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Heritability Analysis of Cytokines as Intermediate Phenotypes of Tuberculosis

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Abstract

Numerous studies have provided support for genetic susceptibility to tuberculosis (TB); however, heterogeneity in disease expression has hampered previous genetic studies. The purpose of this work was to investigate possible intermediate phenotypes for TB. A set of cytokine profiles, including antigen-stimulated whole-blood assays for interferon (IFN)–γ, tumor necrosis factor (TNF)–α, transforming growth factor (TGF)–β, and the ratio of IFN to TNF, were analyzed in 177 pedigrees from a community in Uganda with a high prevalence of TB. The heritability of these variables was estimated after adjustment for covariates, and TNF-α, in particular, had an estimated heritability of 68%. A principal component analysis of IFN-γ, TNF-α, and TGF-β reflected the immunologic model of TB. In this analysis, the first component explained >38% of the variation in the data. This analysis illustrates the value of such intermediate phenotypes in mapping susceptibility loci for TB and demonstrates that this area deserves further research.

Tuberculosis (TB) poses a major global public health threat [1]. In recent decades, the number of reported cases has increased in both industrialized and developing nations [2]. The natural history of TB follows a variable course after initial infection; only 10% of those infected with *Mycobacterium tuberculosis* develop clinical disease [3]. Although comorbidities, medical treatments, and malnutrition, may increase the risk for developing disease, these conditions, except for human immunodeficiency virus (HIV) infection, account for only a small proportion of TB cases today. There is a growing body of evidence that susceptibility to TB may be affected by host genetic factors. Twin studies [4,5], animal models (reviewed in [6,7]), candidate gene studies [8–21], segregation analysis [22], linkage analyses [22–25], and fine-mapping studies [26] all provide support for host genetic susceptibility. However, the results of these studies differ greatly; therefore, the genetic factors contributing to susceptibility remain unclear.

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Written informed consent was obtained from the head of the household, all adults, and parents or guardians of the children in the household, according to the policies of the institutional review board at Case Western Reserve University and the Ugandan National AIDS Research Subcommittee.

One inherent limitation in previous genetic studies of human TB has been the definition of the disease phenotype. TB is a heterogeneous disease, which presents most often as pulmonary disease but is able to affect nearly all organ systems. Even for pulmonary TB, the presentation may differ between people with progressive primary disease and those with reactivation of a latent form of infection. Because it is such a heterogeneous disease, it is difficult to define a reliable phenotype for genetic analysis. Moreover, TB may develop at any time after infection, and active disease may not be present during data collection but may develop at a later time. In other words, current TB status does not reflect long-term disease risk. Another approach has been to define "liability classes" on the basis of known risk factors for TB, such as age, tuberculin skin test reactivity, and previous infection. However, because these definitions differ among studies, they do not provide a consistent picture of the genetic basis of TB.

One solution to this problem is to study related quantitative traits as the phenotype of interest. This choice is justified because binary traits are often functions of multiple quantitative traits [27]. Also, binary traits, such as disease status (presence and/or absence of TB), may provide less information about a gene or set of genes than a quantitative trait. A well-chosen quantitative trait may be more informative than a clinical diagnosis or a threshold scale and therefore may be more closely tied to gene expression [28]. Analysis of simulated data has shown that there is considerably more information for linkage in quantitative variation than there is in dichotomous traits [29]. For all these reasons, it is of interest to investigate intermediate phenotypes for TB, to gain more information about possible genetic factors involved in disease development.

Because of the limitations of studying disease status alone, we propose that quantitative traits that are based on a model for immunity to TB would offer insights into the genetic determinants of TB. In this study, we investigated 3 cytokines produced in whole-blood culture assays as intermediate phenotypes for TB: interferon (IFN)–γ, tumor necrosis factor (TNF)–α, and transforming growth factor (TGF)–β. IFN-γ is a proinflammatory cytokine produced by T cells that appears to be necessary for the containment of mycobacterial infections [30]. TNF-α is another proinflammatory cytokine modulated by T cell–macrophage interaction and is involved in granuloma formation and containment of TB infection [31]. TGF-β is a dominant inhibitory cytokine that keeps in check the inflammatory responses and promotes fibrosis [32]. In a study population of TB index-case subjects and their household contacts, we estimated heritability for these measured traits. We also derived a trait combining multiple cytokines and evaluated the heritability of this factor.

SUBJECTS, MATERIALS, AND METHODS

Data collection

Between October 1995 and February 1999, consecutive TB index-case subjects and their household contacts were enrolled in a prospective cohort study. The TB index-case subjects were aged ≥18 years, had sputum smear–positive pulmonary TB, had at least 1 person living with them (household contact), and were identified at the Uganda National Tuberculosis and Leprosy Program treatment center at Old Mulago Hospital in Kampala, Uganda. On the initial home visit, which was made within 2 weeks of the diagnosis of TB in the index-case subject, the home health visitor provided general information about the study and health education about TB to all household contacts. A household contact was defined as an individual who had resided in the same household with the index-case subject for at least 7 consecutive days during the 3 months before the diagnosis of TB in the index-case subject.

Measurements

All household members were evaluated for active pulmonary TB through a standard investigation that included a collection of medical history information, symptom survey, chest radiography, mycobacterial smear, and testing for response to antituberculous therapy, if indicated. To assess latent TB infection, a tuberculin skin test with purified protein derivative (PPD) was performed using the Mantoux method. A tuberculin test was considered to be reactive if the greatest diameter of induration was ≥5 mm. Vaccination with bacille Calmette-Guérin (BCG) was determined by the presence of a characteristic scar on the left deltoid and was verified by use of medical records. HIV serostatus was determined by the ELISA method using commercial diagnostic kits (Cambridge BioScience).

Cytokine responses were measured by use of a whole-blood cytokine assay. In this assay, blood cells were stimulated with *M. tuberculosis* culture filtrate and were incubated for 18–120 h at 37°C. After incubation, the supernatant was removed (at 18 h for TNF-α and at 120 h for IFN-γ and TGF-β). Supernatant cytokine concentrations (ng/mL) were measured using commercial ELISA assays for IFN-γ (Endogen) and for TNF-α and TGF-β (R&D).

Data analysis

The measured trait variables analyzed included levels of IFN-γ, TNF-α, and TGF-β. First, all variables were examined for normality. The raw data were highly skewed, and a log transformation (base 10) of the cytokine variables made them approximately normally distributed. However, there were still some outlying observations, which we chose to exclude from further analyses to prevent erroneously high estimates of variance, which could upwardly bias our estimates of heritability. We excluded raw cytokine variables by use of the following criteria: log IFN-γ value <0.10 (6% of sample), log TNF-α value <1.50 (1% of sample), and log TGF-β value <2.50 (0.5% of sample). Then, each of the traits of interest was adjusted by use of linear regression analysis for the presence of BCG scar, PPD reactivity, sex, HIV status, body mass index (BMI), and age, to remove any confounding environmental effects from the measured traits. Residuals were extracted from these regression models and were used in all future analyses. After adjustment for these covariates, the residuals were approximately normally distributed. Observations outside of 3 interquartile-range lengths of the box plot [33] were excluded from the final analysis; for IFN-γ, TNF-α, and TGF-β, this included <1% of the observations for each variable.

By use of the measured cytokine traits, 2 derived traits also were evaluated: the ratio of IFN-γ to TNF-α and the first principal component (PC) of the residuals for IFN-γ, TNF-α, and TGF-β. PC analysis uses a correlation matrix of the variables of interest to create new uncorrelated variables that are linear combinations of the original variables. The goal of this method is to reduce the total number of variables analyzed by explaining most of the variation in the data by 1 or a few new variables [34]. Past work has shown that PC analysis may be able to identify features of the underlying genetic model [35]. Only the first PC (denoted “PC1”) had an eigenvalue >1, so it was retained for further analysis. Using the PC coefficients and the measured values of cytokines, we calculated a PC score for each study subject. This PC score represented a composite measure of all 3 cytokines. To determine internal validity of the score, mean PC scores were compared among the subjects with and without TB by use of the *t* test.

Finally, the heritability of the 4 cytokines and first PC was estimated in 3 groups of subjects. Heritability is defined as the proportion of variance in a trait attributable to genetic effects and can be estimated by use of relative-pair correlations. The 3 methods used to estimate heritability are detailed in the Appendix. First, all eligible subjects (outliers eliminated) were included in the analysis. Then, the analysis was repeated, including only individuals with

reactive PPD skin tests, to control for the absence of infection in cytokine response. The final analysis included all subjects and was adjusted for disease status (active vs. inactive TB). These separate analyses were done to examine whether our results were confounded by disease status. The results of these adjusted analyses did not differ from the unadjusted analysis; therefore, only the analysis of all eligible individuals is provided here.

RESULTS

Descriptive data on the analyzed sample is provided in table 1. The study identified 285 index-case subjects. Cytokine data were unavailable for 108 of these index-case subjects; thus, this analysis included 177 index-case subjects and their household contacts with available cytokine data. When compared by baseline characteristics, index case subjects with and without available cytokine data were slightly more likely to have a BCG scar ($P = .022$). Household contacts with cytokine data were more likely to be older males with a slightly higher BMI ($P < .001$) than were their counterparts without cytokine data. However, since age, sex, and BMI were adjusted for in the linear regression model, these differences among individuals should not have affected the results. Of the individuals in the sample, 34.8% had active TB, 83.9% were PPD positive, 64.1% had a BCG scar, and 24.4% were HIV positive. Significantly more male subjects had TB than female subjects ($P = .023$; data not shown). The mean age of the participants was 18.88 years; male and female subjects did not differ significantly by age ($P = .140$).

Because factors such as the presence of BCG scar, PPD positivity, sex, HIV status, BMI, and age may confound these immunological variables, adjustment for these potential confounders was done by use of regression analysis (table 2). The R^2 values indicate the percentage of variance in cytokine measurement explained by the confounders in the model. For example, the confounding variables explain 18.1% of the variance of IFN-γ expression. After this adjustment, 80%–90% of the variance was left unexplained for the cytokine variables. This unexplained variance might be due, in part, to genetic factors.

Only PC1 had an eigenvalue >1, and it explained 39% of the variation in the data. For this PC, the score coefficients were 0.567 for IFN-γ, 0.619 for TNF-α, and −0.389 for TGF-β. The PC scores for subjects with active TB were significantly different from those for healthy subjects (−0.215 vs. 0.115, respectively; $P = .0002$), which demonstrates the internal validity of this measure in distinguishing between healthy subjects and those with disease.

All relative-pair correlations (table 3) were positive, which indicates that relatives tend to be alike for these variables, a sign that these traits may be in part genetically determined. In this set of pedigrees, TNF-α had the highest parent-offspring correlation (0.3373). Sibling correlations were highest for TNF-α, TGF-β, and PC1. Spousal correlations were >0; this observation, along with the relatively high sibling correlations, suggests shared environmental exposure. Thus, it is important to account for these high correlations in our estimates of heritability.

After accounting for spousal and sibling correlations, the estimated heritability values (table 4) were >10%, with the greatest heritability estimate for TNF-α (68%). The heritability estimate for PC1 also was high (33%). The estimates of heritability determined by simply multiplying the parent-offspring correlation by 2 were not appreciably different from those derived by use of equation 1 in the Appendix. The heritability estimates calculated by use of the formulation by Rice et al. [38] were consistently higher than those calculated by our formulation and served as an upper limit for the heritability value.

DISCUSSION

Infection by *M. tuberculosis* is a necessary, but not sufficient, cause of clinical TB. According to our current understanding of the infection, only 10% of individuals infected by *M. tuberculosis* actually develop clinical disease, which suggests that there are other factors involved in disease susceptibility. Environmental conditions, such as poor economic conditions, malnutrition, stress, and overcrowding, do not fully explain the increased susceptibility of some populations over others, nor do underlying comorbidities or their treatment. Although several studies [4,5,8–26] support genetic factors in human susceptibility to TB, no coherent model for the genetic susceptibility of TB to date has emerged, which may, in part, be due to the heterogeneity of disease expression.

For this reason, we sought to analyze intermediate phenotypes of TB, because they may be more closely tied to gene expression [28]. Complex traits like TB may be the end result of the combined effects of multiple genes, leading to a continuous distribution [27]. There is considerably more information available in the continuous scale of measurement [29], which is amenable for linkage mapping. Thus, identification of an informative intermediate trait will facilitate future approaches in gene mapping, including segregation and linkage analyses.

Of the intermediate traits that we investigated, TNF-α had the greatest heritability (68%). For reference, the heritability of height is ~65%; therefore, the estimated heritability of TNF-α found here suggested a strong genetic component in susceptibility to TB. This finding is greater than the IFN-γ heritability of 39% estimated in a recent twin study from The Gambia [39]. Polymorphisms in IFN-γ, TNF-α, and TGF-β genes have been studied in various infectious and autoimmune disorders. Deficiencies in the IFN-γ receptor 1 gene have been associated with mycobacterial [18] and BCG [19] infection, and mutations in this and the IFN-γ gene have been associated with susceptibility to hay fever, systemic lupus erythematosus (SLE), and rheumatoid arthritis (RA) [40–42]. TB is lethal in TNF-α–deficient mouse strains [30]. Mutations within the TNF-α gene or its promoter region have been associated with increased risk in humans for infectious diseases, such as malaria, leprosy, *Helicobactor pylori* infection, HIV disease progression, and septic shock, as well as autoimmune disorders, such as asthma, SLE, RA, Crohn disease, sarcoidosis, psoriasis, and diabetes [40–42]. In addition, polymorphisms in the TGF-β1 gene have been associated with asthma and diabetes, as well as other chronic disorders [40–42]. To summarize, the literature suggests that IFN-γ, TNF-α, and TGF-β genes may be related to dysfunction of the immune system in general, and, therefore, further study with respect to TB susceptibility is warranted.

Although environmental conditions may confound the results, there are 2 reasons why this effect is minimal. First, the supernatant for TNF-α was harvested after only 18 h of stimulation, so that levels represent early, possibly innate, immune responses to mycobacterial antigens. Second, the estimate has been adjusted for common environmental factors that might affect TNF-α responses, such as HIV status, BCG vaccination, and age. Adjustment for these common environmental factors left 80%–90% of the original variability in the cytokines intact, which suggests that a strong genetic component underlies each of these variables. This finding lends support to the role of TNF-α in host immunity to TB.

Although IFN-γ and TGF-β were both familial traits in this analysis, their degree of heritability was not as great as that of TNF-α. This finding suggests that environmental factors may play a predominant role in the expression of these cytokines. However, when information from all cytokines was combined into a single trait by use of PC analysis, a

pattern emerged that was consistent with our underlying model for immunity to TB. This is similar to a previous study, in which PC analysis illustrated characteristics of the underlying genetic model [35]. The magnitude and sign of the score coefficient corresponded with the effect of each cytokine on the regulatory interaction between macrophages and T cells. The coefficients of both IFN-γ and TNF-α were positive and of similar magnitude, which suggests that they had a stimulatory effect in the immune response; the coefficient of TGF-β was negative, which is consistent with the inhibitory effect of this cytokine [43]. Thus, as a derived trait, the PC score was internally consistent with our immune model. PC1 had the second highest heritability estimate of 33%. Furthermore, the mean PC score was significantly lower in TB index-case subjects than in household contacts, which suggests either a deficiency of IFN-γ and TNF-α relative to TGF-β or a greater inhibitory effect of TGF-β in general. These imbalances in cytokine responses may be clues to the increased susceptibility to TB.

Our study had a number of inherent limitations. First, only *M. tuberculosis* culture filtrate was used to stimulate cells in the whole-blood cytokine assay. *M. tuberculosis* culture filtrate contains a mixture of T cell antigens and macrophage-activating molecules; therefore, cells were stimulated simultaneously with a broad array of *M. tuberculosis* antigens. Moreover, *M. tuberculosis* culture filtrate is an antigen preparation enriched for soluble proteins that stimulate primarily $CD4^+$ T cell subsets. This may not be representative of the sequence of events in a natural infection. Further studies of this nature are needed in which a panel of antigens that includes intact organisms is used. Second, the underlying biologic relationships in the pedigrees were not confirmed with genotyping, because informed consent was not given for this evaluation. Special efforts were made, through multiple interviews, to verify relationships. In addition, where there was any uncertainty in the relationships within a pedigree, that individual was deleted from the analysis.

Our analysis reaffirmed the idea that susceptibility to TB may be under some genetic control. Examination of relative-pair correlations portrayed the environmental component to TB, for which we have accounted both by adjusting for ascertainment in our computation of relative-pair correlations and by incorporating the spousal correlation into our calculation of heritability. These traits, particularly TNF-α expression and the PC derived from regulatory cytokine responses, are worthy of further investigation. A segregation analysis of these traits would begin to elucidate their genetics; furthermore, they could be used as quantitative traits in linkage analysis to search for TB susceptibility loci. The present analysis suggests that the evaluation of other quantitative variables may provide insights into genetic factors involved in TB susceptibility.

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APPENDIX

If the spousal correlation is negligible, then heritability can be estimated as twice the parent-offspring correlation. However, if the spousal correlation is nonzero, failure to account for it can lead to overestimation of heritability [36]. To adjust for nonzero spousal correlation that could be caused by shared common environment, we assumed the following model:

$$\begin{aligned}\text{Cov (parent, offspring)} &= \tfrac{1}{2}\sigma_g^2,\\ \text{Cov (siblings)} &= \tfrac{1}{2}\sigma_g^2+\sigma_c^2,\end{aligned}$$

and

$$\text{Cov (spouse)}=\sigma_c^2,$$

where σ_c^2 is a common environmental variance, the same for siblings and spouses, and σ_g^2 is the genetic variance. Denoting parent-offspring correlation by r(po), sibling correlation by r(siblings), and spousal correlation by r(spouse), we can estimate heritability and its variance by the following formula (equation 1):

$$h^2=2\,[w_1 r(\text{po})+w_2 r\,(\text{diff})] \tag{1}$$

and $\sigma_{h^2}^2=4\,\{w_1^2\sigma_{r(\text{po})}^2+w_2^2\sigma_{r(\text{diff})}^2+w_1 w_2\,\text{Cov}\,[r(\text{po}), r(\text{diff})]\}$, where r(diff) = r(siblings) – r(spouse) and the weights w_1 and w_2 are inversely proportional to the variances of these components:

$$w_1=[1/\sigma_{r(\text{po})}^2]/[1/\sigma_{r(\text{po})}^2+1/\sigma_{r(\text{diff})}^2],$$

and

$$w_2=[1/\sigma_{r(\text{diff})}^2]/[1/\sigma_{r(\text{po})}^2+1/\sigma_{r(\text{diff})}^2].$$

The REGC package of S.A.G.E. version 3.1 [37] gives estimates of the sibling, parent-offspring, and spousal correlation and the corresponding variance-covariance matrix of the correlation estimates using large sample likelihood theory. This program also includes an ascertainment correction that conditions on the trait values of the index case. Using these estimates, variances, and covariances, we can estimate heritability and its SE from equation 1. Rice et al. [38] gave another equation for calculating heritability when the spousal correlation is nonzero. This third formulation is considered a “maximal” heritability and also incorporates relative-pair correlations as follows:

$$h^2=\frac{[r(\text{siblings})+r(\text{po})][1+r(\text{spouse})]}{1+r(\text{spouse})+2\,[r(\text{spouse})\,r(\text{po})]}.$$

Heritability estimates from all 3 methods are provided in table 4.

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Table 1

Description of analysis sample of tuberculosis (TB) index case subjects and their household contacts.

Group	Subjects, no. (%)
Pedigrees	177
All subjects	554
Sex, female/male	245/309
Sibling pairs	212
Avuncular pairs	22
Grandparental pairs	14
Half-sibling pairs	32
Active TB	193 (34.8)
PPD positive	465 (83.9)
BCG scar	355 (64.1)
HIV infected	135 (24.4)

NOTE. BCG, bacille Calmette-Guérin; HIV, human immunodeficiency virus; PPD, purified protein derivative of tuberculin.

Table 2

Linear regression models of cytokine traits adjusted for presence of bacille Calmette-Guérin scar, purified protein derivative reactivity, sex, human immunodeficiency virus status, body mass index, and age.

Trait	R^2	*P*
IFN-γ	0.181	$<10^{-10}$
IFN-γ/TNF-α	0.156	$<10^{-10}$
TNF-α	0.024	.001
TGF-β	0.012	.026

NOTE. IFN, interferon; TGF, transforming growth factor; TNF, tumor necrosis factor.

Table 3

Relative-pair correlations for each trait with SDs.

	Relative-pair type		
Trait	**Parent-offspring**	**Sibling-sibling**	**Spouse-spouse**
IFN-γ	0.1169 (0.0654)	0.2034 (0.0632)	0.2287 (0.0986)
IFN-γ/TNF-α	0.1454 (0.0747)	0.1841 (0.0695)	0.3078 (0.1074)
TNF-α	0.3373 (0.0688)	0.5860 (0.0581)	0.2404 (0.0968)
TGF-β	0.0960 (0.0681)	0.3856 (0.0680)	0.1363 (0.0867)
PC1	0.1942 (0.0851)	0.4039 (0.0767)	0.3052 (0.1148)

NOTE. IFN, interferon; PC1, first principal component; TGF, transforming growth factor; TNF, tumor necrosis factor.

Table 4

Heritability estimates for cytokine traits using 3 different methods.

Trait	Equation 1[a]	Rice et al. [38][b]	2[*r*(po)][c]
IFN-γ	0.1720 (0.1068)	0.3070	0.2338 (0.1210)
IFN-γ/TNF-α	0.1478 (0.1282)	0.3084	0.2908 (0.1494)
TNF-α	0.6795 (0.1119)	0.8165	0.6746 (0.1376)
TGF-β	0.2795 (0.0936)	0.4708	0.1920 (0.1362)
PC1	0.3317 (0.1426)	0.5483	0.3884 (0.1620)

NOTE. IFN, interferon; PC1, first principal component; TGF, transforming growth factor; TNF, tumor necrosis factor.

[a] See Appendix.

[b] Rice et al. [38] did not offer an estimator of SE for heritability.

[c] *r*(po) is the parent-offspring correlation.