Identification of Macrophage and Stress-induced Proteins of *Mycobacterium tuberculosis*

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Abstract

Using phosphorimager technology to quantitate differences in protein expression, we have investigated the modulation of protein synthesis by *Mycobacterium tuberculosis* in response to intracellular residence in human macrophages and, for comparison, in response to various stress conditions during extracellular growth. Proteins of *M. tuberculosis* growing intracellularly in human THP-1 cells and extracellularly in broth were labeled with [^{35}S]methionine; during intracellular growth, host cell protein synthesis was inhibited with cycloheximide. The metabolically labeled proteins were separated by two-dimensional gel electrophoresis and quantitatively analyzed. Intracellular residence in macrophages induced a profound change in the overall phenotype of *M. tuberculosis*. The expression of at least 16 *M. tuberculosis* proteins was induced (at least a twofold increase compared with growth in broth) and 28 proteins repressed (at least a twofold decrease). Many of the phenotypic changes in protein expression induced during intracellular growth occurred during extracellular growth in response to stress conditions including heat-shock, low pH, and H_2O_2. However, the pattern of induced and repressed proteins was unique to each stress condition. Of the 16 macrophage-induced proteins, 6 were absent during extracellular growth under both normal and stress conditions. Such proteins are potential virulence determinants and/or they may be important in the cell-mediated and protective immune response to *M. tuberculosis* infection. (*J. Clin. Invest.* 1995. 96:245–249.) Key words: phosphorimager • tuberculosis • heat-shock • oxidative stress • acidification

Introduction

Tuberculosis is a major cause of morbidity and mortality worldwide. It is estimated that one-third of the world's population is infected with *Mycobacterium tuberculosis* (1). The incidence of tuberculosis in the United States, which had been declining, has been increasing in recent years primarily because of a high rate of infection in AIDS patients (2, 3). Compounding the problem, outbreaks of tuberculosis caused by multidrug-resistant strains of *M. tuberculosis* have recently occurred (4). Such cases are difficult or impossible to treat and threaten to become widespread.

The resurgence of tuberculosis has highlighted the need for new strategies to combat *M. tuberculosis*. To develop such strategies, more needs to be learned about the pathogenesis of *M. tuberculosis* infection. The investigation reported here was undertaken to enhance our understanding of one key aspect of pathogenesis—the interaction of *M. tuberculosis* with the human macrophage, the cell within which the organism resides and multiplies in the host.

To survive intracellularly, *M. tuberculosis* must adapt to its intracellular environment in the macrophage, including any stresses it encounters in a host cell armed with a powerful array of antimicrobial defenses. The resistance involves a number of genes. The protein products of these genes are not only potential virulence determinants, but because of the likelihood that they are processed and presented by infected macrophages, these proteins are also potentially important in the cell-mediated and protective immune response to *M. tuberculosis* infection. For these reasons, identifying and characterizing macrophage-induced proteins of *M. tuberculosis* is potentially of great importance to our understanding of the pathogenesis of tuberculosis.

To identify macrophage-induced proteins, we infected THP-1 cells, a human macrophage line, with virulent *M. tuberculosis* Erdman strain. We then labeled bacterial proteins with [^{35}S]-methionine while inhibiting host cell protein synthesis with cycloheximide (5, 6). For purposes of comparison, we also labeled proteins of *M. tuberculosis* growing extracellularly in broth culture in the presence or absence of various stress treatments including heat-shock, low pH, and H_2O_2. The labeled proteins of *M. tuberculosis* growing intracellularly or extracellularly were then separated by two-dimensional (2-D)[1] gel electrophoresis and quantitatively analyzed by phosphorimager.

Methods

Bacteria. Virulent *M. tuberculosis* Erdman strain (35801; American Type Culture Collection, Rockville, MD) was maintained by passage through guinea pigs. For preparation of bacterial stocks, *M. tuberculosis* organisms recovered from the lung of infected guinea pigs were grown for 7 d on Middlebrook 7H11 agar as described (7), scraped from the plates, suspended in 7H9 medium containing 10% glycerol at a concentration of 10^8 bacterial particles per milliliter, and stored at −70°C until use.

Cell culture and differentiation and infection with M. tuberculosis. THP-1 cells, a human acute monocytic leukemia line, were obtained from the American Type Culture Collection. Cells were maintained in RPMI-1640 (Irvine Scientific, Santa Ana, CA) containing 10% FCS, 2 mM L-glutamine, and gentamicin (GIBCO BRL, Gaithersburg, MD).

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1. *Abbreviations used in this paper:* 2-D, two-dimensional; hsp, heat-shock protein

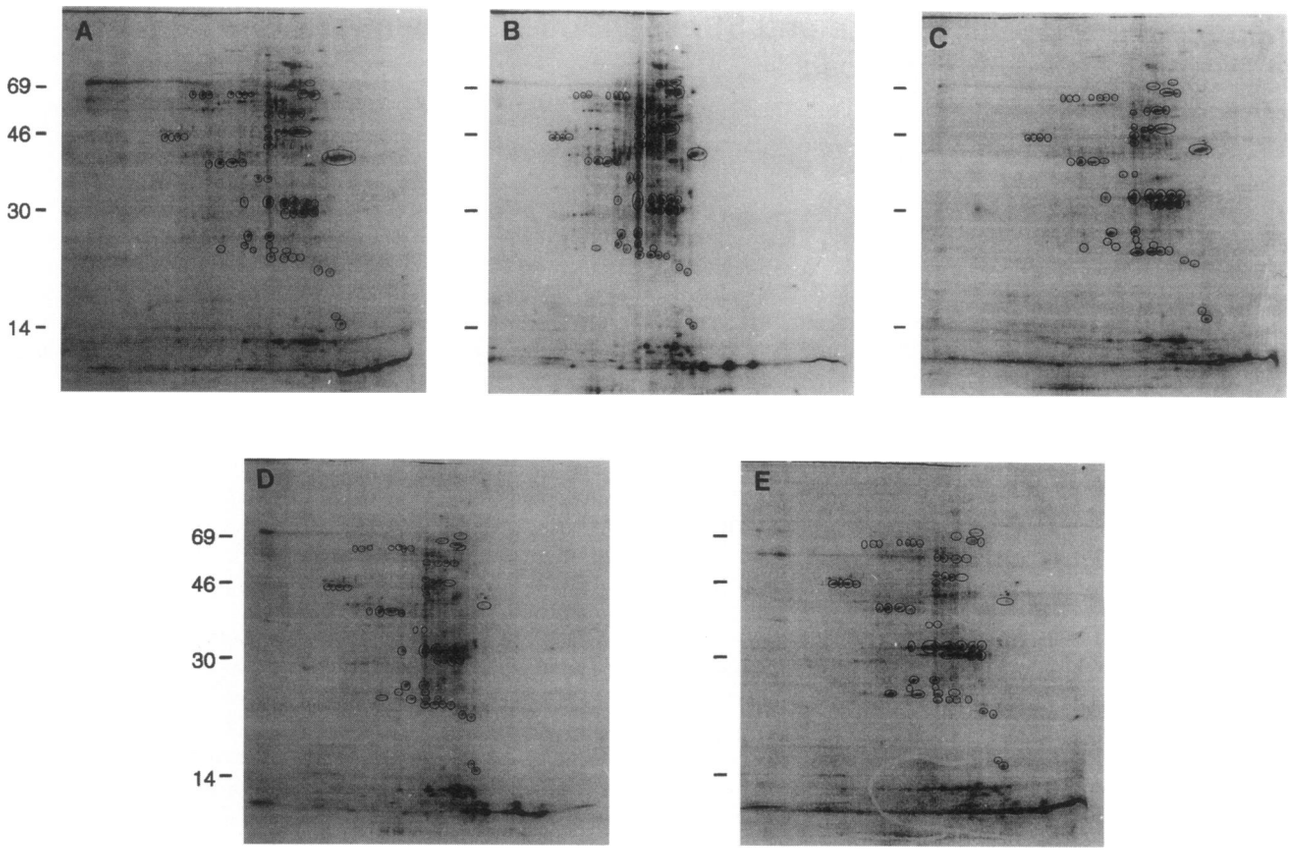

Figure 1. Proteins produced by *M. tuberculosis* growing extracellularly under normal and various stress conditions. Bacteria were labeled with [^{35}S]methionine (100 μCi/ml) for 20 h at 37°C (*A*), 42°C (*B*), in the presence of 2 mM H_2O_2 (*C*), at pH 6.0 (*D*), or at pH 5.5 (*E*). Radiolabeled bacterial protein samples containing equivalent amounts of radioactivity were separated by isoelectric focusing in the first dimension (pH 4–7 from right to left) and by SDS-PAGE in the second dimension. The circled proteins were quantitated by phosphorimager (See Fig. 2). Molecular mass standards in kilodaltons are to the left of each figure. Similar results for each stress condition were obtained in at least three independent experiments.

Cell cultures were incubated at 37°C in a humidified 5% CO_2 atmosphere. For infection experiments, THP-1 cells were resuspended at a density of 10^6 per milliliter in culture medium in the absence of antibiotics, and were differentiated with 10^{-7} M PMA (Sigma Chemical Co., St. Louis, MO) for 48 h. The differentiated and adherent cells were washed and incubated at 37°C for another 48 h before infection with *M. tuberculosis*. The bacteria, freshly grown from stock cultures on 7H11 agar plates for 7 d, were resuspended in RPMI-1640 supplemented with 20% normal human serum type AB, 2 mM L-glutamine, and 10 mM Hepes, and the number of bacterial particles determined by Petroff–Hauser counting. The serum opsonized bacteria were added to the differentiated THP-1 cells at a ratio of 10:1 (bacteria/cells) and incubated for 90 min at 37°C. Extracellular bacteria were then removed by extensive washing with RPMI-1640. Bacterial growth in THP-1 cells was monitored daily by lysing and plating infected cell cultures on 7H11 agar and enumerating colony forming units after 10-d incubation at 37°C.

Stress conditions. Bacteria grown on 7H11 agar plates for 7 d were resuspended in 7H9 medium, pH 6.7 to an optical density of 0.1 at 540 nm and cultured at 37°C in 5% CO_2 for another 7 d to reach the exponential phase of growth. The bacteria were then pelleted and resuspended at an optical density of 0.4 at 540 nm in fresh 7H9 medium at standard pH (6.7) or at pH 6.0 or 5.5 (acid-shock). Cultures at standard pH were either heat-shocked by incubation at 42°C or subjected to oxidative stress by incubation with H_2O_2 (2 mM).

Radiolabeling of M. tuberculosis proteins. To radiolabel *M. tuberculosis* proteins produced by the organism in broth cultures under various stress conditions, we incubated the bacteria for 3 h under the stress condition and then added 100 μCi/ml of [^{35}S]methionine (sp act 1,000 Ci/mmol; Amersham International, Little Chalfont, United Kingdom), to each culture for 20 h. Bacteria were pelleted, washed, and stored at −70°C. To radiolabel *M. tuberculosis* proteins produced by the bacteria in THP-1 cells, we replaced the culture medium 60 h after infection with L-methionine–deficient RPMI-1640 containing 5% FCS, 2 mM L-glutamine, 10 mM Hepes and 50 μg/ml cycloheximide (Sigma Chemical Co.), incubated the cultures for 3 h, and then added [^{35}S]methionine to a final concentration of 100 μCi/ml. After 20 h, the infected monolayer was lysed with 0.1% SDS, and the bacteria were harvested by centrifugation at 5,000 rpm for 30 min. The bacterial pellet was washed once with Dulbecco's phosphate buffered saline and stored at −70°C.

2-D gel electrophoresis. Radiolabeled bacteria were resuspended in sample buffer containing 2% SDS, 5% 2-mercaptoethanol, and 10% glycerol, and heated at 95°C for 10 min. The solubilized proteins (10^6 cpm/gel for broth-grown organisms and 5×10^5 cpm/gel for macrophage-grown organisms) were subjected to electrophoresis as described by O'Farrell (8). The first dimension gels contained pH 4–6 ampholytes and pH 3–10 ampholytes (1:4). The second dimension slab was a discontinuous SDS-PAGE system, Protean IIxi (BioRad, Richmond, CA), containing 12.5% acrylamide.

Quantitation. Protein spots on 2-D gels were quantitated by phosphorimager (Molecular Dynamics, Inc., Sunnyvale, CA). To assess quantitatively changes in protein expression under different conditions of bacterial growth, we included, in the second dimension of each gel electrophoresis, ^{14}C-methylated proteins (Amersham International) with a standard amount of radioactivity. The intensity of each protein spot on each 2-D gel was first normalized by reference to the intensities of ^{14}C-labeled protein standards on the gel. (The intensities of proteins for

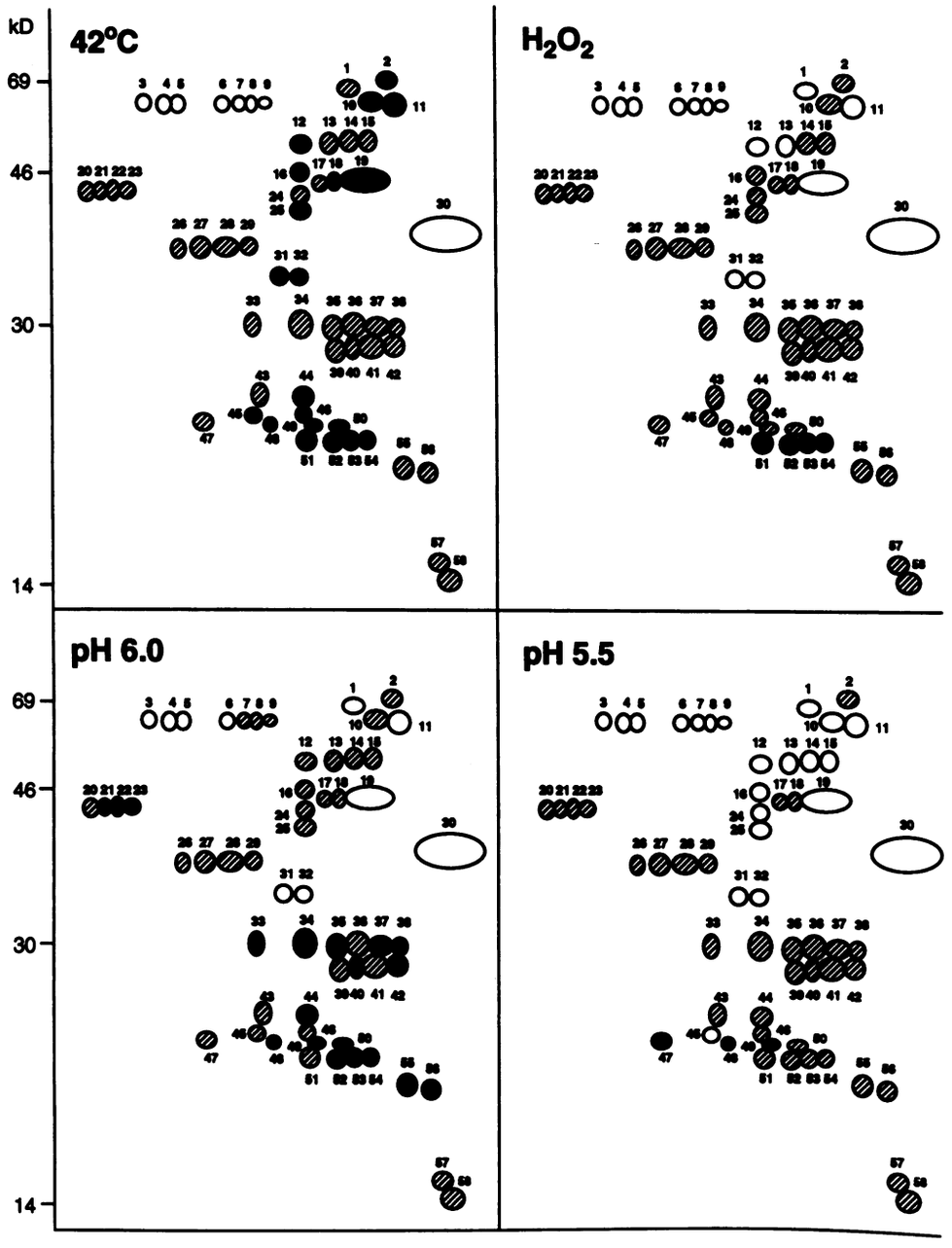

Figure 2. Quantitative analysis of ^{35}S-labeled 2-D gel protein profiles of *M. tuberculosis* growing extracellularly under various stress conditions. The intensities of protein spots of *M. tuberculosis* radiolabeled under various stress conditions were quantitated by phosphorimager, normalized as described in Methods, and compared with the intensities of corresponding protein spots of *M. tuberculosis* radiolabeled under normal culture conditions (37°C, pH 6.7). Stress-induced proteins were defined as proteins whose intensity was at least twofold higher under the stress condition than under normal conditions. Stress-repressed proteins were defined as proteins whose intensity was at least twofold lower under the stress condition than under normal conditions. Solid circles represent stress-induced proteins. Open circles represent the stress-repressed proteins. Striped circles represent proteins whose expression is unchanged under the stress condition. Proteins numbered 2 and 10 are the hsp70 and hsp65 proteins, respectively. Proteins numbered 33–42 are members of the antigen 85 complex of 30/32-kD proteins.

macrophage-grown organisms were doubled to correct for the fact that half as many counts were loaded on to gels as for broth-grown organisms). Then the intensities of protein spots on different 2-D gels were compared. Stress- or macrophage-induced proteins were defined as proteins with intensity at least twofold higher under the stress condition than under normal conditions. Stress- or macrophage-repressed proteins were defined as proteins with intensity at least twofold lower under the stress condition than under normal conditions.

Results

Protein modulation by M. tuberculosis growing under stress conditions. Proteins of *M. tuberculosis* were radiolabeled in broth under various stress conditions including heat-shock at 42°C, exposure to H_2O_2, mild acidification (pH 6.0), and intense acidification (pH 5.5) for 20 h. Radiolabeled *M. tuberculosis* proteins were separated by 2-D gel electrophoresis (Fig. 1). 58 distinct proteins of *M. tuberculosis* radiolabeled under various stress conditions were quantitated by phosphorimager and compared with the corresponding proteins of *M. tuberculosis* radiolabeled under normal culture conditions (37°C, pH 6.7) (Fig. 2). Under heat-shock conditions, the expression of 20 proteins was increased at least twofold and the expression of 8 proteins was decreased at least twofold. With exposure to 2 mM H_2O_2, the expression of 4 *M. tuberculosis* proteins was increased and 15 *M. tuberculosis* proteins was decreased. With mild acidification, the expression of 19 *M. tuberculosis* proteins was increased and 10 *M. tuberculosis* proteins decreased. With intense acidification, expression of 3 proteins was increased and 22 proteins decreased. Thus, the expression of *M. tuberculosis*

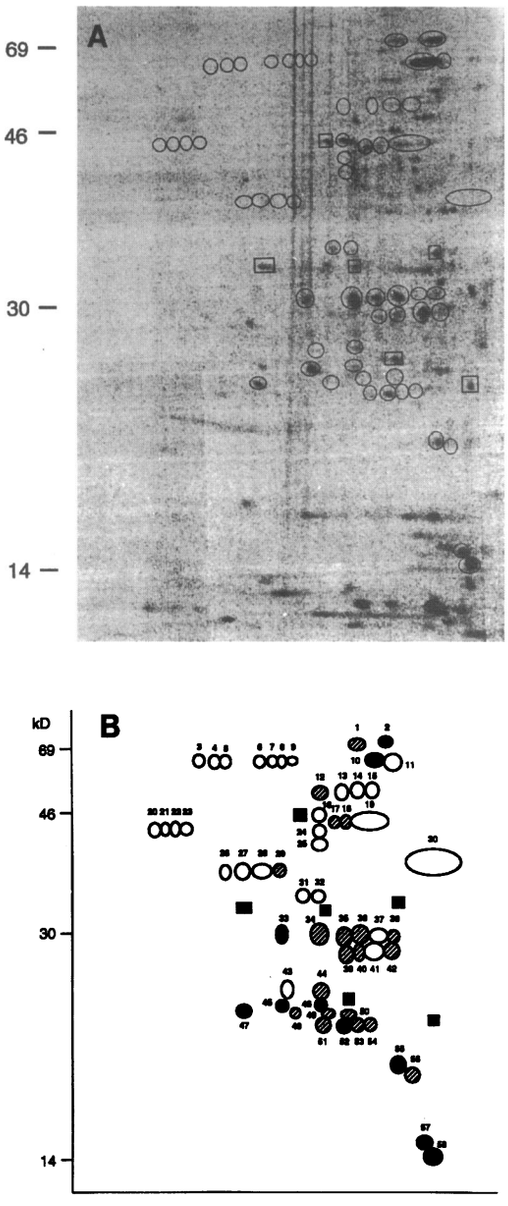

Figure 3. Protein expressed by *M. tuberculosis* in macrophages. Proteins produced by *M. tuberculosis* growing intracellularly were labeled with [^{35}S]methionine, analyzed by 2-D gel electrophoresis (*A*), and quantitated by phosphorimager (*B*). Circles show locations of proteins expressed by *M. tuberculosis* growing in broth culture under normal conditions. Squares show proteins expressed by *M. tuberculosis* growing in THP-1 cells but not by *M. tuberculosis* growing in broth culture under normal conditions (37°C, pH 6.7). Molecular mass standards in kilodaltons are to be left of the Fig. 3 *A*. The intensity of each protein spot was quantitated by phosphorimager, normalized as described in Methods, and compared with the intensity of the corresponding protein spot of radiolabeled proteins produced by *M. tuberculosis* growing in broth culture. Macrophage-induced proteins were defined as proteins whose intensity was at least twofold higher for *M. tuberculosis* growing in macrophages than in broth culture under normal conditions. Repressed proteins were defined as proteins whose intensity was at least twofold lower for *M. tuberculosis* growing in macrophages than in broth culture under normal conditions. Solid circles represent proteins whose expression is increased in bacteria growing in macrophages. Solid squares show proteins expressed by *M. tuberculosis* growing in THP-1 cells but not expressed at a detectable level by *M. tuberculosis* growing under normal conditions (37°C, pH 6.7). Open circles represent the macrophage-repressed proteins. Striped circles represent *M. tuberculosis* proteins whose expression is unchanged in bacteria growing in macrophages. The same protein pattern was observed in three independent experiments.

proteins in broth culture is profoundly altered by stress conditions. About half of the proteins present under normal conditions of broth culture are induced or repressed under stress. The pattern of induced and repressed proteins is unique to each stress condition. Proteins were more often induced than repressed by heat-shock and mild acidification (pH 6.0). Proteins were more often repressed than induced by oxidative stress (H_2O_2) and intense acidification (pH 5.5). Some proteins (No. 44, 48–54) were induced under more than one stress condition. Other proteins, however, were induced under one stress condition and unchanged (No. 2, 18, 21–23, 33–35, 37, 38, 40, 42, 46, 47, 55, 56) or even repressed (No. 11, 19, 31, 32) under another.

Protein modulation by M. tuberculosis growing in THP-1 cells. Proteins of *M. tuberculosis* growing intracellularly were labeled with [^{35}S]methionine, while macrophage protein synthesis was completely inhibited with cycloheximide. Large scale cultures of *M. tuberculosis*–infected mononuclear phagocytes were needed to obtain an adequate signal from radiolabeled proteins produced by *M. tuberculosis* growing intracellularly. We therefore utilized a human macrophage cell line for these studies. We chose THP-1 cells (9, 10) because the growth rate of *M. tuberculosis* in these cells (1 log in 3 d) was comparable to that in human monocytes and superior to that in the other macrophage cell lines that we tested (U-937 and HL-60 cells). Moreover, *M. tuberculosis* interacts similarly with THP-1 cells and human monocytes. In both cell types, *M. tuberculosis* (*a*) induces the release of tumor necrosis factor after phagocytosis (11); (*b*) inhibits phagosome–lysosome fusion (Clemens, D. L., and M. A. Horwitz, unpublished results); and (*c*) resides in a phagosome that stains weakly with the lysosome-associated membrane glycoprotein CD63 (Clemens, D. L., and M. A. Horwitz, unpublished results).

To analyze macrophage-induced *M. tuberculosis* proteins, we infected differentiated THP-1 cells in the absence of antibiotics. We minimized the number of extracellular bacteria in the cultures by extensively washing THP-1 monolayers after a 90-min period of phagocytosis at the start of the experiment and by removing culture supernatant fluid and harvesting only macrophage-associated organisms at the end of the experiment. We did this in lieu of using antibiotics to inhibit extracellular bacteria because antibiotics consistently suppressed *M. tuberculosis* growth in THP-1 cells and reduced the signal from radiolabeled proteins below the level of detection.

M. tuberculosis proteins produced during intracellular and extracellular growth were analyzed by 2-D gel electrophoresis (Fig. 3). In comparison with the protein pattern of *M. tuberculosis* growing in broth culture under normal conditions, synthesis of 28 *M. tuberculosis* proteins was repressed and synthesis of 16 *M. tuberculosis* proteins was induced by growth within macrophages. Of the 16 macrophage-induced proteins, 8 proteins were induced (increased by at least twofold) in *M. tuberculosis* growing in broth culture by one or another stress condition, two proteins (No. 57 and 58) were present under normal conditions of broth growth but not increased under stress conditions, and

six proteins were absent under both normal and stress conditions. No proteins were detected in cycloheximide-treated THP-1 cells which were not infected with *M. tuberculosis*. The hsp65 (No. 10) and hsp70 (No. 2) heat-shock proteins and members of the 30/32-kD protein complex (No. 33–36, 40–41) were among the more abundant proteins expressed by *M. tuberculosis* in macrophages. The identity of these proteins was confirmed by Western blot analysis and by colocalization with the purified *M. tuberculosis* proteins on 2-D gels.

Discussion

This paper demonstrates that the expression of a large number of *M. tuberculosis* proteins is significantly modulated by intracellular residence in human macrophages. The use of phosphorimager technology in this study, its first application to a study of this type, allowed us to quantitate changes in protein expression by *M. tuberculosis* in response to various environmental conditions including intracellular residence. Even conservatively defining a change in protein expression as one involving at least a twofold increase or decrease in expression, we identified 44 *M. tuberculosis* proteins whose expression was modulated within macrophages, 16 up-regulated and 28 down-regulated. This indicates that intracellular residence induces a profound change in the overall phenotype of this pathogen.

Many of the phenotypic changes in *M. tuberculosis* protein expression that occurred in response to intracellular residence also occurred in response to "stresses" in the extracellular environment including heat-shock, exposure to hydrogen peroxide, and acidification. However, none of the stress conditions reproduced completely the intracellular phenotype. Indeed, eight proteins induced by intracellular residence were not induced under any of the stress conditions. This suggests that certain gene products presumably required for adaptation to the intracellular environment are not induced by global stress signals, at least the ones used in this study. This is not surprising, given the complexity of the intracellular environment.

The intracellular environment of macrophages has been characterized as a hostile one, and consistent with this idea, so-called stress proteins were found to be up-regulated in macrophages infected with *M. tuberculosis* in this study and with *Legionella pneumophila* and *Salmonella typhimurium* in studies by other investigators (5, 6). However, many intracellular pathogens require host mononuclear phagocytes to multiply in vivo and both *M. tuberculosis* and *L. pneumophila* utilize host cell receptors and host proteins to gain access to mononuclear phagocytes (12, 13). Moreover, the growth rate of *M. tuberculosis* is fivefold greater and the growth rate of *L. pneumophila* twofold greater in the supposedly inhospitable intracellular environment of the macrophage than in broth media especially designed to support maximal rates of growth of these organisms (14–16). This calls into question the notion that the intracellular environment is hostile. In any case, the capacity of intracellular pathogens to thrive intracellularly demonstrates their exquisite adaptation to the intracellular milieu. Our study indicates that this adaptation includes the modulation of a large number of gene products.

The 30/32-kD complex of proteins were prominent among the proteins of *M. tuberculosis* expressed during intracellular residence in macrophages. These proteins are the major secretory proteins of *M. tuberculosis*, and they are prominent in the cell-mediated and protective immune response (15, 17). Their abundance in infected macrophages, coupled with their extracellular release and hence availability for antigen processing and presentation, likely underlies their immunoprotective capacity (15).

Little is known regarding virulence determinants of *M. tuberculosis*, especially proteins that allow the organism to parasitize mononuclear phagocytes. In this regard, the six macrophage-induced proteins not present in organisms growing in broth under any of the conditions tested, would appear to be excellent candidates as virulence determinants.

Acknowledgments

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References

1. Kochi, A. 1991. The global tuberculosis situation and the new control strategy of The World Health Organization. *Tubercle.* 72:1–6.

2. Center for Disease Control. 1993. Tuberculosis morbidity in the United States - 1992. *MMWR (Morb. Mortal. Wkly. Rep.).* 42:696–704.

3. Chalsson, R. E., and G. Slutkin. 1989. Tuberculosis and human immunodeficiency virus infection. *J. Infect. Dis.* 159:96–100.

4. Fischl, M. A., G. L. Daikos, R. B. Uttmchandani, R. B. Poblete, J. N. Moreno, R. R. Reyes, A. M. Boota, L. M. Thompson, T. J. Cleary, S. A. Oldham, et al. 1992. Clinical presentation and outcome of patients with HIV infection and tuberculosis caused by multiple-drug resistance bacilli. *Ann. Intern. Med.* 117:184–190.

5. Buchmeier, N. A., and F. Heffron. 1990. Induction of *Salmonella* stress proteins upon infection of macrophages. *Science (Wash. DC)* 248:730–732.

6. Kwaik, Y. A., B. I. Einstein, and N. C. Engleberg. 1993. Phenotypic modulation by *Legionella pneumophila* upon infection of macrophages. *Infect. Immun.* 61:1320–1329.

7. Pal, P. G., and M. A. Horwitz. 1992. Immunization with extracellular proteins of *Mycobacterium tuberculosis* induces cell-mediated immune responses and substantial protective immunity in a guinea pig model of pulmonary tuberculosis. *Infect. Immun.* 60:4781–4792.

8. O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* 250:4007–4021.

9. Tsuchiya, S., M. Yamabe, Y. Yamaguchi, Y. Kobayashi, T. Konno, and K. Tada. 1980. Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). *Int. J. Cancer.* 26:171–176.

10. Tsuchiya, S., Y. Kobayashi, Y. Goto, H. Okumura, S. Nakae, T. Konno, and K. Tada. 1982. Induction of maturation in cultured human monocytic leukemia cells by a phorbol diester. *Cancer Res.* 42:1530–1536.

11. Friedland, J. S., D. G. Remick, R. Shattock, and G. E. Griffin. 1992. Secretion of interleukin-8 following phagocytosis of *Mycobacterium tuberculosis* by human monocyte cell lines. *Eur. J. Immunol.* 22:1373–1378.

12. Payne, N. R., and M. A. Horwitz. 1987. Phagocytosis of *Legionella pneumophila* is mediated by human monocyte complement receptors. *J. Exp. Med.* 166:1377–1389.

13. Schlesinger, L. S., C. G. Bellinger-Kawahara, N. R. Payne, and M. A. Horwitz. 1990. Phagocytosis of *Mycobacterium tuberculosis* is mediated by human monocytes complement receptors and complement component C3. *J. Immunol.* 144:2771–2780.

14. Horwitz, M. A., and S. C. Silverstein. 1980. Legionnaires' disease bacterium (*Legionella pneumophila*) multiplies intracellularly in human monocytes. *J. Clin. Invest.* 66:441–450.

15. Horwitz, M. A., B.-W. E. Lee, B. J. Dillon, and G. Harth. 1995. Protective immunity against tuberculosis induced by vaccination with major extracellular proteins of *Mycobacterium tuberculosis. Proc. Natl. Acad. Sci. USA.* 92:1530–1534.

16. Clemens, D. L., and M. A. Horwitz. 1995. Characterization of the *Mycobacterium tuberculosis* phagosome and evidence that phagosomal maturation is inhibited. *J. Exp. Med.* 18:257–270.

17. Havlir, D. V., R. S. Wallis, W. H. Boom, T. M. Daniel, K. Chervenak, and J. J. Ellner. 1991. Human immune response to *Mycobacterium tuberculosis* antigens. *Infect. Immun.* 59:665–670.