MAJOR ARTICLE

Independent Association between Rate of Clearance of Infection and Clinical Outcome of HIV-Associated Cryptococcal Meningitis: Analysis of a Combined Cohort of 262 Patients

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Background. Progress in therapy for cryptococcal meningitis has been slow because of the lack of a suitable marker of treatment response. Previously, we demonstrated the statistical power of a novel endpoint, the rate of clearance of infection, based on serial quantitative cultures of cerebrospinal fluid, to differentiate the fungicidal activity of alternative antifungal drug regimens. We hypothesized that the rate of clearance of infection should also be a clinically meaningful endpoint.

Methods. We combined data from cohorts of patients with human immunodeficiency virus–associated cryptococcal meningitis from Thailand, South Africa, and Uganda, for whom the rate of clearance of infection was determined, and clinical and laboratory data prospectively collected, and explored the association between the rate of clearance of infection and mortality by Cox survival analyses.

Results. The combined cohort comprised 262 subjects. Altered mental status at presentation, a high baseline organism load, and a slow rate of clearance of infection were independently associated with increased mortality at 2 and 10 weeks. Rate of clearance of infection was associated with antifungal drug regimen and baseline cerebrospinal fluid interferon-γ levels.

Conclusions. The results support the use of the rate of clearance of infection or early fungicidal activity as a means to explore antifungal drug dosages and combinations in phase II studies. An increased understanding of how the factors determining outcome interrelate may help clarify opportunities for intervention.

Acute mortality due to human immunodeficiency virus (HIV)–associated cryptococcal meningitis remains unacceptably high [1–5], and, consequently, cryptococcal disease remains a leading cause of death in cohorts of HIV-infected individuals in Africa and Asia [6–8]. The urgent need to improve the acute management of cryptococcal meningitis is further reinforced by expanding access to antiretroviral therapy (ART). With access to ART, these patients have a good long-term prognosis, provided they survive the initial critical months [4].

After landmark studies in the 1990s [9, 10], recent progress in therapy for cryptococcal disease has been slow, because of the large numbers of patients needed for clinical endpoint trials and the lack of a suitable marker of treatment response. However, in a study carried out in Thailand, we demonstrated the feasibility and statistical power of a novel endpoint, the rate of clearance of cryptococcal colony-forming units from cerebrospinal fluid (CSF), based on serial quantitative

Received 4 March 2009; accepted 23 April 2009; electronically published 17 July 2009.

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Clinical Infectious Diseases 2009; 49:702–9

1058-4838/2009/4905-0005$15.00
DOI: 10.1086/604716

culture of CSF from lumbar punctures during the initial 2 weeks of treatment [1]. The use of a summary statistic (ie, the rate of decrease in colony-forming units), based on repeated quantitative measures in individual patients, means this endpoint is statistically more powerful than other markers of response previously used, for example, the proportion of patients with a positive CSF culture result at 2 weeks [11]. In the study, the fungicidal activity of alternative combinations of antifungal drugs could be differentiated with only 16 patients enrolled per arm [1].

We hypothesized that the rate of clearance of infection should also be a clinically meaningful endpoint. The organism load at baseline is a powerful prognostic factor [1], and sterilization of the CSF at 2 weeks has previously been shown to be associated with clinical outcome at 10 weeks [12]. Herein, we investigate the association of rate of clearance of infection and acute mortality due to HIV-associated cryptococcal meningitis, using data from a combined cohort of 262 patients from the Thai study and subsequent studies in Cape Town, South Africa, and Mbarara, Uganda.

PATIENTS AND METHODS

Data for our study were collected from 4 trials of initial antifungal therapy for treatment of HIV-associated cryptococcal meningitis [1, 4, 13, 14]. These studies were approved by the ethical and scientific review subcommittee of the Thai Ministry of Public Health, by the research ethics committee of the Faculty of Health Sciences, University of Cape Town, and the Medicines Control Council of South Africa, by the ethics committee of the University Hospital of Mbarara, Uganda, and by Wandsworth local research ethics committee, covering St. George's Hospital in London, England. They are as follows:

1. A randomized study of 63 ART-naive HIV-seropositive patients treated with amphotericin B (AmB) 0.7 mg/kg per day, alone or in combination with flucytosine (100 mg/kg per day), fluconazole (400 mg per day), or both, in Ubon Ratchathani in Northeast Thailand [1].
2. An observational study of 54 ART-naive or -experienced patients treated with AmB 1 mg/kg per day or with fluconazole 400 mg per day, according to local protocol, in Cape Town, South Africa [4].
3. Randomized studies of ART-naive patients who were receiving AmB-based combination therapy in Cape Town, South Africa (International Standard Randomized Controlled Trial Number [ISRCTN] 68133435) [13]. In step 1, the use of 1 mg/kg per day of AmB was compared with the use of 0.7 mg/kg per day of AmB, both with flucytosine 100 mg/kg per day (64 patients). In step 2, the use of AmB 1 mg/kg per day plus flucytosine was compared with the use of AmB 1 mg/kg per day plus fluconazole (at 800 or 1200 mg per day, 21 patients, recruitment ongoing).
4. A cohort, dose-escalation study of initial therapy with fluconazole at Mbarara University Hospital, Uganda, where standard treatment was with fluconazole at 400 mg per day. Thirty patients were treated with fluconazole at 800 mg per day, and 30 patients were treated with fluconazole at 1200 mg per day [14].

For the randomized studies of combination therapy in Thailand and Cape Town, the exclusion criteria were an alanine aminotransferase level >5 times the upper limit of normal (ie, >200 IU), an absolute neutrophil count of $<500 \times 10^6$ cells/L, a platelet count of $<50{,}000 \times 10^6$ platelets/L, pregnancy, lactation, previous serious reaction to AmB, flucytosine, or fluconazole, and patient already on ART. In Mbarara, Uganda, the exclusion criteria were an alanine aminotransferase level >5 times upper limit of normal (>200 IU), pregnancy, and prior ART.

In the above-mentioned trials 1, 3, and 4, after 2 weeks, 400 mg per day of fluconazole was used for 8 weeks, and 200 mg per day of fluconazole was used thereafter. In trial 2, AmB was given to patients for a median of 7 days prior to switching to fluconazole. After initial inpatient treatment, patients continued to be followed up in established HIV outpatient clinics at the study sites. At the time of the trial in Thailand, ART was not generally available in that country. In all the more recent trials, patients have been counselled and started on ART at a median interval of 47 days (Cape Town) and 38 days (Mbarara) after starting antifungal therapy.

Lumbar punctures were done on days 1, 3, 7, and 14, for quantitative culture to assess the rate of clearance of infection. Quantitative culture of CSF was performed as previously described elsewhere [1, 4]. The rate of decrease in $\log_{10}$ cfu/mL of CSF per day was derived from the slope of the linear regression of $\log_{10}$ cfu against time for each patient [1, 4]. Population modelling confirmed that a linear model best fitted the colony-forming unit clearance data, and gave results consistent with the individual patient analysis. CSF cytokine levels (interferon [IFN]–γ, tumor necrosis factor [TNF]–α, and interleukin [IL]–6) were determined using the Luminex multianalyte system (Luminex), cytokine kits (Bio-Rad), and a separate enzyme-linked immunosorbent assay (Quantikine; R&D Systems), as previously described elsewhere [4, 15]. Plasma viral load and CSF cytokine levels were not available for patients enrolled in Uganda.

Plasma viral load, baseline CSF colony-forming units, and cytokine data were log transformed. Continuous data were categorized into equal-sized groups and analyzed by use of the χ^2 test. Rate of clearance of infection was analyzed both as a continuous variable and categorized into quartiles. Survival analysis was conducted using Cox regression, with time calculated from

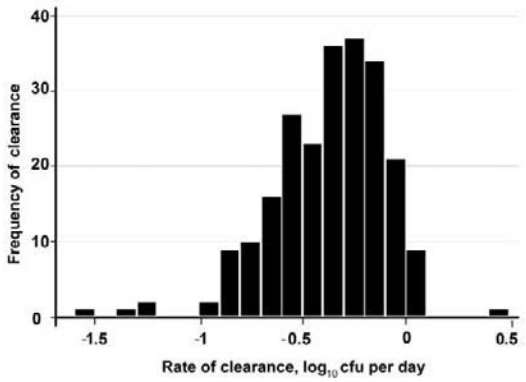

Figure 1. Frequency distribution for the rate of clearance of infection (log_{10} colony-forming units [cfu] per mL of cerebrospinal fluid per day) in the combined cohort of 262 patients.

the date treatment was started to either 14 or 70 days, depending on the analysis, the date last seen, or the date of death. Models were compared using the likelihood ratio test. Additional analyses were done with patients who were lost to follow-up classified as having died. The findings from these analyses were similar, and so only the data with subjects who were lost to follow-up censored when last seen are presented here. Linear regression was used to explore associations of other variables with the rate of clearance of infection [15] and the Spearman rank correlation coefficient, to examine the association between cytokines and CD4 cell count. Analyses were conducted using Stata, version 10 (StataCorp).

RESULTS

Table 1 shows the characteristics at baseline of the 262 subjects recruited from the 4 cohorts. Five subjects (2%) were lost to follow-up within 2 weeks, and an additional 38 subjects (15%) died. By 10 weeks, 9 subjects (3%) were lost to follow-up, and an additional 81 subjects (31%) had died. The rate of clearance of infection could not be measured for 31 subjects who died or were lost to follow-up before a second measurement of colony-forming units; for the remaining 231 subjects, the rate of clearance of infection was approximately normally distributed with a mean (± standard deviation [SD]) clearance of -0.37 ± 0.27 log_{10} cfu per day (figure 1).

Associations with mortality at 2 and 10 weeks. Consistent

Table 1. Baseline Clinical and Laboratory Characteristics and Clinical Outcomes of the Combined Cohort

Characteristic	Patients (n = 262)
Sex	
Female	143 (55)
Male	119 (45)
Age, years	33 (29–38)
Weight, mean kg (±SD)	52 ± 11
Abnormal mental status	65 (25)
CD4 cell count, 10^6 cells/L	26 (9–56)
Viral load, copies/mL	150,997 (480,00–372,475)
CSF data at baseline	
Opening pressure,[a] cm H_2O	27 (17–36)
WBC count, cells/μL	14 (1–54)
Fungal burden, cfu/mL of CSF	271,371 (38,963–1,112,423)
IFN-γ level, pg/mL	30 (7–96)
TNF-α level, pg/mL	9 (3–22)
IL-6 level, pg/mL	192 (43–1417)
Mean rate of clearance of infection (±SD), log_{10} cfu per day	-0.37 ± 0.27
Deaths[b]	
At 2 weeks	38 (15)
At 10 weeks	81 (31)

NOTE. Data are no. (%) of patients or median values (interquartile range), unless otherwise indicated. Data on viral load and cytokine levels were not available from the Uganda cohort. Cfu, colony-forming units; CSF, cerebrospinal fluid; IFN, interferon; IL, interleukin; SD, standard deviation; TNF, tumor necrosis factor; WBC, white blood cell.

[a] Normal range, 7–18 cm H_2O.

[b] Five patients were lost to follow-up before 2 weeks, and 9 patients were lost to follow-up before 10 weeks.

Table 2. Variables Associated with Mortality at 2 and 10 Weeks in Both Univariate and Multivariate Analysis

	2-week mortality[a]					10-week mortality[b]				
		Univariate		Multivariate			Univariate		Multivariate	
Variable, value	Patients	HR (95% CI)	*P*	HR (95% CI)	*P*	Patients	HR (95% CI)	*P*	HR (95% CI)	*P*
Colony-forming units at baseline										
<4.95	3/85 (4)	1.0	<.001	1.0	.006	17/85 (20)	1.0	<.001	1.0	.02
4.95–5.87	12/86 (14)	4.1 (1.2–14.5)		8.2 (1.0–68.5)		26/86 (30)	1.6 (0.9–3.0)		1.8 (0.9–3.6)	
>5.87	22/85 (26)	8.3 (2.5–27.6)		11.3 (1.4–90.6)		36/85 (42)	2.7 (1.5–4.8)		2.3 (1.2–4.5)	
Altered mental status										
No	16/197 (8)	1.0	<.001	1.0	.01	43/197 (22)	1.0	<.001	1.0	<.001
Yes	22/65 (34)	4.9 (2.6–9.4)		3.4 (1.3–8.5)		38/65 (58)	3.9 (2.5–6.0)		3.4 (2.0–5.8)	
Slope										
<−0.55	2/57 (4)	1.0	<.001	1.0	.04	9/56 (16)	1.0	<.001	1.0	.02
−0.55 to −0.33	2/59 (3)	1.0 (0.1–6.8)		0.6 (0.08–4.3)		10/59 (17)	1.1 (0.4–2.7)		0.9 (0.4–2.3)	
−0.33 to −0.18	5/58 (9)	2.5 (0.5–12.8)		1.3 (0.2–7.0)		18/55 (33)	2.1 (1.0–4.7)		1.7 (0.8–3.8)	
>−0.18	13/57 (23)	7.0 (1.6–31.2)		2.6 (0.5–12.4)		25/56 (45)	3.6 (1.7–7.7)		2.1 (0.9–4.7)	
CD4 cell count, 10^6 cells/L										
⩽25	14/113 (12)	1.0	.06	…		34/112 (30)	1.0	.2	…	
>25	6/113 (5)	0.41 (0.16–1.07)		…		27/109 (25)	0.73 (0.44–1.20)		…	

NOTE. Data are proportion (%) of patients. The *P* values refer to tests for trend. CI, confidence interval; HR, hazard ratio.

[a] For slope fitted on a continuum scale, the univariate HR was 1.52 (95% CI, 1.24–1.85; $P < .001$), for each 0.1 log-unit decrease in rate of clearance. After adjusting for the baseline count and altered mental status, the HR was 1.34 (95% CI, 1.06–1.68; $P = .01$). The CD4 cell count was not statistically significant in multivariate analysis ($P = .8$).

[b] For slope fitted on a continuum scale, the univariate HR was 1.27 (95% CI, 1.13–1.43; $P < .001$), for each 0.1 log-unit decrease in rate of clearance. After adjusting for the baseline count and altered mental status, the HR was 1.18 (95% CI, 1.04–1.33; $P = .008$). The CD4 cell count was not statistically significant in multivariate analysis ($P = .5$).

with prior studies [1, 9, 12], altered mental status (defined as a Glasgow coma score of <15) at presentation and high baseline organism load were associated, very significantly, with mortality (table 2; figure 2). In addition, a slow rate of clearance of infection was associated with mortality in both univariate and multivariate analysis (table 2; figure 2). None of the other variables examined (CD4 cell count, sex, age, weight, CSF opening pressure, CSF white cell count, or viral load) were associated with mortality.

The mean (±SD) rate of clearance of infection for those who died and those who survived was −0.17 ± 0.27 and −0.40 ± 0.27 $\log_{10}$ cfu per day, respectively, at 2 weeks, and −0.27 ± 0.27 and −0.41 ± 0.26 $\log_{10}$ cfu per day, respectively, at 10 weeks. These rates were significantly less rapid for those who died, compared with those who survived, at both 2 and 10 weeks (difference in mean rate of decline per day, −0.23 $\log_{10}$ cfu per day [95% confidence interval {CI}, −0.35 to −0.11 $\log_{10}$ cfu per day] vs. −0.15 $\log_{10}$ cfu per day [95% CI, −0.22 to −0.069 $\log_{10}$ cfu per day]; $P < .001$, for both cases).

In multivariate models that included altered mental status, baseline organism count, and rate of clearance of infection, all 3 factors remained independently associated with mortality. When the rate of clearance was fitted onto a continuum scale, after adjusting for baseline count and altered mental status, the hazard ratio for each 0.1 log-unit decrease in the rate of clearance of infection was 1.34 (95% CI, 1.06–1.68; $P = .01$) at 2 weeks and 1.18 (95% CI, 1.04–1.33; $P = .008$) at 10 weeks (table 2).

We also fitted the serial colony-forming unit counts as a time-dependent variable. After adjusting for altered mental status at baseline, the hazard ratios for death were 1.91 (95% CI, 1.28–2.85; $P < .001$) at 2 weeks and 1.14 (95% CI, 1.01–1.30; $P = .04$) at 10 weeks, for each unit increase in the last $\log_{10}$ cfu count. When we also adjusted for the baseline colony-forming unit count (in addition to altered mental status), these hazard ratios were 1.82 (95% CI, 1.12–2.96; $P = .007$) at 2 weeks and 1.13 (95% CI, 0.65–1.32; $P = .14$) at 10 weeks, for each unit increase in the last $\log_{10}$ cfu count.

Associations of other variables with rate of clearance of infection. In previous work based on the Thai study, only the antifungal drug regimen and the baseline CSF IFN-γ level were independently associated with the rate of clearance of infection [15]. Patients in this larger cohort were treated with a total of 9 different regimens containing AmB and 3 regimens of fluconazole. In univariate analysis, the strongest associations with the rate of clearance of infection were with treatment that included AmB, CSF IFN-γ level, and baseline organism count ($P < .001$, for all 3 associations). Consistent with the prior analysis, in a multivariate model that included all 3 factors, only treatment with AmB and CSF IFN-γ level remained independently associated with the rate of clearance. In a final model

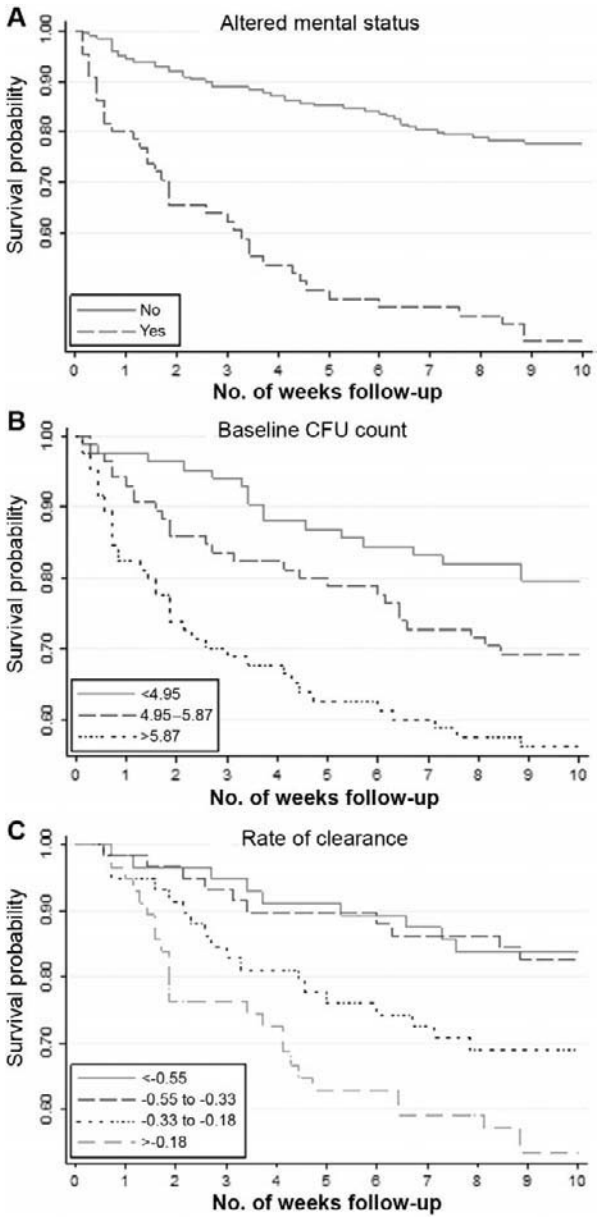

Figure 2. Kaplan-Meier survival curves by *(A)* altered mental status at presentation (yes or no), *(B)* baseline cerebrospinal fluid (CSF) organism load (categorized into tertiles: <4.95, 4.95–5.87, and >5.87 $\log_{10}$ colony-forming units [cfu] per mL of CSF), and *(C)* rate of clearance of infection (categorized into quartiles: >−0.18, −0.18 to −0.33, −0.33 to −0.55, and <−0.55 $\log_{10}$ cfu per mL of CSF per day).

that included these 2 factors, the mean rate of decrease in CSF $\log_{10}$ cfu counts was more rapid for AmB-containing regimens, compared with fluconazole regimens (difference, 0.45 $\log_{10}$ cfu per day; 95% CI, 0.26–0.63 $\log_{10}$ cfu per day; $P < .001$). The $\log_{10}$ IFN-γ level was significantly associated with a more rapid clearance; the increase in the rate of fall in colony-forming units for each unit increment in $\log_{10}$ IFN-γ level was 0.11 $\log_{10}$

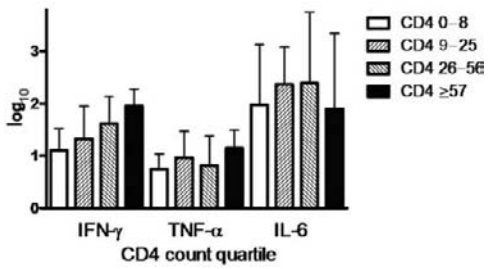

Figure 3. Association of median baseline cerebrospinal fluid cytokine levels (interquartile range) and CD4 cell counts. CD4 cell counts were categorized into quartiles: first quartile, 0–8 $\times$ 10^6 cells/L; second quartile, 9–25 $\times$ 10^6 cells/L; third quartile, 26–56 $\times$ 10^6 cells/L; and fourth quartile, $\geq$57 $\times$ 10^6 cells/L. IFN, interferon; IL, interleukin; TNF, tumor necrosis factor.

cfu/mL of CSF per day (95% CI, 0.06–0.15 $\log_{10}$ cfu/mL of CSF per day; $P < .001$).

Correlations between CSF cytokines and CD4 T cell count. There was a positive correlation between CD4 cell count and log CSF IFN-γ levels ($r = 0.4$; $P < .0001$; figure 3) and between CD4 cell count and CSF TNF-α levels ($r = 0.3$; $P = .001$). CSF IL-6 levels were not correlated with CD4 cell count. In this data set, CSF IFN-γ and TNF-α levels remained strongly positively correlated ($r = 0.7$; $P < .001$), but, in contrast to earlier analysis [15], there was no statistically significant correlation between IFN-γ and IL-6 levels ($r = 0.1$; $P = .08$).

DISCUSSION

In this cohort of 262 patients, we have demonstrated an association between the rate of clearance of infection and survival, independent of the other 2 major prognostic factors (ie, altered mental status at presentation and baseline organism load). The strength of the association in multivariate analysis was stronger with survival at 2 weeks than at 10 weeks. This may reflect the fact that deaths within 2 weeks are nearly all related to cryptococcal infection, whereas, after this time point, deaths are increasingly related to other complications of late-stage HIV infection. The results lend strong support to the use of the rate of clearance of infection as both a statistically powerful and clinically relevant marker of treatment response. The shape of this relationship, whether it is linear or whether there is a cutoff above which more rapid clearance has little further benefit, remains to be defined by analysis of larger cohorts, although the data do suggest that there may be a lesser impact on outcome at the more rapid rates of clearance. Larger phase III cohorts, with larger numbers of patients on particular drug regimens, will also be needed to test whether the rate of clearance fulfills the additional criteria of a surrogate marker of treatment response [16]. Larger cohorts will also be needed to explore with adequate power the possible effect of additional factors, such as fungemia (not examined in this study), on mortality.

Given the dependence of the rate of clearance of infection on antifungal regimen, it is not possible to completely exclude the possibility that an association between rate of clearance and outcome could be observed in this cohort if fluconazole therapy were associated with higher mortality by a separate unknown mechanism, independent of its association with a slow clearance of infection. However, it seems more likely that a prolonged exposure to the organism, as a result of a high organism load at baseline and a slow clearance, had a direct impact on outcome,

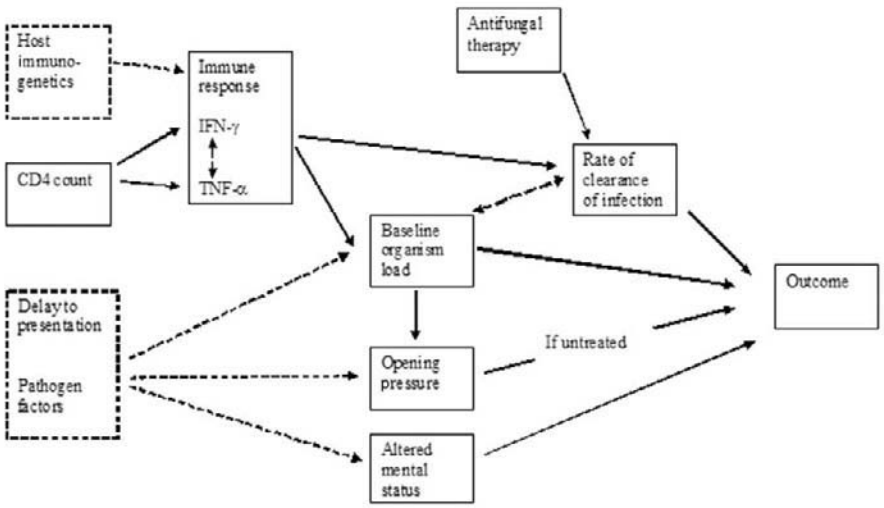

Figure 4. Model illustrating possible relationships between factors associated with rate of clearance of infection and survival. Proposed causal links are shown with solid arrows, noncausal associations with long-dashed arrows, and speculative associations with short-dashed arrows. IFN, interferon; TNF, tumor necrosis factor.

as suggested by an examination of prior trials [9, 10, 12, 17] and our analysis.

The associations between variables in the cohort have led us to propose a model for how the factors determining the rate of clearance of infection and mortality may interrelate (figure 4). The proposed causal nature of the associations in the model remain speculative, although in one instance (ie, the association between IFN-γ level and rate of clearance of infection), causality could be tested by intervention studies, such as those published and ongoing to examine the effects of adjunctive therapy on IFN-γ level (ISRCTN72024361) [18].

Notable was the fact that, in this cohort, we could not demonstrate an association between baseline CSF opening pressure and survival, as has been found in some prior studies [19]. Efforts were made to ensure accurate measurement of opening pressure in all patients, and in all trials, patients had a minimum of 4 lumbar punctures, according to protocol, and further lumbar punctures if the opening pressure was raised. This aggressive approach to management of increased CSF pressure may have reduced its effect on outcome [20]. The size of this cohort enabled us to demonstrate an association between CSF IFN-γ level (but not IL-6 level) and CD4 cell count, consistent with the known reduction in IFN-γ level and the preservation of IL-6 release in late-stage HIV infection [21, 22].

An increased understanding of the factors determining the rate of clearance of infection and outcomes increases our ability to examine the impact of individual components. Thus, for example, variations in the immunity (eg, CSF IFN-γ level) of individual patients can be controlled for in trials examining the effect of novel drug regimens on clearance of infection. An increased understanding of how the factors determining outcome interrelate may also help clarify opportunities for intervention—for example, more rapidly active drug combinations, adjunctive immunotherapy, or earlier diagnosis and treatment—and may help prioritize research questions—for example, understanding the pathophysiological basis of increased CSF pressure and altered mental status.

Our study demonstrates that the rate of clearance of infection is not only a statistically powerful endpoint but also a clinically meaningful one. The results support the use of rate of clearance, or early fungicidal activity, as a means to explore antifungal drug dosages and combinations in phase II studies that can prioritize novel regimens for testing in phase III clinical endpoint trials.

Acknowledgments

We thank Supraphada Pinpraphaporn, Bina Maharjan, Anna Checkley, Vanaporn Wuthiekanun, Premjit Amornchai, Nongluk Getchalarat, Pissamai Manupan, Jintana Suwanpruek, Nick Day, and Sharon Peacock for help with the study at Sappasithiprasong Hospital; Nomqondiso Sidibana, Tom Crede, Vanessa Burch, Anthony Williams, Noxolo Mahlaza, and Elma de Vries for help with the studies at GF Jooste hospital; and James Mwesigye, Joselyne Rwebembera, Ali Chakera, Emma Wall, and Irene Andia for help with the study at Mbarara hospital.

Financial support. This work was supported by the UK Medical Research Council (grant G0501476), a British Infection Society Fellowship to T.B., and a Wellcome Trust Training Fellowship in Tropical Medicine to A.E.B. (grant 069991) and was part of the Wellcome Trust–Mahidol University–Oxford Tropical Medicine Research Programme.

Potential conflicts of interest. All authors: no conflicts.

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