Extrapulmonary Dissemination of *Mycobacterium bovis* but Not *Mycobacterium tuberculosis* in a Bronchoscopic Rabbit Model of Cavitary Tuberculosis

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The rabbit model of tuberculosis is attractive because of its pathophysiology resembling the disease in humans. Rabbits are naturally resistant to infection but may manifest cavitary lung lesions. We describe here a novel approach that utilizes presensitization and bronchoscopic inoculation to reliably produce cavities in the rabbit model. With a fixed inoculum of bacilli, we were able to reproducibly generate cavities by using *Mycobacterium bovis* Ravenel, *M. bovis* AF2122, *M. bovis* BCG, *M. tuberculosis* H37Rv, *M. tuberculosis* CDC1551, and the *M. tuberculosis* CDC1551 ΔsigC mutant. *M. bovis* infections generated cavitary CFU counts of 10^9 to 10^9 bacilli, while non-*M. bovis* species and BCG yielded CFU counts that ranged from 10^7 to 10^8 bacilli. Extrapulmonary dissemination was almost exclusively noted among rabbits infected with *M. bovis* Ravenel and AF2122. Though all of the species yielded secondary lesions at intrapulmonary sites, *M. bovis* infections led to the most apparent gross pathology. Using the *M. tuberculosis* icd and dosR-gene expression patterns as molecular sentinels, we demonstrated that both the cavity wall and cavity lumen are microenvironments associated with hypoxia, upregulation of the bacterial dormancy program, and the use of host lipids for bacterial catabolism. This unique cavitary model provides a reliable animal model to study cavity pathogenesis and extrapulmonary dissemination.

Tuberculosis (TB) continues to be a global public health problem, with approximately one-third of the world’s population latently infected (14). Nine million active infections are diagnosed annually, and approximately two million people died of the disease last year (33). Cavitary pulmonary lesions are key means of disease transmission (1, 21). The lesions present an ideal environment for tremendous bacillary growth, which, under the correct conditions, may liquefy and cavitate. Early investigations by Wells and Lurie yielded cavity formation with virulent and attenuated strains of mycobacteria. Gross pathology, histopathology, CFU counts of lung and extrapulmonary lesions, and *M. tuberculosis* gene expression patterns were explored in selected rabbits.

**MATERIALS AND METHODS**

**Microorganisms.** Cultures for bronchoscopic infection were prepared by thawing frozen stock aliquots of *M. bovis* Ravenel, *M. bovis* AF2122, *M. bovis* BCG, *M. tuberculosis* H37Rv, *M. tuberculosis* CDC1551, and the *M. tuberculosis* CDC1551 ΔsigC mutant. The *M. tuberculosis* CDC1551 ΔsigC mutant is a gene replacement mutant that showed a modest degree of attenuation in the mouse model (28). Mycobacteria were grown in 7H9 Middlebrook liquid medium supplemented with oleic acid albumin, dextrose, and catalase (Becton Dickinson, Inc., Sparks, MD), 0.5% glycerol, and 0.05% Tween 80. The glycerol-containing medium, as opposed to an a pyruvate carbon source, was not found to limit the growth of *M. bovis* strains.

**Animals and infection.** Thirty pathogen-free outbred New Zealand White rabbits (2.5 to 3.5 kg) were obtained from Covance Research Products, Inc. (Denver, PA). Animals were maintained in standard cages under biosafety level 3 conditions. All animals were maintained in accordance with protocols approved by the Institutional

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Animal Care and Use Committees of Johns Hopkins University. Sensitization was performed by administration of five subcutaneous injections of $10^7$ heat-killed M. bovis cells in incomplete Freund’s adjuvant 3 to 4 days apart. An intradermal skin test with 0.1 ml of Old Tuberculin (Sibiotics Corp., Kansas City, MO) was given 25 days after the last sensitization injection. Old Tuberculin, as opposed to purified protein derivative, was chosen secondary to the enhanced ability to detect delayed-type hypersensitivity (DTH) in rabbits. The tuberculin reaction was read 48 to 72 h later to confirm successful acquisition of DTH immunity. Measurements of skin fold thickness were taken in two dimensions, and the results were calculated by using the formula for the volume of an oval spheroid. Rabbits were anesthetized with xylazine (5 to 10 mg/kg) and ketamine (15 to 25 mg/kg). Yohimbine (0.1 to 0.2 mg/kg) was used to reverse excessive sedation. A 3.0-mm flexible Pentax FB-8V pediatric bronchoscope (Pentax Medical Company, Montvale, NJ) was wedged into the right basal lobe of the lung. A total of 0.3 ml of a bacillary suspension containing $10^9$ to $10^8$ CFU was delivered via the bronchoscope insertion port. Confirmation of the number of CFU delivered was done via plating of serial dilutions of the inoculated suspension.

Clinical assessment. After infection, the rabbits were monitored twice weekly for clinical appearance, weight, and rectal temperature.

Necropsy. Rabbits were observed for a minimum of 30 days and a maximum of 105 days after infection. Criteria for euthanasia included signs of respiratory distress (dyspnea) and/or significant loss of weight (150 to 200 g). Rabbits were euthanized with intravenous Euthasol (Virbac Corporation, Fort Worth, TX). At necropsy, samples were obtained from the lungs and extrapulmonary sites. Cavity specimens that represented the primary lesion included the (i) lumen contents, (ii) wall, and (iii) surrounding inflammatory tissue. Grossly visible secondary lesions of the ipsilateral lung, contralateral lung, and extrapulmonary sites were noted. Extrapulmonary sites included the (i) lymph nodes (mediastinal, thoracic, mesenteric, Peyers’s patches in the small intestine, and mesoentery), (ii) spleen, (iii) liver, (iv) kidney (bilatetal), (v) ovary, (vi) adrenal, (vii) bone marrow, and (viii) ecum.

Scoring of gross pathology and cavity histopathology. At necropsy, grossly visible pulmonary primary lesions in the right lower lobe and secondary lesions in the ipsilateral and contralateral lungs were scored according to their prevalence. Grossly visible extrapulmonary lesions were scored according to both prevalence and location. Paraffin-embedded tissue sections were stained with hematoxylin and eosin and by the Ziehl-Neelsen carbol-fuchsin method (Becton Dickinson and Company, Franklin Lakes, NJ). All slides were examined with a light microscope. Paraffin-embedded tissue sections were stained with hematoxylin and eosin and by the Ziehl-Neelsen carbol-fuchsin method.

Determination of bacterial counts. CFU counts were measured at all of the predetermined pulmonary and extrapulmonary sites of each rabbit infected. Tissue samples from each site were homogenized, and aliquots were plated on selective 7H11 agar supplemented with oleic acid albumin, dextrose, and catalase. CFU counts were enumerated on days 14, 21, and 28.

RNA isolation and transcript analysis. For transcriptional analyses, total RNA was isolated from scraping of caseating lesions (homogenized inner cavitory contents), fragments of the cavitary wall (homogenized segment of the fibrous cavitary wall) of rabbits infected with M. tuberculosis CDC1551 or M. bovis AF2122, and in vitro-grown log-phase (A590 of 0.8) and stationary-phase (A590 of 2.0) cultures of the two organisms by the Trizol method according to the supplier’s instructions (Invitrogen Corporation, Carlsbad, CA). Enrichment for bacterial RNA isolated from tissues of infected animals was carried out with the MICROBEnrich kit (Ambion Inc., Austin, TX) in accordance with the manufacturer’s protocol. To quantify the level of expression of each transcript, RNA was treated with RNase-free DNase (Ambion), and 1 μg of RNA was subjected to reverse transcription with the iScript cDNA synthesis kit (Bio-Rad Laboratories). This was followed by real-time quantitative PCR with the SYBR Green Supermix (Bio-Rad Laboratories) and gene-specific primers ([5' GAG CTTGACGTCTTAGTGTGA 3'], [5' AGATCGGCTGCTATACCGAG 3'], [5' AGGATGACGGCGCCGACTG 3'], and [5' GACGACACGGGTATCGTCTG 3']). The n-fold differences in transcript levels were derived by comparing the cycle threshold values of the test samples with those of samples from cultures grown to log phase, following normalization to the M. tuberculosis sigA transcript (amplified with primers sigA-F [5' CGATAGCGACCGAGAGAGAGA TGCC 3'] and sigA-R [5' CAGGCTACCTTGGCCGATCG 3'])

Statistical analysis. Data are reported as mean values unless otherwise stated. Mean paired values of intrathoracic sites were compared to cavity center values by two-tailed t tests. The level of significance was set at $P < 0.10$.
Necropsy specimens obtained later in infection (>50 days) were more likely to fall into types C and D of Yamamura’s spectrum of lung cavity classification: type A, necrosis or loss of tissue substances in the central part accompanied by no granulation tissue in the surrounding region; type B, caseation and liquefaction in the central part with beginning of fibroblastic proliferation around a lesion; type C, caseation and liquefaction in the central part accompanied by the fibroblastic proliferation and connective tissue formation in surrounding tissue; type D, greater fibroblastic proliferation and connective tissue formation (35).

**M. bovis uniquely generates extrapulmonary dissemination.** *M. bovis* Ravenel and AF2122 both uniquely disseminated to all observed intrapulmonary and extrapulmonary sites (Fig. 3). Despite the cavity formation seen when other mycobacterial species and strains were used, uniform spreading from primary lesions was seen only in rabbits infected with *M. bovis*. Although *M. bovis* Ravenel and AF2122 led to numerous visualizable extrapulmonary tuberculomas, splenic lesions were seen in only one *M. bovis* Ravenel-infected rabbit and two *M. bovis* AF2122-infected rabbits on gross pathology (Fig. 4). However, CFU counts were most abundant in the spleen and thus confirmed the early extrapulmonary dissemination of tubercle bacilli through the blood and key reticuloendothelial organs (6). Visible tuberculomas and histopathologic granulomas were noted in the bilateral kidneys, cecum, and appendices. Few abdominal lesions were seen in the gastrointestinal tract, with the exception of the appendiceal/cecal area. A stomach wall granuloma was seen in a rabbit infected with *M. bovis* Ravenel and AF2122. CFU counts confirmed gross pathological findings, with elevated counts measured in the liver, kidney, and selected gastrointestinal sites (cecum and appendix). The organs and sites that showed the lowest CFU counts were the liver, ovaries, bone marrow, and feces.

Limited extrapulmonary dissemination was noted at the gross pathology level among rabbits infected with *M. tuberculosis* and *M. bovis* BCG. Microbiology of CFU counts in non-*M. bovis*-infected rabbits corresponded relatively well to the absence of extrapulmonary gross pathology. Two notable exceptions included two *M. tuberculosis* CDC1551-infected rabbits that had CFU counts of 2- to 3-log units in the normal-appearing abdominal lymph nodes that were sampled. *M. tuberculosis* H37Rv also resulted in CFU counts of approximately 3-log units in a grossly unremarkable kidney of the single rabbit that died prematurely of pneumothorax.

**Both *M. bovis*-infected and non-*M. bovis*-infected rabbits show intrapulmonary dissemination.** Multiple lesions were noted at pulmonary sites of the ipsilateral (right upper and middle lobes) and contralateral (left) lung segments of *M. bovis*-infected rabbits. CFU counts of the intrathoracic compartments confirmed gross pathological findings (Fig. 5). *M.
TABLE 1. Bacterial gene expression in the cavity microenvironment

<table>
<thead>
<tr>
<th>Gene</th>
<th>Log-phase in vitro culture</th>
<th>Stationary-phase in vitro culture</th>
<th>Intraluminal contents of cavitary cavity</th>
<th>Cavity wall</th>
</tr>
</thead>
<tbody>
<tr>
<td>icl</td>
<td>1</td>
<td>0.86 ± 1.26</td>
<td>83.69 ± 40.3</td>
<td>25.56 ± 10.4</td>
</tr>
<tr>
<td>dosR</td>
<td>1</td>
<td>261.23 ± 171.9</td>
<td>14.48 ± 9.5</td>
<td>5.53 ± 4.13</td>
</tr>
</tbody>
</table>

* Relative icl and dosR transcript levels in intraluminal contents of cavitary cavities, and fragments of the granuloma walls of *M. tuberculosis* CDC1551-infected rabbits are shown. The transcript levels of the respective genes in log phase were assigned a value of 1. The results are representative of measurements from two infected rabbits, with at least two replicates in each set.

bovis-infected rabbits yielded greater than 3- to 4-log unit CFU counts at all selected pulmonary sites. *M. bovis* Ravenel- and AF2122-infected rabbits displayed similar CFU count profiles. The highest total CFU counts were measured in the inner cavitary contents, which yielded greater than 10^6 bacilli in *M. bovis* CDC1551-infected rabbits and greater than 10^7 bacilli in *M. bovis* AF2122-infected rabbits.

All non-*M. bovis* bacilli showed intrapulmonary dissemination to the ipsilateral and/or contralateral upper lobe lung segments. *M. tuberculosis* CDC1551 and H37Rv were the only non-*M. bovis* bacilli to yield a majority of infected organisms to spread to the thymus and mediastinal lymph nodes. Cavitary lesions found in non-*M. bovis* bacilli showed colony counts that ranged from 10^4 to 10^6 CFU. The highest CFU counts were found in the cavity centers within the softened "solid" caseum. Difficulty in dissecting the cavity wall from the inner cavity contents was also evident in these rabbits, and therefore the cavity CFU counts may have been partially reflective of both anatomical sites. Granulomas were also visualized inside a uniquely thick cavity wall (intramural cavity lesion) in two *M. tuberculosis* CDC1551-infected rabbits and three of four *M. tuberculosis* CDC1551 ΔsigC-infected rabbits. Despite the suspected decreased virulence, ΔsigC mutants were the only non-*M. bovis* bacilli to yield a majority of infections with this thick-walled pattern of gross pathology.

Gene expression of bacilli in rabbit cavitary lesions. To define the microenvironment in the cavitary lesions, the transcriptional profiles of the Rv0467 (icl) and Rv3133c (dosR) genes were evaluated in RNA samples isolated from log-phase and early stationary-phase cultures of in vitro-grown *M. tuberculosis* CDC1551. The icl gene, which encodes isocitrate lyase, is involved in utilizing lipids as a carbon source and, along with its isoform icl2, is required for in vivo growth and virulence (19, 20). The two-component response regulator DosR is hypothesized to play a leading role in the adaptation of *M. tuberculosis* to hypoxia/anaerobic growth (22, 25). Both of these genes are involved in modulating functions believed to be closely associated with the establishment and maintenance of chronic and/or latent *M. tuberculosis* infection in the human host. Transcript levels of these genes were assessed in two rabbits infected with *M. tuberculosis* CDC1551 in both cavity luminal contents and wall samples. The in vivo expression levels were compared to those of in vitro-grown bacteria in log phase and stationary phase. As shown in Table 1, levels of both icl and dosR were significantly upregulated in the cavity and wall specimens. This observation suggests that the intragranulomatous milieu is hypoxic and also that the bacilli are expressing enzymes of the glyoxylate shunt, essential for carbon anaplerosis in the Krebs cycle during growth on substrates containing C_2 units such as fatty acids. A modest increase in the levels of these transcripts was observed in the cavity and wall specimens obtained from *M. bovis* AF2122-infected rabbits as well (data not shown).

**DISCUSSION**

This report describes a reproducible animal model of TB cavity pathogenesis and disseminated secondary lesions. Patients with cavitary TB are the key contributors to TB transmission (13). In humans, cavitary disease has also been found to be an independent risk factor for disease relapse following 6 months of directly observed therapy. The baseline risk of relapse was 2%, while those with cavitary disease and sputum smear positivity after 2 months of therapy (intensive phase) had a relapse rate of 22% (29). Moreover, elevated bacillary titers in cavities increase the probability of establishing drug-resistant bacterial populations (2, 9). Lung cavities are thus the biological foundation for multidrug-resistant and extensively drug-resistant TB. Therefore, this model may have considerable value for assessing strategies to attenuate disease transmission and also for testing the efficacy of chemotherapeutic regimens to prevent the emergence of resistance.

In our study, all rabbits underwent presensitization with five subcutaneous injections with 10^7 heat-killed *M. bovis* bacilli in incomplete Freund's adjuvant prior to infection. Through an elegant series of experiments, Yamamura et al. showed the importance of sensitization to reduce the time and improve the probability of cavity formation. A mixture that included heat-killed bovine tubercle bacilli was injected subcutaneously at various intervals over a period of 5 to 7 days. Rabbits were inoculated by infections through the thoracic wall after confirming the acquisition of a cutaneous DTH reaction with bovine- and human-type mycobacterial bacilli. Cavities were noted to be generated in less time and more reliably in animals that underwent presensitization (34, 35). Investigations into the individual components of tubercle bacilli leading to cavity formation showed that a mixture of mycobacterial lipids and proteins could generate cavities. With proteins alone, phenotypically distinct cavities were generated if rabbits were properly presensitized. Yamamura et al. later showed that mycobacterial proteins combined with synthetic adjuvants could predictably generate cavity formation (38). Interestingly, he also demonstrated that desensitization to such mycobacterial lipoproteins inhibited the creation of these cavities (37).

In our study, we infected rabbits with inocula of 5,000 to 18,000 bacilli. Such high-dose infection may raise questions regarding the specificity of the cavitation response and whether the pulmonary cavities would have formed regardless of presensitization. Three rabbits were selected to undergo high-dose infection with either *M. bovis* AF2122 or Ravenel (greater than 10^4 bacilli) without presensitization (unpublished results). These rabbits, although they did not form cavities, underwent greater intra- and extrapulmonary dissemination than their sensitized counterparts. Pulmonary involvement in all specimens yielded massive amounts of caseation throughout the region of inoculation in the right lung with sparing of solely the apical regions that contained multiple granulomas.
Our study also showed that bacterial CFU counts were most abundant in the center of the cavities. This location of increased multiplication is logical since the host immune response is limited, if not absent, in the liquefied caseum (5, 7, 17). Due to reduced macrophage function and entry in the liquefied meniscum, excessive bacillary growth can result in secondary pulmonary and extrapulmonary dissemination (6, 37). Approximately 10^4 to 10^8 bacilli can be routinely cultured from a single human pulmonary cavity (4). In our case, M. bovis infections generated cavitary CFU counts of 10^6 to 10^9 bacilli, while M. bovis BCG and non-M. bovis species showed CFU counts that ranged from 10^4 to 10^6 bacilli. The fewest cavity CFU were observed in rabbits infected with the attenuated ΔsigC mutant. The diminished CFU counts obtained with the ΔsigC mutant suggest that transcriptional adaptation by the bacterium is required for full survival in the cavity environment.

It is well known that the virulence of M. bovis is far greater than that of M. tuberculosis in rabbits challenged with low-dose infection (8). Rabbits are relatively resistant to M. tuberculosis and are better able to contain the infection via host immune response (12, 18, 39). Such distinctions in the pathogenesis of mycobacterial species was confirmed in our study in which rabbits were infected with high doses of both M. bovis and M. tuberculosis bacilli. Extrapulmonary dissemination was almost exclusively noted among rabbits infected with M. bovis Ravanel and AF2122. Indeed, with limited exceptions, non-M. bovis bacilli generally did not show extrapulmonary dissemination. Nonetheless, intrapulmonary secondary lesions could be generated with all of the mycobacteria used in this model. On gross pathology, it was the ipsilateral and contralateral lungs’ secondary lesions due to the more virulent M. bovis species that were most apparent. All M. bovis species uniformly led to greater than 10^4 bacilli in the lungs of every infected rabbit.

To determine the nature of the intragranulomatous environment in cavitary lesions, we carried out a transcriptional analysis of two bacterial genes likely to be central to the regulation of M. tuberculosis physiology in human TB. Both the icl and dosR transcripts were found to be substantially induced, consistent with the above hypothesis. Lipids have long been thought to play key roles in the pathogenesis of M. tuberculosis. There is growing evidence that host-derived lipids provide nutrition whereas pathogen-derived lipids mediate immune suppression (10). It has been suggested that fatty acids are likely to be a major carbon source for energy metabolism in M. tuberculosis in chronically infected lung tissues (3). The more recent observation of the impaired persistence of an M. tuberculosis Δicl mutant highlighted the role of the glyoxylate shunt, an anaplerotic pathway that bypasses the CO2-generating steps of the Krebs cycle and enables bacteria to synthesize carbohydrates and replenish Krebs cycle intermediates from fatty acid-derived acetyl coenzyme A (19). Hypoxic conditions within the human host have been closely linked to the phenomenon of TB latency (31). Ry3133c/DosR, a transcription factor of the two-component response regulator class, has been shown to be the primary mediator of a hypoxic signal within M. tuberculosis, and its role in dormancy has been extensively studied (15, 22, 24, 25, 26, 31). We therefore conclude from the transcription analyses that bacilli in the cavitary environment experience low oxygen tension and are probably utilizing fatty acids as an energy source (30).

One limitation of our work was that we did not investigate the necessity of infection with live bacilli for the formation of lung cavities. Future experiments that measure cavity formation with either heat-killed mycobacteria or antigen-coated polystyrene beads may be insightful for our rabbit model. Such experiments would allow us to determine the contribution of the host immune response to lesion development. The host’s involvement in cavitary pathogenesis is well known from studies showing attenuation of cavity formation with the use of immunosuppressive agents (36). Another limitation was the sole focus on cavitary lesions for gene expression data, as opposed to such sites as solid granulomas. Future studies may include comparing the molecular environment of granulomas and cavitary lesions to determine the factors that contribute to cavity formation.

In summary, this is the first study to use a bronchoscopic form of infection to produce cavities in the rabbit model. Through the use of presensitization and direct bronchoscopic instillation of bacilli, we observed cavities with M. tuberculosis, M. bovis, and mutants of these parental strains. Attenuated mutant strains (M. tuberculosis CDC1551 ΔsigC and M. bovis BCG) even displayed classic cavity formation, with notably fewer culturable bacilli, which resembled the gross pathology and histopathology of more classically virulent species like M. bovis. Moreover, M. bovis species could be phenotypically differentiated from non-M. bovis species by their unique capacity for extrapulmonary dissemination.

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REFERENCES


