Inhibition of Mycobacterial Growth *In Vitro* following Primary but Not Secondary Vaccination with *Mycobacterium bovis* BCG

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Despite the widespread use of the *Mycobacterium bovis* BCG vaccine, there are more than 9 million new cases of tuberculosis (TB) every year, and there is an urgent need for better TB vaccines. TB vaccine candidates are selected for evaluation based in part on the detection of an antigen-specific gamma interferon (IFN-γ) response. The measurement of mycobacterial growth in blood specimens obtained from subjects immunized with investigational TB vaccines may be a *in vitro* correlate of *in vivo* vaccine efficacy. We performed a clinical study with 30 United Kingdom adults who were followed for 6 months to evaluate the abilities of both a whole-blood- and a novel peripheral blood mononuclear cell (PBMC)-based mycobacterial growth inhibition assay to measure a response to primary vaccination and revaccination with BCG. Using cryopreserved PBMCs, we observed a significant improvement in mycobacterial growth inhibition following primary vaccination but no improvement in growth inhibition following revaccination with BCG (*P* < 0.05). Mycobacterial growth inhibition following primary BCG vaccination was not correlated with purified protein derivative (PPD) antigen-specific IFN-γ enzyme-linked immunospot (ELISPOT) responses. We demonstrate that a mycobacterial growth inhibition assay can detect improved capacity to control growth following primary immunization, but not revaccination, with BCG. This is the first study to demonstrate that an *in vitro* growth inhibition assay can identify a difference in vaccine responses by comparing both primary and secondary BCG vaccinations, suggesting that *in vitro* growth inhibition assays may serve as better surrogates of clinical efficacy than the assays currently used for the assessment of candidate TB vaccines.

There are more than 9 million new cases of tuberculosis (TB) every year, primarily affecting young adults in the most productive years of their lives. Despite the existence of effective tools for the diagnosis and treatment of TB, the global incidence remains high, driven primarily by the increase in HIV-associated TB in Africa (1). The only available vaccine for TB, *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), was first used as a human vaccine in 1921 and is one of the most widely administered vaccines in the world. Although widely practiced, the administration of BCG to newborns as recommended by WHO, while affording protection against severe infant TB, leads to protection against later pulmonary disease that is variable and mostly poor (2). There is an urgent need to develop an improved vaccine. Over the last decade, 16 candidate vaccines have entered clinical trials (3). However, the progression of candidate vaccines from preclinical studies and early phase I trials to larger and more expensive efficacy trials is frustratingly slow. In part, this is due to the nature of the disease, as only 1 in 10 people infected with TB develop disease, which may occur decades after initial infection. It is therefore necessary to prioritize which candidate vaccines are to be tested in efficacy trials at the available field sites. The selection of candidate vaccines for efficacy testing is based in part on safety and immunogenicity in early clinical trials. The identification of a biomarker or biosignature of protective efficacy that could be used to select vaccine candidates for further study would greatly facilitate vaccine development and would have a major impact on the field. Several groups have demonstrated the potential use of *ex vivo* mycobacterial growth inhibition assays (MGIA) in both *Mycobacterium tuberculosis*-exposed and BCG-vaccinated cohorts (4–9). However, despite more than a decade of use, these assays have not been adopted by TB vaccine developers, in part because of concerns regarding reproducibility of the assays. To date, TB vaccine developers have focused on the discovery of T-cell signatures of vaccine effectiveness. However, due to the recent failure of T-cell signatures to differentiate protected from nonprotected individuals (10), there has been a resurgence of interest in the utility of MGIA as an alternative route to assessing vaccine-induced protection.

Here, we describe the findings of a clinical study to evaluate the abilities of both a whole-blood- and a peripheral blood mononuclear cell (PBMC)-based MGIA to measure a response to primary and repeat BCG vaccination in United Kingdom adults. The use of functional assays for the early identification of promising vaccine...
candidates is essential if we are to expedite the development of a new TB vaccine and use limited resources most effectively.

**MATERIALS AND METHODS**

**Ethics statement.** Participants were recruited under a protocol approved by the Oxfordshire Research Ethics Committee (OxREC A). Written informed consent was obtained from all individuals prior to enrollment in the trial.

**Study design and participants.** This was a nonrandomized, clinical study in healthy adults with (i) no history of BCG vaccination or (ii) a history of prior BCG vaccination more than 6 months before study enrollment (Fig. 1). Volunteers were screened and enrolled only if routine hematologic and biochemistry measures were within the normal range and if they were negative on serology for HIV, hepatitis B virus (HBV), and hepatitis C virus (HCV). Volunteers were aged 18 to 50 years with no evidence of latent *M. tuberculosis* infection, as determined by an PPD (SSI). Positive-control staphylococcal enterotoxin B (SEB) and negative-control (medium-only) wells were included for each participant.

**Bacterial strains and culture.** We used BCG Pasteur (a donation from Ann Rawkins, Health Protection Agency, Porton Down, United Kingdom) as the immune target in our growth inhibition assays. BCG Pasteur was cultured in 6X Bactec mycobacteria growth indicator tubes (MGIT) for 8 days. Mycobacterial cultures were then pooled, aliquoted, and frozen at −80°C, as previously described, to provide stocks for use in the study (4).

**Whole-blood growth inhibition assay.** The whole-blood growth inhibition assay was performed using blood from 18 of the 30 volunteers, as previously described (4). Duplicate tubes containing 300 μl of whole blood were incubated on a rotator at 37°C with 300 μl RPMI seeded with ~150 CFU of mycobacterial stock culture (BCG Pasteur) for 4 days. The blood cells were then lysed with sterile water, and the lysate containing mycobacteria was transferred to a Bactec MGIT supplemented with PANTA antibiotics and oleic acid-albumin-dextrose-catalase (OADC) enrichment broth (all from Becton, Dickinson). The tube was placed in a Bactec MGIT 960 and incubated until growth was detected (time to positivity [TTP]). Use of a standard curve enables conversion of the TTP of a sample tube into an initial mycobacterial inoculum volume (see Fig. S1 in the supplemental material). This inoculum volume is then converted to CFU (50 CFU of inoculum = 3.50E+04 CFU/ml). Duplicate MGIT are seeded with ~150 CFU of mycobacterial stock culture and placed directly into the Bactec MGIT 960 on day 0 of the blood culture to be used as growth control tubes. The CFU count of each sample tube is divided by the CFU count of the growth control tube, divided by 4 days, and log converted. This gives the delta log growth per day for each sample tube. Software to calculate the change in bacillary viability based on MGIT TTP written by one of the authors (R.S.W.) is available on request. (For an example showing data expressed as TTP and delta log growth per day in one case, see Fig. 4 and compare it with Fig. S2 in the supplemental material.)

**Immunological assays.** PBMCs were isolated from heparinized whole blood, and the ex vivo IFN-γ ELISPOT was performed as previously described (11). PBMCs were isolated by centrifugation of whole blood over 15 ml LymphoPrep (Axis-Shield) in a Leucoat tube (Greiner Bio-One) according to the manufacturer’s instructions. The ex vivo IFN-γ ELISPOT assay was used to assess antigen-specific responses by incubating PBMCs (0.3 × 10^6) overnight for 18 h with 20 μg/ml purified protein derivative (PPD) from *M. tuberculosis* (SSI). Positive-control staphylococcal enterotoxin B (SEB) and negative-control (medium-only) wells were included for each participant.

**Whole-blood growth inhibition assay.** The whole-blood growth inhibition assay was performed using blood from 18 of the 30 volunteers, as previously described (4). Duplicate tubes containing 300 μl of whole blood were incubated on a rotator at 37°C with 300 μl RPMI seeded with ~150 CFU of mycobacterial stock culture (BCG Pasteur) for 4 days. The blood cells were then lysed with sterile water, and the lysate containing mycobacteria was transferred to a Bactec MGIT supplemented with PANTA antibiotics and oleic acid-albumin-dextrose-catalase (OADC) enrichment broth (all from Becton, Dickinson). The tube was placed in a Bactec MGIT 960 and incubated until growth was detected (time to positivity [TTP]). Use of a standard curve enables conversion of the TTP of a sample tube into an initial mycobacterial inoculum volume (see Fig. S1 in the supplemental material). This inoculum volume is then converted to CFU (50 CFU of inoculum = 3.50E+04 CFU/ml). Duplicate MGIT are seeded with ~150 CFU of mycobacterial stock culture and placed directly into the Bactec MGIT 960 on day 0 of the blood culture to be used as growth control tubes. The CFU count of each sample tube is divided by the CFU count of the growth control tube, divided by 4 days, and log converted. This gives the delta log growth per day for each sample tube. Software to calculate the change in bacillary viability based on MGIT TTP written by one of the authors (R.S.W.) is available on request. (For an example showing data expressed as TTP and delta log growth per day in one case, see Fig. 4 and compare it with Fig. S2 in the supplemental material.) The number of viable mycobacteria recovered following incubation of PBMCs was lower than expected due to the effect of residual penicillin and streptomycin in culture media. The predicted growth was 2.5 × 10^8 CFU, and the actual growth was 250 CFU, a difference of approximately 3 log units (0.9013 delta log growth per day) (see Fig. S3 in the supplemental material). To calculate growth inhibition in PBMC samples, 0.9013 delta log growth per day was subtracted from the control tube to better reflect the number of viable mycobacteria used in the assay.

**PBMC-based growth inhibition assay.** We adapted the whole-blood assay described above for use with cryopreserved PBMCs. PBMCs from 19 of the 30 volunteers were thawed and rested overnight at 37°C in RPMI containing benzonase (10 U/ml; Novagen), 10% pooled human AB serum, L-glutamine, penicillin, and streptomycin. After the overnight rest, the cells were counted, washed, and resuspended in the above-mentioned medium without benzonase or antibiotics but with HEPES. The percent viability of recovered cells was 70 to 90% per vial. Duplicate 2-ml screw-cap tubes containing 1 × 10^6 PBMCs in 600 μl of medium were incubated on a rotator at 37°C with ~600 CFU of BCG Pasteur stock for 4 days. The PBMCs were then lysed with sterile water, and the lysate containing mycobacteria was transferred to a Bactec MGIT supplemented with PANTA antibiotics and OADC enrichment broth. The tube was placed in a Bactec MGIT 960 and incubated until growth was detected (time to positivity). The data were analyzed as described above for the whole-blood assay.

**Data analysis.** Statistical analysis was performed using SPSS and Graphpad Prism. One-way analysis of variance (ANOVA) and Students *t* test.
tests were used to determine significant differences in growth inhibition. Mann-Whitney and Wilcoxon signed-rank tests were used to determine differences in ELISPOT responses. Spearman’s rho was used to determine correlations between growth inhibition and immune response. Assay variability was assessed by calculating the coefficient of variation (CV).

RESULTS

Study participants. There were 30 participants enrolled in the study, 15 of whom had been previously vaccinated with BCG (a mean of 17.8 years prior to enrollment). The remaining 15 had no history of BCG vaccination (Fig. 1). All volunteers received a single intradermal immunization with BCG-SSI. The demographic characteristics of the study participants are summarized in Table 1 and are similar to those reported in previously published studies (11, 12).

IFN-γ ELISPOT response to PPD in BCG-vaccinated volunteers. In order to compare immunological responses in the BCG-vaccinated subjects with growth inhibition, IFN-γ responses were first measured by ELISPOT. There was a significant increase in the magnitude of the IFN-γ ELISPOT response to PPD following both primary vaccination and revaccination with BCG (P < 0.0001) (Fig. 2A and B). The peak IFN-γ ELISPOT response was at 4 weeks following immunization, and it had returned to baseline by 24 weeks for both groups (Fig. 2A and B). When the two groups were compared, we found that the IFN-γ ELISPOT responses were significantly higher in those volunteers revaccinated with BCG at baseline, week 4, and week 24 (P < 0.05) (Table 2). This is consistent with PPD IFN-γ ELISPOT responses observed following BCG vaccination in previously published studies (11).

Measurement of growth inhibition following BCG vaccination. A mycobacterial growth inhibition assay using a Bactec MGIT to detect growth (13) was modified (see Materials and Methods) and used to measure growth inhibition using both whole blood and cryopreserved PBMCs from volunteers previously vaccinated with BCG and volunteers who had no history of BCG vaccination. Growth inhibition was measured in 9 volunteers using the whole-blood assay and in 10 volunteers using the PBMC assay, with 5 volunteers tested using both assays. Using whole blood, there was a trend toward enhanced growth inhibition when BCG-vaccinated and naive subjects were compared, but the difference was not significant (Fig. 3A). Using cryopreserved PBMCs, there was significantly more mycobacterial growth inhibition when PBMCs from BCG-vaccinated subjects were compared with those from BCG-naive subjects (P < 0.05) (Fig. 3B).

Following primary BCG vaccination, we observed enhanced mycobacterial growth inhibition compared with prevaccination responses in whole blood collected 8 weeks following vaccination in 7 of 9 volunteers tested (P < 0.05; t test), although the response was not significant when tested using one-way ANOVA (Fig. 3A; for comparison with time-to-positivity data, see Fig. S2A in the supplemental material). In subjects with a history of BCG vaccination, we did not detect increased mycobacterial growth inhibition in whole blood after BCG revaccination (Fig. 4B; for comparison with time-to-positivity data, see Fig. S2B in the supplemental material).

Using cryopreserved PBMCs from the same volunteers, we observed significantly enhanced mycobacterial growth inhibition at both 4 and 8 weeks following primary vaccination with BCG (P < 0.05; Wilcoxon signed-rank test) (Fig. 4C). All volunteers displayed enhanced mycobacterial growth inhibition following BCG immunization; for 6 volunteers, this occurred at both weeks 4 and 8. In subjects with a history of BCG vaccination, we did not detect increased mycobacterial growth inhibition in cryopreserved PBMCs after BCG revaccination (Fig. 4D).

TABLE 1 Demographics of volunteers enrolled in the study

| Characteristic          | Value for each groupa |
|-------------------------|------------------------|
|                         | BCG (n = 15)           | BCG-BCG (n = 15)         |
| Male [no. (%)]          | 5 (33)                 | 8 (53)                   |
| Median age [yr (range)] | 28 (18–55)             | 27.5 (19–53)             |
| Avg time since BCG      |                        |                         |
| vaccination [yr (range)]| 17.8 (3–38)            |                         |

a BCG, primary vaccination; BCG-BCG, revaccination.

FIG 2 T-cell responses in adults receiving primary vaccination with BCG or BCG revaccination. PPD antigen-specific T cells were detected in PBMCs from healthy, BCG-naive (A) or previously BCG-vaccinated (B) adults receiving ~2 × 106 to 8 × 106 CFU of BCG-SSI. The T cells were stimulated overnight with PPD, and the responses were detected using an ex vivo IFN-γ ELISPOT assay. Values were determined by a Wilcoxon matched-pairs signed-rank test compared to the baseline (before BCG vaccination). SFC, spot-forming cells. Dots indicate individual data points, and the line indicates the median response for the group.

TABLE 2 Comparison of immune responses between groups of volunteers receiving primary BCG vaccination or revaccination with BCG

| Wk no. | No. of SFC/106 PBMCs [median (IQR)] for group (n = 15)a | P value (Mann-Whitney; BCG vs BCG-BCG) |
|--------|----------------------------------------------------------|----------------------------------------|
| 0      | 37 (14–60)                                               | <0.005                                 |
| 4      | 210 (79–516)                                             | <0.05                                  |
| 8      | 185 (83–858)                                             | NSa                                    |
| 24     | 53 (32–117)                                              | <0.05                                  |

a SFC, spot-forming cells; IQR, interquartile range; BCG, primary vaccination; BCG-BCG, revaccination.

b NS, not significant.
The magnitude of observed growth inhibition was significantly greater using cryopreserved PBMCs than using whole blood at both weeks 4 and 8 following vaccination with BCG. To confirm the ability of the PBMC assay to detect a difference pre- and post-vaccination with BCG, we repeated the experiment using PBMCs from the same 8 volunteers we used in the whole-blood assay. We also ensured that the PBMCs were not exposed to antibiotics at any point in the procedure, as was the case with the PBMC data shown in Fig. 3B. Differences pre- and postvaccination with BCG were observed only when we lowered the inoculum volume to 250 \( \times 10^5 \) CFU in 1 \( \times 10^6 \) PBMCs (Fig. 5). This suggests that BCG vaccine-mediated control of mycobacterial growth may be overwhelmed at higher doses of viable mycobacteria and that growth inhibition in the whole-blood assay may be further improved if a lower dose of mycobacteria is used.

The reproducibility of the growth inhibition assay in these studies was assessed as described in Materials and Methods for both whole blood and cryopreserved PBMCs using samples collected in 3 consecutive weeks at baseline in our trial (Fig. 1). The mean CVs and 95% confidence intervals for cryopreserved

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**FIG 3** In vitro growth inhibition in BCG-vaccinated and BCG-naive subjects using both whole blood and cryopreserved PBMCs. (A) The abilities of whole blood from 8 BCG-naive and 10 previously BCG-vaccinated volunteers to inhibit mycobacterial growth at baseline were compared. There was a trend toward enhanced mycobacterial growth inhibition in the BCG-vaccinated group, but it was not significant. (B) The abilities of cryopreserved PBMCs from 9 BCG-naive and 10 BCG-vaccinated volunteers to inhibit mycobacterial growth were assessed. Significantly more growth inhibition was observed in the PBMC cultures from previously BCG-vaccinated subjects \((P < 0.05; t\text{ test})\). The box plots show the lowest data point of the 25th quartile and the highest data point of the 75th quartile (Tukey).

**FIG 4** Mycobacterial growth in the blood and PBMCs of adults receiving primary vaccination with BCG or following revaccination with BCG. Mycobacterial growth was measured in whole blood (A and B) and cryopreserved PBMCs (C and D) from healthy, BCG-naive (A and C) or previously BCG-vaccinated (B and D) adults receiving \( \sim 2 \times 10^3 \) to \( 8 \times 10^3 \) CFU of BCG-SSI. Mycobacterial growth in samples collected at baseline (before BCG vaccination) was compared to growth in samples collected at 4, 8, and 24 weeks postvaccination. One-way ANOVA was used to test for significance, followed by a paired \( t\) test. The box plots show the lowest data point of the 25th quartile and the highest data point of the 75th quartile (Tukey).
DISCUSSION

Using a mycobacterial growth inhibition assay, we observed that subjects with a history of BCG vaccination control the growth of mycobacteria more effectively than those who have never been immunized. In addition, primary BCG vaccination leads to inhibition of mycobacterial growth for up to 8 weeks following immunization, whereas revaccination with BCG does not improve, and may reduce, the ability of cells to control mycobacterial growth. In the United Kingdom, primary vaccination with BCG protects with an efficacy of 80% (14). There have been no clinical trials of intradermal BCG revaccination in the United Kingdom, but the lack of change in TB incidence following withdrawal of BCG revaccination (Finland) (15) and lack of efficacy in clinical trials of BCG revaccination in other countries (Malawi, Brazil, Chile, and Hong Kong) (16–20) have shown that revaccination with BCG does not improve protection from TB disease. Our in vitro mycobacterial growth inhibition data are consistent with the epidemiological data, which indicate that primary vaccination, but not revaccination, with BCG can improve protection against TB disease.

There is an urgent need for a biomarker that can be used to identify potentially protective vaccines or vaccine combinations early in clinical development, which could accelerate regulatory approval of clinical studies and ultimately the approval of new TB vaccines. The capacity of these assays to detect inhibition of mycobacterial growth in both historically and recently BCG-vaccinated subjects warrants further evaluation in different study populations and in phase I and II clinical trials of candidate TB vaccines. An assay used for regulatory approval would have to be validated according to the principles of the International Conference on Harmonization (ICH) (ICH Harmonized Tripartite Guidelines), which encompass accuracy, precision (repeatability, intermediate precision, and reproducibility), specificity, and linearity. To begin to determine if it would be possible to validate an MGIA, we determined the intra-assay variability of both the
whole-blood- and PBMC-based MGIA. Tuomela et al. suggest that for assay validation a CV of less than 50% is acceptable variation for the measurement of a bacterial target of a cell-based assay (21). In this report, both the whole-blood and the PBMC MGIA had a CV of less than 50% over repeated sampling prior to immunization. Volunteers were recruited over a 12-month period, and multiple aliquots of frozen mycobacterial stock were used in the whole-blood assay. Variations in mycobacterial stock viability and in volunteer blood and variability in the week-to-week performance of the assay could have contributed to the overall higher variability of the whole-blood assay compared to the PBMC assay, which was run in just two batches in this study. We saw the greatest improvement in growth inhibition at 8 weeks following primary vaccination with BCG, indicating that this is the optimum time point for measurement of growth inhibition following vaccination with live, replicating mycobacteria. We could not detect growth inhibition at 24 weeks. It is probable that the optimum time for measurement of growth inhibition will have to be determined during early-phase clinical trials for each candidate TB vaccine.

Earlier studies assessing four mycobacterial growth inhibition assays, two whole-blood- and two PBMC-based assays, observed optimum growth inhibition at 8 weeks and 6 months following revaccination with BCG (6, 22). Differences between our study and those of Hoft et al. and Cheon et al. include the use of a different growth inhibition assay protocol and a different BCG vaccine strain, enrollment of a United Kingdom study population in which primary BCG vaccination is known to have an efficacy of 80%, and a longer interval between primary vaccination and revaccination with BCG. Cheon et al. found that blood from only 4 of 10 subjects from the United States inhibited mycobacterial growth following primary vaccination with BCG, whereas PBMCs from 7 of the 8 United Kingdom subjects with no history of BCG vaccination tested in our study inhibited mycobacterial growth 8 weeks following primary BCG vaccination.

To determine if a BCG vaccine-induced immune response was correlated with growth inhibition, we compared the PPD antigen-specific IFN-γ T-cell response, measured using an ex vivo ELISPOT assay, with mycobacterial growth inhibition. Revaccination with BCG induces a greater number of PPD antigen-specific T cells than primary vaccination with BCG, yet this greater number of T cells does not result in an improved capacity to control mycobacterial growth in vitro. This is consistent with other reports that have found that IFN-γ is not correlated with mycobacterial growth inhibition following BCG vaccination of either infants or adults (6, 7). The peak immune response was at 4 weeks following revaccination with BCG, and this was the only time point at which the IFN-γ T-cell response was associated with control of mycobacterial growth in PBMCs. It is thought that prior exposure to environmental mycobacteria or M. tuberculosis can interfere with the establishment of protective immunity following immunization with BCG (13, 23). An immune response to PPD can be induced by previous BCG vaccination, exposure to environmental mycobacteria, or exposure to M. tuberculosis itself. Barreto et al. reported that the efficacy of BCG revaccination was 33% in a population in which there was low exposure to environmental mycobacteria and M. tuberculosis (the Brazilian city of Salvador) but that efficacy was lost in older children, who were more likely to have a response to PPD at the time of vaccination, and was also lost in cities where there was higher exposure to environmental mycobacteria and M. tuberculosis (23). These findings are consistent with our growth inhibition assay findings, in which we saw that a higher baseline immune response to PPD was associated with lack of control of mycobacterial growth at 24 weeks following revaccination. In this study, we have not identified the immune mechanism of in vitro growth inhibition but, consistent with other reports, we have shown that measurement of vaccine-induced IFN-γ-secreting T cells alone does not reflect the overall capacity of cells from vaccines to control mycobacterial growth.

Due to the lack of an immune correlate for protection against TB, IFN-γ remains the primary measure of TB vaccine immunogenicity in humans. Vaccine candidates are mostly selected for further development based on efficacy in preclinical animal models, safety, and the induction of an IFN-γ response in early clinical trials (24). In this report, we describe an alternative assay that could potentially allow direct comparison of vaccines across clinical trials and animal species. As this assay requires no specific immune reagents or antigen stimulation, it could be used for the early assessment of a wide range of candidate TB vaccines. Since the Bactec MGIT is found in many clinical laboratories, an automated version of the growth inhibition assay can be implemented by investigators performing TB vaccine studies in human subjects as described in this report, as well as in animal models of TB (25). Furthermore, as this assay appeared to mimic the findings of clinical-efficacy trials, detecting improved capacity to control mycobacterial growth following primary immunization but not revaccination with BCG, it may be a better surrogate for clinical efficacy than existing assays used for the assessment of candidate TB vaccines.

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