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Immunology

Activation of the interleukin 6 gene by *Mycobacterium tuberculosis* or lipopolysaccharide is mediated by nuclear factors NF-IL6 and NF-κB

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ABSTRACT **The host response to *Mycobacterium tuberculosis* includes granuloma formation at sites of infection and systemic symptoms. Cytokines have been identified by immunohistochemistry in granulomas in animal models of bacillus Calmette–Guérin (BCG) infection and are released by mononuclear phagocytes upon stimulation by mycobacterial proteins. In this regard, the cytokine interleukin 6 (IL-6) may play a role in the clinical manifestations and pathological events of tuberculosis infection. We have demonstrated that lipoarabinomannan (LAM) from the mycobacterial cell wall, which was virtually devoid of lipopolysaccharide (LPS), stimulated mononuclear phagocytes to release IL-6 in a dose–response manner. LAM and LPS were potent inducers of IL-6 gene expression in peripheral blood monocytes. Both LAM- and LPS-inducible IL-6 promoter activity was localized to a DNA fragment, positions −158 to −49, by deletion analysis and chloramphenicol acetyltransferase assay. Two nuclear factor NF-IL6 (positions −153 to −145 and −83 to −75) and one nuclear factor NF-κB (positions −72 to −63) motifs are present within this fragment. Site-directed mutagenesis of one or more of these motifs within the IL-6 promoter demonstrated that each has positive regulatory activity and that they could act in a function- and orientation-independent manner. Deletion of all three elements abolished inducibility of IL-6 promoter activity by both LAM and LPS. We conclude that the NF-IL6 and NF-κB sites mediate IL-6 induction in response to both LPS and LAM, acting as bacterial or mycobacterial response elements.**

The variety of human cell-mediated immune defenses against *Mycobacterium tuberculosis* is reflected in the wide range of clinical manifestations of active disease (1, 2). Ineffective cellular immunity results in the failure of localization of *M. tuberculosis* infection with absence of granuloma formation. Tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β), and interferon γ are thought to play a part in human cell-mediated immune response to infection with many facultative intracellular pathogens, including *Listeria monocytogenes, Leishmania, Candida*, and mycobacteria (3–6). TNF-α is also required for localization of mycobacterial infection and granuloma formation (7, 8). Interleukin 6 (IL-6) is a multifunctional cytokine regulating immune responses and the acute-phase protein response (9–11). In active tuberculosis, the role of IL-6 may be predominantly negative. This is supported by several facts: (*i*) IL-6 promotes the growth of mycobacteria in peripheral blood monocytes (12, 13). (*ii*) IL-6 inhibits the production of TNF-α and IL-1β, which may enhance intracellular killing of microorganisms and development of granulomata (14, 15).

M. tuberculosis components stimulate the release of both IL-1β and TNF-α from mononuclear phagocytes (16–21). TNF-α has been shown to accumulate in granulomas of experimental models of tuberculosis infection, and pretreatment with anti-TNF-α antibody results in poorly formed granulomas, dissemination of the mycobacteria, and death of the animal (8). TNF-α and IL-1β induce IL-6 in human fibroblasts by activating nuclear factors that bind to a NF-κB-like sequence (22). IL-6 may be a negative modulator of TNF-α and IL-1β in active tuberculosis favoring dissemination of the disease. In this regard, in human immunodeficiency virus-infected individuals, hypergammaglobulinemia and expansion of B cells may be likely a result of IL-6 (23). Tuberculosis in human immunodeficiency virus-infected individuals takes on a more aggressive course with dense aggregates of organisms in infected tissue, poorly formed and fewer granulomata, and more frequent miliary presentation (24–26).

Alveolar macrophages phagocytose mycobacteria but have difficulty with intracellular killing. The cell wall of mycobacteria does not contain lipopolysaccharide (LPS), which is a well known inducer of IL-6. However, lipoarabinomannan (LAM), a complex lipid glycoprotein anchored in the cell membrane by phosphatidylinositol, shares many physicochemical properties with LPS (27, 28). To determine whether purified LAM, virtually devoid of LPS contamination, could induce the production of IL-6, we measured IL-6 protein and mRNA levels after exposure of human peripheral blood monocytes to LAM. Not only was IL-6 protein and mRNA increased but also IL-6 promoter activity was markedly enhanced by LAM or LPS.

MATERIALS AND METHODS

LAM from laboratory-attenuated *M. tuberculosis* H37Ra was kindly provided by P. Brennan (Colorado State University, Ft. Collins). The LAM had been passed through a Detoxi-Gel column using sterile pyrogen-free water and stored in pyrogen-free vials from which any contaminating LPS had been removed. Only pyrogen-free water was used in reconstitution of this material. Evaluation of tuberculosis reagents including LAM for the presence of Gram-negative bacterial endotoxin was done with the amebocyte lysate assay (E-toxate kit, Sigma). Six lots of LAM obtained between 1990 to 1993 contained <10 pg of LPS per μg of LAM per batch.

Isolation of Human Monocytes from Peripheral Blood. Mononuclear cells in peripheral blood from purified-protein-derivative-negative donors were separated by centrifugation over lymphocyte-separation medium (Flow Laboratories).

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Abbreviations: IL-6, interleukin 6; LAM, lipoarabinomannan; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor α; IL-1β, interleukin 1β; DTT, dithiothreitol; CAT, chloramphenicol acetyltransferase.

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The cells were seeded onto the plastic surface of a 175-cm^2 flask to allow monocytes to adhere. Human myelomonocytic leukemic cell line THP-1 (ATCC) or the isolated human peripheral blood monocytes were cultured in RPMI 1640 medium supplemented with 10% (vol/vol) fetal bovine serum. IL-6 protein levels in cell supernatants were measured with a commercial ELISA kit (Biokine, Cambridge, MA).

Isolation of RNA and Northern Blot Analysis. Human peripheral blood monocytes were treated with test reagents, collected by centrifugation, and lysed by addition of 5.5 M guanidinium isothiocyanate buffer. Total RNA was isolated through $CsCl_2$ gradient ultracentrifugation. An equal amount of RNA was fractionated by electrophoresis through a 1% agarose/6% formaldehyde denaturing gel and transferred onto a nitrocellulose filter (BA 85, Schleicher & Schuell). The baked filter was prehybridized at 42°C for 6–12 hr. For hybridization, the IL-6 cDNA (kindly provided by J. Vilcek, New York University) was nick-translated with [α-^{32}P]dCTP (specific activity, 3000 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear). The hybridization was carried out at 42°C for 10 hr. The filter was then washed in 2× standard saline citrate (SSC)/0.5% SDS at room temperature for 20 min followed by 0.1× SSC/0.5% SDS at 65°C for 30 min. The filter was then exposed to film at −70°C.

Plasmid Construction and Site-Directed Mutagenesis. Serial deletion mutant constructs of IL-6 5′ flanking region were as described (22).

Site-directed mutagenesis was carried out according to directions in the Bio-Rad site-directed mutagenesis kit. The mutant oligonucleotides synthesized were as follows: NF-IL6 (positions −153 to −145) to 5′-CCGTACAAT-3′, NF-IL6 (positions −83 to −75) to 5′-CCATAAAAT-3′, and NF-κB (positions −72 to −63) to 5′-CTCATTTTCC-3′. The IL-6 fragment, positions −224 to +11, was used as the template. The achieved mutant IL-6 DNA sequences were verified by DNA sequencing. Seven single or combined mutations were obtained: single NF-IL6 site mutation, double NF-IL6 site mutations, a combined NF-IL6 and NF-κB site mutation, and triple (two NF-IL6 and one NF-κB sites) mutations.

Transient Transfection of Human Suspension Cultures and the Assay of Chloramphenicol Acetyltransferase (CAT) Activity. THP-1 cells were transfected with various IL-6 DNA constructs in the plasmid pTK(−)CAT by the DEAE-dextran method (29). Briefly, 10^7 cells were washed in STBS (25 mM Tris, pH 7.4/137 mM NaCl/5 mM KCl/0.6 mM Na_2HPO_4/0.7 mM $CaCl_2$/0.5 mM $MgCl_2$), transfected with 10 μg of $CsCl_2$-purified plasmids in 1 ml of DEAE-dextran (400 μg/ml) at 37°C for 90 min. The transfected cells were then incubated in complete medium for 24 hr in the absence or presence of inducing agents. The transfected cells were lysed by three freeze–thaw cycles, and equal amounts of protein from different cell extracts were assayed for CAT activity (30).

Preparation of Nuclear Extracts and DNA Mobility Shift Assay. Cell extracts were prepared from THP-1 cells by using the method of Dignam *et al.* (31). Cultures (5 × 10^8 cells) were lysed by homogenization in buffer A [10 mM Hepes, pH 7.9/1.5 mM $MgCl_2$/10 mM KCl/0.5 mM dithiothreitol (DTT)/0.5 mM phenylmethylsulfonyl fluoride] and centrifuged at 1000 × *g* for 10 min at 4°C to separate the nuclei from the cytosolic fraction. The nuclear proteins were extracted in buffer C [20 mM Hepes, pH 7.9/25% (vol/vol) glycerol/0.42 M NaCl/1.5 mM $MgCl_2$/0.2 mM EDTA/0.5 mM DTT/0.5 mM phenylmethylsulfonyl fluoride] on ice for 30 min with shaking. The extracted nuclear proteins were dialyzed against buffer D (20 mM Hepes, pH 7.9/20% glycerol/0.1 M KCl/0.2 mM EDTA/0.5 mM DTT/0.5 mM phenylmethylsulfonyl fluoride) for 5 hr and frozen in liquid nitrogen in aliquots.

DNA mobility shift assay was carried out as described by Sen and Baltimore (32). The DNA probes were end-labeled by using polynucleotide kinase and 100 μCi of [γ-^{32}P]ATP. Probes (10,000 cpm) were incubated with 1 μg of cell extracts under the binding conditions [10 mM Tris, pH 7.5/50 mM NaCl/1 mM EDTA/0.1 mM DTT/2 μg of poly(dI-dC)/10% glycerol] in a total volume of 20 μl. The reactions were carried out at room temperature for 15 min, and products were electrophoresed on a 5% polyacrylamide gel. The gel was analyzed by autoradiography.

RESULTS

Mycobacterial Cell Wall Component LAM Stimulates the Release of IL-6 Protein and Expression of IL-6 mRNA from Peripheral Blood Monocytes. Monocytes released a significant amount of IL-6 in the presence of LAM at 0.1 ng/ml over a 24-hr period with a maximal response at 2 μg/ml (Fig. 1). The half-maximal effect occurred at 500 ng/ml (ED_{50}), which was chosen as the dose for subsequent experiments. Live *M. tuberculosis* H37Ra and a clinical isolate of *M. tuberculosis* exhibited a parallel dose–response stimulation of IL-6 release. The effect of LAM was not due to contamination by LPS because the LPS content in 1 μg of LAM was <10 pg in all LAM aliquots, as determined by *Limulus* amebocyte assay, and LPS at 10 pg/ml could not stimulate the production of IL-6 as detected by ELISA. Polymyxin B at 10 μg/ml inhibited the LPS effect, whereas it had no effect on the ability of LAM to stimulate IL-6 release.

To examine whether the increased release of IL-6 protein was in parallel to the expression of IL-6 mRNA, we measured the levels of IL-6 mRNA by Northern blot analysis. In this experiment, we used peripheral blood monocytes and the human myelomonocytic leukemic cell line THP-1, which was used for transient transfection experiments shown later. The IL-6 mRNA levels were significantly increased in blood monocytes and THP-1 cells after stimulation with LAM or LPS for 4 hr (Fig. 2). Fig. 2 *C* and *D* demonstrates equal amounts of RNA in each lane (ethidium bromide-stained gels). This suggested that the increased IL-6 mRNA level contributes to the enhanced release of IL-6 protein detected in the cell culture supernatants.

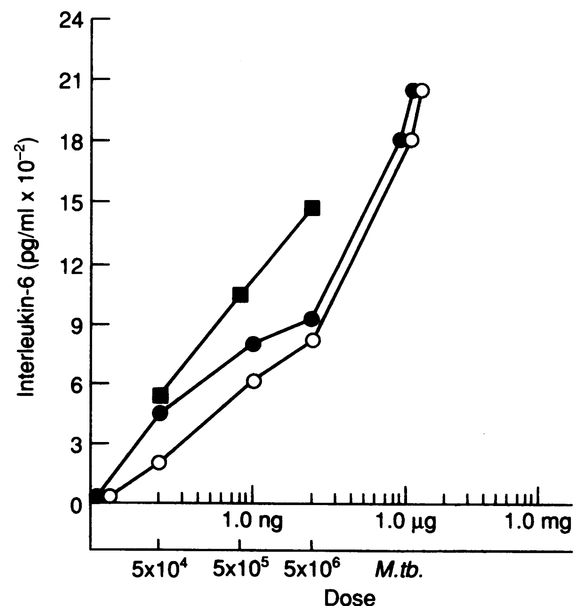

FIG. 1. Release of IL-6 from peripheral blood monocytes after stimulation with LAM, LPS, or live *M. tuberculosis (M.tb.)* H37Ra. ○, LPS; ●, LAM; ■, *M. tuberculosis* H37Ra.

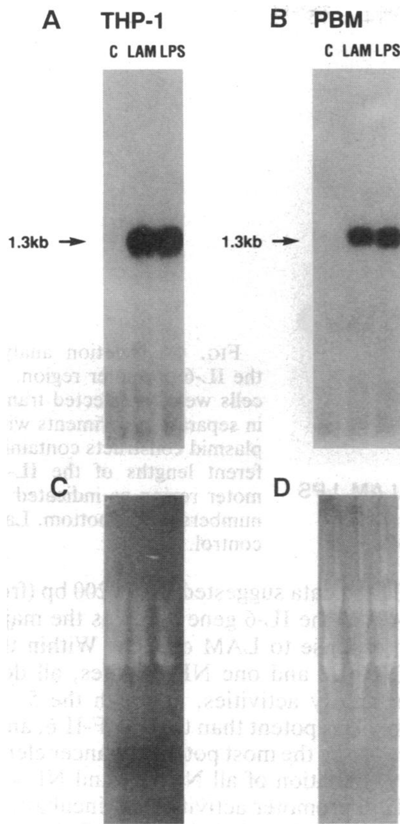

FIG. 2. Northern blot analysis of IL-6 mRNA in peripheral blood monocytes (*B*) and THP-1 cells (*A*) after LAM or LPS stimulation. Peripheral blood monocytes or THP-1 cells were incubated for 4 hr with or without stimuli (LAM at 500 ng/ml or LPS at 500 ng/ml). (*C* and *D*) Ethidium bromide-stained gels demonstrating equal amounts of RNA in each lane. Lanes C, control.

Regulation of IL-6 Gene Transcription by LAM and LPS in THP-1 Cells. To further investigate the molecular mechanisms by which the expression of the IL-6 gene is regulated upon exposure to *M. tuberculosis*, we mapped DNA regulatory elements on the 5′ flanking region of the IL-6 gene that responded to LAM and compared them with LPS, a well known inducer of IL-6. Serial IL-6 deletion mutant constructs were transiently transfected into THP-1 cells by the DEAE-dextran method, and CAT assay was carried out (Fig. 3, schematic of IL-6 promoter elements and deletion mutants). CAT activity was induced with the IL-6 construct, positions −1158 to +11, after stimulation with LAM or LPS (Fig. 4). There was increased CAT activity after stimulation by LAM or LPS with the constructs, positions −224 to +11, positions −158 to +11, and positions −109 to +11. Further deletion to position −49 totally abolished the induction of CAT activity after stimulation with LAM or LPS. Sequence analysis demonstrated that deletion from positions −158 to −109 resulted in removal of a NF-IL6 binding site (positions −153 to −145), and further deletion to position −49 led to removal of another NF-IL6 binding site (positions −83 to −75) and a NF-κB binding site (positions −72 to −63).

To test whether NF-IL6 [consensus sequence, T(T/G)NNGNAA(T/G)] and NF-κB binding sequences contribute to the stimulatory activities by LAM or LPS, we performed site-directed mutagenesis on the IL-6 DNA fragment from positions −224 to +11, in which seven mutation constructs were made and tested in the presence or absence of LAM or LPS (Fig. 5). The CAT activity produced with the wild-type fragment from positions −224 to +11 is shown in Fig. 4. Mutation of the 5′ NF-IL6 site with intact 3′ NF-IL6 and NF-κB sites resulted in inducible CAT activity that was similar to that seen with the unmutated construct. With a mutation of the 3′ NF-IL6 site alone or after a single mutation of the NF-κB site, IL-6 promoter activity was still inducible by LAM or LPS, albeit at a somewhat reduced level. Mutation of two of the three sites showed inducibility to be 5-fold with NF-κB intact, 4-fold with 3′ NF-IL6 intact, and 3-fold with 5′ NF-IL6 intact. The fold increase from the construct with the 3′ NF-IL6 intact was 3.8 and 3.7, respectively, after stimulation with LAM and LPS, which was notably greater than the 2.9- and 2.7-fold increase seen with the construct with only the 5′ NF-IL6 intact. Mutation of all three nuclear binding elements abolished activation of IL-6 promoter activity by LAM or LPS. These experiments suggested that the two NF-IL6 sites and the NF-κB site on the IL-6 gene functioned as positive regulatory sequences in response to the induction with LAM or LPS. Each site can function in an independent manner after mutation of the other two sites, but the relative importance of each of the three elements is different.

LAM and LPS Stimulate Interaction Between Nuclear Protein(s) and NF-IL6 or NF-κB Sequences. The deletion analysis and site-directed mutagenesis experiments described above suggest that the NF-IL6 and NF-κB sites are the positive regulatory elements for LAM or LPS stimulation. To determine whether these sequences interact specifically with nuclear proteins, DNA mobility shift assay was performed. Incubation of nuclear extracts from LAM- or LPS-stimulated cells with the 5′ NF-IL6 probe led to the appearance of a protein–DNA complex (Fig. 6, lanes 2 and 3, respectively). The formation of the complexes demonstrated by DNA

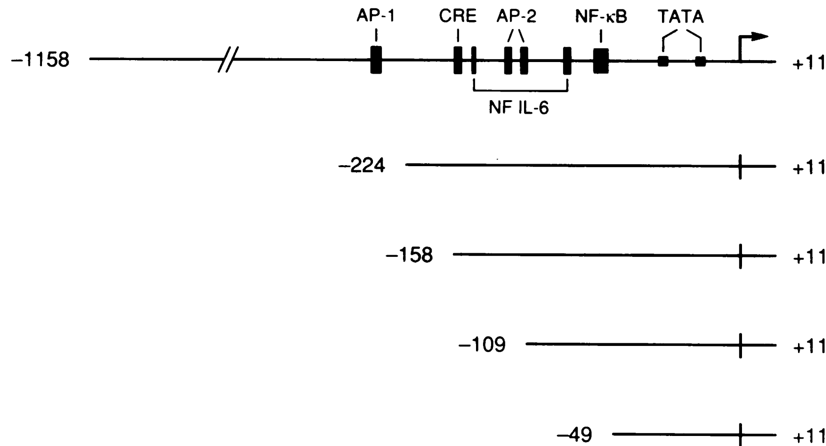

FIG. 3. Schematic of the IL-6 gene and deletion analysis of the 5′ flanking sequences.

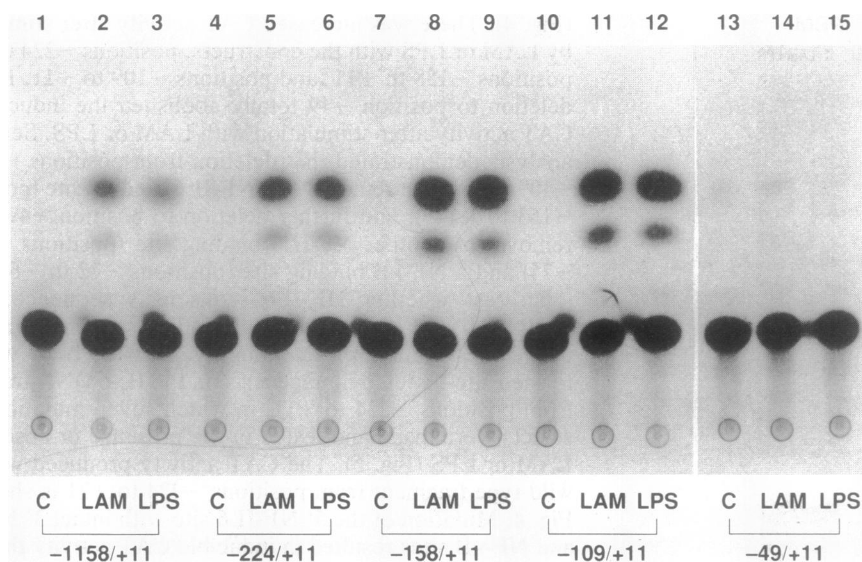

FIG. 4. Deletion analysis of the IL-6 promoter region. THP-1 cells were transfected transiently in separate experiments with four plasmid constructs containing different lengths of the IL-6 promoter region as indicated by the numbers on the bottom. Lanes C, control.

mobility shift assay was specific, because the appearance of the complex was abolished in the presence of 100× molar excess of the unlabeled wild-type NF-IL6 probe but not abolished by competition with 100× molar excess of unlabeled mutant NF-IL6 probe (Fig. 6, lanes 4 and 5 and lanes 6 and 7). Similar results are shown with the NF-κB probe. Experiments not shown demonstrated similar results with DNA mobility shift assay with the 3′ NF-IL6 probe.

DISCUSSION

In this report, we investigated the molecular mechanisms by which IL-6 gene expression is regulated upon exposure of mononuclear phagocytes to *M. tuberculosis* or its components (33). LAM purified from the cell wall and live *M. tuberculosis* appeared to be significant enhancers of IL-6 expression, and LAM and LPS shared common mechanisms for the activation of IL-6 promoter elements. The LAM had been shown to be LPS-free and LPS inhibitors did not affect the results (19, 34): both targeted inducing activity on NF-IL6 and NF-κB motifs. NF-IL6 or the NF-κB site can function independently in response to LAM or LPS stimulation because mutation of either did not interfere with the function of the other. These data suggested that <200 bp (from positions −158 to −49) of the IL-6 gene contains the major promoter activity in response to LAM or LPS. Within this fragment were two NF-IL6 and one NF-κB sites, all demonstrating positive regulatory activities, although the 5′ NF-IL6 was demonstrably less potent than the 3′ NF-IL6, and the NF-κB site appeared to be the most potent enhancer element (Fig. 5). Importantly, mutation of all NF-IL6 and NF-κB sequences abolished IL-6 promoter activity after incubation with LAM or LPS. LAM or LPS could induce specific binding of nuclear proteins to the NF-κB or NF-IL6 wild-type target sequences. These data suggest that binding of the LAM- or LPS-induced transcription factors to the IL-6 regulatory region mediated transcriptional activation of IL-6. This report also demonstrates that LPS from Gram-negative bacteria and LAM from mycobacteria function through similar mechanisms: they both activate NF-IL6 and NF-κB on the IL-6 gene. We propose NF-IL6 or NF-κB are important mycobacterial or bacterial response elements and may be relevant to other host cytokines.

Previous investigators have evaluated the IL-6 5′ flanking region enhancer/promoter sites that responded to IL-1β. Ray *et al.* (35) identified a 115-bp fragment (positions −225 to

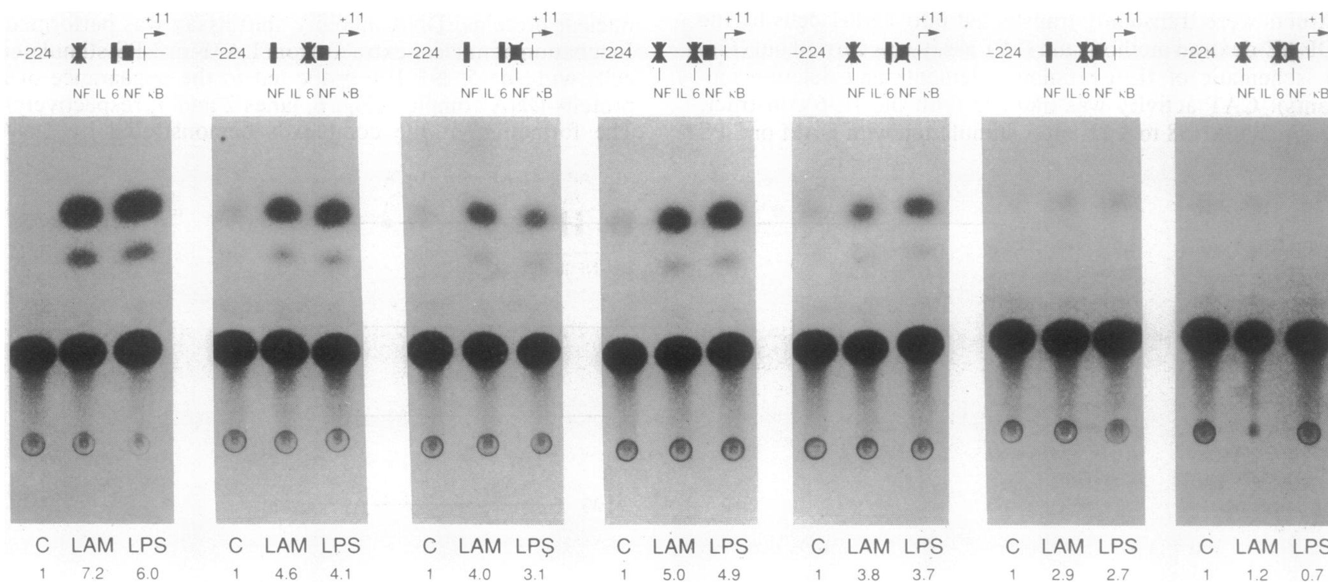

FIG. 5. Analysis of the IL-6 promoter region by site-directed mutagenesis. The schematics at the top show the combinations of mutations. The X marks the mutated motif. The fold increase in CAT activity after stimulation with LAM or LPS is shown at the bottom. Lanes C, control.

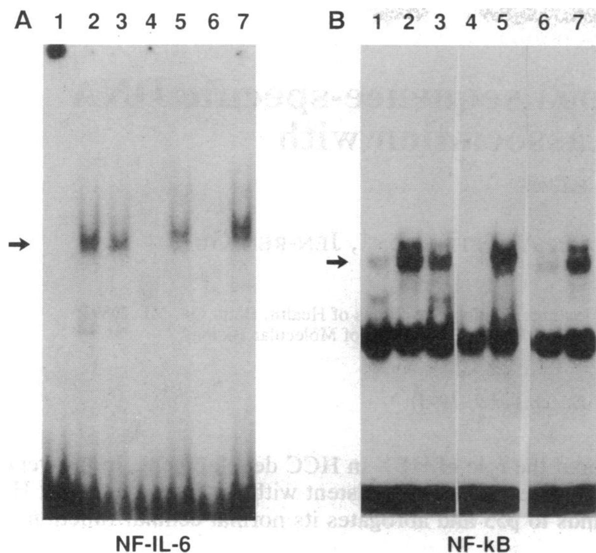

FIG. 6. Induction of the binding of nuclear protein(s) to the 5′ NF-IL6 or NF-κB sequence. (*A*) The 5′ NF-IL6 probe. Lanes: 1, unstimulated control; 2, LAM (500 ng/ml); 3, LPS (500 ng/ml); 4, LAM-stimulated nuclear extracts in the presence of a 100-fold molar excess of unlabeled authentic wild-type NF-IL6 DNA; 5, LAM-stimulated nuclear extracts in the presence of a 100-fold molar excess of unlabeled synthetic mutant NF-IL6 sequence; 6, LPS-stimulated nuclear extracts in the presence of a 100-fold molar excess of unlabeled authentic wild-type NF-IL6 sequence; 7, LPS-stimulated nuclear extracts in the presence of a 100-fold molar excess of synthetic mutant NF-IL6 sequence (5′-CCGTACAAT-3′). (*B*) NF-κB probe. Lanes: 1, unstimulated control; 2, LAM stimulation; 3, LPS (500 ng/ml); 4, LAM-stimulated nuclear extracts in the presence of a 100-fold molar excess of unlabeled authentic wild-type NF-κB sequence; 5, LAM-stimulated nuclear extracts in the presence of a 100-fold molar excess of synthetic mutant NF-κB sequence; 6, LPS-stimulated nuclear extracts in the presence of a 100-fold molar excess of unlabeled authentic wild-type NF-κB sequence; 7, LPS-stimulated nuclear extracts in the presence of a 100-fold molar excess of synthetic mutant NF-κB sequence (5′-CTCATTTTCC-3′).

−111) that had promoter activity with a 23-bp element (positions −173 to −151) that responded to multiple stimuli and overlapped 2 bp of our 5′ NF-IL6 site. However, Lieberman and Baltimore (36) and others (20, 37) provided convincing evidence including site-directed mutagenesis that a NF-κB site (positions −73 to −63) was the key IL-1β and TNF-α responsive enhancer/promoter element on IL-6. Interestingly, Lieberman and Baltimore (36) stated that there was a nearby unidentified sequence that could act synergistically with NF-κB. Close inspection of our data reveals synergism between our 3′ NF-IL6 site and NF-κB (Fig. 5). Isshiki *et al.* (38) identified a 14-bp palindromic sequence that overlapped our 5′ NF-IL6 site that was an IL-1 responsive element. This was consistent with our data demonstrating that the 5′ NF-IL6 and the other two enhancer/promoter sequences acted independent of each other and orientation. Akira *et al.* (39) cloned the NF-IL6 gene and observed that it had high sequence homology with C/EBP, a family of rat liver nuclear factors. They noted that NF-IL6 bound to the regulatory regions of several genes involved in the acute-phase response and present in macrophages including TNF-α, IL-8, IL-1β, albumin, haptoglobin, granulocyte colony-stimulating factor, and C reactive protein.

Clinical investigations of peripheral blood monocytes and bronchoalveolar lavage cells will help clarify the presence of IL-6 in active tuberculosis infection and determine whether modulating IL-6 has a role in assisting host defense mechanisms as a potential therapeutic strategy.

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