Leucine Auxotrophy Restricts Growth of *Mycobacterium bovis* BCG in Macrophages

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The ability of slow-growing mycobacteria to replicate within host mononuclear phagocytes is thought to be central to the pathogenesis of mycobacterial infection. However, because of the lack of a mycobacterial mutant defective for intracellular replication, it has not been possible to test this hypothesis directly. Previously, we showed that a BCG leucine auxotroph with a transposon disruption of the *leuD* gene is unable to grow in mice. Here we demonstrate that this mutant is also incapable of replicating within cultured macrophages in vitro. Complementation of the *leuD* mutation with the *leuCD* genes of *Escherichia coli* restored wild-type levels of growth in macrophages, establishing that the defect for intracellular replication was due to leucine auxotrophy per se and not to a polar effect of the transposon insertion on an adjacent gene. These results suggest that the inability of the leucine auxotroph to grow in mice was due to its sequestration, after phagocytosis, in an intracellular compartment from which it could not obtain leucine.

Virulence and pathogenesis of *Mycobacterium tuberculosis* are clearly related to the ability to survive and grow inside host cells (9). For intracellular pathogens other than mycobacteria this important parasite-host relationship has been investigated by generating mutants that are auxotrophic for certain nutrients such as amino acids, purines, or pyrimidines. In vivo and in vitro experiments have shown that the inability of auxotrophic strains to replicate inside cells could be correlated with avirulence in animal models. Thymine auxotrophs of *Shigella flexneri* were attenuated in monkeys (1, 30). Fields and coworkers and Leung and Finlay identified auxotrophs of *Salmonella typhimurium* requiring purines, pyrimidines, aromatic amino acids, or histidine; all of these auxotrophs were attenuated in mice (14, 22). Bacon and colleagues demonstrated that purine and *p*-aminobenzoic acid auxotrophs and a leucine auxotroph of salmonellae were avirulent in mice (5, 6). Infection in cell culture using auxotrophic strains helped to elucidate the conditions for and mechanisms involved in intracellular growth (24, 27). *Listeria monocytogenes* is naturally auxotrophic for certain amino acids (31). Wild-type *L. monocytogenes* escapes from the phagosome and replicates within the cytoplasm. Hemolysin-minus mutants of *L. monocytogenes*, which are unable to escape from the phagosome, do not grow inside cells (16, 32). These in vitro experiments suggested that the cytoplasm behaves like rich media, as it provides amino acids and other essential nutrients. There are other mechanisms for intracellular pathogens to access intracellular nutrients. For example, the parasitophorous vacuolar membranes of both *Toxoplasma gondii* and *Plasmodium falciparum* contain a pore which allows access to small cytosolic constituents (13, 20).

The intracellular mode of survival of *M. tuberculosis* in mononuclear phagocytes remains a controversial issue. Studies by D'Arcy Hart and colleagues with *Mycobacterium microti* and *M. tuberculosis*-infected macrophages initially suggested

that there is inhibition of lysosome-phagosome fusion (2, 3, 12, 17). In contrast, recent studies with *M. tuberculosis* have found fusion of thoria-containing lysosomes with *M. tuberculosis*-containing phagosomes (26). In studies of *Mycobacterium avium* infection, fusion was observed but was selective in that the proton pump required for endosomal acidification was not found in the vacuoles containing mycobacteria (35). Earlier work using transmission electron microscopy indicated that *M. tuberculosis* could escape from membrane-encoded vesicles into the cytoplasm (28). Later studies indicated that while *Mycobacterium bovis* BCG remained within vacuoles clearly bound by membranes over a period of 4 days, *M. tuberculosis* appeared to progress from fused phagolysosomes to vacuoles with tightly apposed membranes, and finally bacilli were found with no discernible membrane (26). In contrast, studies using freeze electron microscopy found that all *M. tuberculosis* bacilli were surrounded by an endosomal membrane (10, 36).

Previous studies of the pathogenesis of mycobacteria and their interactions with macrophages were confined to comparisons of different strains of *M. tuberculosis* such as H37Rv and H37Ra or different species such as *M. tuberculosis* and *M. bovis* BCG (29), which upon culturing for many years have been attenuated by undefined numbers of mutations. Only recently the generation of auxotrophs of mycobacteria has been reported (18, 19, 25). Our laboratory used transposon mutagenesis to engineer a BCG leucine auxotroph and compared growth of the auxotrophic strain with that of the parental strain in mice (25). As opposed to wild-type BCG, the BCG leucine auxotroph, which carried a disruption of the *leuD* gene, did not grow and the infection was cleared within 7 weeks.

In this study we addressed the question of whether the attenuated phenotype of the leucine auxotroph in mice (in vivo) might be explained by its failure to grow inside macrophages (in vitro). We looked for growth of the BCG leucine auxotroph inside the human monocyte/macrophage cell line THP-1. We compared the intracellular growth of the BCG leucine auxotroph complemented with a *leuD* gene with that of the BCG parental strain to determine whether complementation for leucine alone could fully restore the growing phenotype inside macrophages.

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MATERIALS AND METHODS

Bacterial strains, media, and cultivation of mycobacteria. BCG (Pasteur vaccine strain; Statens Serum Institute) was grown in Middlebrook 7H9 broth (Difco BRL) with 0.2% glycerol, 0.05% Tween 80, and 10% OADC (0.5% bovine albumin fraction V, 0.2% glucose, 0.006% oleic acid, 140 mM NaCl), referred to as minimal medium. The BCG leucine auxotroph was propagated on minimal medium supplemented with 50 μ g of leucine per ml. Construction of the BCG leucine auxotroph and the complemented mutant has been described previously (25). Briefly, the BCG leucine auxotroph was engineered by insertional mutagenesis using IS1096-derived transposons containing a kanamycin resistance gene. A transposon was inserted in the *leuD* gene of BCG, and the resulting auxotrophic strain was designated mc²798. The *leuCD* genes were amplified from *Escherichia coli* by the PCR and cloned into the pMV361 expression vector. The resulting plasmid was transformed into mc²798, and strains were plated on minimal medium containing 20 μ g of kanamycin per ml. The BCG leucine auxotroph complemented with the *leuCD* genes was grown in minimal medium with 20 μ g of kanamycin per ml, whereas the BCG leucine auxotroph was grown in minimal medium supplemented with 20 μ g of kanamycin per ml and 50 μ g of leucine per ml.

Infection of the human leukemia cell line THP-1. THP-1 macrophages (ATCC TIB 202) were propagated in RPMI 1640 (Difco BRL) supplemented with 10% fetal calf serum (FCS) (Sigma). Mycobacterial strains were grown to an optical density at 600 nm (OD₆₀₀) of 0.5 to 0.7. Before adsorption, bacterial cells were sonicated (Braun Sonic U) and filtered through a Cameo 25NS syringe filter with a pore size of 5.0 μ m (Fisher Scientific) to obtain a suspension of single nonclumped cells. Before adsorption, bacteria and THP-1 cells were washed separately in RPMI 1640 with 10% FCS. Then, 10⁶ THP-1 cells were infected with a multiplicity of infection between 20 and 50 bacteria per cell and activated with 15 ng of phorbol 12-myristate 13-acetate (PMA) (Sigma). We tried to improve efficiency of phagocytosis by activation of macrophages with PMA 12 h before adsorption. Whereas this did not improve the uptake of bacteria, a higher multiplicity of infection or a longer adsorption period resulted in multiply infected macrophages. However, we aimed for singly infected macrophages to better discriminate intracellular growth from intracellular nongrowth. Once activated with PMA, cells became adherent after 12 to 18 h and stopped replicating. With respect to slow-growing mycobacteria, the latter feature was important to avoid dilution effects during the 8-day course of infection.

After adsorption in 15-ml conical tubes (Falcon) (tubes inverted every 60 min) for 6 h at 37°C and 5% CO₂, noninternalized bacteria were separated from macrophages by low-speed centrifugation at 500 $\times$ g. Cells were washed and vortexed vigorously four times with cold medium and resuspended in medium supplemented with 10% FCS and 15 ng of PMA. On glass coverslips (Erie Scientific, Portsmouth, N.H.) pretreated with acetone and fitted to a 12-well plate (Falcon) cells were allowed to adhere overnight. At different time points, cells on the coverslips were washed with phosphate-buffered saline (PBS) and fixed with 10% formalin. For staining, auramine and rhodamine were used according to standard procedures. As a counterstain we used 0.1 g of nuclear fast red (Sigma) dissolved in 5% aluminum sulfate (Sigma). Coverslips were mounted on microscope slides by using Permount mounting fluid (Fisher Scientific). The infection was evaluated by using fluorescence microscopy.

The infection of macrophages had been carried out in RPMI 1640 containing 50 μ g of leucine per ml (according to the manufacturer) supplemented with 10% FCS. The medium was changed every 48 h during the 8 days of infection to ensure that the extracellular concentration of leucine was always close to 50 μ g/ml. It is noteworthy that 50 μ g of leucine per ml was sufficient to allow growth of the auxotrophic strain on synthetic medium. For infection of macrophages in RPMI 1640 without leucine (Difco BRL) and supplemented with 10% FCS, the procedure was essentially the same as above. However, bacteria were washed and grown in leucine-free medium for 12 h prior to adsorption. Plating for CFU showed that viability of the BCG leucine auxotroph was not affected at the beginning of adsorption by growing the strain in leucine-free medium for 12 h. Adsorption, washing, and subsequent incubation were done in leucine-free medium supplemented with 10% FCS.

Evaluation of bacillary growth. (i) Inside macrophages. At indicated times, preparations were visualized under the microscope to count and calculate (i) the percentage of macrophages that were infected and (ii) the number of acid-fast bacilli per macrophage. For the former, five fields of 100 macrophages each were counted per time point and the mean percentage of macrophages infected was calculated. For the latter, three fields of 50 infected macrophages each were counted per time point. By using a counting method described earlier (11), the counts were classified in groups containing 1 (score = 1), 2 to 5 (score = 3.5), 6 to 20 (score = 13), and clumps (score = 30) of bacilli. These groups provided the data for calculating the mean number of acid-fast bacilli per infected macrophage.

(ii) After growth in RPMI 1640 only. For growth of mycobacteria in RPMI 1640 only, equal numbers of bacteria were washed once in medium, sonicated, and inoculated in 30-ml culture bottles. The bottles were incubated standing under the same conditions as those for infected macrophages (37°C, 5% CO₂). At different time points, aliquots were taken, mixed 1:1 with 7H9 medium containing 0.5% Tween 80, and sonicated 20 times for 1 s each (Braun Sonic U) and serial dilutions were plated on appropriate synthetic media.

FIG. 1. Intracellular growth of the BCG leucine auxotroph (mc²798) and the complemented mutant [mc²798(*pleuCD*)] in PMA-activated THP-1 macrophages. After adsorption for 6 h, macrophages were separated from nonphagocytized bacteria by low-speed centrifugation. Cells were allowed to adhere to glass coverslips overnight. Cells were stained for acid-fast bacilli (AFB) with rhodamine-auramine. Infected macrophages were visualized under a fluorescence microscope, and intracellular bacteria were counted and calculated as mean acid-fast bacilli per infected macrophage at 18 h and 4, 6, and 8 days (left to right).

RESULTS

In a previous study, McAdam and colleagues generated a leucine auxotroph of BCG by transposon mutagenesis and demonstrated that this mutant, which carried a transposon insertion in the *leuD* gene, failed to grow in mice (25). In order to determine whether the failure of the BCG *leuD* mutant to grow in vivo could be ascribed to a defect in intracellular growth, we asked whether the *leuD* mutant was capable of replicating in cultured human macrophages in vitro. As transposon mutagenesis can cause polar effects on downstream genes in the same operon, we compared growth of the *leuD* mutant complemented with the wild-type *leuCD* genes from *E. coli* with that of the parental BCG strain. The LeuD protein is known to interact with the LeuC protein to generate isopropyl malate isomerase activity; because it seemed possible that the *E. coli* LeuD protein might interact poorly with the LeuC protein of BCG, we provided both genes from *E. coli* to ensure full restoration of leucine prototrophy. Indeed, when expressed from the mycobacterial *hsp60* promoter, the *leuCD* genes of *E. coli* fully restored the ability of the *leuD* mutant to grow on synthetic media (25). For the intracellular replication assays, we chose the human leukemia cell line THP-1 because, compared with other human myeloid cell lines, the phenotype of differentiated THP-1 is closest to that of native monocyte-derived macrophages (reviewed in reference 4). Furthermore the cell line has been shown to be permissive for BCG and *M. tuberculosis* (15, 34).

Bacteria were adsorbed to THP-1 cells in suspension for 6 h. THP-1 cells were separated from nonphagocytized bacteria by low-speed centrifugation and extensive washing. Figure 1 shows that there was a substantial difference in growth inside macrophages between the BCG leucine auxotroph and the complemented strain. Over 8 days of infection the number of bacilli of the complemented strain per infected macrophage

FIG. 2. Replication of the BCG leucine auxotroph (mc²798), the complemented mutant [mc²798(pleuCD)], and the BCG wild type in RPMI 1640. After inoculation, aliquots were plated in serial dilutions for determination of CFU. Growth is expressed as fold increase of CFU at 18 h and 4, 6, and 8 days (left to right).

increased more than 20-fold. In contrast, there was only a slight increase in the number of BCG leucine auxotrophic bacilli per macrophage and the growth of bacteria seemed to have stopped completely by day 6 of infection. Upon extension of infection up to 12 days, we saw the same initial growth for the first 5 days and no replication at all after that (data not shown). In order to test whether the difference in growth was due to internalization of bacteria, we inoculated the auxotrophic strain, the complemented strain, and the BCG wild type directly in RPMI 1640. Aliquots were plated for CFU at 18 h and 4, 6, and 8 days after inoculation (Fig. 2). Growth is depicted as the fold increase relative to the inoculation dose. Both the BCG leucine auxotroph and the complemented strain showed an equal rate of replication, which matched that of the BCG wild type. Thus, upon culturing in cell medium directly, no differences in replication between the auxotrophic strain, the complemented strain, and the BCG wild type could be detected. These results suggested that the difference in growth between the BCG leucine auxotroph and the complemented strain when infected into THP-1 macrophages was due to internalization and subsequent sequestration in a leucine-free compartment.

The initial intracellular growth of the leucine auxotroph might have been due to cophagocytosis of leucine-containing medium during the infection. Thus, we infected THP-1 cells in RPMI 1640 without leucine and supplemented with 10% FCS. It is interesting that virulent auxotrophic *L. monocytogenes* continued to grow intracellularly when host cells were deprived of specific amino acids required by both the host cell and the intracellular pathogen (24). As *L. monocytogenes* escapes into the cytoplasm, these results suggested that even under starvation conditions the cytoplasm of eucaryotes provides enough amino acids for *Listeria* auxotrophs to grow. During the 8 days of infection the number of PMA-activated macrophages attached to glass coverslips remained unchanged. The viability of THP-1 macrophages did not seem to be affected in medium without leucine, probably because of residual leucine in the FCS and/or sufficient intracellular leucine stores. To deplete

FIG. 3. Intracellular growth of the BCG leucine auxotroph (mc²798) and the complemented mutant [mc²798(pleuCD)] after infection in leucine-free medium. Adsorption, washing, and subsequent incubation were done in leucine-free medium supplemented with 10% FCS. AFB, acid-fast bacilli. Data are shown for 18 h and 4, 6, and 8 days (left to right).

intracellular leucine in the bacteria prior to infection, the BCG leucine auxotroph was grown in leucine-free 7H9 medium for less than one generation cycle (12 h). We controlled for equal viability of both the auxotrophic and the complemented strain before the beginning of infection by plating for CFU. Under these conditions no growth at all inside macrophages could be detected for the BCG leucine auxotroph (Fig. 3). Figure 4 shows THP-1 macrophages with intracellular bacteria (arrows) from the same experiment. These results led us to conclude that the initial growth described above was due to leucine-rich medium, which had been cophagocytized, rendering the auxotrophic strain capable of growing for a couple of generations. Ultimately, with depletion of leucine, the initial intracellular replication ceased.

In order to rule out polar effects on genes downstream from the *leuD* gene caused by the insertional mutagenesis, we asked whether the growth rate of the complemented BCG leucine auxotroph inside macrophages was equal to that of the parent BCG wild type. We infected THP-1 cells with the BCG leucine auxotroph, the complemented mutant, and the BCG wild type (Fig. 5). Figure 5A shows that between 5 and 15% percent of macrophages were infected. Within 8 days of infection, there was no significant change in the percentage of infected macrophages, indicating that no lysis of macrophages from any of the three strains had occurred. No replication of the BCG leucine auxotroph was detected. However, with respect to the complemented mutant, the mean number of bacilli within macrophages increased equally to that of the BCG wild type (Fig. 5B). As the number of infected macrophages had not been increased by lysis and reinfection, both the complemented mutant and the BCG wild type remained within the first round of infection. Thus, we concluded that upon providing the *leuCD* genes on a plasmid, the ability of the BCG leucine auxotroph to replicate inside macrophages could be fully restored and the growth kinetics matched that of the BCG wild type. Therefore, a polar effect affecting genes downstream from the *leuD* gene and causing the nonreplicating phenotype could be ruled out.

FIG. 4. Intracellular acid-fast bacilli in THP-1 macrophages: comparison of the BCG leucine auxotroph (mc^{2798}) and the complemented mutant ($mc^{2798}[pleuCD]$). Pictures were taken at 18 hours, 4 days, and 8 days after infection. Bacilli are marked with arrows.

FIG. 5. Intracellular growth of the complemented mutant [mc²798(pleuCD)] and BCG wild type. (A) Percentage of infected macrophages at 18 hours and 4, 6, and 8 days after infection (left to right). (B) Mean numbers of acid-fast bacilli (AFB) per macrophage at 18 h and 4, 6, and 8 days after infection (left to right).

DISCUSSION

In this study we investigated a BCG leucine auxotroph with respect to its growth inside the monocytic macrophage cell line THP-1. The results showed that the BCG leucine auxotroph was unable to grow inside THP-1 macrophages. The nongrowing phenotype was complemented with the *E. coli* *leuCD* genes and matched that of the BCG wild type. In addition, the auxotrophic phenotype was used as a marker to ensure that the infection technique presented here resulted in an efficient internalization of bacteria by the macrophages.

Fields et al. and Leung and Finlay demonstrated that restriction for growth inside macrophages (in vitro) of a variety of *S. typhimurium* auxotrophs is associated with attenuation in mice (in vivo) (14, 22). However, attenuation of intracellular pathogens in vivo may be caused by factors other than the failure of intracellular growth, for example, serum resistance or adherence to and entry of host cells. Our laboratory has shown recently that the BCG leucine auxotroph, in contrast to the parent BCG strain, showed an inability to grow in vivo and was cleared from the lungs and spleen of intravenously injected

C57BL/6 mice (25). In this study we showed that the BCG leucine auxotroph also failed to grow inside macrophages. The ability of BCG to grow within and destroy host cells is probably of significance in determining the fate of infection in vivo, since destruction of the phagocytic cells may facilitate the spread of infection. Our results suggest that the attenuated phenotype of the BCG leucine auxotroph in C57BL/6 mice resulted from its inability to replicate inside macrophages.

The failure of the BCG leucine auxotroph to grow in mice raised the interesting question of whether the leucine auxotrophy itself was responsible for the nongrowing phenotype. Although applying an extensive screen for mutants of *S. typhimurium* growth defective in vitro, Fields et al. and Leung and Finlay failed to isolate a leucine auxotroph (14, 22). The BCG leucine auxotroph used in this study was generated by transposon mutagenesis. The IS1096 transposon could have caused polar mutations. We fully restored for growth inside macrophages by introducing the *leuCD* genes on a plasmid into the BCG leucine auxotroph. As the failure to grow in mice is most likely caused by the inability of the BCG leucine auxotroph to replicate inside cells, we conclude that restriction for growth inside macrophages (in vitro) and in mice (in vivo) is due to the insertional disruption of the BCG *leuD* gene alone and not to polarity affecting genes downstream of the *leuD* gene. Whether growth in vivo is fully restored by providing the *leuCD* genes on a plasmid into the BCG leucine auxotroph remains to be tested.

L. monocytogenes and *S. flexneri*, two intracellular pathogens, have been shown to escape into the cytoplasm, which contributes to the virulence of both pathogens (32, 33). *L. monocytogenes* is naturally auxotrophic for leucine (31). A hemolysin-minus mutant of *L. monocytogenes*, which is not able to escape into the cytoplasm, does not grow within the cell (32). Escape of *M. tuberculosis* into the cytoplasm was initially reported by Myrvik and colleagues (28), but there remains uncertainty whether the organism remains in a vacuole with a modified membrane not visible by transmission electron microscopy or whether some organisms dissolve the membrane and enter the cytoplasm. In contrast there is no evidence to date that BCG resides in anything other than the phagolysosome within infected macrophages. A hemolysin has been cloned recently in *M. tuberculosis* (21), but it remains to be established that it has a role in pathogenesis or escape from phagocytic vacuoles. Our results suggest that the BCG leucine auxotroph is unable to access intracellular leucine. It is intriguing to speculate that if *M. tuberculosis* could enter the cytoplasm, an *M. tuberculosis* leucine auxotroph might not be attenuated for growth inside macrophages. Work is in progress to further elucidate the nutritional requirements of BCG and *M. tuberculosis* inside cells of the mononuclear phagocytic lineage.

There is an increasing interest in obtaining an attenuated BCG or *M. tuberculosis* strain to be used as a live vaccine in immunocompromised hosts. One approach to this problem would be to create BCG or *M. tuberculosis* auxotrophs. An ideal vaccine might be a double mutant in which the probability of reversion to a fully viable organism is remote and which is attenuated in the host (8). We showed here that for an auxotrophic BCG strain, attenuation in the animal was correlated with nongrowth inside macrophages. This in vitro system could be used to evaluate large numbers of auxotrophic strains before testing them in the animal.

The macrophage is certainly one of the most challenging environments to select for genes which are exclusively turned on during infection. Based on a technique developed by Mahan and coworkers to look for in vivo-induced genes (23), we have developed a vector system to be used for studying in vivo expression in mycobacteria. Using the promoterless *leuCD*

genes and *lacZ* gene as reporters in fusion with a genomic library of *M. tuberculosis*, we intend to select for growth inside macrophages, applying the system described in this study. Using this defined system one can modify the conditions of infection such as activation of macrophages with gamma interferon or tumor necrosis factor alpha.

In conclusion, we presented evidence that the BCG leucine auxotroph, which failed to grow and survive in mice, did not multiply in THP-1 macrophages. Our data indicate that the attenuated phenotype in mice is due to the inability to replicate inside macrophages. The BCG leucine auxotroph could not access intracellular leucine, which is consistent with BCG remaining inside the phagolysosome. Whether an *M. tuberculosis* leucine auxotroph (7) is able to access leucine and grow intracellularly by a possible escape into the cytoplasm is currently being investigated. By this means the genetic dissection of virulence mechanisms in BCG and *M. tuberculosis* might be possible.

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