Mycobacterium tuberculosis CDC1551 Induces a More Vigorous Host Response In Vivo and In Vitro, But Is Not More Virulent Than Other Clinical Isolates

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Mycobacterium tuberculosis CDC1551 Induces a More Vigorous Host Response In Vivo and In Vitro, But Is Not More Virulent Than Other Clinical Isolates

Claudia Manca,* Liana Tsenova,† Clifton E. Barry III,‡ Amy Bergtold,* Sherry Freeman,* Patrick A. J. Haslett,* James M. Musser,§ Victoria H. Freedman,* and Gilla Kaplan*

Mycobacterium tuberculosis CDC1551, a clinical isolate reported to be hypervirulent and to grow faster than other isolates, was compared with two other clinical isolates (HN60 and HN878) and two laboratory strains (H37Rv and Erdman). The initial (1–14 days) growth of CDC1551, HN60, HN878, and H37Rv was similar in the lungs of aerosol-infected mice, but growth of Erdman was slower. Thereafter, the growth rate of CDC1551 decreased relative to the other strains which continued to grow at comparable rates up to day 21. In the lungs of CDC1551-infected mice, small well-organized granulomas with high levels of TNF-α, IL-6, IL-10, IL-12, and IFN-γ mRNA were apparent sooner than in lungs of mice infected with the other strains. CDC1551-infected mice survived significantly longer. These findings were confirmed in vitro. The growth rates of H37Rv and CDC1551 in human monocytes were the same, but higher levels of TNF-α, IL-10, IL-6, and IL-12 were induced in monocytes after infection with CDC1551 or by exposure of monocytes to lipid fractions from CDC1551. CD14 expression on the surface of the monocytes was up-regulated to a greater extent by exposure to the lipids of CDC1551. Thus, CDC1551 is not more virulent than other M. tuberculosis isolates in terms of growth in vivo and in vitro, but it induces a more rapid and robust host response. The Journal of Immunology, 1999, 162: 6740–6746.

Tuberculosis (TB),1 the primary cause of mortality from an infectious agent in the world, accounts for ~3 million deaths per year. Efforts to eradicate the disease are hindered by several factors, including the prevalence of the disease in socioeconomic populations that have limited access to diagnosis and treatment and the development of multidrug-resistant Mycobacterium tuberculosis strains. Outbreaks of drug-sensitive TB can involve infection with strains of M. tuberculosis with widely different rates of transmission (1). In 1995, an outbreak occurred in a rural area in the United States considered to be at low risk for TB (2). The strain, designated CDC1551 or CSU 93 by the Centers for Disease Control and Prevention (CDC) attracted special attention because of an unusually high rate of transmission as evaluated by skin test conversion. In addition, infected subjects had a very large skin test response to purified protein derivative of tuberculin (PPD). Infection with CDC1551 was not associated with an obvious increase in active TB cases, nor did any patients have extrapolmonary disease. However, when the growth of CDC1551 was evaluated in lungs of mice at 20 days after aerosol infection, 100-fold higher numbers of bacilli were isolated compared with numbers of bacilli isolated from the lungs of mice infected with the M. tuberculosis laboratory strain Erdman (2). This led investigators to conclude that CDC1551 had “increased virulence,” and this clinical isolate was selected for sequencing by the National Institutes of Health (3).

In the present study, we have used in vivo and in vitro models of infection to investigate whether CDC1551 is indeed more virulent than other M. tuberculosis strains. Using an aerosol infection model, we compared the growth rates of two recent clinical isolates and two laboratory strains of M. tuberculosis in the lungs, spleen, and liver of infected mice. Simultaneously, we monitored the granulomatous response in the infected lungs, the relative cytokine mRNA levels expressed in the infected lungs, and the clinical response to infection. To confirm that the host response observed in the mouse is analogous to that of humans, we examined mycobacterial growth and the cell surface markers and cytokine response following infection of human monocytes in vitro with M. tuberculosis CDC1551 and H37Rv.

Materials and Methods

M. tuberculosis strains

The experiments involved laboratory strain H37Rv (Trudeau Institute, Saranac Lake, NY); strain CDC1551 (Dr. T. M. Shinnick, CDC, Atlanta, GA); strain Erdman (provided as multiple vials of stock by Dr. J. Belisle, Colorado State University, Fort Collins, CO); clinical isolates HN60 and HN878 recovered from patients in Houston, TX; H37Rv and the three clinical isolates were grown for 7 days in Middlebrook 7H9 medium (Difco, Detroit, MI) containing 0.05% Tween 80 (Sigma, St. Louis, MO) at 37°C with daily agitation. All stocks at 10⁵–10⁶ bacilli/ml were stored frozen at −70°C until use. All procedures were performed in a laminar flow hood in a biosafety level III laboratory.

Preparation of mycobacterial fractions

Proteins. Cultures of H37Rv and CDC1551 (100 ml) grown for 7 days from a starting OD of 0.1 at 650 nm in GAS media (glucose-alanine salts at pH 6.6 contains 0.3 g/L Difco Bacto Casein, 0.05 g/L ammonium
iron(III) citrate, 4.0 g/L K$_2$HPO$_4$, 2.0 g/L citric acid, 1.0 g/L L-alanine, 1.2 g/L MgCl$_2$·6H$_2$O, 0.6 g/L K$_2$SO$_4$, 2.0 g/L NH$_4$Cl, 1.80 ml of 10 M NaOH, 10.0 ml of glycerol. The filtrate was concentrated by ultrafiltration through a 10,000-m.w. cutoff filter (Millipore, Bedford, MA) to a final volume of 2.0 ml (secreted proteins). The harvested cells were then suspended in 2 ml of PBS (pH 7.5) and combined with 0.5 of 0.1-mm-diameter glass beads and homogenized in a minibeat bead apparatus (BioSpec Products, Bartlesville, OK) for 3 min. The 10,000 × g supernatant was collected (cytoplasmic proteins). The remaining cell debris was suspended in 2 ml of 0.1% Triton X-100 (Sigma), mixed for 30 min at room temperature, and then centrifuged for 2 min (cell wall-associated proteins).

**Lipids.** Cultures of H37Rv and CDC1551 (100 ml) were grown in Middlebrook 7H9 media to an OD (650 nm) of 0.5 (8.7 × 10$^{13}$ CFU), recovered by centrifugation at 12,000 × g for 20 min, suspended in 20 ml of methanol, 0.3% aqueous sodium chloride (100:10), and extracted with 10 ml of petroleum ether for 15 min. The petroleum ether layer was removed, transferred to a glass vial, and dried under nitrogen. Two milliliters of PBS were added to the dried residue, and the vial was sonicated in a bath sonicator for a total of 5 min in 1-min bursts (apolar lipids). The remaining cells were extracted twice with 17.3 ml of chloroform, methanol, 0.3% aqueous sodium chloride (90:100:30). Centrifugation was followed by drying of the organic layer under vacuum. The residue was resuspended in 2 ml of PBS and sonicated as above (polar lipids).

**Reagents**

OADC supplement (sodium chloride, bovine albumin fraction V, dextrose, catalase, oleic acid) was from Becton Dickinson (Cockeysville, MD); human serum (pooled AB+) was from Gemini Bio Products (Calabasas, CA).

**In vivo studies**

**Mice.** Female 8-week-old (C57BL/6 × DBA/2)F1 (B6D2F1) mice, free of common viral pathogens were from Charles River Laboratories (Wilmington, MA).

**Aerosol Infection.** For each experiment, a vial of stock bacilli was sonicated in a water bath sonicator (Laboratory Supplies, model G112SPIT) for 1 hour, MA). Common viral pathogens were from Charles River Laboratories (Wilmington, MA).

**CFU assays.** The growth of M. tuberculosis in the lungs, spleen, and liver of infected mice was evaluated by homogenizing the right lung, the whole liver, and spleen in saline plus 0.04% Tween 80 and plated 10-fold serial dilutions of the homogenate on Middlebrook 7H11 agar (Difco). Histology and morphometry. The lungs and the upper lobe of the left lung of each infected mouse were fixed in 10% formalin, embedded in paraffin, and processed. Sections were stained with hematoxylin-eosin and Ziehl-Nielsen for light microscopy. Morphometry was performed with Microcomp, a computer-based image analysis system (Southern Micro Instruments, Atlanta, GA). A calibration micrometer slide was used to determine the area evaluated (square micrometers).

**RT-PCR for cytokine mRNA detection in lung homogenates.** The lower lobe of the left lung of each infected mouse was removed and immediately frozen. Tissues were homogenized in 3 ml of RNAzolB (Cinna/Biotecx, Houston, TX) with a tissue Polytron homogenizer. RNA was extracted according to the manufacturer’s instructions. The RT-PCR was conducted as described (5). Density of the amplification bands was conducted using a Phosphorimager (Molecular Dynamics). Results were normalized to the densitometry of β-actin and expressed as relative units or fold increase over baseline (uninfected control mouse lung).

**In vitro studies**

**Human monocytes.** PBMCs were isolated from fresh human blood (buffy coat) (New York Blood Center, New York, NY) and from healthy PPD-positive donors by centrifugation on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) as described previously (6). The cells were resuspended in RPMI 1640 (Life Technologies, Gaithersburg, MD), supplemented with 1% AB human serum (R1) (Gemini Bio Products, Calabasas, CA) and adjusted to 6 × 10$^6$/ml; 0.5 ml were plated in 24-well Falcon tissue culture dishes (Becton Dickinson Labware, Lincoln Park, NJ). Adherent cells were cultured in RPMI 1640 with 20% human serum (R20) at 37°C and 5% CO$_2$. **Infection of human monocytes.** Human monocytes were infected with M. tuberculosis H37Rv and CDC1551 on day 0. Before infection, aliquots of bacteria were probe-sonicated for 20 s at low output power (model 60 sonic dismembrator, Fisher Scientific, Springfield, NJ) to disperse clumped bacilli. All the procedures involving probe sonication of M. tuberculosis were conducted within a Biological Safety Cabinet Class II located in a Biosafety Level III Laboratory. All procedures used were approved by the Laboratory Safety Committee at The Rockefeller University. The bacterial suspension, diluted in R20, was added at a multiplicity of infection of 1 viable bacillus per cell to monolayers of 3 × 10$^6$ monocytes in R20 medium and cultured for 4 days (8). At the designated time points, PBS containing 0.016% digitonin (Sigma) and 0.25% Tween 80 (Sigma) was added to each well to release mycobacteria from the cells. After 10 min at 37°C, the cultures were probe sonicated, and mycobacteria were plated on Middlebrook 7H10 agar supplemented with OADC, as described (9).

**Cytokine determination.** Culture supernatants from infected monocytes or from monocytes incubated with vortexed 1:50 dilutions of polar or apolar lipids obtained from M. tuberculosis H37Rv and CDC1551 were harvested, frozen at −70°C, and then assayed with commercial ELISA kits (Endogen, Boston, MA) according to the manufacturer’s instructions. Spontaneous release of cytokines was monitored by culturing monocytes in the absence of experimental infection or stimulation.

**mAbs.** The following mAbs were used for flow cytometry: FITC-anti-CD3, PE-anti-CD14, PE-anti-CD80 (B7.1), PE-anti-HLA-DR, and PE-anti-γ$_c$ (isotype) control (Becton Dickinson, San Jose CA); FITC-anti-CD40 and PE-anti-CD86 (B7.2) (PharMingen, San Diego, CA).

**Flow cytometric analysis.** Monocytes stimulated with polar lipids (see above) were incubated for 30 min on ice and then treated for 30 min with cold PBS containing 0.2% EDTA (pH 7.2). Detached cells were washed three times with cold RPMI, and the pellet was resuspended in 700 μl of R1. Approximately 1.3 × 10$^6$ cells were incubated with 10 μl of conjugated mAbs in the dark at 4°C for 15 min; washed once in cold PBS containing 1% FCS, 0.1% sodium azide; fixed overnight with 1% paraformaldehyde; and protected from light at 4°C until flow cytometry (FACScan, Becton Dickinson).

**Statistical analysis.** The in vitro cytokine data were analyzed by a paired t test. Kaplan-Meier analysis was used to determine statistical significance of the differences in survival of mice; 95% confidence indices and the log rank test were used.

**Results**

We compared in vivo infection of mice with M. tuberculosis strains CDC1551, HN878, HN60, H37Rv, and Erdman. HN878 is a recent clinical isolate from Houston which has 20 hybridizing copies of IS6110 and is a member of M. tuberculosis principal genetic group 1 (10). DNA fingerprint 033 organisms have caused 60 cases of TB in Houston since 1995 and have been responsible for at least three known disease outbreaks. HN60 is another recent clinical isolate representative of Houston IS6110 fingerprint 085. It has 5 hybridizing copies of IS6110 and is a member of M. tuberculosis principal genetic group 3 (10). DNA fingerprint 085 organisms have caused five cases of TB in Houston since 1995 but have not been responsible for disease outbreaks in this community. M. tuberculosis H37Rv is the most commonly studied laboratory strain, and its genome was recently sequenced (11).

**Growth of M. tuberculosis strains in the lungs of mice infected by aerosol**

The course of lung infection following aerosol exposure of mice to either M. tuberculosis H37Rv, Erdman, or CDC1551 was followed for 60 days (Fig. 1A) and to M. tuberculosis HN60 and HN878 for 28 days (Fig. 1B). All strains except for the Erdman strain grew immediately in the lungs. By day 14 postinfection, H37Rv had multiplied from log$_{10}$ 2.2 ± 0.1 to log$_{10}$ 5.8 ± 0.5, CDC1551 from log$_{10}$ 2.4 ± 0.4 to log$_{10}$ 6.6 ± 0.4, HN60 from log$_{10}$ 2.9 ± 0.3 to log$_{10}$ 6.8 ± 0.3, and HN878 from log$_{10}$ 3.0 ± 0.3 to log$_{10}$ 7.1 ± 0.2. In contrast, the Erdman strain showed a delay in initial growth and then multiplied from log$_{10}$ 2.4 ± 0.2 to log$_{10}$ 4.1 ± 0.4 by day 14 (Fig. 1A). In these experiments, the estimated doubling times during the first 14 days of infection were similar for CDC1551, HN60, HN878, and H37Rv, but the doubling time was twice as long for Erdman (Table I). From 14 to 21 days, the CDC1551
strain grew more slowly than the other strains, suggesting that CDC1551 was subject to earlier growth restriction by the immune response of the host. This is supported by the change in doubling time from 25 h (0–14 days) to 105 h (14–21 days). The other M. tuberculosis strains continued growing at about the same initial rates up to day 21 postinfection (Table I). CDC1551, H37Rv, HN60, and HN878 CFU in the lungs decreased slightly from day 21 while Erdman continued growing albeit very slowly (see Fig. 1, A and B, and Table I).

For all five strains CFU were detected in spleen and liver at 7 days. The Erdman strain grew slower in both organs than the other strains, reaching log_{10} 3.6 in the spleen and log_{10} 2.6 in the liver 28 days after infection. The CFU for CDC1551, HN60, HN878, and H37Rv, both in the spleen and in the liver were always 1–2 log_{10} higher than Erdman; no significant differences were seen in the CFU among CDC1551, HN60, HN878, and H37Rv in these organs at any time point.

Granulomatous response in the lungs

Granuloma formation in the lungs infected with each of the strains was then examined. Small, well-organized granulomas were observed in histological sections of the lungs at day 14 only in CDC1551-infected mice (Fig. 1C). The calculated volume of these granulomas was ~0.03 mm³. In contrast, H37Rv-, HN60-, HN878-, and Erdman-infected mice did not have organized granulomas. Rather, the lungs of these animals had small cell aggregates of about 0.01, 0.008, 0.001, and 0.003 mm³, respectively. At 14 days postinfection, the total number of granulomas, cell aggregates detected in all histological sections of lungs was 2-fold higher for the CDC1551-, HN60-, and HN878-infected mice than for the mice infected with H37Rv (13.3 ± 6.4 for CDC1551, 16.6 ± 8 for HN60, 15.3 ± 9 for HN878, and 6.3 ± 2 for H37Rv).

By 21 and 28 days, the size and numbers of granulomas in the lungs of mice infected with H37Rv, HN60, and HN878 were comparable with those seen in the lungs of CDC1551-infected mice (Fig. 1, C and D). Well-formed, differentiated granulomas containing heavily infected macrophages and cuffs of lymphocytes were observed in the lungs by 28 days. In contrast, in the lungs of mice infected with Erdman, granulomas developed much more slowly and did not achieve the size observed in the lungs of mice infected with the other strains (60-day experiment) (Fig. 1, C and D).

Survival of mice

To determine whether there were differences in survival, mice were infected via aerosol with CDC1551, HN60, HN878, or H37Rv and monitored for >250 days. Mice infected with HN60 and HN878 began to die at about day 30, whereas mice infected with H37Rv did not begin to die until about day 80 (Fig. 2). The mean survival times were 177 days for mice infected with HN60 and 126 days for HN878 (95% confidence intervals 151–202 days and 99–153 days, respectively). The mean survival time for mice infected with H37Rv was 185 days (95% confidence intervals 164–205 days). Survival of mice infected with HN878 was significantly shorter than survival of mice infected with HN60 (p = 0.001). In

### Table I. Doubling time (h) of M. tuberculosis strains in the lungs of B6D2F₁ mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>Days 0–14</th>
<th>Days 14–21</th>
<th>Days 21–60</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDC1551</td>
<td>25 ± 0.8</td>
<td>105 ± 28</td>
<td>Neg</td>
</tr>
<tr>
<td>H37Rv</td>
<td>28 ± 2.5</td>
<td>36 ± 11</td>
<td>Neg</td>
</tr>
<tr>
<td>Erdman</td>
<td>56 ± 5.1</td>
<td>68 ± 15</td>
<td>554 ± 24</td>
</tr>
<tr>
<td>HN60</td>
<td>26 ± 0.1</td>
<td>30</td>
<td>ND</td>
</tr>
<tr>
<td>HN878</td>
<td>25 ± 0.8</td>
<td>42</td>
<td>ND</td>
</tr>
</tbody>
</table>

*Mice were exposed to aerosol infection, resulting in implantation of ~2.2–2.7 log_{10} organisms into the lungs. Doubling times were calculated from the mean number of CFU ± SD obtained from three experiments for CDC1551, H37Rv, and two experiments for Erdman, HN60, and HN878 (three to four mice per group per experiment).

*Neg, negative growth: a decrease in the total number of CFU was observed.*
contrast, mice infected with CDC1551 survived significantly longer than mice infected with the other strains, with a mean survival time of 250 days (median of 257, 95% confidence interval 239–275 days) (p < 0.001 compared with all strains).

Cytokine mRNA expression in the lungs of infected mice

To further evaluate the host immune response in the lungs, cytokine production was measured with a semiquantitative assay of specific mRNA levels (RT-PCR). We compared cytokine mRNA expression in lungs of mice infected with each of the five strains. To visualize the kinetics of changes in the cytokine-specific mRNA levels over the experimental period, results were expressed as fold increase over uninfected controls (baseline) assayed at the same time. Fig. 3 shows that there is earlier expression (day 7) of cytokine mRNA for TNF-α, IL-6, and IL-12 in CDC1551-infected mice. This pattern of early cytokine expression was not seen in response to infection with the other strains. By day 14, expression of all cytokines had increased in the CDC1551-infected mice. Thereafter, there was a transient reduction in cytokine expression (day 21–28) followed by a return to higher levels by day 60, with especially high levels of IL-10 and IFN-γ. In contrast, cytokine levels increased more slowly following infection with H37Rv, HN60, HN878, or Erdman (Fig. 3). Fig. 4 shows a direct comparison of the actual cytokine levels, assayed simultaneously at 14 and 60 days after infection with CDC1551, H37Rv, and Erdman. At day 14 postinfection, only CDC1551 elicited considerable cytokine levels in the lungs. TNF-α, IL-6, IL-10, IL-12, and IFN-γ were all expressed at higher levels than in the lungs of mice infected with H37Rv and Erdman. By 60 days postinfection, TNF-α levels were similar for the three strains. The other cytokines had increased in the lungs of H37Rv- and Erdman-infected mice, although the extent of increase following infection with Erdman was lower (Fig. 4).

In vitro infection studies

We then examined the response of human monocytes to infection with selected strains of M. tuberculosis. H37Rv was selected for these studies because it is the strain recently sequenced and therefore of interest for detailed biochemical and genetic analyses (11). The completion of the sequence of CDC1551 will enable direct comparison of these two strains (3).

Intracellular mycobacterial growth rates. To compare the growth of CDC1551 with that of the laboratory strain H37Rv, human monocytes were infected with each strain at a multiplicity of infection of 1:10 as described in Materials and Methods.

FIGURE 3. Kinetics of cytokine expression in lungs of mice infected with M. tuberculosis CDC1551 (left cross-hatched column), H37Rv (center open column), Erdman (center closed column), HN60 (right open column), and HN878 (right closed column). Results are expressed as fold increase over specific mRNA obtained from uninfected control lungs, normalized to the amount of β-actin mRNA. Data are means of two experiments with three to four mice per time point.

FIGURE 4. mRNA cytokine levels in lungs of mice infected by aerosol with M. tuberculosis CDC1551 (⊙), H37Rv (□), and Erdman (○). RNA extracted from uninfected lungs was used as controls (■). Results are expressed as mean ± SD of relative density units obtained from one representative experiment of three (three to four mice per time point per strain) calculated as described in Materials and Methods. To assure equivalent PCR conditions, the assays for the mRNA obtained from the lungs of the mice infected with the three M. tuberculosis strains were conducted simultaneously.
TNF-α levels were also determined in the same supernatants. Unlike in the absence of experimental infection, IL-10 and IL-12 elicited higher TNF-α levels than H37Rv at all time points (p < 0.01) (Fig. 6) and IL-6 (p < 0.01) (not shown). The differences in levels of IL-10 and IL-12 induced by apolar lipids of either H37Rv or CDC1551 were consistent but were not statistically significant (p = 0.09). Thus, the differences in cytokine production observed following infection with CDC1551 and H37Rv may be due to differences in the lipid components of these *M. tuberculosis* strains.

In addition, polar lipids affected the levels of CD14 expression on monocytes (Table III). The lipid fraction from CDC1551 induced higher levels of CD14 expression than did lipids of H37Rv. CD3, CD80, CD86, HLA-DR, and CD40 levels were unaffected.

## Discussion

In 1995, an outbreak of TB was traced to a strain of *M. tuberculosis* CDC1551 with an unusually high skin test conversion rate and unusually large PPD reactions (2). PPD conversion was reported even after only a single contact with the infected index case of infection of one viable bacillus per cell. In fresh human monocytes, both *M. tuberculosis* strains grew at a similar rate (Fig. 5A). The calculated doubling time of H37Rv during the 4 days of culture was 31 ± 3.8 h. CDC1551 grew within monocytes with a doubling time of 24 ± 3.9 h.

### Cytokine induction following infection of human monocytes

We next compared the ability of the two *M. tuberculosis* strains to induce host cells to release cytokines at various time points after infection. For both strains, maximal concentration of TNF-α was detected at 24 h following infection. *M. tuberculosis* CDC1551 elicited higher TNF-α levels than H37Rv at all time points (p < 0.05) (Fig. 5B). TNF-α was not detectable in the culture supernatant in the absence of experimental infection. IL-10 and IL-12 levels were also determined in the same supernatants. Unlike TNF-α, the peak of IL-10 production was observed at 96 h after infection with either strain (Fig. 5C). IL-10 concentrations induced by CDC1551 were higher than those induced by H37Rv. A small increase in spontaneous release of IL-10 into culture supernatant was detected 96 h after infection. In preliminary studies, IL-12 levels were maximal at 24 h postinfection of the monocytes. Higher levels of this cytokine were induced by infection of the monocytes with CDC1551 compared with H37Rv (Fig. 5D). IL-12 was not detectable in the culture supernatant of uninfected cells. Thus, in spite of similar numbers of intracellular organisms, monocytes infected with *M. tuberculosis* CDC1551 produced higher amounts of cytokines. Therefore, the stronger cytokine response following infection of human monocytes with CDC1551 compared with H37Rv, was analogous to the results obtained in vivo in mice, where CDC1551 induced higher levels of cytokine mRNA in the infected lungs.

### Cytokine induction and CD14 up-regulation by polar and apolar lipid fractions of *M. tuberculosis* CDC1551 and H37Rv in human monocytes

Previous studies have shown that mycobacterial proteins and lipids play an important role in inducing the host response (13, 14). Since the cytokine responses to infection with these two strains were different, we examined whether the two *M. tuberculosis* strains contained components with differing capacities to induce monocyte cytokines.

Mycobacterial fractions (polar and apolar lipids, secreted proteins, cell wall-associated components, and lysate components) were tested for their ability to induce monocyte cytokine production in vitro. The relative levels of monocyte TNF-α and IL-12 induction by the different fractions of CDC1551 are shown in Table II. The lipid fractions were found to induce 70–75% of the total activity. Therefore, the two lipid fractions (polar and apolar) prepared from the same number of either *M. tuberculosis* H37Rv or CDC1551 were compared for induction of TNF-α, IL-10, IL-12, and IL-6.

Fig. 6 shows the production of TNF-α and IL-12. For all cytokines tested, the response to the polar lipid fraction of CDC1551 was higher (p ≤ 0.01) than the response to the polar lipid fraction of H37Rv. The response to the CDC1551 apolar lipid fraction was higher for TNF-α (p = 0.01) (Fig. 6) and IL-6 (p ≤ 0.01) (not shown). The differences in levels of IL-10 and IL-12 induced by apolar lipids of either H37Rv or CDC1551 were consistent but were not statistically significant (p = 0.09). Thus, the differences in cytokine production observed following infection with CDC1551 and H37Rv may be due to differences in the lipid components of these *M. tuberculosis* strains.

In addition, polar lipids affected the levels of CD14 expression on monocytes (Table III). The lipid fraction from CDC1551 induced higher levels of CD14 expression than did lipids of H37Rv. CD3, CD80, CD86, HLA-DR, and CD40 levels were unaffected.

## Table II. Monocyte cytokine induction by *M. tuberculosis* CDC1551 fractions (fold increase over secreted protein-induced cytokine levels*)

<table>
<thead>
<tr>
<th>Lipid Fraction</th>
<th>TNF-α Fold Change</th>
<th>IL-12 Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apolar Lipid</td>
<td>10 ± 2</td>
<td>8 ± 2</td>
</tr>
<tr>
<td>Polar Lipid</td>
<td>13 ± 3</td>
<td>7 ± 2</td>
</tr>
<tr>
<td>Cell Wall</td>
<td>1.6 ± 0.4</td>
<td>1.4 ± 0.1</td>
</tr>
<tr>
<td>Lysate</td>
<td>3.4 ± 0.7</td>
<td>5.9 ± 1.5</td>
</tr>
<tr>
<td>Protein</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

* Cytokine levels induced by the secreted protein fraction were designated as 1 U and were used to calculate the relative amount of cytokines induced by the other fractions. Results are the mean ± SEM of four separate experiments.

**Fig. 6.** Cytokine release by cultured human monocytes. Monocytes were stimulated with 10 μl of apolar and polar lipid fractions of *M. tuberculosis* CDC1551 (1) and H37Rv (2). Results, expressed as nanograms per ml, are means ± SEM of three (apolar) and five (polar) experiments. A paired t test was used for statistical analysis. *p ≤ 0.01.
Table III.  CD14 expression on human monocytes

<table>
<thead>
<tr>
<th>Time in Culture (h)</th>
<th>Polar Lipid Fraction</th>
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<tbody>
<tr>
<td></td>
<td>CDC1551</td>
</tr>
<tr>
<td>24</td>
<td>3.1 ± 0.2</td>
</tr>
<tr>
<td>48</td>
<td>3.9 ± 0.5</td>
</tr>
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</table>

* Results are means of two experiments expressed as fold increase in fluorescence intensity of the experimental over the untreated monocytes.

...patient. In experimental murine infection studies, CDC1551 was reported to have a faster growth rate than the laboratory strain M. tuberculosis Erdman. It was therefore claimed that the CDC1551 strain may be more virulent in humans. In the present study, we characterized CDC1551 and compared it with two laboratory strains of M. tuberculosis, as well as with two recent clinical isolates of M. tuberculosis. We studied the growth of these strains in vivo and in vitro and the host response to these organisms.

Our studies confirmed that the CDC1551 strain grew faster in mice than the Erdman strain did. However, our results showed that two additional recent clinical isolates and the commonly used laboratory strain, H37Rv, grew similarly in mice with a generation time of 25–28 h (measured from 0 to 14 days after infection) compared with the 25-h generation time observed for CDC1551 (Fig. 1, Table I). Therefore, the growth rate of CDC1551 is not unusually fast. In vitro in human monocytes, H37Rv and CDC1551 showed a 20% difference in the doubling times (31 and 24 h, respectively) (Fig. 5A). Thus, if the growth rate of mycobacteria is considered to be an indicator of virulence (15), then CDC1551 is not more virulent than other recent clinical isolates or than the H37Rv laboratory strain of M. tuberculosis. It does, however, appear to be more virulent than the Erdman strain.

The results reported here suggest that if CDC1551 differs significantly from other strains of M. tuberculosis, the difference is in the ability of this strain to induce a host immune response. In the mouse, CDC1551 induces granulomatous differentiation in the lungs at an earlier time point than that for the other clinical isolates and H37Rv and Erdman (Fig. 1B). In spite of what appear to be relatively small differences in the volume of the cellular aggregates induced in the lungs of infected mice (3–30-fold), cytokine mRNA levels expressed in the lungs are higher and appear sooner in infection with CDC1551 (Figs. 3 and 4). Consequently, this prompt host response is associated with earlier control of CDC1551 growth in the lungs, as shown by the rapid (~14 days) establishment of chronic stable infection with no increase in CFU in the lungs. Although these differences are apparent for only a few weeks, they are critical for the long-term outcome, since CDC1551-infected mice survive significantly longer than mice infected with H37Rv, HN60, and HN878 (Fig. 3) or Erdman (not shown). In addition, the results obtained in vitro show that the differences in the host response observed in mice are also observed in human monocytes, thereby confirming that CDC1551 induces an accelerated and more robust cytokine response.

The mechanism underlying the better protective immune response to CDC1551 infection and improved outcome is suggested by the results of our experiments. IFN-γ is an important mediator of macrophage activation, and the regulation of this cytokine has been considered to be central to the protective immune response (16–18). IL-12 induces IFN-γ production by lymphoid cells (19, 20). The early expression of IL-12 during T cell differentiation has been shown to favor the development of a Th1-type protective cytokine response over a Th2-type cytokine response (21–23). In our studies, IL-12 was expressed in the lungs of mice infected with CDC1551 by day 7 postinfection, long before it appeared in the lungs of mice infected with the other M. tuberculosis strains. It appears, therefore, that the early induction of IL-12 and the relatively high levels of IFN-γ expressed in the lungs of these mice throughout the first 60 days of infection may be responsible for the prompt local control of mycobacterial growth and the longer survival of mice infected with CDC1551. In our monocyte infection studies conducted in vitro, CDC1551 induced substantially higher IL-12 production at 24 h than the level induced by H37Rv. This in vitro observation confirms our in vivo observations. That IL-12 is actually involved in the human protective immune response to mycobacteria is indicated by studies in humans with a genetic absence of an intact IL-12 signaling pathway. In these individuals, monocyte costimulation for IFN-γ production is defective, resulting in disseminated Mycobacterium avium infections (24).

In addition, TNF-α has also been shown to be required for the generation of the protective host response as well as for the generation and maintenance of granulomas (25, 26). In our studies, earlier expression of TNF-α in the lungs of CDC1551-infected mice was associated with earlier appearance of organized granulomas. Since the granulomatous response plays a critical role in control of TB, early production and higher levels of TNF-α in the lungs may also contribute to the better survival of the CDC1551-infected mice.

Our observation that CDC1551 infection of mice and human monocytes induces an earlier and more robust host response may partially explain the observation that individuals exposed even casually to CDC1551 showed a converted skin test response with an unusually long induration. What regulates the size of the PPD response is not known (27, 28). The intradermal injection of PPD to previously sensitized individuals induces the expression of a series of physiological changes, including the emigration to and activation of lymphocytes and monocytes at the dermal site (29). In some individuals, the tuberculin test may result in a large, blistering, necrotic lesion. Ag-specific T cell activation has been shown to be necessary for PPD skin test conversion to occur. In addition, monocyte cytokine production, particularly TNF-α, has been implicated as a determinant of size and tissue damage of the PPD response (30). We suggest that even small numbers of infecting CDC1551 bacilli induce a cytokine response large enough to stimulate granuloma formation and T cell migration and activation at the site of infection. This is reflected by the high frequency of skin test conversion. If these individuals develop active disease, we would predict well-differentiated granulomas in their lungs. In addition, there would be up-regulation of CD14 on the monocytes of exposed individuals, “priming” these to respond more vigorously to future exposure to M. tuberculosis lipid products. These cells may release higher TNF-α levels, resulting in larger PPD responses upon skin testing. Increased TNF-α responses following BCG sensitization have been reported in mice and in humans (31, 32).

The differential cytokine-inducing capacity of CDC1551 appears to be a property of that strain. When crude lipid fractions of the bacilli were tested in monocytes, they induced significantly higher TNF-α and IL-12 production and higher CD14 expression than similar fractions prepared from H37Rv. This finding suggests that polar and/or apolar lipids of M. tuberculosis CDC1551 may be responsible for the differential response. At present, we do not know whether the lipid fractions of CDC1551 are qualitatively or quantitatively different from those of H37Rv. It is also not clear which molecule(s) may be responsible for these differences. Studies have shown that lipoarabinomannan, a major cell wall component of M. tuberculosis, induces monocyte cytokine production possibly via a CD14-dependent pathway (13, 33). However, other lipids...
may signal monocyte cytokine production through a CD14-dependent pathway (34). Future studies will be directed toward fractionating the lipids, characterizing the active components, and identifying the specific molecules which mediate the effects, including CD14 up-regulation, reported here. In addition, with the completion of the sequence of CDC1551, a direct genetic comparison of this strain with H37Rv (3) will be possible. This information should help explain the special properties of CDC1551.

In summary, the studies presented here provide experimental evidence indicating that \textit{M. tuberculosis} CDC1551 is not unusually virulent but rather more immunogenic and that it induces a rapid and vigorous cytokine response which may account for the very high frequency and large size of PPD responses following exposure to patients infected with this \textit{M. tuberculosis} isolate.

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\textbf{References}


