Characterization of monoclonal antibody MEST-2 specific to glucosylerceramide of fungi and plants

Marcos S. Toledo, Erika Suzuki, Steven B. Levery2, Anita H. Straus, and Helio K. Takahashi1

Department of Biochemistry, Universidade Federal de São Paulo/Escola Paulista de Medicina, Rua Botucatu 862, São Paulo, SP, 04023–900, Brazil, and 2The Complex Carbohydrate Research Center, University of Georgia, 220 Riverbend Road, Athens, GA 30602, USA

Received on May 22, 2000; revised on September 21, 2000; accepted on September 25, 2000

An IgG2a monoclonal antibody anti-glucosylerceramide was established and termed MEST-2. High performance thin layer chromatography immunostaining, and solid-phase radioimmunoassay showed that MEST-2 reacts with glucosylerceramide from yeast and mycelium forms of Paracoccidioides brasiliensis, Histoplasma capsulatum, and Sporothrix schenckii; from hyphae of Aspergillus fumigatus; and from yeast forms of Candida albicans, Cryptococcus neoformans, Cryptococcus laurentii, and Cryptococcus albidus. Studies on the fine specificity of MEST-2 showed that it recognizes the β-glucose residue, and that the 2-hydroxy group present in the fatty acid is an important auxiliary feature for the antibody binding. It was also demonstrated that phosphatidylcholine and ergosterol modulate MEST-2 reactivity to glucosylerceramide, by solid-phase radioimmunoassay. Indirect immunofluorescence showed that MEST-2 reacts with the surface of yeast forms of P. brasiliensis, H. capsulatum and S. schenckii. Weak staining of mycelial forms of P. brasiliensis and hyphae of A. fumigatus was also observed. The availability of a monoclonal antibody specific to fungal glucosylerceramide, and its potential use in analyzing biological roles attributed to glucosylerceramide in fungi are discussed.

Key words: 2-hydroxy fatty acid/glucosylerceramide/monoclonal antibody/Paracoccidioides brasiliensis/pathogenic fungi

Introduction

As a part of a systematic study of fungal glycosphingolipids (GSLs), detailed characterizations of the neutral GSLs present in different pathogenic and nonpathogenic fungi were carried out. These studies showed that all fungi studied so far show as neutral GSLs only monohexosyl ceramide (CMH) (Toledo et al., 1995, 1999, 2000; Suzuki et al., 1997; Levery et al., 1998, 2000).

Common features of fungal cerebrosides include a ceramide containing (4E,8E)-9-methyl-4,8-sphingadienine base and 2-hydroxy fatty acids, which may also be modified by (E)-Δ3 unsaturation (Mizushina et al., 1998). So far, the latter appears to be a modification unique to fungal cerebrosides.

Recently, we reported the detailed structural analysis of CMHs from yeast and mycelium forms of the thermally dimorphic mycopathogen, Paracoccidioides brasiliensis, and from hyphae of Aspergillus fumigatus. It demonstrated the presence of both saturated and (E)-Δ3 unsaturated 2-hydroxy fatty acids in their ceramide moieties (Toledo et al., 1999). Two strains of A. fumigatus were analyzed, and it was observed that they express both glucosylerceramide (GlCer) and galactosylerceramide (GalCer). The differential synthesis of these two compounds seems to be related to the degree of Δ3 unsaturation in the 2-hydroxy fatty acids. In addition, a clear difference was also demonstrated in the amount of Δ3 unsaturation when comparing GlCer from mycelium or yeast forms of P. brasiliensis (Toledo et al., 1999). The role of such chemical dimorphism in CMH in P. brasiliensis is still unknown, but may be related to signaling processes associated with morphological transitions in P. brasiliensis.

GlCer is now recognized as an important compound related to fructification (Kawai and Ikeda, 1983, 1985; Kawai, 1989), membrane maintenance at low temperatures (Levery et al., 1998), fungal dimorphism (San-Blas and San-Blas, 1985), mitogenesis (Marchell et al., 1998), or signal transduction (Patton and Lester, 1992; Boldin and Futerman, 2000). Thus, the availability of a monoclonal antibody (MAb) specifically directed to GlCer containing 2-hydroxy fatty acids will be an effective tool leading to new approaches aiming to a more accurate understanding of the role and organizational pattern of GlCer in the membrane/cell wall in different fungi.

Results

Characterization of MAb MEST-2

Among a few hybridomas showing reactivity with GlCer of P. brasiliensis, a clone secreting IgG2a monoclonal antibody was successfully established after repeated subcloning by limited dilution, and termed MEST-2. By high performance thin layer chromatography (HPTLC) immunostaining, it was verified that MEST-2 reacted with GlCer from yeast and mycelium forms of P. brasiliensis, H. capsulatum, and S. schenckii; mycelia of Aspergillus fumigatus; yeast forms of Candida albicans, Cryptococcus neoformans, Cryptococcus...
Sylceramide (glucosylceramide (Gaucher’s spleen) and 2-hydroxy fatty acid) was further analyzed by solid-phase RIA using mixtures of glucosylceramide (GlcCer) from Gaucher’s spleen or bovine buttermilk which present non-hydroxylated fatty acids, but reacted effectively with soybean GlcCer, comprised primarily (>95%) of ceramide containing 2-hydroxy fatty acids (Sullards et al., 2000) (Figures 1, 2). These results suggest that the 2-hydroxy group of the fatty acid (f.a.) is an important feature for the MEST-2 binding to GlcCer. The importance of 2-hydroxy fatty acids in the MEST-2 binding was further analyzed by solid-phase RIA using mixtures containing different molar proportion of non-hydroxylated f.a. glucosylceramide (Gaucher’s spleen) and 2-hydroxy f.a. glucosylceramide (P. brasiliensis), thus varying the levels of 2-hydroxy fatty acid GlcCer. As expected, it was observed a decrease of MEST-2 reactivity to glucosylceramide when the plates were adsorbed with a mixture 2-hydroxy f.a. glucosylceramide and non-hydroxylated f.a. glucosylceramide (1:1 and 1:9), a shift to the right of the binding curve was observed. However, as shown in Figure 3B, if the binding curves are calculated considering only the amount of the 2-hydroxy fatty acid GlcCer present in each mixture the curves are exactly the same as the one with only 2-hydroxy fatty acid glucosylceramide alone. This experiment clearly demonstrate that: (1) glucosylceramide without 2-hydroxy fatty acid do not compete or enhance the MEST-2 binding to glucosylceramide presenting 2-hydroxy fatty acids and (2) a direct relationship between the MEST-2 reactivity and the amount of glucosylceramide presenting 2-hydroxy fatty acid.

**Effect of temperature and lipids on MEST-2 binding**

In order to determine whether other lipids could affect the conformation of glucosylceramides and therefore modulate the MEST-2 reactivity, a binding assay was also carried out at different temperatures using lipid mixtures containing different molar ratios of cerebrosides, phospholipids, and fungal sterols. As shown in Figure 4, by solid-phase RIA, MEST-2 reacts only with fungal and soybean glucosylceramide (Figure 4A) and does not react with yeast glucosylceramide (Figure 4B). Temperature did not alter the MEST-2 binding to GlcCer alone. On the other hand, it was observed an increase of MEST-2 reactivity to fungi GlcCer at 37°C in presence of phosphatidylcholine (Figure 4A). MEST-2 reactivity to fungi and soybean glucosylceramide increased to

---

**Fig. 1.** HPTLC pattern and immunostaining with MEST-2 of CMH from different sources. Fungal Glc/GalCer were purified by a combination of chromatography in DEAE-Sephadex and silica gel 60, and preparative HPTLC in solvent B. About 3 µg of each purified CMH were applied on HPTLC plate and developed in solvent B. (A) Stained with orcinol/H2SO4 and (B) immunostaining with MEST-2. Lane 1, GlcCer from mycelium forms of P. brasiliensis; lane 2, GlcCer from yeast forms P. brasiliensis; lane 3, GalCer from mycelium of A. fumigatus; lane 4, GlcCer from mycelium of A. fumigatus; lane 5, GlcCer from mycelium forms of A. niger; lane 6, GlcCer from mycelium forms of H. capsulatum; lane 7, GlcCer from yeast forms of H. capsulatum; lane 8, GlcCer from mycelium forms of S. schenckii; lane 9, GlcCer from yeast forms of S. schenckii; lane 10, GalCer from yeast forms of S. schenckii; lane 11, GlcCer from yeast forms of C. albicans; lane 12, GlcCer from yeast forms of C. neoformans; lane 13, GlcCer from yeast forms of C. laurentii; lane 14, GlcCer from yeast forms of C. albicus; lane 15, GlcCer from Gaucher’s spleen; lane 16, GlcCer (2-OH fatty acid) from soybean.

**Fig. 2.** Binding specificity of monoclonal antibody MEST-2 to GlcCer from various sources. GlcCer, GalCer and CDH (first well 0.5 µg) were serially double diluted in ethanol and adsorbed on a 96-well plates. MEST-2 (100 µl) was added and incubated overnight at 4°C. The amount of antibody bound to GSLs was determined by incubation with rabbit anti-mouse IgG (2 h) and 10⁵ c.p.m. of ¹²⁵I-labeled protein A in 1% BSA. GlcCer from yeast (solid squares) and mycelium (open squares) forms of P. brasiliensis; (open triangles) GlcCer from soybean; (open circles) GlcCer from human Gaucher’s spleen; (inverted triangles) GlcCer from bovine buttermilk; (crossed squares) galactosylcerebrosides from bovine brain; (crossed circles) CDH and CTH from human erythrocytes.
Monoclonal antibody directed to glucosylceramide

about 80% in the presence of phosphatidylcholine, at the same molar concentration of CMH, at 37°C. Conversely, presence of equimolar amount of ergosterol decreased the MEST-2 reactivity to fungi and soybean glucosylceramide to about 40% and 70%, respectively. No significant reactivity was detected with GlcCer from Gaucher’s spleen and with GalCer from Aspergillus fumigatus at different temperatures even in the presence of different molar ratios of phosphatidylcholine or mixtures containing ergosterol and phosphatidylcholine.

Inhibition assays of antibody binding using methyl-glycosides

The importance of the sugar moiety in the reactivity of MEST-2 with GlcCer was confirmed by an inhibition assay using different methyl-glycosides. The assay was carried out by solid-phase RIA on 96-well plates coated with highly purified fungi GlcCer, using as inhibitors several methyl-α- and β-D-glycosides (glucopyranoside, galactopyranoside, and mannopyranoside) in concentrations ranging from 293 nM to 75 mM. Only methyl-β-D-glucoside at a concentration of 37.5 mM, was able to inhibit about 85% of the binding of MEST-2 to GlcCer isolated from the different fungi (Figure 5).

Indirect immunofluorescence with MEST-2

As shown in Figure 6, indirect immunofluorescence studies with MEST-2 showed clear staining of the surface of yeast forms of P. brasiliensis and H. capsulatum. Weak staining of the surface of the mycelial forms of P. brasiliensis and H. capsulatum and of hyphae of A. fumigatus was also observed. In A. fumigatus, it a remarkable difference was detected in the fluorescence pattern with MEST-2 between hyphae and the conidiophore, that is, hyphae presented only a weak fluorescence whereas the conidial heads were highly reactive with MEST-2. Also, as expected, yeast forms of S. schenckii gave strong immunofluorescence with MEST-2, whereas mycelium forms reacted only weakly with the antibody (data not shown). On the other hand, yeast forms of C. albicans, C. albidos, C. laurentii, and C. neoformans showed no immunofluorescence with MEST-2, although GlcCer isolated from these fungi was reactive with MEST-2 by HPTLC immunostaining or solid-phase RIA.

Discussion

An increasing number of studies has pointed to possible roles for cerebrosides in biological processes of various organisms. For example, physiological activities described for GlcCer include stimulation of mitogenesis in mammalian cells (Marchell et al., 1998) and induction of fruiting body formation in fungi such as Schizophyllum commune and Coprinus cinereus (Kawai and Ikeda, 1983, 1985; Kawai, 1989; Mizushina et al., 1998). A MAb directed to GlcCer containing 2-hydroxy fatty acid represents a relevant reagent for immunochemical and biological studies of GlcCer in different fungi.

The fine specificity of MEST-2 was assessed by inhibition assays using five different methyl-glycosides, and only β-D-Glc was able to inhibit, by about 85%, MEST-2 binding to GlcCer of P. brasiliensis, H. capsulatum, S. schenckii, A. fumigatus, and C. albicans. However, the striking lack of reactivity of MEST-2 with Gaucher’s spleen GlcCer led us to analyze other structural features involved in the recognition of fungal GlcCer.
by this antibody. Structures such as the 9-methyl group and $\Delta^8$ unsaturation of the sphingosine are likely to be embedded in the cell membrane, and they presumably do not have any influence in the interaction between MEST-2 and fungal GlcCer. Also, the $\Delta^3$ unsaturation of C-18 2-hydroxy fatty acid, described in fungal cerebrosides, seems not to be relevant to MEST-2 recognition of fungal GlcCer, since no difference was observed in MEST-2 reactivity with GlcCer isolated from yeast or mycelium forms of *P. brasiliensis*, which present 15% and 55% of $\Delta^3$ unsaturation, respectively (Toledo et al., 1999).

One likely key feature for the MEST-2 reactivity with fungal and plant GlcCer appeared to be the 2-hydroxy group of the fatty acid, which is absent in Gaucher’s GlcCer (Koerner et al., 1999) and in commercial buttermilk GlcCer (Matreya, Inc.). In contrast, by $^{+}$ESI-MS, the ceramide present in the soybean GlcCer standard employed for this study is >95% N-2′-hydroxyhexadecanoyl-(4E,8E)-sphinga-4,8-dienine (data not shown), essentially as described by Shibuya et al. (1990) and Sullards et al. (2000). Detailed mass spectrometric characterization of all fungal cerebrosides used in this study, as their Na+ and/or Li+ adducts, by both $^{+}$ESI-MS and $^{+}$ESI-MS/collision induced decomposition-mass spectrometry (CID-MS), have been or will be described elsewhere (Levery et al., 1998, 2000; Toledo et al., 1999, and unpublished observations). These studies confirm that the major ceramides in all cases consist of (>95%) N-2′-hydroxyalkanoyl- or N-2′-hydroxy-(E)-3-alkenoyl-(or both)-(4E,8E)-9-methyl-sphinga-4,8-diene. The possibility of the 2-hydroxy group being the primary epitope recognized by MEST-2 was discarded by the fact that this antibody does not react with GalCer of *A. fumigatus* which presents 2-hydroxy fatty acids (Toledo et al., 1999). Therefore, the minimum epitope required for optimum binding of MEST-2 with GlcCer would comprise primarily the $\beta$-D-Glc residue and a secondary site represented by the 2-hydroxy group of the fatty acid, which present a direct relationship to MEST-2 binding capacity, as determined by solid-phase RIA using different

Fig. 4. Binding specificity of monoclonal antibody MEST-2 to cerebrosides (CMH) immobilized in lipid mixed monolayer and effect of temperature; 25 µl of 32 µM solutions of phosphatidylcholine (PC), ergosterol (ERG) and phosphatidylcholine mixed to ergosterol (PC+ERG) were serially double diluted on a 96-well plates, in presence 25 µl of different cerebrosides (16 µM): (A) GlcCer from *P. brasiliensis* yeast forms; (B) GlcCer from soybean; (C) GlcCer from Gaucher’s spleen; and (D) GalCer from *A. fumigatus*. MEST-2 (100 µl) was added to each well and incubated for 2 h at different temperatures, 4°C (open); 24°C (light shadow); and 37°C (heavy shadow). The amount of antibody bound to CMH was determined by incubation with rabbit anti-mouse IgG (2 h) and $^{[125]}$-labeled protein A. (C) Control, well adsorbed only with CMH (0.4 nmol); 1, CMH (0.4 nmol) + lipids (0.1 nmol); 2, CMH (0.4 nmol) + lipids (0.2 nmol); 3, CMH (0.4 nmol) + lipids (0.4 nmol); and 4, CMH (0.4 nmol) + lipids (0.8 nmol).
proportions of glucosylceramides containing hydroxy and non-
hydroxy fatty acids.

The immunofluorescence profile of mycelium forms of
P. brasiliensis with MEST-2 suggested that the organization
of GlcCer or cell wall is different from that observed in yeast
forms. Similarly, in hyphae of A. fumigatus, MEST-2 reacted
almost exclusively with the head of the conidiophore. In
general, the immunofluorescence pattern of the various yeast
forms with MEST-2 studied in this work is consistent with a
model where GlcCer is distributed evenly throughout the fungi
surface in yeast forms, and accessible to MEST-2, whereas in
mycelium forms the presentation of GlcCer is not favorable to

interact with this antibody. Since both forms of dimorphic
fungi present CMH in similar amounts, as previously reported
(Toledo et al., 1995, 1999, 2000), the strong reactivity of
MEST-2 with CMH of yeast forms possibly is associated with
the cell wall organization and morphological transition of
mycelium to yeast forms (San-Blas, 1985). The results obtained
by solid-phase RIA using mixtures of GlcCer of different
sources, ergosterol and phosphatidylcholine, indicate that the
antibody binding to glucosylceramide containing 2-hydroxy
fatty acids is favored by a specific conformation induced by
phosphatidylcholine or a mixture of phosphatidylcholine and
ergosterol. On the other hand, the reactivity of MEST-2 to
fungi and soybean GlcCer decreased significantly in the presence
of ergosterol alone. It should be noted that different temperatures
(4°C to 37°C) did not alter the specificity of MEST-2. These
results are consistent with the notion that the surface phos-
lipids and fungal sterols may modulate the MEST-2 reactivity/
accessibility, but they do not alter the MEST-2 specificity.

Fig. 5. Inhibition of monoclonal antibody MEST-2 binding to GlcCer from
different sources by methyl-glycosides. 96-well plates were adsorbed with
different fungal CMH: (A) GlcCer from yeast forms of P. brasiliensis;
(B) GlcCer from mycelium forms of P. brasiliensis; (C) GlcCer from yeast
forms of S. schenckii; (D) GlcCer from yeast forms of H. capsulatum;
(E) GlcCer from C. albicans and (F) GlcCer from A. fumigatus. Different
methyl-glycosides (first well 150 mM) were serially double diluted with PBS
and preincubated with MEST-2. The inhibition assay was carried out as
described in Materials and methods. The effects of the methyl-glycosides are
expressed as percentages of inhibition of MEST-2 binding to the GlcCer. Solid
squares, methyl-β-D-glucopyranoside; open squares, methyl-α-D-
glucopyranoside; inverted triangles, methyl-β-D-galactopyranoside; open
circles, methyl-α-D-galactopyranoside; and diamonds, methyl-α-D-
mannopyranoside.

Fig. 6. Indirect immunofluorescence of different fungi with MAb MEST-2.
(A) and (B) yeast forms of P. brasiliensis; (C) and (D), mycelium forms of
P. brasiliensis; (E) and (F) mycelium forms of A. fumigatus; (G) and (H) yeast
forms of H. capsulatum; (I) and (J) mycelium forms of H. capsulatum. (A, C,
E, G, and I), fluorescence. (B, D, F, H, and J), phase contrast.
MAbs MEST-1 (an IgG3 monoclonal antibody directed to terminal residues of galactofuranose linked in β1→3 or β1→6; see Suzuki et al., 1997) and MEST-2 should be effective reagents in studies for the distribution of glycosinositol-phosphoryl ceramides and GlcCer in the plasma membrane/cell wall of yeast and mycelium forms of different fungi by electron microscopy, thus providing more accurate information regarding GSLs expression in the processes of growth, morphological transition, and infectivity.

Materials and methods

Fungal isolates and growth conditions

Paracoccidioides brasiliensis strain Pb18 was provided by Dr. C. Fava-Netto, Sao Paulo, Brazil; strain B339 was provided by Dr. A. Restrepo-Moreno, Medellin, Colombia. Yeast and mycelium forms of the P. brasiliensis were grown at 37°C and 25°C, respectively, in PGY (peptone 5 g/l, glucose 15 g/l, yeast extract 5 g/l) using 2.5 l Fernbach flasks in a 9197) mycelia were grown in malt extract (20 g/l) at 25°C. Histoplasma capsulatum strain 496 from human pulmonary lesion and Sporothrix schenckii strain 65 from Human foot cutaneous lesion, were kindly provided by Dr. O. Gompertz, Sao Paulo, SP, Brazil. Yeast and mycelium forms of both fungi were grown in brain heart infusion (BHI) (37 g/l) at 37°C and 25°C, respectively. The yeast form of Candida albicans, strain ATCC 10231 was grown in modified PGY supplemented with malt extract (peptone 5 g/l, glucose 10 g/l, yeast extract 3 g/l, and malt extract 3 g/l) at 28°C. Yeast forms of Cryptococcus neoformans, strain 512 VFSB; Cryptococcus laurentii, strain 40043; and Cryptococcus albidus, strain 40077 were grown in PGY at 25°C. After 1 week both yeast and mycelium forms of the various fungi were inactivated with 0.1% of thimerosal, and after an additional 48 h the fungi were collected by filtration on Whatman no. 1 filter paper, except for yeast forms of S. schenckii and H. capsulatum, which were harvested by centrifugation at 5000 r.p.m. for 20 min.

Extraction and purification of glycosphingolipids (GSLs)

GSLs were extracted by homogenizing yeast or mycelium forms (~30 g) in an Omni-mixer (Sorvall Inc. Wilmington, DE), three times with 200 ml of isopropanol/hexane/water (IHW, 55:20:25, v/v/v, upper phase discarded), and twice with 200 ml of chlorofom/methanol (CM, 2:1, v/v). The five extracts were pooled, dried on rotary evaporator, dialyzed against water, and lyophilized. The CMHs used in this study were purified by a methodology avoiding acetylation/Florisil chromatography/deacetylation (Saito and Hakomori, 1971), in order to allow immunochemical analysis of fungal CMHs as close as possible to their native composition and state. Neutral and acidic GSLs were separated in a DEAE-Sephardex A-25 column as described by Yu and Ledeen (1972). The neutral GSL fraction was further purified from other contaminants by Folch’s partitioning (Folch-Pi et al., 1951), and chromatography on silica gel 60 using a step-wise gradient of chloroform/methanol from 9:1 to 1:1 (v/v) (Sweetley, 1969). Fractions containing CMHs, were assessed by HPTLC on silica gel 60 plates (E. Merck, Darmstadt, Germany) using solvent A: chloroform/methanol/CaCl₂ 0.02% (60:40:9; v/v/v), and stained with orcinol/H₂SO₄. For preparative-scale HPTLC separated GSL bands were visualized under UV after spraying with primulin 0.01% in 80% aqueous acetone (Straus et al., 1993). Pure GlcCer was obtained from CMH fraction (separated from GalCer and other contaminants) by using solvent B: chlorofom/methanol/NH₄OH/NH₄Cl 0.83% (50:36.8:6:7.2; v/v/v/v). GSLs were isolated from silica gel scraped from the plates by repeated sonication in IHW, as described previously (Straus et al., 1997). Galactocerebrosides were purchased from Sigma (St. Louis, MO). Ergosterol, GlcCer from Human Gaucher’s spleen, soybean GlcCer, bovine buttermilk GlcCer from Matreya, Inc. (Pleasant Gap, PA).

Production of hybridomas

About 300 ng of GlcCer purified from mycelium forms of P. brasiliensis was dissolved in 1.5 ml of distilled water and mixed with 1.2 mg of acid-treated, heat-inactivated Salmonella minnesota. Aliquots (100 µl) of this suspension containing 20 µg of the antigen were used to immunize 6-week-old BALB/c mice, by i.v. route, through the caudal vein once a week, over 4 weeks. After a rest period of 30 days, the immune response was boosted with 200 µl of the immunogenic complex. Three days later, the mice were sacrificed and their spleen removed. The lymphocytes were fused with NS-1 myeloma cells and placed in 96-well plates. Hybrids secreting immunoglobulins reacting with GlcCer were detected by solid-phase RIA. Only clones showing strong reactivity with GlcCer of mycelium forms of P. brasiliensis were cloned by limited dilution as described previously (Takahashi et al., 1988; Straus et al., 1992).

Binding assay

Various GSLs were adsorbed on 96-well plates (Falcon Micro-test III flexible assay plates, Oxnard, CA). Solutions (25 µl/well, 300 ng/first well) in ethanol of different GSLs were serially diluted, dried at 37°C and the wells blocked with 1% bovine serum albumin (BSA) in 0.01 M phosphate-buffered saline (PBS), pH 7.2 (200 µl) for 2 h, and sequentially incubated with MAb MEST-2 (100 µl) overnight at 4°C, rabbit anti-mouse IgG (50 µl) for 2 h, and with 50 µl of 125I-labeled protein A in PBS with 1% of BSA (about 10⁶ c.p.m./well) for 1 h. The amount of MAB MEST-2 bound to GlcCer was determined by measuring the radioactivity in each well in a gamma counter (Suzuki et al., 1997).

MEST-2 reactivity to mixtures containing different molar proportion of non-hydroxylated fatty acid glucosylceramide (Gaucher’s spleen) and 2-hydroxy fatty acid glucosylceramide (P. brasiliensis) was carried out as described before, where 25 µl of glucosylceramide from Gaucher or P. brasiliensis (0.32 mg/ml) or mixtures containing 2-hydroxy f.a. glucosylcera-

MEST-2 binding assay to immobilized CMH in a mixed lipids monolayer

Twenty-five microliters of solutions of phosphatidylycholine, ergosterol, and a mixture of phosphatidylycholine with ergosterol in ethanol (32 µM/first well) were serially diluted on 96-well
plates. Immediately, 25 µl of a CMH solution at 16 µM were added to each well and mixed. The plates were dried at 37°C and the wells blocked with 1% BSA in PBS (200 µl) for 2 h, and incubated with MAb MEST-2 (100 µl) for 2 h at 4°C, 25°C, and 37°C. The amount of antibody bound to CMH was determined as described in Binding assay.

**Methyl-glycosides**

Methyl-α- and β-D-galactopyranoside, methyl-α- and β-D-glucopyranoside and methyl-α-D-mannopyranoside were purchased from Sigma.

**Inhibition of antibody binding by different methyl glycosides**

Initially, 75 µl of a 150 mM solution of different methyl glycosides were serially diluted with PBS in a 96-well plate. Each methyl glycoside solution was incubated with 75 µl of MEST-2 at room temperature (Straus et al., 1993). After 2 h, aliquots of 100 µl were taken and incubated overnight at 4°C in 96-well plates pre-coated with GlcCer (40 ng/well) essentially as described under Binding assay.

**High performance thin layer chromatography (HPTLC) immunostaining**

CMHs purified from different fungi were separated by HPTLC, and the immunostaining of the plates was performed by the procedure of Magnani et al. (1980). GSLs (3 µg) were separated in solvent A or B, after development, the plates were dried soaked in 0.5% polymethacrylate in hexane, dried, and separated in solvent A or B, after development, the plates were immunostained with rabbit anti-mouse IgG and 125I-labeled protein A (2 × 107 c.p.m./50 ml of BSA/PBS).

**Indirect immunofluorescence**

Fungi were fixed with 1% formaldehyde in PBS for 10 min. Cells were washed and resuspended in 1 ml of PBS, and 20 µl of the solution was added to a coverslip pretreated with poly-lysine 0.1% during 1 h. Air-dried preparations were soaked for 1 h in PBS containing 5% of BSA, and incubated subsequently with culture supernatant of MEST-2 MAb (2 h), biotin-conjugated goat anti-mouse IgG (1 h), and avidin-conjugated fluorescein (1 h). After each incubation the coverslips were washed five times with PBS. The coverslips were examined with an epifluorescence microscope (Straus et al., 1993). Control experiments for each fungus were carried out in the presence of an irrelevant monoclonal antibody (BM-8, IgG2a isotype), and no fluorescence was observed.

**Electrospray ionization mass spectrometry (ESI-MS) and tandem collision-induced dissociation mass spectrometry (ESI-MS/CID-MS)**

Mass spectrometric analysis was performed on a Sciex API-III (Concord, Ontario, Canada) tandem quadrupole instrument equipped with a standard IonSpray source; samples were introduced into the source by direct infusion of dilute solutions in 100% methanol. Molecular ion profiles of glyco- and galactocerebrosides samples used in this study were obtained by positive ion mode ESI-MS, either as their Na+ adducts, under conditions described by Toledo et al. (1999, 2000), and/or as their Li+ adducts as described by Levery et al. (2000). Analysis of individual unit-mass resolved cerebroside components by positive ion mode ESI-MS/CID-MS was carried out on the Li+ molecular ion adducts as described by Levery et al. (2000).

**Acknowledgments**

This work was supported by FAPESP, CNPq, and PRONEX (Brazil; M.S.T., E.S., A.H.S., and H.K.T.); a Glycoscience Research Award from Neose Technologies, Inc. (S.B.L.); and the National Institutes of Health Resource Center for Biomedical Complex Carbohydrates (NIH #5 P41 RR05351, S.B.L.).

**Abbreviations**

BSA, bovine serum albumin; BHI, brain heart infusion; CID-MS, tandem collision-induced dissociation mass spectrometry; CM, chloroform/methanol; CMH, monohexosyl ceramide; ERG, ergosterol; *ESI-MS, positive electrospray ionization mass spectrometry; f.a., fatty acid; GalCer, galactosylceramide; GlcCer, glucosylceramide; GSL, glycosphingolipid; HPTLC, high performance thin layer chromatography; IHW, isopropanol/hexane/water; MAB monoclonal antibody; PBS, 0.01 M phosphate-buffered saline; PC, phosphatidylcholine; PGY, peptone, glucose, yeast extract; RIA, radioimmunoassay.

**References**


Saito, T., and Hakomori, S. (1971) Quantitative isolation of total glycosphin-
San-Blas, G. (1985) *Paracoccidioides brasiliensis*: cell wall glucans, patho-
Szaniszlo, P.J., and Harris, J.L. (eds.), *Fungal Dimorphism*. Plenum Press,
New York, pp. 93–120.
Shibuya, H., Kawashima, K., Sakagami, M., Kawanishi, H., Shimomura, M.,
Chemical structures and ionophoretic activities of soya-cerebrosides I and
Straus, A.H., Travassos, L.R., and Takahashi, H.K. (1992) A monoclonal anti-
Straus, A.H., Levery, S.B., Jasiulionis, M.G., Salyan, M.E., Steele, S.I.,
glycosphingolipids from amastigote forms of *Leishmania (L.) amazonesis*.
Immunogenicity and role in parasite binding and invasion of macrophages.
Straus, A.H., Valero, V.B., Taktizowa, C.M., Levery, S.B., Toledo, M.S.,
Suzuki, E., Salyan, M.E.K., Hakomori, S., Barberi, C.L., and Takahashi, H.K.
(1997) Glycosphingolipid antigens from *Leishmania (L.) amazonensis*
amastigotes. Binding of anti-sphingolipid monoclonal antibodies in
determination of soya bean and wheat glucosylcereamides by tandem mass
monoclonal antibody directed to terminal residue of β-galactofuranose of a
glycolipid antigen isolated from *Paracoccidioides brasiliensis*: cross
reactivity with *Leishmania major* and *Trypanosoma cruzi*. *Glycobiology*,
**7**, 463–468.
monoclonal antibody directed to Tn antigen (tumor associated α-N-acetyl-
galactosaminyi epitope) that does not cross-react with blood group A antigen.
phic expression of cerebrosides in the mycopathogen *Sporothrix schenckii*.
Toledo, M.S., Levery, S.B., Straus, A.H., Suzuki, E., Momany, M., Glushka, J.,
lipids from mycopathogens: factors correlating with expression of 2-hydroxy
fatty acyl (E)-Δ3-unsaturation in cerebrosides of *Paracoccidioides brasiliensis*
lipids from *Paracoccidioides brasiliensis*. Isolation of a galactofuranose-
containing glycolipid reactive with sera of patients with paracoccidioido-