

Mycobacterium tuberculosis CDC1551 Is Resistant to Reactive Nitrogen and Oxygen Intermediates In Vitro

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Resistance to reactive oxygen intermediates and reactive nitrogen intermediates in vitro of a clinical isolate of *Mycobacterium tuberculosis* (CDC1551) that caused a large outbreak of tuberculosis was compared to that of *M. tuberculosis* strains CB3.3, H37Rv, H37Ra, Erdman, RJ2E, C.C. 13, and C.C. 22 as well as *M. bovis* strains Ravenel and BCG. CDC1551 and CB3.3 were significantly more resistant to both hydrogen peroxide (H₂O₂) and acidified sodium nitrite than were the other strains tested. This biological phenotype may serve as an in vitro marker for clinical strains of *M. tuberculosis* likely to cause a large outbreak of tuberculosis.

A strain of *Mycobacterium tuberculosis* (CDC1551 or CSU 93) was responsible for a recent outbreak of tuberculosis (TB) in a rural area near the Kentucky-Tennessee border (29). In a population at low risk for TB, 21 persons were diagnosed with active TB between 1994 and 1996. Among 429 contacts of TB index patients, 72% had positive tuberculin reactions, including 20% with documented skin test conversions (29). Because of the high number of positive tuberculin reactions, skin test conversions, and infected casual contacts, it was suggested that CDC1551 is an exceptionally virulent strain of *M. tuberculosis* (29).

The role of reactive oxygen intermediates (ROI) and, more recently, reactive nitrogen intermediates (RNI) in host defense against *M. tuberculosis* has been studied in various murine models (2, 3, 5–7, 10, 11, 13, 19, 28). Although the importance of RNI defense against *M. tuberculosis* has been well established in the murine model, accumulating evidence suggests that RNI may play a role in host defense against *M. tuberculosis* in humans (16, 22, 30). Numerous in vitro studies demonstrate that strains of *M. tuberculosis* vary with regard to their susceptibility to ROI (15, 18, 20) and RNI (12, 24, 26, 31). In addition, several *M. tuberculosis* genes associated with RNI resistance have been recently described (4, 8, 27). One example of an RNI-resistant *M. tuberculosis* strain is the drug-susceptible, clinical strain CB3.3 (C strain) that was involved in a New York City outbreak between 1991 and 1994. Investigations of CB3.3 found that it was more resistant to acidified sodium nitrite (ASN), a chemical reaction that converts nitrite to reactive nitrogen species, than were H37Rv and several other clinical *M. tuberculosis* isolates (12). Infection with strain CB3.3 was epidemiologically associated with injection drug users with tuberculosis in New York City (12).

In order to determine if the clinical strain CDC1551 exhibited characteristics similar to those of CB3.3, we compared its RNI and ROI resistance patterns to those of other clinical isolates, including CB3.3 (from New York City), RJ2E (one of the predominant *M. tuberculosis* strains circulating in Rio de

Janeiro, Brazil) (9), and C.C. 13 and C.C. 22 (from northern California) as well as virulent (H37Rv, Erdman, and *M. bovis* Ravenel) and attenuated (H37Ra and *M. bovis* BCG) reference laboratory *M. tuberculosis* complex strains.

All strains of mycobacteria were grown in Middlebrook 7H9 broth (Difco Laboratories, Detroit, Mich.) with 0.5% glycerol, 0.02% Tween 80, and ADC enrichment (Difco Laboratories) to mid-log phase. Multiple cultures from the original slant of each strain were grown to similar absorbances, and then single cell suspensions of each isolate were prepared according to a previously published method (14). Multiple aliquots of each strain's single cell suspension were stored at –80°C for no longer than 3 months before use. The approximate number of organisms per frozen suspension was quantified by enumeration of CFU of serial dilutions plated on Middlebrook 7H11 agar. Survival was determined by placing 0.1 ml of thawed suspension into 0.9 ml of 7H9 broth (pH 5.4 for ASN assays or pH 7.0 for H₂O₂ assays) containing various concentrations of either sodium nitrite (Sigma, St. Louis, Mo.) or H₂O₂ (Sigma). As a control, a 0.1-ml aliquot from the same thawed suspension was added to 0.9 ml of 7H9 broth at the appropriate pH without sodium nitrite or H₂O₂. Each suspension was incubated at 37°C for 16 h, plated onto Middlebrook 7H11 agar (Difco Laboratories), and incubated at 37°C for 3 weeks. Resistance to the reagents was expressed as a proportion of the number of CFU recovered from isolates exposed to either ASN or H₂O₂ to the number of CFU recovered from the same isolates not exposed to these reagents. Each strain was tested similarly in triplicate. The data represent results from triplicate experiments from a single cell suspension per culture from at least three separate cultures for each strain. Statistical analysis was performed with the Student *t* test with the *P* value adjusted to <0.0055 by using Bonferroni's correction for multiple comparisons.

Aside from *M. tuberculosis* H37Ra and C.C. 22, most strains were relatively resistant to low concentrations of either H₂O₂ (2 mM) or ASN (3 mM) (Fig. 1 and 2). At 5 mM H₂O₂, CDC1551 was significantly (*P* < 0.0055) more resistant than were H37Rv, H37Ra, BCG, C.C. 13, and C.C. 22 (Table 1). Although not statistically significant, CDC1551 demonstrated greater resistance than did CB3.3, RJ2E, Erdman, and Ravenel (Fig. 1). Survival at 6 mM ASN indicated that CDC1551

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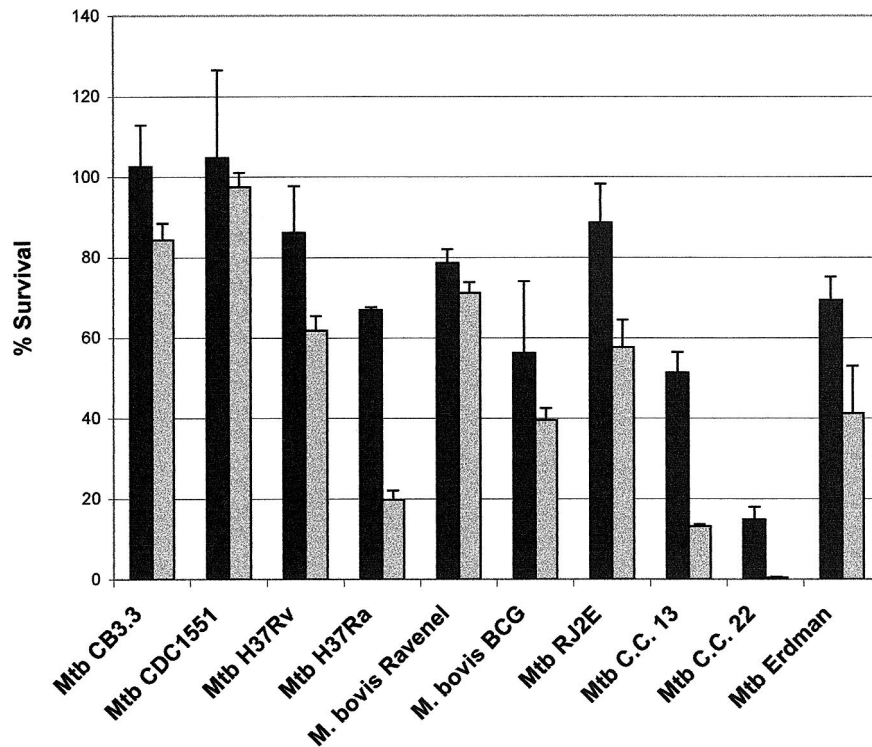


FIG. 1. Effect of H₂O₂ on mycobacterial viability in Middlebrook 7H9 medium at 2 mM (black bars) and 5 mM (gray bars) concentrations. Data shown are representative of triplicate experiments. Results are expressed as percents survival relative to baseline \pm standard errors of the means and are mean values of triplicate cultures for each strain.

was significantly ($P < 0.0055$) more resistant than were H37Ra, BCG, and C.C. 22 (Table 1). In addition, CDC1551 demonstrated enhanced resistance in 6 mM ASN compared to that of H37Rv, C.C. 13, and Erdman, although the levels were not statistically significant ($P = 0.0068, 0.0059, \text{ and } 0.006$, respectively) (Table 1). Moreover, the percent survival of both CB3.3 and CDC1551 in 5 mM H₂O₂ and 6 mM ASN was higher than that of the eight other strains tested in this study (Fig. 1 and 2).

It was initially reported that mice infected with CDC1551 contained 10-fold-more bacilli per lung after 10 days and 100-fold-more bacilli per lung after 20 days than did mice infected with *M. tuberculosis* strain Erdman (29). Another study, however, observed that CDC1551 had similar growth rates in mouse lungs over 20 days of infection as H37Rv and *M. bovis* Ravenel and, therefore, concluded that CDC1551 is not necessarily a more virulent strain in the mouse model (23). More recent studies have demonstrated that CDC1551 grows similarly in vitro (18, 20, 21) and in vivo (1, 17, 21, 23) as H37Rv. In addition, a study by Orme demonstrated that CDC1551 grows similarly in mice as other clinical *M. tuberculosis* isolates associated with outbreaks (25). However, it has been demonstrated elsewhere that CDC1551 induces granulomas in mouse lungs earlier than does H37Rv, Erdman, or two clinical isolates and that higher levels of tumor necrosis factor alpha, interleukin 10 (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 (21). Consequently, it was suggested that CDC1551 was not more virulent than other *M.*

tuberculosis isolates in terms of growth in mice but might be more immunogenic (21).

RNI are an important determinant of infection outcome in the interaction between *M. tuberculosis* and the mouse macrophage. Mice disrupted in the inducible nitric oxide synthase (NOS2) gene have increased susceptibility to *M. tuberculosis* infection (19). More recently, Thoma-Uszynski et al. demonstrated that the activation of toll-like receptor 2 (TLR2) leads to killing of intracellular *M. tuberculosis* in both human and murine macrophages (28). They suggested that killing in murine macrophages is mediated by the nitric oxide-dependent pathway but that in human macrophages it is independent of this pathway (28). Consequently, the role of RNI in controlling *M. tuberculosis* infection in the human host continues to be debated, although several studies have provided evidence that RNI are expressed by *M. tuberculosis*-infected human alveolar macrophages (16, 22, 30).

The possible epidemiological importance of RNI resistance in *M. tuberculosis* was first observed with the demonstration that the most common drug-susceptible strain of *M. tuberculosis* circulating in New York City during the early 1990s was highly resistant to RNI (12). A recent study compared CDC1551 to seven clinical isolates of *M. tuberculosis* for susceptibility to chemically generated RNI in vitro and was unable to demonstrate any significant differences regarding RNI susceptibility among these strains (32). It should be noted that a laboratory reference strain (e.g., H37Rv) or a clinical strain

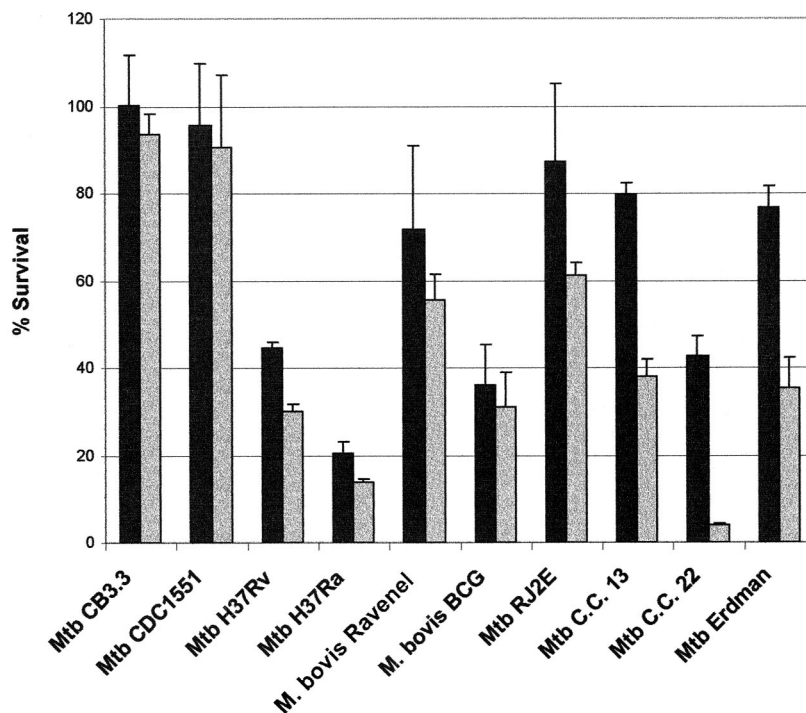


FIG. 2. Effect of ASN on mycobacterial viability in Middlebrook 7H9 medium at 3 mM (black bars) and 6 mM (gray bars) concentrations. Data shown are representative of triplicate experiments. Results are expressed as percents survival relative to baseline \pm standard errors of the means and are mean values of triplicate cultures for each strain.

with known levels of RNI resistance was not included in that study for comparison.

Our results demonstrate that, at lower concentrations of ASN and H₂O₂ (3 and 2 mM, respectively), most strains of mycobacteria were relatively resistant, except for the attenuated, laboratory strain of *M. tuberculosis* (H37Ra) and a clinical isolate of *M. tuberculosis* (C.C. 22). These results are consistent with those of Rhoades and Orme (26), who demonstrated that certain strains could resist some degree of RNI generated in vitro. At higher concentrations of ASN (6 mM), however, the CDC1551 strain was as resistant as the previously described RNI-resistant clinical isolate CB3.3 from

New York City (12). The percent survival of CB3.3 as well as H37Rv at 6 mM ASN was in the same range as that reported for these strains by Friedman et al. (12), thus supporting the reproducibility of the ASN assay. Moreover, CDC1551 was the most resistant strain among the 10 strains tested when exposed to 5 mM H₂O₂. Although our observation is preliminary, based on a small number of isolates, high-level resistance to RNI and ROI may serve as a convenient marker of *M. tuberculosis* strains with an epidemic potential. Opportunities to examine this suggestion may arise with large outbreaks of tuberculosis that may occur in the future.

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TABLE 1. Comparison (*P* value) of resistance to ASN and H₂O₂ between CDC1551 and other mycobacterial strains^a

Strain	<i>P</i> value with reagent at concn			
	ASN		H ₂ O ₂	
	3 mM	6 mM	2 mM	5 mM
CB3.3	*	*	*	*
H37Rv	*	*	*	0.0016
H37Ra	*	0.0013	*	<0.001
Ravenel	*	*	*	*
BCG	*	0.0049	*	0.003
RJ2E	*	*	*	*
C.C. 13	*	*	*	<0.001
C.C. 22	*	<0.001	*	<0.001
Erdman	*	*	*	*

^a *P* values were determined by using Student's *t* test (significance levels were adjusted with Bonferroni's correction for multiple comparisons). *, not significant (*P* > 0.0055).

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