Evaluation of Mycobacterium tuberculosis Genes Involved in Resistance to Killing by Human Macrophages

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A coinfection assay was developed to examine Mycobacterium tuberculosis genes suspected to be involved in resistance to killing by human macrophages. THP-1 macrophages were infected with a mixture of equal numbers of recombinant Mycobacterium smegmatis LR222 bacteria expressing an M. tuberculosis gene and wild-type M. smegmatis LR222 bacteria expressing the xylE gene. At various times after infection, the infected macrophages were lysed and the bacteria were plated. The resulting colonies were sprayed with catechol to determine the number of recombinant colonies and the number of xylE-expressing colonies. M. smegmatis bacteria expressing the M. tuberculosis glutamine synthetase A (glnA) gene or open reading frame Rv2962c or Rv2958c demonstrated significantly increased survival rates in THP-1 macrophages relative to those of xylE-expressing bacteria. M. smegmatis bacteria expressing M. tuberculosis genes for phospholipase C (plcA and plcB) or for high temperature requirement A (htrA) did not.

It is estimated that Mycobacterium tuberculosis, the causative agent of tuberculosis, infects about one-third of the world’s population, and about three million people die of tuberculosis each year (24). M. tuberculosis is an intracellular pathogen which survives and replicates within cells of the host immune system, primarily macrophages. Following phagocytosis into the macrophage, M. tuberculosis prevents acidification of the phagosome and fusion with lysosomes by altering the maturation of the phagosome (1, 5, 8, 17, 29). The precise survival strategies used by M. tuberculosis and the genes required for intracellular survival remain to be elucidated, although several candidate genes and activities, such as superoxide dismutase and catalase/peroxidase, have been proposed to be involved (6, 21, 23, 32).

To examine the potential involvement of M. tuberculosis genes in survival in macrophages, a coinfection assay was developed in which the survival of a recombinant Mycobacterium smegmatis strain relative to that of a wild-type strain could be directly measured. For these assays, cells of THP-1, a human monocyte-derived macrophage line (2), were used. The THP-1 cells were maintained in RPMI 1640 medium (Gibco BRL, Gaithersburg, Md.) containing 10% fetal calf serum (FCS) (Gibco BRL) at 37°C in 5% CO₂ and differentiated into macrophage-like cells by treatment with 10 μM phorbol myristate acetate (Sigma Chemical Company, St. Louis, Mo.) as previously described (2). A coinfection assay was necessary because wild-type M. smegmatis bacteria are rapidly killed in the first few hours after phagocytosis by differentiated THP-1 macrophages (Fig. 1). By 24 h postinfection, less than 0.01% of the phagocytized bacteria are viable (<100 CFU per well). The determination of the precise kinetics of survival of the bacteria is confounded by well-to-well variability in the numbers of bacteria phagocytized and the small numbers of CFU recovered. This results in large standard deviations which might mask relatively small differences in survival rates.

In order to distinguish the two strains in the coinfection, the wild-type strain was engineered to express catechol 2,3-dioxygenase. The xylE gene of Pseudomonas putida was isolated from the plasmid pTKmx (14) and cloned into the expression vector pHIP, and the construct was electroporated into wild-type M. smegmatis LR222 (18). Colonies of mycobacteria expressing the xylE gene turn yellow when sprayed with catechol due to the conversion of catechol to 2-hydroxymuconic semialdehyde by catechol 2,3-dioxygenase (33). By measuring the ratio of white to yellow colonies over time in macrophages infected with a mixture of nonexpressing bacteria (white) and xylE-expressing bacteria (yellow), the survival rates of the two strains can be directly compared.

In this study, we investigated six genes that have been suggested to contribute to the ability of M. tuberculosis to survive in the macrophage: glnA, plcA, plcB, htrA, Rv2962c, and Rv2958c. In pathogenic mycobacteria, glutamine synthetase A catalyzes the extracellular synthesis of i-glutamate, an important cell wall component (10). Phospholipase C might enable the tubercle bacillus to escape from the phagosome into the cytoplasm, as has been observed in some studies (17, 20). In Listeria monocytogenes, two phospholipase C proteins are produced, one of which is involved in escaping the phagosome while the other is involved in cell-to-cell spread (16, 27). The M. tuberculosis high temperature requirement A gene (htrA) encodes a protein that has homology with the HtrA family of serine proteases (30). Disruption of the htrA genes of Yersinia enterocolitica and Salmonella enterica serovar Typhimurium results in decreased rates of survival of the bacteria in cultured murine macrophages relative to those of wild-type bacteria (13, 22, 31). The precise role of HtrA in intracellular survival of these bacteria is not known. M. tuberculosis open reading frames (ORF) Rv2962c and Rv2958c display homology to a Mycobacterium leprae ORF that was shown to confer increased survival in J774 macrophages on both Escherichia coli and M. smegmatis recipients (19).

To construct M. smegmatis bacteria expressing the M. tuberculosis glutamine synthetase A (glnA), phospholipase C A (plcA), phospholipase C B (plcB), or high temperature requirement A (htrA) gene or Rv2962c or Rv2958c, each ORF was PCR amplified from M. tuberculosis H37Rv (28) genomic DNA and cloned individually downstream of the M. tuberculosis...
diluted to approximately 5 in inoculum (mine the number of viable bacteria of each strain in the initial tryptic soy agar (TSA) plates (Difco Laboratories) to determine the ratios at time zero and the subsequent time points are recovered at that time by the number of CFU recovered at time zero (1.35 × 10^6 CFU) and multiplying by 100. Error bars represent the standard deviations for three replicate cultures. Percent survival at 12 h was 0.67 ± 0.32, and that at 24 h was 0.015 ± 0.005.

The wells which were assayed immediately after the addition of bacilli secrete the GlnA protein and that both the \( hsp65 \) gene (yellow). The ratio of white to yellow colonies is shown for 37°C, after which each well was washed twice with RPMI 1640–10% FCS, and resuspended in RPMI 1640–10% FCS at a concentration of 1.5 \( \times 10^6 \) bacteria/ml. Equal volumes of the two bacterial suspensions were mixed, and a portion of the combined suspension was plated onto tryptic soy agar (TSA) plates (Difco Laboratories) to determine the number of viable bacteria of each strain in the initial inoculum (−2 h time point). The combined suspension was diluted to approximately 5 \( \times 10^7 \) bacteria/ml and 3 ml was added to each well of THP-1 macrophages (−1 \( \times 10^6 \) cells per well), giving a multiplicity of infection of 50 bacteria per macrophage. Phagocytosis of the bacteria was allowed to proceed for 2 h at 37°C, after which each well was washed twice with RPMI 1640–10% FCS. This results in approximately one phagocytized bacterium per THP-1 macrophage. To kill remaining extracellular bacteria, 3 ml of fresh medium containing 200 μg of amikacin (Sigma) per ml was added to each well. Cultures were incubated at 37°C in 5% CO₂. At various times after phagocytosis, the medium was removed from each of three wells and 1 ml of 0.1% (vol/vol) Triton X-100 in H₂O was added to each well to lyse the macrophages. Each lysate was diluted as necessary and portions were plated onto TSA plates. The wells which were assayed immediately after the addition of amikacin served as the standard for measuring the number and ratio of phagocytized bacteria; the time at which these wells were assayed was considered time zero. The TSA plates from each time point were incubated at 37°C for 3 days and then stored overnight at 4°C. The following day, the plates were sprayed with 0.5 M catechol in 50 mM potassium phosphate (pH 7.5) to distinguish the xylE-expressing colonies (yellow) from the recombinant colonies (white). Storing the plates overnight at 4°C results in a stronger yellow color.

To determine if the expression of the xylE gene or pHIP vector genes affected survival, THP-1 macrophages were infected with a mixture of recombinant \( M. smegmatis \) bacteria expressing xylE and wild-type \( M. smegmatis \) bacteria by using a multiplicity of infection that results in uptake of about one bacterium per macrophage. The ratio of xylE-expressing colonies (yellow) to wild-type colonies (white) was 1:1 at 0 h and remained 1:1 throughout the experiment (Fig. 2), indicating that the two strains are phagocytized and survive equally well and that the xylE-expressing strain is a suitable reference or internal control for comparison of the survival rates of other \( M. smegmatis \) strains.

During the course of an infection of THP-1 macrophages with a mixture of \( M. smegmatis \) bacteria expressing the \( M. tuberculosis \) glnA gene and wild-type \( M. smegmatis \) bacteria expressing the xylE gene, both strains of mycobacteria were rapidly killed but the ratio of glnA-expressing colonies to wild-type colonies increased from 1:1 at 0 h (9.6 \( \times 10^4 \) white to 9.8 \( \times 10^4 \) yellow colonies) to 3:2:1 at 12 h (4.6 \( \times 10^4 \) white to 1.4 \( \times 10^4 \) yellow colonies) to 6:1 at 24 h (150 white to 25 yellow colonies) after phagocytosis (Fig. 2). The differences between the ratios at time zero and the subsequent time points are statistically significant (\( P < 0.005, \) two-sample \( t \) test) for all time points.

Previous studies (10, 11) demonstrated that expression of the \( M. tuberculosis \) glnA gene in \( M. smegmatis \) results in a recombinant protein that is identical to the \( M. tuberculosis \) protein and that both the \( M. smegmatis \) recombinant and \( M. tuberculosis \) bacilli secrete the \( M. tuberculosis \) GlnA protein. The \( M. smegmatis \) GlnA protein is not secreted. One possible
explanation for the ability of the secreted \textit{M. tuberculosis} GlnA protein to enhance the survival of \textit{M. smegmatis} is that the GlnA protein may modulate the pH of the phagosome by altering the levels of phagosomal ammonia (9, 10). So, by secreting the \textit{M. tuberculosis} GlnA protein, the recombinant \textit{M. smegmatis} bacteria may interfere with the acidification of the phagosome and thereby delay fusion with lysosomes and exposure to the antimicrobial activities in the lysosome. Expression of GlnA is not sufficient, however, to prevent acidification and fusion, because the recombinant \textit{M. smegmatis} bacteria are still efficiently killed by the macrophages. Indeed, no viable mycobacteria were recovered from samples harvested 48 h postinfection.

The \textit{M. tuberculosis} ORFs \textit{Rv}2962c and \textit{Rv}2958c were identified through their homology to an ORF of \textit{M. leprae} and wild-type bacteria expressing (19). The expected to be involved in survival in murine J774 macrophages \textit{M. smegmatis} secreting the GlnA protein may modulate the pH of the phagosome by explanation for the ability of the secreted \textit{M. tuberculosis} to survive in the human macrophage. The analysis of \textit{M. tuberculosis} strains with disruptions in the glnA, \textit{Rv}2962c, or \textit{Rv}2958c genes is needed to determine these genes’ roles in intracellular survival. Finally, the ability to preferentially recover recombinant strains with a greater resistance to killing by macrophages suggests that the culture system could be modified to enrich for recombinants with increased survival rates from a library of \textit{M. smegmatis} bacteria carrying large fragments of \textit{M. tuberculosis} DNA, perhaps thereby allowing identification of additional \textit{M. tuberculosis} genes involved in resistance to killing by macrophages.

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