Differences in Rate and Variability of Intracellular Growth of a Panel of *Mycobacterium tuberculosis* Clinical Isolates within a Human Monocyte Model

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Significant differences were observed in the capacities of *Mycobacterium tuberculosis* clinical isolates to grow within human monocytes. Genotyping indicated that the four most rapidly growing isolates were members of the Beijing strain family. *M. tuberculosis* strain H37Rv provided more reproducible infection than the clinical isolates or *M. tuberculosis* Erdman.

For many years, the marked variability in the course of various tuberculosis cases has fueled speculation regarding the respective contributions of host resistance and bacterial virulence to the outcome of infection with *Mycobacterium tuberculosis* (9). Only recently have advances in the molecular biology of mycobacteria provided tools such as restriction fragment length polymorphism (RFLP)-based molecular epidemiology (1, 8, 24) and the capacity for directed disruption of mycobacterial genes (20) that may provide the means for clarifying differences in the virulence of *M. tuberculosis* isolates.

Virulence of *M. tuberculosis* strains has traditionally been assessed in terms of the ability of bacilli to replicate within specific organs of mice and guinea pigs following aerosol infection. Such studies are time-consuming and expensive, however. Furthermore, differences among species in the course of *M. tuberculosis* infection and in the protective mechanisms required for containment of the organism may limit the applicability of these studies to the understanding of virulence as it relates to human tuberculosis (6, 10–12, 21).

We previously described a method for the low-level infection of human blood monocytes (MN) in which patterns of intracellular growth of virulent *M. tuberculosis* H37Rv, attenuated vaccine strain *Mycobacterium bovis* BCG, and avirulent *M. tuberculosis* H37Ra correlated with the virulence of these reference strains as previously defined in animal models (22). In the present study, we sought to determine the applicability of this model to the in vitro characterization of the virulence of *M. tuberculosis* clinical isolates. We assessed the reproducibility and the levels of growth exhibited by nine such isolates following MN infection and compared these findings with previously described results of murine infection with the organisms and with genotypic analysis of the isolates.

Infection with H37Rv is more reproducible than that with strain Erdman or the clinical isolates of *M. tuberculosis*. A panel of *M. tuberculosis* isolates was obtained in a blinded fashion from John Belisle (Colorado State University [CSU]) under the TB Research Materials and Vaccine Testing Contract sponsored by the National Institutes of Health. The isolates were identified to us only by code numbers and patterns of antibiotic resistance. For safety considerations, we chose to work only with the nine strains that were reported as having no significant resistance to isoniazid. Upon removal of the blinding condition, we learned that this set of isolates comprised the virulent Erdman reference strain as well as organisms used in an analysis of the capacity of drug-resistant *M. tuberculosis* isolates to replicate within mice (19). Our studies also included strain CDC1551 (also identified as CSU 93, provided by Thomas Shinnick of the Centers for Disease Control), an extensively transmitted isolate of *M. tuberculosis* (26), and the virulent reference strain *M. tuberculosis* H37Rv (catalog number 25618; American Type Culture Collection, Manassas, Va.). Bacterial stocks were prepared as described previously (23). To minimize clumping and allow for accurate quantification, cultures were subjected to repeated centrifugations and mechanical disruptions as described previously (23).

MN were isolated as previously described from the peripheral blood of healthy volunteers between the ages of 22 and 50 years (23). MN were aliquoted into round-bottomed 96-well plates (10^5 MN/well) and incubated overnight at 37°C. MN from each of the 11 subjects were infected with each of the 11 study organisms. Organisms were incubated with plated MN in a 1:1 ratio for 1 h as previously described. The burden of intracellular bacteria was determined by direct counting of the CFU of MN lysates plated immediately following infection and on days 4 and 7 of culture (23).

Figure 1 illustrates the intracellular CFU for each *M. tuberculosis* strain observed in the MN of each subject studied. For some time points, fewer than 11 individual CFU results are displayed, indicating that some plates could not be counted because of bacterial or fungal contamination. As illustrated, the individual data indicate the substantial variability in initial infectious burden and subsequent intracellular growth dis-
played by the various *M. tuberculosis* strains. Most strikingly, although MN from the same donors were infected with each organism, both initial intracellular CFU and subsequent growth were markedly more reproducible for H37Rv than for the other organisms studied. However, the statistical analysis of the raw data displayed in Table 1 indicates that the intracellular burdens of *M. tuberculosis* immediately following infection were equivalent for the majority of the isolates. The mean intracellular CFU at time zero for each isolate and reference strain, expressed as log CFU (per 10⁶ MN), was 4.75 (±0.43). For each of the nine clinical isolates, the mean initial CFU was within 0.5 log of the mean for both strain Erdman and H37Rv and within 0.5 log of the mean for the initial CFU for the entire study, as shown in Fig. 1. No differences in the intracellular growth of the isolates were observed by day 4. By day 7, however, significant differences were apparent, as described below.

Table 1 also indicates the variability in the intracellular CFU observed for each organism, as represented by the calculated coefficient of the variation of each strain at each time point. Infection with H37Rv resulted in the lowest mean coefficient of variation (2.90) and was significantly more reproducible than infection with Erdman (mean coefficient of variation, 7.46) and with six of the clinical isolates studied (CSU 20, CSU 23, CSU 24, CSU 25, CSU 26, and CDC1551, *P* = 0.05). Clinical isolates of *M. tuberculosis* display different capacities for intracellular growth within human MN. The capacity for intracellular growth of each isolate was calculated as the mean log increase in numbers of intracellular CFU within MN for each subject between days 0 and 7 of culture. To allow for

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**FIG. 1.** Clinical isolates of *M. tuberculosis* display differences in magnitude and variability of intracellular growth within human MN. MN of 11 subjects were incubated with each of the 11 study organisms using a 1:1 multiplicity of infection. Numbers of intracellular CFU were determined immediately following infection as well as 4 and 7 days later. Isolates obtained from CSU are identified by previously published CSU reference numbers. The mean growth (solid lines) and individual CFU results (open circles) are displayed for each clinical isolate as well as for the virulent *M. tuberculosis* laboratory reference strains Erdman and H37Rv. As illustrated, the capacity for intracellular growth of the clinical isolates varied considerably and growth rates that were both lower and higher than those of the reference strains were observed. The data for individual subjects also illustrate that the variability of intracellular growth of both Erdman and the clinical isolates was substantially greater than that of H37Rv.
correlation with murine studies in which Erdman was used as the reference strain, the growth of each strain was compared statistically to that of Erdman by means of a mixed-effect linear regression model that controls for donor-to-donor variability (15).

As illustrated in Fig. 2, CSU 25 was the only isolate found to display significantly greater intracellular growth than the Erdman strain. H37Rv and CSU 24 both tended toward greater growth than Erdman (0.05 > P > 0.10). The growth levels of CDC1551, CSU 28, CSU 19, and CSU 20 were comparable to that of Erdman whereas clinical isolates CSU 23, CSU 27, and CSU 26 each displayed significantly less growth than the Erdman strain (P < 0.001). Of note, CSU 23 was the only strain to display a persistent change in growth rate following its passage through human cells. Growth of CSU 23 on Middlebrook 7H10 plates following the lysis of infected MN was markedly slower than that observed during the initial quantification of bacterial stocks.

The previously described patterns of growth of the organisms within a murine infection model (19, 26) are also represented in Fig. 2. As illustrated, only limited correlations were observed between our findings and those observed following the aerosol infection of mice. Specifically, CSU 25 grew more rapidly than Erdman both in mice and within human MN, whereas the growth of CSU 20 was similar to that of the reference strain in both infection systems. CSU 23 did not grow as well as Erdman by either measure. On the other hand, CSU 19, CSU 28, CDC1551, and CSU 26 all exhibited growth similar to or more limited than that of Erdman within MN, despite having grown more rapidly than the reference strain in mice, as shown in Fig. 2. Likewise, CSU 24 grew somewhat more rapidly than Erdman and CSU 27 displayed significantly less capacity for growth than Erdman within MN, although both exhibited growth similar to that of Erdman in previous murine studies.

Correlation of intracellular growth with clinical data and strain characterization of *M. tuberculosis* isolates. Determination of whether capacity for growth within MN reflects the virulence of *M. tuberculosis* strains for humans would ideally involve correlation with specific clinical information regarding these isolates. Such information is available for CDC1551. This strain was associated with an outbreak of tuberculosis in the southeastern United States in which a remarkably high percentage of contacts of the index case developed positive tuberculin skin tests (26). Although CDC1551 was reported to grow to a substantially greater extent than Erdman in mice (26), we found it to be less capable of intracellular growth than Erdman within human MN. Two other groups of investigators have likewise reported that CDC1551 does not grow unusually rap-
M. tuberculosis strain or isolateabcdef

<table>
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<tr>
<th>M. tuberculosis strain or isolateabcdef</th>
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<th>Country of origin</th>
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<th>Spoligocodeabcdef</th>
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a Strains are listed in the order of their growth within MN.
b Numbers of bands identified by RFLP.
c IS6110 patterns indicate distinct genotypes in which more than one pattern containing the same number of bands was observed within the set of isolates (2).
d Spoligotypes are depicted as 15-digit spoligocodes in which the pattern observed for each set of three nucleotide spacers is represented by a digit from 1 to 9 (7).
e A review of the results of previous drug susceptibility testing of the CSU isolates performed specifically for the preparation of this paper revealed several errors in the calculation and recording of the original data. Accordingly, the corrected susceptibility results presently reported differ somewhat from those previously reported (19).

Although previous studies have assessed the intracellular growth of selected clinical isolates of M. tuberculosis (13, 29), the present study represents the first examination of a panel of clinical isolates of M. tuberculosis obtained in a blinded fashion. Our findings indicate that, despite its laboratory cultivation for nearly a century (25), H37Rv displays an in vitro phenotype that is at least as robust and significantly more reproducible than that of both Erdman and a variety of clinical isolates of M. tuberculosis, suggesting that H37Rv should remain a preferred reference strain for many types of in vitro studies.

Unlike the results of our earlier studies involving laboratory strains of mycobacteria, the intracellular growth of the clinical isolates presently studied showed only partial correlation with previous findings in a murine model of infection. The capacity for short-term intracellular growth may not fully approximate the outcome of M. tuberculosis infection over a longer period as assessed in the 60-day murine model. However, the discrepancies observed could also reflect differences in host-pathogen interactions relevant to virulence for humans as opposed to mice (6, 10–12, 21). Although detailed clinical information would be invaluable in clarifying these issues, strain analysis of the organisms supports the relevance of the MN model. Specifically, the four isolates that grew most rapidly all share both the spoligotype and high IS6110 copy number characteristic of the Beijing family. Beijing strain organisms account for a majority of isolates in the People’s Republic of China and are found to a lesser extent in surrounding Asian nations (28). In addition, Beijing family strains have been found to be responsible for tuberculosis outbreaks in a variety of countries and clinical settings (4). These observations suggest that the Beijing genotype may confer a selective advantage causing human disease.

The clinical isolates that displayed antibiotic resistance (CSU 20, CSU 19, CSU 24, and CSU 25) also displayed more rapid growth within MN than did the susceptible organisms in this study. Although the organisms we evaluated exhibited unusual patterns of drug resistance, our observations should nevertheless add a note of caution against the presumption that drug-resistant M. tuberculosis isolates are inherently less virulent than fully susceptible organisms (17, 18). Indeed, our findings suggest that the genetic background of these isolates, as members of the Beijing family, may predominate over the acquisition of drug resistance as a determinant of their virulence. This possibility is supported by the observation that large tuberculosis outbreaks in the United States have been attributed to Beijing family organisms that are both drug sus-
ceptible, such as *M. tuberculosis* strain 210 (3), and multidrug resistant, such as the W strain family (5).

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REFERENCES


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