Induction of the Antigen 85 Complex of *Mycobacterium tuberculosis* in Sputum: A Determinant of Outcome in Pulmonary Tuberculosis Treatment

Robert S. Wallis, Mark Perkins, Manijeh Phillips, Moses Joloba, Barbara Demchuk, Alice Namale, John L. Johnson, Donna Williams, Kathy Wolski, Lucileia Teixeira, Reynaldo Dietze, Roy D. Mugerwa, Kathleen Eisenach, and Jerrold J. Ellner

Case Western Reserve University, Cleveland Ohio; Universidade Federal do Espirito Santo, Vitoria, Brazil; Makerere University, Kampala, Uganda; Duke University Medical Center, Durham, North Carolina; University of Arkansas Medical Center, Little Rock, Arkansas

Sputum quantitative culture, acid-fast smear, days-to-positive by BACTEC, and *Mycobacterium tuberculosis* antigen 85 complex were monitored during therapy in 42 patients with pulmonary tuberculosis (TB). By BACTEC, 4 patients were persistently positive on days 90–180, and treatment ultimately failed in 2 of these. Antigen 85 expression increased in subjects in whom disease persisted (persisters) from days 0 to 14 when the difference between persisters and nonpersisters was statistically significant ($P = .002$). Only antigen 85 complex values at day 14 suggested TB persistence at or after day 90. All subjects with day 14 antigen 85 complex values <60 pg/mL responded rapidly to treatment and were cured. Of those with values >60 pg/mL, in 33% TB persisted at or after day 90 and treatment failed in 17%. Biologic factors expressed early in therapy, not related to compliance or resistance, may exert a substantial influence on outcome. The antigen 85 complex is critical in cell wall biosynthesis and is induced by isoniazid in vitro. Its induction may represent an adaptive transition to a persistent state during therapy.

Mycobacterium tuberculosis has a remarkable ability to persist under adverse conditions, often resisting both the host immune response and prolonged combination chemotherapy. Mycobacterial survival in conditions of low oxygen tension is enhanced by induction and accumulation of the heat-shock protein α-crystallin in the cell wall [1, 2]. The mechanisms involved in persistence during therapy are less well understood, as there are no satisfactory models to study this phenomenon in vitro. However, it is generally believed that most actively replicating bacilli are killed early in therapy and that prolonged treatment is required to eradicate persisting organisms exhibiting reduced or altered metabolism. As a consequence, tuberculosis (TB) therapy is more prolonged and more complex than that of nearly all other infections.

Several studies have suggested that the kinetics of the early response to TB therapy provide important clues regarding the likelihood of relapse. By meta-analysis, Mitchison [3] found that regimens with superior sterilizing activity at 1–2 months of treatment had lower relapse rates [3]. Epstein et al. [4] reported that quantitative assessment of viable mycobacteria in sputum (as days to positive in mycobacterial growth indicator tube [MGIT] cultures) during therapy predicted outcome in individual patients [4]. In that study, all 13 in whom treatment failed but none of 13 who were cured had ⩾1 positive MGIT culture within 20 days by day 40 of therapy. These reports suggest it may be possible to use early markers to individualize TB therapy, potentially offering superior outcomes and greater efficiency in resource utilization.

In the present study, sputum $\log_{10}$ *M. tuberculosis* colony-forming units (cfu) and quantitative microscopy (acid-fast bacilli [AFB]), days-to-positive (DTP) by BACTEC, and antigen 85 complex expression were evaluated prospectively during early multidrug therapy of smear-positive pulmonary TB. The antigen 85 complex comprises 3 30- to 32-kDa related proteins that are mainly localized to the extracellular space during in vitro growth of *M. tuberculosis* and are secreted in an energy-dependent process involving an upstream signal sequence [5, 6]. The proteins, mycolyl transferases, are essential for cell wall biosynthesis [7]. Expression of the antigen 85 complex is induced by isoniazid in what has been interpreted as a response by damaged mycobacteria to maintain the integrity of a partially compromised cell wall [8]. Thus, analysis of antigen 85 expression during therapy might provide insights into mechanisms involved in mycobacterial persistence.

Methods

Subjects and treatment regimen. Patients with cough were prospectively recruited at TB control clinics in Kampala, Uganda, and Vitoria, Brazil. TB was diagnosed on the basis of a positive acid-fast smear of sputum and a compatible chest radiograph and was confirmed by culture. Each subject was counseled before and after testing for human immunodeficiency virus (HIV). Serology for

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All subjects gave written informed consent in their local language for study participation and for testing for human immunodeficiency virus. The study protocol was approved by institutional review boards at Case Western Reserve University, University Hospitals of Cleveland, Universidade Federal do Espirito Santo, Duke University Medical Center, and Makerere University.

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Reprints or correspondence: Dr. R. S. Wallis, Case Western Reserve University School of Medicine, BRB 1037, 10900 Euclid Ave., Cleveland, OH 44106-4984 (rsw2@po.cwru.edu).

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HIV-1 was done for all subjects; those identified as HIV-1 seropositive were excluded from further study. Subjects were also excluded from this analysis if their isolates were identified as resistant to isoniazid, rifampin, pyrazinamide, or ethambutol or they were followed <180 days.

Radiographic extent of disease. The radiographic extent of disease was classified in accordance with National Tuberculosis and Respiratory Disease Association criteria [9] into one of three categories: minimal, moderately advanced, or far advanced disease. In persons with minimal disease, lesions are noncavitary with slight-to-moderate density and involve a small area of one or both lungs (total extent is less than the volume of one lung above the second chondrosternal junction and the spine of the fourth or body of the fifth thoracic vertebrae). In persons with moderately advanced disease, slight to moderately dense lesions occupy a total volume of one lung or less and dense lesions are less than one-third the volume of one lung; cavitary lesions are <4 cm in diameter. In far advanced disease, lesions are more extensive than in moderately advanced disease.

After collection of baseline specimens, subjects were treated daily with isoniazid, rifampin, ethambutol, and pyrazinamide at standard doses for 2 months and then with isoniazid and rifampin daily for 4 months. Patients were hospitalized for the initial 2 weeks of treatment to facilitate frequent sputum collection. During that time, therapy was directly observed. Thereafter, therapy was self-administered. Compliance was assessed by review of dispensing records, clinic attendance, and by urinary isoniazid metabolite testing. Subjects were classified as having treatment failure or relapse if *M. tuberculosis* was repeatedly identified on days 120–180 or after day 180, respectively, in conjunction with symptoms and radiographic findings consistent with active TB.

Specimen processing. Three sputum specimens were obtained prior to initiation of therapy. Two specimens were obtained on days 2, 4, 7, 14, 30, and monthly thereafter. Specimens were digested at room temperature for 1 min with 1 mL (25 mg/mL) of *N*-acetyl cysteine (NALC) in phosphate buffer (pH 6.8). Specimens were vortexed with several 4-mm glass beads. An aliquot of 0.25 mL was removed and frozen for antigen analysis. A 2.5-mL aliquot of the remaining specimen was decontaminated by addition of 2.5 mL of a 1:1 mixture of 4% NaOH and 2.9% sodium citrate for 15 min. The specimen was then washed by addition of 45 mL of phosphate buffer. Bacilli were sedimented, diluted serially in 0.25% Tween-80 (Sigma, St. Louis) in 0.9% NaCl, and plated on Middlebrook Cohn 7H10 agar medium, placed in BACTEC culture, and examined by acid-fast microscopy. Even though decontamination of sputum with NaOH reduces cfu by 80% [10], we used this method because, in a pilot study, NaOH resulted in less contamination than did an antibiotic-containing medium and because the reduction in cfu levels due to use of NaOH was consistent among specimens.

85 ELISA. Culture filtrate of *M. tuberculosis* H37Rv was prepared from spent Proskauer-Beck medium as previously described [11]. The antigen 85 complex represented 10% of the protein content of the filtrate by densitometry and Western blot analysis with monoclonal antibody TBC-27 [12]; this antibody was also used for antigen capture. TBC-27 was purified from hybridoma cells grown in serum-free medium, biotinylated using sulfo-*N*-hydroxysuccinimide biotin (Pierce, Rockford, IL), and fixed to avidin-precoated plates (Pierce). Nonspecific protein binding was inhibited with Superblock (Pierce). Samples were diluted in an equal volume of Superblock, added to single ELISA wells, and incubated overnight. Antigen was detected using rabbit bacille Calmette-Guérin antiserum (Dako, Carpinteria, CA) that had been preabsorbed using a column of immobilized TBC-27. Bound rabbit immunoglobulin was detected with alkaline phosphatase–conjugated anti-rabbit immunoglobulin (Pierce). After being washed, the alkaline phosphatase signal was amplified by a recycling NADH method described by Self [13]. Amplifier (0.02 m*M* $NAPD^+$ in 50 m*M* diethanolamine, 1 m*M* $MgCl_2$, 0.1 m*M* $ZnCl_2$, 15 m*M* NaN_3, pH 9.5) was added to each well. After 10 min, a diaphorase-dehydrogenase mixture was added (2 mg/mL alcohol dehydrogenase, 1.5 mg/mL diaphorase, ethanol 4%, 0.55 m*M* *p*-iodonitrotetrazolium violet, and 5 mg/mL bovine serum albumin, pH 7.2). OD at 492 nm was determined after an additional 5 min. The results from the specimens were compared to those of a serially diluted standard at 1–1000 pg/mL, using four-parameter analysis (Softmax; Molecular Devices, Menlo Park, CA). The calculated values were

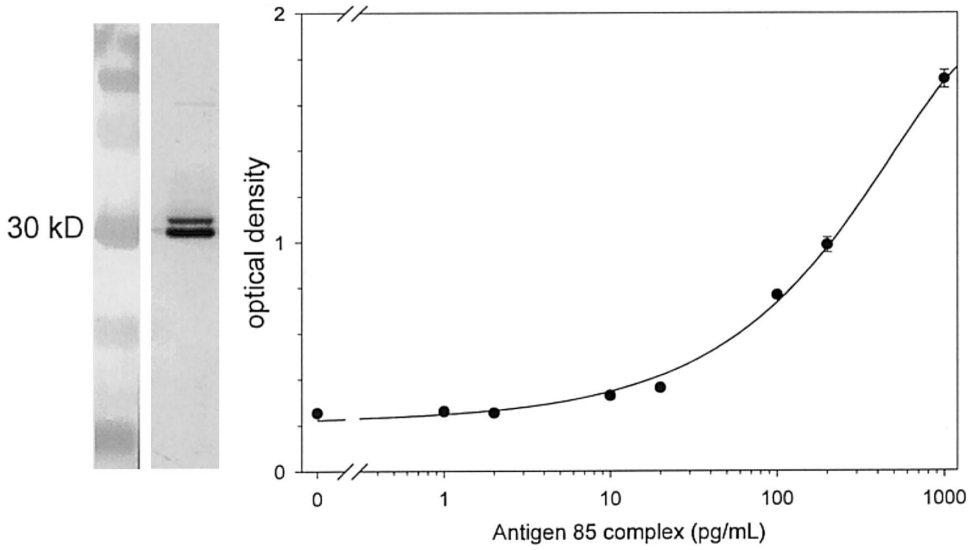

Figure 1. Left, Western blot of *M. tuberculosis* filtrate with TBC-27 (monoclonal antibody used for antigen capture). Right, representative standard curve.

Table 1. Baseline parameters in subjects grouped according to study site and microbiologic response to TB treatment.

	Location			Disease		
	Brazil (n = 21)	Uganda (n = 21)	P	Nonpersistent (n = 38)	Persistent (n = 4)	P
Age (years)	34.8 ± 13	25.8 ± 7	.01	30.6 ± 11	27.8 ± 10	.64
Body mass (kg)	56.3 ± 12	50.4 ± 7	.06	53.2 ± 9.1	55.0 ± 17	.74
Extent of disease (%)						
Minimal	0	35	.01	16.7	25	.62
Moderate	15	10		11.1	25	
Far advanced	85	55		72.2	50	
85 (pg/mL)	34.0 ± 31	41.8 ± 29	.41	39.8 ± 31	23.8 ± 10	.31
$\log_{10}$ AFB/mL	6.1 ± .86	6.4 ± .88	.27	6.29 ± .82	5.42 ± 1.1	.06
$\log_{10}$ cfu/mL	5.9 ± .84	5.6 ± .73	.20	5.79 ± .78	5.40 ± .99	.36
BACTEC DTP	2.6 ± 1.4	2.9 ± 1.4	.49	2.47 ± 1.3	3.63 ± 2.3	.13
Persistence ⩾90 days (%)	4.8	14.3	.61			

NOTE. In subjects with persistent disease, cultures obtained at or after day 90 had growth detected by BACTEC within 20 days. Data are mean ± SD or %. AFB, acid-fast bacilli; cfu, colony-forming units; DTP, days to positive.

adjusted to correct for dilution by NALC by the formula: (original specimen volume + 1)/(original specimen volume).

Data analysis. Values from multiple specimens from each subject on a given day were reduced to a single mean value to avoid statistical errors arising from repeated sampling. Comparisons between groups were done using 2-tailed Student's t test or Fisher's exact test for continuous or binary data, respectively, using RS1 (BBN, Boston). Cultures without growth were coded as 99 or 0 for DTP or cfu, respectively. Zero values and negative cultures were excluded in calculations of the coefficient of variation (SD/mean). The correlation between variables was measured using the Pearson product method. Logistic regression was done using SigmaStat (SPSS, Chicago).

Results

Test characteristics. A Western blot of *M. tuberculosis* culture filtrate using the capture monoclonal antibody TBC-27 is shown in figure 1. The lower band represents 85B; 85A and C are in the upper band. Recognition of A, B, and C by TBC-27 was confirmed by 2-D Western blot (not shown). Figure 1 also shows a typical standard curve of the assay. The detection threshold of the assay was 2–10 pg/mL. The coefficient of variation of duplicate wells of *M. tuberculosis* filtrate was 1%–5%.

Distribution of persistent TB and treatment failure. Forty-two subjects were studied (21 in Brazil, 21 in Uganda) with follow-up of 308 ± 70 days (mean ± SD). The baseline characteristics of the subjects are described in table 1. Brazilian subjects were older and had more advanced disease by chest radiography but did not differ by any microbiologic parameter.

The duration of TB persistence was defined as the last day during therapy in which BACTEC cultures became positive in <20 days. This criterion was selected on the basis of results reported by Epstein et al. [4], who found that a similar parameter—the duration of MGIT culture positivity within 20 days—predicted outcome. Four subjects (1 Brazilian, 3 Ugandans) were identified as having persistent disease; each had ⩾1 BACTEC culture at or after day 90 of therapy that become positive within 20 days. An additional group of 8 subjects had a somewhat delayed response to therapy and remained rapidly positive through day 60. The frequency distribution of persistence is shown in figure 2.

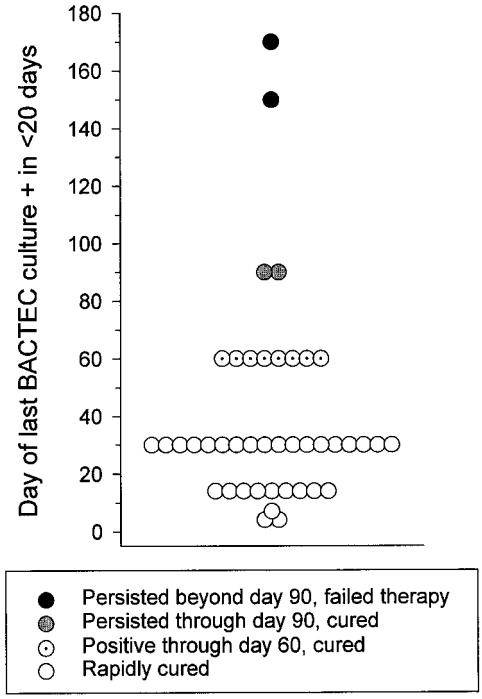

Figure 2. Frequency distribution of TB persistence (day of last BACTEC culture positive within 20 days) and relationship to outcome in pulmonary TB.

Table 2. Microbiologic parameters of individual sputum specimens during therapy in 2 subjects in whom treatment failed.

	Subject 29 (Brazil)				Subject 80730 (Uganda)			
Day	AFB	cfu	DTP	Antigen 85	AFB	cfu	DTP	Antigen 85
0	5.74	5.97	1	20	5.78	4.53	7	40
0	6.38	7.13	1		6.27	4.7	C	0
0	4.38	6.60	1		5.4	3.98	6	0
2	7.53	6.38	2	0	6.11	C	8	76
2	5.63	4.81	6	26	6.89	4.06	7	76
4	6.64	5.56	6		5.42	3.37	9	79
4	7.11	5.89	4	55	6.19	3.59	9	
7	7.25	5.83	2	99	5.36	3.03	10	75
7	7.00	5.54	2	75	5.56	3.01	9	56
14	6.09	4.42	7	60	6.05	3.78	9	90
14	6.74	5.06	6	77	6.06	3.66	9	69
30	5.29	3.11	12	0	5.2	0	18	82
30	5.42	3.23	12	21	4.9	0	C	
60	2.81	0.00	25	0	0	0	NG	47
60	0	0.00	33	0	0	0	C	149
90	0	0.00	NG		0	0	NG	0
90	0	0.00	NG		0	0	NG	
120	0	0.00	NG	24	0	0	NG	12
120	0	0.00	NG	26				
150					**5.36**	**0**	**3**	**81**
170	0	0.00	NG					
170	**0**	**0.00**	**18**					
180	0	0.00	NG		**0**	**0**	**C**	**58**
180	0	0.00	NG		**0**	**0**	**18**	**103**
270					**6.54**	**>3**	**2**	
270					**6.71**	**>3**	**2**	
360	**3+**	**>3**						
360	**3+**	**>3**						

NOTE. AFB, acid-fast bacilli (mL); cfu, colony-forming units (mL); DTP, days to positive; antigen 85 (pg/mL); C, contaminated; NG, no growth. Specimens leading to classification of subjects as having persistent disease are shown in bold.

Two treatment failures were identified, 1 at each site. The overall treatment failure rate was 4.8%, but it was 50% (2/4) in subjects who were positive at or after day 90 versus 0/36 in all other subjects ($P = .007$). Both patients with failed treatment were culture- and smear-negative for ⩾2 months in midtherapy but became positive late in therapy (table 2). In subject 29, failure was attributed to complete noncompliance during the last 2 months of therapy. The other subject (80730) appeared to have reduced compliance during this time as evidenced by delayed clinic attendance; however, he had a positive urinary isoniazid metabolite test at each visit. In neither case was 1° or 2° drug resistance detected. The results of the cultures leading to the classification of the treatment failures as persistent disease were not available until late in therapy (days 188 and 153). Thus, this parameter would not have been directly useful for clinical case management.

Baseline evaluations. Subjects who ultimately persisted as positive at or after day 90 of treatment could not be distinguished from other subjects by any baseline clinical or microbiologic parameter (table 1). Baseline AFB, cfu, and DTP measures were all highly intercorrelated (all $P <$ .0001), but none of these parameters correlated with pretreatment antigen 85 complex scores ($P = .1–.3$). The cfu were 0.45 $\log_{10}$ U lower than corresponding AFB scores ($P <$ .0001), in part reflecting the loss of viability due to processing with NaOH for bacterial decontamination. The mean coefficients of variation of antigen 85 complex, cfu, AFB, and DTP in multiple specimens obtained on the same day from the same subject were 72%, 16%, 14%, and 24%, respectively. The large coefficients of variation of the antigen 85 ELISA may reflect the combined effects of sputum inhomogeneity and small sample volume (100 μL).

Changes during therapy. Cumulative expression of antigen 85 during the first 30 days (estimated as area under the curve) was greater in subjects in whom TB persisted at or after day 90 than in all other subjects ($P = .005$) (figure 3). Neither AFB, cfu, nor DTP differed in cumulative expression between subjects in whom TB persisted and others during this time ($P = .15$, .45, and .56, respectively). Pretreatment values generally did not correlate with subsequent values for any parameter. However, day 2 values correlated with subsequent values through day 14 for 85B and DTP ($P <$

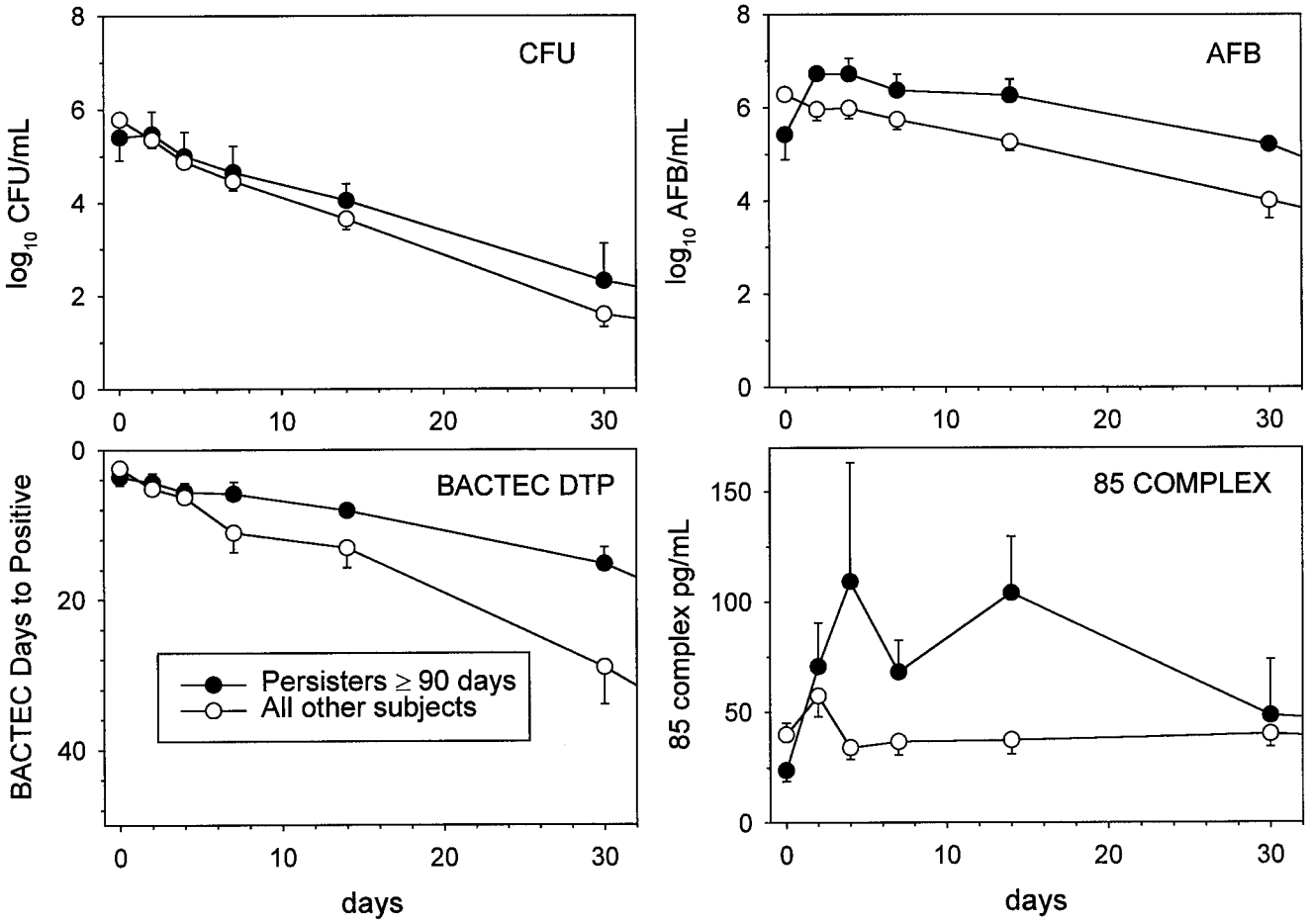

Figure 3. Sputum *M. tuberculosis* colony-forming units (cfu), acid-fast bacilli (AFB), days-to-positive (DTP) by BACTEC, and antigen 85 complex measurement during pulmonary TB therapy. Subjects in whom TB persisted had ⩾1 positive culture by BACTEC within 20 days of day 90 or later. Symbols indicate mean and SE.

.006) and through day 7 for AFB and cfu ($P < .01$). Such correlation over time within individuals is generally seen in most biologic processes.

Expression of antigen 85 complex increased significantly from day 0 to days 7 and 14 only in the subjects in whom TB persisted ($P = .05$). No significant increase from baseline was observed in either group for any other parameter, although a trend toward an increase was noted in AFB from days 0–2 in those with persistent disease ($P = .067$). The difference in antigen 85 expression between persisters and nonpersisters reached $P = .002$ by day 14. The difference did not appear to be an artifact related to the small number of those with persistent TB, as the difference remained significant if the definition of persistence was broadened to include subjects who persisted positive at or after day 60 ($n = 12$, $P = .012$). The difference was also not a reflection of differences between Brazilian and Ugandan subjects, as these were equal on that day ($P = .15$). Induction of the antigen 85 complex resolved by day 30, although in 1 subject in whom treatment failed (80730), high level antigen 85 expression continued through day 60. As indicated in table 2, high level antigen 85 expression recurred in this subject at the time that treatment failed.

Predicting persistence. Logistic regression analysis was performed to determine the extent to which day 7–30 values of all early markers predicted persistence to day 90 or beyond. As indicated in table 3, a significant relationship was identified only for day 14 antigen 85 complex. A scatter plot of the relationship between outcome and day 14 85 complex is shown

Table 3. Univariate logistic regression analysis of the determinants of TB persistence at or after day 90 during therapy.

Variable	Coefficient	*P*	OR (95% CI)
85 complex pg/mL, day 14	0.0336	.026	1.034 (1.004–1.065)
$\log_{10}$ acid-fast bacilli/mL, day 14	1.179	.093	3.250 (0.820–12.877)
BACTEC days-to-positive, day 30	−0.0518	.431	0.950 (0.835–1.080)

NOTE. Odds ratio (OR) reflects change in risk attributed to a 1 U change in variable. Relative risk reflecting change of Δx may be calculated as $e^{(x*\text{coefficient})}$. CI, confidence interval. No other parameters tested approached statistical significance for days 7–30.

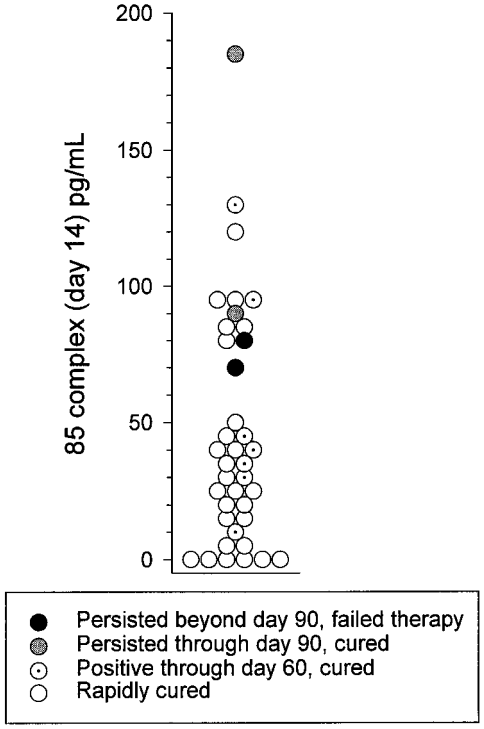

Figure 4. Relationship of antigen 85 complex concentration in sputum on day 14 of therapy to outcome in pulmonary TB.

in figure 4. All subjects with day 14 antigen 85 levels <60 pg/mL (68% of the total) had clear cultures by day 90 and were cured. Of the subjects with values >60 pg/mL, 33% persisted positive at or after day 90 and for 17% therapy failed.

Discussion

TB is unique among chronic infections in that despite substantial interpatient variation, treatment is based on a standardized regimen applied with little individual adjustment (directly observed short-course chemotherapy). Our study suggests that biologic factors expressed early in therapy, not related to patient compliance or traditional measures of drug resistance, exert a substantial influence on outcome. Expression of the *M. tuberculosis* antigen 85 complex increased during the first 2 weeks of therapy in subjects whose cultures persisted rapidly positive late in therapy. Ultimately, therapy failed in half of this group. Its concentration on day 14 was the only predictor of persistence to day 90 or beyond. These observations have practical implications for assessing patient responses to TB treatment and for more fundamental questions regarding mycobacterial persistence.

The antigen 85 complex is essential for synthesis of trehalose dimycolate (cord factor), a glycolipid that defines many of the physical characteristics of the mycobacterial cell wall (including acid fastness), and is a marker for virulence [7, 14, 15]. Isoniazid inhibits mycobacterial mycolic acid synthesis, in part through inhibition of 2-trans-enoyl-ACP reductase [16], thus causing a rapid decrease in the mycolic acid content of the mycobacterial cell wall [17]. Garbe et al. [8], using metabolic radiolabeling, found that isoniazid rapidly induces antigen 85 synthesis in vitro. The dependence of induction of the 85 complex by isoniazid on new protein synthesis has been confirmed in our laboratory (not shown). In addition, recent studies of gene expression using the microarray method identified the 85C gene as one of several induced by isoniazid (Wilson M, Stanford University, personal communication). These findings raise the possibility that induction of the antigen 85 complex may be a component of an adaptive transition to a persistent state during isoniazid therapy. This hypothesis is indirectly supported by the apparent increase in acid-fast organisms that accompanied increased antigen 85 expression. It is unlikely that this reflected a true 20-fold increase in organisms in such a short interval. Rather, it may reflect increased trehalose dimycolate accumulation and increased acid fastness of persisting bacilli.

The present study did not distinguish between cell wall–associated enzyme and that cleaved free of its signal peptide nor did it differentiate between the three members of the 85 complex. Some of the antigen detected may represent its accumulation on the cell wall as has been described with α-crystallin [2]. Further studies are needed to determine the identity, localization, and molecular mechanisms for the increased protein expression observed in this study.

Our observations also raise questions regarding the potential role of isoniazid in promoting persistence. Although isoniazid previously was considered to be bactericidal against *M. tuberculosis,* that activity is markedly reduced when stationary-phase bacilli are studied [18, 19] or if the duration of culture is extended beyond a few days [20]. Mitchison and Nunn [21] concluded that isolated isoniazid resistance has little effect on clinical outcome in standard short-course TB therapy and that the sterilizing activity of isoniazid in vivo is substantially less than that of rifampin and pyrazinamide. Isoniazid was antagonistic to the bactericidal activity of rifampin plus pyrazinamide in two studies in mice [22, 23]. Although an adverse pharmacokinetic interaction between isoniazid and rifampin was identified in the latter report, the authors noted that the magnitude of that interaction did not appear to be sufficient to explain the antagonism observed since blood levels of rifampin remained 80 times the MIC for *M. tuberculosis* [23]. Thus, further research is warranted to determine whether induction of enzymes for cell wall biosynthesis by isoniazid contributes to persistence of disease and treatment failure during in human TB. Studies are also warranted to determine whether the observed intersubject variation in TB persistence is due to host or microbial factors.

From a practical perspective, early stratification of patients by sputum 85 complex may aid in the cost-efficient allocation of resources in TB control programs and in the preliminary evaluation of new drugs. Future research might revisit detection of mycobacterial antigens in sputum for rapid TB diagnosis.

Despite numerous prior studies, we believe ours to be the first in which a mycobacterial antigen was detected in unconcentrated specimens [24–27]. The simplified protocol for specimen processing described here may be particularly suited to busy clinics in low-income countries in which TB diagnosis in a single clinic visit is a major but as of yet unmet goal.

In summary, monitoring of *M. tuberculosis* antigen 85 complex in sputum during early TB therapy may aid, along with quantitative culture, in evaluating the response to treatment. Additional studies are warranted to improve and extend the tools available for antigen measurement and to explore the application of this approach in the clinic and in basic scientific research.

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