

Urease Activity Does Not Contribute Dramatically to Persistence of *Mycobacterium bovis* Bacillus Calmette-Guérin

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Multiplication of BCG_{ure-}, an isogenic urease-negative mutant of *Mycobacterium bovis* BCG constructed by allelic exchange (J. M. Reyrat, F. X. Berthet, and B. Gicquel, Proc. Natl. Acad. Sci. USA 92:8768–8772, 1995), was examined in human macrophages and mice. Although ureolytic activity was not essential to BCG_{ure-} growth, a slight decrease in the multiplication and persistence of the mutated strain compared with wild-type BCG was observed in lungs of infected mice.

Tuberculosis is a major threat to humans, responsible for more deaths than any other single infectious disease. The etiological agents of tuberculosis, *Mycobacterium tuberculosis*, *M. bovis*, and *M. africanum*, are facultative intracellular pathogens able to replicate inside macrophages. During their intracellular life cycle, pathogenic mycobacteria reside in a weakly acidified membrane-bound phagosome (3, 5). After phagocytosis, mycobacteria may selectively inhibit fusion of its vacuoles to proton-ATPase-positive vesicles, inducing the lack of acidification of the mycobacterial phagosomes (20). Ammonia production by *M. tuberculosis* may also contribute to its intracellular survival inside the macrophage by inhibiting phagosomal maturation (10). Urease, a nickel-containing enzyme (EC 3.5.1.5) that hydrolyzes urea into carbon dioxide and ammonia (for a review, see reference 14), might then play a role in modulating the pH of the phagosome and inhibiting the maturation of the phagolysosome. Ureasases, via ammonia production, have been implicated as virulence factors in a number of other microbial pathogens, either by promoting direct toxicity to renal epithelium and stone formation, as for *Proteus mirabilis*, or by allowing colonization of the acidic environment of the stomach, as for *Helicobacter pylori*. Recently, the urease locus of *M. tuberculosis* has been cloned and sequenced (4, 18). This locus, mapped between the *adhA* (alcohol dehydrogenase A) and *bfr* (bacterioferritin) genes, consists of five genes: *ureA*, *ureB*, and *ureC*, encoding urease subunits (18), and *ureF* and *ureG*, encoding urease accessory polypeptides (4). Urease enzymatic activity is heat stable and is induced upon nitrogen deprivation (4).

The only vaccine currently available against tuberculosis is bacillus Calmette-Guérin (BCG), a live attenuated strain of *M. bovis* obtained during the 1920s (2). Although attenuated, BCG is able to replicate inside macrophages in vivo. In inoculated mice, BCG growth occurs in target organs such as lungs, spleen, or liver but is restricted, and the infection is cleared. However, numerous mycobacterium-host interactions studies have been performed with BCG, which is a safe organism and does not require biohazard containment laboratories. Recently, we reported the construction of a urease-negative iso-

genic mutant strain of BCG by allelic exchange involving replacement of the *ureC* gene with a kanamycin-disrupted copy (*ureC::aph*) (18). In this study, we compared the persistence of *M. bovis* BCG_{ure-} and parental wild-type BCG both in vitro in human macrophages and in vivo in BALB/c mice.

In vitro analysis. Survival of mutant BCG_{ure-} in macrophages, the target cell for mycobacterial infections, was compared with the parental Pasteur BCG 1173P2 strain in THP-1 cells (ATCC TIB 202), a human monocytic cell line. This cell line was chosen because, upon stimulation with tetradecanoyl phorbol acetate, it differentiates into cells closest to the native monocyte-derived macrophages (21). Moreover, this cell line has been used as an in vitro model in mycobacterial infections (1, 8). Given that mycobacteria may interact differently with mononuclear phagocytes depending on whether they derive from mice or men (7), we chose an in vitro model with cells of human origin. Cells were allowed to adhere to 24-well plates by stimulation with 10 nM phorbol myristate acetate (Sigma, St. Louis, Mo.) 48 h before the infection. Cultures of both strains were obtained as described previously (9), and aliquots were kept in 50% glycerol stocks at -20°C. Prior to infection, the aliquots were grown to a final optical density at 600 nm of 0.7 to 0.9 (5×10^6 to 3×10^7 CFU/ml) in 5 ml of 7H9 medium enriched with ADC Middlebrook and 0.05% Tween 80. The inoculum was prepared in cell culture medium (RPMI 1640, 5% fetal calf serum, 20 mM glutamine) and added to the macrophages (10^6 per well) in 500 μ l. The multiplicity of infection was 5 to 10 mycobacteria per cell, and the infection was carried out in 3 h. Immediately after infection, viable counts of BCG CFU were determined by plating serial dilutions of supernatants and cell lysates on 7H10 medium supplemented with Middlebrook enrichment medium (OADC). BCG_{ure-} and wild-type BCG levels of uptake by THP-1 cells were 24 and 30%, respectively, with no statistical difference ($P > 0.1$ [analysis of variance, $n = 6$]). Infected monolayers were then washed, and 100 μ g of amikacin per ml was applied for 24 h to clear extracellular bacteria; then, the medium was changed, and the incubations were maintained for 2 weeks. Intracellular persistence of both BCG strains was analyzed by counting viable CFU after 1, 8, or 15 days of infection. As depicted in Fig. 1, both strains failed to grow, and they were killed at similar rates by the macrophages. These results for wild-type BCG intracellular kinetics agree with those from previous studies (13). Direct observation of the cells done in parallel on Lab-Tek chambers with acid-fast staining (Kinyoun) demon-

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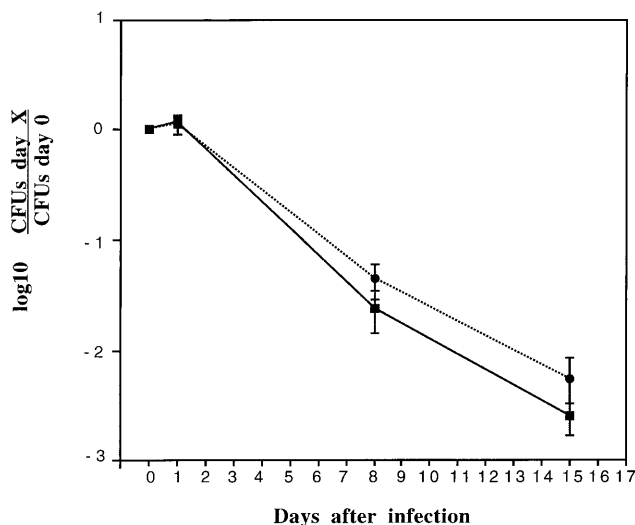


FIG. 1. Survival of wild-type BCG 1173P2 (circles) and the BCGure- mutant (squares) in THP-1 cells. After a 3-h infection, BCG CFU counts were determined from infected-cell lysates and were monitored from day 0 to day 15. The data are means with standard deviations ($n = 4$).

strated that neither the percentage of cells associated with mycobacteria (30 to 40%) nor the number of mycobacteria per cell varied significantly between the two strains during the incubations. Taken together, these results show that absence of urease activity does not significantly affect BCG growth inside cultured macrophages.

In vivo analysis. The growth of the BCGure- mutant was analyzed in the well-characterized mouse model of *M. tuberculosis* complex infection (16). Suspensions of parental Pasteur BCG 1173P2 and mutant BCGure- were prepared (9), and it should be noted that no differences in growth rates between BCGure- and BCG 1173P2 were observed in vitro, in 7H9 or Sauton's culture medium. One million CFU was injected in the tail vein in 6- to 8-week-old female BALB/c mice. At various time intervals after inoculation, five animals in each group were sacrificed and spleen, liver, and lungs were homogenized (Stomach Lab-Blender 80; Bioblock). Suitable dilutions were plated onto Middlebrook 7H11 medium supplemented with OADC to enumerate BCG CFU 17 days later. Urease phenotype was checked on several BCGure- or wild-type BCG colonies at this stage, and no reversion was observed. The experiment was performed twice, and the results in Fig. 2 are the most representative. The BCGure- mutant shows growth characteristics identical to the parental strain in spleen and liver in BALB/c mice carrying *bcg^s/lsh^s/ity^s*, a gene expressed in macrophages implicated in control of intracellular parasite multiplication (22). However, in lungs, decreases of about half a log in both multiplication and persistence of the BCGure- mutant were observed. Compared with growth defects in mice for the leucine-auxotrophic BCG mutant strain (12), the effect of urease gene inactivation is weak. However, this growth defect is statistically significant ($P < 0.005$ [paired *t* test, $n = 2$]), and interestingly enough, it is observed in lungs, the target organ for tuberculosis. Recently, virulence ranking of pathogenic mycobacterial strains in mice was correlated to the degree of lung pathology observed (6). Oxidative burst in pulmonary macrophages is more active than in spleen macrophages or Kupffer cells from the liver, rendering pulmonary macrophages more potent at eliminating intracellular bacteria. One hypothesis might be that urease gene disruption renders

BCGure- more sensitive than wild-type BCG to the active pulmonary-macrophage killing mechanisms. This might thus explain why the slight BCGure- growth defect is observed only in lungs.

According to our findings, urease activity is not essential to the persistence of *M. bovis* BCG either in cultured macrophages or in BCG-sensitive BALB/c mice, even though a slight decrease of BCGure- growth was observed in lungs of infected mice. However, *M. bovis* BCG is not virulent. Attenuation of BCG is characterized by its limited growth in all organs of inoculated mice because of inhibitory action of acquired immunity (15, 17). The situation is different for a fully virulent mycobacterial strain like *M. tuberculosis*, which replicates to a high degree especially in lungs of infected mice (16). In a study we performed in the microbiology department of a city hospital, mycobacterial samples from patients treated for tuberculosis during a 2-year period were characterized, and their urease activities were tested. Six of 101 *M. tuberculosis* infectious isolates were urease negative. Noteworthy, patients infected with urease-negative isolates of *M. tuberculosis* did not share any characteristics such as immunosuppression, age, or geographic origin. Together with the high percentage (6%) of clinical isolates presenting a urease-negative phenotype, this indicates that urease is not a virulence factor per se.

In this study, we did not observe any measurable impact of the absence of urease activity on the intracellular survival of BCG in cultured macrophages. Nevertheless, the capacity of BCG to replicate inside cultured macrophages is also impaired compared with virulent *M. tuberculosis* (13), and the situation for pathogenic strains of mycobacteria might be different. Schlesinger (19) has demonstrated that attenuated and nonattenuated strains of *M. tuberculosis* interact differently with host cell receptors, and in fact, uptake pathways are determinantal for the intracellular fate of pathogens (11). Although the role of urease activity in the pathogenesis of tuberculosis might be more important, the data we present here indicate that this enzyme is not critical to the maintenance of the vaccine BCG strain in human-derived macrophages or in mouse target organs.

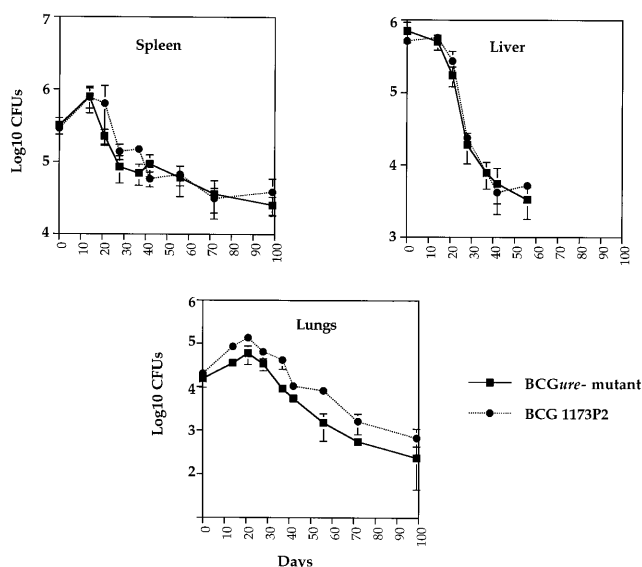


FIG. 2. Total CFU counts of wild-type BCG 1173P2 and BCGure- in spleen, liver, or lungs were determined, and means with standard deviations are shown.

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