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Solid-Phase Competitive-Binding Radioimmunoassay for Detecting Antibody to the M Antigen of Histoplasmin

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A radioimmunoassay (RIA) was designed and compared with complement fixation and immunodiffusion tests for their relative ability to detect antibodies in sera of histoplasmosis patients. M antigen, purified from histoplasmin, was fixed to microtiter wells as the solid phase, and specific rabbit ^{125}I-labeled anti-M globulin was the source of indicator antibodies. The optimal concentrations for the competitive-binding assay were 1.6 ng per well for M antigen and 650 ng per well for the ^{125}I-labeled anti-M globulin. A panel of sera from 29 histoplasmosis patients and from patients with other mycoses was screened for RIA activity and in complement fixation and immunodiffusion tests that used histoplasmin and *Histoplasma capsulatum* yeast-form antigens. The sera of 22 histoplasmosis patients reacted in the RIA, 21 in the complement fixation, and 16 in the immunodiffusion tests. Sera of patients with other mycotic infections did not react in the RIA, with the exception of those of one blastomycosis patient and one candidiasis patient. The RIA could be modified to quantitate M antigen; as little as 125 pg could be detected. The evaluation of this panel of histoplasmosis patients' sera showed that the RIA was about equivalent in sensitivity to the complement fixation test. Some advantages of the RIA over the complement fixation test were that RIA was less prone to cross-reactions and gave better quantitation of low-titered sera. The RIA was a 1-day test, was not hindered by the anti-complementary activity of some sera, and could be modified to quantitate minute amounts of M antigen.

Complement fixation (CF) and immunodiffusion (ID) tests with histoplasmin, the culture filtrate of the mycelial form of *Histoplasma capsulatum,* have proven valuable in the diagnosis of histoplasmosis (1, 5, 7, 12, 15). In the past few years, the H and M antigens of histoplasmin have been separated and partially purified by gel permeation and ion-exchange chromatography (2).

The M antigen was characterized as a glycoprotein containing galactose, glucose, mannose, and glucosamine. Although antibodies to both H and M antigens may appear as a consequence of infection with *H. capsulatum,* antibodies to M antigen may also appear in sensitized normal persons after skin tests with histoplasmin (5). Of the serological reactions that occur in histoplasmosis patients, the most prevalent antibodies are formed towards the M antigen. This antibody appears early in the acute stage of the illness and may persist for years. Antibodies to the H antigen are not as frequently encountered, titers are rarely as high as those observed with anti-M, and anti-H antibodies decline more rapidly. The H antigen, however, is associated with acute illness and has diagnostic significance.

The CF test, with histoplasmin and yeast-form antigens, detects disease in over 90% of histoplasmosis patients (8). However, some positive CF reactions with these antigens may not be due to infection with *H. capsulatum* but to infections caused by other pathogens (12). Cross-reactions may occur in the CF test with sera of patients with candidiasis, blastomycosis, pulmonary sporotrichosis, cryptococcosis, paracoccidioidomycosis, and coccidioidomycosis. ID tests with unpurified histoplasmin may detect disease in 82 to 90% of the histoplasmosis patients (1, 8). Although the ID test is entirely specific for *H. capsulatum* antibodies (1, 9), it is less sensitive than the CF test.

With the production of the purified M antigen and, most recently, of antiserum specific for the M antigen (3), it appeared that suitable reagents were now available for the development of a more sensitive and specific immune assay for detecting antibodies in histoplasmosis patients. The matter of sensitivity was approached by evaluating radioimmunoassay (RIA) as the test system. A modified RIA, the competitive bind-

ing assay, should directly measure a patient's M antibody level by competing with radiolabeled rabbit anti-M globulin for a limited number of antigenic sites. We hoped that the use of the purified M antigen coupled with a more specific anti-M serum would minimize the cross-reactions that are frequently encountered with sera of patients with heterologous mycoses and would lend itself to more accurate quantitation when low levels of complement-fixing antibody were encountered.

The potential benefits of adapting the RIA for detecting *H. capsulatum*-specific antibodies are that (i) the test, unlike the CF and ID tests, which require overnight incubation, can be performed in 1 day, and (ii) it can be used to test serum specimens that could not be tested by CF because of anti-complementary activity. It may be argued that the purified fraction of histoplasmin is less apt to detect antibodies than unfractionated, presumably polyvalent histoplasmin and that, therefore, a reduction in overall sensitivity might offset the benefit of using radiolabeled globulins. In the absence of any previous information about the reactivity of histoplasmin antigens in the standard RIA, the optimal conditions and combining proportions of purified M antigen and ^{125}I-labeled rabbit anti-M globulins had to be determined. The practicality of this test was then evaluated by using panels of sera from patients with histoplasmosis and other systemic mycoses.

MATERIALS AND METHODS

M antigen. Histoplasmin from *H. capsulatum* 6624 (Center for Disease Control Mycology Division Culture Collection) was concentrated 20 times in dialysis tubing against carboxymethyl-cellulose and was purified by molecular-sieve chromatography by using Sephadex G100. M antigen was separated from H antigen on a diethylaminoethyl (DEAE)-cellulose column with 0.05 M tris(hydroxymethyl)aminomethane buffer (pH 7.0; 2). M antigen at 0.01 ml gave a strong ID and capillary precipitin reaction with specific rabbit antiserum to M antigen, but not with rabbit antiserum to H antigen or to *Blastomyces dermatitidis* yeast-form fractions. Analysis by immunoelectrophoresis (IEP) of this antigen, however, revealed an entity (Bd) that reacted with rabbit antiserum to yeast-form *B. dermatitidis* fractions. In addition, a small amount of non-M antigen (3) was demonstrated. Similarly, the rabbit antiserum to purified M antigen had non-M antibody and antibody to the Bd antigen demonstrated by IEP. The M antigen was stored at 5°C in aqueous solution containing 0.02% NaN_3 and 0.01% sodium ethylmercurithiosalicylate (Merthiolate, Aldrich Chemical Co., Inc., Milwaukee, Wis.) as preservatives, since some bacterial growth occurred in preparations stored in azide alone. The protein content of M antigen was determined with Folin phenol reagent (13).

Rabbit anti-M globulins. Rabbit anti-M serum was supplied by James Green and Knox Harrell, Center for Disease Control, Atlanta, Ga. This antiserum was produced in rabbits immunized with M antigen-containing IEP precipitin arcs (3). Ammonium sulfate precipitation was used to partially purify the rabbit immunoglobulins (4). An equal volume of filtered 70% saturated $(NH_4)_2SO_4$ was slowly added to a 10-ml portion of rabbit antiserum, and the suspension was centrifuged at 20,000 × *g* for 20 min. The precipitate was dissolved in 10 ml of deionized water and a second identical $(NH_4)_2SO_4$ precipitation was carried out. The residue was dissolved in water and diafiltered in a 50-ml magnetically stirred ultrafiltration cell (Amicon Corp., Lexington, Mass.). Filtration was carried out at an N_2 pressure of 34 lb/in^2 for 3 h over a PM 30 membrane (Amicon) against 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 8.2) containing 0.11 M NaCl, which was the buffer for subsequent column chromatography. After 100 ml of filtrate had passed through the membrane, the concentrated globulin solution was applied to a DEAE-Sephadex A50 column. A bed volume of 30 ml of DEAE-Sephadex in a column (2-cm ID) was first equilibrated with tris(hydroxymethyl)aminomethane-hydrochloride buffer–0.11 M NaCl having a conductivity at 25°C of 14,000 reciprocal Ω as measured in a Beckman model RC-16C conductivity bridge (Beckman Instruments, Cedar Grove, N.J.). After the sample was applied, the flow rate of the column was adjusted to 0.5 ml/min, the effluent was monitored at 280 nm with an ultraviolet flow analyzer (Uvicord II, LKB Instruments, Bromma, Sweden), and 5-ml fractions were collected with a Racetrack Fractionator (Gilson Medical Electronics, Middleton, Wis.). Protein-containing fractions were pooled and diafiltered over a PM 30 membrane against 0.05 M potassium phosphate buffer (pH 7.5) containing 0.02% NaN_3. The retentate then was concentrated to 4.9 ml and stored at 5°C. The extent of purification of immunoglobulin G (IgG) at each stage of antiserum fractionation was determined by cellulose-acetate strip electrophoresis and detected with a scanning densitometer. Serum from a normal rabbit devoid of complement-fixing and precipitating antibodies to fungus antigens was used as a control and was treated identically to the anti-M serum. The final protein concentration was determined with Folin phenol (13). The purity of the globulin fraction obtained after DEAE-Sephadex chromatography, as judged by cellulose-acetate strip electrophoresis, was 98.1% IgG for the anti-M globulins and 93.7% IgG for the normal rabbit serum. The cellulose-acetate strip electrophoresis profile at different stages of fractionation is shown in Fig. 1. The IgG broke through the DEAE column in the first 50 ml of effluent. A summary of the yield of protein obtained after each stage of fractionation is in Table 1.

After DEAE-Sephadex chromatography and diafiltration, 5 mg of the rabbit anti-M IgG was iodinated with carrier-free Na ^{125}I (11 to 17 mCi/μg; Amersham/Searle, Arlington Heights, Ill.) by the chloramine-T procedure (6). The protein-bound radioactivity was measured (6), and the iodinated globulins were stored at 5°C in 0.05 M potassium phosphate buffer (pH 7.5) containing 0.02% NaN_3. The typical

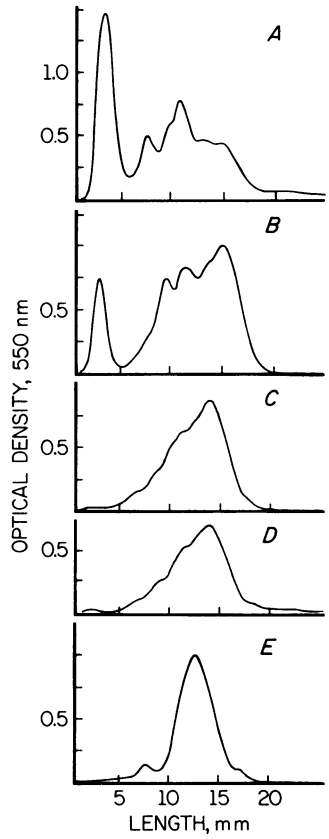

FIG. 1. *Densitometric recordings of cellulose-acetate strip electrophoresis of rabbit globulins at various stages of purification. (A) Serum; (B) first ammonium sulfate precipitate; (C) second ammonium sulfate precipitate; (D) after diafiltration; (E) after DEAE-Sephadex chromatography.*

radiolabeling efficiency obtained was 83 to 89% protein-bound ^{125}I, and the specific activities were 157 to 180 μCi/mg of protein. These values are for freshly iodinated proteins. Because of radioactive decay, the iodinated globulins were not used after 2 months.

RIA procedure. The RIA reaction was carried out in polyvinyl microtiter plates containing 96 wells, which were coated with silicone and dried (16). The diluent employed was phosphate-buffered saline (PBS; pH 7.2) composed of 0.0081 M Na_2HPO_4, 0.0015 M KH_2PO_4, 0.137 M NaCl, and 0.0027 M KCl. The wetting agent Tween 20, polyoxyethylene sorbitan monolaurate (Fisher Scientific Co., Pittsburgh, Pa.), was added to PBS where specified to give a 1% (vol/vol) solution. The M-antigen stock solution, containing 2.5 mg of protein per ml, was diluted 1:80,000 in PBS. Either 50 μl of this solution, containing 1.6 ng of protein, or buffer alone was added to each well and dried at 26°C overnight. The antigen was fixed by addition of 0.15 ml of neutral-buffered Formalin for 15 min and then was washed twice with PBS-Tween. Serial twofold dilutions of patients' sera were made in PBS-Tween containing 10% (vol/vol) fetal calf serum, and 25-μl samples were applied in triplicate to antigen-coated microtiter wells. Controls were duplicate samples of identical dilutions of patients' sera in wells with no antigen and 16 samples per plate of PBS-Tween-fetal calf serum with no patient sera. The plates were then incubated for 1.5 h at 37°C and washed twice with PBS-Tween. The ^{125}I-labeled rabbit anti-M globulin (0.83 mg of protein per ml; specific activity, 180 μCi/mg) was diluted to 1:32 with PBS-Tween containing 1% fetal calf serum. Each well received 25 μl of diluted globulin solution, was incubated for 1.5 h at 37°C, and then was washed three to four times with PBS-Tween. Residual buffer was removed by vigorously striking the plate on an absorbent paper-lined metal tray. The dry wells were cut out, transferred to test tubes (15 by 125 mm), and screened for 1 min for radioactivity in an automatic gamma scintillation spectrometer.

The inhibition of ^{125}I-labeled anti-M globulin binding to the antigen was determined by comparing the radioactivity (counts per minute) bound by controls with radioactivity (counts per minute) bound by the human serum specimens. The fractional values of the mean counts per minute of triplicate samples at each dilution of patients' sera, divided by the mean counts per minute of 16 replicate controls obtained with PBS-Tween-fetal calf serum and no sera were calculated. This fraction, x, was converted to percent inhibition, $100(1 - x)$. The antibody titer reported as the end point was that serum dilution giving greater than 10% inhibition.

The RIA was modified to quantitate M antigen. Twofold dilutions of the antigen in 0.5 ml were combined with an equal volume of ^{125}I-labeled anti-M globulin (1:64 dilution) and were incubated for 1 or 3 h at 37°C. Then 25 μl of this solution was added to microtiter wells previously coated with 3.1 ng of M-antigen protein. After a second incubation for 1.5 h at 37°C, wells were washed, and the adsorbed radioactivity was counted; the fractional binding was expressed as mean counts per minute of six replicates at each preincubated M-antigen concentration divided by the counts per minute obtained with wells preincubated with ^{125}I-labeled anti-M globulin alone.

Serological tests. MicroCF and ID tests employing histoplasmin and other fungus antigens were performed according to Kaufman et al. (10, 11). Sera used in this study were obtained from the Fungus Immunology Branch, Center for Disease Control.

TABLE 1. *Protein content of normal rabbit globulins and anti-M rabbit serum before and after fractionation*

State of extraction	Total protein (mg)	
	Normal serum	Anti-M serum
Whole serum[a]	ND[b]	463
First $(NH_4)_2SO_4$ precipitate	108	239
Second $(NH_2)_4SO_4$ precipitate	67	185
Diafiltration	42	179
DEAE-Sephadex	31	108

[a] Initial volume, 10 ml.
[b] ND, Not determined.

Those sera described as being from clinically diagnosed histoplasmosis were obtained from patients from whom *H. capsulatum* was isolated or was demonstrated in histological sections. A panel of sera from histoplasmosis patients was kindly provided by Zell McGee, Vanderbilt University Hospital, Nashville, Tenn., (patients 2, 3, and 22 to 29).

RESULTS

Determination of combining proportions of M and ^{125}I-labeled anti-M. In a competitive-binding assay, the sensitivity of the assay is increased by using a minimal quantity of antigen for combination with the unknown antibody. Hence, the optimal amounts of M antigen and ^{125}I-labeled anti-M globulin yielding significant binding ratios (counts per minute of ^{125}I-labeled anti-M bound divided by counts per minute of background) in the standard assay were determined. The antigen was diluted in threefold decrements beginning at 1:300 (416 ng per well) to 1:72,900 (1.72 ng per well). Each antigen concentration was reacted with varying concentrations of ^{125}I-labeled anti-M, from 5.2 μg per well to 81.2 ng per well (1:4 through 1:256, respectively), in twofold decrements. The results showed that all antigen concentrations, including 1.72 ng per well (1:72,900), yielded significant ratios (>2.0) at all ^{125}I-labeled anti-M concentrations (Fig. 2). These data suggested that lower concentrations of antigen would also produce significant ratios.

At the lowest concentration of antigen, 1.72 ng per well, the ratio counts per minute of antigen/counts per minute of control increased as the amount of ^{125}I-labeled anti-M added to each well was decreased from 5.8 μg per well (1:4 dilution) to 650 ng per well (1:32 dilution). Additional dilution of ^{125}I-labeled anti-M yielded decreasing ratios of adsorption. On the basis of these results, the optimal concentration of antigen was 1.72 ng per well, and the concentration of ^{125}I-labeled anti-M was 650 ng per well (1:32 dilution). At these combining proportions, the mean of 26 replicates of M-antigen ^{125}I-labeled anti-M was 3,402 ± 260 cpm when a freshly iodinated protein was used. For convenience in the competitive-binding assays, the antigen was diluted to 1.57 ng per well (1:80,000). Later experiments proved that 1.24 ng per well (1:100,000) of the antigen increased the sensitivity of the assay.

Effect of adding Tween 20 to the diluent. Tween 20, a wetting agent, was added to the diluent in an attempt to reduce the nonspecific adsorption of ^{125}I-labeled anti-M to the microtiter wells containing saline and no antigen. Adding 1% Tween 20 to the buffer used for diluting ^{125}I-labeled anti-M and for rinsing the microtiter plates reduced the nonspecifically adsorbed radioactivity from 521 ± 90 cpm (12 replicates) for buffer without Tween 20 to 77 ± 19 cpm (16 replicates).

Evaluation of sensitivity and specificity of *H. capsulatum* competitive-binding RIA. Four sera with varying antibody levels to M antigen were selected for graphically depicting the competitive-binding RIA (Fig. 3). Serum titrations were plotted as the percentage of inhibition on the ordinate and the reciprocal serum dilution on the abscissa. An arbitrary value of 10% inhibition was chosen as the titration end point. Three typical anti-M-positive serum titrations and a -negative serum titration are illustrated in Fig. 3. Sera from 29 patients with clinically diagnosed histoplasmosis were tested by RIA. The results were compared with those obtained by CF and ID (Table 2). The 29 sera that were screened for anti-*H. capsulatum* antibodies were also tested against a battery of

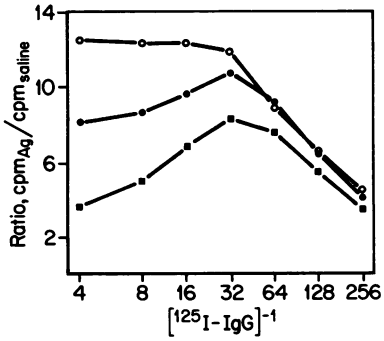

FIG. 2. *Binding curves for ^{125}I-labeled anti-M as a function of M-antigen concentration.* (○) *15.5 ng of antigen per well;* (●) *5.15 ng per well;* (■) *1.72 ng per well.*

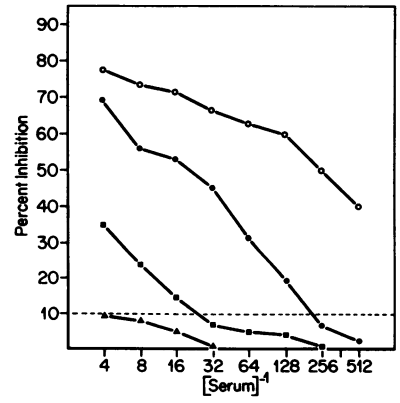

FIG. 3. *RIA activity curves of four histoplasmosis patients' sera.* (○) *Patient 11;* (●) *patient 3;* (■) *patient 2;* (▲) *patient 19.*

TABLE 2. *Comparison of RIA with CF and ID tests for detecting antibodies in human histoplasmosis case sera*

Patient no.	Antibody titer^{-1}				
	RIA	CF			ID
		H[a]	Y[b]	B[c]	
1	4	64	0	0	H,M
2	16	AC[d]			M
3	256	1,024	512	16	H,M
4	64	512	128	0	H,M
5	16	32	32	16	H,M
6	16	32	32	8	H,M
7	128	128	32	0	H,M
8	0	8	0	0	0
9	16	512	64	32	0[e]
10	8	16	16	8	M
11	>512	256	256	64	0[e]
12	8	16	64	0	M
13	8	8	16	8	M
14	16	16	16	8	H,M
15	8	8	16	0	H,M
16	32	8	0	0	H,M
17	8	16	32	0	H,M
18	32	8	8	0	H,M
19	0	0	128	0	H,M
20	0	0	0	0	0
21	0	0	0	0	0
22	8	0	0	0	0
23	0	AC			0
24	16	0	8	8	0
25	32	8	64	32	0
26	8	AC			0[e]
27	0	0	0	0	0
28	0	0	8	8	0[e]
29	4	0	0	0	0

[a] H, Histoplasmin.
[b] Y, *H. capsulatum* yeast forms.
[c] B, *B. dermatitidis* yeast forms.
[d] AC, Anti-complementary.
[e] Positive precipitin arc in IEP versus histoplasmin. Patients 9, 11: M; patients 26, 28: nature of arc undetermined.

CF antigens consisting of histoplasmin, Merthiolate-killed yeast forms of *H. capsulatum*, whole-cell homogenate of *B. dermatitidis*, and coccidioidin. The results obtained with coccidioidin were uniformly negative. The reactions occurring with *B. dermatitidis* antigen in CF tests are a measure of serum cross-reactivity, which in some instances makes these results equivocal.

Of 29 human histoplasmosis patients' sera, 22 reacted in the RIA test (76%), 21 reacted with the histoplasmin or *H. capsulatum* yeast-form CF antigens (72%), and 16 (55%) produced an immune precipitate with the M antigen in ID (Table 2). The relationship between RIA and an M line in ID was as follows: 15 sera of histoplasmosis patients were both RIA and M-precipitate positive, 7 sera were RIA positive and M-precipitate negative, and 1 serum was RIA negative and M-precipitate positive (patient 19). Of the 13 sera that did not react in ID, 4 produced a precipitin arc in IEP with histoplasmin, leading to the conclusion that the absence of ID reactivity was probably due to a lack of antigen-antibody equivalence. Three sera nonspecifically bound complement; of these, two reacted in RIA and one produced an immunoprecipitin arc with M antigen in ID. The RIA titers, or end-point serum dilutions resulting in greater than 10% inhibition of adsorbed counts per minute, were generally lower than the CF titers for unfractionated histoplasmin. One notable exception was the serum from patient 11. This was the most RIA reactive (RIA $\geqq$ 1:512) serum examined, but the histoplasmin CF titer was lower (1:256). Although the standard ID reaction failed to show an M line with this serum, an M-precipitate arc was seen by IEP. Some patients with histoplasmosis (nos. 20, 21, 23, and 27) were serologically unresponsive in all tests. Patient 19 had Hodgkin's disease, was being treated with immunosuppressive drugs, and was diagnosed as having the unusual type of histoplasmosis referred to as endogenous reinfection, or reactivation of previously walled-off lesions. The extent of cross-reactions in the CF test observed with the panel of sera (Table 2) and *B. dermatitidis* antigen was 11 of 29 (38%).

Sera from patients with heterologous mycoses were tested for their reactivity in the histoplasmin RIA. Of 21 sera obtained from patients with blastomycosis, candidiasis, coccidioidomycosis, cryptococcosis, paracoccidioidomycosis, or pulmonary sporotrichosis, one serum of candidiasis and one of blastomycosis cross-reacted with histoplasmin M antigen in the RIA (Table 3). The serum of one blastomycosis patient that gave a strong cross-reaction in RIA (end point, 1:32) was anti-complementary and produced a line in ID with *B. dermatitidis* antigen but not with histoplasmin. This patient lived in Georgia, where *H. capsulatum* can be isolated from soil,

TABLE 3. *Reactivity in the RIA test of human sera from patients with histoplasmosis and heterologous mycoses*

Mycosis	Total no. of tested case sera	No. reactive in RIA	Reactive (%)
Histoplasmosis	29	22	76
Blastomycosis	3	1	33
Coccidioidomycosis	3	0	0
Cryptococcosis	5	0	0
Candidiasis	5	1	20
Paracoccidioidomycosis	2	0	0
Pulmonary sporotrichosis	3	0	0

and therefore may have become exposed to both fungi. As a control, sera of 10 normal individuals were operated in the RIA. These persons had blood drawn at physical examinations before employment at the Center for Disease Control. None of these sera was reactive in the RIA.

Modification of RIA for quantitation of M antigen. In determining that M antigen was not modified upon fixation to the wells of microtiter plates, the RIA method was altered to measure the quantity of M antigen that blocked the reactivity of the affixed M antigen with ^{125}I-labeled anti-M globulin. The results showed that preincubating small quantities of M antigen for 1 to 3 h with the ^{125}I-labeled anti-M globulin, followed by adding this solution to the M antigen-coated microtiter wells, resulted in inhibition of bound counts per minute; as little as 125 pg of M caused 16% inhibition (Table 4). It was determined that a quantity of antigen equal to that affixed to the microtiter wells (3.1 ng per well) still permitted binding of the ^{125}I-labeled anti-M globulin to the affixed antigen. Hence, drying and affixing the M antigen to the microtiter wells did not appear to modify the antigen adversely.

DISCUSSION

Purified M antigen derived from histoplasmin and ^{125}I-labeled anti-M globulins was used in this RIA test. Others (14) have suggested that antigens of lesser purity than are used in CF or hemagglutination tests can be used in RIA, provided the labeled IgG is specific for the antigenic component in question. Although the M antigen was purified, it still contained small amounts of antigens that cross-reacted with rabbit anti-*B. dermatitidis* serum in IEP. Because of its heat stability, we believe the antigen involved in the latter case may be Heiner's C antigen (5). The rabbit anti-M globulins likewise reacted with a *B. dermatitidis* antigen preparation. Although rabbit anti-M may react with *B. dermatitidis* antigen, this cross-reactivity should not enhance the cross-reactivity of a patient's serum, but serum from a blastomycosis patient may react in this RIA test. For this reason, it may be desirable in the future to use convalescent human anti-*H. capsulatum* serum as a source of IgG for labeling purposes. In that event, however, antibodies to other *H. capsulatum* components in addition to anti-M may occur in the labeled reference globulins.

TABLE 4. *Modification of RIA for the quantitation of M antigen*

M antigen (pg)	cpm[a]	% Inhibition
0	1,482 ± 117	0
125	1,248 ± 99	16.0
156	1,166 ± 70	21.3
208	1,071 ± 67	27.7
313	962 ± 82	35.1
500	802 ± 84	45.9
781	718 ± 26	51.6
1,563	591 ± 40	60.1
3,126	362 ± 25	75.6

[a] Mean of six replicates ± standard deviation.

In sera from patients with proven histoplasmosis, RIA detected 76% of the cases, the CF test detected 72%, and the ID test detected only 55%. These results show that, as far as detection of positive histoplasmosis sera is concerned, the RIA procedure approximates the CF test. Since RIA measurements are based on an action curve of percent inhibition, titers are more accurate for sera of low antibody activity (patients 2 and 18, Fig. 2) than are determinations made by CF; in this regard, the RIA test is better and more reliable. The question that must be answered regarding the RIA test is whether the sensitivity can be increased, without affecting specificity, by using a lower base line cutoff for RIA inhibition.

Concerning specificity, it was clear, within the limits imposed by the availability of a very small number of sera from blastomycosis patients, that sera from patients with other systemic mycoses were not prone to cross-react in the RIA. The clinical relevance of the M antigen is underscored by the fact that the RIA test with purified M and reference antibody of narrow specificity achieved parity with the CF test, which employs heterogeneous antigens.

Titers in RIA were generally lower than those obtained in the histoplasmin CF test, with one notable exception, patient 11, whose RIA titer was higher, suggesting the presence of antibodies specific for M that did not fix complement. In this case, the amount of M antibody was so high that it was not detected by ID, presumably because of antibody excess; the specific M antibody was observed, however, by IEP. In the CF test, the antigen may also have been at a suboptimal concentration for this patient's antibody. The problem of apparent serological inertness in sera from some histoplasmosis patients is evident (Table 2). When it was defined as CF titers of 1:16 and below and an absence of precipitins, eight sera fell into this antibody-poor category; of these, four were detected in RIA. Failure to add Merthiolate (final concentration, 0.01%) as preservative may account for some of the negative sera. A genuine antibody-poor state at the time blood was drawn may account for other negative sera. Information on the state of immunocompetence of the patients

was not available. In immunosuppressed patients, there is the possibility of reactivating calcified pulmonary foci. In such instances, modification of RIA to detect M antigenemia or soluble immune complexes could be most useful.

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