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# Differential expression of a unique protein by intracellular *Mycobacterium tuberculosis* complex

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**We have investigated the changes in the protein synthesis pattern in guinea pig peritoneal macrophages following infection with virulent *Mycobacterium tuberculosis* H37Rv *in vitro*. By $^{35}$S methionine labelling of the newly synthesized proteins followed by ultracentrifugation, SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) and autoradiography, the protein synthesis pattern of the control uninfected macrophages and the infected macrophages *in vitro* were compared. By adding cycloheximide to the macrophage cultures, the protein synthesis of macrophages was inhibited and the protein synthesis pattern of *M. tuberculosis* has been analysed. We have identified a mycobacterial protein of molecular weight 17 kDa which was expressed exclusively in the cytosolic fraction of *M. tuberculosis*-infected guinea pig macrophages *in vitro*.**

TUBERCULOSIS is a major cause of morbidity and mortality worldwide. There is an enormous increase in the incidence of tuberculosis due to HIV epidemic and rise in multi-drug resistance. *Mycobacterium tuberculosis* is one of the successful intracellular pathogens infecting over one-third of the world's population and causing 8 million new cases of active tuberculosis annually[1]. The resurgence of tuberculosis has highlighted the need for new strategies to combat *M. tuberculosis*. To develop such strategies, one needs to learn more about the pathogenesis of *M. tuberculosis* infection.

*M. tuberculosis* is an intracellular pathogen. The macrophage fails to eliminate this pathogen in spite of the powerful array of antimicrobial defences it puts forth, along with other components of the immune system. This shows that *M. tuberculosis* adopts itself to the intracellular environment by mounting a response appropriate to ensure its survival. The resistance by *M. tuberculosis* would involve a number of genes. The protein products of these genes could not only be potential virulent determinants but also important in cell-mediated and protective immune response to *M. tuberculosis*, because they are processed and presented by infected macrophages. So it becomes imperative to identify and characterize *M. tuberculosis* proteins which would help us to understand the pathogenesis of tuberculosis and in turn to develop novel drug targets and attenuated vaccines.

To understand these mechanisms, the bacterial gene products that are specifically required at each stage of the infection process have to be identified. It has been demonstrated by Buchmeir and Heffron[2] that virulent Salmonella strains upon phagocytosis by a macrophage cell line express at least 30 different proteins. Monahan

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*et al.*[3] studied the protein synthesis profile in response to peroxide and demonstrated the induction of heat shock proteins (hsp) 60, 70 and 90 with minor changes, when *M. bovis* BCG was cultured in Dubos broth using metabolic labelling, SDS-PAGE and autoradiography.

It is therefore possible to analyse the protein synthesis patterns associated with host–pathogen interaction and study the host factors that induce an adaptive pathogen response. This approach might be helpful to define the molecular mechanisms involved in host–pathogen interactions.

We undertook the study to identify the protein products which are uniquely expressed by *M. tuberculosis* in the intracellular environment of the guinea pig macrophages *in vitro*. We chose guinea pigs because the tuberculosis disease in this animal model mimics the disease in humans.

Eight-week-old inbred guinea pigs were injected intraperitoneally with 2% sterile starch. Seventy-two hours later, the peritoneal cavity was washed with ice-cold Hanks balanced salt solution (HBSS) (Bio Whittakor, Walkersville, MD, USA). The number of macrophages in the peritoneal lavage was ascertained by neutral red staining (approximately 90–95% were macrophages).

Macrophages recovered from the peritoneal cavity were suspended in RPMI medium (Sigma Chemical Co, St. Louis, MO, USA) without methionine. Macrophages were cultured in 12 well-tissue culture plates (1 million/well). The cells were allowed to adhere at 37°C, 5% $CO_2$, in a humidified atmosphere for 60 min. The non-adherent cells were removed by gentle washing and the adherent macrophages were cultured in RPMI (without methionine) containing 5% foetal calf serum (FCS) (Sigma Chemical Co, USA).

Starch-elicited guinea pig peritoneal macrophages *in vitro* were infected with *M. tuberculosis*, *M. bovis* BCG, heat killed *M. tuberculosis*, yeast and *Listeria monocytogenes* separately. The macrophage to organism ratio was adjusted to 1 : 10 (i.e. for every single macrophage, there were ten organisms). One hour after infection, the unphagocytosed organisms were removed by gentle washing. $^{35}S$ methionine (Board of Radiation and Isotope Technology, Mumbai, India) (10 µCi/well) was added to the infected macrophages.

The cultures were terminated 48 h after infection. The macrophages were lysed with ice-cold lysis buffer (1% CHAPS [3-cholamidopropyl dimethylammonio] 1 propane sulphate), 3 mM EDTA (ethylene diamine tetraacetic acid, 2 mM PMSF (phenyl methyl sulphonyl fluoride), 1.7 TIU/ml aprotonin and 100 mM iodo acetamide in PBS (Sigma Chemical Co, USA). The lysate was centrifuged at 5000 rpm for 10 min. The supernatant was ultracentrifuged at 45,000 rpm for 45 min. The supernatant obtained from ultracentrifugation represents cytosolic fraction of the macrophages. The proteins in the supernatant were resolved by SDS-PAGE and transferred onto nitrocellulose paper (NCP)[4]. The NCP was exposed to an X-ray film, which was later developed.

*M. tuberculosis* H37Rv was cultured in RPMI without methionine with 5% FCS for one week. The organisms were pelleted by centrifugation at 10,000 rpm for 10 min. The pellet was suspended in 2 ml of RPMI. The suspension was subjected to sonication by ultrasonic vibration (soniprep 150 MSE, UK) for 10 min in ice. The viability of *M. tuberculosis* in the sonicate preparation was confirmed by plating in 7H11 with oleic acid dextrose complex (OADC) (Difco Laboratories, Detroit, MI, USA). The culture filtrate antigen was prepared as follows: *M. tuberculosis* was cultured in RPMI without methionine with 5% FCS for one week. The organisms were pelleted by centrifugation at 10,000 rpm for 10 min. The cell-free supernatant was lyophilized. The lyophilized pellet fraction was made up to 200 ml with normal saline.

*M. tuberculosis* H37Rv and *M. bovis* BCG were cultured in Dubos broth containing Tween 80. A single-cell suspension of mycobacteria at peak log phase of growth was used for infection. At the time of infection, the bacterial cell suspension was washed three times in HBSS to remove Tween 80. The bacterial cell suspension was subjected to mild sonication, to disaggregate the clumps. The number of mycobacteria in the suspension was ascertained by counts in Thoma counting chamber. Heat-killed *M. tuberculosis* cell suspension was prepared by incubating *M. tuberculosis* cell suspension at 80°C, in water bath for 20 min.

Heat-killed yeast particles were suspended in normal saline. At the time of infection, the yeast particles were sonicated to disaggregate the clumps and the total number of yeast particles in the suspension was ascertained by counts in Neubauer counting chamber.

*Listeria monocytogenes* was cultured in blood agar in the presence of carbon dioxide. Single colonies of *L. monocytogenes* were recovered from blood agar and washed three times in HBSS. The clumps were dispersed by vigorous vortexing. The number of organisms present in the suspension was ascertained by counts in Thoma counting chamber.

More than 40 bands were observed both in the cytosolic and membrane fraction from the infected and uninfected macrophages (data not shown). More strikingly, one of the proteins of cytosolic fraction from *M. tuberculosis*-infected macrophages was highlighted prominently compared to the cytosolic fraction from uninfected macrophages *in vitro* (Figure 1). This protein has been identified consistently on five occasions, when the experiment was repeated.

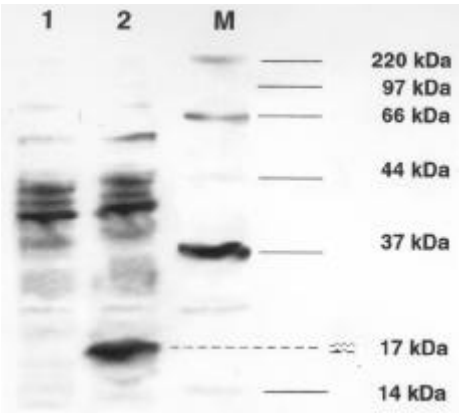


**Figure 1.** Unique protein of the cytosolic fraction from *M. tuberculosis*-infected macrophages as shown by autoradiogram. Lanes 1 and 2 represent cytosolic fractions from uninfected macrophages and *M. tuberculosis*-infected macrophages, respectively. Lane M represents the molecular weight marker. The dotted line points to the unique protein expressed in *M. tuberculosis*-infected macrophages. Equivalent counts (cts) (270,000 cts $min^{-1}$) were applied to each lane.

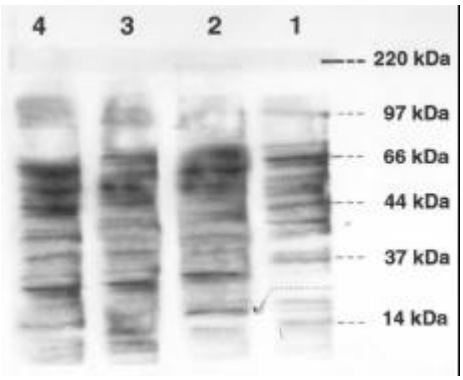


**Figure 2.** Differential expression of a protein by intracellular *M. tuberculosis* compared to cell-free system. Lanes 1 to 4 represent cytosolic fractions from uninfected macrophages and *M. tuberculosis*-infected macrophages, sonicate antigen and culture filtrate antigen from *M. tuberculosis*, respectively. Arrow points to the unique protein expressed by *M. tuberculosis* infected macrophages. Equivalent counts (cts) (250,000 cts $min^{-1}$) were applied to each lane.

The unique protein was not expressed in a cell-free system where *M. tuberculosis* is cultured in RPMI or Dubos broth without macrophages. Figure 2 shows the pattern obtained with sonicate and culture filtrate antigen from $^{35}S$ methionine-labelled bacilli resolved in SDS-PAGE, in parallel with the cytosolic fraction from infected and uninfected macrophages. The sonicate and culture filtrate antigen does not show the unique band of molecular weight around 17 kDa, implicating that this protein is produced by *M. tuberculosis* on entry into the macrophages.

Further, to analyse whether viable bacilli alone are capable of inducing this protein, macrophages were infected with heat-killed *M. tuberculosis*. The cytosolic fraction of the macrophages infected either with heat-killed *M. tuberculosis* or heat-killed yeast did not express this protein (Figure 3). Other intracellular pathogens like *L. monocytogenes* also did not induce this protein on phagocytosis. *M. bovis* BCG-infected macrophages induced this protein to a lower level (data not shown).

The next question was to find out the origin of the cytosolic protein; whether it is synthesized by macrophages upon infection with *M. tuberculosis* or synthesized by *M. tuberculosis* after getting phagocytosed. Cycloheximide is known to inhibit the protein synthesis of eukaryotes. Hence cycloheximide at two different concentrations of 50 and 100 µg/ml was included in the experiment. At a concentration of 100 µg/ml, cycloheximide was able to inhibit the protein synthesis of the macrophages. Among the mycobacterial proteins visible in the autoradiogram, the unique band with a molecular weight of around 17 kDa was also observed only in the infected macrophages in the presence of cycloheximide (Figure 4). This clearly reveals that this protein is produced by *M. tuberculosis* following entry into macrophages.

*M. tuberculosis* infection is usually transmitted via the air-borne route. This intracellular pathogen, after entry into the body, invades the macrophages. The macrophages act as reservoirs, where it can remain for years before embarking an orgy of destruction in the victims lungs, that leads to disease and illness[5]. Our understanding of the pathogenicity has often lagged behind knowledge of host responses to infection. An understanding of the mechanism of bacterial pathogenesis is dependent on the identification and characterization of the microbial gene product that influences the progression of the disease.

Virulent tubercle bacilli multiply freely within the macrophages[6]. Unlike the other intracellular pathogens whose virulence factors are known like adhesins, invasins and hemolysins, we do not know the virulence factor for *M. tuberculosis*. Over the years, a number of systems have been tried for the study of mycobacterial virulence. Arruda *et al.*[7] used a non-invasive strain of *Escherichia coli* as a surrogate host, to identify the DNA fragment responsible for the ability of *M. tuberculosis* to bind to and subsequently invade HeLa cells. Kinger and Tyagi[8] analysed the differences in gene expression between the avirulent H37Ra and virulent H37Rv using susbtractive hybridization. They identified 5 mRNAs present in both strains, but preferentially expressed in the virulent strain. Mundayoor and Shinnick[9] used a library of *M. tuberculosis* DNA in *M. smegmatis* and identified genes that increased the intracellular survival of the bacteria transiently, but the genes were never fully characterized. A macrophage-induced gene or mig was identified. This encodes a 27 kDa protein of unknown function and is absent in the *M. tuberculosis* culture[10]. The study of virulence has also been attempted using a new technology called *in vivo*

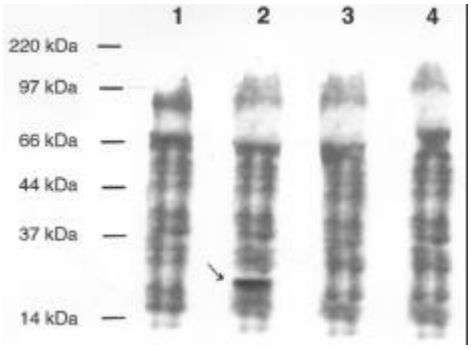


**Figure 3.** Comparison of protein synthesis pattern of *M. tuberculosis*-infected macrophages with those infected with heat-killed *M. tuberculosis* and yeast. Lanes 1 to 4 represent cytosolic fractions from uninfected macrophages and *M. tuberculosis*-infected macrophages, heat-killed yeast-phagocytised macrophages and heat-killed *M. tuberculosis*-phagocytised macrophages, respectively. The arrow points to the unique protein expressed by the cytosolic fraction from *M. tuberculosis*-infected macrophages. Equivalent counts (cts) (280,000 cts $min^{-1}$) were applied to each lane.

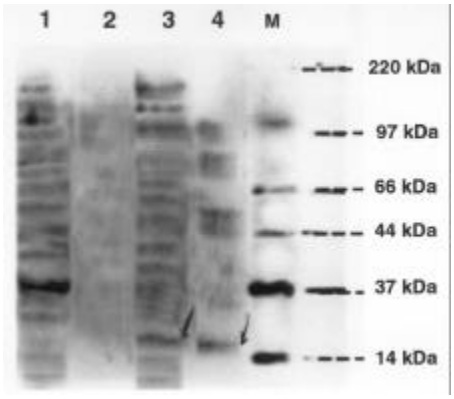


**Figure 4.** SDS-PAGE followed by autoradiogram shows that intracellular *M. tuberculosis* synthesize 17 kDa protein. Lanes 1 to 4 represent cytosolic fractions from uninfected macrophages, cycloheximide-treated uninfected macrophages, *M. tuberculosis*-infected macrophages and *M. tuberculosis*-infected cycloheximide-treated macrophages, respectively. Lane M represents the molecular weight marker. The arrows point to the unique protein expressed by the cytosolic fraction from *M. tuberculosis*-infected macrophages and from *M. tuberculosis*-infected cycloheximide-treated macrophages. Equivalent counts (cts) (300,000 cts $min^{-1}$) were applied to each lane.

expression technology or IVET, which can positively select bacterial genes that are induced within their host and this technique has been successfully used in Salmonella[11]. In spite of similar attempts, the virulence factor of *M. tuberculosis* has not been identified and characterized.

It is possible that tubercle bacilli may undergo important phenotypic changes *in vivo*, which contribute to virulence. So we have used a new strategy of identifying directly, the protein which is differentially expressed by *M. tuberculosis* in the intracellular environment of the guinea pig macrophages *in vitro*. We have attempted to analyse the protein synthesis patterns associated with host–pathogen interactions by labelling the proteins synthesized soon after the entry of *M. tuberculosis* inside the guinea pig macrophages *in vitro* with $^{35}S$ methionine, followed by ultracentrifugation, SDS-PAGE and autoradiography. It will be interesting to further characterize the proteins of *M. tuberculosis* induced inside the macrophages and study their role in the pathogenesis.

We now report a protein of molecular weight of around 17 kDa, produced by *M. tuberculosis* only inside the intracellular environment of guinea pig macrophages cultured *in vitro*. Future experiments will be focused on isolation, purification and characterization of this unique protein using immunological and molecular biological methods and to study whether this protein has any role in the pathogenesis of the disease. Recently, Yuan *et al.*[12] reported a protein of molecular weight 16 kDa limited to the slowly-growing *M. tuberculosis* complex organisms. 16 kDa protein was over expressed during the stationary phase of growth. We have observed earlier that the tubercle bacilli after being phagocytosed by the macrophages remain stationary up to 72 h without multiplication, as shown by our intracellular killing assay (unpublished observation). It will be interesting to see whether this 17 kDa protein has a role to play in the intracellular survival of *M. tuberculosis*.

Demonstration of the presence of a unique putative protein or virulence determinant *in vivo* is one of the difficult tasks. As a first step, we have demonstrated that the unique protein is produced by *M. tuberculosis* in a host environment *in vivo*.

Future research should unravel the role of this protein in the pathogenesis of the disease and may suggest targets for therapeutic intervention to eradicate such organisms.

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