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Effects of Histoplasmin M Antigen Chemical and Enzymatic Deglycosylation on Cross-Reactivity in the Enzyme-Linked Immunoelectrotransfer Blot Method

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The enzyme-linked immunoelectrotransfer blot (EITB) method was evaluated as a suitable method for detecting antibodies against M antigen of *Histoplasma capsulatum* by use of both glycosylated and deglycosylated M protein of histoplasmin (HMIN). Sera from patients with histoplasmosis, paracoccidioidomycosis, blastomycosis, coccidioidomycosis, and aspergillosis were tested by the EITB with glycosylated M protein of HMIN. This assay demonstrated 100% sensitivity with histoplasmosis serum samples, all of which reacted with the 94-kDa glycoprotein (M antigen). Although the EITB is highly sensitive, it is not specific for histoplasmosis when glycosylated M protein is used as an antigen. A total of 81% of paracoccidioidomycosis, 25% of blastomycosis, 33% of coccidioidomycosis, 73% of aspergillosis, and 16% of tuberculosis serum samples cross-reacted with M protein of HMIN and yielded patterns indistinguishable from those obtained with histoplasmosis serum samples. The EITB reactions with both untreated M antigen and M antigen altered by periodate oxidation or by deglycosylation with endoglycosidases were compared. Cross-reactions with heterologous sera in the EITB could be attributed to periodate-sensitive carbohydrate epitopes, as reflected by the increase in the test specificity from 46.1 to 91.2% after periodate treatment of M protein. The EITB for the detection of antibodies to M antigen is a potential diagnostic test for histoplasmosis, provided that periodate-treated M protein is used as an antigen.

Histoplasmosis is a systemic fungal disease caused by *Histoplasma capsulatum* var. *capsulatum*, a dimorphic fungus which grows in the mycelial form at room temperature and in the yeast form at 37°C or in infected tissues. Histoplasmosis is worldwide in distribution, and the clinical presentation mimics those of other serious disease entities (16). It is recognized as a common and serious opportunistic infection in AIDS patients (17, 18). The definitive diagnosis of a *Histoplasma* infection requires the isolation of the organism, which is time-consuming and lacking in sensitivity (3).

Serologic evidence is the prime diagnostic indicator of histoplasmosis. Such evidence may be obtained with several serologic tests (7, 16), especially the immunodiffusion test, which detects precipitins against the species-specific H and M antigens found in histoplasmin (HMIN) (7). In contrast, cross-reactions frequently occur in other methods, such as complement fixation and enzyme immunoassay with sera from patients with other deep mycoses, such as paracoccidioidomycosis, blastomycosis, and coccidioidomycosis (7, 8).

Although the H and M antigens are pluripotent glycoproteins, eliciting both humoral immunity and cell-mediated immunity, M is considered the immunodominant antigen of *H. capsulatum*, since antibodies against M antigen are first to arise in infection and are more commonly present during all phases of histoplasmosis (8, 19). M antigen, even after purification by chromatographic procedures to remove other proteins and extraneous C antigen, cross-reacts in more sensitive antibody detection methods, such as enzyme immunoassay (2, 14, 22). Previous research from our laboratories indicated that this cross-reactivity results from covalent glycosidic epitopes present in M antigen (14, 20).

In the present research, we evaluated the enzyme-linked immunoelectrotransfer blot (EITB) method as a suitable method for detecting antibodies against M antigen and, in particular, the effect of chemical and enzymatic deglycosylation of M antigen as a means of increasing diagnostic specificity.

MATERIALS AND METHODS

Antigens. HMIN was prepared from mycelium-form cultures of *H. capsulatum* CDC6623 (ATCC 26320) as described by Zancopé-Oliveira et al. (20). HMIN was passed through 0.45-μm-pore-size membranes (Nalgene Co., Rochester, N.Y.), concentrated 20-fold in a hollow-fiber ultrafiltration device with an HIP10-43 cartridge (nominal M_r, 10,000; Amicon Corp., Danvers, Mass.), and dialyzed against phosphate-buffered saline (PBS) (0.01 M; pH 7.2). H and M antigens were purified from HMIN by cation-exchange chromatography on CM Sepharose CL-6B columns (Pharmacia-LKB, Piscataway, N.J.). Antigens were eluted from the columns with a step salt gradient consisting of 0.05, 0.5, and 1 M NaCl in 25 mM citrate buffer (pH 3.5). Fractions were pooled according to the antigens present, as detected by rocket immunoelectrophoresis with *H. capsulatum*-positive control rabbit antiserum (lot 81-0176), rabbit anti-M serum (lot 84-0086), and rabbit anti-H serum (lot 84-0085) from the Biological Products Inventory, Centers for Disease Control and Prevention, and concentrated

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10-fold by ultrafiltration. After dialysis against PBS (0.01 M; pH 7.2), each pool received as a preservative a final concentration of 0.05% Merthiolate. Aliquots of the pool rich in M antigen (S-II) were stored frozen (20).

Enzymatic deglycosylation. S-II samples, rich in M antigen, were deglycosylated by the following enzymatic methods: (i) endo-β-*N*-acetylglucosaminidase H (Endo H; Boehringer Mannheim Corp., Indianapolis, Ind.) in 50 mM sodium acetate buffer (pH 5.5), with 50 mU of enzyme per ml of antigen (1 mg/ml); (ii) *N*-glycosidase F (PNGase F; Boehringer Mannheim) at 2 U/ml in S-II fractions (300 μg of protein) first denatured by being boiled for 5 min in 0.5% sodium dodecyl sulfate (SDS) and then suspended in a final concentration of 200 mM sodium phosphate (pH 8.6) containing 1.25% Nonidet P-40; and (iii) endo-α-*N*-acetylgalactosaminidase (*O*-glycanase; Genzyme Corp., Cambridge, Mass.). One hundred micrograms of SDS-denatured S-II was incubated at 37°C for 60 min in a solution containing 10 mM calcium acetate, 20 mM sodium cacodylate buffer (pH 6.0), 1.25% Nonidet P-40, and 1 U of neuraminidase (Genzyme) per ml, and then 50 mU of *O*-glycanase per ml was added to the solution. All of the enzymatic digestions were carried out for 18 h at 37°C in accordance with the manufacturers' instructions.

Periodate oxidation of antigen. An aliquot of S-II (protein concentration, 4,000 μg/ml) was oxidized with 100 mM sodium *meta*-periodate (Sigma) for 18 h at 4°C in the dark. The reaction was stopped by adding an equimolar amount of glycerol and then, after 15 min, 100 mM sodium borohydride (Sigma). After incubation for 2 h at 4°C, the reaction mixture was dialyzed against distilled water at 4°C for 24 h.

MAbs. Ascitic fluids containing murine monoclonal antibodies (MAbs) EC2-EC7 and CA1-CB4, specific for the M and C antigens (14), respectively, were obtained from the Emerging Bacterial and Mycotic Diseases Branch, Centers for Disease Control and Prevention, and used to evaluate deglycosylation procedures.

Sera. A total of 71 serum specimens from Brazilian patients were obtained from the Medical Mycology Laboratory Serum Bank, Hospital Evandro Chagas, Fundação Oswaldo Cruz, Rio de Janeiro. The majority of the serum samples were from patients with proven mycotic diseases (19 histoplasmosis, 16 paracoccidioidomycosis, 15 aspergillosis, and 6 coccidioidomycosis cases). All serum samples were tested by immunodiffusion against *H. capsulatum*, *Paracoccidioides brasiliensis*, and *Aspergillus fumigatus* exocellular antigens produced in the Medical Mycology Laboratory, Hospital Evandro Chagas, Fundação Oswaldo Cruz, by standard protocols (11) and against *Coccidioides immitis* immunodiffusion antigen (Immuno-Mycologics, Inc., Norman, Okla.). All patients presented with various clinical forms of the diseases and were either untreated or in different stages of antifungal therapy. The four blastomycosis serum samples were provided by S. F. Hurst, Centers for Disease Control and Prevention. Serum samples from six tuberculosis patients were obtained from clinically diagnosed patients at Hospital Universitário, Universidade Federal do Rio de Janeiro, and the five control serum samples were obtained from healthy individuals on our laboratory staffs.

SDS-PAGE. Antigens were first dissociated by being heated at 100°C for 5 min in 0.125 M Tris-HCl (pH 6.8) containing 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.025% bromophenol blue. SDS-polyacrylamide gel electrophoresis (PAGE) was then performed with 7.5% resolving and 4% stacking gels by use of a Mini-Protean II electrophoresis cell (Bio-Rad Laboratories, Richmond, Calif.). The electrophoresis conditions were 10 mA of constant current for stacking and 30 mA for separation of the proteins.

TABLE 1. EITB reactions of sera obtained from healthy individuals and patients with mycotic diseases and tuberculosis and tested against untreated and deglycosylated M antigen

Human infection	No. of positive serum samples/no. tested with the following M-antigen treatment:				
	None	$NaIO_4$	Endo H	PNGase F	*O*-Glycanase
Histoplasmosis	19/19	13/13	7/7	7/7	7/7
Paracoccidioidomycosis	13/16	2/7	5/5	4/5	4/5
Blastomycosis	1/4	0/4	1/4	1/4	1/4
Coccidioidomycosis	2/6	0/6	2/2	2/2	2/2
Aspergillosis	11/15	0/6	0/2	0/2	0/2
Tuberculosis	1/6	1/6	1/6	1/6	1/6
None	0/5	0/5	0/5	0/5	0/5
Total	47/71	16/47	16/31	15/31	15/31

EITB. Proteins were transferred to 0.2-μm-pore-size nitrocellulose membranes by electrophoresis for 1 h at 400 mA in a Mini Trans-Blot cell (Bio-Rad) containing 25 mM Tris, 192 mM glycine, and methanol (20% [vol/vol]) (pH 8.3) according to Tsang et al. (15). Transfer efficiency was monitored with prestained marker proteins (106 to 18.5 kDa; Bio-Rad). Then, unoccupied binding sites on the nitrocellulose membranes were blocked with 5% nonfat dry milk in 20 mM Tris-HCl–500 mM NaCl–0.2% Tween 20 (pH 7.5) (TTBS) for 30 min. Nitrocellulose membranes were sliced vertically, and the strips were incubated for 60 min at room temperature with serum specimens diluted 1:100 in TTBS containing 5% nonfat dry milk. After each strip was washed in TTBS four times for 20 min each time, a goat anti-human immunoglobulin G–alkaline phosphatase conjugate optimally diluted in TTBS (Sigma) was added, and the strips were incubated as described above. Blot strips were then washed four times for 20 min each time in TTBS and incubated with a substrate solution consisting of 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitroblue tetrazolium in alkaline phosphatase buffer (100 mM Tris-HCl [pH 9.5], 100 mM NaCl, 50 mM $MgCl_2 \cdot 6H_2O$) (5). After color development, the strips were rinsed exhaustively in distilled water.

RESULTS

Chemical and enzymatic deglycosylation of M antigen. M antigen and its products from periodate oxidation and from enzymatic deglycosylation with Endo H, PNGase F, and *O*-glycanase were analyzed by SDS-PAGE and EITB. When blots were probed with EC2-EC7, a murine MAb specific for M antigen (14), strong reactions were observed with the untreated 94-kDa glycoprotein (M antigen), with an 88-kDa protein from periodate-treated M antigen, and with the 80-, 74-, 88-kDa proteins resulting from Endo H, PNGase F, and *O*-glycanase deglycosylations, respectively. Blots probed with MAb CA1-CB4 (C antigen specific) showed that periodate oxidation abolished the CA1-CB4 epitopes present in M antigen, although the integrity of the protein and its serological reactivity were retained.

EITB. The EITB with homologous histoplasmosis sera and the EITB heterologous sera from patients with diseases other than histoplasmosis were done with untreated M antigen and deglycosylated M antigen (Table 1). Deglycosylated M antigen was tested only with those heterologous sera showing cross-reactivity with untreated M antigen. Antibodies to the M

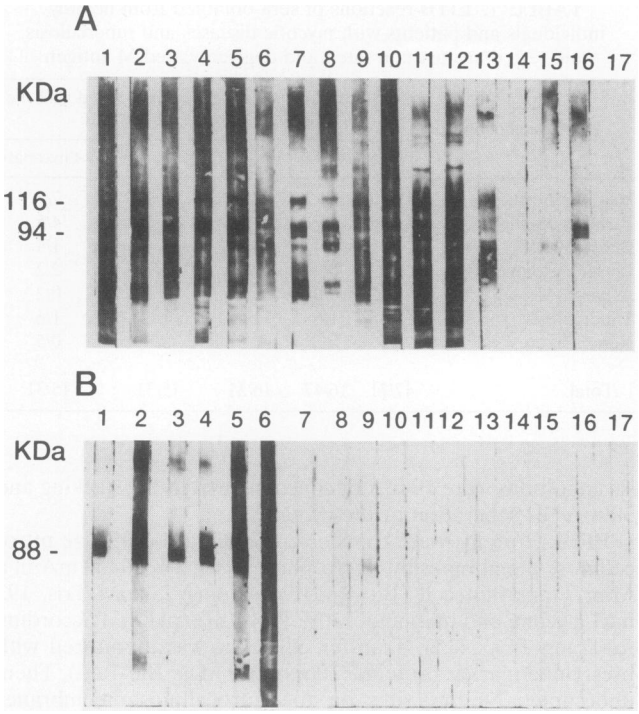

FIG. 1. Representative EITB reactions for untreated M antigen (A) and periodate-treated M antigen (B) probed with sera from the following patients: lanes 1 to 5, histoplasmosis; 6 to 10, paracoccidioidomycosis; 11 and 12, coccidioidomycosis; 13 and 14, aspergillosis; and 15 and 16, tuberculosis. Lane 17, healthy individual.

antigen were detected in all histoplasmosis patient sera probed with glycosylated or deglycosylated M antigen, indicating that the EITB with either type of M antigen is 100% sensitive for proven histoplasmosis. However, in the EITB with untreated M antigen, there was extensive cross-reactivity with sera from patients with heterologous diseases, particularly with sera from patients with other mycoses (Fig. 1). A total of 81% of paracoccidioidomycosis, 25% of blastomycosis, 33% of coccidioidomycosis, 73% of aspergillosis, and 17% of tuberculosis serum samples cross-reacted in the EITB with untreated M antigen (Table 1). Consequently, the EITB has a low specificity (46.1%) when performed with glycosylated M antigen.

To evaluate the possible role of the carbohydrate epitopes of M antigen in the EITB reactions, we tested sera against periodate-treated M antigen and against M antigen deglycosylated by endoglycosidases. A total of 47 serum samples, 13 from histoplasmosis patients and 34 from nonhistoplasmosis patients, were tested against M antigen oxidized by $NaIO_4$. Of these 47 serum samples, 31 were also tested against M antigen digested by endoglycosidases. Although the homologous reactions were somewhat weaker than the ones observed when untreated M antigen was used, all sera from histoplasmosis patients remained positive when tested against chemically or enzymatically deglycosylated M antigen, thus yielding 100% sensitivity. However, most of the heterologous sera did not react with periodate-treated M antigen (Fig. 1), thereby increasing the test specificity to 91.2%. Weak cross-reactivity persisted in two paracoccidioidomycosis serum samples and in serum from a tuberculosis patient (Fig. 1B, lanes 6, 9, and 16). On the other hand, the EITB with M antigen deglycosylated with different enzymes had an average specificity of only 62.5%.

DISCUSSION

The detection of antibodies against the M glycoprotein of HMIN in the serum of histoplasmosis patients is very useful in the diagnosis of this systemic mycosis. An important limitation, however, is misleading positive results in patients with other diseases caused by organisms that cross-react with *H. capsulatum*. This cross-reaction has been attributed to the C carbohydrate, a heat-stable galactomannan shared by the major genera of systemic dimorphic fungi (6, 10, 13), although precipitins against C antigen are rare in humans (6).

Different procedures for purifying HMIN antigens, including methods based on size or charge, have been reported and a number of reactive components have been found (1, 4, 12, 14, 20). Some procedures were able to remove the C antigen from the antigenic mixture (4, 20), leaving the immunodominant M antigen depleted of extraneous C antigen. However, this M antigen still showed cross-reactivity when probed with polyclonal antibodies and MAbs in the EITB, thus leading to the hypothesis that this antigen contains both species-specific protein epitopes and cross-reactive glycosidic epitopes (14, 20). The latter epitopes present an obstacle to diagnostic specificity in sensitive primary binding immunoassays. To confirm this hypothesis, the M antigen was treated with sodium *meta*-periodate, which cleaves vicinal hydroxyl groups on monosaccharides, or was digested with endoglycosidases, which cleave oligosaccharides from glycoproteins and polysaccharides. The oxidation of M antigen with 100 mM periodate eliminated cross-reactions in the EITB with MAb CA1-CB4, specific for the polysaccharide C antigen, without altering its reactions with the M-antigen-specific MAb EC2-EC7 (21).

The results obtained with sera from patients with histoplasmosis and other mycoses in EITBs with glycosylated and deglycosylated M antigens also demonstrated that the glycosidic determinants are responsible for the observed cross-reactivity. The EITB reactions of the histoplasmosis sera were hardly affected by periodate oxidation of the M antigen, indicating that the conditions for periodate oxidation were not harmful to the important protein epitopes. However, the cross-reactions exhibited by most of the heterologous sera were abolished by periodate oxidation of the M antigen. Thus, many of the cross-reactive serum samples appeared to recognize periodate-sensitive carbohydrate epitopes, as reflected by the increase in the test specificity from 46.1 to 91.2%.

Cross-reactivity was observed in two serum samples from paracoccidioidomycosis patients and in one serum sample from a tuberculosis patient when the EITB was performed with periodate-treated M antigen. The possibility of intercurrent and subclinical histoplasmosis cannot be excluded in these patients, since it is well known that histoplasmosis may coexist with other granulomatous diseases of the lungs, including tuberculosis and other mycoses (3, 16). Another possibility is that the antibodies arose from past exposure of the patients to *H. capsulatum*. Most cases of histoplasmosis (95%) are inapparent, subclinical, or completely benign, and anti-M-antigen antibodies can remain elevated for several years following recovery. Such residual precipitins may lead to a misdiagnosis of histoplasmosis in patients with illnesses caused by other infectious agents (16) or even in healthy residents of areas in which histoplasmosis is endemic (2). The high prevalence of histoplasmosis, paracoccidioidomycosis, and tuberculosis in Rio de Janeiro State could explain the presence of three cross-reactive serum samples in our study.

An immunochemical analysis of glycosidic moieties in the H and M glycoproteins showed that the M antigen was only partially deglycosylated by enzymatic treatment, since its elec-

trophoretic mobility had changed but its ability to bind to concanavalin A had been retained (21). This finding might explain the persistence of low-level cross-reactivity in some heterologous sera tested against endoglycosidase-treated M antigen in the EITB. Perhaps higher concentrations of endoglycosidases or even longer exposures to these enzymes could result in more complete digestion of glycopeptide bonds in the M glycoprotein. It is possible that glycopeptide linkages that are occluded or not susceptible to the endoglycosidases that we employed exist.

The EITB for antibodies to the M antigen of *H. capsulatum* has the potential for use as a diagnostic test for histoplasmosis (9). Periodate oxidation, under the conditions used, destroyed cross-reactive carbohydrate epitopes while leaving the protein core of the M glycoprotein apparently intact. Consequently, sera from histoplasmosis patients were able to recognize *H. capsulatum*-specific peptide epitopes within the M antigen.

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