

## Bactericidal Activity in Whole Blood as a Potential Surrogate Marker of Immunity after Vaccination against Tuberculosis

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**The development of new tuberculosis (TB) vaccines will require the identification of correlates of human protection. This study examined the balance between immunity and virulence in a whole blood infection model in which intracellular mycobacterial survival was measured using BACTEC. In the blood of tuberculin-negative donors, counts of *Mycobacterium tuberculosis* H<sub>37</sub>Ra organisms fell by 0.14 log<sub>10</sub> CFU during 96 h of whole blood culture, whereas counts of *Mycobacterium bovis* BCG, *M. tuberculosis* H<sub>37</sub>Rv, and a clinical TB isolate's organisms increased by 0.13, 0.43, and 1.04 log<sub>10</sub> CFU, respectively ( $P < 0.001$ ), consistent with their relative virulence. Inhibition of tumor necrosis factor alpha by the addition of methylprednisolone or pentoxifylline or removal of CD4<sup>+</sup> or CD8<sup>+</sup> T cells by magnetic beads had deleterious effects on immune control of intracellular growth only in the blood of tuberculin-positive donors. Repeated vaccination of eight tuberculin-negative volunteers with *M. bovis* BCG resulted in a 0.3 log (50%) reduction in BCG CFU counts in the model compared to baseline values ( $P < 0.05$ ). Three of the volunteers responded only after the second vaccination. These experiments indicate that whole blood culture may be used to measure immunity to *M. tuberculosis* and that further studies of repeated BCG vaccination are warranted.**

The development of new vaccines for tuberculosis (TB) has become increasingly recognized as a high priority in the effort to control TB worldwide, due to the increasing incidence of drug-resistant disease and the apparent ineffectiveness of BCG in preventing pulmonary TB, the form of the disease from which most new infections arise. New tools will be required to facilitate the clinical evaluation of candidate vaccines, particularly the validation of in vitro correlates of the protection afforded by vaccination.

Whole blood culture may provide one such tool. It has been widely used to evaluate other aspects of immune function, including expression of cytokines and killing of bacteria (10, 14, 21). The simplicity of whole blood culture facilitates its inclusion in clinical trials, as it requires only the mixing of heparinized blood with tissue culture medium and an appropriate stimulus or infectious agent. It has previously been documented that small numbers of mycobacteria undergo nearly complete phagocytosis after addition to whole blood culture, indicating a potential role as an intracellular TB infection model (22). Kampmann et al. have reported superior control of *Mycobacterium bovis* BCG growth by the blood of tuberculin-reactive individuals, indicating the expression of acquired cell-mediated immunity in the model (11). In that report, light production by recombinant BCG *lux* was used to measure mycobacterial growth. In the present report, growth was mea-

sured as days to positivity (DTP) in BACTEC, a modification that permits the study of any isolate, rather than of only a genetically engineered strain. The method was evaluated in the context of a study in which healthy adult volunteers were vaccinated with BCG and then revaccinated after 6 months. The objective was to explore the potential role of this surrogate marker in the evaluation of new TB vaccines.

### MATERIALS AND METHODS

**Isolate preparation and whole blood culture.** *M. tuberculosis* strains H<sub>37</sub>Ra and H<sub>37</sub>Rv and a recent clinical isolate (MP-28) were prepared by propagation to a growth index (GI) of 250 in BACTEC 12B medium (Becton Dickinson, Sparks, Md.). Stocks were frozen at  $-70^{\circ}\text{C}$  until needed. At the first use of each batch of stock, serial 10-fold dilutions (1,000 to 0.1  $\mu\text{l}$ ) were directly inoculated into BACTEC to develop a standard curve relating inoculum size to DTP. Bacilli from 100  $\mu\text{l}$  of these cultures were sedimented at  $6,000 \times g$  for 10 min. This inoculum volume contained approximately  $5 \times 10^3$  CFU and reached a GI of 30 approximately 6 days after inoculation into BACTEC. Bacilli were resuspended in 300  $\mu\text{l}$  of RPMI medium supplemented with glutamine and 25 mM HEPES. An equal volume of blood was added. In each experiment, a day 0 control culture was performed in which a 100- $\mu\text{l}$  volume of the stock culture was inoculated directly into BACTEC.

Studies of the effects of BCG vaccination were performed using *M. bovis* BCG *lux* to permit the direct comparison of BACTEC DTP and light expression within individual whole blood cultures. BCG stock cultures were freshly prepared for each experiment. Light production and bacillary number are directly related (number of CFU = number of relative light units/10) (11). BCG stock cultures were therefore standardized according to light output, as previously described (11). BCG whole blood cultures were performed using a greater total volume (1 ml) and a larger inoculum ( $10^4$  CFU) to permit assessment of BCG growth by both luciferase and BACTEC methods. A standard curve relating BCG inoculum volume to DTP was prepared for each BCG culture batch to ensure consistency in measurement of growth in BACTEC.

**Determination of bactericidal activity.** Replicate whole blood cultures were incubated at  $37^{\circ}\text{C}$  with slow constant mixing. Cultures were harvested at selected

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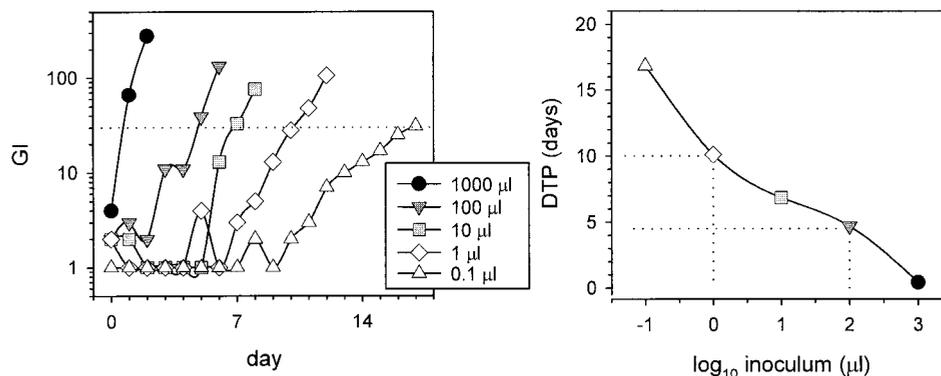


FIG. 1. Relationship between inoculum size and DTP in BACTEC for a representative culture of a recent clinical isolate of *M. tuberculosis*. The threshold for positivity was a GI of 30. Two hypothetical cultures with DTP values of 5 and 10 days (dotted lines, right panel) would differ in apparent inoculum size by 100-fold ( $2 \log_{10}$  CFU).

intervals (0, 24, 72, and 96 h, as indicated) by sedimentation at  $6,000 \times g$  for 10 min. Supernatants were removed. Host cells were disrupted by addition of sterile water. After 10 min, bacilli were sedimented at  $6,000 \times g$  for 10 min, resuspended, and inoculated into a BACTEC 12B bottle. GIs were monitored daily. The fractional number of days required for positivity (GI = 30) was interpolated from daily readings. The volume of stock corresponding to each DTP value was estimated from the standard curve for that stock (Fig. 1). The change in the  $\log_{10}$  number of viable bacilli from day 0 to the harvesting of the culture was determined according to the formula

$$\Delta \log_{10} \text{CFU} = \log_{10} (\text{final}) - \log_{10} (\text{initial})$$

in which initial and final refer to the calculated volumes of the inoculum and completed whole blood culture, respectively. This relationship is mathematically equivalent to  $\log_{10}$  (final/initial). Data management, standard curves, interpolation, and calculation of  $\Delta \log_{10}$  CFU were performed using computer software written by one of the authors (R.S.W.). For clarity, we have included the unit "CFU" when reporting data in this manuscript, recognizing that, as a growth ratio, representation without units may also be appropriate.

**Depletion of T-cell subsets.** In some experiments,  $\text{CD4}^+$  and  $\text{CD8}^+$  T cells were removed from heparinized blood prior to culture, using monoclonal antibodies coupled to magnetic beads (Dynabeads, Lake Success, N.Y.). One milliliter of blood was diluted with an equal volume of cold RPMI 1640 medium and was placed into a polypropylene tube containing 250  $\mu\text{l}$  of washed antibody-coated beads. Cells and beads were mixed at  $4^\circ\text{C}$  for 30 min. Beads were removed using a magnet. The efficiency of depletion was determined by flow cytometry, following immunofluorescent staining with CD3PE+CD4FITC, or CD3PE+CD8PerCP monoclonal antibodies (Becton Dickinson, San Jose, Calif.). The analysis was restricted to lymphocytes based on forward and side light scatter.

**Cytokine inhibition experiments.** In other experiments, methylprednisolone sodium succinate (Pharmacia, Kalamazoo, Mich.) or pentoxifylline (Sigma, St. Louis, Mo.) was added to whole blood cultures 20 min prior to infection. Supernatants were collected after 24 h. Tumor necrosis factor alpha (TNF- $\alpha$ ) expression was measured by enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, Minn.) according to the manufacturer's instructions.

**Statistical analysis.** The difference between paired values was determined by the paired Student *t* test. Differences among repeated measurements within individuals were determined by one- or two-way repeated-measures analysis of variance (RM ANOVA). This test examines the changes within each individual subject to determine whether they are greater than would be expected by chance. Like standard ANOVA, it is appropriate for multiple comparisons; like the paired *t* test, it emphasizes changes within individuals. In those cases in which significant differences were detected by ANOVA, post hoc ("after this") testing was performed using Tukey's test, a conservative method to detect differences between pairs of measurements after ANOVA without overestimating significance due to repeated testing. Correlations were examined by the Pearson product method. These tests were performed using SigmaStat (SPSS, Chicago, Ill.).

**Informed consent.** Informed consent was obtained from subjects according to the guidelines of the U.S. Department of Health and Human Services.

## RESULTS

**Quantitative use of BACTEC.** The inverse relationship between  $\log_{10}$  inoculum size and DTP in BACTEC is illustrated in Fig. 1. This forms the basis of the quantitative use of BACTEC, in which the extent of growth or killing during culture is determined by comparing the DTP values of the completed culture with those of the inoculum, using a standard curve. Results are expressed as  $\Delta \log_{10}$  CFU; positive numbers indicate growth.

**Effect of BCG vaccination on control of intracellular BCG replication.** The effects of BCG vaccination and boosting on control of BCG replication in whole blood culture were studied in 10 healthy adults without known TB exposure or prior purified-protein-derivative (PPD) skin test reactivity, recruited by the St. Louis University Vaccine and Treatment Evaluation Unit. Other laboratory and clinical aspects of the BCG vaccine component of this report have been submitted separately for publication.

Initial experiments examined the extent of BCG growth and its intra- and intersubject variability in triplicate whole blood cultures of these subjects. Prior to vaccination, there was net growth of BCG during whole blood culture, as indicated in Fig. 2. The average variability (standard deviation [SD]) within the triplicate cultures of each subject was  $0.08 \log_{10}$  CFU, whereas that among subjects was  $0.17 \log_{10}$  CFU. Based on this analysis, cultures at subsequent time points were performed by pooling portions of triplicate whole blood cultures to form a single BACTEC culture for each subject.

Subjects then underwent intradermal vaccination with  $3 \times 10^6$  CFU of BCG Connaught, administered as directed by the manufacturer. Eight of the 10 individuals consented to revaccination at 6 months; the results of the two individuals who declined revaccination were censored after the 6-month evaluation. Mean results at each time point are shown in Fig. 2; individual responses are shown in Fig. 3. For clarity, the latter figure shows subjects grouped according to the temporal pattern of responses, using  $-0.25$  (3 SD) as the threshold to define a response. Four subjects showed improved bactericidal activity following the first vaccination (Fig. 3A). Their responses waned at 6 months but recurred after revaccination. Three subjects responded only after the second vaccination

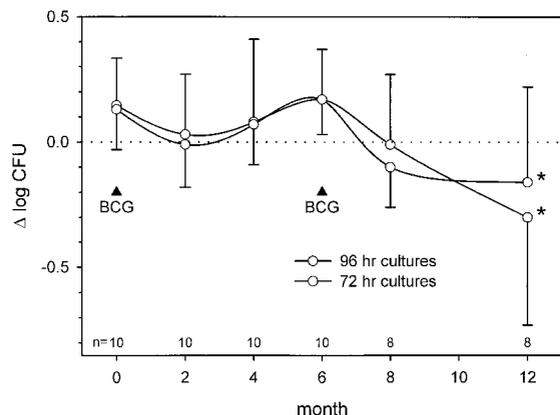


FIG. 2. Effect of BCG vaccination and boosting on growth of *M. bovis* BCG in whole blood cultures of 72- and 96-h duration. Data indicate mean and SD. RM ANOVA revealed significant overall changes ( $P < 0.02$ ); post hoc testing indicated that 12-month values (indicated by asterisks) differed from those for months 0 and 6 (both  $P < 0.05$ ).

(Fig. 3B). No response was evident in the three subjects in Fig. 3C; one member of this group declined repeat vaccination.

A crude statistical measure of the overall effect of each vaccination may be obtained by comparing baseline values to subsequent ones by paired  $t$  test. Such an analysis reveals significant changes only after the second vaccination (month 6 versus month 8,  $P = 0.009$ ; month 6 versus month 12,  $P = 0.04$ ). However, this approach is subject to type 1 error: it may overestimate the likelihood of a true effect due to multiple comparisons. A more accurate assessment of the overall changes occurring during the entire study may be obtained by RM ANOVA, which examines variation within subjects during the entire period of repeated testing. This analysis reveals that the variation was greater than would be expected by chance ( $P = 0.007$ ). Post hoc testing, using the Tukey method to account for multiple comparisons, reveals that this effect is attributable to the 12-month values, which differ significantly from months 0 and 6 (both  $P < 0.05$ ). Cultures at 12 months contained a mean of  $0.3 \log_{10}$  (50%) fewer CFU than those on entry to the study.

Very similar responses were observed in the 96-h cultures, with respect to both the kinetics and magnitude of vaccine effects. RM ANOVA of these data revealed similar significant changes overall ( $P = 0.016$ ) and also identified month 12 as differing from months 0 and 6 in post hoc testing (both  $P < 0.05$ ). The changes from months 0 to 12 in the 96-h cultures were very highly correlated with those of 72-h cultures, as were the changes from months 6 to 12 ( $r > 0.86$ ,  $P \leq 0.001$  for both comparisons). Furthermore, significant correlations were identified at month 12 between  $\Delta \log$  CFU values and corresponding  $\Delta \log$  relative light unit values ( $r = 0.7$ ,  $P = 0.047$  in 72-h cultures; and  $r = 0.8$ ,  $P = 0.01$  in 96-h cultures; luciferase data reported separately). These findings indicate that the variability among the subjects in their responses to vaccination is unlikely to be random.

**Virulence.** Bactericidal activity against *M. bovis* BCG may not necessarily indicate immunity against *M. tuberculosis*: complementary studies were therefore performed in a group of

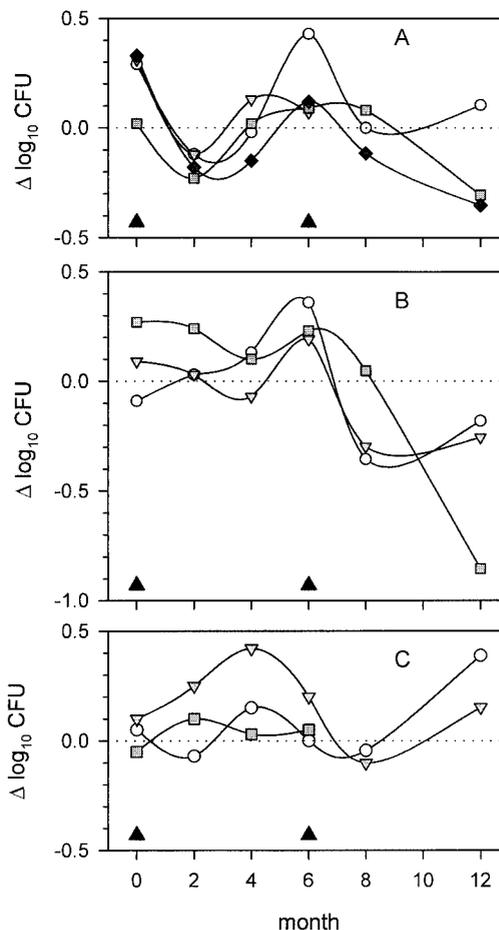


FIG. 3. Effect of BCG vaccination and boosting on growth of *M. bovis* BCG in whole blood cultures of 72-h duration. Vaccine administration (0 and 6 months) is indicated by filled triangles. Each curve represents a single subject. Subjects have been grouped for clarity according to their temporal response to vaccination (defined as a change of  $-0.25$  or more). Those whose results are given in panel A responded to the first vaccination, whereas those with results in panel B responded only to the second. Subjects with results shown in panel C showed no response.

healthy adults in whom tuberculin skin test reactivity reflected likely infection with *M. tuberculosis* rather than vaccination with *M. bovis* BCG. These experiments were performed using three strains of *M. tuberculosis*: H<sub>37</sub>Ra, H<sub>37</sub>Rv, and a randomly selected recent clinical isolate (MP-28). As shown in Fig. 4, the strains differed significantly in their ability to grow in the blood of these donors ( $P < 0.001$  overall by two-way RM ANOVA and  $P < 0.01$  in all post hoc paired comparisons). In skin test nonreactors, counts of H<sub>37</sub>Ra organisms decreased by a mean of  $0.14 \log_{10}$  CFU, whereas those of H<sub>37</sub>Rv and MP-28 organisms increased by  $0.43$  and  $1.04 \log_{10}$  CFU, respectively. A trend was observed in this small sample toward superior killing by skin test reactors of the attenuated strain H<sub>37</sub>Ra, compared to that by nonreactors, that approached statistical significance ( $P = 0.07$ ); however, it was not observed in the other strains. The average intersubject variability (SD) in these cultures was  $0.15 \log_{10}$  CFU.

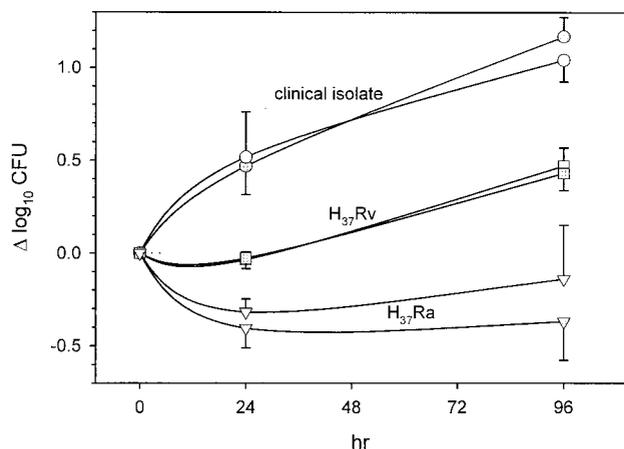


FIG. 4. Kinetics of growth of *M. tuberculosis* strains H<sub>37</sub>Ra and H<sub>37</sub>Rv and of a recent clinical isolate in whole blood of healthy volunteers. Negative values indicate net killing; positive values, growth. Triangles indicate H<sub>37</sub>Ra; squares, H<sub>37</sub>Rv; and circles, a recent clinical isolate (MP-28). Shaded figures indicate PPD skin test reactors (*n* = 4); open figures, nonreactors (*n* = 4). Error bars indicate SD. The difference among strains after 96 h of culture was highly statistically significant in two-way RM ANOVA (*P* < 0.001).

**Cytokine inhibition and T-cell subset depletion.** To define the role of immune mechanisms in this model, experiments were performed in which methylprednisolone (50 μg/ml) or pentoxifylline (1 mM) was added. The selected drug concentrations reduced *M. tuberculosis*-induced TNF-α expression by 90% or greater (Table 1). The addition of either drug resulted in increased growth of *M. tuberculosis* H<sub>37</sub>Ra in PPD-reactive donors (Table 2). No significant effects were observed in PPD nonreactors. Neither drug exerted a significant influence on growth of the clinical isolate.

To assess the role of T-cell subsets in the control of intracellular growth, CD4<sup>+</sup> or CD8<sup>+</sup> T cells were removed from blood using magnetic beads. The efficiency of depletion was assessed by flow cytometry (fluorescence-activated cell sorter) in each experiment; in all cases, the desired cell population was removed with 99% or greater efficiency. A representative fluorescence-activated cell sorter analysis is shown in Fig. 5. The effects of depletion of T-cell subpopulations prior to whole

blood culture are shown in Table 3. Deleterious effects on control of *M. tuberculosis* H<sub>37</sub>Ra were observed in cultures of PPD-positive donors alone. Simultaneous depletion of CD4<sup>+</sup> and CD8<sup>+</sup> T cells produced additive effects. No significant effects were observed on survival of the clinical *M. tuberculosis* isolate, whose vigorous growth was unaffected by the removal of T cells (not shown).

**DISCUSSION**

Vaccination with *M. bovis* BCG produces incomplete and highly variable levels of protection against TB, apparently influenced by epidemiologic, host, and other factors (6). The mechanisms through which these factors influence human cellular immunity to *M. tuberculosis* and thereby affect vaccine efficacy are incompletely understood. Acquired cellular immunity is essential for containment of *M. tuberculosis* infection. Lymphocytes provide essential signals for production of nitric oxide and induction of host cell apoptosis; they may also lyse infected macrophages through cytotoxic mechanisms and may directly kill mycobacteria via release of antibacterial peptides. The relative contributions of these various mechanisms to human immunity and the antigens that trigger them are presently the subjects of considerable research.

The present lack of suitable correlates of human protection that reflect a single pathway has encouraged the development of in vitro models that reflect all possible killing mechanisms. Cheng et al. in 1988 showed superior inhibition of intracellular growth of *Mycobacterium microti* by blood mononuclear cells following vaccination with BCG (5). Silver et al. in 1998 described a similar in vitro model system for study of virulent *M. tuberculosis* (18). More recently, Kampmann et al. reported the adaptation of whole blood culture to the study killing of *M. bovis* BCG, using a luciferase reporter system (11). These models avoid potential biases due to the selection of a single protective antigen, responding cell type, activation marker, or killing mechanism. The whole blood model additionally allows full interplay of cellular and humoral factors; this interaction may be important in TB immunity (8, 9, 20). The main limitation of the model is that blood monocytes may not fully represent the activities of mature lung macrophages and that other cell populations may differ in the two compartments.

TABLE 1. Effect of methylprednisolone or pentoxifylline on TNF-α production in *M. tuberculosis*-stimulated whole blood cultures of 24-h duration<sup>a</sup>

Donor	Strain	Control	TNF-α concn after addition of								
			Methylprednisolone at (μg/ml):					Pentoxifylline at (mM):			
			0.01	0.1	1.0	10	50	0.01	0.1	1.0	
1	H <sub>37</sub> Rv	7158	7,109	4,265	1,115	354	172				
1	H <sub>37</sub> Ra	3979					160				
1	MP-28	971	920	753	4	2	18				
1	MP-28	850			39		25	737	222	24	
2	MP-28	651			27		16	512	111	15	
3	MP-28	386			60		42	163	76	7	
4	MP-28	263			48		37	233	89	0	
5	MP-28	142			22		13	333	21	0	
6	MP-28	138			34		3	31	65	0	

<sup>a</sup> Values indicate TNF-α concentrations in picograms per milliliter.

TABLE 2. Effect of methylprednisolone (50 µg/ml) or pentoxifylline (1 mM) on control of *M. tuberculosis* in 96-h whole blood cultures in subjects grouped according to tuberculin skin test status<sup>a</sup>

Drug used	Effect of drugs on control of growth of <i>M. tuberculosis</i> strain							
	H <sub>37</sub> Ra in:				MP-28 in:			
	PPD <sup>+</sup> donors (n = 5)		PPD <sup>-</sup> donors (n = 3)		PPD <sup>+</sup> donors (n = 6)		PPD <sup>-</sup> donors (n = 7)	
	Mean ± SD	P	Mean ± SD	P	Mean ± SD	P	Mean ± SD	P
No drug	-0.27 ± 0.2		0.25 ± 0.2		1.02 ± 0.1		0.92 ± 0.1	
Methylprednisolone	-0.10 ± 0.1	0.03	0.19 ± 0.2	0.9	1.04 ± 0.1	0.6	1.02 ± 0.1	0.1
Pentoxifylline	-0.12 ± 0.1	0.05	0.06 ± 0.2	0.5	1.08 ± 0.1	0.2	0.95 ± 0.1	0.3
P (overall)		0.02		0.5		0.3		0.1

<sup>a</sup> Data indicate Δlog CFU. Overall significance was determined by RM ANOVA. Comparisons to control cultures without added drug were performed using Tukey's test. PPD<sup>+</sup>, PPD positive; PPD<sup>-</sup>, PPD negative.

Mycobacteria introduced into whole blood culture must undergo complete (or nearly complete) phagocytosis if the model is to serve as one of intracellular infection. Since normal blood contains 285 to 500 monocytes/µl, the cultures in this study contained 1 CFU per 14 to 30 monocytes for both *M. tuberculosis* and BCG. This infection ratio is substantially lower than that of the macrophage model (18). It has been previously documented that this infection method is efficient (as nearly all bacilli are ingested) and robust (as results are unaffected by as much as 10-fold variation in inoculum size) (22). Such low infection ratios must be combined with highly sensitive detection systems, such as BACTEC, to be successful. Detection by BACTEC is unaffected by clumping, a bacillary attribute associated with virulence that may confound conventional CFU counting (3). Estimation of mycobacterial viability in BACTEC is dependent on bacillary replication, however. Specimens in

which bacillary number is preserved but in which lag phase has been prolonged will appear to have fewer viable organisms, a potential artifact. However, as human mycobacterial immunity in vivo may more often reflect containment rather than true eradication of infection, this effect may enhance the ability of the assay to detect a protective effect of vaccination. Further studies will be required to address this question.

The use of BACTEC rather than a reporter phage eliminates constraints on the testing of diverse isolates, thus facilitating studies of mycobacterial virulence in TB outbreaks. We found the rank order of growth (H<sub>37</sub>Ra < BCG < H<sub>37</sub>Rv) to be consistent with estimates of their virulence in other models (15, 19). We also identified a highly virulent clinical isolate (MP-28), whose growth was unaffected by the factors that influenced growth of reference strains. Manca et al. recently characterized a "hypervirulent" isolate that modulated the mu-

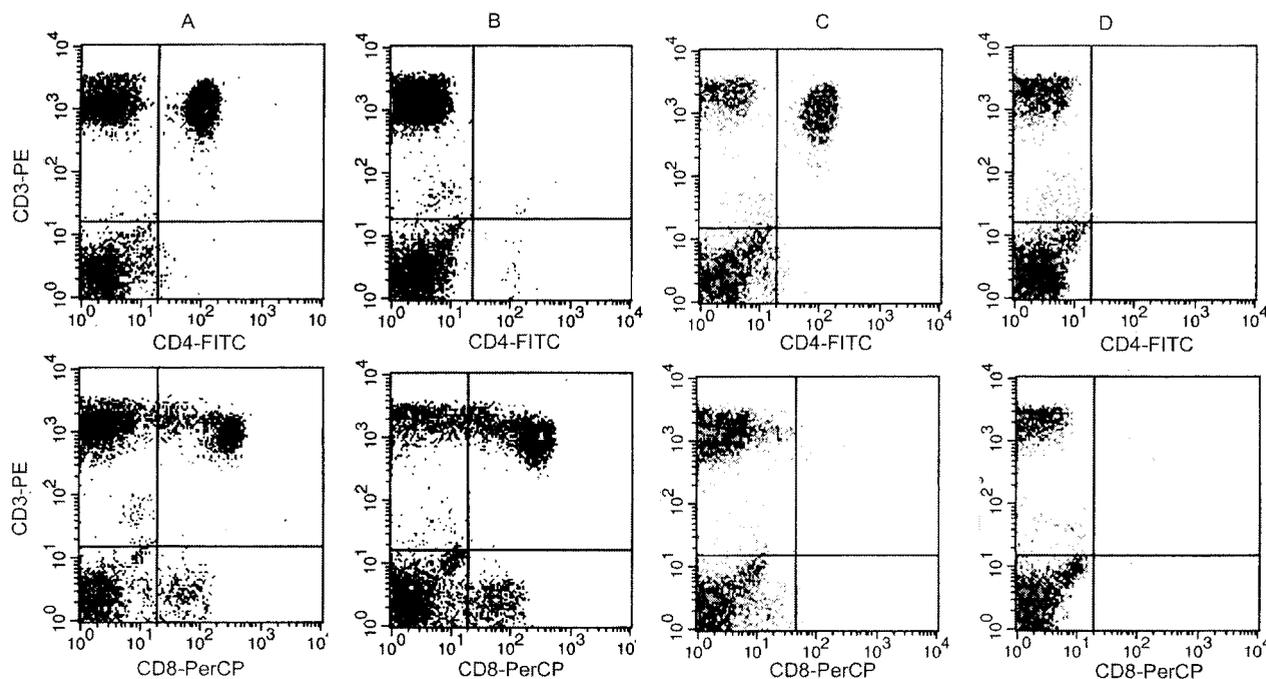


FIG. 5. Efficiency of depletion of T-cell subsets from whole blood using magnetic beads, as determined by flow cytometry. A representative experiment is shown. Column A, untreated blood cells; column B, CD4-depleted cells; column C, CD8-depleted cells; and column D, CD4- and CD8-depleted cells. In all experiments, the target cell population was reduced to 1% or less of total lymphocytes.

TABLE 3. Effect of depletion of CD4<sup>+</sup> and CD8<sup>+</sup> T cells on control of growth of *M. tuberculosis* H<sub>37</sub>Ra in whole blood cultures of 24- and 96-h durations<sup>a</sup>

Cells type depleted	Effect of T-cell depletion (in Δlog <sub>10</sub> CFU) of control of <i>M. tuberculosis</i> growth in:							
	PPD <sup>+</sup> donors (n = 8)				PPD <sup>-</sup> donors (n = 5)			
	At 24 h	P	At 96 h	P	At 24 h	P	At 96 h	P
None	-0.83 ± 0.4		-0.78 ± 0.4		-0.66 ± 0.3		-0.68 ± 0.3	
CD4	-0.68 ± 0.3	0.1	-0.61 ± 0.4	0.2	-0.59 ± 0.3	0.8	-0.50 ± 0.4	0.2
CD8	-0.63 ± 0.3	0.03	-0.61 ± 0.3	0.2	-0.61 ± 0.3	0.9	-0.41 ± 0.4	0.06
CD4 and CD8	-0.60 ± 0.3	0.009	-0.43 ± 0.4	0.001	-0.62 ± 0.3	0.9	-0.49 ± 0.4	0.2
P (overall)		0.009		0.003		0.8		0.07

<sup>a</sup> Significance was determined by RM ANOVA and Tukey's test. PPD<sup>+</sup>, PPD-positive; PPD<sup>-</sup>, PPD-negative.

rine host response to its advantage by stimulating expression of alpha/beta interferon (13). Infection with that strain was associated with higher CFU counts, increased mortality, and decreased expression of TNF-α and gamma interferon. Similar escape from host immune mechanisms may account for the lack of effect of skin test status, T-cell depletion and cytokine inhibition on growth of the MP-28 clinical isolate in this study, which, like that studied by Manca et al., was also a poor TNF-α inducer. Such evasion of immune mechanisms has not been observed in studies of *M. tuberculosis* Erdman or H<sub>37</sub>Rv in macrophages or animal models (15, 19). Further studies will be required to characterize the immune mechanisms that control the growth of H<sub>37</sub>Rv in the whole blood and to determine whether other, yet unidentified, host factors restrict growth of highly virulent clinical strains.

Antigenic variation among TB strains may also affect immune control of *M. tuberculosis* infection. Although little is known regarding the magnitude and frequency of such antigenic differences, Rhee et al. speculated that the extent of relatedness to BCG of prevalent *M. tuberculosis* strains may be an important factor determining vaccine efficacy (17). These points all underscore the potential importance of studying immune responses and bactericidal activity against virulent clinical isolates as well as attenuated strains, as is facilitated by this method.

In this study, the ability to kill *M. bovis* BCG Montreal was measured after vaccination with the closely related Connaught strain (1) to minimize any confounding influence of antigenic variation. The target BCG strain was found to be intermediate in virulence relative to H<sub>37</sub>Ra and H<sub>37</sub>Rv, as judged by its growth in blood of naïve individuals. It is possible that the extent of protection is better assessed using one or more representative virulent clinical isolates of *M. tuberculosis*. Additional studies will therefore be required to determine the extent to which the selection of BCG as a test strain influenced our results.

Repeated vaccination with BCG appeared to boost waning immune responses and facilitate the development of robust responses in this study (as significant responses to vaccination were detected only at the conclusion of this trial, 6 months after boosting). The implications of this observation are somewhat uncertain, since T-cell responses to some mycobacterial antigens have been reported to evolve up to a year postvaccination (16). However, nearly half of the subjects in this small study who ultimately responded to vaccination did so without exhibiting any response within the first 6 months after the first

vaccination. This may indicate a possible advantage to repeated vaccination for TB, a concept supported by animal studies (4, 7). The lack of convincing human data in this respect may reflect the long intervals (years or decades) between vaccinations in such studies (12) (<http://www.who.int/vaccines-documents/DocsPDF99/www9943.pdf> and <http://www.who.int/vaccines-documents/DocsPDF/www9401.pdf>). Modern vaccine administration schedules may therefore have a role in improving the efficacy of BCG.

We observed significant heterogeneity among subjects in their responses to BCG vaccination. Host genetic factors may govern this response much as they govern that to *M. tuberculosis* infection. Unlike natural *M. tuberculosis* infection, BCG vaccination and in vitro challenge can be administered with a high degree of uniformity to many individuals. A study to map the genes regulating these responses may therefore uncover important influences on mycobacterial immunity while avoiding many of the sources of variability that hinder epidemiologic studies of TB immunogenetics.

Brennan et al. have suggested that coordinated phase II studies may help propel new TB vaccines through the regulatory process (2). Such coordinated studies, conducted in 100 to 1,000 subjects, would serve to prioritize candidate vaccines according to immunologic correlates of protection. The most promising candidates would then proceed to large phase III trials with conventional clinical endpoints. The success of this strategy is dependent on the validation of surrogate markers through prospective epidemiologic studies performed in areas of TB endemicity. The whole blood BACTEC model described in the present report should be considered for inclusion for validation in such studies.

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