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Drug-Resistant Strains of *Mycobacterium tuberculosis* Exhibit a Range of Virulence for Mice

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A panel of clinical isolates of *Mycobacterium tuberculosis*, several of which were resistant to one or more antimycobacterial drugs, were tested for their capacity to give rise to active disease following aerogenic infection of normal immunocompetent mice. The panel exhibited a range of virulence in this model, which followed no clear trend in terms of geographical source, degree of drug resistance, or rate of growth in vitro. Several isolates grew very quickly over the first 20 days in mouse lungs before being contained by emerging immunity. In view of this latter observation, we hypothesize that it is possible that such so-called fast growers may be responsible for the rapid fatality sometimes seen in immunocompromised patients with tuberculosis. Moreover, the results of the study do not support the belief that increased drug resistance usually associates with loss of virulence of the isolate.

The management and therapy of drug-resistant tuberculosis (TB) is a growing problem both in the Third World and, more recently, in more-developed countries (2, 10). The evolution of sanatoria, the discovery of effective chemotherapies, and the instigation of notification requirements all led to the steady 5 to 6% decline seen annually in the United States from 1953 to the mid-1980s. The optimism engendered by these data led to the publication of a Centers for Disease Control document (3) in 1989 predicting the reduction of TB cases in the United States to <1 case per million by the year 2010.

In fact, by 1984 the decrease in the case rate had already begun to slow, and by 1990 the case rate had begun to increase. If one extrapolates the decline from 1975 onwards and imagines that this decline had continued, then by 1991 39,000 excess cases of TB had been reported (8, 11). The reasons proposed for this fall under three main categories: (i) immigration of individuals with TB from high-prevalence countries, (ii) the impact of human immunodeficiency virus, and (iii) the increasing incidence in institutions (homeless shelters, inner city schools, prisons, etc.). Moreover, 70% of TB cases now occur in racial and ethnic minority groups.

Coincident with the increased TB case rate, large outbreaks of drug-resistant tuberculosis have occurred (1, 4–7, 16). In fact, in the first 3 months of 1991, 13% of reported cases (8) were resistant to at least one drug (to date, isoniazid resistance is most common). Many of these cases have occurred in human immunodeficiency virus-positive patients, in whom the mortality from TB is very high and in whom it progresses rapidly from diagnosis to subsequent death.

Resistance to drugs in strains of *M. tuberculosis* is believed to arise as a result of a gene point mutation or as a result of gene deletion (8, 12). Because of this, the prevailing wisdom to date has held that such changes may also result in a reduction in virulence of the isolate. This hypothesis originally arises from the classical studies of Mitchison and his colleagues, who observed that a significant number of isoniazid-resistant *M. tuberculosis* strains isolated from patients in India tended to be of low virulence in the guinea pig infection model (13).

In the present study, we examined the capacity of 15 *M. tuberculosis* isolates, selected at random in terms of geographical source or drug susceptibility profile, to grow in the lungs of mice following aerogenic exposure of these animals to a low-dose inoculum of approximately 50 bacilli. The results show that the panel exhibited a wide range of virulence, regardless of the degree of resistance to conventional drugs. Moreover, several isolates exhibited a so-called fast-grower phenotype (abbreviated herein as FGTB, or fast-growing TB, so as to distinguish these from much-faster-growing mycobacterial species), enabling them to reach substantial numbers in mouse lungs prior to the onset of acquired immunity.

A brief description of clinical isolates tested in this study is contained in Table 1. Passage in vitro of each isolate was kept to a minimum and ranged from one to as many as three times; it is theoretically possible that these passages reduced the virulence of some of the isolates. Each isolate was grown to mid-log phase in either glycerol alanine salts medium or Proskauer Beck medium and stored in ampoules frozen at −70°C. Drug susceptibility profiles were determined by a conventional proportion method (9). The presence of catalase was determined by observation of the ability of colonies to produce oxygen from hydrogen peroxide. Some isoniazid-resistant strains were catalase negative, suggesting absence of the *katG* gene (17).

To determine in vitro growth rates, synchronized cultures of bacterial isolates were grown in nutrient 7H9 broth containing 0.05% Tween, and the optical density at 600 nm was determined daily. Generation time (k) was calculated by the formula $k = (\log N_t - \log N_o)/t \log Z$, where N_o is the initial optical density at 600 nm of the log-phase growth, N_t is the optical density of the log phase at time t, and $Z = 2$ (i.e., doubling of population density). For in vivo studies, aliquots were frozen and diluted in sterile pyrogen-free saline to a concentration of 5×10^4 viable bacilli per ml. A volume of 10 ml was then added to the venturi nebulizer unit of a Middlebrook Aerosol Generation device (Glas-Col, Terre Haute, Ind.), and mice were exposed to an aerosol for a 30-min period. This routinely resulted in the implantation of 20 to 50 bacilli in the lungs of

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TABLE 1. Characteristics of *M. tuberculosis* clinical isolates studied

Strain	Resistance (% of inoculum still growing/μg of drug per ml) to indicated antibiotic[a]											Catalase	Source[b]
	AK	CAP	CS	EMB	ETA	INH	KM	PZA	RBT	RIF	SM		
CSU 11	NR	NR	NR	NR	NR	65/1	NR	NR	NR	100/1	NR	+	K
CSU 12	NR	NR	NR	NR	NR	65/0.2	NR	50/25	NR	NR	NR	−	K
CSU 15	NR	NR	NR	NR	NR	40/1	NR	NR	NR	NR	NR	+	K
CSU 17	NR	NR	NR	NR	NR	50/1	NR	NR	NR	NR	NR	−	K
CSU 18	NR	NR	10/60	NR	NR	55/5	NR	NR	NR	NR	NR	+	K
CSU 19	NR	NR	10/60	100/10	NR	NR	NR	100/50	NR	NR	NR	+	C
CSU 20	NR	NR	10/60	100/10	NR	NR	NR	100/50	NR	NR	NR	+	C
CSU 21	NR	NR	10/60	65/7.5	NR	100/5	NR	NR	100/4	100/10	100/10	−	C
CSU 22	100/8	20/10	NR	10/15	10/15	100/1	100/12	NR	NR	100/10	100/10	−	C
CSU 23	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	+	C
CSU 24	NR	NR	100/10	100/10	NR	NR	10/12	NR	30/4	NR	NR	+	C
CSU 25	NR	NR	NR	NR	NR	NR	NR	30/25	10/2	NR	NR	+	C
CSU 26	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	+	C
CSU 27	NR	NR	NR	20/10	NR	NR	NR	NR	NR	NR	NR	+	C
CSU 28	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	+	C
Erdman	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	NR	+	

[a] Numerical values derive from the proportional method, e.g., 10/15 indicates that 10% of the inoculum still grew in 15 μg of drug per ml. NR, no resistance to drugs. Antibiotics: AK, amikacin; CAP, capreomycin; CS, cycloserine; EMB, ethambutol; ETA, ethionamide; INH, isoniazid; KM, kanamycin; PZA, pyrazinamide; RBT, rifabutin; RIF, rifampin; SM, streptomycin.

[b] K, James Kilburn, Centers for Disease Control and Prevention, Atlanta, Ga. (isolates from the United States); C, Ray Cho, Seoul, Republic of Korea (isolates from the subcontinent and the rest of Asia).

these animals. The numbers of bacteria in the lungs of test animals were monitored against time by harvesting of mice by CO_2 inhalation, plating of serial dilutions of individual whole-organ homogenates on nutrient 7H11 agar, and then counting of bacterial colony formation 2 to 3 weeks later after incubation at 37°C in humidified air. For comparison, the growth of the virulent laboratory strain *M. tuberculosis* Erdman was also determined.

The results of these experiments are shown in Fig. 1. The panel was divided into three obvious patterns, with five isolates

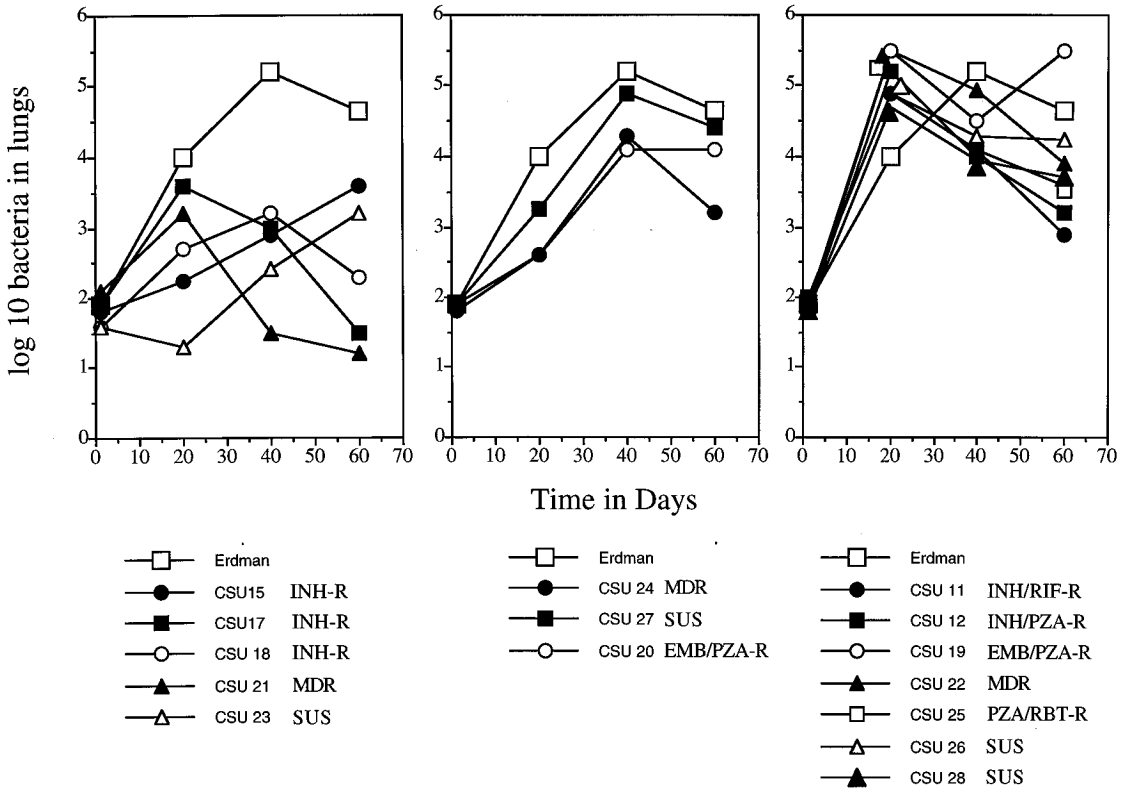

FIG. 1. Growth of *M. tuberculosis* isolates in C57BL/6 mice following exposure to low-dose aerosol infections. Growth patterns fell into three categories, avirulent, virulent, and fast-grower, in comparison with the virulent laboratory strain Erdman. The curve for *M. tuberculosis* Erdman is shown in each panel for comparison. Data shown are mean values for four mice (standard errors of the mean are omitted; they did not exceed 0.35).

tested falling into a low-virulence or avirulent category, in that they grew only marginally or very slowly (as was the case with strain CSU15) in infected mice. Three strains performed better, giving curves similar to that of the laboratory Erdman strain. Finally, seven strains were observed that grew 1.0 to 1.5 log units faster than the Erdman strain (at least over the first 20 days of the infection, after which they were controlled and slowly eliminated by acquired immunity).

When the growth rates of these isolates were tested in vitro, we observed no relationship between generation times in nutrient broth and behavior in the animal model. Strains 15, 18, 20, 24, 25, 27, and Erdman all had generation times of 24 to 35 h, whereas four FGTB strains all grew much more slowly (40 to 90 h).

In summary, therefore, the results revealed no trend in terms of geographical source, degree of drug resistance, or in vitro growth characteristics, in relation to virulence in this mouse infection model. Individual strains could be grouped as of low virulence, as virulent as the laboratory strain *M. tuberculosis* Erdman, or as more virulent, in the sense that certain isolates grew much faster over the first 20 days of the infection. Moreover, in terms of the isoniazid-resistant strains, three were of the avirulent type while two others fell into the latter FGTB category. It will be noted also that 5 of the 11 drug-resistant strains fell into this latter group.

In this regard, it has previously been demonstrated that many isoniazid-resistant strains of *M. tuberculosis* possess reduced expression or lack of catalase-peroxidase activity and that this loss of activity was associated with a lower level of virulence. More recently, at least one form of isoniazid resistance has been shown (17) to be associated with either a deletion or a point mutation in the *katG* gene which encodes the heat-labile catalase-peroxidase enzyme of *M. tuberculosis*. In the context of the present study, however, it still remains to be determined which of the isolates tested here have lost the ability to express the active *katG* gene product and hence whether this loss has any bearing on the virulence of these strains.

It is also generally accepted that the more virulent strains of *M. tuberculosis* have faster generation times, and it has been proposed that this speed may be responsible for their progressive growth in vivo (14). In view of this, one would have expected the FGTB strains to divide more quickly. This did not appear to be the case, however, and suggests that other currently unidentified factors, which hopefully will soon be discovered by molecular genetic approaches (15), also play a role in allowing these isolates to grow rapidly in the lungs.

With regard to the isolates that grew very quickly in the mouse lungs over the initial period of the infection, we should note that probably because of our own concentration on the so-called laboratory strains of *M. tuberculosis*, we have not previously observed this FGTB type. It may be a common trait or may represent a relatively new virulence phenotype. We would be interested to receive comments from our colleagues on this matter.

Interestingly, it has been noted that human immunodeficiency virus-positive individuals often present with a rapidly progressive, fatal form of tuberculosis, with survival of only 4 to 16 weeks (8). It will be evident, therefore, that the FGTB isolates tested in this study, which were contained by the immunocompetent mice after 20 days of the infection, would probably have grown progressively and killed these animals in a relatively short time had they been immunocompromised. It is even possible that rapidly fatal tuberculosis in certain AIDS patients may reflect infection with isolates bearing this particular phenotypic trait, although this remains to be seen.

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