The *In Vivo* Environment Accelerates Generation of Resuscitation-Promoting Factor–Dependent Mycobacteria

To the Editor:

It is believed that one-third of the entire world population is latently infected with *Mycobacterium tuberculosis*, which has previously been attributed to the ability of the tubercle bacillus to survive for years without replication (1). The documented examples of tuberculosis reactivation after years of absence of disease illustrate the capacity of *M. tuberculosis* to reversibly switch from a nonreplicating state to active growth (2). The presence of *M. tuberculosis* in lungs of latently infected patients has been confirmed by molecular and immunological techniques (3), yet growth in culture of the bacteria associated with latent infection has not been demonstrated. Therefore, it has been hypothesized that during latent infection *M. tuberculosis* produces nonreplicating forms, which require resuscitation under specialized cultivation conditions to produce growth (4). However, nongrowing persister-like *M. tuberculosis* can also be detected in sputum collected from patients with active tuberculosis (5–7). The majority of these bacilli required specific growth conditions and could be cultivated only in the presence of recombinant resuscitation-promoting factor (Rpf) or culture supernatant (6). Moreover, these Rpf-dependent mycobacteria were more tolerant to rifampicin and accumulated during chemotherapy while other organisms were eliminated (6), confirming the production of physiologically distinct *M. tuberculosis* forms during tuberculosis infection or during transition to sputum. Because numbers of Rpf-dependent mycobacteria varied between patients (6), it is plausible to suggest the importance of specific host factors for the development of Rpf dependency.

The molecular mechanisms underlying the formation of Rpf-dependent bacteria recovered from sputum remain unknown. Rpf-dependent cells could be generated in loci of infection (e.g., lungs) in high numbers and subsequently gradually released into sputum; alternatively, mycobacteria may rapidly develop Rpf dependency during transition from lung to sputum under the influence of certain, but yet unknown, stimuli. Our previous identification of Rpf-dependent bacteria in patients with active tuberculosis points to the presence of a heterogeneity in growth states within the bacterial populations residing in sputum. However, how and when these adaptions arise remains unknown and in this regard we propose two possibilities: (*1*) Considering that tubercle bacilli traffic out of necrotic cavities and into the airways, the accompanying changes in the extracellular environment and short exposure to sputum may induce an adaptive response that results in Rpf dependency; (*2*) Rpf dependency may arise in response to stresses imposed by the immune system and longer stationary exposure to the tissue or cavity environment. We previously found that direct exposure of *M. tuberculosis* to sputum did not result in Rpf dependency (6), which suggested that the extracellular environment in sputum cannot be the sole inducer of this adaptive response in *M. tuberculosis*. Consequently, in this study we investigated whether Rpf-dependent bacilli are produced in pulmonary infection in an animal model.

Nine-week-old BALB/c mice were infected intranasally with *Mycobacterium bovis* BCG Glaxo at a dose of 2×10^5 bacteria per mouse, and numbers of mycobacteria in lungs were monitored for 6 weeks. For this, we employed growth assays previously developed for investigation of mycobacterial populations in sputum (6). We quantified numbers of mycobacteria that were able to grow either on 7H10 agar (colony-forming unit [CFU] counts) or in liquid 7H9 medium (using the most probable number [MPN] assay). The numbers of Rpf-dependent mycobacteria (RDM) were assessed by MPN assay in liquid 7H9 medium, containing culture supernatant from growing bacteria. At 24 hours postinfection, CFU, MPN, and RDM counts of mycobacteria recovered from lungs of infected animals were not significantly different ($P > 0.05$, one-way analysis of variance), suggesting that the preparation of bacteria for infection and initial adaptation *in vivo* did not induce Rpf dependency. However, during the course of infection there was a dramatic 2.5 $\log_{10}$ reduction in CFU and MPN counts of mycobacteria in the lungs of infected animals (Figure 1A). These results are in good accordance with previously reported survival patterns of *M. bovis* BCG in BALB/c mice (8, 9). In contrast, the number of mycobacteria grown with culture supernatant changed only at the beginning of infection (a 0.5 $\log_{10}$ reduction 1 wk postinfection) and at later stages it remained constant, suggesting that more than 98% of mycobacteria recovered from lungs at 6 weeks postinfection required special conditions for cultivation (Figure 1A). To confirm that bacteria recovered in the presence of culture supernatant were indeed Rpf dependent, further experiments were performed. In these experiments numbers of mycobacteria grown in culture supernatant treated with specific inhibitors of Rpf (10), or in culture supernatant prepared from a quintuple *M. tuberculosis* mutant missing all five Rpfs (11), were assessed. As shown in Figure 1B, both Rpf inhibitors completely eliminated the resuscitation activity of culture supernatant, and Rpf-negative supernatant also failed to resuscitate nonculturable bacteria. Both of these control experiments confirm that the nonculturable mycobacteria recovered were indeed Rpf dependent.

Incubation of mycobacteria in lung homogenates did not result in the development of Rpf dependency (data not shown). We therefore investigated whether exposure of mycobacteria to murine serum would stimulate production of Rpf-dependent forms. We incubated growing *M. bovis* BCG bacteria in phosphate-buffered saline (PBS) containing 25% (vol/vol), 50% (vol/vol), or undiluted murine serum, obtained from mice infected with *M. bovis* BCG for 24 hours, at 37°C without shaking. CFU and MPN counts were taken after 1 and 3 days of incubation. However, incubation of mycobacteria in PBS containing serum did not result in any statistically significant loss of culturability or generation of Rpf-dependent forms (Figure 1C). Sera from uninfected mice showed similar effects. This could be because cell-mediated immunity is essential for the generation of Rpf-dependent bacteria.

This study demonstrates that the *in vivo* environment changes mycobacterial physiological characteristics and accelerates the generation of Rpf-dependent mycobacteria. Our results suggest that Rpf-dependent mycobacteria are generated in murine lungs soon

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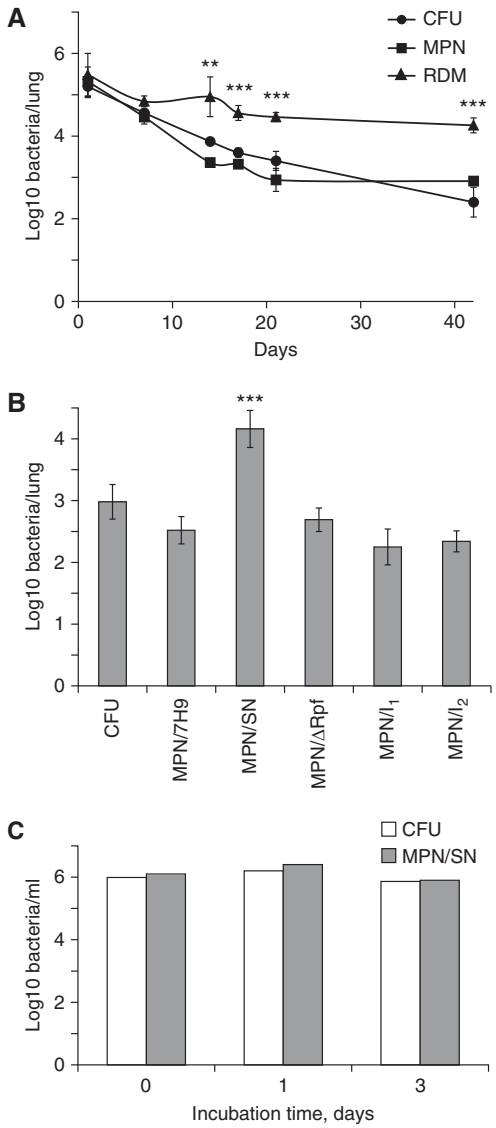

Figure 1. Generation of resuscitation-promoting factor (Rpf)-dependent *Mycobacterium bovis* (BCG) in murine lungs. (*A* and *B*) Nine-week-old BALB/c mice were infected intranasally with 2×10^5 bacterial colony-forming units (CFUs) per animal. (*A*) Homogenized tissue samples were used for assessment of CFU counts (*circles*) or most-probable number assay (MPN) counts in liquid 7H9 medium (*squares*) or Rpf-dependent mycobacteria (RDM) counts in 7H9 medium supplemented with culture supernatant (*triangles*). A PANTA antimicrobial mixture (Becton, Dickinson and Co., Franklin Lakes, NJ) was added to all cultivation media. Average values for four independent animals are presented; *error bars* indicate standard deviations. **RDM values were significantly different from CFU and MPN counts ($P < 0.01$, *t* test); ***RDM values were significantly different from CFU counts ($P < 0.001$, *t* test). (*B*) Control experiments. CFU and MPN counts were determined in lung homogenates from mice 3 weeks postinfection. The Rpf inhibitors NTB (3-nitro-4-thiocyanato-benzonitrile), designated as I_1, and NTPPM [(3-nitro-4-thiocyanato-phenyl)-phenyl-methanone], designated as I_2, did not inhibit growth of active *M. bovis* BCG *in vitro* at the concentration used in these experiments (5 μg/ml). SN = culture supernatant. (*C*) Effect of murine serum on *M. bovis* BCG viability. Bacteria from the logarithmic phase were exposed to 20% (vol/vol) murine serum in phosphate-buffered saline. CFU and RDM counts were determined after 1 and 3 days of exposure.

after infection and represent significant proportions of the bacteria present. Therefore it is plausible to suggest that Rpf-dependent mycobacteria recovered from the sputum of infected patients are generated in the lungs during infection rather than during the transition into sputum.

The precise factors responsible for the formation of Rpf-dependent bacteria remain unknown. Previously, it was demonstrated that *M. tuberculosis* bacilli developed Rpf dependency during prolonged incubation in the stationary phase (11) or on gradual acidification of medium (12). Nonculturable and Rpf-dependent cells of *M. tuberculosis* have been recovered from peritoneal macrophages of infected mice after several days of infection (13). However, exposure of mycobacteria to murine serum did not stimulate Rpf dependency, suggesting that a yet unknown combination of environmental conditions not identified (or present) in *in vitro* experiments or certain immune factors may trigger transition to the Rpf-dependent state. Our findings have important implications for the diagnosis of tuberculosis (14, 15) and development of models for testing the bactericidal activity of novel drugs. The presence of a high number of Rpf-dependent bacteria *in vivo* must be taken into account in the design of drugs for the treatment of tuberculosis, and compounds should also be tested for activity against Rpf-dependent forms. Our data suggest that significant proportions of mycobacteria may remain undetected in animal infection experiments and during clinical trials of new treatments. ■

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Caffeine Therapy for Apnea of Prematurity: Long-Term Effect on Sleep by Actigraphy and Polysomnography

To the Editor:

Marcus and colleagues checked the long-term effects of caffeine therapy for apnea of prematurity by using actigraphy, polysomnography, and parental sleep questionnaire (1). Although the authors concluded that therapeutic neonatal caffeine administration had no long-term effects on sleep duration or sleep apnea during childhood, there was a discrepancy in sleep duration between results from actigraphy and polysomnography. Melzer and colleagues mentioned that actigraphy showed poorer specificity (to detect wake), and each researcher should adjust the scoring algorithm/sensitivity depending on age and the level of sleep disturbance (2). I suppose that the authors understand the limitation of actigraphy for sleep evaluation, but there was no precise description on the adjustment of actigraphy in the text or the online supplement. Meltzer and colleagues reported that actigraphy should be used with caution to keep specificity and sensitivity by adjusting the scoring threshold of sensitivity of Actiwatch 2 (Philips Respironics, Bend, OR) (2, 3), which could be done by the setting through software. As Marcus and colleagues conducted the validation study with an epoch-by-epoch comparison of actigraphy and simultaneous ambulatory polysomnography in a random sample of 20 subjects, I want to know the level of sensitivity for Actiwatch 2.

Kushida and colleagues found the best accuracy and specificity between Actiwatch and polysomnography when the "high-sensitivity" setting (20 cpm) was used (4). Peterson and colleagues adopted the default sensitivity setting (40 cpm) of Actiwatch and found overestimation of total sleep time and underestimation of wake after sleep onset (5). There is a difference between brain activity and physical movement during sleep, and the discrepancy of sleep parameters between polysomnography and actigraphy is obvious for subjects with insomnia (6). Sitnick and colleagues also described the limitation of actigraphy for detecting night waking (7).

I appreciate the study design by Marcus and colleagues, but methodological validation is important for the evaluation of caffeine therapy for apnea of prematurity and subsequent sleep problems at school age. ■

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Reply

From the Authors:

Dr. Kawada has raised an important question about the sensitivity setting of the actigraphy used in our study (1). For this study, we chose to use the medium sensitivity threshold.