Histoplasmosis is an important systemic fungal infection, particularly among immunocompromised individuals living or travelling in areas of endemicity, who, without antifungal therapy, may develop a progressive disseminated fatal infection. For such patients, the detection of antibody responses by immunodiffusion or complement fixation test is of limited use. In contrast, the detection of Histoplasma capsulatum circulating antigens may provide a more practical approach to the rapid diagnosis of the disease. Accordingly, an inhibition enzyme-linked immunosorbent assay (ELISA) for the detection of a 69- to 70-kDa H. capsulatum-specific determinant and incorporating a species-specific murine monoclonal antibody was developed. With sera from patients with different forms of the disease (n = 35), the overall sensitivity of the test was found to be 71.4%, while the specificity was found to be 98% with normal human sera from areas of endemicity (n = 44) and 85.4% with sera from patients with other chronic fungal or bacterial infections (n = 48). This novel, highly specific ELISA provides a significant addition to the existing diagnostic tests for the detection of histoplasmosis.

Histoplasma capsulatum var. capsulatum is a pathogenic dimorphic fungus of worldwide distribution which is endemic to the Ohio and Mississippi river valleys in the United States and to certain regions of Central and South America (22). Histoplasmosis is the most common systemic mycosis in North America, causing progressive disease particularly in immunocompromised individuals (10, 21), and it is either the first or second most prevalent systemic mycosis in areas of Central and South America (1, 18, 19). The clinical spectrum of histoplasmosis ranges from asymptomatic infection to severe disseminated forms of disease (3, 10, 24). The majority of the exposed population have a mild self-limiting or subclinical form of infection (9); however, infants, the elderly, and immunocompromised individuals may contract acute symptomatic or progressive life-threatening disseminated forms of the disease (2, 9, 10, 30). The definitive diagnosis of histoplasmosis relies on the isolation of H. capsulatum var. capsulatum by culture from clinical specimens (3, 23). Microscopic identification of yeast cells in tissue is possible in approximately 60% of the cases of disseminated disease (50 to 75% of bone marrow samples and 10 to 50% of peripheral blood samples) (3, 23). However, microscopic identification of the fungus in localized forms of infection is often extremely difficult or may require invasive procedures (3, 22). In addition, differentiating H. capsulatum var. capsulatum yeast cells from Candida glabrata or Pneumocystis carinii (21) cells or from Penicillium marneffei (12) cells may constitute a problem. Isolation of H. capsulatum by culture provides a higher sensitivity, and reported isolation rates vary from 58% for localized forms to 80% for disseminated forms (3, 21). However, cultures often require a 2- to 4-week incubation period before the identification of the fungus is possible (21).

Serological methods offer a rapid alternative to microbiological techniques, and the detection of antibodies to H. capsulatum by immunodiffusion and complement fixation test is often used (3, 15, 16). However, anti-H. capsulatum antibody titers remain elevated months or even years after successful therapy, and as a result it may not be possible to differentiate between subacute or inactive infections, chronic active forms, and relapses (3, 9, 10); the latter are particularly frequent among AIDS patients (24, 25, 34). In addition, false-negative results are often seen since antibody titers may be low or even absent in immunocompromised patients (26) and those with chronic disseminated disease. Finally, false-positive results from antibody detection testing can also arise as a result of serological cross-reactivity with other fungi such as Blastomyces dermatitidis, Coccidioides immitis, Paracoccidioides brasiliensis, Cryptococcus neoformans, Aspergillus spp., and Candida spp. (17); such results are also possible with Mycobacterium tuberculosis (17, 27). However, it is also possible that tuberculosis patients living in areas of endemicity had also been exposed to H. capsulatum var. capsulatum, which could explain their seropositivity.

A more rational approach to the diagnosis of histoplasmosis and the follow-up of patients may be the detection of H. capsulatum var. capsulatum antigen in body fluids. Polysaccharide antigen has been detected successfully by radioimmunoassay (RIA) (6, 21, 25, 33), and RIA can be used to detect antigen in urine, serum, and cerebrospinal and bronchoalveolar lavage fluids, particularly from patients with the disseminated forms of the disease (28, 33). The detection of polysaccharide antigen is more sensitive with urine than with sera, and antigenuria has been detected in 92, 21, and 39% of samples from patients with disseminated, chronic pulmonary, and self-limited forms of the disease, respectively (33). Antigenuria decreases concurrently with effective therapy, making it feasible to monitor treatment responses (21, 25, 28, 32). However, there are problems associated with the use of RIA, notably relating to cross-reactivity with B. dermatitidis (8, 21), Coccidioides immitis, Paracoccidioides brasiliensis, and Penicillium marneffei (31) antigens. Currently, the RIA uses rabbit polyclonal antisera, although monoclonal antibodies have been used in an attempt to replace...
the polyclonal sera. However, the results arising from this modification were not reported as being satisfactory (5).

In this paper we report on the development of a different H. capsulatum var. capsulatum antigen detection test which utilizes a species-specific murine monoclonal antibody (MAb) to a 69- to 70-kDa antigenic determinant in an inhibition ELISA. The test was assessed retrospectively by testing 35 sera from patients with different forms of histoplasmosis, in whom the diagnosis had been established by microbiological and/or serological techniques. Sera from patients with paracoccidioidomycosis, aspergillosis, cryptococcosis, sporotrichosis, and tuberculosis, as well as sera from healthy individuals living in areas of endemity, were used as controls to assess specificity.

MATERIALS AND METHODS

Fungal isolates and antigen preparations. The following pathogenic fungi were cultured and harvested as previously described (4, 11): H. capsulatum var. capsulatum, CIB (Corporacion para Investigaciones Biologicas, Medellin, Colombia); Hc1980, H. capsulatum var. capsulatum CIB Hc11265, Paracoccidioides brasiliensis CIB 339, B. dermatitidis, NCPF (National Collection of Pathogenic Fungi, Colindale, London, United Kingdom) 4076, Sporothrix schenckii Sist (St. John's Institute of Dermatology, London, United Kingdom); and Penicillium marneffei NCPF 4160. Cytoplasmic yast (CYA) and culture filtrate antigens were prepared as described elsewhere (4, 11).

MAb production. The modification by Hamilton et al. (4, 11) of the Matthew and Eichmann protocol (14) was used. Briefly, 10 BALB/c mice were inoculated intraperitoneally (i.p.) with 100 μl of an equal mixture of Paracoccidioides brasiliensis CIB 339 CYA and B. dermatitidis NCPF 4076 CYA, made up 1:1 in Freund's incomplete adjuvant (Difco, East Molesey, Surrey, United Kingdom) and at a final protein concentration of 50 μg per 100 μl. Cyclodextrinphosphamide (Sigma, Poole, United Kingdom) at a dose of 50 mg per kg of body weight was given i.p. to each mouse 15 min, and 24 and 48 h later. Ten days later mice were inoculated i.p. with 100 μl of an equal mixture of H. capsulatum var. capsulatum CIB Hc11980 CYA and Paracoccidioides brasiliensis CIB Hc11265 culture filtrate antigens made up 1:1 in Freund's incomplete adjuvant at a final concentration of 50 μg per 100 μl. A second dose of 100 μl of the H. capsulatum antigen mixture was given again i.p. the day following. After a further 3 days, mice were bled and sera were used in an enzyme-linked immunosorbent assay (ELISA) test (4, 11) to determine the mouse with the highest differential response to H. capsulatum antigens. This mouse received a further intravenous dose of 50 μg of the H. capsulatum antigen mixture in sterile phosphate-buffered saline (PBS) and was used for the fusion protocol 3 days later. Spleen cells from the donor mouse were fused with Sp2/0 murine myeloma cells, and hybridomas were produced as previously described (4).

Characterization of MAb s. The specificities of MAb s were assessed by ELISA and Western blotting using antigens from a range of pathogenic fungi as previ- ously described (4). MAb culture supernatants were concentrated 100-fold by ammonium sulfate precipitation, and MAbs were subclassed with a Serotec protocol 3 days later. Spleen cells from the donor mouse were fused with Sp2/0 murine myeloma cells, and hybridomas were produced as previously described (4).

Inhibition ELISA. The inhibition ELISA was performed with both serum and culture filtrate antigens. Polystyrene 96-round-well microtiter plates (NUNC, A/S, Kamstrup, Denmark) in PBS-Tween and 1% (wt/vol) BSA, and the plate was washed three times in PBS-Tween and blocked by incubation with 20% (wt/vol) BSA per well made up in PBS-Tween for 2 h at 37°C. After the washings in PBS-Tween, 100-μl aliquots of each serum or urine from patients and controls made up 1:2 in the respective diluting buffers were added. Subsequ- ently, 100-μl aliquots of MAb HIC made up 1:1,600 in diluting buffer were added to the wells containing patient samples. Plates were mixed on a shaker for 30 min at room temperature and subsequently incubated overnight at 4°C.

Inhibition standard. An inhibition standard curve was constructed for each plate by adding 100-μl aliquots of a 1:1,600 solution of MAb HIC to 100 μl of pooled NHS (or pooled NHU) containing decreasing concentrations of H. capsulatum CIB Hc1980 CYA (from 60 μg to 4 μg per ml) made up 1:2 in diluting buffer. NHS and NHU made up 1:2 in diluting buffer (100 μl) were used as negative controls. Plates were mixed and incubated overnight as described above.

Testing of all the standards, samples, and controls was performed in duplicate.

Reaction plate. Each well of 96-well microtiter plates (Maxisorp; NUNC A/S) was coated with 0.5 μg of H. capsulatum CIB Hc1980 CYA in 0.06 M carbonate buffer (pH 9.6). Plates were left to stand at room temperature for 30 min and then were incubated overnight at 4°C. After overnight incubation, plates were washed three times in PBS-Tween and blocked by incubation with 200 μl of 1% (wt/vol) BSA per well in PBS-Tween for 1 h at 37°C; after three further washes, 100-μl aliquots of the contents of each well in the inhibition plate (containing a mixture of MAb HIC-circulating antigen complexes and free MAb HIC) were transferred to the respective wells in the reaction plate and allowed to react for 2 h at 37°C. Plates were then washed three times, each well was coated with 100 μl of a 1:2,500 dilution of goat anti-mouse immunoglobulin G (IgG) (Paj, Jackson, West Grove, Pa.) in PBS-Tween and 1% (wt/vol) BSA, and the plate was incubated for a further hour at 37°C. Plates were then washed, and 100 μl of α-phenylenediamine (0.2 mg per ml)-0.005% H2O2 in 0.1 M sodium citrate buffer (pH 5.0) per well was used as the enzyme substrate. Plates were incubated for 15 min in the dark, and the reaction was stopped with 0.4 N H2SO4 (100 μl per well). OD490 was then measured on an ELISA plate reader (Bio-Rad, Hemel Hempstead, United Kingdom). The cutoff point was established as the upper limit of the 90% least significant difference confidence interval of the OD490 values obtained with the negative controls (NHS or NHU; see below).

Subjects. Serum and urine samples were all taken from patients diagnosed between January 1991 and October 1996 at the Mycology Laboratory of the Corporacion para Investigaciones Biologicas). Only samples from those patients with a confirmed diagnosis whose full clinical notes were available were included in the study. A total of 35 serum and 16 urine specimens from patients with different clinical forms of histoplasmosis (9, 29) were used (see Table 1). The mean age of these patients was 27.25 years (standard deviation [SD], 5 years); two-thirds (68.6%) were males (male/female ratio, 2.2:1). However, all patients presenting with the disseminated form and AIDS were males; these patients had a mean age of 30.36 years (SD, 6.93 years).

Sera from patients with tuberculosis, together with sera and some urine from a range of patients with other systemic mycoses were also tested (Table 1). Serum (n = 48) and urine (n = 20) samples from healthy volunteers living in areas in which histoplasmosis is endemic were used as negative controls.

Statistical analysis. Statistical analysis was performed with Statgraphics Plus, release 2.0, Statgraphics Corp., Rockville, Md.). The inhibition standard curves were constructed in duplicate in at least four independent assays. A regression model was constructed by using the reciprocal values of antigen concentrations and the values of OD490 obtained. Comparisons were done by the one-way analysis of variance. Intergroup comparisons were performed by the multiple-range test with the least significant difference (90%).

| TABLE 1. Clinical classification of histoplasmosis patients and patients with other infectious diseases included in this study |
| Patient group | No. of: |
| | Sera | Urine samples |
| Histoplasmosis | 35<sup>a</sup> | 16<sup>b</sup> |
| Acute | 9 | |
| Disseminated | 8 | |
| Disseminated and AIDS | 11 | |
| Chronic pulmonary | 7 | |
| Paracoccidioidomycosis | 10 | 11 |
| Aspergillosis | 10 | 0 |
| Cryptococcosis | 10 | 0 |
| Sporotrichosis | 9 | 0 |
| Tuberculosis | 9 | 0 |
| NHU | 44 | 16 |

Total | 127<sup>a</sup> | 43<sup>b</sup> |

<sup>a</sup> Total number of sera from histoplasmosis patients.

<sup>b</sup> Total number of urine samples from patients with histoplasmosis.
RESULTS

MAb production. After successive subclonings, a panel of six different hybridoma lines specific to \textit{H. capsulatum}, which included MAb H1C, were produced. Figures 1 and 2 demonstrate the reactivities by ELISA and by Western blotting of MAb H1C culture supernatant against antigen preparations from different dimorphic fungi. As indicated by ELISA, MAb H1C recognizes an antigen which is specific to \textit{H. capsulatum} (Fig. 1); this antigen was found to have a reduced molecular mass of 69 to 70 kDa by Western blotting (Fig. 2). MAb H1C was a member of the IgG1 subclass (data not shown) and was used to develop the inhibition ELISA.

Detection of \textit{H. capsulatum} antigenemia by inhibition ELISA. Figure 3 demonstrates the standard inhibition curve constructed with known quantities of \textit{H. capsulatum} CYA. The correlation coefficient of the curve \((r)\) was 0.9897. The curve was used to determine the \textit{H. capsulatum} antigen concentration in each sample tested. The cutoff point for positivity was fixed as the upper limit of the 90% confidence interval of the readings of the negative controls; this corresponded to an antigen concentration above 1.09 \(\mu\)g per ml.

Overall, 71.4% of the histoplasmosis serum samples had circulating antigen levels above the cutoff point (see Fig. 4), with a mean antigen level of 9.519 \(\mu\)g per ml. Table 2 shows the results observed when samples from histoplasmosis patients were analyzed separately according to the different clinical forms of the disease; 88.9% of the patients with acute pulmonary histoplasmosis had detectable levels of circulating antigen. Similarly, 62.5% of patients with disseminated forms, 72.7% of patients with AIDS and disseminated histoplasmosis, and 57.1% of patients with the chronic pulmonary form had detectable antigenemia. The mean \(\text{OD}_{490}\) obtained with the 44 NHS samples corresponded to an antigen concentration of 0.11 \(\mu\)g per ml (10-fold below that of the cutoff point, 1.10 \(\mu\)g per ml), and only 1 (2.27%) of the NHS samples gave an \(\text{OD}_{490}\) reading above the cutoff point (false positive, corresponding to an antigen concentration of 1.16 \(\mu\)g per ml). Cross-reactivity

FIG. 1. Differential reactivities of decreasing dilutions of culture supernatant of MAb H1C, as determined by ELISA, against \textit{H. capsulatum} CIB Hc1980 CYA (■), \textit{Paracoccidioides brasiliensis} CIB 339 CYA (○), \textit{B. dermatitidis} NCPF 4076 CYA (□), and \textit{S. schenckii} Ss17 CYA (●).

FIG. 2. Reactivities of culture supernatant of MAb H1C, as determined by Western blotting, against antigen preparations from different dimorphic fungi. Lane A, \textit{H. capsulatum} CIB Hc1980 CYA; lane B, \textit{Paracoccidioides brasiliensis} CIB 339 CYA; lane C, \textit{B. dermatitidis} NCPF 4076 CYA; lane D, \textit{S. schenckii} Ss17 CYA; lane E, \textit{Penicillium marneffei} NCPF 4160 CYA.

FIG. 3. Standard inhibition curve for MAb H1C. The curve was constructed from a regression model for absorbances (OD\(_{490}\)) obtained against known inhibitory concentrations of \textit{H. capsulatum} CIB Hc1980 CYA diluted in NHS, as determined by the inhibition ELISA test. The inhibition curve was constructed in duplicate from at least four independent assays. The correlation coefficient \((r)\) was 0.9897.

FIG. 4. Inhibition ELISA for the detection of circulating antigen with MAb H1C in sera from patients with different infectious diseases and from normal controls from areas of endemicity. The dotted line represents the cutoff point equivalent to an antigen concentration of 1.09 \(\mu\)g/ml. Subjects' groups: 0, NHS \((n = 44)\); 1, histoplasmosis patient sera \((n = 35)\); 2, paracoccidioidomycosis patient sera \((n = 10)\); 3, aspergillosis patient sera \((n = 10)\); 4, cryptococcosis patient sera \((n = 19)\); 5, sporotrichosis patient sera \((n = 9)\); 6, tuberculosis patient sera \((n = 9)\).
was seen in a total of 14.6% of the sera from patients with other infectious diseases (Fig. 4). Specifically, it was seen in two paracoccidioidomycosis sera (mean concentration, 2.70 µg per ml), two aspergillosis sera (mean concentration, 14.93 µg per ml), one cryptococcosis serum (concentration, 12.31 µg per ml), and two tuberculosis sera (mean concentration, 10.23 µg per ml). None of nine sporotrichosis sera exhibited cross-reactive levels of antigenuria (mean concentration, 3.04 µg of antigen per ml). In addition, 3 of 16 (18.7%) urine samples from normal subjects from areas of endemicity gave a false-positive reaction (mean concentration, <0.01 µg of antigen per ml). There were no statistically significant differences found when the mean antigenuria values for histoplasmosis and paracoccidioidomycosis patients and those for normal controls were compared.

**DISCUSSION**

Cyclophosphamide has previously been incorporated in standard immunization protocols to produce specific MABs directed against fungal antigens (4, 11). This methodology has now been successfully applied to produce MAB H1C, which recognizes a 69- to 70-kDa species-specific antigenic determinant of *H. capsulatum*. MAB H1C has been used as the basis of a highly specific inhibition ELISA for the detection of antigenemia. Using Western blot analysis of histoplasmosis patient sera, Torres et al. in 1993 described human humoral responses against a 70-kDa *H. capsulatum* antigenic determinant (20). It is possible that this 70-kDa antigen is the same determinant recognized by MAB H1C; ongoing studies involving the purification and characterization of this 69- to 70-kDa antigen may resolve this question.

The overall sensitivity of the inhibition ELISA was 71.4% for all clinical forms of histoplasmosis. Interestingly, detectable circulating antigen levels varied for the different clinical forms of the disease, and sensitivity was highest (at 88.9%) for those patients with acute histoplasmosis. For this group of patients the observation and isolation of the fungus in clinical specimens are rare (3, 9), and antibody detection offers no advantage since antibody titers often become detectable late in the course of infection. Consequently, the high percentage of positivity observed when our antigen test was used for this group of patients, which are often difficult to diagnose, is of particular interest. Sensitivity fell to between 73 and 63% for disseminated disease in the presence or absence, respectively, of concomitant AIDS and was at its lowest (57.1%) in cases of chronic localized disease. These figures compared well with the documented sensitivity of the existing RIA which detects polysaccharide antigen and whose sensitivity varies from 92% for the disseminated forms down to 21% for the chronic pulmonary forms (33).

Perhaps as significant, this novel inhibition ELISA demonstrates high specificity (98%) when NHS from individuals from

**TABLE 2.** Detection of *H. capsulatum* 69- to 70-kDa circulating antigen by inhibition ELISA with MAb H1C in sera from histoplasmosis patients and from patients with other infectious diseases, and in NHS

<table>
<thead>
<tr>
<th>Patient group</th>
<th>No. of sera</th>
<th>% Positive</th>
<th>Mean Aga concn (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>Negativea</td>
<td>Positive</td>
</tr>
<tr>
<td>Acute histoplasmosis</td>
<td>9</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>Disseminated histoplasmosis</td>
<td>8</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Disseminated histoplasmosis and AIDS</td>
<td>11</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>Chronic pulmonary histoplasmosis</td>
<td>7</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Paracoccidioidomycosis</td>
<td>10</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>Aspergillosis</td>
<td>10</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td>Cryptococcosis</td>
<td>10</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>Sporotrichosis</td>
<td>9</td>
<td>9</td>
<td>0</td>
</tr>
<tr>
<td>Tuberculosis</td>
<td>9</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>NHS</td>
<td>44</td>
<td>43</td>
<td>1</td>
</tr>
</tbody>
</table>

a Antigen concentration of <1.10 µg/ml.

*Ag, antigen.*
areas of endemicity is used; this value fell to 86% when testing sera from patients with fungal and bacterial infections which have exhibited serological cross-reactivity in previous studies (17, 27). Some cross-reactivity was detectable with sera from patients with aspergillosis, tuberculosis, paracoccidioidomycosis, and cryptococcosis. However, future studies using larger series of sera will elucidate the extent of cross-reactivity that may be expected when using this test.

It is of note that the existing RIA uses anti-H. capsulatum rabbit polyclonal IgG, both as the capture and the detector antibodies, and therefore variability from different batches of rabbit antisera might be expected. Furthermore, interassay variability when using the RIA has also been reported (25). In contrast, the use of MAb HIC in the inhibition ELISA described in this communication is likely to reduce such variability since MAbs are readily available in unlimited quantities as appropriate and since MAbs do not exhibit batch variability.

It is perhaps surprising that the results of the inhibition ELISA for the quantification of urinary antigen are less promising than those obtained when using sera, with a lower overall sensitivity (44%) and lower specificity (81.3% for NHU from normal hosts). Medicine of Buenos Aires, during 1975–1994. Rev. Inst. Med. Trop. Sao Paulo. 37: 531–535.


