Lipopolysaccharide Is a Potent Monocyte/Macrophage-specific Stimulator of Human Immunodeficiency Virus Type 1 Expression

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Summary

Lipopolysaccharide (LPS) potently stimulates human immunodeficiency virus type 1–long terminal repeat (HIV-1–LTR) CAT constructs transfected into monocyte/macrophage-like cell lines but not a T cell line. This effect appears to be mediated through the induction of nuclear factor κB (NF-κB). Electrophoretic mobility shift assays demonstrate that LPS induces a DNA binding activity indistinguishable from NF-κB in U937 and THP-1 cells.

LPS is also shown to dramatically increase HIV-1 production from a chronically infected monocyte/macrophage-like cloned cell line, U1, which produces very low levels of HIV-1 at baseline. The stimulation of viral production from this cell line occurs only if these cells are treated with granulocyte/macrophage colony-stimulating factor (GM-CSF) before treatment with LPS. This stimulation of HIV-1 production is correlated with an increase in the level of HIV-1 RNA and an activation of NF-κB. LPS is not able to induce HIV-1 production in a cloned T cell line. The effect of LPS on HIV-1 replication occurs at picogram per milliliter concentrations and may be clinically significant in understanding the variability of the natural history of HIV-1 infection.

Control of the replication of HIV-1 involves both virus-encoded proteins and cellular factors activated by a variety of exogenous stimuli (1–3). The role of cellular events is evident because stimulation of the HIV-1 promoter within the long terminal repeat (LTR)[1] is produced by various cellular stimuli including phorbol esters, TNF-α, and heterologous viral proteins (3). Many of these agents appear to function through the activation of nuclear factor κB (NF-κB) which binds to two 11 bp motifs in the HIV-1 LTR and consequently activates LTR-driven RNA transcription (3–7). The effects of certain cellular stimuli on HIV-1 replication have been studied in transient transfection assays of the HIV-1–LTR linked to reporter genes and by utilizing chronically infected cell lines and primary cells (3–14).

HIV-1 growth in infected people is difficult to study directly and so must be modeled by examining influences on cells in culture. Factors that stimulate HIV-1 replication in cell culture may also regulate HIV-1 expression in CD4 lymphocytes and monocytes/macrophages in vivo (1–3). These are the two major cell types known to harbor HIV-1 (1). In the periphery, CD4 lymphocytes carry most of the virus (15), but the monocyte/macrophage may be the major cell type infected in the central nervous system and other tissues (1, 16, 17). The monocyte/macrophage may act as a "Trojan Horse," concealing the virus from the immune system, and it may be among the first cells infected by HIV-1 in patients (1, 17).

Many agents that stimulate HIV-1 replication do so in both T cells and monocyte/macrophages (6–8). With the exception of granulocyte/macrophage colony stimulating factor (GM-CSF), agents that induce HIV-1 replication only in monocyte/macrophages are not well described (9, 12, 14). Stimuli that selectively affect HIV-1 replication in monocyte/macrophages might, therefore, hold significant clinical importance.

The monocyte/macrophage can be studied either directly after culturing or through cell line models. Primary monocyte/macrophages have thus far been refractory to transfection with significant efficiency, and therefore, are not suitable for the study of gene expression and regulation. We have thus chosen to study the U937 and THP-1 monocyte/macrophage-like cell lines as model systems.

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[1] Abbreviations used in this paper: CAT, chloramphenicol acetyl transferase; GM-CSF, granulocyte/macrophage colony-stimulating factor; LTR, long terminal repeat; NF-κB, nuclear factor κB.

In this paper, we demonstrate that LPS or endotoxin, a constituent of the cell wall of gram-negative bacteria, stimulates gene expression directed from the HIV-1–LTR in monocyte/macrophage-like cell lines but not in T cell lines. LPS has this effect at physiologically significant concentrations and appears to do so through the activation of NF-κB, which in turn binds to two κB enhancer elements in the U3 region of the HIV-1–LTR (4).

Contrary to a previous report (7), we find no correlation between the state of differentiation of the available monocyte/macrophage-like cell lines and the level of constitutive expression of activated NF-κB. However, we do find that activation of NF-κB by LPS and consequent stimulation of HIV-1 from a cloned monocyte/macrophage-like cell line (9) occur only after treatment with GM-CSF. This suggests that the developmental maturity of HIV-1–infected monocyte populations may determine whether they respond to LPS with an increase in HIV-1 production.

Materials and Methods

Cells. A monoblastoid cell line, U937, was maintained in RPMI 1640 media supplemented with 10% FCS, penicillin (250 U/ml), and streptomycin (250 μg/ml) (18). The cell line THP-I, derived from monocytic leukemic cells, was maintained as above (19). A human T cell line Jurkat was also maintained as previously described (20).

A subclone of HIV-1–infected U937 cells, U1, was donated by Thomas Folks and was obtained from the AIDS Research and Reference Program, NIAID, National Institutes of Health, Bethesda, MD. This cell line shows a minimal amount of constitutive expression of HIV-1 (9).

A variant of the CEM T-cell line, A3.01, was infected with the LAV strain of HIV-1 and a subclone, ACH-2, was obtained that exhibits minimal amounts of constitutive expression of HIV-1 (10). This cell line was donated by Thomas Folks and was obtained from the AIDS Research and Reference Program, NIAID, NIH.

Plasmids. The plasmid HIV-CAT, containing the HIV-1–LTR upstream of the chloramphenicol acetyl transferase (CAT) gene has been described (4). The plasmid, HIV NF-κB Δ CAT, with mutations in the NF-κB sites, rendering these sites unable to bind the NH-κB protein, has also previously been described (4).

A plasmid (J18) containing two κB sites from the κ enhancer which were cloned into Hind III and Sal I sites upstream of a truncated mouse c-fos promoter linked to the CAT gene, was kindly provided by J. Pierce (21). The c-fos promoter was truncated to nucleotide 56 to remove phorbol ester and serum-responsive elements. A plasmid lacking κB sites but retaining the truncated c-fos promoter was also obtained from J. Pierce, Whitehead Institute, Cambridge, MA (21).

Transfections and CAT Assays. All cell lines were transfected using DEAE-dextran. In brief, cells were washed twice with PBS and resuspended in serum-free suspension Tris-buffered saline (22). 10^7 cells were then treated with DEAE-Dextran at 200 μg/ml (Pharmacia, Uppsala, Sweden) containing 10 μg of plasmid in 1 ml. Cells were treated 90 min at 37°C with frequent mixing. A 10% DMSO shock was performed and the cells were washed twice with PBS and then resuspended to a concentration of 5 × 10^5 cells/ml in RPMI 1640 media with 10% FCS.

24 h after transfection, the cells were exposed to either LPS *Escherichia coli* 0127:B8 (Difco Laboratories, Detroit, MI), recombinant

human TNF-α (Genzyme Corp., Cambridge, MA), or PMA (Sigma Chemical Co., St. Louis, MO) and PHA (Sigma). Some transfections received no treatments. A rabbit anti–human TNF-α neutralizing antibody (1 μl neutralizes 10^3 U of TNF-α) was also added to some transfections (Genzyme Corp.). 44 h after transfection, cell extracts were prepared for CAT assay and protein concentrations were determined by the method of Bradford (23) (Bio-Rad Laboratories, Richmond, CA). The quantity of protein used per assay was normalized (70 μg for U937 and THP-1 cells and 100 μg for Jurkat cells). CAT assays were performed according to the method of Gorman et al. (24). Percent conversion of acetylated products by liquid scintillation was calculated.

Transfections were performed between two and six times and results are reported as the arithmetic mean + SEM.

Cellular Extracts and Electrophoretic Mobility Shift Assays (EMSA). Cellular extracts were prepared from 5 × 10^7 cells with or without treatment for various time periods with GM-CSF (Genetics Institute, Cambridge, MA), LPS, or PMA plus PHA according to a protocol, with some modifications, described by Baeuerle and Baltimore (25). Cells were washed twice with ice-cold PBS and then resuspended in lysis buffer that consisted of 20 mM Hepes (pH 7.9), 0.35 M NaCl, 20% glycerol, 1% NP-40, 1 mM $MgCl_2$, 1 mM DTT, 0.5 mM EDTA, 0.1 mM EGTA, 1 mM PMSF, 0.3 μg/ml antipain (Sigma), and 0.3 μg/ml leupeptin (Sigma). After lysis on ice for 10 min, particulate material was removed by centrifugation at 11,000 g for 15 min at 4°C. The supernatant's protein content was measured by an assay using bicinchoninic acid according to the manufacturer's instructions (Pierce Chemical Co., Rockford, IL). Electrophoretic mobility shift analyses to detect NF-κB binding were carried out as previously described (26).

The ^{32}P-labeled DNA probe was a 44-bp HindIII-SalI fragment cut from a plasmid (J10), containing a single κB enhancer site upstream of the mouse c-fos promoter (21). This fragment was end-labeled with [^{32}P]CTP and [^{32}P]ATP (New England Nuclear, Boston, MA) using the Klenow fragment of *E. coli* DNA polymerase I (Boehringer-Mannheim Biochemicals, Indianapolis, IN).

Bands other than free probe and NK-κB activity are noted with cellular extracts made with this method. They represent cytoplasmic DNA binding proteins and/or proteolytic digestion products of nuclear proteins (Feinberg, M., and D. Baltimore, manuscript in preparation).

The competition experiments were performed with various nanogram amounts of an unlabeled double-stranded oligonucleotide containing both HIV-1 NF-κB-binding motifs and an oligonucleotide with mutations in the HIV-1 NF-κB-binding motifs, previously shown to not bind NF-κB (4) (kindly provided by Dr. M. Lenardo). HIV-1 κB oligonucleotide: 5'TCGACAAGGGACTTTCCGCTG-GGGACTTTCCAGGGC3'. Mutant HIV-1 κB oligonucleotide: 5'TCGACAACTCACTTTCCGCTGCTCACTTTCCAGGGC3'.

Stimulation of HIV-1 Expression from U1 and ACH-2 Cell Lines. 5 × 10^5 were seeded in 2 ml of medium in 24-well plates (Costar, Cambridge, MA). They were either left untreated or a variety of agents were added including TNF-α, LPS, and PMA plus PHA. After 24 h, samples of supernatants were removed daily for 3 d. The p24 antigen level was measured using a sensitive ELISA method (Dupont, Wilmington, DE) following the manufacturer's instructions. Some cultures were pretreated with GM-CSF for 24 h before other agents were added. Anti-TNF-α neutralizing antibody (Genzyme Corp.) was added where indicated.

Each experiment was repeated at least twice using duplicate or triplicate samples per experiment. Data presented represent the arithmetic mean ± SEM.

Results

Activation of HIV-1-LTR by LPS in Monocyte/Macrophage-like Cell Lines. To examine the effect of LPS on HIV-1-LTR–directed gene expression, various cell lines were transfected with an HIV-1-LTR CAT construct. LPS (10 µg/ml) greatly increased the amount of CAT activity produced from this HIV-1-LTR CAT construct after transient transfection of both U937, and the phenotypically more mature THP-1 cell lines (Fig. 1, *A* and *B*). PMA plus PHA (50 ng/ml and 2 µg/ml) and TNF-α (1,000 U/ml) also stimulated CAT production in both cell lines, but to lesser degrees than LPS (Fig. 1, *A* and *B*). Our ability to demonstrate HIV-1-LTR CAT stimulation by PMA and PHA in THP-1 cells is at variance with a previous report (7). A concentration of TNF-α of 1,000 U/ml, as illustrated in Fig. 1, resulted in the greatest level of stimulation in a dose-response curve with this cytokine. Above this level cytotoxicity was evident (data not shown). Mock transfections consistently gave percent conversion levels of <1%.

To determine whether LPS could induce CAT activity in T cells, Jurkat cells were transfected with the HIV-1-LTR CAT plasmid. CAT activity was not stimulated by LPS (10 µg/ml) in Jurkat cells, although PMA and PHA, as previously demonstrated (4), stimulated CAT activity in this cell line (Fig. 1 *C*). TNF-α was also demonstrated to stimulate the HIV-1-LTR–directed CAT gene in Jurkat cells (data not shown).

To determine whether LPS might stimulate HIV-1-LTR CAT at physiologically significant concentrations, various amounts of LPS were added to transient transfections. LPS was able to stimulate HIV-1-LTR CAT in U937 cells to a significant degree at concentrations as low as 10 pg/ml. This effect was not evident at 1 pg/ml (Fig. 2). Stimulation decreased over the range of LPS concentrations from 100 ng to 10 pg/ml.

To examine whether the κB sites in the HIV-1-LTR were necessary for LPS-induced CAT stimulation, an HIV-1-LTR CAT construct with mutant NF-κB binding sites (4) (HIV-NF-κB Δ CAT) was transfected into U937 and THP-1 cells. LPS (10 µg/ml), when added 24 h after transfection, led to no significant stimulation in CAT activity in U937 or THP-1 cells (Fig. 1, *A* and *B*). CAT activity from this construct was also not stimulated above baseline by TNF-α or PMA and PHA treatment in the U937 and THP-1 cell lines (data not shown).

To examine whether regions other than the κB sites in the HIV-1-LTR were required for LPS-induced CAT stimulation, a plasmid (J18) containing two κB sites from the κ enhancer
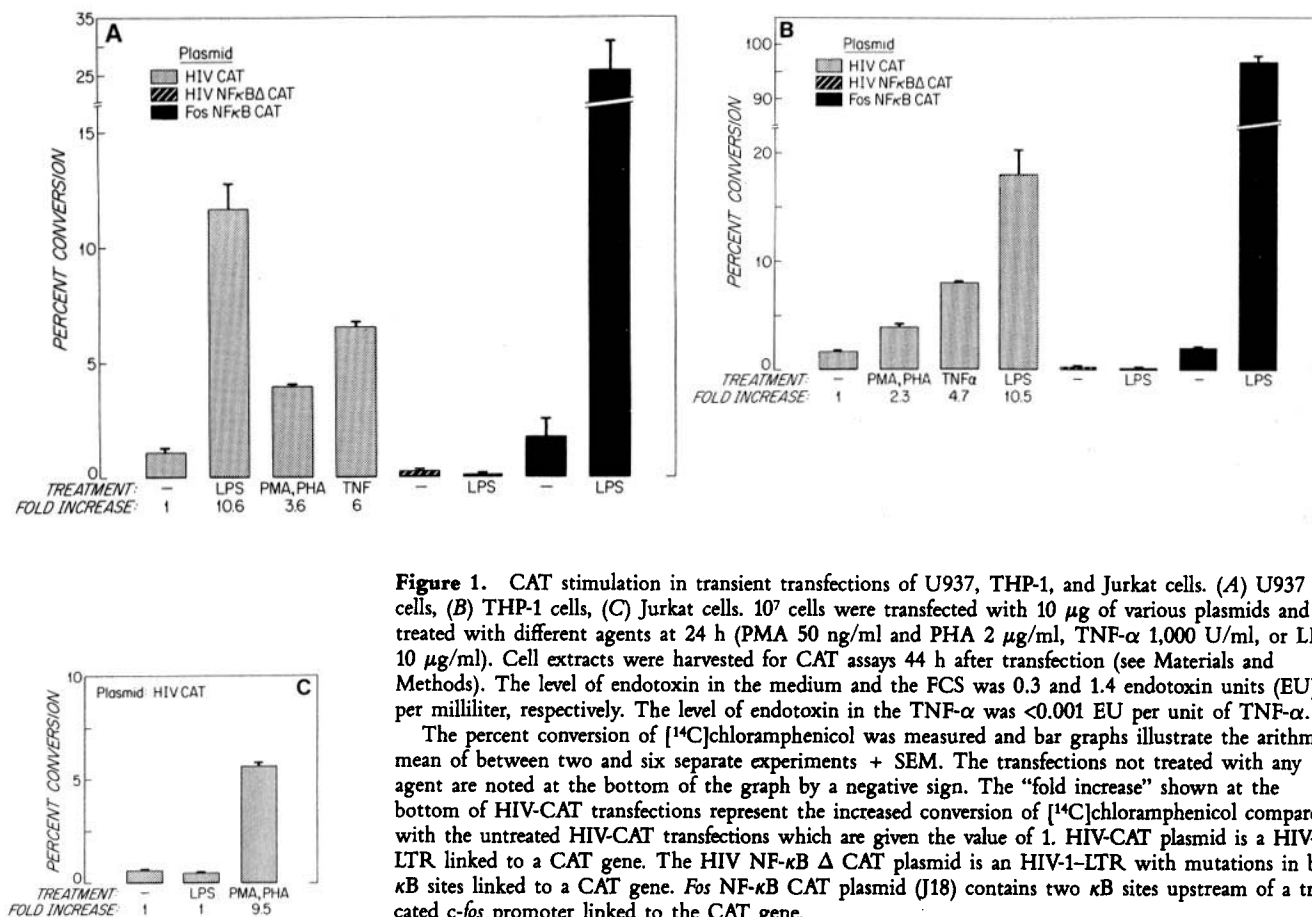

Figure 1. CAT stimulation in transient transfections of U937, THP-1, and Jurkat cells. (*A*) U937 cells, (*B*) THP-1 cells, (*C*) Jurkat cells. 10⁷ cells were transfected with 10 µg of various plasmids and treated with different agents at 24 h (PMA 50 ng/ml and PHA 2 µg/ml, TNF-α 1,000 U/ml, or LPS 10 µg/ml). Cell extracts were harvested for CAT assays 44 h after transfection (see Materials and Methods). The level of endotoxin in the medium and the FCS was 0.3 and 1.4 endotoxin units (EU) per milliliter, respectively. The level of endotoxin in the TNF-α was <0.001 EU per unit of TNF-α.

The percent conversion of [¹⁴C]chloramphenicol was measured and bar graphs illustrate the arithmetic mean of between two and six separate experiments + SEM. The transfections not treated with any agent are noted at the bottom of the graph by a negative sign. The "fold increase" shown at the bottom of HIV-CAT transfections represent the increased conversion of [¹⁴C]chloramphenicol compared with the untreated HIV-CAT transfections which are given the value of 1. HIV-CAT plasmid is a HIV-1-LTR linked to a CAT gene. The HIV NF-κB Δ CAT plasmid is an HIV-1-LTR with mutations in both κB sites linked to a CAT gene. *Fos* NF-κB CAT plasmid (J18) contains two κB sites upstream of a truncated c-*fos* promoter linked to the CAT gene.
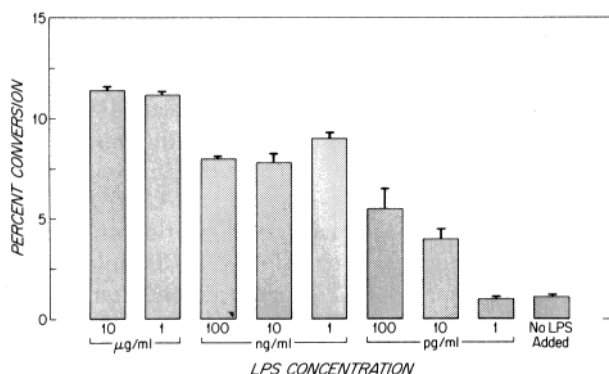
Figure 2. Dose response of HIV-CAT stimulation by LPS in U937 cells. 10^7 U937 cells were transfected with 10 μg of the HIV-CAT plasmid. 24 h after transfection, various amounts of LPS were added to give differing final concentrations of LPS in the medium. Cell lysates were harvested 44 h after transfection. Percent conversions of [^{14}C]chloramphenicol are the arithmetic means + SEM of three independent transfections.

upstream of a truncated c-*fos* promoter linked to a CAT gene (21) was transfected into U937 and THP-1 cells. When treated with LPS (10 μg/ml), a large stimulation of CAT activity in U937 and THP-1 cells was demonstrated (Fig. 1, *A* and
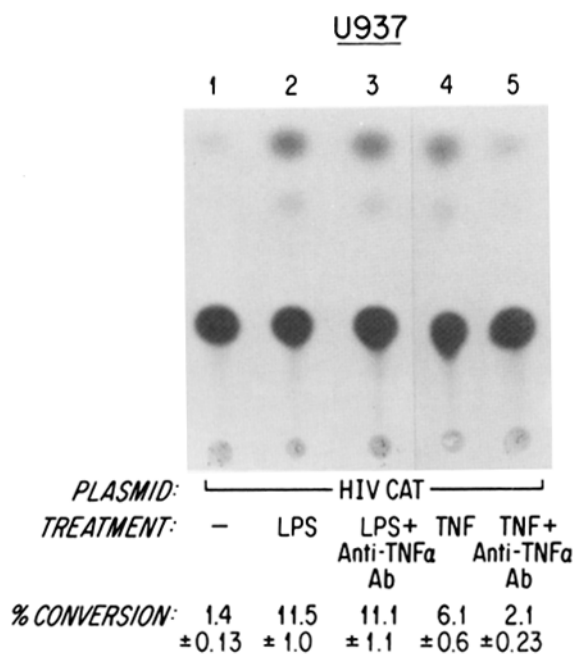

Figure 3. Anti-TNF-α antibody treatments of HIV-CAT transfections in U937 cells. U937 cells were transfected with 10 μg of HIV-CAT plasmid and treated with LPS or TNFα, with or without anti-TNFα antibody, 24 h after transfection. 20 μl of anti-TNF-α antibody, which neutralizes 2×10^4 U of TNF-α, were added to appropriate transfections. (Lane *1*) No treatment, (lane *2*) LPS (10 μg/ml), (lane *3*) LPS + anti-TNF-α antibody, (lane *4*) TNF-α (1,000 U/ml), and (lane *5*) TNF-α + anti-TNF-α antibody. The percent conversions of [^{14}C]chloramphenicol are the arithmetic means $\pm$ SEM of three independent transfections. The autoradiograph shown is a representative example of these experiments.

B). A plasmid lacking the κB sites but retaining the c-*fos* promoter linked to a CAT gene (21) was not stimulated by LPS in either cell line (data not shown).

Transfections of the HIV–NF-κB Δ CAT and J18 plasmids indicate that the LPS stimulation of the HIV-1–LTR CAT in the U937 and THP-1 cell lines is based on the induction of NF-κB and its binding to κB enhancer motifs in the LTR.
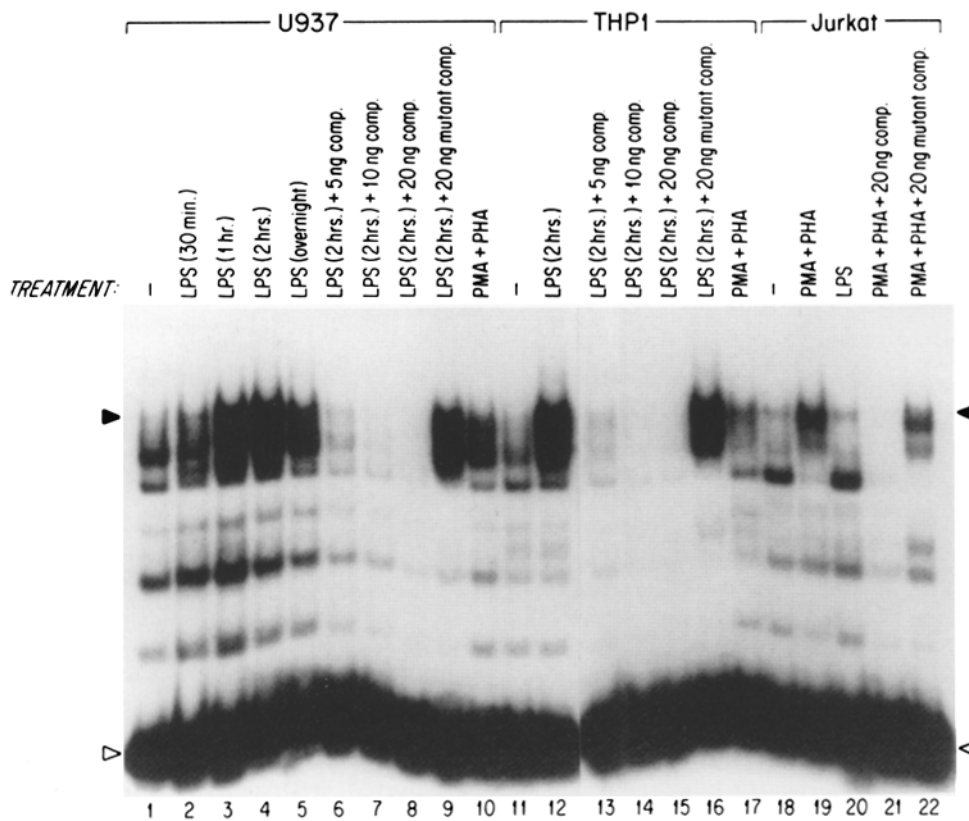
To ascertain if the LPS-induced CAT stimulation was caused by TNF-α released from cells, HIV-1–LTR CAT constructs were transfected into U937 cells, which were then either treated with LPS (10 μg/ml) plus or minus anti-TNF-α antibody, TNF-α (1,000 U/ml) plus or minus anti-TNF-α antibody, or left untreated (Fig. 3). Anti-TNF-α antibody had no significant effect on LPS-induced stimulation of HIV-1–LTR CAT but reduced by ~86% the level of CAT activity induced by TNF-α (Fig. 3). Anti-TNF-α antibody was also unable to block LPS-induced CAT stimulation in the THP-1 cell line (data not shown).

LPS Induces an Activity in Monocyte/Macrophage-like Cell Lines that Binds to κB sites on EMSA. Cellular extracts were prepared by a brief protocol (25), rather than the nuclear extracts prepared by the method of Dignam et al. (27), for EMSA. This method gave more background bands but allowed the use of small amounts of cells and minimized proteolysis common in the manipulation of monocyte/macrophage-like cell lines. A binding activity, indistinguishable from NF-κB, was found in extracts of U937 and THP-1 cells treated with LPS but not Jurkat cells.

This binding activity was induced after only 30 min of LPS treatment of U937 cells and increased over treatment periods up to 2 h (Fig. 4, lanes *2–4*). Prolonged treatment with LPS showed persistent but reduced levels of NF-κB-like binding activity (Fig. 4, lane *5*).

NF-κB-like binding activity was also detected in THP-1 cells stimulated with LPS (Fig. 4, lane *12*), but contrary to a previous report (7), little of this activity was found in our unstimulated THP-1 cells (Fig. 4, lane *11*). This LPS-inducible complex comigrated with a PMA plus PHA–inducible complex in U937, THP-1, and Jurkat cells (Fig. 4, lanes *10, 17, 19*). Peak levels of NF-κB-like activity were induced by PMA/PHA in U937 and THP-1 cells with overnight treatment, while PMA/PHA treatment gave its greatest effect in Jurkat cells after 2 h (data not shown). LPS is a much more potent stimulus for NF-κB-like binding activity in U937 and THP-1 cells than PMA plus PHA. LPS was unable to stimulate NF-κB-like binding activity in Jurkat cells (Fig. 4, lane *20*).

The LPS-inducible complex was specifically competed away by an unlabeled fragment composed of both κB sites from the HIV-1–LTR in reactions with cellular extracts from both U937 and THP-1 cells (Fig. 4, lanes *6–8* and *13–15*). An unlabeled fragment with mutations in the κB sites of the HIV-1–LTR was unable to compete with this binding activity from LPS-stimulated U937 and THP-1 cells (Fig. 4, lanes *9* and *16*). The unlabeled competitor fragment effectively competed with binding activity found in PMA plus PHA-treated Jurkat cell extracts, while the mutant unlabeled fragment showed

Figure 4. Electrophoretic mobility shift assays of U937, THP-1, and Jurkat cells. 5×10^7 cells in 50 ml of medium were treated with various agents over differing time periods. Cellular extracts were prepared and EMSAs performed as described in Materials and Methods.

(Lane 1) U937, no treatment (noted by negative [-] sign). (Lanes 2–5) U937 + LPS (10 μg/ml) over various stimulation time periods. (Lanes 6–8) U937 + LPS and differing amounts of unlabeled competitor (comp). (Lane 9) U937 + LPS and 20 ng of mutant unlabeled competitor. (Lane 10) U937 + PMA (50 ng/ml) plus PHA (2 μg/ml) overnight stimulation. (Lane 11) THP-1, no treatment. (Lane 12) THP1 + LPS, 2 h stimulation. (Lane 13–15) THP-1 + LPS and differing amounts of unlabeled competitor. (Lane 16) THP-1 + LPS and 20 ng of mutant unlabeled competitor. (Lane 17) THP-1 + PMA and PHA overnight stimulation. (Lane 18) Jurkat, no treatment. (Lane 19) Jurkat + PMA and PHA, 2 h stimulation. (Lane 20) Jurkat + LPS, 2 h stimulation. (Lane 21)

Jurkat + PMA and PHA 20 ng competitor. (Lane 22) Jurkat + PMA and PHA and 20 ng mutant competitor. The autoradiograph illustrated was slightly overexposed to demonstrate changes in the NF-κB band in lanes where various amounts of unlabeled competitor were added. (Filled arrowheads) NF-κB band; (open arrowheads) unbound probe.

no competition with this binding activity (Fig. 4, lanes 21 and 22). The above data demonstrate that LPS induces a binding activity indistinguishable from NF-κB in monocyte/macrophage-like cell lines.

Stimulation of HIV-1 Production by LPS in U1 Cells Pretreated with GM-CSF. The U1 cell line is a subclone of HIV-1–infected U937 cells which constitutively produce very low amounts of virus. These cells can be stimulated to produce much greater amounts of HIV-1 by treatment with TNF-α, PMA, and GM-CSF (7, 9).

We were able to reproduce these findings. We noted a 320- and 27-fold increase in p24 antigen levels over baseline with TNF-α and GM-CSF, respectively (Fig. 5, A and B). PMA plus PHA induced p24 antigen levels to >100 ng/ml by day 3 (data not shown). LPS alone, however, failed to yield a consistent increase in p24 antigen levels (Fig. 5 A). There was <0.2 ng/ml of p24 antigen in the supernatant of unstimulated U1 cells by the third day in culture.

Reasoning that the U1 cell line might be a relatively immature subclone of U937 cells, we attempted to differentiate this cell line using GM-CSF before treatment with LPS. After treatment for 24 h with GM-CSF, LPS led to a 200-fold increase in p24 antigen levels in the supernatant compared with untreated cultures, and a 7.4-fold increase compared with cultures treated solely with GM-CSF by day 3 after LPS treat-

ment (Fig. 5 B). We were further able to demonstrate that after GM-CSF pretreatment, HIV-1 production could be further augmented in the U1 cell line with concentrations of LPS as low as 100 pg/ml (Fig. 6). All of the stimulatory effects on HIV-1 production in the U1 cell line resulted in increased levels of HIV-1-specific RNA (Pomerantz, R.J., D. Trono, M. Feinberg, and D. Baltimore, manuscript submitted for publication).

To examine whether the LPS-mediated stimulation of HIV-1 in U1 cells was due to the release of TNF-α from the GM-CSF–treated U1 cells, we added anti-TNF-α neutralizing antibody to cultures treated with GM-CSF plus LPS. This antibody had a minimal effect on the level of virus production in GM-CSF pretreated cells to which LPS was added, although it was able to totally ablate the stimulation of virus production induced by 1,000 U/ml of TNF-α (Fig. 5, A and B).

A chronically HIV-1-infected T cell line, ACH-2, which constitutively produces small amounts of virus, was also treated with a variety of agents. Although TNF-α led to a significant increase in viral production, LPS alone, GM-CSF alone, and GM-CSF plus LPS had no effect on viral replication (Fig. 5 C).

Stimulation of NF-κB-like Binding Activity in U1 Cells by LPS after Pretreatment with GM-CSF. To demonstrate that the LPS-mediated increase in HIV-1 production in U1 cells pretreated with GM-CSF was correlated with the activation of NF-κB,
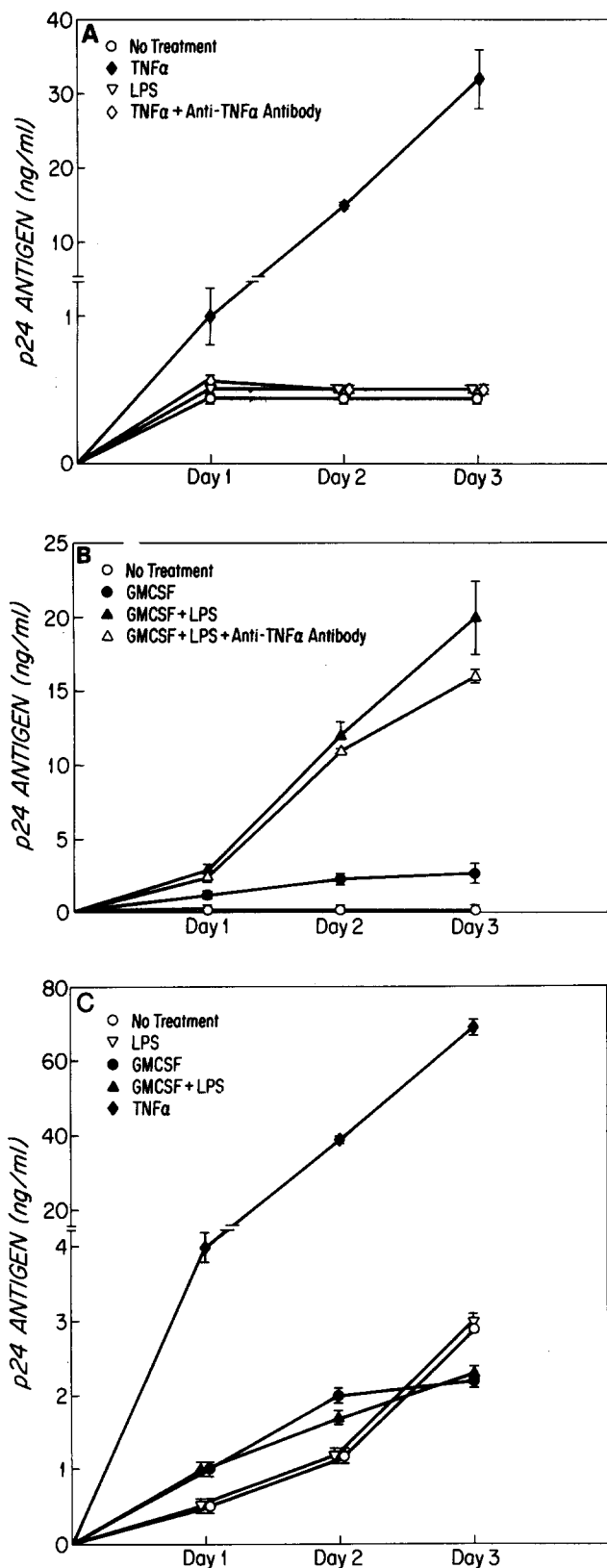
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cellular extracts were made after a variety of treatment protocols and the binding reactions to a labeled probe with 2 κB sites were studied by EMSA.

LPS alone was unable to induce NF-κB-like activity in U1 cells (Fig. 7, lane 2). NF-κB-like activity was not stimulated by LPS alone in U1 cells after 1, 2, 6, 24, and 36 h of treatment (data not shown). As previously reported (7), a small amount of NF-κB-like activity was induced in the U1 cell line after 24 h of treatment with PMA and PHA (Fig. 7, lane 4).

As reported for other cell lines (6), treatment of the U1 cell line for 48 h with GM-CSF did not induce NF-κB-like activity (Fig. 7, lane 3). GM-CSF did not induce NF-κB-like activity in U1 cells when treated for time periods of between 2 and 36 h (data not shown). LPS, though, was able to induce a small quantity of NF-κB-like activity after the U1 cells were pretreated for 24 h with GM-CSF (Fig. 7, lane 5). LPS induced NF-κB-like activity in GM-CSF pretreated U1 cells only if added to the medium for 24 h or longer (data not shown).

This NF-κB-like activity comigrated with a band induced by PMA plus PHA in Jurkat cells (Fig. 7, lane 9) and was specifically competed away with an unlabeled DNA fragment with two κB sites from the HIV-1-LTR but not a fragment with mutations in these κB sites (Fig. 7, lanes 6 and 7). This DNA binding activity was, therefore, indistinguishable from NF-κB. In multiple experiments, only relatively small quantities of NF-κB were induced by PMA plus PHA or GM-CSF plus LPS in the U1 cell line compared with levels induced in uncloned and uninfected U937 cells.

To further demonstrate that NF-κB was activated by PMA plus PHA and GM-CSF plus LPS in U1 cells, these cells were transfected with the Fos NF-κB CAT (J18) plasmid. Treatment of these treatment transfections with PMA plus PHA or GM-CSF plus LPS led to a significant stimulation of CAT activity. Treatment with GM-CSF alone or LPS alone did not stimulate CAT activity (data not shown).

Discussion

We have demonstrated here that LPS potently activates the HIV-1-LTR in monocyte/macrophage-like cell lines but not in a T cell line. The LPS effect appears to be mediated through NF-κB. LPS increased HIV-1 production from a cloned monocyte/macrophage-like cell line which was pretreated with GM-CSF. These LPS effects were demonstrated at picogram per milliliter concentrations.

LPS, which is a major constituent of the cell walls of gram negative bacteria, has a plethora of effects on a variety of cells. The release of TNF-α from monocyte/macrophages by LPS

Figure 5. Production of HIV-1 from the U1 and ACH-2 cell lines. (A and B) U1 cells, (C) ACH-2 cells. 5 × 10⁵ cells were placed in 2 ml of medium in 24-well plates. Different agents were added and p24 antigen levels in the supernatants were determined daily for 3 d. The treatment regimens included LPS (10 μg/ml) with or without

anti-TNF-α antibody (4 μl), TNF-α (1,000 U/ml) with or without anti-TNF-α antibody, GM-CSF (500 U/ml) alone, and GM-CSF (500 U/ml) for 24 h followed by LPS (10 μg/ml). Certain cultures were left untreated. The level of endotoxin in the GM-CSF was <0.001 EU per unit of GM-CSF. Data points represent the arithmetic means ± SEM of two to three separate experiments performed in duplicate.
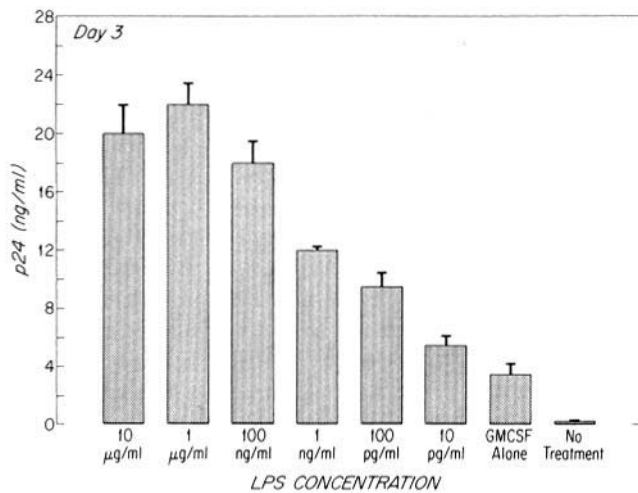
Figure 6. Dose response of HIV-1 production in GM-CSF pretreated U1 cells by LPS. Various amounts of LPS were added to U1 cells pretreated with GM-CSF (500 U/ml) for 24 h. p24 antigen levels were measured from these supernatants on the third day after LPS was added. Each bar is the arithmetic mean + SEM of two experiments performed in duplicate.

leads to many but not all the pathophysiologic effects noted during endotoxemia (28, 29). LPS activates macrophages and induces Interleukin I, Interleukin 6 and Interferon γ production (29, 30). LPS is also a murine B lymphocyte mitogen and will activate protein kinase C and induce NF-κB in pre-B cells although it has little effect on T lymphocytes (31). Many of these LPS-induced effects are seen with nanogram or picogram per milliliter concentrations (28, 29). LPS may not activate NF-κB in T cells for lack of an LPS receptor or for other reasons.

Since LPS induces production of TNF-α (28) and TNF-α was able to activate transcription of our HIV-1-CAT construct, it could be that the effect of LPS on HIV-1-LTR–driven transcription is indirect. An antibody to TNF-α did not block the LPS-induced HIV-1–LTR CAT stimulation. Moreover, EMSAs demonstrated that LPS activation of NF-κB begins within 30 min or less of treatment. It is not likely, therefore, that an LPS-induced increase in production or release of TNF-α is the major mechanism underlying this effect. The TNF-α promoter itself has κB-like regions (32), and consequently, both stimulation of TNF-α and stimulation of HIV-1 may be independent results of LPS-mediated NF-κB activation.

A recent report has demonstrated that LPS-treated primary macrophages produce cytokines, including TNF-α, which stimulate HIV-1 production in T lymphocytes (10). This is certainly a possible mechanism by which LPS stimulation of some monocyte/macrophage populations might affect not only HIV-1 production directly in monocyte/macrophages but also have an indirect effect on HIV-1 replication in T lymphocytes in vivo.

It is instructive to note that a significant effect on HIV-1 production was not demonstrated when the U1 cell line was treated with LPS alone. Only after pretreatment with GM-
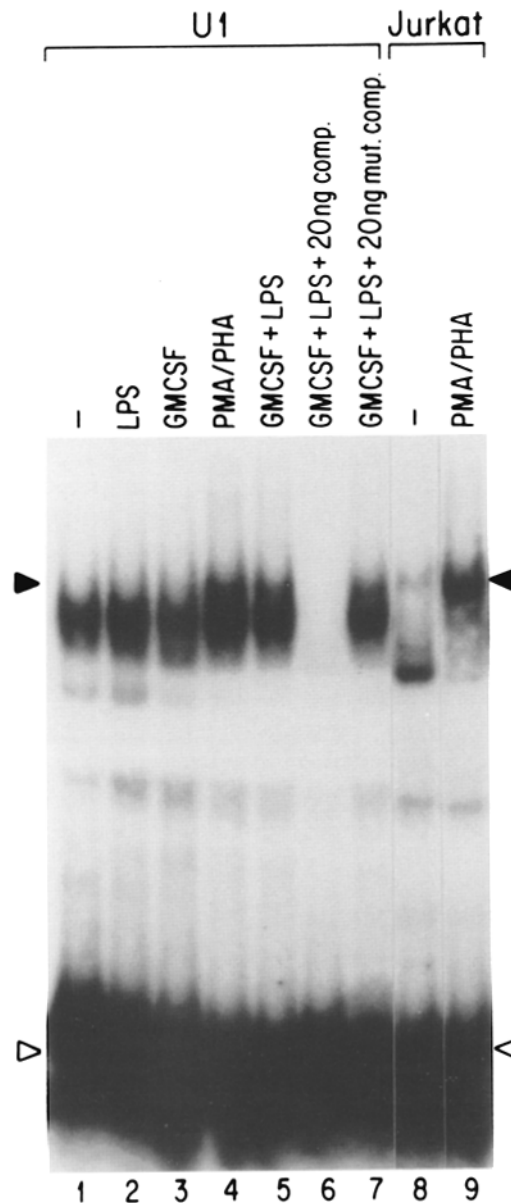

Figure 7. EMSA of U1 cells. (Lane 1) U1 cells, no treatment. (Lane 2) U1 cells treated with LPS (10 μg/ml) for 48 h. (Lane 3) U1 cells treated with GM-CSF (500 U/ml) for 48 h. (Lane 4) U1 cells treated with PMA (50 ng/ml) and PHA (2 μg/ml) for 24 h. (Lane 5) U1 cells treated with GM-CSF for 24 h then LPS for 24 h. (Lanes 6 and 7) U1 cells pretreated with GM-CSF for 24 h and then LPS for 24 h plus 20 ng of unlabeled competitor (comp.) or mutant competitor (mut. comp.), respectively. (Lane 8) Jurkat, no treatment. (Lane 9) Jurkat plus PMA and PHA for 2 h. This representative autoradiograph was slightly overexposed to properly illustrate the small quantity of NF-κB that was activated in U1 cells (uppermost band in lanes 4, 5, and 7). (Closed arrowheads) NF-κB band; (open arrowheads) unbound probe.

CSF, which has been noted to induce differentiation and maturation in monocytic precursor cells (33), was LPS able to stimulate high levels of HIV-1 production. It appears that the U1 cell subclone of HIV-1-infected U937 cells may be quite im-

mature and either lack an LPS receptor or some component of the proximal NF-κB activation pathway induced by LPS. Only after maturation by GM-CSF can the U1 cell line respond to LPS with an increase in HIV-1 production from its integrated provirus. As with the HIV-1–LTR CAT transfections, the LPS-effect on viral production is largely a direct one and is not mediated via a release of TNF-α. An anti-TNF-α antibody was able to decrease only slightly the level of HIV-1 production in the U1 cell line pretreated with GM-CSF to which LPS was added. Only if endogenously produced TNFα is more difficult to neutralize compared with exogenous TNF-α might a portion of the LPS effect still be mediated through TNF-α.

A recent study has demonstrated that pretreating primary macrophages with LPS may protect them from HIV-1 infection, possibly through activation (13). This study is not at variance with our data. Treatment of macrophages with LPS before infection with HIV-1 may have a different effect on HIV-1 production than treatment of chronically HIV-1-infected monocyte/macrophages. That LPS may protect macrophages from HIV-1 infection and increase HIV-1 production from chronically infected monocyte/macrophages suggests that, in vivo, the effects of LPS on HIV-1 may be complex.

The induction of NF-κB as the cellular mediator of the LPS effect on HIV-1 production in monocyte/macrophage-like cells is yet another system controlled by this transcriptional regulator (for review see reference 34). The induction of NF-κB does not require protein synthesis, and probably occurs through the release of an inhibitor (IκB), bound to NF-κB (25). The free form of NF-κB may then migrate to the nucleus where it binds to 10 or 11 bp enhancer-motifs present in a variety of promoters. These include HIV-1, CMV, IFN-β, IL-2, and the human Igκ enhancer, to name but a few (34).

NF-κB activation takes place via more than one pathway

(Ghosh, S., personal communication). That LPS and phorbol esters have very different time courses to induce NF-κB in U937 and THP-1 cell lines might suggest a different pathway, as has recently been considered for TNF-α and phorbol esters (6).

LPS has recently been shown to minimally increase (1.5-fold) HIV-1 production in chronically infected THP-1 cells (35). We have confirmed these findings (data not shown), but would suggest that the extremely low HIV-1-producing monocyte/macrophage-like cell line, U1, is a better model in which to study the control of HIV-1 production. Most investigators have been unable to demonstrate consistent increases in HIV-1 production, by a variety of agents, in uncloned, chronically infected cell lines (11). This appears to be due to the vast heterogeneity of these cell populations and the relatively high level of viral production noted in such chronically infected cell lines even at baseline levels (11).

Many patients with or at risk for HIV-1 infection have episodes of localized bacterial infections or frank bacteremias (36). Endotoxin might reach levels, in various locations of the body, demonstrated to induce HIV-1 production in monocyte/macrophage-like cells in this study. In normal individuals, endotoxin is cleared by the reticuloendothelial system of the liver (37). The small amount of endotoxin made by gram-negative bacteria always found in the gut is continuously cleared by these mononuclear phagocytic cells of the liver (37). Our data demonstrate that LPS at concentrations as low as 10 pg/ml might affect HIV-1 replication. In HIV-1-infected individuals, this amount of LPS might stimulate HIV-1 production from monocyte/macrophages located in liver, as well as in other organs.

Monocyte/macrophages may be the major cell type infected with HIV-1 in the central nervous system (16). Whether exposure of these cells to LPS may lead to increased HIV-1 production in the central nervous system, and subsequent clinical deterioration, warrants future studies.

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