Evaluation of Human Antimycobacterial Immunity Using Recombinant Reporter Mycobacteria

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A novel in vitro whole blood model was developed to study human antimycobacterial immunity. Recombinant reporter mycobacteria were used to enumerate the bacteria, and interactions between host immune cells and mycobacteria were studied using whole blood rather than cell fractions. The ability of healthy tuberculin-positive and tuberculin-negative individuals to restrict mycobacterial growth was compared. Growth of luminescent mycobacteria was significantly lower in blood samples of tuberculin-positive individuals than in blood samples of tuberculin-negative individuals (P = .005). Restricted mycobacterial growth was associated with significantly higher production of tumor necrosis factor (TNF)–α and interferon (IFN)–γ (P = .01 and .004, respectively). Inhibition of the TNF-α and IFN-γ response pathways by neutralizing monoclonal antibodies increased mycobacterial growth in whole blood. This model is the first functional assay in which individual variations in cell-mediated immunity are shown to correlate with differences in ability to control mycobacterial growth. It provides a new tool for studying human mycobactericidal mechanisms and, potentially, for the evaluation of improved vaccines.

Mycobacterial disease remains a leading cause of morbidity and mortality worldwide [1]. After decades of declining incidence, the number of infected individuals is increasing once again in developing as well as developed countries [2], with the spread of multidrug-resistant tuberculosis making treatment of the disease increasingly difficult [3].

Vaccination with the live attenuated vaccine strain, Mycobacterium bovis bacille Calmette-Guérin (BCG), has been a component of childhood immunization schedules in most developing countries for many years [4], but the current vaccine is inconsistent in its ability to protect against the predominant pulmonary disease in adults [5]. A major impediment to the development of improved vaccines is the inadequate understanding of the mechanisms underlying protective immunity. Most individuals infected with M. tuberculosis or vaccinated with BCG develop an immune response that can be detected in the form of a delayed-type hypersensitivity reaction to intradermal injection of a crude mixture of M. tuberculosis antigens (tuberculin). Although the tuberculin response provides evidence of previous exposure to mycobacteria, it does not distinguish individuals who will go on to develop progressive disease from those who can successfully contain the infection [6]. Improved understanding of the mechanisms responsible for containing mycobacterial growth in humans is essential for the development of improved vaccines [7].

Immunity to mycobacterial infection has been studied extensively in mice, but the extent to which these observations can be extrapolated to humans is unclear. Murine macrophages are able to restrict the growth of mycobacteria in vitro by a pathway that depends on generation of reactive nitrogen intermediates [8, 9] and is enhanced by the addition of interferon (IFN)–γ [10, 11] and tumor necrosis factor (TNF)–α [12] and by the presence of interleukin (IL)–12 [13]. Multiple subsets of T cells are essential for protective immunity in mice [14, 15], but no single immune activity is, in itself, sufficient to confer optimal protection.

The increased susceptibility to mycobacterial infections in humans with mutations that affect the IFN-γ receptor [16, 17] and the IL-12 receptor [18] emphasizes the crucial role these pathways play in humans as well. Conflicting data on the role of T cells [19], macrophages [20], and immune effector mechanisms [21] in the human host have been published, and it is likely that a complex interaction of cellular and humoral factors...
[22] is required to contain the growth of mycobacteria [23]. However, there is general agreement that the ultimate factor determining immunity is the ability of the human immune system to prevent growth of intracellular mycobacteria within the target cell population—that is, the macrophage.

The mechanisms involved in restriction of mycobacterial growth have been difficult to study because of the slow growth of the organism; 2–4 weeks are required for formation of colonies according to conventional culture protocols. Expression of recombinant luciferase enzymes has been widely used as a reporter, to monitor changes in cell viability in bacterial and eucaryotic systems. In mycobacteria, luciferase reporters have been used mainly to monitor the effect of drugs on viability [24], but they have also shown some promise in detecting immune-mediated killing in animal models [25, 26]. We postulated that the reporter gene strategy could be used to study human mycobactericidal mechanisms in whole human blood, which contains all the cellular and humoral components potentially required for control of mycobacterial growth.

Materials and Methods

Human subjects. Response to 10 U of purified protein derivative (PPD; tuberculin PPD 100 U/mL; Evans Medical Ltd., Leatherhead, UK) was assessed in 20 healthy adult volunteers 3–6 months prior to collection of blood samples. Groups of 10 tuberculin-negative individuals with an induration <5 mm and 10 tuberculin-positive individuals with an induration >15 mm were recruited for the study. Peripheral blood obtained by venipuncture was diluted with an equal volume of RPMI 1640 (Life Technologies Ltd., Paisley, UK), supplemented with 1% l-glutamine and heparin (10 U/mL; Monoparin 1000 U/mL; CP Pharmaceuticals Ltd., Wrexham, UK). Part of the blood sample was centrifuged for 10 min at 1200 g to obtain plasma; the remainder was used directly in the whole blood assay.

Reporter mycobacteria. M. bovis BCG, Montreal strain, was transformed with the shuttle plasmid pSMT1, as described elsewhere [26]. pSMT1 carries the luxAB genes from Vibrio harveyi, under the control of the mycobacterial GroEL (hsp60) promoter. The promoter is constitutively active, showing little or no response to stress in the plasmid construct [27]. Recombinant mycobacteria (BCG lux) were grown into the logarithmic phase in Middlebrook 7H9 culture medium with 10% ADC supplement (Becton Dickinson, Oxford, UK) and hygromycin (50 µg/mL; Roche, Lewes, UK). The culture was dispensed into vials; glycerol was added, to a final concentration of 25%; and the vials were frozen at −70°C on a platform shaker (model STR6; Stuart Scientific, Stone, UK) set at 20 rev/min. Triplicate samples were analyzed for mycobacterial viability at the time of inoculation and at time points up to 96 h. Parallel infection experiments were set up, using plasma samples from the same donor, and were processed in the same way as the whole blood assays.

At each time point, Bijou tubes were centrifuged for 10 min at 2000 g. Supernatant (0.4 mL) was aspirated from each tube and was immediately frozen at −70°C for subsequent cytokine measurements. RPMI (0.4 mL) was then added to each tube, and the contents were mixed and transferred into 20-mL Universal containers (SLS). Red blood cells were lysed by the addition of 8 mL of distilled water and incubated at room temperature for 10 min. Bijou tubes were rinsed with a further 2 mL of distilled water to remove any residual material, which was then added to the corresponding Universal container. After lysis, the samples were centrifuged at 2000 g for 10 min, the supernatant was discarded, and the remaining pellet of bacteria and cells was resuspended in 1 mL of PBS. Serial dilutions (10−1 and 10−2) were prepared, and luminescence was measured, as described above. Readings were carried out in duplicates for each individual tube and were expressed as the mean of triplicate samples. Where appropriate, 0.1-mL aliquots of serial dilutions were tested for colony-forming units, as described above.

Cytokine assays. Supernatants for cytokine assays were thawed, and serial dilutions were prepared in PBS/10% fetal calf serum (Life Technologies). IFN-γ, TNF-α, and IL-10 were measured by ELISA, using antibody pairs, provided by BD Pharmingen (Becton-Dickinson, Oxford, UK), according to the manufacturer’s recommended protocols. Standard curves were generated, using reference cytokines provided by BD Pharmingen. For each of the cytokines, the detection limit was 50 pg, and a linear response was observed for amounts up to 5000 pg. Results are expressed as the mean of duplicate readings for each sample, all of which were assayed in triplicate. For cytokine inhibition assays, monoclonal antibodies (MAbs) against TNF-α (5 µg/mL), IFN-γ (17.5 µg/mL), and IL-12 (10 µg/mL; all from R&D Systems Europe Ltd., Abingdon, UK) and against IFN-γ receptor (2.5 µg/mL; Genzyme Diagnostics, Cambridge, MA) as well as isotype controls (goat IgG for anti–TNF-α and anti–IFN-γ and mouse IgG, for anti–IFN-γ receptor and anti–IL-12, in the same concentrations as neutralizing antibodies), were added to aliquots of blood from tuberculin-positive individuals prior to inoculation with BCG lux, following the manufacturer’s recommendations. At the concentrations used, each of the neutralizing antibodies was shown, by ELISA, to completely block detection of the target cytokine.

Statistical methods. Nonparametric statistical tests were used to avoid assumption of normally distributed data sets. To deter-
Figure 1. Growth of bacille Calmette-Guérin (BCG) lux, measured by relative light units (RLUs; □) and colony-forming units (cfu; ○). A. Growth in mycobacterial culture medium: $10^5$ RLU ($10^3$ cfu) of BCG lux was inoculated into 7H9 mycobacterial culture medium, and the number of mycobacteria was measured over 18 days. At each time point, 100 µL was removed from the culture, and RLUs and colony-forming units were assessed in triplicate samples. B. Growth in whole blood: $10^5$ RLU ($10^4$ cfu) of BCG lux was inoculated into triplicate samples of whole blood of a tuberculin-positive individual, and mycobacterial numbers were measured over 96 h. At each time point, the RLUs and colony-forming units were assessed in triplicate samples, following the described method. The means of the triplicate samples are shown alongside the error bars, which represent SDs.

Figure 2. Growth of bacille Calmette-Guérin (BCG) lux in whole blood (A) and plasma (B), comparing blood (●) and plasma (○) of a tuberculin-positive individual and blood (■) and plasma (□) of a tuberculin-negative individual: 100 µL of BCG lux, containing $5 \times 10^7$ relative light units (RLUs), was inoculated into 0.9 mL of whole blood or plasma from a tuberculin-positive and a tuberculosis-negative individual, and the RLUs were measured over 96 h. Symbols represent means of triplicate samples; the error bars represent SDs.

Results

Reporter mycobacteria in a whole blood infection model. To validate the use of bioluminescence to monitor the growth of mycobacteria during interaction with immune cells in whole blood, we inoculated aliquots of fresh, anticoagulated blood from volunteers with $10^5$ RLU/mL of luciferase-labeled BCG (BCG lux). Figure 1 illustrates the growth of BCG lux, as measured by RLUs and colony-forming units, in bacterial culture medium (figure 1A) and in whole blood of a tuberculin-positive donor (figure 1B). In a series of initial experiments, we observed a similar pattern of growth, assessed by colony-forming units or RLUs. A reading of 10 RLU was found to correspond to $\geq 1$ cfu (Spearman’s correlation coefficient, $r = 1$; $P = .0001$). The more rapid and technically simpler luminescence assay was adopted for subsequent analysis of multiple samples from a panel of 20 volunteers.

Growth in whole blood and plasma. To determine whether the cellular component of human blood affected mycobacterial viability, growth of BCG lux in whole blood was compared with growth in plasma samples from which blood cells had been removed by centrifugation prior to

mine whether 2 sets of paired data were significantly different for a given variable, the Wilcoxon matched pairs signed-rank tests were used. All data were analyzed by using Stata statistics/data analysis (Stata Corp., College Station, TX).
Figure 3. Growth of bacille Calmette-Guérin (BCG) lux in whole blood of 10 tuberculin-positive and 10 tuberculin-negative individuals. The growth of BCG lux was determined at 96 h in 10 tuberculin-positive and 10 tuberculin-negative individuals. Growth of BCG lux is expressed as a growth ratio, using the formula: [(RLU of BCG lux at 96 h) – (RLU of BCG lux in inoculum)]/(RLU of BCG lux in inoculum). Each point represents the mean of triplicate determinations; horizontal lines show median values. For tuberculin-positive individuals, median = 1.8, range = 0.3–6; for tuberculin-negative individuals, median = 4.9, range = 2.2–10 (P = .005, Wilcoxon signed-rank test).

infection. Figure 2 shows a representative experiment, comparing growth of BCG lux in whole blood (figure 2A) and plasma from a tuberculin-positive and a tuberculin-negative individual (figure 2B). For each individual, mycobacterial growth in whole blood was lower than that in plasma, which suggests that the presence of cellular elements was limiting the growth of the mycobacteria.

Mycobacterial growth in whole blood of tuberculin-positive and tuberculin-negative individuals. To test whether mycobacterial viability in whole blood was influenced by immune status, the growth of BCG lux was compared in whole blood of tuberculin-positive and tuberculin-negative individuals. In all of the experiments in which paired samples were studied, less growth was observed in the whole blood of tuberculin-positive than in that of tuberculin-negative individuals. The combined results from all 20 individuals are shown in figure 3, expressed as a growth ratio based on the following formula: [(RLU of BCG lux at 96 h) – (RLU of BCG lux in inoculum)]/(RLU of BCG lux in inoculum).

At 96 h, the growth of BCG lux was significantly lower in blood from tuberculin-positive individuals (median, 1.8; range, 0.3–6) than in blood from tuberculin-negative individuals (median, 4.9; range, 2.2–10; P = .005, Wilcoxon signed-rank test). In contrast, no difference in growth was detected in the plasma samples from the 2 groups (P = .54; data not shown).

Associated cytokine production. Because IFN-γ and TNF-α have been shown to be critical for inhibiting mycobacterial growth in mice, we studied the production of IFN-γ, TNF-α, and IL-10 during growth of BCG lux in whole blood. The time course for production of each of these cytokines was determined over the duration of the experiment. Peak levels of TNF-α and IL-10 were observed at 72 h, whereas IFN-γ peaked at 96 h. The results for the panel of 20 volunteers, based on cytokine measurements at the peak time of production, are summarized in table 1.

Significantly more IFN-γ and TNF-α were produced during growth of BCG lux in the blood of tuberculin-positive individuals than in the blood of tuberculin-negative individuals (P = .005 for both). There was no significant difference in the amount of IL-10 produced (P = .65).

To evaluate the relationship between mycobacterial growth and cytokine production, we separated subjects into those showing restricted growth of BCG lux (defined as a growth ratio below the median of 1.8 for tuberculin-positive individuals [n = 6]) and those whose blood supported mycobacterial growth (a growth ratio above the median [n = 14]). Subjects with restricted growth produced significantly more TNF-α than those with unrestricted growth produced (median, 734 vs. 144 pg/mL, respectively; P = .02). The production of IFN-γ was also significantly higher in the group with restricted growth (median, 329 vs. 18 pg/mL, respectively; P = .011), whereas no significant differences were seen in the production of IL-10 (median, 254 vs. 243 pg/mL; P = .5).

Effect of neutralizing cytokines. To assess whether the differences in cytokine production between tuberculin-positive and tuberculin-negative individuals were responsible for the observed differences in growth of BCG lux, neutralizing antibodies were used to modulate the activity of key cytokines.

MAbs against TNF-α, IFN-γ, IL-12, IFN-γ receptor, and their isotype controls were added to aliquots of blood from tuberculin-positive individuals prior to inoculation with 1 × 10⁷ RLU/mL of BCG lux. Control samples from the same individuals, not containing neutralizing antibody but with the same inoculum of BCG lux, were analyzed at the same time points. Results are shown in figure 4 as increased or decreased growth in comparison with the antibody-negative control. Each

| Table 1. Peak concentrations (pg/mL) of tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and interleukin (IL)-10 in supernatants during growth of bacille Calmette-Guérin (BCG) lux in whole blood. |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Cytokine measured | Tuberculin positive | Tuberculin negative |
|                 | (n = 10)         | (n = 10)         |                  |                  |
|                 | Median   | Range       | Median   | Range       | P     |
| TNF-α           | 584     | 112–1187   | 138     | 40–535      | .005  |
| IFN-γ           | 270     | 53–898     | 0       | 0–117       | .005  |
| IL-10           | 254     | 8–936      | 243     | 0–1636      | .65   |

NOTE. Cytokine concentrations were measured during growth of BCG lux in whole blood of 10 tuberculin-positive and 10 tuberculin-negative individuals. Peak TNF-α and IL-10 concentrations were measured at 72 h and peak IFN-γ concentration at 96 h. Medians and ranges are based on triplicate samples. Concentrations of IFN-γ and TNF-α were significantly higher in plasma of tuberculin-positive individuals than in plasma of tuberculin-negative individuals (P = .005 by Wilcoxon signed-rank test).
anti–tumor necrosis factor (TNF–a) individuals ( ) were incubated with neutralizing monoclonal antibodies (MAbs) to TNF–a, interferon (IFN–g), anti–IFN–g receptor, anti–interleukin (IL–12), and isotype control antibodies. Growth of BCG lux was measured at 96 h. The growth of BCG lux in the presence of an inhibitory antibody is expressed as a percentage of the growth in the control (100%), which did not contain inhibitory antibodies. ● Mean of triplicate determinations for a single individual; ▲, mean for each group. Error bars show SDs.

Figure 4. Effect of neutralizing monoclonal anticytokine antibodies on growth of bacille Calmette-Guérin (BCG) lux in whole blood of tuberculin-positive individuals. Blood samples from tuberculin-positive individuals (n = 5) were incubated with neutralizing monoclonal anti–tumor necrosis factor (TNF–a), anti–interferon (IFN–g), anti–IFN–g receptor, anti–interleukin (IL–12), and isotype control antibodies. Growth of BCG lux was measured at 96 h. The growth of BCG lux in the presence of an inhibitory antibody is expressed as a percentage of the growth in the control (100%), which did not contain inhibitory antibodies. ● Mean of triplicate determinations for a single individual; ▲, mean for each group. Error bars show SDs.

Discussion

The vast majority of individuals exposed to infection with Mycobacterium tuberculosis mount an immune response that prevents progression to primary disease. However, ~10% of infected individuals go on to develop disease either following initial infection or at a later stage in life [28]. It is currently impossible to determine whether disease results from a poor initial response to infection or from a subsequent impairment of an initially protective response. The inability to distinguish individuals with lifelong immunity from those who are at risk of progressive disease presents a fundamental obstacle to the design of tuberculosis control strategies and to the rational development of improved vaccines.

Recognizing that tuberculin skin test reactivity does not provide a reliable indicator of protective immunity, many investigators have tried to identify individual cellular components, reactivity to specific antigens, or patterns of cytokine expression that might be associated with protective immune responses [23].

Previous attempts to use mycobacterial viability as a means of assessing immunity in humans have focused on use of isolated cell fractions. Cheng et al. [29] assessed mycobacterial growth in peripheral blood–derived human monocytes obtained from individuals before and after BCG vaccination. Boney et al. [30] similarly used blood–derived monocytes to assess the effects of addition of a variety of cytokines and used a luciferase reporter system to monitor effects on mycobacterial viability.

The experiments described in the present study have focused on use of BCG lux as a reporter. Whereas this system has obvious practical advantages over the use of M. tuberculosis itself in relation to safety, it is possible that different mechanisms are required for control of the virulent and attenuated strains. Preliminary studies with M. tuberculosis lux have shown a pattern of growth in the blood of tuberculin-positive and tuberculin-negative individuals similar to that seen with BCG lux (authors’ unpublished observations), but further detailed studies in the whole blood model will be required to establish whether growth of the 2 bacteria is controlled in the same way.
Constructs in which luciferase expression is placed under the control of promoters that are differentially regulated during the course of the infection represent a possible approach to further enhancement of the discriminatory ability of the whole blood assay. Although it could be argued that whole blood is not the ideal system for studying mycobacterial immunity, lymphohematogenous spread occurs during early dissemination of mycobacteria [32], and bacteremia is increasingly recognized, particularly in the immunocompromised [33]. The use of whole blood in a functional assay that assesses mycobacterial growth as the final effector function is, therefore, a relevant model for understanding key elements of protective immunity.

Current research efforts toward the development of a more effective vaccine for the prevention of tuberculosis are driven by the availability of the genome sequence for M. tuberculosis [34]. Screening of the resulting panel of subunit and live-attenuated candidates currently relies on extensive testing in mouse and guinea pig models, which may only partially reflect vaccine activity in humans. Once identified, promising candidates will have to undergo long and expensive prospective clinical trials to determine their protective efficacy in human populations. A human in vitro assay able to detect potential protective responses elicited by a vaccine candidate in preliminary immunogenicity trials would be of great benefit for rational vaccine development. In this context, the whole blood assay might provide an important new tool.

References

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