Mycobacterium tuberculosis Enhances Human Immunodeficiency Virus-1 Replication by Transcriptional Activation at the Long Terminal Repeat

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Abstract

Tuberculosis has emerged as an epidemic fueled by the large number of individuals infected with the human immunodeficiency virus, especially those who are injecting drug users. We found a striking increase from 4- to 208-fold in p24 levels in bronchoalveolar lavage fluid from involved sites of *Mycobacterium tuberculosis* infection vs uninvolved sites in three HIV+ patients. We used an in vitro cell culture model to determine if tuberculosis could activate replication of HIV-1. Mononuclear phagocyte cell lines U937 and THP-1 infected with HIV-1_{JR-CSF}, in vitro and stimulated with live *M. tuberculosis* H37Ra, had a threefold increase in p24 in culture supernatants. Using the HIV-1 long terminal repeat with a chloramphenicol acetyltransferase (CAT) reporter construct, live *M. tuberculosis* increased transcription 20-fold in THP-1 cells, and cell wall components stimulated CAT expression to a lesser extent. The nuclear factor-κB enhancer element was responsible for the majority of the increased CAT activity although two upstream nuclear factor-IL6 sites may also contribute to enhanced transcription. Antibodies to TNF-α and IL-1 inhibited the increase in CAT activity of the HIV-1 long terminal repeat by *M. tuberculosis* from 21-fold to 8-fold. Stimulation of HIV-1 replication by *M. tuberculosis* may exacerbate dysfunction of the host immune response in dually infected individuals. (*J. Clin. Invest.* 1995. 95:2324–2331.) Key words: tuberculosis • lipoarabinomannan • human immunodeficiency virus

Introduction

Tuberculosis has emerged as an epidemic since its steady decline began to plateau in 1985 resulting in an excess of ~ 60,000 cases by 1992 compared to the numbers expected if the decline had continued (1–3). The tuberculosis epidemic has been concentrated in inner cities, e.g., cities > 100,000 with 26% of the United States population account for 53% of tuberculosis morbidity (4). There is a striking correlation between the epidemiology of AIDS and tuberculosis: cities with the highest AIDS case rates also have the highest rates of tuberculosis, e.g., matches of AIDS case registries with reported tuberculosis cases have found 12% of AIDS patients infected with *Mycobacterium tuberculosis* and 40% of tuberculosis patients infected with HIV-1 (3, 5, 6). Selwyn et al. (7) has demonstrated a striking risk of 11% per year for developing active tuberculosis among HIV positive, purified protein derivative–positive injecting drug users enrolled in a methadone maintenance program compared to the 10% lifetime risk in purified protein derivative–positive individuals without HIV-1 infection. HIV-1 infected individuals have a high likelihood of developing progressive pulmonary and/or extrapulmonary tuberculosis upon new infection with *M. tuberculosis.* (8, 9). Tuberculosis has recently been added to the case definition of AIDS. As HIV-1 infection progresses and CD4+ T lymphocytes decline in number, there is a concomitant increase in the percentage of patients with extrapulmonary tuberculosis and mycobacteremia (10).

Tuberculosis may well accelerate the course of HIV infection (8, 11, 12). Mortality of dually infected HIV/tuberculosis individuals has been reported to be as high as 50% at 12 mo in New York compared to < 10% due to *M. tuberculosis* alone, and mortality among HIV-1–infected individuals with multidrug-resistant *M. tuberculosis* can be as high as 90% at 9 mo (13). Toosi et al. (14) reported an enhanced susceptibility of blood monocytes from patients with tuberculosis to productive infection with HIV-1.

Alveolar macrophages are primary players in host defense phagocytosing *M. tuberculosis,* and are capable of being infected by HIV-1. At sites of active *M. tuberculosis* infection, bacilli-laden macrophages may be HIV-infected as well. Infection with *M. tuberculosis* stimulates the release of mononuclear phagocyte cytokines including IL-1 (IL-β or α), TNF-α, and IL-6 in animal models, in in vitro cell culture, and in humans with active tuberculosis (15–23). *M. tuberculosis* up-regulates the genes for these cytokines in peripheral blood monocytes (PBM)[1] in vivo from patients with active tuberculosis, and release of these cytokines is enhanced when these cells are cultured in vitro (23, 24). These cytokines enhance replication of HIV-1 in vitro using several cell lines (25). The mechanism of this stimulation has been localized to the 5′ long terminal repeat (LTR) of HIV-1, and specifically to the nuclear factor (NF)-κB transcription enhancer site (26–32). We hypothesized that *M. tuberculosis* and its components may enhance HIV-1 replication, and do so analogous to cytokines by acting on 5′ LTR transcription. We found that *M. tuberculosis* and its major antigenic cell wall component, lipoarabinomannan (LAM), were potent inducers of HIV-1 replication and LTR

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1. *Abbreviations used in this paper:* BAL, bronchoalveolar lavage; CAT, chloramphenicol acetyltransferase; C/EBP, CCAAT/enhancer binding protein; HRP, horseradish peroxidase; LAM, lipoarabinomannan; LM, lipomannan; LTR, long terminal repeat; NF, nuclear factor; PBM, peripheral blood monocytes; PIM, phosphoinositolmannoside.

transcription via NF-κB and possibly NF-IL6 sites, and that this effect was partially mediated by the cytokines IL-1 and TNF-α.

Methods

Materials. Live *M. tuberculosis* H37Ra was from American Type Culture Collection, Rockville, MD. LAM, lipomannan (LM), phosphoinositolmannoside (PIM), and deacylated LAM were from a laboratory-attenuated strain of *Mycobacterium smegmatis* provided by P. Brennan, Colorado State University, Ft. Collins, CO (33). These reagents had been eluted through Detoxi-Gel (Pierce Chemical Co., Rockford IL) columns using sterile, pyrogen-free water, and were 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 an Amebocyte lysate assay (E-Toxate kit; Sigma Chemical Co., St. Louis, MO). All lots of LAM, LM, and PIM were tested for LPS contamination and were found to contain < 5 pg of LPS per microgram test reagents.

The pHIVICAT plasmid construct was obtained from Dr. B. M. Peterlin, University of California, San Francisco, CA (34).

Polyclonal antibodies against TNF-α, IL-1β, and IL-1α and an isotype control antibody were from R & D Systems, Inc. (Minneapolis, MN).

Bronchoalveolar lavage. Bronchoalveolar lavage (BAL) was approved by the New York University and Bellevue Hospital Human Subjects Review Committees. We performed BAL with a fiberoptic bronchoscope under local anesthesia, instilling three, 50-ml aliquots of normal saline into a radiographically involved and uninvolved site sequentially. The fluid was filtered through sterile gauze, centrifuged for 5 min, and the BAL supernatants frozen at −70°C. The BAL supernatant was concentrated 10 times by centrifugation at 40,000 *g* for 1 h. HIV test results were performed with ELISA (Abbott Laboratories Diagnostic Division, Abbott Park, IL, detection limit 0.3 pg/ml) confirmed by Western blot. Three patients were diagnosed with pulmonary tuberculosis. Patient 1 had positive sputum for cultures for *M. tuberculosis* and patients 2 and 3 had clinical criteria consistent with pulmonary tuberculosis and were treated empirically; both had a successful clinical response to antimycobacterial therapy.

Cell culture. Human myelomonocytic leukemia cell line THP-1 cells and U937 cells were purchased from the American Type Culture Collection. THP-1 cells or U937 cells were maintained in RPMI 1640 supplemented with 10% FBS. Cells were placed in 24-well plastic tissue culture plates (Falcon Labware, Oxnard, CA) in RPMI 1640 at a density of 10^5 or 10^6 cells/well for specific assays.

In vitro infection of U937 and THP-1 cells with HIV-1. Mononuclear phagocyte U937 cells or THP-1 cells (10^5 cells/ml) were cultured in 24-well tissue culture plates in 1 ml of complete RMPI 1640 and incubated at 37°C in 5% CO_2. U937 or THP-1 cells were washed with RPMI 1640 twice and 1 ml of complete RPMI 1640 added containing 10^3 tissue culture infectious dose/ml of HIV-$1_{JR\text{-}CSF}$ (AIDS Research and Reference Reagent Program, Bethesda, MD), and incubated overnight at 37°C in 5% CO_2. Infected cell lines were washed with RPMI 1640 two times, and stimulated with *M. tuberculosis* H37Ra (10^3, 10^4, and 10^5 bacilli/ml) or various concentrations of LAM for 4–10 d. Assays for p24 HIV antigen were performed on the supernatants using a standard ELISA (Abbott Laboratories Diagnostic Division). The detection limit of the p24 assay was 0.3 pg/ml. All experiments were performed at least three times.

Site-directed mutagenesis and transient transfection of THP-1 cells and assay of chloramphenicol acetyltransferase (CAT) activity. The HIV-1 LTR in the pHIVCAT plasmid was mapped and two NF-IL6 and double NF-κB sites identified. Site-directed mutagenesis (Bio-Rad Laboratories, Richmond, CA) was carried out according to the manufacturer's directions. The following mutant oligonucleotides were synthesized: mutation of NF-IL6 (−491/−483) 5′ TTTAGATAT 3′ to 5′ AATTACATAT 3′; NF-IL6 (−300/−292) 5′ TTGAGCCAGA 3′ to 5′ AAGACCCAGA 3′; and double NF-κBs (−104/−81); 5′ GGGACTTTCCGCTGGGGACTTTCC 3′ to 5′ GGGACTTTAGGCTGCTCACTTTCC 3′. Site-directed mutagenesis was then carried out. The achieved mutant sequences were verified by DNA sequencing. Seven different single or combined mutations were obtained: single NF-IL6 mutations; double NF-IL6 mutations; combined NF-IL6 and NF-κB mutations; NF-κB mutations; and triple mutations. To test activities of NF-IL6 or NF-κB sites in the front of a heterologous promoter, synthetic double strand oligonucleotides containing NF-IL6 sites or the NF-κB sites were subcloned into polylinker sites of pTK.CAT plasmid (34).

THP-1 cells were transfected with serially constructed plasmids by the DEAE-Dextran method (35, 36). Briefly, 10^7 cells were washed in STBS solution (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 cesium chloride ultracentrifugation–purified plasmids in 1 ml DEAE-Dextran solution at 400 μg/ml at 37°C for 90 min. The transfected cells were incubated in complete medium for 24 h in the absence or presence of inducing agents. The CAT assays were carried out with commercially available kits from Boehringer Mannheim Biochemicals (Indianapolis, IN). For the assay, the transfected THP-1 cells were broken by three cycles of freeze-thawing. The whole cell extract was heated to 60°C for 10 min and spun in a microfuge for 5 min. The clear supernatant was quantitated for protein concentration with Bio-Rad reagents and 300 μg protein from each sample was used for the CAT assay. For the CAT assay, 100-μl cell extracts containing 300 μg protein were added to wells of rigid flat-bottomed microtiter plates coated with murine mAb to CAT. After incubation of the samples and throughout the wash of the wells, horseradish peroxidase (HRP)-conjugated anti-CAT antibody was added to the test wells. After a second incubation, the excess of the HRP-conjugated antibody was removed by washing. The substrate of HRP was then added, and the color intensity was measured with a microtiter plate reader at 405 nm. The experiments were done in triplicate. The amount of CAT activity was calculated based on a standard curve generated using purified CAT protein. Results represent the means of at least three independent transfections. Within each transfection duplicate samples varied < 10%.

Preparation of nuclear extracts and DNA mobility shift assay. Cells were prepared from THP-1 cells by using the method first described by Dignam et al. (35). Cultures (~ 5×10^8 cells) were lysed by homogenization in buffer A (10 mM Hepes [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM PMSF) and centrifuged at 1,000 *g* for 10 min at 4°C. The nuclei were washed with buffer A once. The nuclear proteins were extracted in buffer C (20 mM Hepes [pH 7.9], 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF) 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 PMSF) for 5 h and were frozen in liquid nitrogen in aliquots.

The DNA mobility shift assay was carried out as described by Sen and Baltimore (37). The NF-κB DNA probe was end-labeled by using polynucleotide kinase in the presence of [γ-^{32}P]ATP. The probe (10,000 cpm) was incubated with 2 μg of nuclear extracts under binding conditions (10 mM Tris [pH 7.5], 50 mM NaCl, 1 mM EDTA, 0.1 mM DTT, 2 μg of poly[dI-dC]), and 10% glycerol in a total volume of 20 μl at room temperature for 15 min, and then the probe was electrophoresed on a 4% polyacrylamide gel (1× TBE buffer pH 8.3 in the gel). The gel was analyzed by autoradiography and experiments were performed at least three times.

Results

Increased p24 release at local sites of M. tuberculosis disease in patients infected with AIDS. We performed bronchoalveolar lavage on three individuals who were HIV+ and presented with

Table I.

Patient	Age	CD4+ cells/μl	Chest radiograph	p24 (picograms/milliliter)		
				Plasma	Involved BAL	Uninvolved BAL
1	32	102	Right upper lobe cavity	3.1	62.5	< 0.3
2	39	89	Right perihilar infiltrate	31.1	16.4	< 0.3
3	43	190	Bilateral apical nodular infiltrate	29.2	1.2	< 0.3

symptoms typical of active tuberculosis (cough, sputum production, fever, night sweats, and weight loss). Since chest radiographs revealed localized disease in each, we lavaged the involved lobe and uninvolved sites separately, e.g., upper lobe vs lower lobe. There was a striking increase in p24 levels in the lavage fluid from the involved site compared to the uninvolved site in each patient (Table I). In patient 1, the involved site was 20-fold higher than that in plasma.

Increased p24 release by mononuclear phagocytes infected with HIV-1 by M. tuberculosis H37Ra. We cultured U937 or THP-1 cells, infected them with HIV-$1_{JR\text{-}CSF}$, followed by live *M. tuberculosis* H37Ra, and observed a dose–response increase in p24 levels in culture supernatants (Fig. 1). The most striking increase (threefold) was in U937 cells that peaked at 10^4 bacilli/ml at 4 d; increases were less at 7 and 10 d. We also found that the mycobacterial cell wall component, LAM, was able to stimulate p24 release at doses of 0.1 μg/ml and 0.5 μg/ml peaking at the higher dose at 4 d in U937 cells (data not shown).

Stimulation of HIV-1 LTR activity by M. tuberculosis and its cell wall components. To investigate the mechanism by which *M. tuberculosis* and its cell wall components enhance HIV-1 replication, the pHIVICAT plasmid containing the LTR sequence in front of the CAT-structural gene was transfected into THP-1 cells. Stimulation for 24 h with live *M. tuberculosis* H37Ra, resulted in a striking 19-fold increase in CAT activity over control (Fig. 2). The LAM, LM, and PIM were also strong stimuli ranging from 7- to 14-fold over control CAT activity, but the deacylated LAM stimulation was not different from control. Previously, Barnes et al. have demonstrated the importance of the acyl moiety of LAM as being necessary for cytokine release from PBM (20).

Evaluation of NF-κB and NF-IL6 sites from the HIV-1 LTR as transcription enhancer sites from a heterologous promoter for M. tuberculosis stimulation. To test whether each individual NF-IL6 sequence or NF-κB sequences were responsive to *M. tuberculosis,* the synthetic double strand NF-IL6 or NF-κB sequences were subcloned in front of the heterologous thymidine

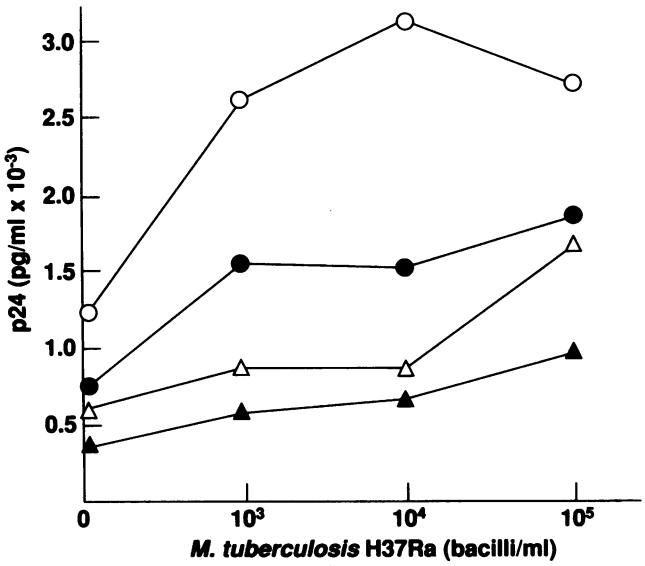

Figure 1. Increased HIV-1 p24 levels from U937 and THP-1 cells following stimulation by *M. tuberculosis* H37Ra. Mononuclear phagocytes were washed in RPMI 1640 twice and plated at 10^5/well with $HIV_{JR\text{-}CSF}$ at 1,000 tissue culture infectious dose/well for 24 h. Cells were washed and cultured in the presence of *M. tuberculosis* H37Ra at doses indicated for 7 d. Supernatants were tested for HIV-1 p24 antibody by ELISA. Shown is a representative experiment from three similar results. (○) U937 cells at 4 d, (△) U937 cells at 7 d, (●) THP-1 cells at 4 d, (▲) THP-1 cells at 7 d.

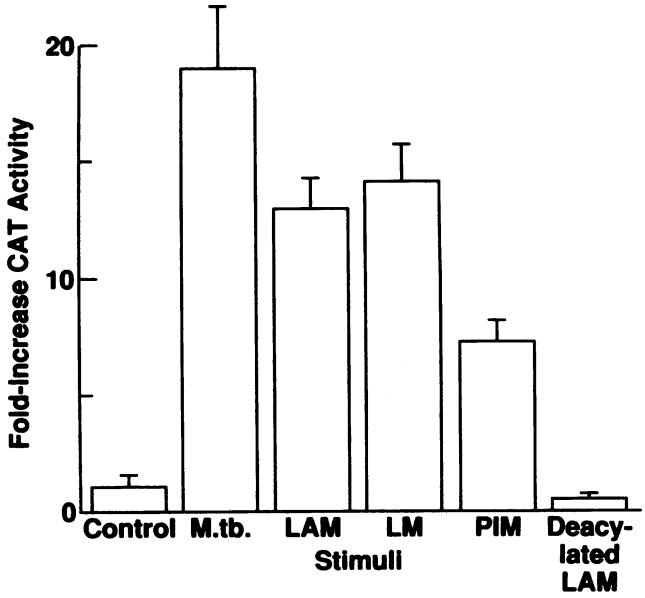

Figure 2. Activation of HIV-1 long terminal repeat region by *M. tuberculosis* (*M.tb.*) and its cell wall components. The plasmid pHIVCAT containing HIV-1 LTR was transfected into THP-1 myelomonocytic leukemia cell line by the DEAE-dextran method. Cells were stimulated for 24 h by live *M. tuberculosis* H37Ra and purified cell wall components LAM, LM, PIM, and deacylated LAM. The cell extracts were evaluated for CAT activity by ELISA and the fold increase over control (unstimulated cells) was determined. Only 10^5 bacilli per 5×10^6 THP-1 cells were used representing a multiplicity of infection of one mycobacterium per 50 cells. Results are representative of three separate experiments.

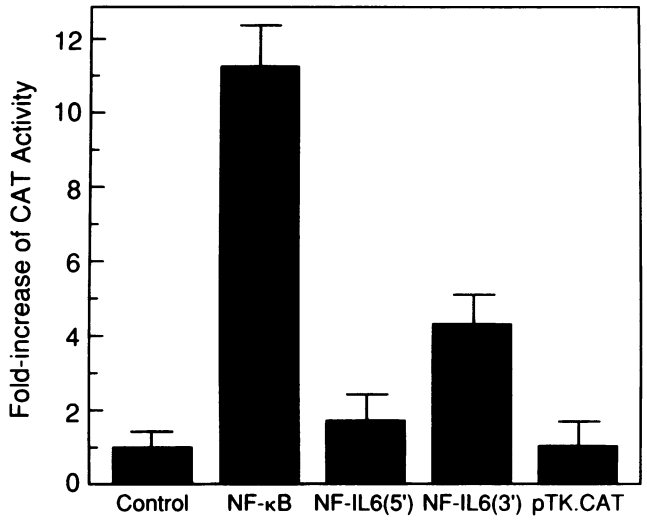

Figure 3. Activation of NF-κB or NF-IL6 sites by *M. tuberculosis* or LAM. The double NF-κB (−110/−75) or single NF-IL-6 sites (−495/−478) and (−300/−286) were synthesized and subcloned in front of the thymidine kinase promoter linked to CAT structure gene (pTK.CAT). Plasmids were transfected into THP-1 cells and stimulated for 24 h with live *M. tuberculosis* H37Ra. The cell lysates were assayed for CAT activity by ELISA and the fold increase compared to unstimulated THP-1 control cells. Results are representative of the three separate experiments.

kinase promoter at polylinker sites in pTK.CAT. The recombinant plasmids were transfected into THP-1 cells and CAT activity measured after stimulation with live *M. tuberculosis* H37Ra. The NF-κB site was stimulated 11-fold in CAT activity over control by live *M. tuberculosis* (Fig. 3). After stimulation of transfected THP-1 cells by *M. tuberculosis* H37Ra, the 5′ NF-IL6 site had a twofold increase in CAT activity, and the 3′ NF-IL6 site had fourfold increase in CAT activity. As a negative control, CAT activity of the pTK.CAT vector plus *M. tuberculosis* was not different from control. This suggests that the NF-κB and the NF-IL6 sites from the HIV-1 LTR can drive transcription from a heterologous thymidine kinase promoter.

Effect of mutations of transcription enhancer elements on the stimulation of HIV-1 LTR by M. tuberculosis and LAM. To evaluate the function of NF-κB's on the LTR, site directed mutagenesis was performed mutating both NF-κBs on the pHIVICAT plasmid leaving the two NF-IL6 sites intact (Fig. 4 *A*). Transient transfection of THP-1 cells, *M. tuberculosis* stimulation, and CAT assay revealed a fourfold increase using the mutant pHIVICAT reflecting the importance of the NF-κB sites on the LTR. LAM also induced increased CAT activity by wild-type and mutant pHIVICAT, but levels were three-fourths that of live *M. tuberculosis,* and deacylated LAM was inactive.

Further mutations were performed on pHIVICAT of two NF-IL6 sites (−491/−483, −300/−292) leaving the double NF-κB site intact, and CAT activity after *M. tuberculosis* stimulation was reduced from 13-fold to 10-fold (Fig. 4 *B*). A similar reduction was observed after LAM stimulation. Individual NF-IL6 mutations combined with double NF-κB mutations on pHIVICAT followed by transient transfection in THP-1 cells and *M. tuberculosis* stimulation resulted in a 1.8-fold increase in CAT activity for NF-IL6 (5′) intact (Fig. 4 *C*) and 2.5-fold increase in CAT activity for NF-IL6 (3′) intact (Fig. 4 *D*) over control. These data suggest a modest role for NF-IL6 sites in addition to the very striking increase for the double NF-κB motif in mediating 5′ LTR transcription after *M. tuberculosis* or LAM stimulation. Again, there was no increase in activity with decylated LAM.

DNA mobility shift assay demonstrates LAM enhances interaction between nuclear protein(s) and the NF-κB sequence. To determine whether the double NF-κB sequence interacted specifically with nuclear protein(s) after LAM stimulation of THP-1 cells, DNA mobility shift assay was performed. Nuclear proteins isolated from LAM-treated or control THP-1 cells were incubated with end-labeled synthetic double-stranded NF-κB probe in the presence or absence of specific or nonspecific competitors. After treatment with LAM, a dramatically enhanced binding of nuclear protein(s) to the NF-κB site was observed (Fig. 5, lane *2*, compared to unstimulated cells in lane *1*). The protein-DNA interaction was still present after a competition experiment with 100-fold molar excess cold mutated NF-κB probe (Fig. 5, lane *3*). However, binding of nuclear protein(s) to the NF-κB site was abolished by competition performed with 100-fold excess cold wild-type NF-κB probe (Fig. 5, lane *4*). To test whether LAM increases the binding of nuclear proteins to NF-IL6 sites, DNA mobility shift assay was carried out. We failed to find differences between unstimulated and LAM-stimulated cells.

Effect of anti–TNF-α or IL-1 antibodies on the activation of the HIV-1 LTR region by M. tuberculosis. Both TNF-α and IL-1 are known to activate transcription of the HIV-1 LTR, and both cytokines are released when mononuclear phagocytes are stimulated by M. tuberculosis or its components. Therefore, to determine if *M. tuberculosis* or LAM activation of the LTR of HIV-1 is mediated by these cytokines, we transiently transfected THP-1 cells with pHIVICAT in the presence of antibodies to these cytokines (Fig. 6). We observed that with *M. tuberculosis* stimulation, anti–TNF-α antibody reduced the increase in CAT activity from 21-fold to 10-fold, that anti–IL-1α plus IL-1β antibodies reduced the increase in CAT activity from 21-fold to 14-fold, and that all three antibodies reduced the increase in CAT activity by *M. tuberculosis* from 21-fold to 8-fold (Fig. 6). Results with an irrelevant IgG_1 isotype control antibody demonstrated no decrease in the CAT activity. Similar reductions were found using LAM as a stimulus and deacylated LAM was inactive. These data suggest that activation of LTR of HIV-1 by *M. tuberculosis* is mainly mediated by secondary release of cytokines TNF-α and IL-1β.

Discussion

M. tuberculosis disease complicates AIDS in being a virulent pathogen leading to dissemination of bacilli with increased percentage of patients with miliary presentation, tuberculosis meningitis (38), and extrapulmonary tuberculosis (2, 6, 8). An important question is whether dually infected patients have a synergistic interaction between the two organisms. Toosi et al. have demonstrated that peripheral blood monocytes from active tuberculosis patients are more receptive to productive HIV infection (14). Monocytes from five tuberculosis patients produced greater HIV-1 p24 levels in supernatants compared to matched controls after infection with $HIV\text{-}1_{JR\text{-}FL}$, and four of five had a > twofold increase in HIV-1 mRNA transcripts detectable by PCR (14).

We found that BAL fluid from sites of active *M. tuberculosis*

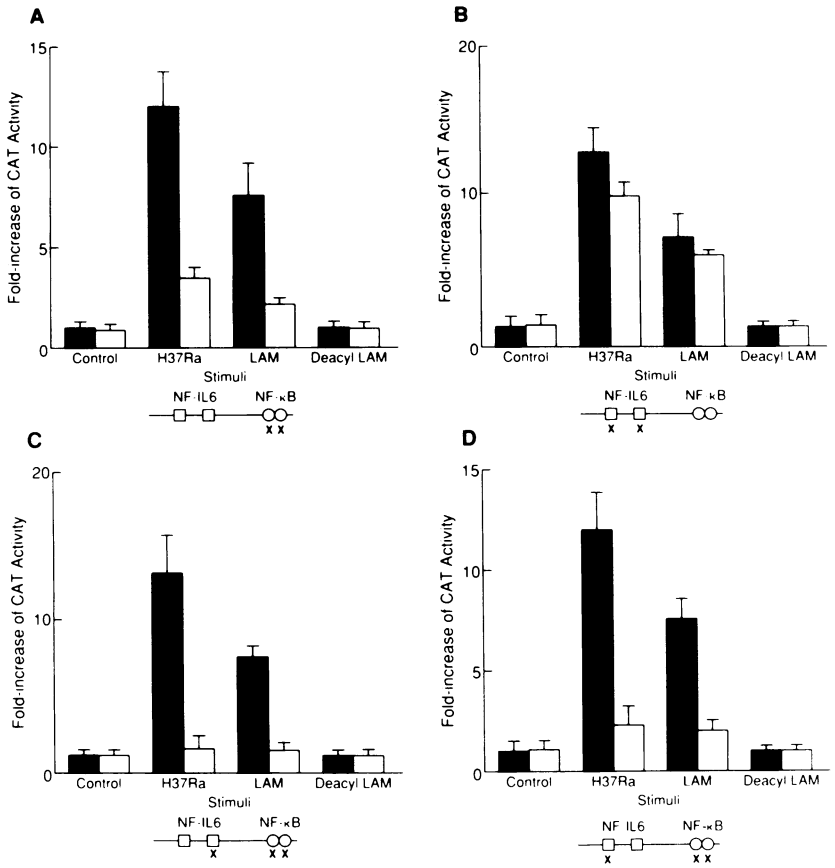

Figure 4. Evaluation of transcription enhancer activity in pHIVCAT after mutations and stimulation by *M. tuberculosis* or LAM. Site-directed mutagenesis was performed on NF-κBs and two NF-IL6 sites as described in Methods. The wild-type (■) or mutant (□) plasmids were transfected into THP-1 cells and stimulated for 24 h with live *M. tuberculosis* H37Ra, LAM, or deacylated LAM. The cell extracts were assayed for CAT activity by ELISA and the fold increase compared to unstimulated THP-1 control cells. (*A*) Effect of mutation of NF-κB sites on the HIV-1 LTR. (*B*) Effect of mutation of both NF-IL6 sites on the HIV-1 LTR. (*C*) Effect of mutation of NF-κB and NF-IL6 (3′) on the HIV-1 LTR. (*D*) Effect of mutation of NF-κB and NF-IL6 (5′) on the HIV-1 LTR. Results are representative of three separate experiments.

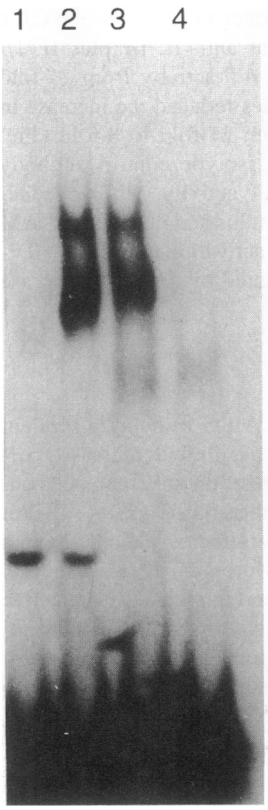

Figure 5. Induction of nuclear protein binding to NF-κB analyzed by DNA mobility shift assay. THP-1 cells were stimulated with LAM (500 ng/ml) for 3 h and the nuclear proteins extracted as described in Methods. 2-μg nuclear extracts were incubated with ^{32}P end-labeled synthetic double NF-κB probe in the absence or presence of competitors. The reaction mixtures were then analyzed on 4% polyacrylamide gel, which was dried and exposed to x-ray film. (Lane *1*) Unstimulated nuclear extracts; (lane *2*) LAM-stimulated nuclear extracts; (lane *3*) LAM-stimulated nuclear extracts in the presence of 100-fold molar excess unlabeled synthetic mutant (same as in Fig. 4) NF-κB sequence; (lane *4*) LAM-stimulated nuclear extracts in the presence of 100-fold molar excess unlabeled synthetic wild-type NF-κB DNA. Results presented are representative of three separate experiments.

disease in three patients with AIDS have a striking increase in p24 levels compared to uninvolved sites. Since the saline in BAL fluid dilutes the epithelial lining fluid ~ 100-fold (39), levels of p24 in involved sites may actually be much higher than in plasma consistent with enhanced local HIV-1 replication in vivo. There are at least two mechanisms whereby *M. tuberculosis* could enhance HIV-1 replication. First, alveolar macrophages could be coinfected, especially at sites of disease where there are large numbers of bacilli where alveolar macrophages are also actively phagocytosing the organism. However, we have demonstrated a very low copy number of HIV-1 DNA in alveolar macrophages from AIDS patients with normal chest radiographs (40). A second mechanism is for macrophages to be stimulated by *M. tuberculosis* to release cytokines that enhance HIV-1 replication. In this regard, we have also demonstrated exaggerated release of IL-1β, TNF-α, and IL-6 from BAL cells into 24-h culture supernatants in vitro from involved sites of patients with pulmonary tuberculosis vs uninvolved sites (41).

The second important finding of our report is that live *M. tuberculosis* stimulates the HIV-1 LTR, and that this occurs mainly via the *cis*-acting NF-κB transcription enhancer. We also observed that LAM could also stimulate HIV-1 transcription but was weaker than the live bacilli. Deacylated LAM had no effect on the HIV-1 LTR emphasizing the importance of the inositol-phosphate backbone of LAM in producing biologic activity. Alterations of LAM by removing carbohydrate moieties, e.g., lipomannan, or removing glycolipids, e.g., phosphoinositolmannoside, still retained biologic activity (20). We attempted to dissect the promoter (−640/+81) of the HIV-1 LTR using a

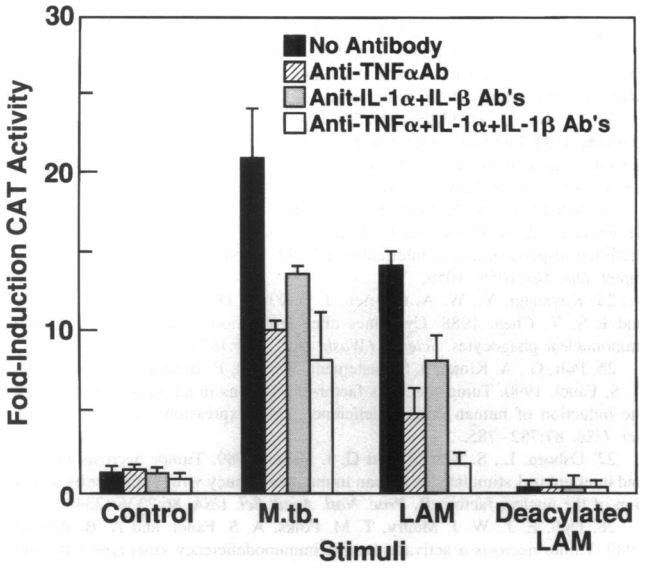

Figure 6. Effect of anti–TNF-α or anti–IL-1β and/or anti–IL-1β and/or anti–IL-1α antibodies on the activation of the HIV-1 LTR region. THP-1 cells were transfected with pHIVICAT followed by stimulation for 24 h by live *M. tuberculosis* (*M.tb.*) H37Ra, LAM, or deacylated LAM in the presence of anti–TNF-α antibody (10 μg/ml) (■), combined anti–IL-1α plus anti–IL-1β antibodies (each 10 μg/ml) (■), triple-combined anti–TNF-α antibody plus anti–IL-1α and anti–IL-1β antibodies (each 10 μg/ml) (□) or no antibody (■). Results with an irrelevant isotype IgG_1 control antibody was the same as no antibody. Control is THP-1 cells transfected with pHIVICAT without stimulation by *M. tuberculosis* or its components but with antibodies as above. Results presented are representative of three separate experiments.

plasmid containing double NF-κB and two NF-IL6 sites cloned into pTK.CAT that responded to *M. tuberculosis* with enhanced CAT activity. Mutations of NF-κB on pHIVICAT revealed a modest amount of CAT increase after *M. tuberculosis* stimulation which may be due to the two NF-IL6 sites or others (e.g., SP1) but mutations of one or more NF-IL6 sites clearly demonstrated that the majority of the CAT activity increase was due to the double NF-κB site. The mutated NF-κB plasmid was unable to compete for labeled oligonucleotide probe in our electrophoretic mobility shift assay demonstrating inability to bind nuclear proteins and efficacy of the mutations. The consensus sequence for NF-IL6 5′ T(^{T}/G)NNGNAA(^{T}/G) 3′ was identified at the −491/−483 and −300/−292 sites. We have previously described two NF-IL6 sites on IL-1β (−90/−82, −40/−32) that are responsive to *M. tuberculosis* components (42) and two NF-IL6 sites located 5′ to a NF-κB site on the IL-6 gene that are all responsive to LAM (43). Mutations of these sites abrogated cytokine stimulation by *M. tuberculosis*. Interestingly there was evidence of synergism between the NF-κB site and the proximal NF-IL6 site on the IL6 promoter after LAM stimulation. In this regard, Matsusaka et al. (44) have also reported that transcription factors NF-IL6 and NF-κB act synergistically in activating the inflammatory cytokines IL6 and IL-8. Cotransfection of NF-IL6 with the NF-κB p65 subunit resulted in synergistic activation of an IL-6 promoter construct, and the basic leucine-zipper domain interacted with the Rel homology domain of p65 (44). NF-IL6 may be a mycobacterial transcriptional response element but is also responsible for other acute phase proteins, cytokines (IL-1β, IL-6, IL-8, TNF-α), albumin, c-*fos*, G-CSF, and several viral proteins (44). It is possible that the enhanced transcription of the HIV-1 LTR by *M. tuberculosis* is due to synergistic interaction of NF-IL6 and NF-κB because mutation of one or the other sites reduced CAT activity. NF-IL6α and β are members of the CCAAT/enhancer binding protein (C/EBP) family of transcription factors (C/EBPβ and C/EBPδ, respectively). Cooperative binding of NF-κB and C/EBP to their adjacent binding sites has been demonstrated on the IL-8 promoter (45). We propose that similar to IL-8 and IL-6, NF-κB plays the more important role in HIV-1 LTR gene regulation but that its activity may be enhanced by C/EBPβ. Further, C/EBPβ specifically bound to and activated the TNF-α promoter at a site that C/EBPα could not bind to (46). A dominant negative version of C/EBPβ blocked TNF-α promoter activation in myeloid cells (46).

The NF-κB transcription-enhancer element on the HIV-1 LTR is responsive to the cytokines IL-1, TNF-α and IL-6 (26–30, 47–49). All three of these cytokines are also stimulated in vitro in PBM by *M. tuberculosis* and its components, especially LAM (16, 18–20, 22). Although these cytokines may only be transiently released in the blood, we have demonstrated up-regulation of the mRNA for these cytokines in PBM from HIV–active tuberculosis patients compared to normal controls (24). Denis and Ghadirian recently demonstrated that alveolar macrophages lavaged from HIV+ individuals spontaneously released more IL-1β, TNF-α, and IL-6 than normal controls, and infecting these macrophages in vitro with *M. avium* augmented cytokine release (50). Importantly, they coinfected alveolar macrophages in vitro with HIV-1 and *M. avium* finding higher reverse transcriptase activity from dually infected experiments than HIV-1 alone over a 5–15-d culture period. In addition to cytokines, the activation of the HIV promoter in the viral LTR has been observed by mitogens, phorbol esters, retinoic acid, ionizing radiation (51), ultraviolet light (52), T_3 receptor (53), and viruses (herpes virus, cytomegalovirus, human T lymphocyte virus-1 [54], papovaviruses and others) (29, 30). We demonstrated that more than half of the stimulation of the HIV-1 LTR by *M. tuberculosis* could be blocked by the combined use of anti–TNF-α, anti–IL-1α, and anti–IL-1β suggesting roles for these cytokines in stimulation (26). IL-6 also has been reported to synergize with TNF-α in the induction of latent HIV-1 expression by acting both at transcription and posttranscriptionally (47). HIV-1 in human alveolar macrophages is latent in vivo but replicates after in vitro stimulation over 6 d in the presence of TNF-α and GM-CSF (55). Although we have focused on the mononuclear phagocyte, dendritic cells in the lung are also a reservoir for HIV-1 infection, and are a major presentor of *M. tuberculosis* antigens to T lymphocytes (56). Coinfection of these cells may cause further disarray of the host immune defense. Whalen et al. recently reported that dually infected HIV+/tuberculosis patients matched by CD4+ counts to HIV+ patients have a significantly reduced 1 yr survival (65% vs 90%, $P = 0.0$) (57).

In summary, *M. tuberculosis* and its components may activate HIV-1 replication through enhanced transcription of the LTR via the NF-κB motif which may be modulated by C/EBPβ (NF-IL-6α), and the cytokines, TNF-α and IL-1β, are partially responsible for this effect. Therapeutic strategies using negative regulators, e.g., thalidomide to block TNF-α, or activating cytotoxic T cells, may be useful in modulating effects of the dual infection (58, 59).

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