



Catalase–peroxidase activity has no influence on virulence in a murine model of tuberculosis

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KEYWORDS

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Murine model;
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Summary The capacity to generate a chronic and persistent infection in the experimental murine model of tuberculosis induced aerogenically by a low-dose inoculum was determined in eight isoniazid-resistant clinical strains of *Mycobacterium tuberculosis* showing different catalase–peroxidase (C–P) activities. Determination of bacillary concentration in lung and spleen and the percentage of pulmonary parenchyma occupied by granulomas were monitored. Data showed no relation between the lack of C–P activity and the ability to develop a persistent infection, highlighting the potential of C–P negative strains to spread through the community. © 2003 Elsevier Ltd. All rights reserved.

Introduction

In the last decade, interest on drug-resistant *Mycobacterium tuberculosis* has peaked because of the marked incidence increase observed in the United States of America.^{1,2} This fact has posed interesting questions on the capacity of those strains to spread through the community. Recent studies have demonstrated that drug-resistant tuberculosis is still a serious problem in countries of Eastern Europe, including Russia. Additionally, a larger incidence has recently been identified in heavily populated areas of Asia, such as China and Iran.^{3,4} The high prevalence of the strain Beijing is an explanation of this phenomenon in the latter geographic areas. This strain has a high capability for transmission in the population and has a high

tendency to carry drug resistance.^{5,6} On the other hand, some studies have shown that drug resistance is not a major public health problem in countries with efficient national control programs, such as the US and Western Europe. In fact, recent studies have demonstrated a limited spread of drug-resistant strains in Los Angeles County⁷ and Texas.⁸

Isoniazid is the only antituberculous drug for which the relation between acquisition of resistance and lack of virulence has been explained. In 1953, only 1 year after the introduction of isoniazid in the treatment of tuberculosis, Middlebrook and Cohn⁹ demonstrated that isoniazid-resistant (INH^R) strains were less virulent when tested in the guinea pig experimental model of infection. This fact seemed to be related to a lack of catalase–peroxidase (C–P) activity.¹⁰ Since the production of reactive oxygen intermediates (ROIs) by mononuclear phagocytes was identified as a mechanism of killing phagocytized bacilli,¹¹ the absence of virulence was feasibly ascribed to the lack of enzymes such as C–P, which are potentially able

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to avoid the toxic effect of ROIs. In this regard, Jaccett et al.¹² related H₂O₂ susceptibility to low virulence in native and INH^R strains, although resistance to H₂O₂ was not the sole determinant of virulence in tubercle bacilli. In 1992, Zhang et al.¹³ showed that deletions in the *katG* gene, encoding for C–P activity, conferred isoniazid resistance. That study also revealed that C–P activity loss is more consistent with a high level of isoniazid resistance, that is usually the consequence of a previous treatment (acquired resistance). This observation lended support to the notion that these strains were not able to disseminate through the community. Soon after, Wilson et al. showed that integration of a functional *katG* gene into a catalase-negative avirulent strain of *M. bovis* restored its virulence in the guinea pig model.¹⁴ Other authors observed that transformation of *M. tuberculosis* or *M. intracellulare katG* genes into a *M. tuberculosis* INH^R defective strain restored C–P activity and the ability of recombinants to persist in spleens of mice and guinea pigs.¹⁵

The aim of this work was to study the virulence of *M. tuberculosis* INH^R clinical strains showing different C–P activities. The ability of these strains to induce a chronic persistent infection in mice following aerogenic exposure with a low-dose inoculum was assessed trying to find valuable insights related to their ability in spreading through a human population.

Methods

Clinic, phenotypic and genotypic characteristics of the clinical strains

All clinical strains were obtained from cases treated between July 1995 and June 1997 in a multicenter drug resistant study at the Unit for the Investigation of Tuberculosis in Barcelona (UITB).¹⁶ Our study included eight representative isolates. Standard strains NC007416 (H37Rv) and NC007417 (H37Ra) were obtained from the National Collection of Type Cultures, London, UK.

Tuberculin skin test was done using 5 UI of PPD RT-23. IS6110 restriction fragment length polymorphism (RFLP) profiling and spoligotyping were performed by an international standardized method.^{17,18} Drug susceptibility test was carried out using Bactec radiometric method (Becton-Dickinson, Franklin Lakes, NJ, USA) for INH, ethambutol, rifampin, streptomycin and pirazinamide at a final concentration of 0.1, 7.5, 2, 6 and

100 µg/ml, respectively. Minimal inhibitory concentration (MIC) to INH was determined testing 0.1–32 µg/ml in 7H10 medium (Difco, Detroit, MI, USA). C–P routine activity was determined for each strain. Briefly, a loopful of 2-week-old culture in Löwenstein–Jensen media was placed in each screw cap-tube with 0.5 ml of distilled sterile water. One milliliter of Bogen reagent (1.25 ml of Tween 80, 0.5 ml of hydrogen peroxide 30% in vol, 0.1 g of bromocatechin in 100 ml of distilled water) was added. One hour later, the bubble ring thickness in millimetres provided the catalase assay value. Formation of a black precipitate 18 h later was considered positive for peroxidase activity.¹⁹ The entire *katG* gene (six fragments; 2200 bp) and specific regions of the *oxyR-ahpC* (105 bp) and *inhA* genes (249 bp) that are related to isoniazid resistance were PCR amplified by using specific primer pairs as previously described.²⁰ DNA sequencing was performed with the fmol DNA Cycle System (Promega Corporation, Madison, WI, USA) with ALF Express II (Amersham Pharmacia Biotech).

Quantitative C–P activity detection by spectrophotometric methods

Previously described^{21,22} methodology was followed. Briefly, lysis was done using a FastPrep™ System silica–ceramic matrix (Bio 101, Vista, CA, USA) and a FastPrep FP120 equipment (ThermoSavant, Holbrook, NY, USA). Catalase and peroxidase activities were determined spectrophotometrically, following the changes in H₂O₂ concentration ($\epsilon_{240} = 0.0435 \text{ mM}^{-1} \text{ cm}^{-1}$) at pH 7.0 and *o*-dianisidine ($\epsilon_{460} = 11.3 \text{ mM}^{-1} \text{ cm}^{-1}$) at pH 5.5, respectively, in the presence of 10 mM H₂O₂ at 25°C. In both cases, the lineal part of the curve was used for rate quantification. Protein concentrations were determined by the method of Lowry. Specific activities were calculated by dividing the observed rate among the protein concentration in the crude extracts. Specific activities were expressed as U/mg, considering 1 unit (U) of enzymatic activity as the amount of enzyme that catalyzes the transformation of 1 µmol of substrate per minute. Both determinations were run three times for each strain (Fig. 1).

Mice

Specific pathogen-free female (*spf*) C57BL/6 Jico mice, 6 weeks old, were purchased from Charles River (St. Germain sur l'Arbresle, France). They were housed under *spf* conditions and provided with sterile water and food ad libitum. All

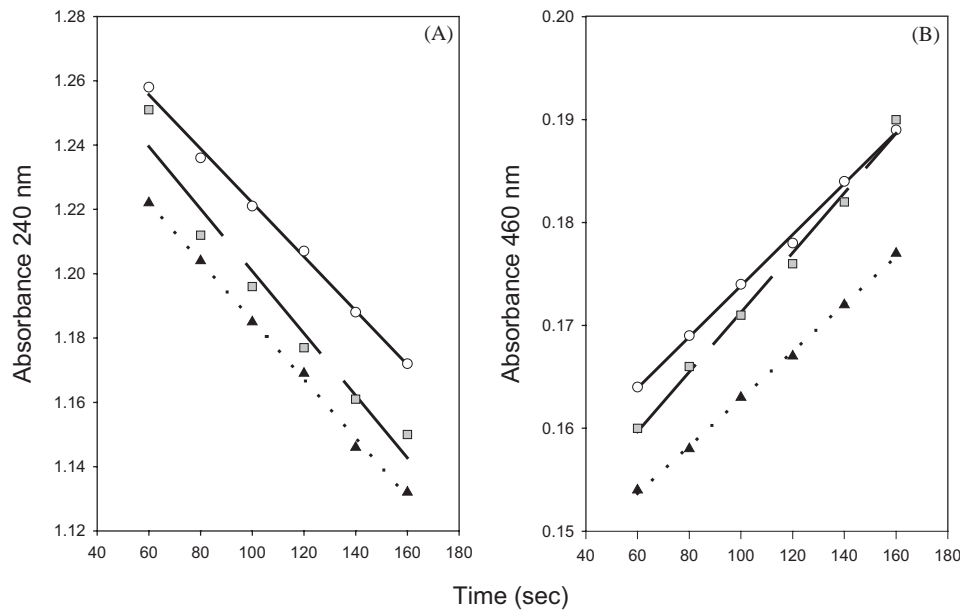


Figure 1 Spectrophotometric assays to detect catalase (A) and peroxidase (B) activities from *M. tuberculosis* H37Rv. (A) shows decrease in absorbance at 240 nm of H_2O_2 and (B) increase of oxidized *o*-dianiside at 460 nm in the linear part of the curve. The assay was repeated three times for each strain.

experimental proceedings were approved and supervised according to the European Union Laws for protection of experimental animals and conformed to NIH guidelines.

Experimental infection

Procedures were performed as published elsewhere.²³ Briefly, *M. tuberculosis* strains were grown in Proskauer Beck medium containing 0.05% Tween 80 to mid-log phase and kept at -70°C until use. Mice were infected by chamber exposure in an airborne infection device (Glas-col Inc., Terre Haute, IN, USA). Four mice were used for every time-point in each experimental group. Lungs and spleens were homogenized and plated on nutrient Middlebrook 7H11 agar on weeks 0, 3, 9, 18, and 22. Colony forming units (CFUs) were counted after 4 weeks incubation at 37°C for the strains with C-P activity and 8 weeks for the strains with low or no C-P activity.

Morphometry

Procedures are inspired by the previous work.²⁴ Two right lung lobes from each mouse were fixed in buffered formalin and subsequently embedded in paraffin. Five-micrometer-thick sections from each specimen were stained with hematoxylin-eosin and photographed at $50\times$ using an Eclipse E400 microscope (Nikon, Kanagawa, Japan) and a

Coolpix 990 digital camera (Nikon). Three sections cut at $10\mu\text{m}$ intervals were studied in each case. Using an appropriate software (SigmaScan, SPSS Software, San Rafael, CA, USA), the area of each single lesion and the ratio between the area occupied by granulomas and the total tissue area was determined on photomicrographs from the three sections of eight lung lobes at each time point (Fig. 2). Sections were blindly evaluated in order to perform a more objective measurement.

Statistical analysis

Sigma Stat (SPSS Software, San Rafael, CA, USA) was used to compare differences among groups by means of all pairwise multiple comparison procedure (Student–Newman–Keuls method), as well as to determine linear regressions through the Pearson product moment correlation coefficient. Differences were considered to be significant when p was $<0,05$.

Results

Quantification of C–P activity yielded a wide range of values. The standard strains show the highest values. No activity was found in multidrug-resistant strains having the highest MIC against INH (Fig. 3). Comparison with the semiquantitative routine assay (Table 1) revealed some activity degree

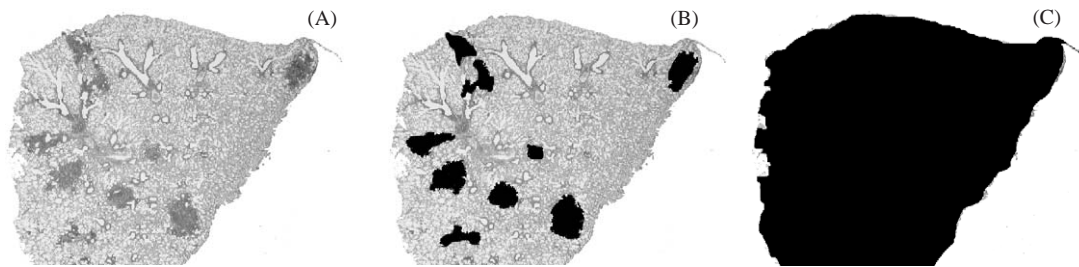


Figure 2 Morphometry of pulmonary lobes. The ratio between the area occupied by granulomas and the total tissue area was obtained by assembling digital photomicrographs pictures obtained at $50\times$ (A). Measurement of the granulomatous infiltration was done semiautomatically by outlining the affected areas and obtaining the area through specific software (B). The total area of every single lobe was obtained through the same procedure (C).

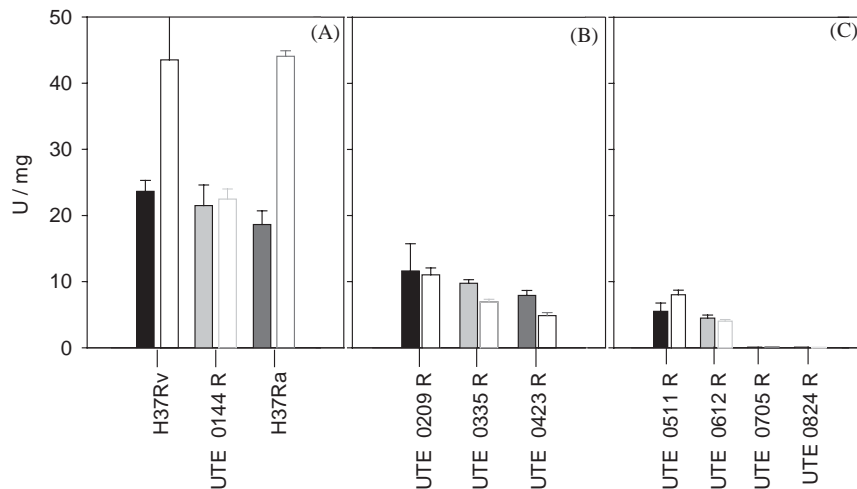


Figure 3 Enzymatic activities for catalase and peroxidase are represented by closed and open bars, respectively. Data show average and standard deviation of three independent determinations for each strain, in three groups of strains, with high (A), middle (B) and low (C) catalase and peroxidase activities.

divergences between the two methods, but both techniques matched in the cases with no activity (Table 1). Bacillary concentrations followed the usual kinetics in all the strains.²³ Fig. 4 shows data from week 3, when highest levels were obtained in this model, and from week 22 to the end of the experiment, when the capacity for persistent infection was evident. Bacillary concentrations in the whole lung 1 h after aerogenic inoculation showed a range between 15 and 50 CFUs (data not shown). In all cases, bacillary concentrations were significantly higher in the clinical strains than in the avirulent standard strain (H37Ra). None of the strains showed significantly lower levels than the virulent standard strain (H37Rv). On the contrary, lung concentrations in six strains were significantly higher in week 3, although only one strain (UTE 0423R) was able to maintain this difference in week 22. On week 22 spleen concentrations exhibited significant differences only for the UTE 0423R and UTE 0335R strains.

Ratios between granuloma-involved areas and global tissue areas in the lungs showed a marked linear relationship along time (Fig. 5), with $p < 0.0005$ in all cases (data not shown). This relation displayed extensive dissemination of granulomas that progressively took place throughout the lung parenchyma for every strain, independent of the strain's C-P activity. In fact, strains showing the vast spreading (UTE 0335R and UTE 0423R) exhibited an intermediate level of C-P activity. All the other strains, including the C-P negative ones, showed similar ratios. As expected, the H37Ra strain did not reveal any granulomatous lesions.

Discussion

The capacity to establish a chronic infection in the aerosol-induced murine model of tuberculosis of eight INH^R clinical strains of *M. tuberculosis* with different degrees of C-P activity was tested. Data

Table 1 Clinic, phenotypic, and genotypic characteristics of the clinical strains.

Strain	Gender	Age	Disease	Other R's	Previous treatment/prophylaxis	VH	Other risk factors	CD4 (cells/ml)	TST	Catalase (mm)	Peroxidase	Isoniazid MIC (µg/ml)	katG	inhA	ahpC	Cluster ^a
UTE 0144 R	F	31	Pulmonary	E, P, R	No	+	IDU + prison	16	+	2	+	8	315 AGC-ACC	399 GAA-GAG	W	II
UTE 0209 R	M	32	Pulmonary	S	No	+	IDU + prison	627	-	2	+	0,25	W	W	-12 GxA	No
UTE 0335 R	M	37	Pulmonary	-	No	-	Alcoholism	ND	+	<1	+	8	315 AGC-ACC	W	W	No
UTE 0423 R	F	31	Pulmonary	-	No	+	-	163	-	3	0	8	315 AGC-ACC	W	W	I
UTE 0511 R	M	29	Vertebral	-	Prophylaxis	+	IDU + alcoholism	ND	ND	4	+	0,5	W	T-GATAGGT	-38 and -39 Ins. C	No
UTE 0612 R	F	27	Lymphoid	-	No	-	Indian immigrant	ND	+	3	0	8	315 AGC-ACC	463 CGC-CTG	-45 and -46 Ins. AT	No
UTE 0705 R	M	67	Pulmonary	R	Treatment	ND	-	ND	ND	0	0	>32	W	W	W	No
UTE 0824 R	M	69	Pulmonary	R	Treatment	-	DM II	ND	ND	0	0	>32	W	W	-12 GxA	No

M: male; F: female; E: ethambutol; P: pirazinamide; R: rifampin; S: streptomycin; IDU: injection drug user; DM II: diabetes mellitus II; TST: tuberculin skin test; ND: not done; NK: not known. MIC: minimal inhibitory concentration; Ins: insertion; R's: resistances; W: wild type.

^aNon-Beijing patron was detected.

conclusively showed that there was no difference among the strains related to the virulent standard, with regard to the evolution of the infection in the lungs, the bacillary concentrations reached or the capacity to occupy lung parenchyma progressively. On the other hand, all the clinical strains with some C-P activity reached higher bacillary concentrations in the lungs in week 3 than the standard H37Rv, although this difference was only maintained by one of them in week 22. Bacillary concentrations in the spleen did not show any difference among the strains related to their capacity for systemic spreading, although two of them (UTE 0335R and UTE 0423R) showed a slight increase on week 22.

Study of virulence in drug-resistant clinical strains in the experimental model of tuberculosis infected through aerogenic exposure to a low-dose inoculum has already been performed.²⁵ According to that study, catalase activity did not seem to have a meaningful impact on the degree of virulence exhibited by the strains. In fact, two of the four catalase-negative strains were determined as "fast-growers" because they were able to grow faster than the Erdman strain, used as the reference strain in that case. At any rate, the Erdman strain cannot be considered as too virulent.²⁶ Hence the relative meaning of the term "fast grower" in this setting. On the contrary, our study focused on the ability of the strains to generate a chronic, stable, and persistent infection in the host, in contrast to their capacity for fast growth at the beginning of the infection. Briefly, we intended to explore whether these strains are able to induce a latent infection in immunocompetent hosts.

The major concern with this kind of studies is the validity of the experimental model used in order to extrapolate data obtained to humans. Key problems in human tuberculosis are the low inoculum dose needed for aerogenic transmission in the vast majority of the cases and its capacity to become a latent infection for years. In this regard, our model resembles natural tuberculous infection in humans, not only because of low-dose airborne inoculation but also because it allows one to assess the capacity to establish a chronic persistent infection. Route of inoculation has a paramount role in the outcome of this experimental infection. Different groups have demonstrated that intravenous inoculation is less pathogenic than aerogenic inoculation in mice²⁷ as a consequence of the faster promotion of systemic immunity in the former.²⁶ Subsequently, trying to evaluate the pathogenicity of different strains by using intravenous¹⁵ or intraperitoneal²⁸ inoculation models must be handled carefully

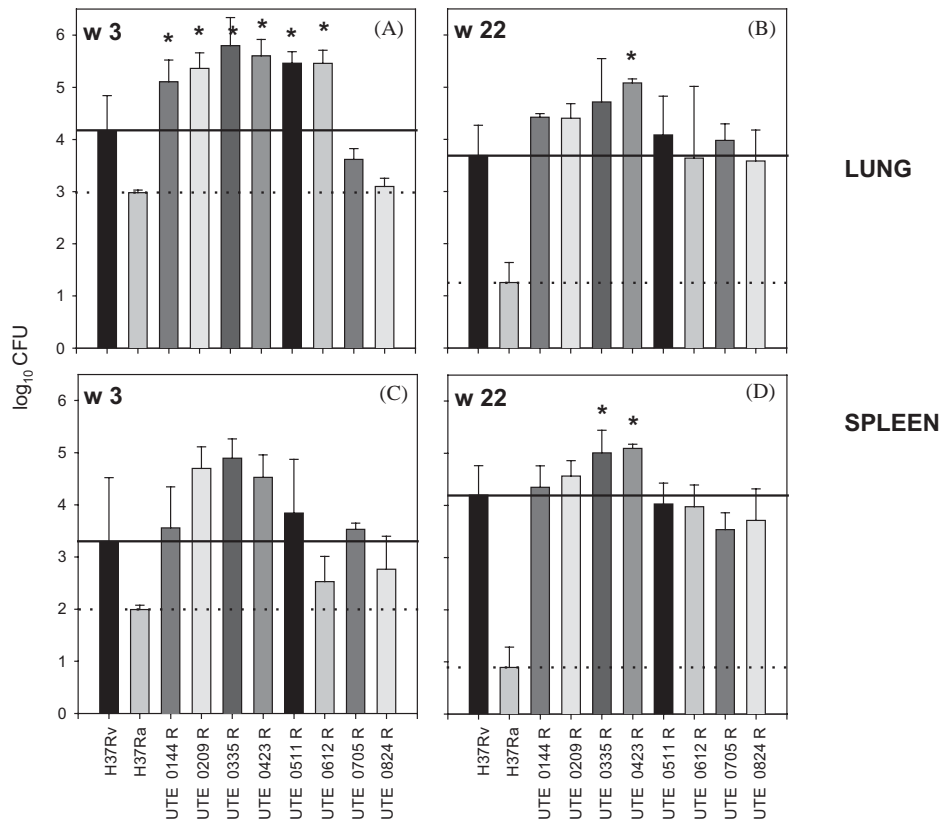


Figure 4 Bacillary concentration of each strain in the lungs (A and B) and spleens (C and D) on week 3 (A and C) and week 22 (B and D). A continuous line is drawn to reflect the level of the virulent standard strain (H37Rv). The dotted line reflects the level of the avirulent standard strain (H37Ra). Data exhibit average and standard deviation of the \log_{10} CFUs obtained for each strain. Differences with the H37Rv strain were determined with all the pairwise multiple comparison procedure (Student–Newman–Keuls method) and marked with an asterisk when significant ($p < 0,05$). All values were significantly higher when related to H37Ra strain.

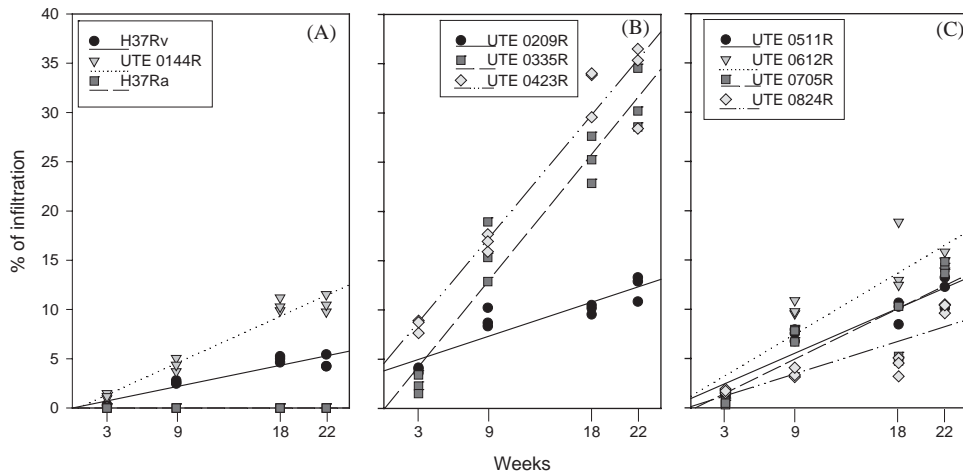


Figure 5 Evolution of the granulomatous infiltration in the lungs. Data show the ratio between granuloma-involved areas divided among global tissue area in two lung lobes for each mouse, in three groups of strains, with high (A), middle (B) and low (C) catalase and peroxidase activities. Three sections cut at 10 μm intervals were studied in every case. Linear regressions were determined through the Pearson product moment correlation coefficient ($p < 0.0005$ in all the cases).

because the chances of bacilli to get firmly established in tissues are lower. Intramuscular inoculation in the guinea pig¹⁵ is even more controversial, since regional lymph nodes might affect the course of infection before reaching the systemic circulation.

The host is another very important issue to take into account. We used mice because they are more resistant than guinea pigs to *M. tuberculosis* infection, and therefore closer to humans in this regard.²⁹ On the other hand, our group previously demonstrated that the continuous flow of infected foamy macrophages from the granuloma and the host inability to build a fibrous capsule around the lesion may favor the steady dissemination of the infection through the lungs.²³ These findings show that the mouse is not the so-called resistant host against *M. tuberculosis* infection, compared to humans, who are capable of developing a much stronger inflammatory and immune response that effectively controls the infection by confining bacilli into the granulomas.³⁰

Previous work on granuloma dissemination in the lung favors the view of a steady process that would result in the complete occupation of pulmonary parenchyma by the time the host finally dies.²⁷ This interpretation is opposed to the idea of chronic infection based on the evolution of bacillary concentration in our experimental model.²⁶ Our data strongly suggest the need for measuring the affected lung parenchyma at several timepoints along the infection. We believe that this approach provides a more accurate assessment of the problem, as opposed to just monitoring bacillary concentrations. In any case, the results of the present study indicate that C–P activity has no apparent relation with the capacity to originate a chronic persistent infection in mice.

These data are very well supported by recent studies run with the murine model induced by aerosol in which phagocyte oxidase knockout mice have been used, specifically p47^{phox} KO³¹ and gp91^{phox} KO.³² The lack of respiratory burst in those mice affected only the very first period of the infection, between days 15 and 30, increasing markedly in the bacillary concentration (10 times) in one of the studies³¹ when compared with wild-type control mice, although no difference could be detected in the other experience. This fact might explain why C–P-negative strains reached lower concentrations in the lung besides the other clinical strains. In any case, both studies detected no difference afterwards, when the Th1-specific immunity and secretion of interferon-gamma controlled the infection, as it has been described widely. In this regard, such studies give support to

the hypothesis that ROIs had just a minor role in the control of *M. tuberculosis* settlement in the lungs, but not in the establishment of a chronic and persistent infection, and this raises the question of the role of C–P activity as a virulence factor.

Regarding C–P activity, we want to stress the point that although data obtained with qualitative and quantitative assays did not always match, both assay types detected a complete lack of activity in the UTE 0705R and UTE 0824R strains. This may reflect the inaccuracy of the routine assay, which is nevertheless useful as a qualitative analysis in the clinical laboratories. Another striking point is the lack of *katG* gene mutations observed in both C–P negative strains. Other authors have already demonstrated high-level INH resistance in clinical strains (including C–P-negative strains) without any *inhA* or *katG* gene alteration. This suggests existence of additional INH resistance mechanisms or that certain regulatory defects involving *katG* or *inhA* expression remain unknown.^{33,34}

Our findings lend support to the idea that *M. tuberculosis* does not need to show C–P activity to be virulent. Two hypotheses try to explain this fact. The first one proposes the evolutionary acquisition of a constitutive oxidative stress response by *M. tuberculosis*³⁵ and the second one points to the possibility that pathogenic mycobacteria may reside within a privileged compartment of the macrophage, thus avoiding exposure to ROIs.³⁶ The first hypothesis is based on the observation that the *oxyR* gene is nonfunctional in the *M. tuberculosis* complex.³⁶ OxyR is both a sensor of reactive oxygen species and a transcriptional activator, inducing expression of detoxifying enzymes such as C–P or alkyl hydroperoxidase (AhpC), and it is a crucial component of the oxidative stress response in Gram-negative bacteria. During INH activation by C–P, the making of ROIs inside the cell cytoplasm causing damage in multiple targets must be highlighted.³⁷ The loss of the OxyR function and lower levels of AhpC might explain why *M. tuberculosis* is so sensitive to INH.³⁸ Besides, *oxyR* and *ahpC* mutants of *Escherichia coli* are sensitive to INH, but wild-type *E. coli* is resistant.³⁹ The lack of such system has suggested that *M. tuberculosis* must be inherently more resistant to ROIs coming from outside the cell. Some components of *M. tuberculosis* cell wall may act as scavengers of ROIs (lipoarabinomannan and phenolic glycolipids)^{40,41} or may confer resistance to ROI-mediated damage (cyclopropanated mycolic acids).⁴²

In conclusion, the lack of C–P activity in INH^R clinical strains exerts no influence on their ability to cause persistent chronic infection in mice following aerogenic exposure to a low-dose

inoculum. According to these findings, it seems reasonable to postulate that C–P negative strains of *M. tuberculosis* may be able to cause such persistent infection in humans, although no information is available on their capacity to reactivate and induce disease in people.

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