

Effect of *Mycobacterium tuberculosis* and its components on macrophages and the release of matrix metalloproteinases

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Background – Pulmonary tuberculosis is associated with caseating necrosis, parenchymal lung destruction, and cavity formation. It was hypothesised that tuberculous lung destruction is mediated, at least in part, by the participation of matrix metalloproteinases released by mononuclear phagocytes.

Methods – Cells of the myelomonocytic leukaemia cell line THP-1 were incubated with lipoarabinomannan (LAM), the major antigenic cell wall component, and with *Mycobacterium tuberculosis* and analysed by Northern blot analysis. Two patients with active cavitary tuberculosis also underwent bronchoalveolar lavage and the cells were analysed by Northern blotting.

Results – Incubation of THP-1 cells with LAM resulted in the stimulated release of matrix metalloproteinase-9 (MMP-9), a 92 kDa gelatinase, by 24 hours in a dose-dependent fashion. In addition, Northern analysis revealed that LAM upregulated the gene for MMP-9 by 24 hours, but not the gene for the 72 kDa gelatinase MMP-2. Heat killed *M tuberculosis* H37Ra also upregulated the MMP-9 gene. Bronchoalveolar lavage of the two patients with active cavitary tuberculosis showed striking upregulation of the MMP-9 gene compared with a normal control using Northern analysis. LAM also upregulated the type I interstitial collagenase (MMP-1) gene by 24 hours in both THP-1 cells and peripheral blood monocytes.

Conclusions – These data suggest that *M tuberculosis* and its major cell antigenic component, LAM, stimulate the release of MMP-9 and upregulate the expression of genes for MMP-1 and MMP-9. It is possible that *M tuberculosis* and its components contribute directly to cavity formation by their ability to stimulate macrophages to release matrix metalloproteinases that digest collagens I-IV, and indirectly by stimulating the release of the cytokines interleukin 1 β and tumour necrosis factor α that induce fibroblasts to amplify the release of matrix metalloproteinases.

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to destruction of lung tissue with cavity formation. The granulomatous lesion is typically a cell mediated response involving macrophages and lymphocytes as the immunoresponsive cells. Fibroblasts, one of the major connective tissue cells, are also involved in the course of the acute and chronic phase of granulomatous inflammation as evidenced by the deposition of collagen fibres in the granulomatous lesion.¹ Advanced pulmonary tuberculosis is associated with a locally destructive process of cavitary lesions which plays an important part in transmission of disease. The pathogenesis of the destructive lung disease caused by *Mycobacterium tuberculosis* is still poorly understood. Dannenberg² suggested that the tissue damage such as liquefaction, caseation, and cavity formation was the result of a hypersensitivity reaction – that is, excess bacillary antigen causing host tissue or cells to produce cytotoxic cytokines, oxygen radicals, hydrolytic enzymes and eventual cell death.³ Since the degradation of connective tissue matrix involves an initial extracellular cleavage of insoluble proteins mediated by neutral proteinases such as elastases and collagenases,⁴ it would be important to assess neutral matrix metalloproteinase activity in host cells during tuberculosis infection.⁵ To test the hypothesis that *M tuberculosis* or its components modulate the synthesis of matrix metalloproteinases, especially collagenases, we have studied the effects of *M tuberculosis* and lipoarabinomannan (LAM), the major antigenic component of the mycobacterial cell wall,^{6,7} on the stimulation of matrix metalloproteinases in human peripheral blood monocytes and a myelomonocytic leukaemia cell line (THP-1).⁷ Bronchoalveolar lavage (BAL) was also performed in two patients with active cavitary tuberculosis to obtain BAL cells to assess expression of the matrix metalloproteinase-9 gene. Since we⁸ and others⁹⁻¹³ have shown that LAM can stimulate the release of tumor necrosis factor α (TNF- α) and interleukin 1 β (IL-1 β) from mononuclear phagocytes, and since these two cytokines are known to stimulate collagenase production in synovial cells,^{14,15} we evaluated gene expression of the type I interstitial collagenase following stimulation of human lung fibroblasts.

Methods

CELL CULTURE AND STIMULATION

Lipoarabinomannan (LAM) from laboratory attenuated *Mycobacterium* spp was kindly provided by Dr P Brennan, Fort Collins, Colorado,

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Central caseating necrosis is the hallmark of tuberculous granulomas. Tissue caseation leads

USA. Evaluation of tuberculosis reagents for the presence of Gram negative bacterial endotoxin was performed with the amoebocyte lysate assay (E-Toxate Kit, Sigma). A lipopolysaccharide (LPS) standard curve was generated with an assay limit of 1 pg/ml LPS. Six different batches of LAM from *Mycobacterium* spp were tested and all contained <5 pg/ml LPS or <5 pg/ μ g test reagent. The LAM had been passed through a Detoxigel column to remove any potential LPS. Lipopolysaccharide from *Escherichia coli* was purchased from Sigma; *M. tuberculosis* H37Ra (heat killed) was obtained from American Type Culture Collection (ATCC), Rockville, Maryland; human lung fibroblasts (CCL 190) and the myelomonocytic leukaemia cell line (THP-1) were obtained from ATCC; human collagenase cDNA (pC11ase 1) was obtained from ATCC and was cloned from TPA treated human skin fibroblasts from a healthy 29 year old man; the 92 kDa and 72 kDa cDNAs for matrix metalloproteinases were a gift of Dr Gregory Goldberg, Washington University, St Louis, Missouri; and the pHe7 cDNA probe was used as a control housekeeping gene.

THP-1 cells were grown in RPMI 1640 medium at 37°C, 95% air/5% carbon dioxide supplemented with 10% fetal calf serum and 100 U/ml penicillin plus 100 μ g/ml streptomycin. Peripheral blood monocytes were isolated from human buffy coat obtained from tuberculin negative individuals. Mononuclear cells were isolated from blood components by lymphocyte separation medium (LSM, Teknita Corp, Durham, North Carolina), centrifuged and washed three times with RPMI 1640. Mononuclear cells were then suspended in RPMI supplemented with 10% fetal calf serum and antibiotics. The cells were plated in a 250 ml culture flask at 2×10^6 cells/ml. After allowing the cells to adhere for 1.5 hours at 37°C the cells were washed three times with ice cold phosphate buffered saline (PBS). The adherent cells, which consisted of more than 95% monocytes, were exposed to test reagents for up to 24 hours in serum-free media.

Human lung fibroblasts were cultured at 37°C in humidified 90% air/10% carbon dioxide in DMEM supplemented with 10% fetal calf serum and penicillin plus streptomycin. At confluence, fibroblasts were treated with IL-1 α (5 U/ml) or TNF- α (10 ng/ml) in serum-free medium.

BRONCHOALVEOLAR LAVAGE

Bronchoalveolar lavage was performed with a flexible fiberoptic bronchoscope with local xylocaine anaesthesia. Normal saline (3×50 ml aliquots) was instilled into the radiographically involved site in tuberculosis patients, and 5×20 ml aliquots were instilled in three different sites and pooled for the normal volunteer. The recovered fluid was filtered through sterile gauze. A total cell count was performed in a haematocytometer and cell viability was determined by trypan blue exclusion. Bronchoalveolar lavage cells were immediately placed in 5.5 M guanidinium isothiocyanate buffer and

frozen at -70°C. Two patients with active pulmonary tuberculosis confirmed by sputum culture of *M. tuberculosis* with radiographic cavities and one normal volunteer were lavaged. All three individuals were HIV negative and both patients with active tuberculosis had received isoniazid, rifampicin, pyrazinamide, and ethambutol for two weeks before lavage. The clinical research protocol had been approved by the human subjects review committees of New York University Medical Center and Bellevue Hospital.

NORTHERN ANALYSIS

Preparation of total RNA from monocytes, THP-1 cells, or bronchoalveolar lavage cells was carried out according to the guanidinium isothiocyanate method. Briefly, cells were washed three times and lysed by 5.5 M guanidinium solution containing 5 mM sodium citrate, 0.5% Sarkosyl, and 1% β -mercaptoethanol. The RNA was then pelleted through a caesium chloride gradient and dissolved in RNA suspension buffer (0.5% SDS, 10 mM EDTA, 10 mM Tris pH 8.0, 0.2 M NaCl).

Electrophoresis of the sample was performed in a 1% agarose/7% formaldehyde gel and the gel was transferred to a NYTRAN membrane (Schleicher and Schuell, Keene, New Hampshire, USA) by capillary blotting. NYTRAN filters were hybridised with respective cDNAs labelled with 32 P using random primer labelling (Boehringer Mannheim). Hybridisations were carried out for 20 hours at 42°C and washing was performed three times for 30 minutes in $2 \times$ SSC/0.1% SDS at 22°C and then twice for 30 minutes in $0.1 \times$ SSC/0.1% SDS at 65°C. Filters were exposed to Kodak X-Omat AR film with intensifying screens at -70°C for 2-8 days.

SUBSTRATE GEL ELECTROPHORESIS

Proteinase profiles were determined by substrate gel electrophoresis using 8% acrylamide gels containing gelatin at a final concentration of 4.75 mg/ml.¹⁶ Conditioned media from THP-1 cells that had been stimulated with varying concentrations of LAM or LPS (500 pg/ml, 1 ng/ml, 500 ng/ml, 1 μ g/ml) were subjected to SDS-PAGE under non-reducing conditions at 22°C. Each lane was loaded with 30 μ g protein. After electrophoresis the gels were washed twice for 30 minutes with 2.5% Triton-X-100 to remove SDS. Gels were then briefly rinsed with deionised water and then incubated overnight at 37°C in buffer containing 50 mM Tris HCl, 150 mM NaCl, and 5 mM CaCl₂ (pH 7.4). Following incubation, gels were stained with 0.1% Coomassie brilliant blue and destained. Proteolytic activity appeared as clear zones against a dark blue background.

Results

DEMONSTRATION OF MMP-9 AND MMP-2

Substrate gel electrophoresis demonstrated a gelatinase with an apparent molecular mass of

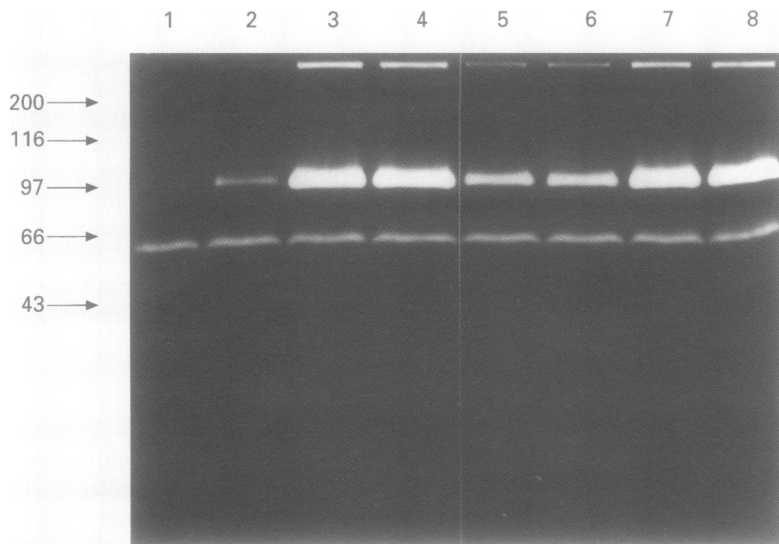


Figure 1 Substrate gel electrophoresis of conditioned media collected at 24 hours from THP-1 cells. Lane 1, medium alone (negative control); lane 2, LAM (500 pg/ml); lane 3, LAM (500 ng/ml); lane 4, LAM (1 μ g/ml); lane 5, LPS (500 pg/ml); lane 6, LPS (1 ng/ml); lane 7, LPS (500 ng/ml); lane 8, LPS (1 μ g/ml). Molecular weight markers (kDa) are indicated along the margins.

92 kDa that was stimulated by LAM and LPS in supernatants from THP-1 cells. The 92 kDa gelatinase (MMP-9) was barely detectable by two hours (data not shown) but was prominent at 24 hours (fig 1). A dose-response analysis revealed that the gelatinase was discernible at LAM doses of 500 pg/ml and maximally stimulated at 500 ng/ml. Other matrix metalloproteinases detected in the THP-1 supernatants were a 230 kDa gelatinase which may be a higher molecular weight aggregate of MMP-9. The 72 kDa gelatinase (MMP-2) was constitutively expressed.

NORTHERN ANALYSIS OF MMP-9 AND MMP-2

Incubation of THP-1 cells for 24 hours with LAM (2 μ g/ml) or LPS (1 μ g/ml) revealed upregulation of MMP-9 by LAM or LPS by 24 hours in comparison with the unstimulated control (fig 2); there was no detectable upregulation at two hours. Equal amounts of total RNA were loaded in each lane as shown by the pHe 7 housekeeping gene. These data are consistent with the increased protein release and time course after LAM or LPS stimulation of THP-1 cells by substrate gel electrophoresis. MMP-2 was constitutively expressed at the gene level (fig 2), similar to the observation made with the zymogram.

Incubation of THP-1 cells with *M tuberculosis* H37Ra (heat killed) revealed upregulation of MMP-9 similar to that with LPS (fig 3).

Northern analysis of bronchoalveolar lavage cells from two patients with active cavitory pulmonary tuberculosis and one normal volunteer demonstrated striking upregulation of the gene for MMP-9 in both tuberculosis patients compared with the normal volunteer (fig 4). A small amount of constitutive expression of MMP-9 could be detected in the normal volunteer and equal amounts of total RNA were loaded in each lane as demonstrated by the β -actin housekeeping gene.

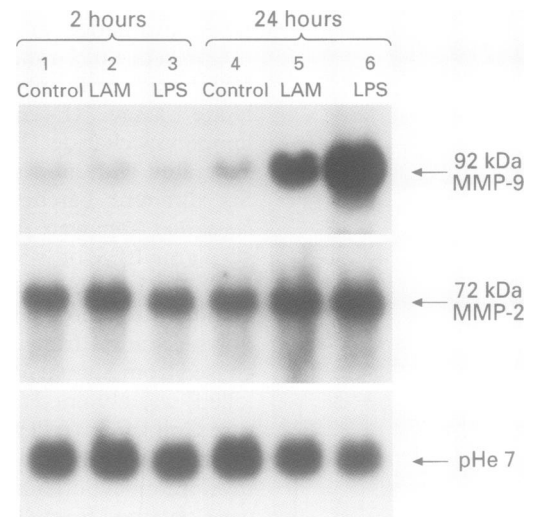


Figure 2 Northern analysis of 92 kDa and 72 kDa matrix metalloproteinases (MMP-9 and MMP-2). THP-1 cells were incubated with LAM (2 μ g/ml) or LPS (1 μ g/ml) for two hours (lanes 1-3) or 24 hours (lanes 4-6). Lanes 1 and 4, unstimulated cells; lanes 2 and 5, LAM; lanes 3 and 6, LPS.

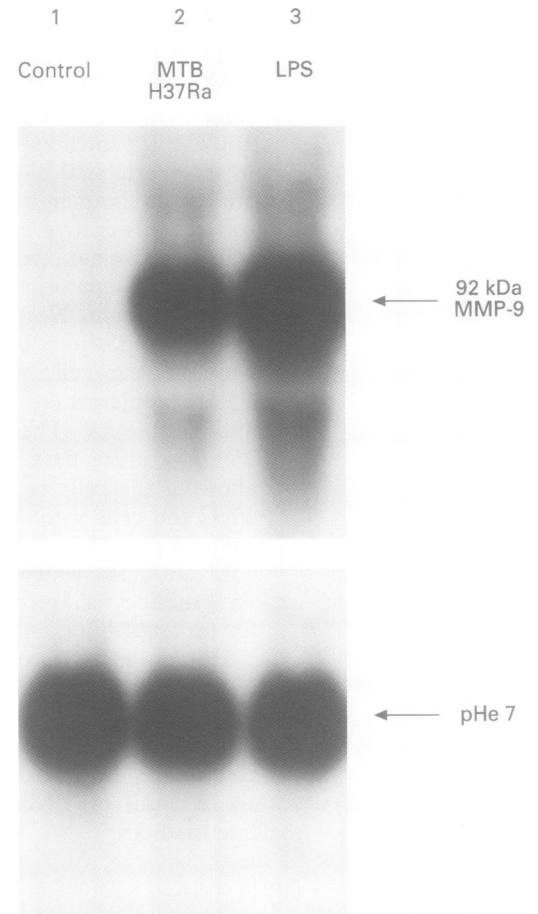


Figure 3 Northern analysis of MMP-9 in THP-1 cells. THP-1 cells were incubated with *M tuberculosis* H37Ra (1 μ g/ml) or LPS (1 μ g/ml) for 24 hours and total RNA extracted. Lane 1, unstimulated cells; lane 2, *M tuberculosis* H37Ra; lane 3, LPS.

NORTHERN ANALYSIS OF MMP-1

Incubation of THP-1 cells with LAM induced an increase in type I interstitial collagenase (MMP-1) mRNA compared with unstimulated cells as early as two hours (fig 5A) and was significantly upregulated at 24 hours (fig 5B) and persisted up to 48 hours of incubation.

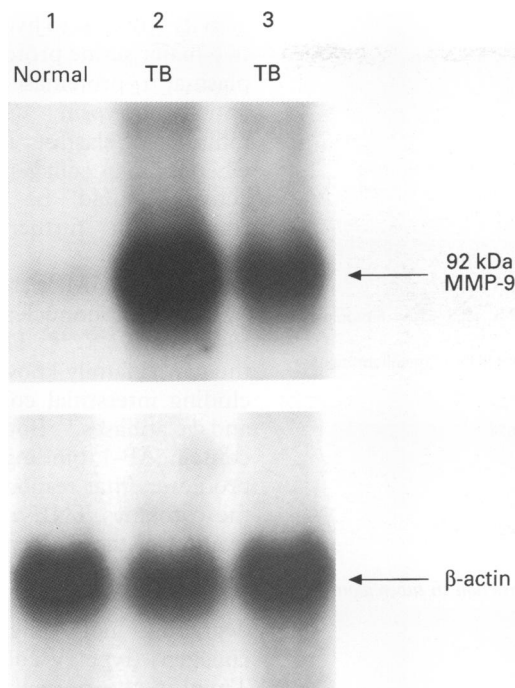


Figure 4 Northern analysis of bronchoalveolar lavage (BAL) cells. Total RNA from BAL cells was run on an agarose-formaldehyde gel and filters were probed with MMP-9 cDNA. Lane 1, normal volunteer; lanes 2 and 3, tuberculosis patients.

Equal amounts of RNA were loaded in each lane as demonstrated by ethidium bromide staining of the gel. The ability of LAM to induce MMP-1 mRNA expression was similar to that with LPS. Incubation of peripheral blood monocytes with LAM induced an increase in MMP-1 mRNA at 24 hours (fig 6). Ethidium bromide stained gels demonstrated equal amounts of RNA in each lane. Incubation of human lung fibroblasts with TNF- α , IL-

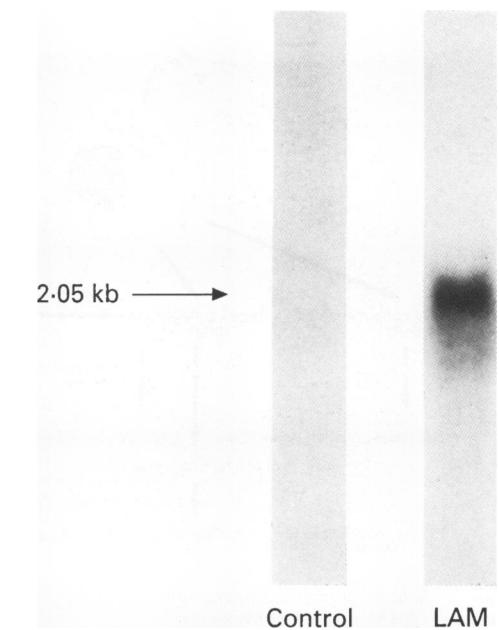


Figure 6 Analysis of steady state MMP-1 mRNA levels in peripheral blood monocytes. Human peripheral blood monocytes were stimulated for 24 hours with LAM (2 μ g/ml) and equal amounts of total RNA (35 μ g) were extracted as determined by ethidium bromide gel staining.

1 α , or tetradecanoyl-phorbol-13-acetate (TPA) induced an increase in MMP-1 mRNA (data not shown).

Figure 7 illustrates a proposed scheme of how *Mycobacterium* spp could stimulate mononuclear phagocytes to upregulate the matrix metalloproteinase genes in macrophages and release human proteinases. Macrophage release of TNF- α and IL-1 β can lead to fibroblast synthesis of MMP-1 which amplifies the degradation of collagen types I-IV. MMP-1 inhibits α_1 -antitrypsin leading to enhanced elastolytic activity with digestion of elastin and fibronectin resulting in tissue destruction, caseation necrosis, and cavity formation.

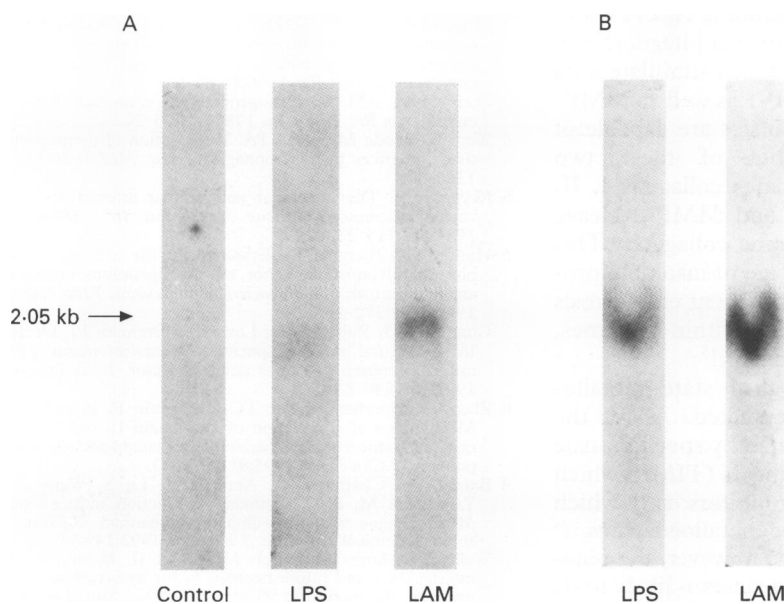


Figure 5 Northern analysis of type I interstitial collagenase gene (MMP-1) for (A) two hours and (B) 24 hours with LPS (1 μ g/ml) and LAM (2 μ g/ml). THP-1 cells were grown to a density of 5×10^5 cells/ml in 10% fetal calf serum and treated with stimuli for various time points. Total RNA was then isolated and an equal amount of RNA from each sample, as determined by ethidium bromide gel staining, was electrophoresed through a 1% denaturing agarose gel containing 7% formaldehyde. A 2.05 kb mRNA transcript for MMP-1 was noted at two hours. Upregulation of the MMP-1 gene by LPS or LAM was seen at two hours and persisted to 24 hours.

Discussion

We have shown that *M. tuberculosis* H37Ra and LAM can induce the formation of the 92 kDa gelatinase MMP-9 in THP-1 cells and the type I interstitial collagenase MMP-1 mRNA in THP-1 cells and human peripheral blood monocytes. In two patients with active cavitary tuberculosis we found striking upregulation of the MMP-9 gene in bronchoalveolar lavage cells. MMP-9 can be upregulated by peptide growth factors and cytokines in mononuclear phagocytes.^{16,17} The induction of MMP-1 mRNA by LAM in mononuclear phagocyte cells is also considered significant for the following reasons: (a) unstimulated cells do not express the type I interstitial collagenase gene¹⁸; (b) several studies have shown that immature mononuclear phagocytes (U-937 cells and peripheral blood monocytes) under basal conditions contain predominantly neutrophil elastase and maturation of these cells into resident macrophages by phorbol ester is associated with an increased synthesis of matrix metalloproteinases which include MMP-1, MMP-2, and MMP-9, and the stromelysins

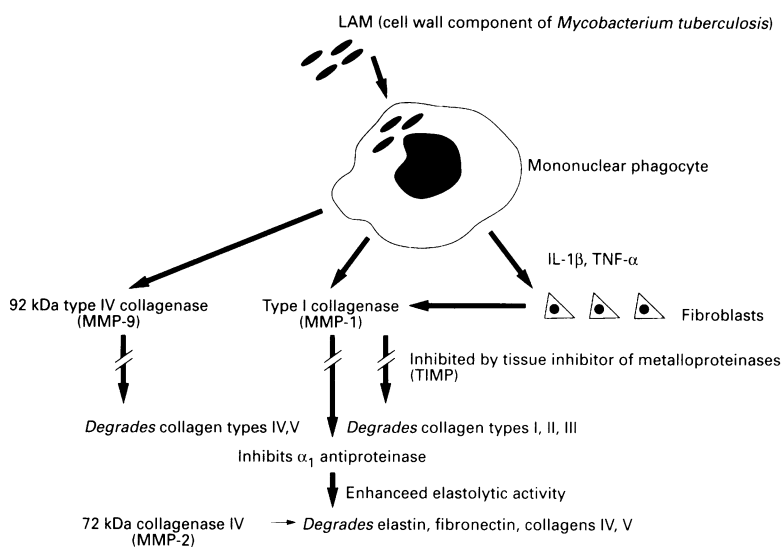


Figure 7 Scheme of proposed mechanisms of parenchymal destruction in tuberculous granuloma. LAM = lipoarabinomannan.

MMP-3, MMP-10 and MMP-11¹⁹⁻²¹; (c) monocytes secrete much smaller quantities of procollagenase upon stimulation than do macrophages.²² All of the matrix metalloproteinases cleave gelatin and fibronectin at the same rate but MMP-1 is unique in its ability to cleave interstitial collagens.¹⁷ Thus, *M tuberculosis* and LAM are potent agents for the induction of MMP-1 mRNAs and MMP-9.

LAM, the major antigenic cell wall component in *Mycobacterium* spp,⁷⁻⁹ can induce MMP-1 mRNA indirectly in human lung fibroblasts through the release of TNF- α and IL-1 β from monocyte macrophages. The released collagenases may participate in the destruction of lung extracellular matrix by the following mechanism. MMP-9 can degrade several native collagens including types IV and V. LAM stimulates mononuclear phagocytes to release IL-1 β and TNF- α which stimulate lung fibroblasts to release MMP-1 as well as MMP-2 and, interestingly, fibroblasts are capable of releasing greater quantities of these two MMPs.^{16,17} MMP-1 degrades collagens I, II, III, and X and MMP-9 and MMP-2 cleave type IV basement membrane collagen.²³ This initial collagenolytic cleavage of insoluble protein is essential for the subsequent endocytosis and completion of digestion within lysosomes, followed by tissue remodelling.^{4,5}

In a normal physiological state metalloproteinases are tightly regulated. All of the metalloenzymes are inhibited by specific tissue inhibitors of metalloproteinases (TIMP) which are ubiquitous natural inhibitors and which form complexes with these metalloenzymes.^{5,24} In pulmonary tuberculosis, however, the regulatory mechanism of collagenases is likely to be altered in favour of collagenase activity. TNF- α and IL-1 β stimulate MMP gene expression¹⁷ but do not affect the level of TIMP.²⁵ This is in contrast to the effect of TPA which stimulates both collagenase and TIMP in skin fibroblasts.²⁶ In addition to collagenolytic activity, MMP-1 has been shown to express serpinase

activity – that is, to hydrolyse and inactivate the two major serine proteinase inhibitors found in plasma, α_1 -proteinase inhibitor (α_1 PI) and α_1 -antichymotrypsin.²⁷ Since α_1 PI is the major inhibitor of elastase, in the microenvironment where human collagenases are present elastase activity would be relatively uninhibited, resulting in further destruction of lung parenchyma.

All of the MMPs are expressed and secreted by human mononuclear phagocytes in response to stimuli.^{16,17,20,22,28} There are 11 members of the MMP family known as endopeptidases including interstitial collagenases, stromelysins, and gelatinases.¹⁷ Both MMP-1 and MMP-9 contain AP-1 binding sites in their 5' DNA promoters that respond to phorbol esters and the cytokines TNF- α and IL-1 β through activation of the *jun* gene.^{17,29} MMP-9 is readily secreted by monocytes.¹⁶ It has been referred to as type IV collagenase and is capable of degrading not only basement membrane collagens (type IV collagen) but also elastin.²⁸ Thus, in the presence of uninhibited matrix metalloproteinases, the destruction of lung matrix is the sum of collagenolytic and elastolytic activities. We propose that *M tuberculosis* and its major cell wall antigen LAM play an important part in the pathogenesis of lung damage associated with pulmonary tuberculosis through the stimulation and release of collagenases by mononuclear phagocytes and, indirectly, by fibroblasts.

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