized as antigenic molecules possibly involved with fungal growth or differentiation in *S. commune* (42), *C. neoformans* (102), *P boydii* (89), *C. albicans* (89), *A. nidulans* (54), *A. fumigatus* (53) and *F. pedrosoii* (78).

In *P. brasiliensis,* two glycosylinositolphosphorylceramide (GIPC) antigens (acidic GSLs), termed Pb-1 and Pb-2, are present. Only the GSL Pb-1 antigen, which presents the carbohydrate structure Gal*f*ß1-6(Manα1-3) Manß1, is reactive with the PCM patient sera. The PCM patient sera did not react with Pb-2, which lacks the Gal*f* residue and which is considered the biosynthetic precursor of Pb-1, indicating that the Gal*f* residue is essential for antibody reactivity. The Pb-1 glycolipid from nontreated patients elicited a primary immune response with immunoglobulin M (IgM) production and subsequent switching to IgG1 production. The IgG1 titer increase after the start of antifungal treatment, and general decreases in the anti-Pb-1 antibody titers are observed after 5 months of treatment. Probably, the Pb-1 antigen, an acidic GSL with terminal Gal*f* residue, has potential application as an elicitor of the host immune response in patients with PCM (8). da Silva *et al.* (2007, unpublished results) investigated the antibody responses against monohexosyl ceramide and acidic glycolipids from *P. brasiliensis* and their involvement in phagocytosis of yeast forms. Polyclonal antibodies from mice immunized with a crude lipid mixture and patient's sera recognized mainly CMH and one fraction of acidic glycolipid. Anti-CMH and anti-acidic glycolipid Abs were used as opsonizing agents in phagocytosis assays increasing the internalization of the yeast cells and production of NO by macrophages. A protective role of these Abs is thus suggest.

A monoclonal antibody to the glucosylceramide synthesized by *P. brasiliensis* was produced and demonstrated to react

with the reproductive structures (conidiophore) of *A. fumigatus* (120) This evidence supported the idea that CMHs are preferentially accumulated in surface sites related to fungal growth, but also suggested that they are involved in the differentiation process. Accordingly, the same research group reported the involvement of GlcCer in fungal development on *A. fumigatus* and *A. nidulans* using a family of compounds known to inhibit GlcCer synthase in mammals. Two analogs inhibited germination and hyphal growth. Neutral lipids from *A. fumigatus* cultured in the presence of these inhibitors displayed a significantly reduced GlcCer/GalCer ratio. These results suggest that synthesis of GlcCer is essential for normal development of these species (53).

Pseudallescheria boydii is a fungal pathogen that causes disease in immunocompromised patients. Ceramide monohexosides were isolated and purified from mycelia of *P. boydii* and their structures were determined by chemical, spectrometric, and spectroscopic methods. This fungus appears to synthesize only glucosylceramides containing 9-methyl-4-8-sphingadienine as long chain base. The different molecular species can thus be attributed to CMH molecules differing only in the chain length of hydroxylated fatty acids (16:0 and 18:0).This molecule was recognized by serum of an infected rabbit, confirming that antibodies to cerebrosides are producing by *P. boydii*. (89) Hydroxylation at position 2 of the fatty acid is apparently important for antigenicity of the CMH (73,134), and possible epitopes involve both glucose and the hydroxylated fatty acid, with modulation by the sphingosine-derived base. Conformer 4 of glucosylceramide – as studied by Nyholm and Pascher (1993) (80) which is allowed in a membrane layer, and further stabilized by a hydrogen bond between the 2-OH group on the fatty acid and the 6-OH group on the glucose residue, in addition to the hydrogen bond between glucose O5 and the amide hydrogen – is a candidate for epitopes reactive with anti-CMH antibodies. CMH accumulated on the surface of mycelia was recognized by antibodies from rabbits immunized with *P. boydii* whole cells. Interestingly, conidial cells did not react with the antibodies to CMH, suggesting that CMHs are differentially expressed in *P. boydii* according with morphological phase. These antibodies were able to inhibit the formation of germ tube–like structures in *P. boydii,* although they did not influence mycelial growth. We have shown that germ tubes are induced after the contact of *P. boydii* conidia with animal cells, a step preceding efficient fungal invasion. (86). Germ tube formation is also recognized as a crucial event in tissue invasion by *C. albicans* (30), a fungus that synthesizes CMHs (64) structurally similar to those previously described in other fungi and to that characterized from *P. boydii.* In this context, the influence of antibodies to CMH on *C. albicans* differentiation was also evaluated. As with *P. boydii,* anti-CMH antibodies inhibited germ tube formation in *C. albicans* (89). Our most recent results demonstrate that polyclonal and monoclonal antibodies to CMH strongly inhibit the differentiation of the plant pathogen *Colletotrichum gloeosporioides* (19). The mechanism by which anti-CMH antibodies inhibit fungal growth and/or differentiation remain to be established, but there is a possibility that CMHs are associated with enzymes involved in the hydrolysis and synthesis of the cell wall and/or with GPI-anchored precursors during cell differentiation and division. In this context, binding of antibodies to CMHs could impair the action of CMH-associated functional proteins inhibiting cell wall synthesis.

By using various mass spectrometric techniques, a cryptococcal cerebroside was characterized by Rodrigues and coworkers (2000) (102) as a β-glucosylceramide, with the carbohydrate residue attached to 9-methyl-4,8-sphingadienine via an amidic linkage to 2-hydroxyoctadecanoic acid. This molecule was recognized by sera from patients with cryptococcosis and a few other mycoses, indicating that CMHs are immunogenic glycolipids that induce the production of human antibodies during fungal infections.

The presence of CMHs as structural components of the cell wall of *C. neoformans* was demonstrated by electron microscopy of yeast cells labeled with immunogold-antibodies (102). An abundant deposition of gold particles was observed on the cryptococcal wall rather than on the plasma membrane, .indicating that the antibody-reactive epitopes of CMH may be sterically accessible only after transfer of the glycosphingolipids to the cell wall. Labeling was also observed on membrane formations, putatively vesicles, across the periplasmic space, linking the plasma membrane to the inner face of the cell wall (5,78,102) suggesting that cerebrosides can be hydrophobic components involved in the vesicular traffic of surface molecules.

Confocal analysis demonstrated that human anti-CMH antibodies mainly reacted at the cell budding sites of *C. neoformans*, suggesting a relationship between CMH distribution and cell growth (102). To confirm this hypothesis, human antibodies to cerebrosides were added to cultures of *C. neoformans* and yeast growth determined at 12 h intervals. In their presence, Rodrigues and coworkers (2000) (102) observed an immediate arrest of cell growth and budding. Although fungal cultures were also supplemented with human serum, this effect was independent of the action of the complement system. Both acapsular and encapsulated strains of *C. neoformans* had budding and cell growth inhibited by the antibodies. Analysis of antibody-treated cells by transmission electron microscopy revealed intense cellular damage, with organelle destruction, membrane retraction, and increased vacuolization (78).

Infection with *F. pedrosoi,* etiological agent of chromoblastomycosis, begins with traumatic inoculation of conidia or mycelial fragments from the soil, but *in vivo* these

cells differentiate into sclerotic bodies. CMHs from conidial forms of *F. pedrosoi* were purified and characterized as *N*-2'-hydroxyhexadecanoyl-1-β-D-glucopyranosyl-9-methyl-4,8-sphingadienine. However, the major cerebroside species purified from sclerotic cells carries an additional hydroxyl group, bound to its long-chain base (78). The structural difference between cerebrosides from mycelial and sclerotic cells was apparently not relevant for their antigenicity, since they were both recognized at similar levels by sera from individuals with chromoblastomycosis and a monoclonal antibody to a conserved cerebroside structure. *F. pedrosoi* conidia were treated with the antibody to CMH and the growth of antibody-treated cells was analyzed by counting the number of colony-forming units. Treatment with anti-CMH antibody killed at least 60% of the conidial population. The addition of the anti-CMH antibody to conidial cell cultures of *F. pedrosoi* also resulted in inhibition of fungal growth. Fungal cells treated with a monoclonal anti-cerebroside antibody were more efficiently internalized and killed by phagocytes, showing for the first time that, besides their immediate antifungal action, CMH-binding antibodies can help host cells to eliminate internalized fungi. Sclerotic cells display a unique shape, along with a muriform arrangement within the tissue, which impairs an efficient host cell attack and access of anti-fungal drugs (34). Pre-incubation of fungal cells with the antibody had no effect on the interaction of *F. pedrosoi* sclerotic cells with murine macrophages. In addition, sclerotic bodies were completely resistant to the antifungal action of anti-CMH antibodies. Immunofluorescence analysis showed that recognition of sclerotic cells by these antibodies only occurs at cell wall regions in which melanization is not evident. Accordingly, melanin removal with alkali results in an increased reaction of fungal cells with anti-CMH antibodies. These results indicate that cerebroside expression in F. pedrosoi cells is associated with dimorphism and melanin assembly on the fungal cell wall (78).

To understand how cerebrosides influence the biology of fungal cells, a profound knowledge of structural and biosynthetic aspects of these molecules is still required. The development of chemical or immunological agents with unquestionable selectivity to inhibit CMH synthesis and expression is also necessary to evaluate if cerebrosides are in fact good targets for the treatment of fungal infections.

ACKNOWLEDGEMENTS

This work was supported by Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP) grants 05/02776-0 and 05/56161-6. C.P.T and E.B.B. are research fellows of the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). E.B.B. was supported by Fundação de Amparo a Pesquisa do Estado do Rio de Janeiro (FAPERJ).

RESUMO

Glicoconjugados e polissacarídios de parede celular de fungos e ativação do sistema imune

Glicoproteínas, glicoesfingolipídios e polissacarídios, expostos nas camadas mais externas da parede celular dos fungos, estão envolvidos em diferentes tipos de interações com o ambiente extracelular. Essas moléculas são componentes essenciais desses organismos, contribuindo para a estrutura, integridade, crescimento celular, diferenciação e sinalização. Alguns são compostos imunologicamente ativos com potencial para regular a patogênese e a resposta imune do hospedeiro, Algumas dessas estruturas podem ser especificamente reconhecidas por anticorpos presentes no soro de pacientes, sugerindo uma possível utilização como ferramenta no diagnóstico das infecções fúngicas.

Palavras-chave: Glicoproteínas, polissacarídios, glicoesfingolipídios, sistema imune, fungos

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