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Strain specificity of antimycobacterial immunity in whole blood culture after cure of tuberculosis

Robert S. Wallis^{1,2,3}, Solange Vinhas⁴, and Ernestas Janulionis²

¹Pfizer, New London CT

²UMDNJ-New Jersey Medical School, Newark NJ

³CWRU School of Medicine, Cleveland OH

⁴UFES, Vitória Brazil

Abstract

Bactericidal assays have facilitated development of most modern vaccines, and have been proposed as indicators of protection after vaccination against tuberculosis. We examined control of intracellular growth of *Mycobacterium tuberculosis* in whole blood cultures of cured TB patients and tuberculin negative healthy volunteers. Cured patients showed superior restriction of growth of the strain with which they had been infected. They similarly showed superior control of growth of a clinical strain (MP-28) that had become attenuated during passage. However, patients and healthy volunteers did not differ with regard to control of *M. tuberculosis* H₃₇Ra. The ability of cured patients to control growth of the strain with which they had been infected correlated with MP-28, but not with H₃₇Ra. These findings indicate *M. tuberculosis* MP-28 may be suitable to assess mycobacterial immunity as growth inhibition in whole blood culture, whereas H₃₇Ra is not. However, additional studies will be required to confirm these observations in additional patient and microbial populations that are distinct according to geography and microbial and host genetics.

Keywords

tuberculosis; whole blood culture; immunity; specificity; diversity

Introduction

Natural infection with *Mycobacterium tuberculosis* in most instances results in the evolution of protective adaptive immunity marked by the acquisition of delayed type hypersensitivity and T cell reactivity to mycobacterial antigens. Similar responses evolve after vaccination against tuberculosis with *M. bovis* BCG or other TB vaccines, suggesting they may serve as biomarkers for protection. [1,2] However, recent field studies indicate that, like the tuberculin skin test (TST), the frequency of antigen-specific IFN γ producing T cells appears more closely

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Correspondence: Dr. Robert S. Wallis, MD FIDSA Pfizer Global R&D, 50 Pequot Ave., MS6025 B3149, New London CT 06320
rswallis@gmail.com (email) 860 732 2573 (voice).

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linked to TB risk than protection. [3-6] Furthermore, BCG strains that afford significantly different levels of protection against experimental tuberculosis have been reported not to differ with regard to antigen-specific T cell frequencies. [7-9] This conundrum has focused attention on the development of functional assays that may better indicate protection.

We and others have previously described a simplified method to assess the outcome of intracellular mycobacterial infection using whole blood culture. [10] Immune control of growth in the whole blood model is inferior in TST negative persons, improves after BCG vaccination, and is impaired by T cell depletion or addition of methylprednisolone or anti-TNF antibody. [11,12] Virulent isolates, such as those causing outbreaks, show superior growth compared to attenuated strains. [13] The assay is highly reproducible, with standard deviations of $<0.08 \log \text{CFU}$ reported in replicate cultures two studies. These characteristics indicate the potential suitability of the model to assess antimycobacterial activity during vaccine development. However, studies to date using different mycobacterial strains have neither clearly indicated a correlation between the strains nor demonstrated the superiority of one as an overall indicator of immunity. It further would be advantageous if an attenuated strain was found suitable, as this would reduce safety concerns and facilitate assay implementation at field research sites.

We previously have reported the use of the whole blood model to assess the bactericidal activity of administered TB chemotherapy, with each patient being studied with his or her infecting strain. [14] Patients were also examined two weeks after the completion of therapy, using three strains: the infecting strain; the attenuated laboratory *M. tuberculosis* strain H₃₇Ra; and MP-28, a clinical isolate that, like H₃₇Ra, had become attenuated following serial passage. [10,11,13, 14] The present analysis compares the immune control of growth of this set of isolates in two cohorts: the cured TB patients from which the strains were derived, and healthy TST-negative volunteers, in whom the same set of isolates were studied. The objective was to examine antimycobacterial immunity in patients after TB cure, and to determine the potential suitability of attenuated TB strains as indicators of protection.

Methods

Subject selection, isolate propagation, and assessment of whole blood bactericidal activity were performed as previously described. [13,14] Briefly, TB patients (N=32) were 18-60 year-old, HIV-1 seronegative, with newly diagnosed first episodes of sputum smear-positive pulmonary tuberculosis, recruited from the outpatient clinic of the Hospital Universitario Cassiano Antonio de Moraes in Vitória, Brazil. Healthy TST-negative volunteers (N=6, 0 mm induration by Mantoux testing) were recruited at the New Jersey Medical School, Newark NJ. All subjects gave written informed consent according to guidelines of the US Department of Health and Human Services and the Brazilian Ministry of Health. Study protocols were approved by the institutional review boards at the Federal University of Espírito Santo, Case Western Reserve University/University Hospitals of Cleveland, and the University of Medicine and Dentistry of New Jersey. Patients were judged as cured based on the clinical, radiographic, and microbiologic response at the completion of standard short course therapy.

Patient strains were propagated in broth culture and genetically characterized by IS6110 DNA fingerprinting. [11,13,15] The resulting RFLP patterns were compared to each other and to a database of isolates established for that purpose. [16,17] Whole blood bactericidal activity was determined as previously described. [11,13,14] Briefly, cultures consisted of equal volumes of blood and tissue culture medium, and were of 72 hr duration. The BACTEC TB-460 system (Becton Dickinson) was used to prepare inocula, develop standard curves relating days to positive (DTP) to inoculum volume, and to determine the extent of growth or killing during whole blood culture. Change in viability was calculated as $\Delta \log_{10} \text{CFU} = \log_{10}(\text{final}) - \log_{10}(\text{initial})$, where *final* and *initial* represent the volumes of the stock culture with DTP values

equal to the completed whole blood culture and its inoculum, respectively. The two studies differed in that means of two duplicate whole blood cultures were used in the NJ study, whereas single cultures were used in Brazil. Detailed laboratory methods and software are available online at <http://rswallis.com>. Differences and correlations between groups were determined by student's t test and the Pearson product method, respectively, using SigmaStat (Systat).

Results

The results of IS6110 DNA fingerprinting of the patient strains in this study have been reported previously. [13] Two patient isolates were found to be identical, and two additional isolates were found to differ by only one band. No group W-Beijing strains were identified. There was no similarity or identity of any strain to MP-28 or H₃₇Ra.

The antimycobacterial activity expressed in whole blood cultures of cured patients and TB-naïve volunteers is illustrated in figure 1. Results are grouped according to subject status and strain type. The vertical axis in these graphs indicates the net change in mycobacterial viability during 72 hr whole blood culture, with positive values indicating growth. Recent clinical isolates showed the greatest increase in CFU number, and H₃₇Ra, the greatest reduction. This relationship was apparent in both the cured patients and in the TST-negative volunteers. Compared to healthy volunteers, growth of clinical isolates was restricted in the cultures of cured patients from which they were derived ($P=.003$ by paired t test), indicating the expression of acquired antimycobacterial immune functions in these cultures. Data for patient isolates in figure 1 are limited to those strains for which corresponding values are available in healthy volunteers, and are analyzed comparing the mean value for the volunteers to the single corresponding patient value using a paired t test. The distributions of the individual results for each of the patient strains in each of the volunteers were similar to that of MP-28, with an overall mean SD=0.271. An alternative, less conservative statistical analysis was also performed in which the individual values of all strains in healthy volunteers were compared to those of patients using an unpaired t test. This analysis also revealed a highly significant difference between volunteers and patients ($P<.001$).

Cured patients and volunteers also differed with regard to MP-28, which showed net growth during 72 hrs culture in TST-negative volunteers, but net killing in cultures of cured patients ($P=.018$). In contrast, cured patients and TST-negative volunteers did not differ with regard to *M. tuberculosis* H₃₇Ra, which was killed equally well in cultures of both groups ($P=.08$). Indeed, the pattern for H₃₇Ra was reversed, with a trend toward superior killing in volunteers (mean= -0.97) compared to cured patients (mean= -0.54). As a result, there was only a 4% likelihood of superior growth of H₃₇Ra in controls (*i.e.*, $P=.96$) as occurs for other strains. MP-28 and H₃₇Ra were equally attenuated as judged by their survival in cultures of cured patients when examined by repeated measures ANOVA.

Within-subject correlations are shown in figure 2. No correlation was observed between control of H₃₇Ra and the infecting strain ($R=.02$, $P=.9$, panel A), whereas a statistically significant correlation was observed for MP-28 ($R=.36$, $P=.04$, panel B). However, this correlation was weak compared to replicate cultures of the same strain in healthy volunteers ($R=.98$, $P<.001$, panel C). There was no correlation between control of growth H₃₇Ra and MP-28, either in cured TB patients ($R=.06$, $P=.74$) or TST-negative volunteers ($R= -.08$, $P=.88$) (not shown).

Discussion

Immune killing assays such as bacterial opsonization or virus neutralization have facilitated the clinical development of most modern licensed vaccines. The first such exploration in tuberculosis examined the effects of BCG vaccination on the cellular control of intracellular

mycobacterial infection in 1988. [18] That assay required the *in vitro* expansion of autologous antigen-reactive T cells, followed by their addition to *M. microti*-infected monolayers. Hoft *et al* have shown that BCG may be substituted for *M. microti* in this model. [19] However, the antigen repertoire expressed by a particular target strain may limit its use for certain vaccines. BCG, for example, is unlikely to be satisfactory for studies of TB vaccines expressing the RD1 antigens ESAT-6 or CFP-10, which are major T cell antigens recognized following natural human *M. tuberculosis* infection but that are absent in all BCG strains. [3,20,21] BCG would also be anticipated to serve poorly as an indicator of mycobacterial immunity following natural infection in persons in whom protection was mainly due to RD1 responses. These concerns have focused attention on the possible use in killing assays of indicator strains with antigen expression profiles closer to wild-type *M. tuberculosis*.

Patients cured of tuberculosis present a unique opportunity to study the possible strain-specificity of human T cell responses to *M. tuberculosis*. The full extent to which recognized genetic differences among clinical *M. tuberculosis* strains result in differential antigen expression is presently unknown. [22] To our knowledge, this is the first report in which patients were studied using their own strains, or in which cross-strain responses were examined. The main findings were that cured TB patients showed superior control of the *M. tuberculosis* strain with which they had been infected compared to TB-naïve volunteers, and that no correlation was evident between control of these strains and that of the attenuated *M. tuberculosis* reference strain H37Ra. In contrast, patients' control of their own infecting strain did correlate with that of a second attenuated strain, MP-28, although the extent of this correlation was weak compared to replicate cultures of identical strains.

The basis of these observations is uncertain. Attenuated isolates such as H37Ra fail to replicate in whole blood culture. Other assays of mycobacterial growth inhibition that are dependent on mycobacterial growth may fail to detect further inhibition of growth of an already attenuated strain. This is not the case for the whole blood Bactec assay, which can readily measure killing as well as growth. The dynamic range of the assay (approximately +2 to -5 logs) was not exceeded in any of the whole blood cultures in this report, regardless of the subject or strain selected. Thus, lack of growth *per se* cannot account for the lack of correlation with H37Ra. This is underscored by the observation that lack of growth of MP-28 in patient cultures did not prevent the observed correlation with virulent infecting strain results.

Alternatively, differential antigen expression may account for these findings. Unlike BCG, *M. tuberculosis* H37Ra does not lack the genes for ESAT-6 or other RD1 antigens; however, its expression of these antigens is reduced, due to loss of PhoP, required for ESAT-6 export. [23] Specific deletion of RD1 genes from *M. tuberculosis* results in its attenuation. [24,25] It is not known whether loss of ESAT-6 or other RD1 genes might account for the attenuation of MP-28. The strain had been isolated 2 years earlier from sputum of a similar patient at the same research site in Brazil. It originally exhibited growth in whole blood culture significantly superior to both H37Rv and Ra, but it became attenuated with serial passage.

Emerging evidence suggests distinct patterns of mycobacterial virulence and immunogenicity related to strain phylogenetics. Six major lineages of *M. tuberculosis* have been proposed, each with distinct geographic localizations. [22] It has been proposed, but not proven, that antigenic differences among the predominant TB strains in different regions may account for the variability in protection afforded by BCG vaccination in these regions. [26] Although MP-28 does not share an IS6110 DNA fingerprint with any other strains in this study, it is possible that its genetic relatedness to other Brazilian isolates resulted in shared antigenicity and contributed to the correlation we observed. If so, one may anticipate different results when MP-28 is tested in patients and strains of other regions and lineages (*e.g.*, India). Future studies

will be required to test this hypothesis, and to determine the molecular basis of the attenuation of this strain.

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Figure 1.

Control of growth of *M. tuberculosis* H₃₇Ra, MP-28, and clinical isolates, in whole blood cultures of healthy TST negative donors (N=6) and HIV-uninfected patients with sputum smear positive pulmonary tuberculosis from whom they had been obtained (N=32). Positive values on the vertical axis indicate net growth during 72 hr whole blood culture. Each symbol represents the result of one strain tested in one subject except in the case of healthy volunteers tested using patient isolates, for which each symbol represents the mean of 6 subjects, with a line connecting to the corresponding value in a cured patient. Patient isolate data are limited to those strains for which corresponding data are available in healthy volunteers. Cured patients showed superior control of their own isolate and that of isolate MP-28 as compared to healthy TST negative controls, whereas H₃₇Ra did not differ between the two cohorts.

Figure 2.

Correlations within subjects between *M. tuberculosis* strains regarding control of intracellular growth in whole blood culture. Axes represent log change in viability after 72 hr. Solid and dotted lines indicate regression and 95% confidence limits, respectively. Panels A and B indicate correlations between patient isolates and reference strains in cured patients. Panel C indicates the correlation between replicate cultures of patient isolates in healthy volunteers. The control of growth by cured patients of the infecting strain was correlated with MP-28 (B) but not with H₃₇Ra (A). However, the extent of correlation was weak compared to replicate cultures of the same strains (C).