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Elimination of False-Positive Serum Reactivity in Latex Agglutination Test for Cryptococcal Antigen in Human Immunodeficiency Virus-Infected Population

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We recently tested serum from a human immunodeficiency virus-infected patient for the presence of cryptococcal antigen using the Meridian latex agglutination (LA) test (Cryptococcal Antigen Latex Agglutination System). Two pronase-treated serum specimens from the patient had LA titers of 80 and 160, but the patient had no evidence of cryptococcal disease. The serum was negative for rheumatoid factor, a well-documented cause of false-positive LA reactions. Seven blood culture supernatants from the patient were also LA positive, but were culture negative for cryptococcus. When the sera and blood culture supernatants were treated with 0.01 M 2-β-mercaptoethanol (2-ME), the agglutinating activity was ablated. Similar results were seen when the sera were tested by two other commercial LA assays. Serum and cerebrospinal fluid specimens from patients with confirmed cryptococcal disease were treated with 2-ME, and the results were compared with those obtained after pronase (sera) or heat (cerebrospinal fluid) inactivation. The titers were identical ($n = 56$) or within 1 dilution ($n = 3$). One hundred serum specimens from human immunodeficiency virus-seropositive patients with no known history of cryptococcal disease were examined to determine the frequency of false-positive reactivity in this patient population. Of this group, three were positive following pronase treatment. One remained positive after 2-ME treatment; the remaining two were negative. These data indicate that 2-ME can be used to eliminate nonspecific reactivity in the LA test without affecting true-positive results.

Cryptococcus neoformans is a ubiquitous yeast capable of causing disease in healthy as well as immunocompromised hosts (28, 29). Cryptococcosis, the fourth most common opportunistic infection in AIDS patients (11, 23, 34), has a wide spectrum of clinical presentations (22, 29). Therefore, testing for this pathogen has become routine for patients infected with human immunodeficiency virus (HIV). Although the rate of culture positivity is high in this patient population (2–4), growth and identification of the organism require several days. Thus, the development of rapid diagnostic tests has had a great impact on the management of cryptococcosis in HIV-infected individuals.

First described in 1967 by Bloomfield et al. (4), the cryptococcal latex agglutination (LA) assay, a direct antigen detection methodology, is one of the most valuable serodiagnostic tests for fungi performed on a routine basis (12, 17, 32). False-positive LA reactions caused by a variety of interfering factors have been eliminated by several procedural modifications, including specimen pretreatment and the incorporation of control reagents (1, 10, 16, 19, 30, 32).

In this report we describe a false-positive cryptococcal antigen LA test (LAT) result in an HIV-infected male who presented with a pseudomonal rectal abscess. Several negative fungal cultures and his rapid response to antibacterial therapy alerted us to the possibility of a false-positive reaction. Further investigation demonstrated that this cross-reactivity could be eliminated by pretreatment with 2-β-mercaptoethanol (2-ME). This finding prompted an investigation of the incidence of the nonspecific reactivity of the cryptococcal LA assay in an HIV-infected population of subjects.

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MATERIALS AND METHODS

Case report. The patient, a 29-year-old male with HIV infection secondary to severe hemophilia A, presented to the emergency room with a history of fever and chills with night sweats for the previous 6 months and a 3-day history of nausea and vomiting. He had been discharged 3 days previously following treatment for rectal bleeding secondary to a rectal fissure. On admission, he complained of rectal pain, had a fever of 38.9°C, a leukocyte count of 1,000/mm^3 with a CD4 count of 4/mm^3 and a small amount of thrush. He was dehydrated and somnolent. Otherwise, his physical examination was unremarkable. Since the cause of his illness was not clear, the differential diagnosis was very broad. An assay for cryptococcal antigen in serum was performed and was positive, with a titer of 80. In response to that test, a computed tomography scan of his head was done, and the result was within normal limits. A cerebrospinal fluid (CSF) examination revealed two leukocytes per ml, with 13% lymphocytes and 87% monocytes, and normal protein and glucose levels. CSF culture and blood cultures (six sets) were all negative for cryptococcus. The patient was begun on amphotericin B therapy, which he tolerated poorly. He received a total of 341 mg of amphotericin B over the next 10 days. On the day after the CSF examination, one of two blood cultures grew *Pseudomonas aeruginosa*. He received imipenem and an aminoglycoside and his fever defervesced within 24 h. Four subsequent blood cultures were negative. Culture of a specimen from his rectal fissure also grew *P. aeruginosa*. A repeat test for crypto-

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coccal antigen in serum was done 9 days after the original one; the result was again positive, with a titer of 160.

Eight months later, he was admitted to the hospital with cryptococcal meningitis. Lab results included a positive CSF stain and culture, a cryptococcal antigen titer in serum of 400, a CSF antigen titer of 200, and 2 weeks later, a repeat CSF antigen titer of 80.

Patient specimens. A total of five specimens (three serum and two CSF specimens) from the patient described in the case report were submitted to the Clinical Microbiology and Immunology Laboratory of the University of North Carolina Hospitals for detection of cryptococcal antigen. Cryptococcal antigen-positive sera and CSF were from patients for whom specimens had been submitted for detection of antigen and who were known to be culture positive. One hundred serum specimens from HIV-positive patients with no clinical history of cryptococcal disease (and negative cultures) were used to determine the false-positive cryptococcal rates in this patient population. Eight serum specimens positive for rheumatoid factor were also examined for false-positive reactivity.

Cryptococcal antigen tests. (i) CALAS. Unless otherwise noted, the cryptococcal antigen test used in the present study was the Cryptococcal Antigen Latex Agglutination System (CALAS; Meridian Diagnostics, Cincinnati, Ohio). The procedure was performed according to the manufacturer's instructions. All serum specimens were treated with pronase (15 min at 56°C and then boiled for 5 min), and CSF specimens were boiled (5 min) prior to testing. Tenfold serial dilutions of screen-positive specimens were prepared in buffer solution. On the basis of the results of this screening dilution, additional twofold serial dilutions were prepared until the titer of the specimen was determined to the endpoint.

(ii) CRYPTO-LEX. The CRYPTO-LEX System (Trinity Laboratories, Inc., Raleigh, N.C.) was performed according to the manufacturer's recommendations. CRYPTO-LEX is an LAT in which latex particles are sensitized with a monoclonal antibody which reacts with all four serotypes of *C. neoformans*. A control latex reagent is not tested with this system since these monoclonal antibodies do not react with rheumatoid factor. Patient samples (serum and/or CSF) were heat treated in a 56°C heat block for 15 min prior to testing. The titers of positive specimens were determined as described above for CALAS.

(iii) CRYPTO-LA test. The Crypto-LA test (Wampole Laboratories, Cranbury, N.J.) was performed according to the manufacturer's recommendations. Serum and CSF specimens were heat inactivated in a 56°C water bath for 30 min prior to testing.

PREMIER EIA. The Premier Cryptococcal Antigen enzyme immunoassay EIA (Meridian Diagnostics) was performed as described previously (13). All EIA screenings were performed without specimen pretreatment.

2-ME treatment. Serum and CSF specimens that were positive for cryptococcal antigen by CALAS LA assay were treated with 0.01 M 2-ME (Sigma Chemical Co., St. Louis, Mo.) as described previously (30). A 0.1 M solution of 2-ME in phosphate-buffered saline (PBS) was made fresh daily and was kept on ice until use. Two aliquots of 180 μl of CSF or serum which had been boiled (CSF) or treated with pronase and boiled (serum) were placed into separate tubes. Twenty microliters of PBS was added to the aliquot in one tube; 20 μl of 0.1 M 2-ME in PBS was added to the aliquot in the other tube. Specimens were vortexed and incubated for 1 h at 35°C. Both aliquots were examined for the presence of cryptococcal antigen.

Rheumatoid factor. The RapiTex RF (Behring Diagnostics, Inc., Somerville, N.J.) was used for the detection of rheumatoid factor in serum. The LAT kit was used according to the manufacturer's recommendations.

TABLE 1. Case patient's cryptococcal antigen results with and without 2-ME treatment

Date (mo-day-yr)	Specimen type	Cryptococcal antigen titer[a]	
		Untreated	Treated with 2-ME
6-2-92	Serum	80	Negative
6-11-92	Serum	160	Negative
2-12-93	Serum	400	400
2-12-93	CSF	200	200
2-28-93	CSF	80	80

[a] Cryptococcal antigen titer obtained by using CALAS LA reagents.

Blood cultures. Blood cultures were performed by using the BacT-Alert blood culture system (Organon Teknika, Durham, N.C.). Blood was inoculated into trypticase soy broth and was monitored for 7 days. An aliquot of the blood-broth mixture was removed from each of the bottles containing the blood of the patient described in the case report at the termination of the blood culture. The blood-broth mixture was centrifuged to pellet all of the cells. Following pronase treatment, the supernatant was assayed for agglutinating activity with the CALAS LA reagents.

Absorption studies. Attempts were made to characterize the false-positive reactivity via absorption of the interfering substance with *P. aeruginosa* and monoclonal antibodies.

(i) *P. aeruginosa*. The *P. aeruginosa* isolate from the patient described above in the case report was subcultured from an agar plate, inoculated into tryptic soy broth, and incubated overnight at 35°C. A suspension of the overnight culture equivalent to a no. 3 McFarland standard was prepared in saline. Two hundred microliters of the patient's serum was mixed with 200 μl of the *P. aeruginosa* suspension and was rotated for 30 min at room temperature. This mixture was centrifuged, and the supernatant was tested with the CALAS LA reagents.

Monoclonal antibodies. Mouse monoclonal anti-cryptococcal capsular polysaccharide antibodies were a gift from Bruce Clinton (Trinity Laboratories, Inc.). These antibodies were the same as those used to prepare the CRYPTO-LEX LA reagent. Serum specimens from the patient described above in the case report were mixed with equal volumes of 1:10, 1:100, 1:1,000, and 1:10,000 dilutions of the monoclonal antibody. The mixtures were vortexed and incubated overnight at 4°C. The specimens were centrifuged in a microcentrifuge (16,000 × *g*) for 3 min, and the supernatant was assayed for reactivity with the CRYPTO-LEX and CALAS LA reagents.

RESULTS

Table 1 summarizes cryptococcal LA assay results, both with and without 2-ME treatment, for the case patient. Neither of the two specimens tested in June 1992 was culture positive for *C. neoformans*. Approximately 8 months later the patient did develop culture-confirmed cryptococcal meningitis. At that time, two CSF and one serum specimen were CALAS LA assay positive following pronase or 2-ME treatment.

The serum specimen testing false positive was tested by the three LA and the EIA cryptococcal antigen detection systems. The LA assays gave similar results; the pronase-treated aliquots were positive, while the specimens treated with 2-ME

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TABLE 2. Reactivities of various specimens with CALAS LA reagents

Specimen	Reactivity of specimens	
	Untreated	Treated[a]
Patient serum	Positive (1:80)	Negative
Blood culture supernatant[b]	Positive (1:80)	Negative
P. aeruginosa-positive blood culture supernatant	Positive (1:80)	Negative
Uninoculated blood culture broth	Equivocal	Negative
P. aeruginosa in overnight culture of TSB[c]	Negative	ND[d]
P. aeruginosa-absorbed serum[e]	Positive	ND
Rheumatoid factor-positive sera (n = 8)	Negative	ND

[a] Specimens treated with 0.01 M 2-ME.

[b] Six negative blood culture supernatants were tested; the titers in two were determined.

[c] A *P. aeruginosa* isolate recovered from the case patient was grown overnight in trypticase soy broth (TSB) and was tested for reactivity.

[d] ND, not determined.

[e] The case patient's sera were absorbed with a *P. aeruginosa* isolate recovered from his blood.

were negative. Both pronase-treated and 2-ME-treated specimens were positive by the Premier EIA. A variety of specimens were tested by the CALAS LA assay to assess reactivity following treatment with 2-ME. These results are presented in Table 2.

The effects of treatment with 2-ME on serum and CSF collected from patients confirmed to be positive for *C. neoformans* by culture were evaluated. Titers were determined for 38 serum specimens obtained from 20 patients and for 21 CSF specimens from 9 patients with and without 2-ME treatment. The titers were identical for 35 serum specimens and all 21 CSF specimens. The titers for the other three serum specimens were within 1 dilution of those for the untreated serum specimens.

The prevalence of false LAT positivity in sera from an HIV-infected population of subjects was also examined. One hundred serum specimens from HIV-positive patients with no previous history of cryptococcal meningitis were tested for their reactivities with the CALAS LA reagents. Of these, three specimens were positive (titers, 20, 40, and 80). The specimen with a titer of 20 remained reactive at that titer following 2-ME treatment. However, the reactivities of the other two specimens with the CALAS LA reagents were eliminated by 2-ME treatment. Neither of the two patients from whom these two serum specimens were obtained had clinical histories or laboratory data consistent with a diagnosis of cryptococcosis during the subsequent 12 months.

In an attempt to characterize the interfering factor, the case patient's serum was absorbed with 10-fold serial dilutions of a mouse monoclonal anti-cryptococcal capsular polysaccharide antibody (Table 3). The "neutralized" sera were then tested for their reactivities with cryptococcus antigen-coated latex beads from the CALAS LA and CRYPTO-LEX LA assays. The lowest dilutions of antibody (1:10, 1:100) were able to absorb out or block the reactivity present in the serum when tested by the CRYPTO-LEX LA assay. However, higher dilutions (1:1,000, 1:10,000) did not block reactivity. In contrast, the CALAS LA assay demonstrated a strong agglutination reaction (4+) regardless of the absorbing antibody dilution.

TABLE 3. Monoclonal antibody absorption studies on case patient's serum

Monoclonal antibody dilution[a]	Reaction by:	
	CRYPTO LEX LA assay	CALAS LA assay
1:10	Negative	Positive (4+)
1:100	Negative	Positive (4+)
1:1000	Positive (2+)	Positive (4+)
1:10,000	Positive (3+)	Positive (4+)

[a] Mouse monoclonal anti-cryptococcal capsular polysaccharide antibody.

DISCUSSION

The detection of cryptococcal antigen in serum and/or CSF by LA has become an essential laboratory tool in the diagnosis of cryptococcal disease, particularly for HIV-infected individuals (6, 8, 9, 12, 32). Most often, positive antigen tests correlate with subsequently positive cultures. However, the cryptococcal LAT is subject to false-positive results, particularly with serum specimens (1, 31). Although the most common cause of this phenomenon in serum is the presence of rheumatoid factor (1, 4, 10, 12, 17), cross-reactivity has also been associated with malignancy (21, 30), chronic meningitis (24), collagen vascular disease (1), agar syneresis fluid (5, 20), *Capnocytophaga canimorsus* (formerly DF-2) (33), and *Trichosporon beigelii* (25–27). Procedural modifications have been incorporated to alleviate this problem, including pretreatment of specimens with heat (16), EDTA (7, 12), pronase (17, 18, 32), dithiothreitol (16, 19), or 2-ME (30). In addition, many kits use latex beads coated with normal rabbit globulin as a control reagent (1, 3, 10). However, false-positive LAT results accompanied by negative results for reagent controls still occur at a low rate (1, 10, 15, 16).

In the present study, serum specimens collected on two separate occasions from the patient described in the case report yielded positive cryptococcal LAT results, despite several negative fungal cultures. Culture of an abscess specimen and one set of blood cultures were positive for *P. aeruginosa*, and the patient responded to antibacterial therapy. Discussions with the clinician concerning these results prompted the laboratory to investigate the possibility of a false-positive LAT result. The decision to pretreat specimens with 2-ME was based on a previous study of Sachs et al. (30). They reported a patient with squamous cell carcinoma of the lung who had a positive titer for cryptococcal antigen in CSF but negative cultures and India ink preparations. Treatment with 2-ME eliminated the interfering substance, while pronase or dithiothreitol treatment did not. The authors' hypothesis that the patient's malignancy was responsible for the false-positive LAT is consistent with previous reports (10, 21).

In our study pretreatment of the serum specimens with 0.01 M 2-ME eliminated reactivity with the antibody-coated latex beads, thus confirming our suspicions of nonspecific cross-reactivity. The 2-ME acts by reducing the disulfide bonds of proteins (14, 29). Similar treatment of serum and CSF specimens known to be positive for *C. neoformans* demonstrated no loss of reactivity or significant decrease in titer. This assured us that the 2-ME was not simply inactivating the latex reagent. We observed that the interfering substance was not effectively eliminated by 2-ME treatment in the Premier EIA. If the cross-reactivity was due to an anti-idiotypic antibody molecule, 2-ME would abrogate lattice formation in a latex-based system; however, Fab fragments might still be able to bind to the capture antibody used in the EIA.

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The exact cause of the original cross-reactivity remains undefined, although the role of the patient's *P. aeruginosa* blood isolate appears to be negligible (Table 2). The cross-reactivity can be characterized as being heat stable, pronase resistant, and undetectable by normal rabbit globulin controls.

The results of the present study suggest that, in our patient population, as many as 2% of HIV-infected individuals may have positive cryptococcal LAT results in the absence of disease. We demonstrated that this false-positive reactivity, which is resistant to pronase, can be eliminated by treatment with 2-ME. Our retrospective analysis of HIV-positive sera detected one specimen that remained positive (titer, 1:20) even after treatment with 2-ME. This may represent a true-positive result; however, the patient was lost to follow-up.

Finally, the problem of cross-reactivity, particularly in LA assays, highlights an important aspect of all diagnostic testing. That is, laboratory information should be interpreted and used only in the context of clinical information. Open lines of communication between the clinician and laboratory personnel are extremely important because they can identify those results which seem questionable. Treatment of all serum specimens with 2-ME would not be practical in a routine setting; however, 2-ME treatment of specimens with positive antigen titers in high-risk patients without culture or histologic evidence of cryptococcosis can help to detect those with false-positive results. Close clinical follow-up of these patients is necessary since the management of these patients remains unclear.

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