Mycobacterium tuberculosis Invasion and Traversal across an In Vitro Human Blood-Brain Barrier as a Pathogenic Mechanism for Central Nervous System Tuberculosis

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Background. Central nervous system (CNS) tuberculosis is a serious, often fatal disease that disproportionately affects young children. It is thought to develop when Mycobacterium tuberculosis breaches the blood-brain barrier (BBB), which is composed of tightly apposed brain microvascular endothelial cells. However, the mechanism(s) involved in this process are poorly understood.

Methods To better understand these processes, we developed an in vitro model of M. tuberculosis BBB infection using primary human brain microvascular endothelial cells.

Results. M. tuberculosis was found to both invade and traverse the model BBB significantly more than did M. smegmatis (a nonpathogenic mycobacterium). Invasion by M. tuberculosis across the BBB required host-cell actin cytoskeletal rearrangements. By microarray expression profiling, we found 33 M. tuberculosis genes to be highly up-regulated during the early stages of invasion of the BBB by M. tuberculosis; 18 of them belong to a previously described in vivo–expressed genomic island (Rv0960–Rv1001). Defined M. tuberculosis isogenic transposon mutants for the up-regulated genes Rv0980c, Rv0987, Rv0989c, and Rv1801 were found to be deficient in their ability to invade the BBB model.

Conclusions. We developed an in vitro model of M. tuberculosis BBB infection and identified M. tuberculosis genes that may be involved in CNS invasion.

Tuberculosis infects 1.3 million new patients and causes 450,000 deaths among children annually [1]. Tuberculosis of the central nervous system (CNS) is a serious, often fatal disease that disproportionately affects young children [2–5]. CNS tuberculosis is difficult to diagnose and treat; treatment includes 4 drugs that prevent death or disability in fewer than one-half of patients [4–6].

The CNS is protected by the physiological blood-brain barrier (BBB), which is composed of tightly apposed brain microvascular endothelial cells held together by tight junctional complexes [7]. CNS tuberculosis is believed to develop when Mycobacterium tuberculosis breaches this barrier, which leads to the development of parenchymal (cortical) and meningeal tuberculomas [8–13]. Tuberculous meningitis may further develop when mycobacteria are released into the subarachnoid space because of caseation of these tuberculomas [9, 14]. It is clear that M. tuberculosis invades the CNS, but the host and microbial mechanism(s) involved in this process are poorly understood. Theoretically, M. tuberculosis may cross the BBB as free (extracellular) organisms or via infected monocytes/neutrophils. Although the latter hypothesis seems attractive, such cellular traffic is severely restricted into.
the CNS before invasion by an offending pathogen [15, 16]. Intravenous inoculation of free \textit{M. tuberculosis} in guinea pigs and \textit{M. bovis} in rabbits has been shown to produce CNS invasion, as evidenced by the formation of tuberculosis in their brain parenchyma [8–10]. Experiments in CD18 \textsuperscript{−/−} knockout mice have shown that free mycobacteria enter the CNS hemato- genously and that monocytes/neutrophils may not be required for this transport [17]. In vitro studies have demonstrated that free \textit{M. tuberculosis} invades endothelial cells [18, 19], and evidence from human autopsy samples supports this concept [20].

Few in vitro or in vivo models are available to study CNS invasion by \textit{M. tuberculosis}. An in vitro model using human brain microvascular endothelial cells (HBMECs) has been used to determine the mechanisms of CNS invasion by other meningitis-causing organisms [21]. We adapted this model for the study of host and microbial factors associated with CNS tuberculosis.

**MATERIALS AND METHODS**

**Cell-culture media.** HBMECs were grown in RPMI 1640 medium (Cambrex BioScience) supplemented with 10% heat inactivated fetal bovine serum (FBS; Omega Scientific), 10% Nu Serum (BD Biosciences), \(\text{l}\)-glutamine (Irvine Scientific), sodium pyruvate, MEM nonessential amino acids, and MEM vitamins (both Cellgro Mediatech). HBMEC experiments were performed using experimental medium that contained 1:1 Ham’s F12: medium 199 (Invitrogen) supplemented with 5% heat-inactivated FBS and \(\text{l}\)-glutamine. HBMEC monolayers were washed using 1:1 Ham’s F12: medium 199 with \(\text{l}\)-glutamine.

**\textit{M. tuberculosis} strains and media.** \textit{M. tuberculosis} H37Rv, \textit{M. tuberculosis} CDC 1551, \textit{M. bovis} bacille Calmette-Guérin (BCG; Pasteur strain), and \textit{M. smegmatis} were grown to the log phase in plastic roller bottles or as shaken cultures in plastic tubes at 37°C in Middlebrook 7H9 liquid broth (Difco Laboratories) supplemented with oleic acid albumin dextrose catalase (Becton Dickinson), 0.5% glycerol, and 0.05% Tween 80. Colony-forming unit counts were determined by plating mycobacteria onto Middlebrook 7H10 medium (Difco Laboratories). Before the inoculation of HBMECs, all mycobacteria were washed and resuspended in experimental medium at 37°C, and their optical densities at 600 nm were adjusted to achieve the required MOI. In addition, 100 \(\mu\)L from each inoculum was plated to determine the colony-forming unit counts. For the traversal assays, colony-forming unit counts for the infected medium from the upper compartment was also determined at 48 h, to adjust for any mycobacterial replication.

**In vitro model of the human BBB with HBMECs.** Primary HBMECs were isolated, characterized, and purified from the cerebral cortex of a 9-month-old infant, as described elsewhere [22–24]. This process was exempt from institutional review board approval. The in vitro monolayer was created by cultivating HBMECs on collagen-coated wells of a 24-well plate or in semipermeable (5 \(\mu\)m pore size) transwell polycarbonate tissue culture inserts (Costar) (figure 1). The HBMEC mono-

![Figure 1](image-url)
Table 1. Genes up-regulated by at least 8-fold in human brain microvascular endothelial-cell–associated vs. –unassociated \textit{Mycobacterium tuberculosis} H37Rv.

<table>
<thead>
<tr>
<th>H37Rv gene no.</th>
<th>Gene name</th>
<th>Fold change</th>
<th>P</th>
<th>Function/probable function</th>
</tr>
</thead>
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<tr>
<td>Rv0368c</td>
<td></td>
<td>10.42</td>
<td>.0002</td>
<td>Conserved hypothetical protein</td>
</tr>
<tr>
<td>Rv0619</td>
<td>galTb</td>
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<td>.0002</td>
<td>Galactose-1-phosphate uridylyl transferase</td>
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<tr>
<td>Rv0661c</td>
<td></td>
<td>9.45</td>
<td>.0002</td>
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</tr>
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<td></td>
<td>8.24</td>
<td>.0006</td>
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</tr>
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<td>Rv0966c</td>
<td></td>
<td>18.40</td>
<td>.0001</td>
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<td>Rv0967</td>
<td></td>
<td>8.16</td>
<td>.0003</td>
<td>Conserved hypothetical protein</td>
</tr>
<tr>
<td>Rv0968</td>
<td></td>
<td>17.02</td>
<td>.0001</td>
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</tr>
<tr>
<td>Rv0970</td>
<td></td>
<td>9.59</td>
<td>.0002</td>
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</tr>
<tr>
<td>Rv0971c</td>
<td>echA7</td>
<td>8.09</td>
<td>.0002</td>
<td>Enoyl-CoA hydratase</td>
</tr>
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<td>Rv0974c</td>
<td>accD2</td>
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<tr>
<td>Rv0977</td>
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<td>.0002</td>
<td>PE-PGRS family protein</td>
</tr>
<tr>
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<td>.0002</td>
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<td>Rv0982</td>
<td>mprB</td>
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<td>.0002</td>
<td>Two-component sensor kinase</td>
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<td>Rv0983</td>
<td>pepD</td>
<td>18.28</td>
<td>.0001</td>
<td>Serine protease</td>
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<tr>
<td>Rv0984</td>
<td>moaB2</td>
<td>12.01</td>
<td>.0002</td>
<td>Molybdopterin biosynthesis</td>
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<tr>
<td>Rv0986</td>
<td></td>
<td>23.04</td>
<td>.0001</td>
<td>Active transport of adhesion component across membrane, energy coupling to the transport system</td>
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<tr>
<td>Rv0987</td>
<td></td>
<td>10.79</td>
<td>.0002</td>
<td>Active transport of adhesion component across membrane, translocation of the substrate across the membrane</td>
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<td>Rv0989c</td>
<td>grcC2</td>
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<td>.0002</td>
<td>Supplier of polypropenyl diphosphate</td>
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<td>8.54</td>
<td>.0002</td>
<td>Oxireductase</td>
</tr>
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<td>PPE29</td>
<td>9.16</td>
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<tr>
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<td>mce3A</td>
<td>8.56</td>
<td>.0003</td>
<td>Involved in host-cell invasion</td>
</tr>
<tr>
<td>Rv1968</td>
<td>mce3C</td>
<td>19.95</td>
<td>.0001</td>
<td>Involved in host-cell invasion</td>
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<tr>
<td>Rv2318</td>
<td>uspC</td>
<td>8.14</td>
<td>.0003</td>
<td>Involved with active transport of sugars across the cell membrane</td>
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<td>PPE47</td>
<td>8.74</td>
<td>.0002</td>
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<td>.0002</td>
<td>Transposase</td>
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<td>.0002</td>
<td>Conserved hypothetical protein</td>
</tr>
<tr>
<td>Rv3639c</td>
<td></td>
<td>8.22</td>
<td>.0003</td>
<td>Conserved hypothetical protein</td>
</tr>
<tr>
<td>Rv3833</td>
<td></td>
<td>9.18</td>
<td>.0002</td>
<td>Transcription regulatory protein</td>
</tr>
</tbody>
</table>

\textbf{NOTE.} Shaded genes belong to a recently described in vivo–expressed genomic island; genes in bold type are predicted to be nonessential for in vitro growth. CoA, coenzyme A; PE, amino acids Pro-Glu; PGRS, polymorphic GC-rich repetitive sequences; PPE, amino acids Pro-Pro-Glu.

layer on collagen-coated transwell inserts exhibits tight junction formation and polarization and develops high transendothelial electrical resistance (TEER; 300–600 $\Omega/$cm$^2$) [22–24]. Culture medium was changed every 2 days, and confluence was determined by light microscopy or by measuring TEER using a Millicell-ERS apparatus (World Precision Instruments).

\textbf{Invasion assay.} A total of 100,000 HBMECs/well were seeded and were confluent on days 3–4 (500,000 cells/well). This monolayer was infected with the mycobacterial suspension (MOI, 10–50), centrifuged (at 890 $g$ for 10 min), and incubated for 90 min at 37°C in 5% CO$_2$. The monolayer was washed, to remove the extracellular mycobacteria, and incubated with 200 $\mu$g/mL amikacin for 2 h at 37°C in 5% CO$_2$, to kill any remaining extracellular mycobacteria [18]. Our preliminary experiments showed that, under the conditions used in our assay, incubation of mycobacteria with 200 $\mu$g/mL amikacin for 2 h achieves 99% killing. The monolayer was washed again and lysed using sterile water, and colony-forming unit counts were determined for the quantification of intracellular mycobacteria. Invasion was expressed as a percentage of the inoculum.

\textbf{Traversal assay.} A total of 20,000 HBMECs/transwell were seeded and were confluent by day 8 (200,000 HBMECs/transwell). The monolayer was infected by inoculating the mycobacterial suspension (MOI, 10–50) in the upper compartment. The transwells were then incubated for 48 h at 37°C in 5% CO$_2$. Thereafter, all contents of the lower compartment (1 mL) were plated for colony-forming unit counts for quantification of the mycobacteria that traversed the monolayer. Traversal was expressed as a percentage of the inoculum.

\textbf{Visualization by transmission electron microscopy of }\textit{M. tuberculosis} \textit{interaction with HBMECs.} At the end of 48 h, infected transwells from the traversal assay were washed several times and fixed overnight with ice-cold 100 mmol/L cacodylate buffer that contained 3% formaldehyde, 1.5% glutaraldehyde, and 5 mmol/L CaCl$_2$ (pH, 7.4). The monolayers were postfixed in Palade’s OsO$_4$, stained en bloc in Kellenberger’s uranyl ac-
M. tuberculosis bacille Calmette-Guérin (BCG) was not significantly different from that for
4 h at 37°C. The wells were then washed several times, fixed with fresh 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and stained with 1:100 Alexa Fluor 568 phalloidin (Molecular Probes). The coverslips were mounted onto slides, and several fields were visualized using the Zeiss 410 confocal microscope. Image J (version 1.32i; National Institutes of Health) with the Volume J plug-in was used for image reconstruction.

Inhibition assay with cytochalasin D. Cytochalasin D (Calbiochem) was dissolved in dimethyl sulfoxide to make a 10-mmol/L stock solution that was diluted in experimental medium to 0.05 and 0.25 μg/mL. HBMECs were grown to confluence and treated with experimental medium with and without cytochalasin D 1 h before the start of the experiment. The monolayer was infected with M. tuberculosis H37Rv (MOI, ∼40), and the invasion assay was performed as described above. The integrity of the HBMEC monolayer was inspected several times during the experiment and before lysis of the monolayer.

M. tuberculosis genes involved in invasion and intracellular survival in HBMECs. To identify potential M. tuberculosis genes involved in invasion and intracellular survival in HBMECs, we studied the M. tuberculosis gene-expression profile after interaction with these specialized endothelial cells. mRNA was extracted from M. tuberculosis-associated (either internalized or tightly adherent) and HBMEC-unassociated (nonadherent) during the early stage of invasion. HBMECs were grown to confluence in 2 collagen-coated 150-mm tissue-culture plates for each experiment. The M. tuberculosis H37Rv suspension was inoculated on the HBMECs (MOI, ∼40) and incubated for 2 h at 37°C in 5% CO₂. Thereafter, the supernatant associated with unassociated mycobacteria was removed and saved. The HBMEC monolayer was washed several times, to remove nonadherent extracellular mycobacteria, and then lysed with 5 mol/L guanidine isothiocyanate lysis buffer [26]. M. tuberculosis H37Rv was recovered by centrifugation at 1335 g, and RNA was extracted from both HBMEC-associated and -unassociated M. tuberculosis H37Rv using standard methods [27]. Total bacterial RNA extracted from both HBMEC-associated and -unassociated M. tuberculosis H37Rv was treated with DNase, to remove contaminating DNA. These were used to prepare cDNA using the Atlas Fluorescent Labeling Kit (BD Biosciences) and were labeled with Cy3 and Cy5 monofluorescent dyes (Amerham Pharmacia). Equal amounts of Cy5-labeled cDNA, prepared using RNA from HBMEC-unassociated M. tuberculosis H37Rv, and Cy3-labeled cDNA, prepared using RNA from HBMEC-associated M. tuberculosis H37Rv, were cohybridized onto a printed oligonucleotide microarray representing all open reading frames (ORFs) of M. tuberculosis (Operon Biotechnologies). The arrays were scanned using the GenePix Scanner 4000B (Axon Instruments). Five different sets of mRNAs prepared from 5 independent experiments were analyzed, to minimize experimental artifacts and microarray-associated systematic errors. Fluorescence intensities obtained from microarrays were normalized. Significance Analysis of Microarrays software (version 1.21; Stanford University) was used to identify genes that were significantly differentially regulated by at least 8-fold in HBMEC-associated, compared with HBMEC-unassociated, M. tuberculosis across the 5 data sets, using a δ-value of 1.33043 (median false discovery rate, 0).

Mutation of HBMEC-induced M. tuberculosis genes. Random insertion mutagenesis of M. tuberculosis CDC 1551 was performed in our laboratory using the Himar1 transposon (Tn).
as part of a comprehensive insertional mutagenesis [28]. Tn insertion sites were identified by sequencing the insertion junction, as described elsewhere [29–31]. Relevant mutants available to us from our collection of Himar1 Tn insertion mutants were used in the study. HBMEC invasion by mutants for 5 genes (Rv0980c, Rv0987, Rv0989c, Rv0990c, and Rv1801), which were up-regulated at least 8-fold in HBMEC-associated M. tuberculosis H37Rv (table 1), and for Rv0981 were compared with that of an intergenic negative-control Tn mutant with Tn insertion at bp 921350, which falls between annotated genes MT0849 (NT02MT0849) and MT0850 (Rv0829). An invasion assay was performed as described above, except that streptomycin (200 μg/mL) was used instead of amikacin. This is because all the Tn mutants possess kanamycin cassettes that may interfere with amikacin killing [32].

Statistical analysis. All invasion, traversal, cytochalasin D inhibition, and mutant studies were performed at least in triplicate, and groups were compared statistically using the Mann-Whitney U test.

RESULTS

Invasion and traversal of the BBB model by mycobacteria.
We tested whether M. tuberculosis was capable of invasion and traversal of our in vitro BBB model. We used M. smegmatis, a nonpathogenic mycobacterial species, as a negative control in our experiments. In addition, we tested M. bovis BCG and compared its invasion and traversal with those of M. tuberculosis laboratory strains H37Rv and CDC 1551. M. tuberculosis did not cause any noticeable lysis or detachment of the monolayer at the end of the invasion and traversal assays.

Invasion. M. tuberculosis H37Rv and CDC 1551 were found to invade HBMECs at a median invasion fraction of 2.6% (range, 1.0%–4.2%) and 2.7% (range, 0.8%–4.5%), respectively. The median invasion fraction for M. smegmatis was 0.18% (range, 0.0%–0.9%), which was significantly lower than that of M. tuberculosis CDC 1551 or H37Rv (P <.0008) (figure 2). The median invasion fraction for M. bovis BCG was 0.9% (range, 0.1%–2.8%), which was not significantly different from that for M. tuberculosis (P = .07).

Traversal. M. tuberculosis H37Rv and CDC 1551 were found to traverse HBMECs at a median traversal fraction of 3.4 × 10⁻⁴% (range, 0.0–0.1 × 10⁻⁴%) and 0.6 × 10⁻⁴% (range, 0.2–1.1 × 10⁻⁴%), respectively (figure 2). M. smegmatis was unable to traverse the HBMEC monolayer (lower limit of detection, 5.1 × 10⁻⁴%; P < .01). The median traversal fraction for M. bovis BCG was 14.2 × 10⁻⁴% (range, 10.0–18.3 × 10⁻⁴%) and was not significantly different from that for M. tuberculosis (P = .06).

Transmission electron micrographs. On interaction with M. tuberculosis, HBMECs formed microvilli-like protrusions that surrounded and endocytosed mycobacteria (figure 3). This finding suggested that M. tuberculosis invasion and traversal requires actin cytoskeleton rearrangements in HBMECs. We therefore tested this hypothesis by performing colocalization and inhibition studies.

M. tuberculosis invasion and HBMEC actin cytoskeleton rearrangements. GFP-expressing M. tuberculosis (green) were found to colocalize with fluorescent phalloidin-stained actin at the surface of HBMECs (figure 4A–4F) consistently across several fields visualized by confocal microscopy. Treatment of HBMECs with cytochalasin D (an actin polymerization inhibitor) at 0.05 and 0.25 μg/mL did not alter the monolayer integrity; however, it significantly decreased the invasion by M. tuberculosis, compared with untreated HBMEC, by 61.0% and 76.7%, respectively (P = .048) (figure 4G).

M. tuberculosis genes involved with invasion and intracellular survival in HBMECs. We identified 180 M. tuber-
crosis genes that were differentially regulated by at least 8-fold in HBMEC-associated, compared with HBMEC-unassociated, *M. tuberculosis*. The 33 up-regulated genes, with their annotated functions [33], are listed in table 1. The 147 down-regulated genes, with their annotated functions, are listed in table 2.

**Mutation of HBMEC-induced *M. tuberculosis* genes and reduced HBMEC invasion.** To determine whether genes up-regulated in response to contact with HBMECs also play a role in endothelial-cell invasion, we obtained *M. tuberculosis* CDC 1551 mutants in which genes were interrupted by Tn insertions. Invasion by mutants Rv0980c, Rv0987, Rv0989c, and Rv1801 was significantly decreased, by 50.0% (P = .0022), 44.2% (P = .0022), 27.4% (P = .0043), and 66.1% (P = .0022), respectively, compared with the negative-control Tn mutant (figure 5). It should be noted that the Rv0987 mutant used in our experiments has a ΔsigF background that could potentially have confounded our results [34]. We therefore compared HBMEC invasion by the Rv0987 mutant with that of *M. tuberculosis* CDC 1551 ΔsigF and found that invasion by the Rv0987 mutant was significantly decreased, by 34.3% (P = .0022), compared with *M. tuberculosis* CDC 1551 ΔsigF. These results indicate that genes Rv0980c, Rv0987, Rv0989c, and Rv1801 are potentially required for endothelial-cell invasion and/or intracellular survival.

**DISCUSSION**

To produce CNS tuberculosis, *M. tuberculosis* needs to traverse the BBB formed by the specialized endothelial cells lining the brain microvasculature. Using HBMECs, we have been able to detect endothelial invasion and traversal by pathogenic mycobacteria. Unlike macrophages, endothelial cells are nonprofessional phagocytic cells, and they do not engulf extracellular material nonspecifically [35]. This is highlighted by the inability of HBMECs to take up naive carboxylated fluorescent 1-μm beads [36]. Nevertheless, *M. tuberculosis* was found to invade and traverse the HBMEC monolayers, which suggests that *M. tuberculosis* triggers its own uptake by HBMECs. In addition, we found that pathogenic *M. tuberculosis* strains H37Rv and CDC 1551 invade and traverse HBMEC monolayers more than the nonpathogenic species (*M. smegmatis*), which suggests that traversal by *M. tuberculosis* across the BBB (and, possibly, other cellular barriers) may be a specific process that requires particular virulence factors. At a cellular level, differences in invasion and traversal by *M. tuberculosis* and *M. smegmatis* may be explained either at the level of uptake or by initial intracellular survival in HBMECs [37, 38]. Further studies are under way to characterize this process. We observed that invasion and traversal by *M. bovis* BCG was similar to that of *M. tuberculosis*.
This is consistent with the observation that the relative avirulence of BCG is not due to its inability to invade but, rather, to an inability to survive host immune-control mechanisms [39]. Traversal across the BBB requires the additional steps of prolonged intracellular survival and exocytosis from the other (brain) side of the cellular barrier. This is consistent with the much lower traversal rates (vs. invasion) observed by us for all mycobacteria tested—an observation that other researchers have seen in related model studies [18].

*M. tuberculosis* did not cause any noticeable lysis or detachment of the monolayer at the end of the invasion and traversal assays. Furthermore, electron microscopy of infected HBMECs suggested that *M. tuberculosis* invasion and traversal required actin cytoskeleton rearrangements by HBMECs. We tested this hypothesis and found that *M. tuberculosis* colocalized with surface actin of HBMECs and that treatment of HBMECs by cytochalasin D significantly decreased invasion by *M. tuberculosis*. These data suggest that traversal across the BBB might possibly be transcellular. However, concurrent pathogen-triggered paracellular traversal cannot be ruled out. Actin cytoskeleton rearrangements by HBMECs have been shown to be a prerequisite for invasion by several other meningitis-causing organisms via different signaling mechanisms [21]. Furthermore, Menozzi et al. [40] have shown that a 28-kDa *M. tuberculosis* adhesin protein induces actin cytoskeleton rearrangements in confluent bovine brain capillary endothelial cells and that it does not open the tight junctions of the endothelial monolayer. Our study, however, did not identify this gene to be differentially regulated in HBMEC-associated *M. tuberculosis* infection.

We hypothesized that genes significantly up-regulated in HBMEC-associated, compared with HBMEC-unassociated, *M. tuberculosis* are likely to be involved in HBMEC invasion and intracellular survival. Indeed, by *M. tuberculosis* whole-genome microarrays, we identified 33 genes that were up-regulated by at least 8-fold in HBMEC-associated *M. tuberculosis*. Talaat et al. [41] described an in vivo–expressed genomic island (iVEGI), *Rv0960–Rv1001*, that contains 20 *M. tuberculosis* genes that are up-regulated in mouse lungs, compared with in vitro growth. The majority of iVEGI genes are involved in cell-wall biosynthesis and lipid metabolism, and iVEGI genes are present in pathogenic mycobacteria (*M. tuberculosis* H37Rv and CDC 1551 and *M. bovis*) but not in *M. smegmatis*. Of the 33 genes identified by us, 18 belong to the same iVEGI, which suggests their potential role in virulence. Furthermore, 25 of 33 genes identified by us are predicted to be nonessential for in vitro growth, which suggests a potential role in vivo survival and/or virulence.

To determine whether these 33 up-regulated genes also play a role in endothelial-cell invasion, we compared the invasion of their Tn mutants with that of a negative control mutant and found that the invasion by Tn mutants for *Rv0980c, Rv0987, Rv0989c*, and *Rv1801* was significantly decreased. However, the phenotypes displayed by these Tn mutants are relatively modest. This could be explained by partial inactivation of the gene

![Figure 5](image_url)

**Figure 5.** Invasion of the infant human brain microvascular endothelial-cell (HBMEC) monolayer by isogenic *Mycobacterium tuberculosis* mutants (normalized to control). Invasion of HBMECs by isogenic *M. tuberculosis* CDC 1551 Tn mutants *MT1008 (Rv0980c), MT1009 (Rv0981), MT1015 (Rv0987), MT1018 (Rv0989c)*, and *MT1850 (Rv1801)* was significantly decreased, compared with the control mutant (*P* < .0043). The control mutant has an intergenic Tn insertion that falls between annotated genes *MT0849 (MT02MT0849)* and *MT0850 (Rv0823)*. Medians are shown; the error bar represents the third quartile. Groups were compared statistically using the Mann-Whitney *U* test.
by the Tn insertion or by compensation through complementary pathways. In addition, although we believe that these genes are potentially required for endothelial-cell invasion and/or intracellular survival, a polar effect of the Tn insertion on downstream genes may be a possible confounding source. In vitro growth differences among the Tn mutants could also potentially confound our results; however, there were no significant differences in the in vitro growth or colony morphology for the mutants tested in the study. Rv0986c, Rv0987, and Rv0989c belong to the iVEGI described by Talaat et al. Rv0987 belongs to an unclassified ATP binding cassette (ABC) transporter system and is postulated to function along with Rv0986 (Rv0986 and Rv0987 were both highly up-regulated in HBMEC-associated M. tuberculosis). The proteins encoded by Rv0986 and Rv0987 are homologous to the AteE-G proteins of Agrobacterium tumefaciens, which form an ABC transporter in A. tumefaciens and are involved in virulence and bacterial attachment to host cells [42, 43]. Therefore, protein products of Rv0986 and Rv0987 are thought to be involved in M. tuberculosis adhesion and virulence. An Rv0986 Tn mutant has been shown to have decreased survival in macrophages, which supports its possible role in virulence [44]. Incidentally, Rv0986–Rv0987 displays only a weak similarity to any ORF in M. smegmatis (E value, <0.05) [41]. Although HBMEC invasion by Tn mutants for Rv0981 and Rv0990c was not significantly decreased, the Tn insertions in these genes were close to their distal ends (nt 547/693 and 612/656, respectively) and possibly cause partial gene inactivation.

The 147 genes that were down-regulated by at least 8-fold in HBMEC-associated M. tuberculosis H37Rv cluster in functional groups of transcription, protein synthesis, energy metabolism, and other metabolic pathways, which suggests that M. tuberculosis decreases its overall metabolic state very early during infection and internalization by endothelial cells. This relative shutdown of the active metabolism may be a precursor of latency, as has been postulated to occur in endothelial cells in human autopsy samples [20].

Schnappinger et al. [45] identified 601 M. tuberculosis genes that are significantly differentially regulated at 24 or 48 h after infection in either naive or interferon-γ-stimulated wild-type macrophages, using a false-discovery rate of <1%. Four of 454 induced genes (galTb, PPE29, mce3C, and Rv3833) and 37 of 147 repressed genes described by Schnappinger et al. are common to our 33 up-regulated and 147 down-regulated genes, respectively. Although different host cells were studied, it is also likely that experimental differences account for the relative lack of overlap in the patterns of bacterial gene expression between the present study and that of Schnappinger et al. For example, we studied gene expression at 2 h, compared with the 24 or 48 h in the Schnappinger et al. model. In addition, we identified genes that were at least 8-fold up-regulated (with a false discovery rate of 0%), compared with no fold cutoff in the Schnappinger et al. model. Some differences in gene-expression patterns may be related to an intrinsically different microenvironment in primary brain microvascular endothelial cells, which are nonprofessional phagocytic cells and, therefore, are unlike macrophages.

In summary, we have studied the interaction between M. tuberculosis and HBMECs and demonstrated both invasion and traversal of HBMEC monolayers by virulent mycobacteria. This in vitro system was based on the well-established role of HBMECs in the physiologic BBB protecting the CNS. Therefore, the model system may prove to be valuable in identifying mechanisms of M. tuberculosis entry into the CNS and the pathogenesis of tuberculous meningitis. Indeed, we identified 33 M. tuberculosis genes that are up-regulated during bacterial association with HBMECs, and mutants in 4 of these genes showed reduced HBMEC invasion. Further studies are under way to characterize the host-cell molecular pathways that are involved in HBMEC invasion by M. tuberculosis and the specific mechanisms by which the mutants identified in the study lack invasion and/or intracellular survival.

Acknowledgments

We thank Moises Hernandez and Qi-Jian Cheng (Johns Hopkins University, Baltimore, MD), for providing some of the transposon mutants used in the study; and Michael Zilliox (Johns Hopkins University, Baltimore, MD), for help with data analysis.

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