Evaluation of a Western Blot Test in an Outbreak of Acute Pulmonary Histoplasmosis

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A western blot (WB) test was evaluated for detection of antibodies against native glycosylated and chemically deglycosylated M and H antigens of Histoplasma capsulatum in serum obtained from patients during the acute phase of pulmonary histoplasmosis that occurred during an outbreak. Of 275 serum samples tested by immunodiffusion and complement fixation (CF) samples from 40 patients affected during this outbreak and from 37 negative controls were tested by WB test. A group of patients whose sera were negative for CF antibodies and precipitins early in the acute stage of histoplasmosis but who all seroconverted during convalescence 6 weeks later were tested with the WB test. Antibodies against untreated H and M antigens were detected at a 1:100 dilution by WB test in 45% of the 20 acute-phase serum samples and in all 20 of the convalescent-phase specimens. The WB test's specificity for acute-phase specimens increased to 90% (18 of 20 specimens) when H and M antigens were treated by periodate oxidation to inactivate susceptible carbohydrate epitopes. When native glycosylated antigens were used in the WB test, positive reactions were observed in negative control serum specimens (3 of 37 specimens; 8%) and in serum specimens obtained from asymptomatic persons screened as part of the outbreak investigation (13 of 20 specimens; 65%). These positive reactions were also attributed to glycosidic epitopes since the specificity of the WB test increased from 78 to 100% when periodate-treated H and M antigens were used. WB test with deglycosylated H and M antigens of histoplasmin provides a rapid, sensitive, and specific test to diagnose acute pulmonary histoplasmosis before precipitins can be detected.

Histoplasmosis is a systemic fungal disease caused by Histoplasma capsulatum var. capsulatum, a dimorphic soil-dwelling mold which converts to a yeast form after aerosolized microconidia are inhaled. H. capsulatum is found worldwide in soil mixed with avian or bat excrement (15). The spectrum of clinical illness includes asymptomatic, acute pulmonary (the most common form), chronic pulmonary, and disseminated extra-pulmonary infection. Diagnosis is complicated because the clinical presentation of pulmonary histoplasmosis mimics those of other serious diseases including tuberculosis (12, 15). Because H. capsulatum frequently fails to grow from clinical specimens (4, 5), serologic evidence is the mainstay of diagnosis of pulmonary histoplasmosis in the absence of a positive culture, but current methods have shortcomings (15): lack of diagnostic specificity, less than optimal sensitivity early in the acute stage of disease, or reduced sensitivity in disease restricted to the lungs. Often, serologic testing requires acute- and convalescent-phase specimens, resulting in significant delays in diagnosis. The complement fixation (CF) test lacks specificity because whole yeast forms and histoplasmin (HMIN) used as antigens share epitopes with other dimorphic fungal pathogens (5). HMIN is the filtrate of H. capsulatum mycelium-form cultures grown in broth medium. In a comparison of the evolution of positive serologic reactions after exposure to H. capsulatum in immunocompetent patients, CF tests with yeast antigen became positive gradually: 7% of the patients had a positive test 1 week after symptoms appeared, and the percentage increased to 66% by 2 weeks and to 77% by 4 weeks (3). The immunodiffusion (ID) test for precipitins against HMIN is very specific, but in the same study, the M precipitin was detected in 50% of patients 4 weeks after symptoms appeared (3). An alternative approach to immunodiagnosis is to detect heat-stable H. capsulatum polysaccharide (HPA) antigen in urine or serum with a radioimmunoassay (5, 14, 16). This method is well suited for diagnosis of disseminated histoplasmosis, particularly in AIDS patients, but is not as useful in the diagnosis of nondisseminated disease; in a recent study 37% of such patients had a positive HPA test (5). Skin tests with HMIN also have limitations as a diagnostic test for histoplasmosis, particularly in areas of endemicity, where the prevalence of reactions among asymptomatic exposed residents is high. Because of impaired immunity, skin tests are negative in 25 to 50% of patients with more severe forms of histoplasmosis (13).

HMIN contains H. capsulatum species-specific H and M antigens as well as C antigen, a heat-stable galactomannan polysaccharide. C antigen is found in the major genera of primary, systemic, dimorphic fungal pathogens. HMIN also contains less well-characterized antigens that cross-react with other fungi (7, 9). Although definitive structural analysis has not been conducted on the glycosidic moieties of H and M antigens, it is hypothesized that they share epitopes with C antigen. M antigen, a 94-kDa glycoprotein, is an immunodominant antigen of H. capsulatum because it is species specific, and M precipitins are usually the first to arise upon seroconversion (8, 10). Tests with increased sensitivity are needed to improve the early serodiagnosis of acute pulmonary histoplasmosis, but a change from precipitin tests to primary binding assays requires the removal of cross-reactive epitopes and extraneous antigens to maintain or improve specificity. We demonstrated that deglycosylation of M antigen increased the specificity of the enzyme-linked immunoelectrotransfer blot (or Western blot...
outbreak (6). HMIN was produced, and H and M antigens were chromatographed were performed on serum specimens obtained from all patients during the public health laboratory in Illinois, an area of endemicity for histoplasmosis. In addition, 37 negative control serum specimens were obtained from a serum samples but who seroconverted 6 weeks later. Single serum specimens patients, for whom precipitins and CF antibodies were absent in acute-phase evaluated by WB. A total of 40 samples were obtained from 20 symptomatic patients, for whom precipitins and CF antibodies were absent in acute-phase samples but who seroconverted 6 weeks later. Single serum specimens were also evaluated by WB. A total of 40 samples were obtained from 20 symptomatic patients, for whom precipitins and CF antibodies were absent in acute-phase serum samples but who seroconverted 6 weeks later. Single serum specimens were also obtained from 20 persons with no clinical or serologic evidence of the disease. In addition, 37 negative control serum specimens were obtained from a public health laboratory in Illinois, an area of endemicity for histoplasmosis. Serologic tests and antigens. CF and ID tests to detect antibodies to HMIN were performed on serum specimens obtained from all patients during the outbreak (6). HMIN was produced, and H and M antigens were chromatographed purified (17, 18). Chemical deglycosylation was achieved by adding sodium peroxide (NaIO4) to 2-ml aliquots of purified antigens (fraction S-II) (17, 18) to a final concentration of 5% nonfat dry milk in 20 mM Tris·HCl·500 mM NaCl·0·2% Tween 20 (pH 7·5) (TTBS). As controls, rabbit anti-HMIN antibodies (Centers for Disease Control and Prevention) and murine monoclonal antibodies against M antigen (10) and H antigen were used. The monoclonal anti-H-antigen antibodies were a gift from Sandra Bragg, Centers for Disease Control and Prevention. Membranes were sliced vertically, and strips were incubated for 60 min at room temperature with serum specimens diluted 1/100 in TTBS containing 5% nonfat dry milk. Strips were washed in TTBS four times for 20 min each; then goat anti-human immunoglobulin G (IgG)- or anti-human IgM-alkaline phosphatase conjugates (Sigma Chemical Co., St. Louis, Mo.) diluted in TTBS were added nonfat milk. Strips were washed in TTBS four times for 20 min each; then goat anti-human immunoglobulin G (IgG)- or anti-human IgM-alkaline phosphatase conjugates (Sigma Chemical Co., St. Louis, Mo.) diluted in TTBS were added and incubated as described above. Blot strips then were washed and incubated with substrate solution consisting of 5-bromo-4-chloro-3-indolylphosphate (BCIP; 15 mg/ml in dimethylformamide [DMF]) and nitroblue tetrazolium (NBT; 30 mg/ml in 70% aqueous DMF). Substrate stock solutions were diluted 1/100 before use in Tris/NaCl buffer (100 mM Tris·HCl [pH 9·5], 100 mM NaCl, 50 mM MgCl2). After color development strips were rinsed exhaustively in deionized water. RESULTS The results of the WB test reactions of untreated and periodate-treated purified H and M glycoproteins observed with acute- and convalescent-phase serum samples from persons with confirmed pulmonary histoplasmosis and controls are depicted in Fig. 1. Table 1 summarizes and compares results obtained with CF, ID, and WB tests. All 20 acute-phase serum samples from symptomatic patients were negative for H and/or M precipitins on the ID test. A positive CF test was considered to be a reaction at a 1:32 dilution. Samples from 2 of the 20 symptomatic persons showed acute reactions on the CF test at a dilution of 1:16, which rose to 1:32 and 1:512 during convalescence. When untreated (deglycosylated) H. capsulatum antigens were used in the WB test (Fig. 1, bottom), 9 (45%) of the 20 acute-phase serum samples obtained from symptomatic patients showed positive results: two patients had positive tests for anti-H-antigen antibodies only (patients 9 and 20), and seven patients’ WB tests were positive for antibodies against H and M antigens. One patient (patient 3) had only a trace of [WB] test (18, 19). The WB test for antibodies against the M antigen of H. capsulatum became a useful diagnostic test once periodate-treated M glycoprotein was introduced as the antigen because 100% sensitivity was observed with this preparation and test specificity increased from 46·1 to 91·2% (18). In this research report we present the results of a study evaluating the WB test with deglycosylated H and M antigens for the diagnosis of acute pulmonary histoplasmosis. MATERIALS AND METHODS Study population. An outbreak of acute pulmonary histoplasmosis occurred in a correctional facility in Virginia in June 1994 (6), and a total of 151 cases of acute pulmonary histoplasmosis were identified. Acute-phase serum specimens were obtained from patients within a mean interval of 7 days (range, 2 to 10 days) after onset of symptoms, and convalescent-phase serum specimens were obtained 6 weeks later. Confirmation of the diagnosis was obtained by culture and immunohistopathologic studies of lung biopsies performed for two patients, by observation of CF antibody titers of 1·32 or greater, and/or by detection of an M-precipitin band, H-precipitin band, or both in the ID test (7, 8). Of a total of 275 persons from this facility whose sera were obtained during the outbreak and tested by ID and CF tests, 60 serum specimens from 40 persons were also evaluated by WB. A total of 40 samples were obtained from 20 symptomatic patients, for whom precipitins and CF antibodies were absent in acute-phase serum samples but who seroconverted 6 weeks later. Single serum specimens were also obtained from 20 persons with no clinical or serologic evidence of the disease. In addition, 37 negative control serum specimens were obtained from patients whose sera were obtained during the outbreak and tested by ID and CF tests at a 1:32 dilution. Samples from 2 of the 20 symptomatic persons showed acute reactions on the CF test at a dilution of 1:16, which rose to 1:32 and 1:512 during convalescence. When untreated (deglycosylated) H. capsulatum antigens were used in the WB test (Fig. 1, bottom), 9 (45%) of the 20 acute-phase serum samples obtained from symptomatic patients showed positive results: two patients had positive tests for anti-H-antigen antibodies only (patients 9 and 20), and seven patients’ WB tests were positive for antibodies against H and M antigens. One patient (patient 3) had only a trace of acrylamide stacking gels in an electrophoresis cell (Mini-Protean II; Bio-Rad Laboratories, Richmond, Calif.). Each gel was charged with 58 µg of HMIN protein in a 7·3-cm-width well, containing 2·700 rocket electrophoresis units of H antigen and 2·600 units of M antigen (17). Electrophoresis was conducted at 10 mA constant current for stacking and at 20 mA for protein separation. Gel contents were electrotransferred to nitrocellulose membranes in a Mini-Trans-Blot cell (Bio-Rad) containing transfer buffer containing 25 mM Tris·HCl, 192 mM glycine, and methanol (20% [vol/vol]; pH 8·3) and operated at 400 mA for 1 h (11). Free binding sites in the membranes were blocked by incubation for 30 min in 5% (wt/vol) nonfat dry milk in 20 mM Tris·HCl·500 mM NaCl·0·2% Tween 20 (pH 7·5) (TTBS). As controls, rabbit anti-HMIN antibodies (Centers for Disease Control and Prevention) and murine monoclonal antibodies against M antigen (10) and H antigen were used. The monoclonal anti-H-antigen antibodies were a gift from Sandra Bragg, Centers for Disease Control and Prevention. Membranes were sliced vertically, and strips were incubated for 60 min at room temperature with serum specimens diluted 1/100 in TTBS containing 5% nonfat dry milk. Strips were washed in TTBS four times for 20 min each; then goat anti-human immunoglobulin G (IgG)- or anti-human IgM-alkaline phosphatase conjugates (Sigma Chemical Co., St. Louis, Mo.) diluted in TTBS were added and incubated as described above. Blot strips then were washed and incubated with substrate solution consisting of 5-bromo-4-chloro-3-indolylphosphate (BCIP; 15 mg/ml in dimethylformamide [DMF]) and nitroblue tetrazolium (NBT; 30 mg/ml in 70% aqueous DMF). Substrate stock solutions were diluted 1/100 before use in Tris/NaCl buffer (100 mM Tris·HCl [pH 9·5], 100 mM NaCl, 50 mM MgCl2). After color development strips were rinsed exhaustively in deionized water.
detectable antibodies in the acute-phase sample. All 20 convalescent-phase samples were reactive. When periodate-treated (deglycosylated) semipurified HMIN was used in the WB test (Fig. 1, top), sensitivity increased; antibodies reactive with the H and M antigens were detected in 18 (90%) of serum samples from the symptomatic patients sampled at the acute phase and in all 20 (100%) of the convalescent-phase specimens from the symptomatic patients. Variable intensities were obtained for blot strip reactions with acute-phase sera from the symptomatic patients blotted against periodate-treated antigens (Fig. 1). Ten patients' sera evoked equivalent H and M antibody reactions (patients 1, 2, 4, 5, 6, 10, 11, 16, 18, and 19), and four patients had relatively stronger anti-M-antigen reactions (patients 3, 7, 12, and 15), whereas stronger anti-H-antigen binding was observed in three other patients (patients 8, 9, and 17). Only one patient had a single detectable anti-H-antigen reaction (patient 20). These reactions were observed when the anti-IgG conjugate was used. When the samples in the same tests were probed with anti-IgM conjugate all samples were negative.

When blot strips containing glycosylated antigens were used to test 20 seronegative samples from asymptomatic persons sampled during the outbreak and samples from 37 negative controls from Illinois, false-positive results were observed. A total of 13 (65%) serum specimens from the asymptomatic outbreak-related group and 3 (8%) from the 37 negative Illinois controls were positive, which yielded a specificity of 78% with the WB test with glycosylated H and M proteins (Table 2). This reactivity was abolished and specificity was improved to 100% in both control groups when the samples were tested by WB test against NaIO₄-treated antigens.

**DISCUSSION**

The WB test with purified, periodate-treated, HMIN antigens had a high degree (90%) of sensitivity in the early diagnosis of acute pulmonary histoplasmosis in this evaluation, while maintaining excellent specificity. Both H and M antigens were desirable to use on blot strips because patients' early acute-phase reactions to these antigens varied in intensity. Reactions with convalescent-phase sera were predictably stronger, with anti-M-antigen reactions often more intense, even though equivalent antigenic H and M proteins were applied to gels. Although the WB tests were conducted retrospectively, this test would have assisted in making the diagnosis during the early stages of acute disease.

Radioimmunoassays and enzyme immunoassays (EIAs) with HMIN as the antigenic complex have been attempted as more sensitive screening tests for antibodies in histoplasmosis patients, but they showed reduced specificity (1, 13). Efforts to improve the specificity of these assays have been made, and we developed a chromatographic method to purify HMIN antigens (17, 18). Purified M antigen was depleted of extraneous C antigen, although there were persistent cross-reactive covalent glycosidic epitopes that were an obstacle to diagnostic specificity in more sensitive primary binding immunooassays like EIA or WB test (10, 17, 18). Mild periodate oxidation of M antigen inactivated or removed cross-reactive carbohydrate moieties while retaining the protein’s integrity, as measured by its molecular mass and antigenicity (10, 19). This step eliminated cross-reactions in the WB probed with sera from patients with aspergillosis, coccidioidomycosis, paracoccidioidomycosis, and tuberculosis (19). The effect of chemical deglycosylation by NaIO₄ of M antigen on its increased effectiveness in EIA was demonstrated in different studies; in an EIA-inhibition the test sensitivity was increased from 58.3 to 88% (1); in the WB test 100% sensitivity was observed, while the test specificity was improved from 46.1 to 91.2% (19). In the present study, test specificity increased from 78 to 100%. Inactivation of carbohydrate epitopes led to increased WB test sensitivity, possibly because key protein epitopes became more exposed as a result of this change in the M antigen structure.

| Type of human serum | WB test | ID test HMIN antigen | CF test with yeast-form and mycelium-form antigen |
|---------------------|---------|-----------------------|-----------------------------------------------|
| No. positive/total  | No. positive/total | No. positive/total | No. positive/total |
| % positive          | % positive | % positive | % positive |
| Acute phase         | 9/20 | 45       | 18/20 | 90       | 0/20 | 0       | 0/20 | 0       |
| Convalescent phase  | 20/20 | 100     | 20/20 | 100     | 20/20 | 100    | 20/20 | 100    |
| Seronegative asymptomatic | 13/20 | 65    | 0/20 | 0       | 0/20 | 0       | 0/20 | 0       |
| Negative controls   | 3/37 | 8       | 0/37 | 0       | 0/37 | 0       | ND   | ND      |

**TABLE 2.** Sensitivities and specificities of WB tests (with glycosylated and deglycosylated antigens) and CF and ID tests

| Test                  | Sensitivity, % | Specificity, % |
|-----------------------|----------------|---------------|
|                       | Acute          | Convalescent  |
| ID                    | 0              | 100           | 100          |
| CF                    | 0              | 100           | 100          |
| WB, glycosylated antigens | 45          | 100           | 78           |
| WB, deglycosylated antigens | 90          | 100           | 100          |

*Sensitivity calculation was based on data for 20 patients sampled in the early acute and convalescent stages during a histoplasmosis outbreak.

*Specificity calculation was based on data for 20 asymptomatic exposed persons and 37 negative controls from an Illinois endemic area.*
The WB test meets the requirements of a good diagnostic test for acute pulmonary histoplasmosis and is the first such WB test for a primary systemic mycosis to be evaluated with serum samples obtained during an outbreak investigation. The major impediment to the utility of the WB test, a lack of antigens free of cross-reactive extraneous common fungal polysaccharides or covalent cross-reactive glycosidic epitopes, was overcome by a combination of purifying HMIN to remove protein and galactomannan impurities, and the inactivation (by mild periodate oxidation) of residual galactomannan and of protein and galactomannan impurities, and the inactivation (by mild periodate oxidation) of residual galactomannan and of covalent glycosidic epitopes present in M and H antigens. The availability of premanufactured blot strips would contribute quality-controlled reagents and conceivably would increase the use of serology in diagnostic laboratories.

The WB test with NaIO₄-treated H and M antigens is a highly sensitive method for detecting antibodies in serum from persons with early acute pulmonary histoplasmosis. The advantages of the WB test are identification of some cases early in infection, before seroconversion can be detected by CF and ID, a high degree of disease specificity, applicability to serum specimens with anticomplementary activity, and potential for premanufactured blot strips to simplify test performance, thereby avoiding the need for a special apparatus. Current efforts to further refine reagents and tests for the immunodiagnosis of histoplasmosis are centered on the functional expression of the cloned gene encoding M antigen (20).

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