MAJOR ARTICLE

Screening for Cryptococcal Antigenemia in Patients Accessing an Antiretroviral Treatment Program in South Africa

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Background. Cryptococcal meningitis is a leading cause of death in patients with acquired immunodeficiency syndrome and contributes substantially to the high early mortality in antiretroviral treatment (ART) programs in low-resource settings. Screening for cryptococcal antigen in patients who enroll in ART programs may identify those at risk of cryptococcal meningitis and permit targeted use of preemptive therapy.

Methods. In this retrospective study, cryptococcal antigen was measured in stored plasma samples obtained from patients when they enrolled in a well-characterized ART cohort in South Africa. The predictive value of screening for cryptococcal antigen before initiation of ART for development of microbiologically confirmed cryptococcal meningitis or death during the first year of follow-up was determined.

Results. Of 707 participants with a baseline median CD4 cell count of 97 cells/μL (interquartile range, 46–157 cells/μL), 46 (7%) were positive for cryptococcal antigen. Antigenemia was 100% sensitive for predicting development of cryptococcal meningitis during the first year of ART, and in multivariate analysis, it was an independent predictor of mortality (adjusted hazard ratio, 3.2; 95% confidence interval, 1.5–6.6). Most cases (92%) of cryptococcal meningitis developed in patients with a CD4 cell count $\leq$100 cells/μL. In this subset of patients, a cryptococcal antigen titer $\geq$1:8 was 100% sensitive and 96% specific for predicting incident cryptococcal meningitis during the first year of ART in those with no history of the disease.

Conclusions. Cryptococcal antigen screening before initiation of ART in patients with a CD4 cell count $\leq$100 cells/μL is highly effective for identifying those at risk of cryptococcal meningitis and death and might permit implementation of a targeted preemptive treatment strategy.

Cryptococcus neoformans is a major opportunistic pathogen and a leading cause of mortality among patients with AIDS in much of the developing world [1–3]. It is the most common cause of meningitis in central and southern Africa [4–6], accounting for 40% of all cases in a recent study from Malawi [7]. Introduction of antiretroviral therapy (ART) has been associated with a decrease in the incidence of cryptococcal meningitis across the developed world [8]. However, in many low-resource settings, most patients continue to present late to ART programs, with low median CD4 cell counts, high risk of new AIDS events, and high early mortality. Eight percent to 26% of patients die during the first year of ART; most deaths occur during the first few months [9]. Cryptococcal meningitis is a leading contributor to this early mortality, accounting for up to 20% of all deaths [10–13]. This constitutes a heavy burden on health care facilities and accounted for 31% of all inpatient days in a study from South Africa [14]. Treatment of patients presenting both before and after initiation of ART remains inadequate; the acute mortality in unselected series was 20%–50%, even with the best current treatment [15–20].

Prevention of severe disease by routine screening for

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subclinical infection with use of cryptococcal antigen tests and administration of preemptive treatment would therefore be an attractive strategy. The current cryptococcal antigen tests are highly sensitive and specific [21, 22] and are validated for use in patients with late-stage HIV infection [23]. Of importance, in patients with cryptococcal disease, antigenemia is detectable a median of 22 days before symptom onset, and it is detectable >100 days before symptom onset in 11% of patients [1].

The clinical course in patients with asymptomatic antigenemia remains poorly defined, because all such patients who are identified typically receive treatment. However, if left untreated, clinical disease may develop [24, 25] as fungal burden increases in the context of persisting immunodeficiency. Alternatively, if ART is commenced, rapid restoration of pathogen-specific immune responses may cause "unmasking" of subclinical disease. Finally, it is possible that restoration of cell-mediated immunity during ART could lead to clearance of asymptomatic infection. To investigate this and to evaluate the potential use of screening for cryptococcal antigen before ART initiation, we conducted a retrospective study in a well-characterized ART cohort in Cape Town, South Africa. Using a large collection of plasma samples obtained from patients just prior to initiation of ART, we were able to determine the subsequent clinical course in patients with cryptococcal antigenemia. We used this information to test the hypothesis that cryptococcal antigen screening before initiation of ART can be used to identify those individuals at risk of developing clinical cryptococcal disease.

PATIENTS AND METHODS

Participants. The study was approved by the Research Ethics Committee of the University of Cape Town. All patients provided written informed consent for sample collection and research use. Samples were available from sequential patients entering the ART service based at a Cape Town community clinic from September 2002 through April 2005. The clinic serves a population of >300,000; the antenatal HIV seroprevalence at the clinic was 28% in 2003. Patients from local primary care clinics are referred to the program and initiate treatment on the basis of World Health Organization recommendations of 2002 (patients with a prior AIDS diagnosis [stage IV disease] or a CD4 cell count <200 cells/μL).

Procedures. A mean delay of ~1 month from patient enrollment to initiation of ART permitted evaluation and preparation for treatment. Blood samples were obtained at a routine clinic visit during this period to measure CD4 cell count (FACSCount; Becton Dickinson) and plasma viral load (Versant; Bayer Healthcare). Excess EDTA plasma samples were stored at −80°C. For a limited time during recruitment of the cohort, additional blood samples were collected 16 weeks after initiation of ART for CD4 cell count and plasma viral load measurement. Therefore, excess EDTA plasma samples were available at 16 weeks for a consecutive subset of patients.

ART consisting of stavudine, lamivudine, and efavirenz or nevirapine was supplied free of charge. Treatment adherence was good; rates of viral load suppression to <400 copies/mL in the cohort exceeded 90% at the 16-week follow-up visit [26]. Clinic visits were scheduled at 4, 8, and 16 weeks and then every 16 weeks; additional open-access appointments were available. Principal causes of death in the cohort have been described in detail elsewhere [27]. Structured clinical records for all patients were prospectively maintained, and this information was transferred weekly into a computer database.

Cryptococcal antigen testing was performed using the Meridian Cryptococcal Latex Agglutination System (Meridian Bioscience Europe), a simple latex test capable of detecting the capsular polysaccharide of *C. neoformans* in CSF and blood. Plasma samples stored at the time of study enrollment (before initiation of ART)—and after 16 weeks of ART for a subset of patients—were tested. The assay was carefully validated for use on EDTA plasma samples by running paired analyses of serum and EDTA plasma samples from both known cryptococcal antigen–positive patients and control subjects. Concordance in antigen titer between serum samples and EDTA plasma samples was 100% across a wide range of dilutions. Samples were first incubated with Pronase (Roche) at 56°C for 15 min to minimize the number of false-positive results and then were analyzed according to the manufacturers' instructions. All samples were analyzed at a 1:2 dilution; then, positive samples were titered down to a dilution of 1:8192.

Study end points. The main outcomes were detection of cryptococcal antigenemia (with a positive result defined as a dilution $\geqslant$1:2) at baseline and microbiologically confirmed cases of cryptococcal meningitis during the first year of follow-up. In patients with a history of cryptococcal disease before ART, symptomatic relapse after ART initiation was defined as recurrence of typical symptoms, CSF antigen or culture positivity for *C. neoformans,* and no alternative diagnosis. Mortality data and ART treatment response were recorded as additional outcome measures. Information regarding causes of death in the ART program was obtained from the local secondary and tertiary care hospitals, hospital mortality review meetings, and postmortem examinations. The most likely cause of each death was assigned on the basis of all available information, after detailed review by 2 specialists in infectious diseases and HIV medicine [27].

Statistical analysis. Data were analyzed using Stata, version 9.0 (StataCorp). Variables were compared across groups with use of Student's t test, the Mann-Whitney U test, the χ^2 test or Fisher's exact test, and Cuzick's nonparametric test for trend, as appropriate. Statistical significance was defined as $P < .05$.

Hazard ratios with 95% CIs were calculated using Cox proportional hazard modeling.

RESULTS

Patients and antigen screening. A total of 707 patients were eligible for inclusion in the study. The mean age of patients was 33 years, and 185 (26%) were male. The median CD4 cell count in the cohort was 97 cells/μL (interquartile range, 46–157 cells/μL), and the median viral load was 76,803 copies/mL (interquartile range, 33,167–191,030 copies/mL). Fifty-five patients (8%) had World Health Organization stage I disease, 87 (12%) had stage II disease, 366 (52%) had stage III disease, and 199 (28%) had stage IV disease. There was no clinical suspicion of meningitis in any of the patients at the time when blood samples were obtained.

At baseline, 46 patients (7%) had a positive cryptococcal antigen assay result. Patients who were antigen positive had lower baseline CD4 cell counts, were more likely to have a history of cryptococcal disease, and were more likely to develop incident cryptococcal meningitis during ART (table 1).

Antigenemia and mortality. Among the patients with complete follow-up data, 14 (34%) of the 41 cryptococcal antigen–positive patients died during the first year of ART, compared with 64 (11%) of the 574 antigen-negative patients ($P < .001$). In a sensitivity analysis in which all patients lost to follow-up were assumed to have died, the relationship between antigen screening status and death remained highly statistically significant. Causes of death are listed in table 1.

Cryptococcal antigen–positive patients had a far higher risk of mortality than did antigen-negative patients during the 1-year follow-up period (hazard ratio, 4.75; 95% CI, 2.6–8.8; $P < .001$). After adjustment for CD4 cell count, viral load, age, and sex, baseline cryptococcal antigenemia remained a strong independent risk factor for death (adjusted hazard ratio, 3.2; 95% CI, 1.5–6.6; $P < .001$). This relationship was also found when the analysis was restricted to patients with no history of cryptococcal disease (adjusted hazard ratio, 3.1; 95% CI, 1.04–9.15; $P < .001$).

Mortality risk was strongly associated with antigen titer. Among patients with cryptococcal antigen titers of $\leqslant$1:8, 1:16–1:64, 1:128–1:512, 1:1024–1:2048, or $\geqslant$1:4096, deaths occurred in 0 (0%) of 10, 2 (25%) of 8, 4 (31%) of 13, 4 (44%) of 9, and 4 (67%) of 6 patients, respectively ($P = .02$). Mortality was 19% among those with an antigen titer of $\leqslant$1:512 and 53% among those with a titer >1:512 ($P = .04$).

Antigenemia and risk of developing cryptococcal disease. Among the 661 patients with a negative cryptococcal antigen test result, no cases of cryptococcal meningitis developed during the 1-year follow-up period. In contrast, of the 46 patients with a positive cryptococcal antigen test result, 13 (28%) developed new or relapsed clinically apparent cryptococcal meningitis during the follow-up period.

A history of cryptococcal meningitis was recorded for 21 (46%) of the cryptococcal antigen–positive patients; disease onset occurred a median of 140 days (interquartile range, 99–230 days) before enrollment in the ART program. Six (29%) of these 21 patients developed symptomatic relapse of their cryptococcal disease a median of 33 days (range, 5–70 days) after initiation of ART. All had been prescribed fluconazole maintenance therapy, and CSF culture results were negative in

Table 1. Baseline characteristics of and outcomes in all 707 patients, by cryptococcal antigen status.

Variable	Cryptococcal antigen–negative patients (n = 661)	Cryptococcal antigen–positive patients (n = 46)	P
Age, mean years	33	34	.28
Male sex	174 (26)	11 (24)	.72
Baseline CD4 cell count, median cells/μL (IQR)	105 (49–162)	46 (19–77)	<.001
Lost to follow-up	87 (13)	5 (11)	.66
Mortality[a,b]	64/574 (11)	14/41 (34)	<.001
Incident cryptococcal meningitis	0 (0)	13 (28)	<.001
History of cryptococcal disease	3 (0.5)	21 (46)	<.001

NOTE. Data are no. (%) of patients, unless otherwise indicated. IQR, interquartile range.

[a] Excluding patients who were lost to follow-up.

[b] The cause of death was known for 13 of 14 antigen-positive patients: 7 (54%) died of cryptococcal meningitis, 2 (15%) died of tuberculosis, 2 (15%) died of Kaposi sarcoma, 1 (8%) died of pneumonia, 1 (8%) died of gastroenteritis, and 1 (8%) died of HIV dementia. The cause of death was known for 44 of 64 antigen-negative patients: 11 (25%) died of tuberculosis, 8 (18%) died of wasting syndrome, 5 (11%) died of Kaposi sarcoma, 5 (11%) died of pneumonia, 4 (9%) died of unspecified systemic sepsis, 5 (11%) died of chronic respiratory illness, 1 (2%) died of bacterial meningitis, 1 (2%) died of chronic CNS disease, 1 (2%) died of lactic acidosis, 1 (2%) died of renal failure, and 1 (2%) died of Stevens-Johnson syndrome.

all cases. All these patients had baseline antigen titers of ≥1:512 (figure 1*A*). However, there were no statistically significant relationships observed between risk of relapse and time from prior cryptococcal disease to ART initiation, baseline CD4 cell count, viral load, or immunological and virological response to ART at 16 weeks (data not shown).

Of the 25 cryptococcal antigen–positive patients who did not have a history of cryptococcal meningitis, 7 (28%) developed clinically apparent cryptococcal meningitis a median of 35 days (range, 8–188 days) after starting ART. In these patients, risk of developing cryptococcal meningitis was significantly associated with cryptococcal antigen titer. The proportions of patients with antigen titers of ≤1:8, 1:16–1:64, 1:128–1:512, 1:1024–1:2048, and ≥1:4096 who developed symptomatic cryptococcal disease during follow-up were 1 (12.5%) of 8, 1 (17%) of 6, 2 (29%) of 7, 2 (67%) of 3, and 1 (100%) of 1, respectively ($P = .03$) (figure 1*B*).

If cryptococcal antigen screening was to be introduced in routine care in ART programs, patients with a history of cryptococcal meningitis would be excluded on the assumption that they were receiving consolidation treatment or adequate secondary prophylaxis. Therefore, in the 683 patients who had no history of cryptococcal disease, 7 cases could have been detected and potentially averted through use of cryptococcal antigen screening (i.e., 98 patients screened to identify 1 case).

Follow-up antigen testing. Nine cryptococcal antigen–positive patients with no history of cryptococcal meningitis who did not develop clinically apparent cryptococcal disease during the follow-up period had a second sample available for antigen testing after 16 weeks of ART. Of these samples, the antigen titer decreased in 7 (78%) and increased in 2 (22%; from 1:4 to 1:8 and from 1:32 to 1:128). The median change from baseline was a decrease of 1 dilution. Eight patients with a history of cryptococcal meningitis before initiation of ART who did not experience relapse (all of whom were receiving fluconazole maintenance treatment) had paired baseline and week-16 samples available for testing. Titers decreased in 7 (88%) and remained unchanged at a titer of 1:16 in 1 patient. The median change from baseline was a decrease of 2 dilutions.

Use of targeted cryptococcal antigen screening for patients with a CD4 cell count ≤100 cells/μL. When analysis was restricted to the 336 patients with a baseline CD4 cell count ≤100 cells/μL, baseline characteristics of patients (including CD4 cell counts) were similar in subgroups of patients with and without antigenemia (table 2). Forty-two patients (13%) had a positive cryptococcal antigen screening result. Fourteen (33%) of the cryptococcal antigen–positive patients died within the first year of treatment, compared with only 38 (13%) of cryptococcal antigen–negative patients ($P = .002$).

Twenty-one (50%) of the cryptococcal antigen–positive patients did not have a history of cryptococcal meningitis. Of these patients, 6 (29%) developed cryptococcal meningitis; these cases may potentially have been averted by screening and appropriate treatment. Therefore, 6 cases would have been detected by screening the total of 312 patients who did not have a history of cryptococcal meningitis (i.e., 52 patients screened to identify 1 case).

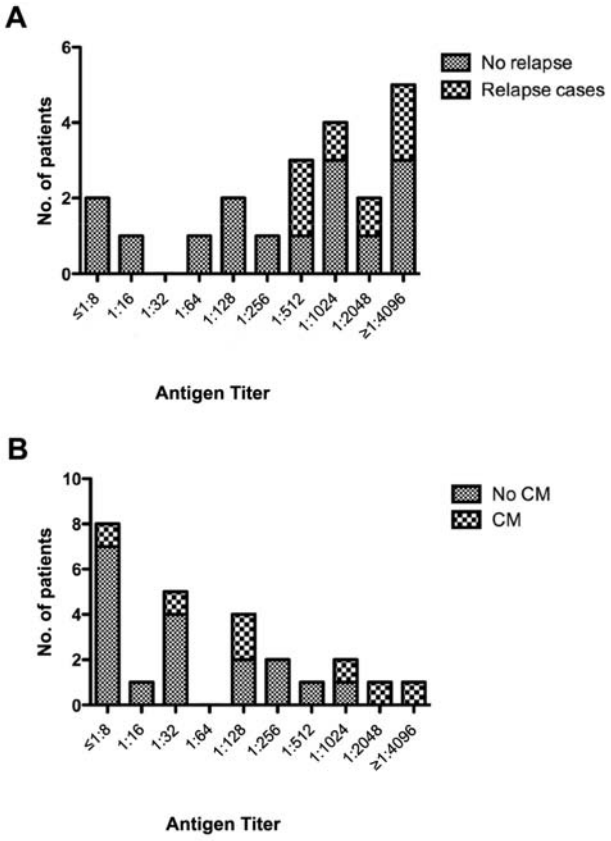

Figure 1. Association between cryptococcal antigen titer and relapse of symptomatic disease in 21 patients with a history of cryptococcal meningitis (CM; *A*). Association between cryptococcal antigen titer and development of incident CM in 25 patients without a history of CM *(B)*.

With use of a cutoff titer of 1:8, cryptococcal antigen screening had 100% sensitivity and 96% specificity for predicting incident cryptococcal disease in patients with baseline CD4 cell counts ≤100 cells/μL and no history of the disease. Thus, in this subgroup of patients, the positive predictive value was 35%, and the negative predictive value was 100%. As would be expected, the sensitivity decreased and specificity increased with increasing antigen titer cutoffs (table 3).

DISCUSSION

We found a high prevalence of cryptococcal antigenemia in patients enrolled in this ART service in South Africa. Thirteen percent of those with a CD4 cell count ≤100 cells/μL were antigen positive at baseline, which was consistent with other

Table 2. Baseline characteristics of and outcomes in patients with a baseline CD4 cell count ⩽100 cells/μL, by cryptococcal antigen status.

Variable	Cryptococcal antigen–negative patients (n = 294)	Cryptococcal antigen–positive patients (n = 42)	P
Age, mean years	34	34	.82
Male sex	86 (29)	11 (26)	.68
Baseline CD4 cell count, median cells/μL (IQR)	48 (23–73)	34 (18–71)	.35
Lost to follow-up	23 (8)	4 (10)	.70
Mortality[a]	38/271 (14)	14/38 (37)	<.001
Incident cryptococcal meningitis	0 (0)	12 (29)	<.001
History of cryptococcal disease	3 (1)	21 (50)	<.001

NOTE. Data are no. (%) of patients, unless otherwise indicated. IQR, interquartile range.

[a] Excluding patients who were lost to follow-up.

studies from sub-Saharan Africa and Southeast Asia, where 10%–18% of HIV-infected first-time clinic attendees have a positive result of routine screening for serum cryptococcal antigen [28, 19, 29]. Antigenemia was not only highly predictive of the development of cryptococcal meningitis but was also an independent predictor of mortality. In addition, both of these outcomes were strongly associated with higher cryptococcal antigen titers. These data suggest that screening for cryptococcal antigen may be useful in this patient group.

The high prevalence of cryptococcal antigenemia among the patients without a history of cryptococcal disease suggests that routine antigen screening may have a role in this context [1, 23]. Evaluation of the possible benefits of such an intervention requires an understanding of the clinical course in patients with antigenemia. Cryptococcal antigenemia in the context of advanced HIV infection has been assumed to indicate disseminated disease, and progression to severe symptomatic cryptococcosis is inevitable unless appropriate antifungal treatment is given [24, 25]. However, the clinical course of untreated antigenemia in patients commencing ART has not previously been described. Liechty et al. [30] (in Uganda) reported that asymptomatic cryptococcal antigenemia in 377 patients commencing ART independently predicted death during the first 12 weeks of treatment. The relative risk of mortality was 6.6 after controlling for CD4 cell count, viral load, and other adverse prognostic markers. However, data on the development of incident cryptococcal disease or the causes of death were not reported [30].

Stored plasma samples from a large patient cohort with comprehensive follow-up data gave us a unique opportunity to investigate the clinical course of untreated cryptococcal antigenemia in HIV-infected patients commencing ART. Of the patients with no history of cryptococcal meningitis who were cryptococcal antigen positive at baseline, 28% developed cryptococcal meningitis, 8% died of causes other than cryptococcosis, and 8% were lost to follow-up; thus, 56% were alive and had not developed cryptococcal disease at 1 year. We speculate that, in many cases, immune reconstitution during ART resulted in effective clearance of asymptomatic infection. This suggestion is supported by the observation that 78% of the antigen-positive patients who did not develop disease had a decrease in antigen titer that was similar in magnitude to that seen with effective antifungal treatment [31–33]. Immune-mediated clearance however, becomes less likely as antigen titer increases. In addition, high antigen titers (⩾1:512) in those with a history of cryptococcal disease were predictive of symptomatic relapse after initiation of ART. In these patients, we suspect that clinical presentation was attributable to immune reconstitution disease [10].

In terms of the potential use of cryptococcal antigen screening for patients commencing ART, the majority of patients (91%) with cryptococcal antigenemia had a CD4 cell count ⩽100 cells/μL (a finding replicated in Cambodia [19]), and all but one case (92%) of incident cryptococcal disease occurred in this subgroup of patients. When we limited analysis to patients with a CD4 cell count ⩽100 cells/μL and used an antigen cutoff titer of ⩾1:8 to minimize the number of false-positive results, antigen screening was highly effective at identifying those at risk of developing cryptococcal disease. The number

Table 3. Sensitivity and specificity of cryptococcal antigen screening in 312 patients with a baseline CD4 cell count ⩽100 cells/μL.

Antigen titer	Sensitivity, %	Specificity, %	Positive predictive value, %	Negative predictive value, %
⩾1:8	100	96.4	35.3	100
⩾1:32	100	96.7	37.5	100
⩾1:128	83.3	98.1	45.5	99.7
⩾1:512	50.0	99.4	60.0	99.0
⩾1:2048	33.3	100	100	98.7

of patients necessary for screening to identify and potentially prevent a case of cryptococcal meningitis was 52. At a cost of ~$3.97 per test (Centers for Disease Control and Prevention estimate [30]), the cost per identified case was $206.44.

Routine primary fluconazole prophylaxis in both developed and developing countries has been shown to reduce the number of cases of cryptococcal meningitis, yet it has not shown a consistent survival benefit [34]. Large numbers of patients require long-term medication, and concerns exist about the development of drug resistance [24]. In Thailand, national guidelines advocate fluconazole for all patients with a CD4 cell count <100 cells/μL. Although such prophylaxis has not been shown to select for fluconazole resistance in cryptococcal isolates from those who subsequently developed cryptococcal disease [35], recent work has nevertheless demonstrated a significant association with development of fluconazole-resistant *Candida* infection [36].

Antigen screening for patients with a CD4 cell count $\leq$100 cells/μL could allow a targeted preemptive treatment strategy, reducing costs and the likelihood of drug resistance. However, important questions remain to be answered. What should be done when an asymptomatic patient has a positive cryptococcal antigen result? Many experts would recommend lumbar puncture to rule out CNS involvement and amphotericin B–based therapy if CNS involvement is found. However, this would entail lumbar punctures in a substantial number of patients before the initiation of ART and would thus place an additional workload on overstretched ART programs and may not be acceptable to all asymptomatic patients. There is limited evidence that, for patients with isolated cryptococcal antigenemia, fluconazole alone is sufficient to prevent clinical disease [24, 25]. It is possible that, below a certain antigen titer, daily fluconazole treatment may be adequate in asymptomatic patients so that lumbar puncture can be avoided. Of note, in a primary prophylaxis strategy, as opposed to preemptive treatment based on antigen screening, these patients would receive intermittent fluconazole. Prospective studies are urgently required to address these issues and to test the benefits of antigen-based screening.

An additional potential limitation of our study was that some of the patients who were lost to follow-up might, in fact, have died without detection by the ART program. However, nondeath losses in this cohort have previously been characterized in detail and were not found to be associated with baseline immunodeficiency and were unlikely to represent unascertained deaths [37]. Overall, loss to follow-up and late mortality rates were low, reflecting excellent treatment response and cohort retention.

In conclusion, cryptococcal antigen screening before initiation of ART in patients with a CD4 cell count $\leq$100 cells/μL is highly effective for identification of patients at risk of cryptococcal meningitis and death and might permit implementation of a targeted preemptive treatment strategy.

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