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Y Zhang, … , B Guillemin, W N Rom

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Research Article

The granulomatous immune response in tuberculosis is characterized by delayed hypersensitivity and is mediated by various cytokines released by the stimulated mononuclear phagocytes, including tumor necrosis factor-alpha (TNF alpha) and IL-1 beta. We have demonstrated that Mycobacterium tuberculosis cell wall component lipoarabinomannan (LAM), mycobacterial heat shock protein-65 kD, and M. tuberculosis culture filtrate, devoid of LPS as assessed by the Amebocyte Lysate assay, stimulate the production of TNF alpha and IL-1 beta proteins and mRNA from mononuclear phagocytes (THP-1 cells). The effect of LAM on the release of these cytokines was specific, as only LAM stimulation was inhibited by anti-LAM monoclonal antibody. Interestingly, we found that LAM and Gram-negative bacterial cell wall-associated endotoxin LPS may share a similar mechanism in their stimulatory action as demonstrated by inhibition of TNF alpha and IL-1 beta release by monoclonal antibodies to CD14. Anti-CD14 monoclonal antibody MY4 inhibited both TNF alpha and IL-1 beta release with LAM and LPS but no effect was observed with other mycobacterial proteins. An isotype antibody control did not inhibit release of cytokines under the same experimental conditions. M. tuberculosis and its components upregulated IL-1 beta and TNF alpha mRNAs in THP-1 cells. Nuclear run-on assay for IL-1 beta demonstrated that LAM increased the transcription rate. The induction of IL-1 beta was regulated at the transcriptional level, in which […]

Mechanisms of Stimulation of Interleukin-1β and Tumor Necrosis Factor-α by *Mycobacterium tuberculosis* Components

Yihong Zhang, Martin Doerfler, Theodore C. Lee, Benedicte Guillemin, and William N. Rom
Division of Pulmonary and Critical Care Medicine, Departments of Medicine and Environmental Medicine and Chest Service, Bellevue Hospital Center, New York University Medical Center, New York 10016

Abstract

The granulomatous immune response in tuberculosis is characterized by delayed hypersensitivity and is mediated by various cytokines released by the stimulated mononuclear phagocytes, including tumor necrosis factor-α (TNFα) and IL-1β. We have demonstrated that *Mycobacterium tuberculosis* cell wall component lipoarabinomannan (LAM), mycobacterial heat shock protein-65 kD, and *M. tuberculosis* culture filtrate, devoid of LPS as assessed by the Amebocyte Lysate assay, stimulate the production of TNFα and IL-1β proteins and mRNA from mononuclear phagocytes (THP-1 cells). The effect of LAM on the release of these cytokines was specific, as only LAM stimulation was inhibited by anti-LAM monoclonal antibody. Interestingly, we found that LAM and Gram-negative bacterial cell wall–associated endotoxin LPS may share a similar mechanism in their stimulatory action as demonstrated by inhibition of TNFα and IL-1β release by monoclonal antibodies to CD14. Anti-CD14 monoclonal antibody MY4 inhibited both TNFα and IL-1β release with LAM and LPS but no effect was observed with other mycobacterial proteins. An isotype antibody control did not inhibit release of cytokines under the same experimental conditions. *M. tuberculosis* and its components upregulated IL-1β and TNFα mRNAs in THP-1 cells. Nuclear run-on assay for IL-1β demonstrated that LAM increased the transcription rate. The induction of IL-1β was regulated at the transcriptional level, in which these stimuli acted through *cis*-acting element(s) on the 5′ flanking region of the IL-1β genomic DNA. *M. tuberculosis* cell wall component LAM acts similarly to LPS in activating mononuclear phagocyte cytokine TNFα and IL-1β release through CD14 and synthesis at the transcriptional level; both cytokines are key participants in the host immune response to tuberculosis. (*J. Clin. Invest.* 1993.91:2076–2083.) Key words: tuberculosis • interleukin-Iβ • tumor necrosis factor-α • lipoarabinomannan • gene regulation

Introduction

Tuberculosis is characterized by cough, sputum production, chest pain, and systemic symptoms such as night sweats, fevers, chills, and weight loss that frequently exceeds 10 kg over several months (1). These symptoms are typical of the acute-phase response and are considered to be mediated by the cytokines tumor necrosis factor-α (TNFα)[1] and IL-1β released by mononuclear phagocytes (2–5). These cytokines have multiple actions that likely play a role in local granuloma formation and inflammation. It is now well established that mycobacteria and mycobacterial proteins can stimulate the release of IL-1β and TNFα (6–16)

Chensue and colleagues (9) reported that peripheral blood mononuclear cells from patients with active tuberculosis released increased amounts of IL-1β, and Takashima et al. (7) showed peripheral blood monocytes from active tuberculosis patients released increased amounts of TNFα in response to a stimulus compared with controls or patients with chronic refractory tuberculosis. TNFα release has also been shown to be higher in tuberculosis patients with fever rather than no fever (17) and was detected in the serum of Brazilian tuberculosis patients (18). In tuberculous pleuritis, high levels of (IFNγ) and TNFα have been found in pleural fluid, and mRNA for both cytokines was detected in pleural tissue by in situ hybridization (6). Moreno and colleagues (8) demonstrated that the mycobacterial cell wall product, purified lipoarabinomannan (LAM), stimulated TNFα from peripheral blood monocytes in a dose–response manner. Importantly, they confirmed that the ability of LAM to stimulate TNFα was apart from any contaminating LPS: they copurified LAM and TNFα-inducing activity over an affinity column containing a monoclonal antibody to LAM; purification over SDS-PAGE demonstrated TNFα-releasing activity in the molecular weight range of LAM; and LAM incubated in weak NaOH that inhibits LPS activity still stimulated TNFα (8). Barnes and colleagues (15) confirmed that weak alkali treatment removing acyl groups from the phosphoinositol membrane backbone of LAM greatly reduced the TNFα-inducing activity of LAM. They demonstrated that LAM and other cell wall products lacking carbohydrate moieties could induce TNFα whereas deacylated LAM could not. Using the polymerase chain reaction, monocytes expressed IL-1α, IL-1β, IL-6, IL-8, IL-10, TNFα, and granulocyte macrophage colony–stimulating factor after LAM stimulation (15). The mycobacterial cytosolic heat shock protein-65 kD (HSP-65kD) has been shown to release TNFα from cytotoxic effector cells (14). Lastly, TNFα has been found to be released by monocytes obtained from leprosy patients, especially those with the tuberculoid form or erythema nodosum leprosum, and *Mycobacterium leprae* proteins stimulated TNFα release in vitro (12, 13). In a murine model of BCG-in-

Address correspondence to Yihong Zhang, Division of Pulmonary and Critical Care Medicine, Bellevue Hospital Center, Rm. 7N24, New York University Medical Center, 550 First Avenue, New York, NY 10016.

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1. *Abbreviations used in this paper:* CAT, chloroamphenicol acetyl transferase; CFL, culture filtrate; HSP-65kD, heat shock protein-65 kD; LAM, lipoarabinomannan; LBP, LPS-LPS-binding protein; STBS, suspension of Tris-buffered saline; TK, thymidine kinase; TNFα, tumor necrosis factor-α.

duced granulomas (19), TNFα synthesis coincided with the development of granulomas. Injection of anti-TNFα antibody blocked accumulation of TNFα mRNA and protein, dramatically reduced the number and size of BCG-induced granulomas, and reduced the accumulation of large epithelioid cells.

We dissected the mechanisms of the host immune response to tuberculosis by evaluating the protein release and gene expression of IL-1β and TNFα in mononuclear phagocyte cells after stimulation with a variety of recombinant or purified proteins from *M. tuberculosis*. We evaluated a cell culture filtrate from *M. tuberculosis* Erdman strain, the purified *M. tuberculosis* cell wall component LAM from strains H37Ra and Erdman, live *M. tuberculosis* H37Ra, and the mycobacterial cytosol recombinant HSP-65kD. All of these mycobacterial products were carefully evaluated to assure that they were devoid of contaminating LPS. An anti-LAM antibody was used to evaluate the specificity of the effect. To determine how these *M. tuberculosis* proteins stimulated cytokines, we compared them to LPS after using anti-CD14 monoclonal antibodies and measured TNFα and IL-1β. Lastly, to determine the mechanism of IL-1β gene regulation, we isolated a 1.4-kb fragment of 5′ genomic DNA and evaluated the responsiveness of this fragment after stimulation with the same stimuli.

Methods

Cell culture and stimulation. Cells from a human myelomonocytic leukemic cell line, THP-1 (American Type Culture Collection, Rockville, MD), were maintained in RPMI supplemented with 10% fetal bovine serum (sterile filtered and LPS free). Cells were then placed in 24-well plastic tissue culture plates (Falcon Labware, Oxnard, CA) with a density of 10^6 cells per well. The cells were stimulated with various test agents for different time periods. The culture supernatant was then collected and stored at −70°C. Stimuli include the following: LPS 055 (*Escherichia coli*) from Sigma Chemical Co. (St. Louis, MO); recombinant human TNFα (sp act, 4.8×10^7 U/mg) kindly supplied by Dr. M. Tsujimoto, Suntory Institute for Biomedical Research (Osaka, Japan); recombinant human IL-1α (sp act 3×10^7 U/mg) kindly provided by Hoffman-LaRoche (Nutley, NJ); LAM from attenuated *M. tuberculosis* H37Ra and Erdman strains kindly provided by P. Brennan, Colorado State University (Ft. Collins, CO); *M. tuberculosis* (Erdman) culture filtrate protein (CFL) that was free of LAM and phosphoinositol mannoside after anion-exchange and molecular sieve chromatography, kindly provided by P. Brennan; live nonattenuated *M. tuberculosis* H37Ra (American Type Culture Collection); and *Mycobacterium bovis* recombinant HSP-65kD kindly provided by R. Van der Zee, National Institute of Public Health and Environmental Protection (Bilthoven, Netherlands). The CFL was devoid of LAM and phosphoinositol mannoside after anion-exchange chromatography that removed carbohydrate moieties and molecular sieve chromatography that excluded LAM on the basis of size. The LAM had been eluted through Detoxi-Gel (Pierce Chemical Co., Rockford, IL) using sterile pyrogen-free water and stored in pyrogen-free vials. Only pyrogen-free water was used in reconstitution of this material. Evaluation of tuberculosis reagents for the presence of Gram-negative bacterial endotoxin was done with the amebocyte lysate assay (E-toxate kit; Sigma Chemical Co.).

The ELISA kit for TNFα assay was from Biokine (Cambridge, MA), and the kit for IL-1β assay was kindly supplied by Dr. R. Dondero, Cistron Biotech (Pine Brook, NJ).

Inhibition of TNFα and IL-1β release by anti-CD14 monoclonal antibodies. Fresh human blood collected in citrate-coated tubes was cultured in 24-well tissue culture plates at a density of 5×10^6 nucleated cells/ml and incubated with LPS, LAM, HSP-65kD, or CFL in the presence or absence of either monoclonal anti-CD14 MY4 (10 μg/ml) or an isotype control (10 μg/ml) for 6 h. MY4 is a murine monoclonal antibody and member of CD14, which is a heterogeneous cluster of monoclonal antibodies recognizing mature monocytes (Coulter Immunology, Hialeah, FL). The isotype control MsIgG2b was obtained from the same source. The anti-CD14 3C10 monoclonal antibody was also of the IgG2 subclass and was a gift of R. Steinman (The Rockefeller University, New York). All of the monoclonal antibodies were used at a concentration of 10 μg/ml. Supernatants were evaluated for release of TNFα and IL-1β by ELISA.

Inhibition of TNFα and IL-1β release by anti-LAM monoclonal antibody. An anti-LAM monoclonal antibody (kindly provided by P. Brennan, Ft. Collins, CO) was added in increasing concentration to THP-1 cells cultured in 24-well tissue culture plates at a density of 10^6 cells/ml with LAM (500 ng/ml). In addition, we evaluated a dose–response of LPS, LAM, HSP-65kD, or CFL in the presence or absence of anti-LAM (3 μg/ml) and measured IL-1β and TNFα release. The anti-LAM monoclonal antibody ML9D3 from ascites fluid was at a titer of 1:1,600 and is a member of the 900 series of IgG3 subclass antibodies described by Gaylord et al. (20).

Isolation of RNA and Northern blot analysis. THP-1 cells were treated with test agents for specified time periods, collected by centrifugation, and lysed by addition of 5.5 M guanidinium isothiocyanate buffer. Cytoplasmic RNA was isolated through $CsCl_2$ gradient ultracentrifugation. Equal amounts of the extracted RNA were fractionated by electrophoresis through a 1% agarose–6% formaldehyde denaturing gel, transferred onto a nitrocellulose filter (BA 85; Schleicher & Schuell, Inc., Keene, NH), and baked at 80°C for 2 h. The baked filter was incubated in 40 ml of prehybridization solution (50% formamide, 0.5% SDS, 10× Denhardt's, 2.5% herring sperm DNA, and 4× SSPE) at 42°C for 12 h. An IL-1β cDNA probe (kindly provided by S. Gillis, Immunex, Seattle, WA), TNFα cDNA probe (Genentech, Inc., South San Francisco, CA), or pHe7 cDNA probe were radiolabeled with α-[^{32}P]dCTP (sp act 3,000 Ci/mmol; New England Nuclear, Boston, MA) by nick translation. Hybridization was carried out at 42°C for 10 h. The filter was then washed in 2× SSC/0.5% SDS at room temperature for 20 min followed by 0.1× SSC/0.5% SDS at 65°C for 30 min. Autoradiography was performed at −70°C for 9–48 h.

Nuclear run-on assay. THP-1 cells were stimulated for 1 h, collected by centrifugation, and washed with cold PBS. The cell pellet was incubated in 4 ml NP-40 lysis buffer (10 mM Tris, pH 7.5; 10 mM NaCl; 3 mM $MgCl_2$; 0.5% NP-40). The lysate was centrifuged for 500 *g* for 5 min at 4°C. The isolated nuclei were then stored in 200 μl glycerol buffer (50 mM Tris, pH 8.3; 40% glycerol; 5 mM $MgCl_2$; 0.1 mM EDTA) after gentle vortexing in liquid nitrogen.

Frozen nuclei (200 μl) were thawed immediately by adding 200 μl 2× reaction buffer (10 mM Tris, pH 8.0; 5 mM $MgCl_2$; 0.3 M KCl) containing nucleotides (1 ml 2× reaction buffer plus 10 μl of 0.1 M ATP, CTP, GTP, and 5 μl 1 M DTT) and 100 μCi α-[^{32}P]UTP (760 Ci/mM). The reaction was carried out at 30°C for 30 min with shaking. DNase I (Boehringer Mannheim Corp., Indianapolis, IN) was added to a final concentration of 40 μg/ml to the labeled nuclei and incubated 5 min at 30°C. To solubilize the nuclei, 200 μl of a mixture of 5% SDS; 0.5 M Tris, pH 7.4; 0.125 M EDTA; and 10 μl proteinase K (20 mg/ml) was added to the sample and incubated at 37°C for 30 min. The samples were then extracted with phenol and precipitated with TCA. The precipitates were treated with DNase I and the filter-binding RNA was eluted at 65°C for 10 min. The eluted RNA was digested again with proteinase K, denatured in NaOH, and precipitated with ethanol. Equal amounts of counts per minute > 3×10^6 cpm) was added to the nitrocellulose filter, on which denatured plasmid DNA was immobilized, in hybridization solution. After hybridization the filters were washed and autoradiography was performed at −70°C for 3 d.

Screening of human genomic library and plasmid construction. A human genomic placental library was purchased from Clontech (Palo Alto, CA). Using a cDNA and synthetic oligonucleotide sequence of IL-1β as probes, 3×10^6 plaques were screened. This resulted in eight positive clones of which one was used for subcloning. An IL-1β DNA fragment from −1130 to +290 was isolated with restriction enzyme

XbaI and subcloned into a polylinker site on plasmid vector pTK.CAT, which contains the herpes simplex virus thymidine kinase promoter and a chloramphenicol acetyl transferase (CAT) structural gene (kindly provided by Dr. Herbert Samuels, New York University Medical Center). The TK promoter was then removed by digestion with BamHI and BglII to produce the plasmid pTK(−)CAT.IL-1β.

Transient transfection of human suspension cultures and the assay of CAT activity. THP-1 cells were transfected with the plasmid pTK(−)CAT.IL-1β by the DEAE-dextran method (21). Briefly, 10^7 cells were washed in suspension of Tris-buffered saline (STBS) solution with the following composition (mM): 25 Tris, pH 7.4; 137 NaCl; 5 KCl; 0.6 Na_2HPO_4; 0.7 $CaCl_2$, 0.5 $MgCl_2$, transfected with 10 μg cesium chloride–purified plasmids in 1 ml STBS solution at 37°C for 90 min, and then shocked with 10% DMSO for 5 min. The transfected cells were then washed with STBS solution and incubated in complete medium for 24 h in the absence or presence of inducing agents. The transfected cells were lysed by three freeze-thawing cycles, and equal amounts of protein from different cell extracts were assayed for CAT activity (22). The protein concentration was determined with reagents (Bio-Rad Laboratories, Richmond, CA). For the CAT assay, 100 μg protein was incubated with 0.1 μCi [^{14}C]chloramphenicol; 250 mM Tris, pH 7.5; and 360 μg/ml acetyl coenzyme A in a total volume of 170 μl at 37°C for 5 h. The reactions were stopped by the addition of 0.5 ml of cold ethyl acetate. After extraction with ethyl acetate, the upper layer was dried and spotted onto a TLC plate. The plate was developed in 95% chloroform/5% methanol, air dried, and exposed to x-ray film. Radioactive spots were removed from the plate for scintillation counting.

Results

Evaluation of M. tuberculosis reagents for LPS contamination. The LAM, HSP-65kD, and CFL used in the experiments were first evaluated using the amebocyte lysate assay for LPS contamination. An LPS standard curve was generated and the limit of the assay was 1 pg/ml of LPS. Six batches of LAM used in these experiments contained < 10 pg LPS/1 μg LAM. The HSP-65kD, CFL, MY4, and C310 anti-CD14 monoclonal antibody contained 1 pg LPS/1 μg test reagent. A dose–response experiment demonstrated no release of TNFα from THP-1 cells at LPS concentrations of 10 pg/ml with a detectable release at 100 pg/ml similar to that shown by others (23).

Release of TNFα and IL-1β by THP-1 cells after stimulation by M. tuberculosis and its components. The human myelomonocytic leukemia cell line THP-1 is known to release cytokines similar to mononuclear phagocytic cells. After 24 h of stimulation with LPS or LAM, the release of TNFα and IL-1β by LPS was approximately one order of magnitude greater than LAM (Fig. 1, *A* and *B*). By contrast, the release of TNFα and IL-1β by HSP-65kD and CFL was almost two orders of magnitude less than LAM. We also confirmed that LAM from the virulent *M. tuberculosis* Erdman strain was barely able to elicit a cytokine response. In comparison with *M. tuberculosis* components, live nonattenuated *M. tuberculosis* H37Ra was able to stimulate a brisk release of TNFα and IL-1β protein. The amount of LAM was ~ 100 ng in 5.8×10^7 *M. tuberculosis* and was ~ 10 ng in 5.8×10^6 *M. tuberculosis* organisms. In addition to a dose–response, a time course demonstrated that approximately two thirds of the total TNFα and IL-1β release occurred over the first 6 h.

Anti-CD14 monoclonal antibodies block TNFα and IL-1β release. The cell surface protein CD14 binds the complex consisting of LPS and the LPS-binding protein (23). To determine whether *M. tuberculosis* components and LPS induce TNFα and IL-1β release by the same or different mechanisms, we compared the blocking ability of anti-CD14 monoclonal antibodies on this activity. Fig. 2, *A–D*, shows that anti-CD14 monoclonal antibody MY4 blocked induction of both TNFα and IL-1β by LPS and LAM from human blood over 6 h. The inhibition occurred over the dose range 100 pg/ml to 10 ng/ml for LPS and up to 100 ng/ml for LAM. An isotype IgG2b control monoclonal antibody had no effect (Fig. 2, *A–D, dotted lines*). As shown in Fig. 2, *C* and *D*, HSP-65kD and CFL were not inhibited by anti-CD14 monoclonal antibody MY4, suggesting a different mechanism of mononuclear phagocyte stim-

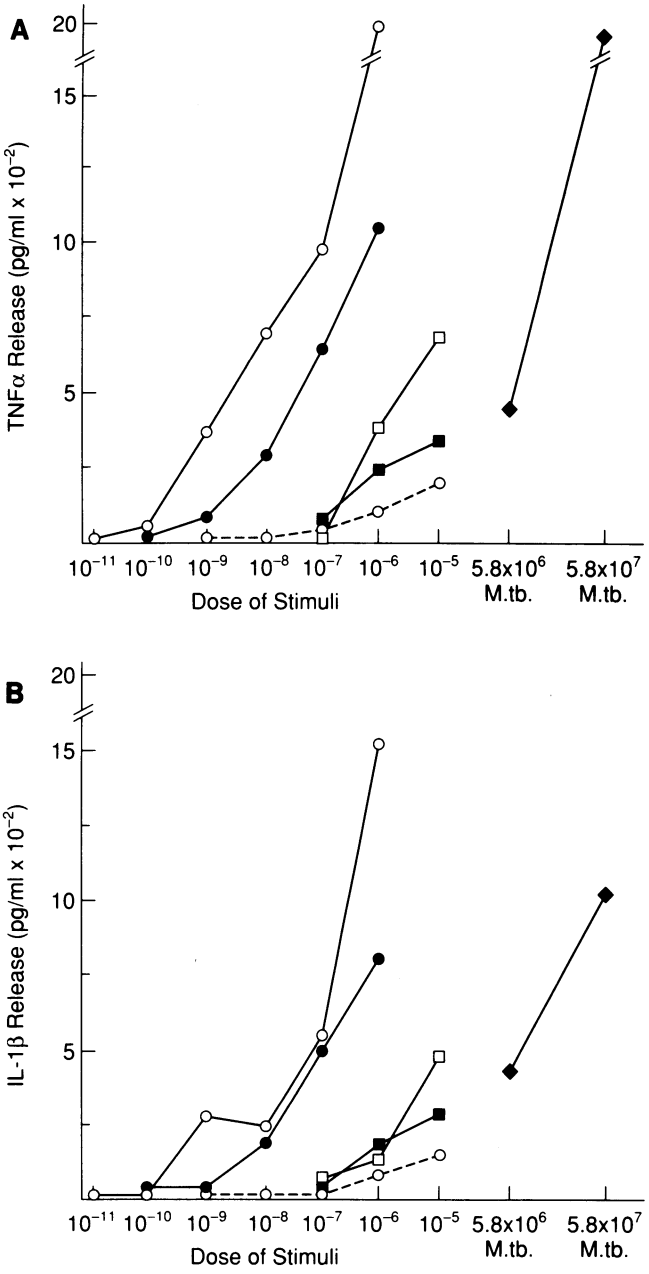

Figure 1. Release of IL-1β or TNFα from THP-1 cells after stimulation. Human myelomonocytic leukemia cell line THP-1 was grown to a density of 10^6 cells/ml in 10% FCS and stimulated for 24 h. Supernatants from the cultures were collected and stored at −70°C until assayed by ELISA. Dose of stimuli expressed in grams. (*A*) TNFα; (*B*) IL-1β. (— ○ —) LPS; (— ● —) LAM H37Ra; (- - ○ - -) LAM Erdman; (□) HSP-65kD; (■) CFL; (♦) *M. tuberculosis* H37Ra.

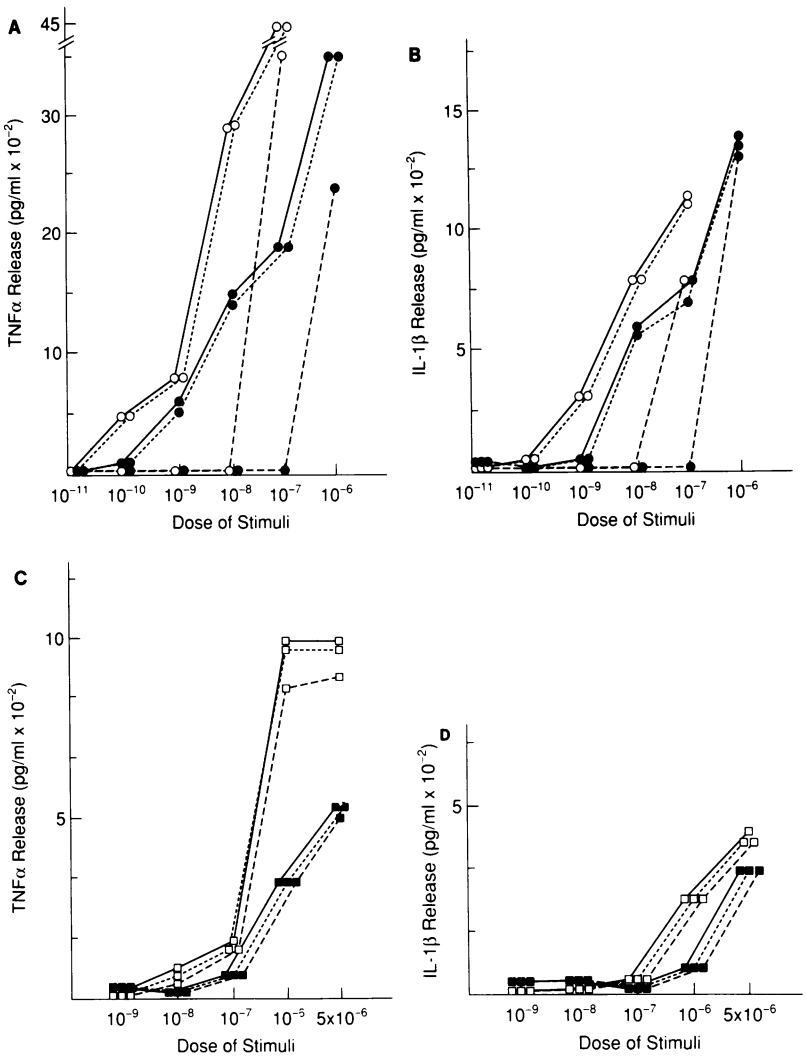

Figure 2. Inhibition of TNFα and IL-1β release by anti-CD14 MY4 monoclonal antibody. Human blood was cultured in 24-well tissue culture plates at a density of 5×10^6 nucleated cells/ml and incubated with stimuli for 6 h. TNFα and IL-1β were measured in supernatants by ELISA. Dose of stimuli is expressed in (*A*) TNFα release after LPS or LAM stimulation. (— ○ —) LPS; (· · · ○ · · ·) LPS plus isotype control IgG2b monoclonal antibody; (– – ○ – –) LPS plus MY4 anti-CD14 monoclonal antibody; (— ● —) LAM; (· · · ● · · ·) LAM plus isotype control IgG2b monoclonal antibody; (– – ● – –) LAM plus MY4 anti-CD14 monoclonal antibody. (*B*) IL-1β release after LPS or LAM stimulation. Symbols are the same as in *A*. (*C*) TNFα release after HSP-65kD or CFL stimulation. (— □ —) HSP-65kD; (· · · □ · · ·) HSP-65kD plus isotype control IgG2b; (– – □ – –) HSP-65kD plus MY4 anti-CD14 monoclonal antibody; (— ■ —) CFL: (· · · ■ · · ·) CFL plus isotype control IgGb; (– – ■ – –) CFL plus anti-CD14 monoclonal antibody. (*D*) IL-1β release after HSP-65kD or CFL stimulation. Symbols are the same as in *C*.

ulation, although doses required for cytokine release by HSP-65kD and CFL were much higher than LAM. We obtained similar results with 3C10, another monoclonal antibody to a different epitope on CD14 (data not shown).

Anti-LAM antibody blocks TNFα and IL-1β release. To determine the specificity of the stimulus by LAM for TNFα or IL-1β release by THP-1 cells, we performed a dose–response evaluation using anti-LAM monoclonal antibody (Fig. 3). The anti-LAM antibody reduced TNFα or IL-1β release from THP-1 cells after stimulation with LAM in a dose-dependent fashion, confirming the specificity of the response. The anti-LAM monoclonal antibody did not reduce TNFα or IL-1β release from THP-1 cells after stimulation with LPS, HSP-65kD, or CFL (Fig. 4, *A* and *B*).

Expression of mRNAs for IL-1β and TNFα in THP-1 cells. After a 5-h exposure with LPS, THP-1 cells expressed the expected 1.6-kb IL-1β and 1.7-kb TNFα mRNA species by Northern blot analysis (Fig. 5). Interestingly, the LPS-free preparation of the *M. tuberculosis* cell wall, LAM, demonstrated identical upregulation of IL-1β and TNFα mRNA as LPS (Fig. 5, *A* and *B*, lanes *2* and *3*). The recombinant HSP-65kD, a cytoplasmic protein ubiquitous among mycobacterial species, and *culture filtrate* CFL shared a similar ability in upregulating IL-1β and TNFα mRNA at 5 h (Fig. 5, *A* and *B*, lanes *4* and *8*). Increased IL-1β mRNA levels were observed within 30 min after stimulation with LPS, LAM, HSP-65kD, and CFL and lasted > 24 h. Equal amounts of mRNA were placed in each lane as demonstrated by the control housekeeping cDNA pHe 7 (Fig. 5 *C*).

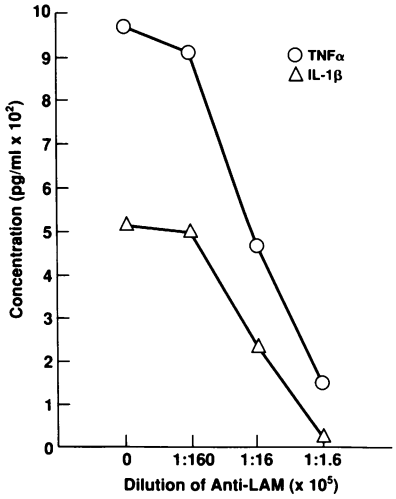

Figure 3. Inhibition of TNFα and IL-1β production by anti-LAM antibody. THP-1 cells were cultured in 24-well tissue culture plates at a density of 10^6 cells/ml with LAM (500 ng/ml) in the presence or absence of an increasing concentration of anti-LAM monoclonal antibody. There was a dose–response reduction of IL-1β and TNFα release with anti-LAM antibody after LAM stimulation.

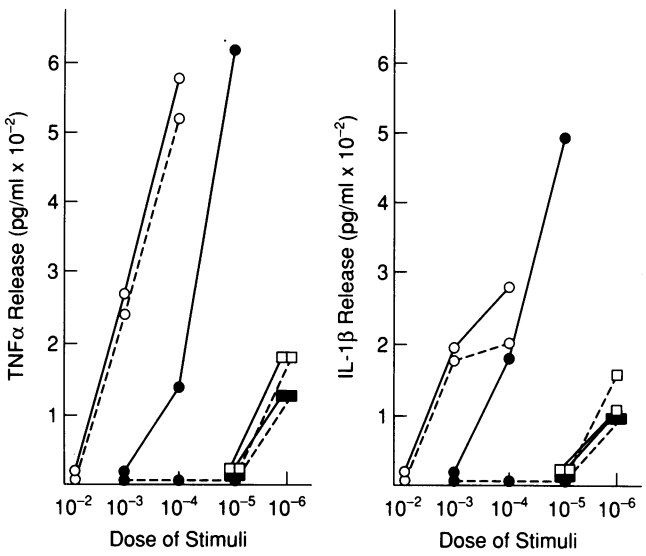

Figure 4. Specificity of anti-LAM effect on IL-1β and TNFα release. Only LAM stimulation was blocked by anti-LAM. There was no effect of anti-LAM antibody on IL-1β or TNFα release after LPS, HSP-65kD, or CFL stimulation. Dose of stimuli is expressed in grams. (*A*) TNFα release. Anti-LAM antibody completely blocked TNFα release by LAM (— ● —, LAM alone; - - ● - -, LAM plus anti-LAM) but had no effect on LPS (— ○ —, LPS alone; - - ○ - -, LPS plus anti-LAM), HSP-65kD (— □ —, HSP-65kD alone; - - □ - -, HSP-65kD plus anti-LAM), or CFL (— ■ —, CFL alone; - - ■ - -, CFL plus anti-LAM. (*B*) IL-1β release. Anti-LAM antibody completely blocked IL-1β released by LAM but had no effect on LPS, HSP-65kD, or CFL stimulation. Symbols are the same as in *A*.

IL-1β gene transcription and regulation by M. tuberculosis. To determine if the transcription rate of mRNA for IL-1β was increased, we used a nuclear run-on assay (Fig. 6). The IL-1β cDNA and housekeeping control gene (β-actin) were fixed to nitrocellulose, and the stimulated cell extracts demonstrated an increased transcription rate after LPS, LAM, or TNFα exposure (Fig. 6, lanes *2, 3,* and *4,* respectively, compared with untreated cells, lane *1*).

To further investigate the molecular mechanisms by which the expression of the IL-1β gene is regulated upon exposure to *M. tuberculosis* and other stimuli, we isolated the IL-1β genomic DNA from a human placenta library. A 1,420-bp IL-1β DNA fragment (−1130/+290) in pTK(−)CAT.IL-1β was evaluated by transient transfection and CAT assay after stimulation with TNFα, LPS, LAM, CFL, and HSP-65kD. The same test agents that upregulated steady state IL-1β mRNA levels (see Fig. 6) enhanced CAT gene expression driven by the IL-1β promoter in THP-1 cells (Fig. 7). By comparison with the unstimulated control, the enhanced IL-1β promoter activity was > 14-fold upon stimulation with LAM or HSP-65kD and was sixfold greater with CFL.

Discussion

Tuberculosis is characterized by recruitment of alveolar and interstitial macrophages and lymphocytes with granuloma formation and systemic symptoms of fever, chills, and night sweats. We as well as others have postulated that local mononuclear phagocyte activation occurs and that cytokines are released (6–16). Using in vitro stimulation by *M. tuberculosis* and its components, we demonstrated increased IL-1β and TNFα protein release, mRNA expression, and showed that activation occurs via the cell surface protein CD14 and that activation for IL-1β occurs at the level of transcription.

LAM has recently been shown to contain a phosphatidylinositol membrane anchor that is altered by deacylation removing palmitic (hexodecanoate) and tuberculostearic (10-methyloctadecanoic) acids (24). Barnes and colleagues (15) demonstrated that deacylated LAM was unable to stimulate cytokines, including IL-1β and TNFα. Chatterjee and colleagues (16) explored the structural basis of the action of LAM, observing that LAM from the virulent *M. tuberculosis* Erdman strain released 100-fold less TNFα from murine macrophages than LAM from the attenuated *M. tuberculosis* H37Ra strain. They postulated that extensive capping of arabannan moieties by mannan in the Erdman virulent strain allowed it to escape eukaryotic host-defense mechanisms, including TNFα release. Our results with the *M. tuberculosis* Erdman strain confirm the results of Chatterjee et al. (16) for TNFα and extend them to IL-1β. LAM has been considered an important virulence factor of *M. tuberculosis* with ability to downregulate IFNγ activation of macrophages, downregulate IFNγ-inducible genes in macrophages, inhibit protein-antigen processing by antigen-presenting cells, scavenge superoxide anion, and decrease protein kinase C activity (25–28).

We carefully evaluated all of our samples of LAM, HSP-65kD, and CFL for LPS contamination using the *Limulus* amebocyte lysate assay, consistently observing < 10 pg/1 μg test reagent, which was below the level that LPS was able to stimulate cytokine release in THP-1 cells. Using the same assay, Chatterjee et al. (16) reported 0.8 ng LPS/μg LAM and Barnes et al. (15) reported 1.6 ng LPS/μg LAM contamination; both were unable to detect an LPS effect in their test systems. Chatterjee et al. (16) as well as others (29) added polymyxin B or the specific LPS inhibitor diphosphoryl lipid A to LAM test wells and obtained the same result. Addition of anti-LAM monoclonal antibody of the IgG3 900 series abrogated the stimulatory activity of LAM for TNFα or IL-1β with no effect on LPS stimulation, further demonstrating that the effect of LAM was not due to contaminating LPS. In addition, anti-LAM had no effect on HSP-65kD or CFL, suggesting that *M. tuberculosis* proteins other than LAM have the ability to stimulate cytokines, although less striking than LAM.

In addition to LAM, we evaluated a CFL of the *M. tuberculosis* Erdman strain that was free of carbohydrate moieties and the recombinant HSP-65kD. The CFL was purified over anion-exchange and molecular sieve chromatography and was free of LAM contamination by Western blot. Mycobacterial culture filtrates have proteins (46 and 20 kD) reported to stimulate TNFα release by peripheral blood monocytes (11). We found that both proteins were weaker stimuli than LAM for IL-1β or TNFα release with the only exception being the transcription experiment using HSP-65kD. HSP-65kD is a cytosolic protein important for intracellular protein assembly, folding, and transport.

We also show that LAM and LPS both stimulate peripheral blood mononuclear cells to release TNFα and IL-1β at low doses (≤ 10 ng/ml) by interacting with the CD14 surface protein. The release of TNFα and IL-1β stimulated by LAM or LPS was blocked by anti-CD14 MY4 antibody but was not blocked by an isotype IgG2b control protein. CD14 is a 55-kD

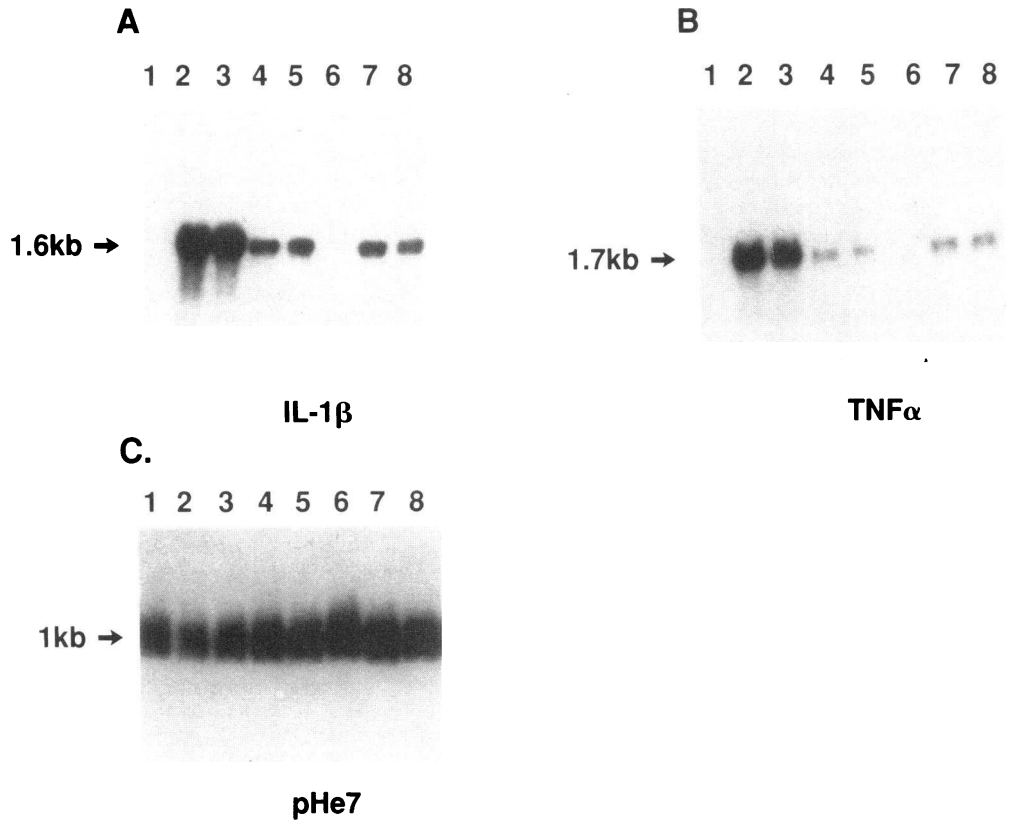

Figure 5. Analysis of steady state IL-1β and TNFα mRNA levels in THP-1 cells. Human myelomonocytic leukemia line THP-1 was grown to a density of 10^6 cells/ml in 10% FCS and treated with different reagents for 5 h. Cytoplasmic total RNA was then isolated and an equal amount of RNA from each sample was electrophoresed through a 1% denaturing agarose gel containing 6% formaldehyde. (*A*) Northern analysis with nick-translated IL-1β cDNA. A single mRNA hybrid of 1.6 kb was identified. The samples were: lane *1*, control without treatment; lane *2*, LPS (100 ng/ml); lane *3*, LAM (100 ng/ml); lane *4*, HSP-65kD (100 ng/ml); lane *5*, TNFα (10 ng/ml); lane *6*, control without treatment; lane *7*, IL-1α (1 ng/ml); lane *8*, CFL (100 ng/ml). (*B*) Northern blot analysis was performed with a nick-translated human TNFα cDNA probe and a 1.7-kb transcript was identified. Lanes are as in 1*A*. (*C*) Northern analysis of the same filter with pHe 7 cDNA housekeeping gene. Lanes are as in *A*.

glycoprotein attached to the membrane via a phosphatidylinositol glycan anchor that interacts with a LPS–LPS-binding protein (LBP) complex (23, 30). It is not known if the LBP can complex LAM but two different monoclonal antibodies to CD14 were able to block the effect of LAM. LBP binds to the lipid A portion of LPS, which may correlate to the important phosphatidylinositol anchor of LAM that is the antigenic component (31). Thus there are structural similarities between LAM and LPS in that a monoclonal antibody to monocytes can recognize similar epitopes and inhibit LPS–LBP or potential LAM–LBP binding to CD14 and subsequent activation of mononuclear phagocytes. Interestingly, the HSP-65kD and CFL act through a different mechanism because the stimulated release was not blocked by anti-CD14. In contrast, at higher doses of LPS or LAM, activation of mononuclear phagocytes to release IL-1β and TNFα occurs despite the presence of anti-CD14 antibodies consistent with an additional mechanism.

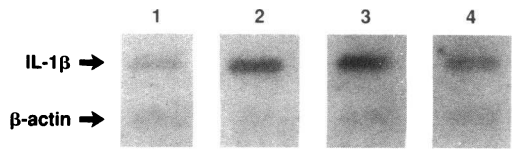

Figure 6. Increased transcription rate of IL-1β gene detected by nuclear run-on assay. THP-1 cells were stimulated for 1 h, washed with PBS 3×, lysed with NP-40, and centrifuged to separate nuclei and cytoplasm. The nuclei were labeled with [^{32}P]UTP, digested with DNase I and proteinase K, and the nascent nuclear RNA chains were separated by TCA precipitation. The labeled RNA were hybridized to nitrocellulose to which plasmids containing cDNAs for IL-1β and β-actin had been fixed. Lane *1*, untreated cells; lane *2*, LPS; lane *3*, LAM; and lane *4*, TNFα.

We demonstrated that the increased steady state level of IL-1β and TNFα mRNA is one of the mechanisms for increased secretion of these proteins. Nuclear run-on assay for IL-1β demonstrated a striking increase in the transcription rate in THP-1 cells after LPS or LAM stimulation. We further investigated the mechanisms by which the IL-1β gene is regulated upon exposure to various stimuli. After transfection of THP-1 cells with the plasmid pTK(−)CAT.IL-1β, the IL-1β promoter activity was dramatically increased by LAM and HSP-65kD and, to a lesser amount, by CFL, as analyzed by CAT assay. This strongly suggests that the IL-1β gene is activated by the mycobacterial components at the transcriptional level and that the *cis*-acting elements in response to the mycobacterial components are located on the IL-1β DNA sequence between −1130 and +290. Two genes encode IL-1α and β with 45% homology in their nucleotide sequences and 26% similarity in their amino acid sequences; both IL-1α and β bind to the same cell surface receptor and have overlapping biological activities (32). Although mononuclear phagocytes secrete much more IL-1β than IL-1α, the mRNA is translated to a 31-kD pro-IL-1β that is cleaved by an IL-1β–converting enzyme releasing the 153 COOH-terminal amino acids (17-kD protein), which constitutes the mature cytokine (33, 34). Whether LAM affects this

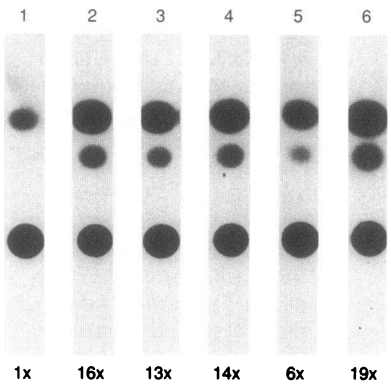

Figure 7. Transient transfection and CAT assay. THP-1 cells were transfected with the plasmid pTK(−)-CAT.IL-1βby the DEAE-dextran method and the CAT activity was determined as described in Methods. The transfected cells were cultured for 24 h with the following stimuli: lane *1*, control; lane *2*, TNFα (10 ng/ml); lane *3*, IL-1β (100 ng/ml); lane *4*, LAM (100 ng/ml); lane *5*, CFL (100 ng/ml); lane *6*, HSP-65kD (100 ng/ml). The number on the bottom of the figure indicates the relative increase (fold induction) in CAT activities calculated by dividing the percent conversion into the acetylated forms of chloramphenicol in extracts from cultures treated with the respective stimuli by that in control extracts. Thus, a value of 1.0 represents no increase over the control.

converting enzyme is not known. The 3′ untranslated region of the IL-1β and TNFα mRNA contains an AU-rich sequence that is implicated in instability and rapid turnover of the message analogous to other genes, including IFNγ, granulocyte/macrophage colony stimulating factor, IL-2, IL-3, *c-fos, c-myc,* and *c-sis* (35). Not only does LAM modulate IL-1β production, but other microbial components do so as well, e.g., the immediate early genes of human cytomegalovirus upregulate expression of the IL-1β gene, and teichoic acid–peptidoglycan complex of pneumoccoccal cell walls stimulate IL-1β release from mononuclear phagocytes (36, 37). Both IL-1β and TNFα are regulated primarily at the level of transcription with stimuli enhancing the duration of increased transcription; in mature macrophages the amount of IL-1β released is less than the amount of TNFα consistent with additional posttranscriptional mechanisms at the level of protein secretion (38).

Since mononuclear phagocytes are also important in the formation of tubercle granulomas, these cytokines may also mediate phagocyte differentiation into epithelioid cells, the release of extracellular matrix, the cellular organization typical of granulomas, and the central caseation necrosis. Further studies of alveolar macrophages and tissue biopsies from patients with active tuberculosis will provide data on the role of IL-1β and TNFα in vivo.

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