

# Production of Tumor Necrosis Factor $\alpha$ and Interleukin $1\beta$ by Monocytic Cells Infected with Human Immunodeficiency Virus

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## Abstract

The production of tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) and IL- $1\beta$  by the monocytic cell line THP-1, productively infected with HIV-1, was investigated using specific RIA and Northern blot analysis. HIV-infected cells, like uninfected cells, did not constitutively produce any detectable amounts of protein or mRNA for TNF $\alpha$  or IL- $1\beta$ . After stimulation with LPS or a combination of LPS plus IFN- $\gamma$ , TNF $\alpha$  and IL- $1\beta$  were detected in tissue culture supernatants and cell lysates and transcripts for both cytokines were seen on Northern blots. No significant difference in production of these two cytokines was observed between uninfected and chronically infected cells. Acutely HIV-infected cells, however, showed phenotypic changes compatible with maturation and an increase in TNF $\alpha$  and IL- $1\beta$  mRNA production, and released significantly higher levels of TNF $\alpha$  and IL- $1\beta$  compared with chronically infected or uninfected cells. Furthermore, LPS stimulation of HIV-infected cells increased virus production. These results suggest that HIV-infected monocytic cells may produce increased amounts of TNF $\alpha$  and IL- $1\beta$  in response to stimuli that could be present in vivo.

## Introduction

Reduced number and function of CD4<sup>+</sup> T lymphocytes after infection by HIV is thought to be the major pathophysiologic event leading to AIDS (1–3). However, the monocyte/macrophage also represents a major target of HIV infection (3–5) and may serve as a reservoir for viral persistence and a vehicle for viral dissemination. Furthermore, certain functions of the monocyte/macrophage may be altered by HIV infection (3, 6, 7). TNF $\alpha$  (also known as cachectin) and IL- $1\beta$  are two cytokines produced by the monocyte/macrophage after various stimuli (8, 9). LPS is one of the major stimuli for TNF $\alpha$  and IL- $1\beta$  production, but viral stimuli are also able to induce production of these cytokines in vitro (10, 11). Tumor necrosis factor  $\alpha$  (TNF $\alpha$ )<sup>1</sup> and IL- $1\beta$  play a major role in tissue remodeling

and are also important immunoregulatory molecules. When produced in large quantities, TNF $\alpha$  appears to be an important mediator in the pathogenesis of septic shock and cachexia. Both molecules are highly inflammatory, can cause fever and loss of appetite, and contribute to a catabolic state (8, 9). The roles played by TNF $\alpha$  or IL- $1\beta$  in the clinical manifestations of HIV infection are not yet clearly defined. Recent studies have reported high levels of TNF $\alpha$  (12, 13) or IL- $1\beta$  (6, 14–16) in sera or in culture supernatants of monocytes from AIDS patients. Although these observations might explain some of the prominent symptoms of fever and wasting in these patients, the regulation of TNF $\alpha$  and IL- $1\beta$  production in this setting has not been defined. Augmented cytokine production may be a direct result of HIV infection of cytokine-producing cells or an indirect result of the various opportunistic infections frequently seen in these patients.

In this study we have investigated the direct effects of HIV infection on in vitro production of TNF $\alpha$  and IL- $1\beta$  by cells of monocyte/macrophage lineage. Given the recognized variability in cytokine production by monocytes from different donors (17), we used the human monocytic cell line THP-1 as a model of TNF $\alpha$  and IL- $1\beta$  production after HIV infection (18, 19).

## Methods

**Cells and virus.** The cell line THP-1, derived from a patient with monocytic leukemia (18), was obtained from the American Type Culture Collection, Rockville, MD (N<sup>o</sup> TIB 202), and grown in RPMI 1640 medium supplemented with Hepses buffer (10 mM), L-glutamine (2 mM), penicillin (250 U/ml), streptomycin (250  $\mu$ g/ml), and fetal bovine serum (10%) at 37°C in 5% CO<sub>2</sub>. HIV-1 IIIB (2) was obtained from Dr. R. Gallo (National Cancer Institute, Bethesda, MD) and viral stocks were propagated in H9 cells as previously described (2); tissue culture supernatants were harvested at peak infectivity and stored in aliquots at –70°C in 60% fetal bovine serum.

**Infection of THP-1 cells.** THP-1 cells (4  $\times$  10<sup>6</sup> cells in 1 ml) were incubated for 1 h at 37°C with 10<sup>3</sup> tissue culture infective doses of HIV in 1 ml (20). The cells were then cultured in 75-cm<sup>2</sup> plastic flasks (Falcon Labware, Oxnard, CA) at a concentration of 4  $\times$  10<sup>5</sup> cells/ml and subsequently split every 4–5 d. Viral infection was monitored by sequential measurement of supernatant reverse transcriptase (RT) activity and detection of viral antigens by indirect immunofluorescence assay (IFA). Five different infections were carried out under these conditions.

**RT assay.** The assay for HIV RT activity was performed as previously described (20). Virus was concentrated from 1 ml of cell-free tissue culture supernatant by precipitation with 0.5 ml polyethylene glycol at 4°C.

**Indirect IFA.** The detection of HIV antigens on the cells was determined by indirect immunofluorescence on methanol-fixed cells using the serum of an AIDS patient (2). Serum from a seronegative donor was used as a negative control.

**Detection of HIV antigen.** HIV p24 antigen was measured in cell-free supernatants by a solid phase antigen capture immunoassay (21) (Abbott Laboratories, North Chicago, IL).

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1. *Abbreviations used in this paper:* GAPD, glyceraldehyde-3-phosphate dehydrogenase; IFA, immunofluorescence assay; NSE, nonspecific esterase; RT, reverse transcriptase; TNF $\alpha$ , tumor necrosis factor  $\alpha$ .

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**Phenotypic studies.** The cell surface expression of CD4 antigen was analyzed using the anti-Leu-3A MAb (Becton Dickinson Immunocytometry Systems, Mountain View, CA) and flow cytometry with a FACS analyzer (Becton Dickinson Immunocytometry Systems) (22). Cytochemical staining for nonspecific esterase (NSE) was performed as previously described (5) using the kit No. 90 (Sigma Chemical Co., St. Louis, MO).

**Stimulation of THP-1 cells.** THP-1 cells were cultured for 24 h in 24-well plates (Costar, Cambridge, MA) at a concentration of  $10^6$  cells/ml with or without LPS (*Escherichia coli* serotype 026:B6; Sigma Chemical Co.) and/or purified recombinant IFN- $\gamma$  (sp act,  $10^7$  U/mg; gift of Genentech Inc., South San Francisco, CA). After preliminary dose-response experiments (0.1–500  $\mu$ g/ml LPS) the dose of 5  $\mu$ g/ml of LPS that induced half-maximal production of TNF $\alpha$  was used. Similarly, the dose of 100 U/ml of IFN- $\gamma$  was used after observing maximal augmentation of LPS-induced cytokine production. Uninfected cells were included in each experiment as controls.

**TNF $\alpha$  and IL-1 $\beta$  assays.** After 24 h of stimulation the cell-free supernatants were harvested and frozen at  $-70^\circ\text{C}$  until assayed. The cell pellets were washed in PBS, pH 7.4, and resuspended in 0.5 ml RPMI 1640 medium. The cells were then frozen and thawed three times and the cell lysates kept at  $-70^\circ\text{C}$  until assayed. Previous experiments have shown that this procedure does not affect the measurement of TNF $\alpha$  or IL-1 $\beta$  and that these cell-associated cytokines are concentrated in the cytosolic compartment (23). RIAs for TNF $\alpha$  and IL-1 $\beta$  have been previously described (24, 25). The only modification was that the pellet obtained after polyethylene glycol precipitation was resuspended in 0.5 ml of 0.5% Na hypochlorite to inactivate HIV. All analyses were performed in duplicate. The IL-1 $\beta$  RIA (95% confidence limit) had a limit of detection of 60 pg/ml. The average limit of detection for the TNF $\alpha$  RIA was 100 pg/ml. When the value obtained in an assay was below the limit of detection, this value was set at the limit of detection for that particular assay. Previous studies have shown that each RIA is specific for the cytokine it measures, does not detect IL-1 $\alpha$ , -2, or -6, GM-CSF, or IFN- $\alpha$ , - $\beta$ , or - $\gamma$ , and correlates well with its respective bioassay (24, 25).

**RNA analysis.** Total cellular RNA was obtained from resting cells or LPS-stimulated cells (5  $\mu$ g/ml of LPS for 3 h) (26) by guanidine isothiocyanate lysis and cesium chloride gradient centrifugation (27, 28). Northern blots were generated using standard methods (27) and transcripts were visualized by autoradiography after hybridization to the following random-primed  $^{32}\text{P}$ -labeled probes: the IL-1 $\beta$  probe, obtained from a 1,075-bp (Pst I-Sst I) fragment of the human pro-IL-1 $\beta$  cDNA clone pGEM2 (29); the TNF $\alpha$  probe, obtained from a 473-bp (Eco RI) fragment of the human TNF $\alpha$  cDNA clone pUC9 (30); and the full 1.9-kb cDNA clone of glyceraldehyde-3-phosphate dehydrogenase (GAPD) (31). Results were quantitated by densitometry (Molecular Dynamics, Sunnyvale, CA).

**Data analysis.** Analysis of variance and two-tailed *t* test were used for statistical comparisons. Results were expressed as mean $\pm$ SEM.

## Results

**HIV infection of THP-1 cells.** THP-1 cells were nonadherent and weakly positive for NSE staining, and grew in clumps with a doubling time of  $\sim 48$  h. More than 90% of the cells expressed CD4 antigen on their surface, the receptor for HIV (32, 33), as determined by FACS analysis (data not shown). HIV infection was readily obtained in each different experiment ( $n = 5$ ) with peak RT activity and viral antigen expression occurring between days 10 and 13 of the culture (Fig. 1). No gross cytopathic effects were noted at this time, but changes consistent with differentiation and/or activation were observed, associated with a decrease in viability (70–80% viable cells as detected by trypan blue exclusion). Indeed, cell growth stopped and nearly all cells became adherent to plastic and

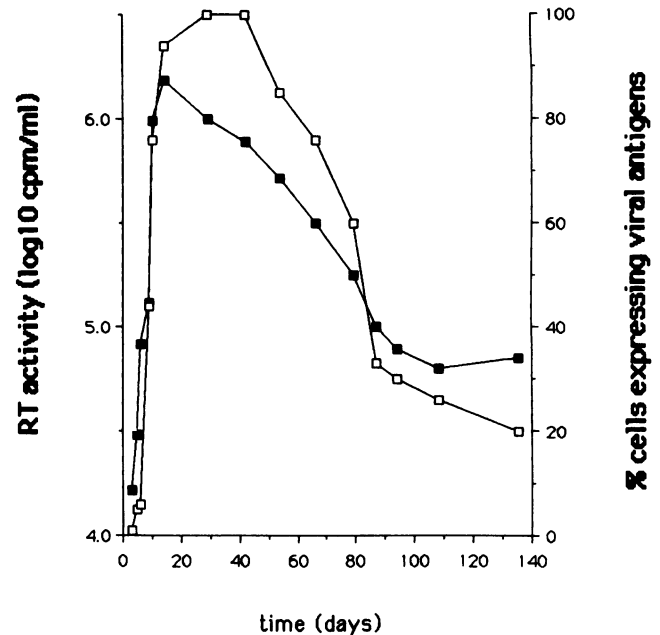
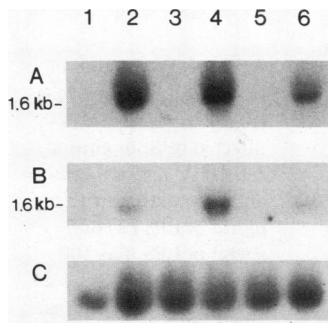


Figure 1. Time course of RT activity (■) and viral antigen expression (□) after HIV infection of THP-1 cells.

strongly positive by NSE staining (data not shown). The cells harvested 1–3 d (e.g., days 8–10) before this differentiated stage, which we term “acutely infected,” were  $> 90\%$  positive for viral antigen expression and  $> 85\%$  viable. After days 10–13 of culture nonadherent cells could be recovered that were easily propagated with cell viability  $> 95\%$  and growth characteristics similar to uninfected cells. However, the expression of CD4 antigens on the surface of these cells was downregulated to 0–6% of cells by FACS analysis (data not shown). These cells, which we termed “chronically infected,” initially released high RT activity and all expressed viral antigens (100% positivity by IFA), but demonstrated over a period of several weeks a progressive decrease of viral antigen expression and RT activity (Fig. 1).

**Production of TNF $\alpha$ .** In the absence of stimulation, the production of TNF $\alpha$  in the supernatant of uninfected, acutely infected, or chronically infected cells was either below or just at the limit of detection of the assay (100 pg/ml) and no detectable TNF $\alpha$  was found in the cell lysates (data not shown). No TNF $\alpha$  mRNA was seen on Northern blots under these conditions (Fig. 2 A). After stimulation with LPS (5  $\mu$ g/ml) for 24 h production of TNF $\alpha$  was observed with  $> 85\%$  of TNF $\alpha$  released in the tissue culture supernatant, and TNF $\alpha$  transcripts were detected (Figs. 2 A and 3 A). Stimulation of the cells with both LPS (5  $\mu$ g/ml) and IFN- $\gamma$  (100 U/ml) further increased the production of TNF $\alpha$  (Fig. 3 B). There was a higher production (two- to sevenfold) of TNF $\alpha$  in the supernatant of acutely infected cells when compared with uninfected cells or chronically infected cells (Fig. 3, A and B). An increase in TNF $\alpha$  mRNA transcription was also noted in LPS-stimulated, acutely infected cells when compared with chronically infected and uninfected cells (when signal intensity was normalized for the constitutively expressed GAPD probe by densitometric analysis; Fig. 2 A). However, the production of TNF $\alpha$  by uninfected and chronically infected cells (with high or low levels of virus replication) was not significantly different (Fig. 2, A



**Figure 2.** Northern blot analysis of TNF $\alpha$  (A), IL-1 $\beta$  (B), and GAPD (C) gene expression in THP-1 cells. Lane 1, resting uninfected cells; lane 2, LPS-stimulated uninfected cells; lane 3, resting acutely infected cells; lane 4, LPS-stimulated acutely infected cells; lane 5, resting chronically infected cells; lane 6, LPS-stimulated chronically infected cells. LPS stimulation consisted of 5

$\mu\text{g/ml}$  of LPS for 3 h. Approximately 10  $\mu\text{g}$  of total RNA was loaded per lane.

and B), and was similar to the production observed after LPS stimulation of cells during the first week of infection when only a few percent of the cells were expressing viral antigens with a low release of RT activity in the supernatant (data not shown).

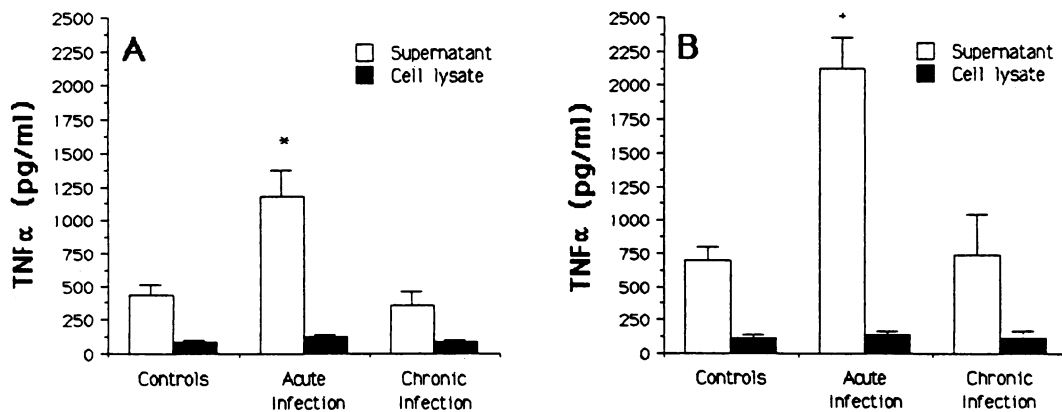
**Production of IL-1 $\beta$ .** Without stimulation the production of IL-1 $\beta$  and IL-1 $\beta$  mRNA by uninfected cells or HIV-infected cells was not detectable (Fig. 2 B and data not shown). After LPS stimulation IL-1 $\beta$  transcripts were observed (Fig. 2 B) and IL-1 $\beta$  was detected in approximately equal amounts in culture supernatants and cell lysates of uninfected or chronically infected cells (Fig. 4 A). Higher production was observed when the cells were stimulated with LPS plus IFN- $\gamma$  (Fig. 4, A and B). An increase of IL-1 $\beta$  mRNA and IL-1 $\beta$  production (2.5- to 13-fold) was found in acutely infected cells with 85% of IL-1 $\beta$  production found in the supernatant (Figs. 2 B and 4, A and B). No significant difference, however, was observed between uninfected cells, chronically infected cells (with high or low level of virus replication), or cells in the first week of infection (Fig. 4, A and B and data not shown).

**RT activity and HIV p24 antigen levels after LPS stimulation.** Chronically HIV-infected cells were stimulated for 24 h with varying doses of LPS. A significant dose-dependent increase in RT activity and HIV p24 antigen was found in the tissue culture supernatants with the percentage increase ranging from 20 to 100% (Fig. 5 and data not shown). Similar results were obtained with acutely infected cells before the peak of RT activity (data not shown). However, no significant change in the number of cells expressing viral antigen was observed by IFA.

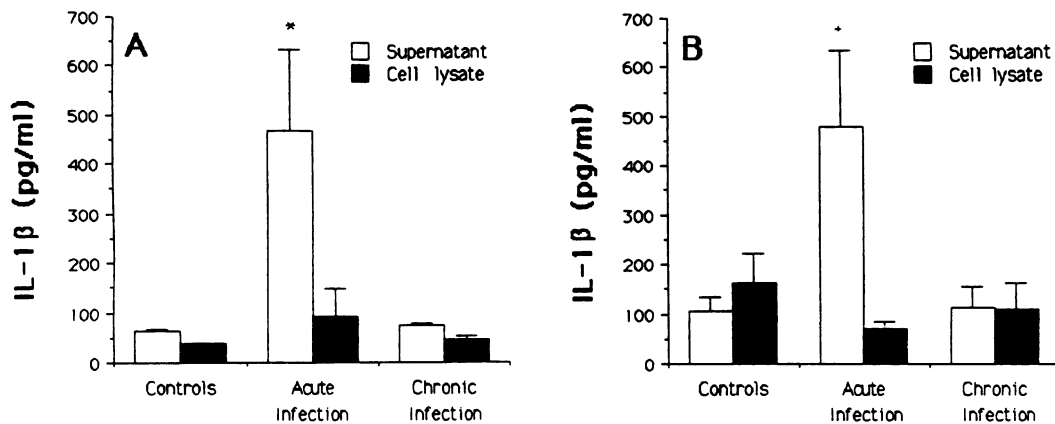
## Discussion

After HIV infection, THP-1 cells did not constitutively produce any detectable amount of TNF $\alpha$  or IL-1 $\beta$  or any mRNA for either cytokine. This observation does not support a direct role for HIV in modulation of TNF $\alpha$  or IL-1 $\beta$  production by resting monocytic cells. Stimulation with LPS, however, resulted in substantial production of these cytokines by HIV-infected and uninfected cells. Further increases in TNF $\alpha$  and IL-1 $\beta$  production were noted after the addition of IFN- $\gamma$ . However, no significant differences in TNF $\alpha$  or IL-1 $\beta$  production were observed between uninfected or chronically HIV-infected cells. These results further support the hypothesis that HIV infection alone was not sufficient to alter cytokine production by these monocytic cells. Folks et al. (34) recently reported increased expression of membrane-bound IL-1 $\beta$  in the U1 monocytic clone chronically infected with HIV-1 after stimulation by PHA-induced supernatant of mononuclear cells. The parental uninfected U937 cells did not show increased IL-1 $\beta$  expression after stimulation. The discrepancy between these observations and ours might be due to comparison between an HIV-infected cell clone (U1) with the uninfected parental cell line (U937) rather than the uninfected cell clone. Furthermore, U937 cells may differ in cytokine modulation after HIV infection from THP-1 cells due to differentiation stage or other factors.

We observed a significant increase in TNF $\alpha$  (2- to 7-fold) and IL-1 $\beta$  (2.5- to 13-fold) production in the supernatant of acutely HIV-infected cells compared with uninfected or chronically infected cells after stimulation with LPS or LPS plus IFN- $\gamma$ . An increase in TNF $\alpha$  and IL-1 $\beta$  mRNA was also observed, suggesting a regulation at the transcriptional level. These acutely infected cells exhibited phenotypic changes suggestive of HIV-induced differentiation or activation as manifested by adherence to plastic, increased NSE staining, and cessation of growth. Similar effects of HIV infection leading to maturation and differentiation have been reported in the monocytic cell line U937 (35). The mechanism by which HIV may induce these phenotypic changes has not been yet investigated. One potential mechanism would be that, as described for HTLV-I (36), the product of a viral gene could regulate the transcription of cellular genes and lead to differentiation. Monocytes are primed after maturation and/or activation and can release increased amounts of TNF $\alpha$  and IL-1 $\beta$  under LPS exposure (37). Since monocyte precursors from the bone marrow can be infected with HIV-1 in vitro (38), a model for HIV



**Figure 3.** Production of TNF $\alpha$  by uninfected cells (controls), acutely HIV-infected cells, or chronically HIV-infected cells after stimulation with 5  $\mu\text{g/ml}$  of LPS (A) or 5  $\mu\text{g/ml}$  of LPS plus 100 U/ml of IFN- $\gamma$  (B) for 24 h. Data are presented as mean  $\pm$  SEM and are the result of five separate experiments. \* $P < 0.001$ ; † $P < 0.001$ .



**Figure 4.** Production of IL-1 $\beta$  by uninfected cells (controls), acutely HIV-infected cells, or chronically HIV-infected cells after stimulation with 5  $\mu$ g/ml of LPS (A) or 5  $\mu$ g/ml of LPS plus 100 U/ml of IFN- $\gamma$  (B) for 24 h. Data are presented as mean $\pm$ SEM and are the result of five separate experiments. \* $P < 0.01$ ; + $P < 0.05$ .

modulation of TNF $\alpha$  and IL-1 $\beta$  production in monocytes may be proposed. Infection by the retrovirus may induce monocyte differentiation and prime these cells, rendering them more sensitive to a second stimulus such as LPS to produce TNF $\alpha$  or IL-1 $\beta$ . A variety of microbial infections occur in patients with AIDS and exposure to LPS could be a common stimulus for enhanced cytokine production by HIV-infected monocytes in vivo. The previously observed increase in TNF $\alpha$  and/or IL-1 $\beta$  production in AIDS patients may be explained by repeated LPS stimulation of HIV-infected, and therefore more susceptible, monocytes.

The roles of TNF $\alpha$  and IL-1 $\beta$  in the clinical manifestations of HIV infection remain to be determined. The fever and the wasting syndrome seen in AIDS patients may be related to enhanced production of these two cytokines. Furthermore, elevated production of IL-1 $\beta$  or TNF $\alpha$  might also activate T cells, increasing their susceptibility to HIV infection. The role of IL-1 $\beta$  inhibitors produced by macrophages infected with HIV (14, 39), which may also contribute to immune dysfunction in AIDS, was not investigated in our study because the RIAs used to measure IL-1 $\beta$  and TNF $\alpha$  are not affected by the presence of inhibitors.

Interestingly, LPS stimulation was also found to increase

the release of HIV by infected THP-1 cells, as measured by supernatant RT activity and p24 antigen levels. This observation may support the hypothesis of enhancement of retroviral infection after opportunistic infections. Further studies are needed to define the mechanisms, direct or indirect, by which LPS stimulation may upregulate HIV expression. Recently Fauci reported enhancement of HIV replication by TNF $\alpha$  in monocytic and lymphocytic T cell lines (40), and we are currently investigating the possibility that in our model the upregulation of HIV expression after LPS stimulation might be related to TNF $\alpha$  production.

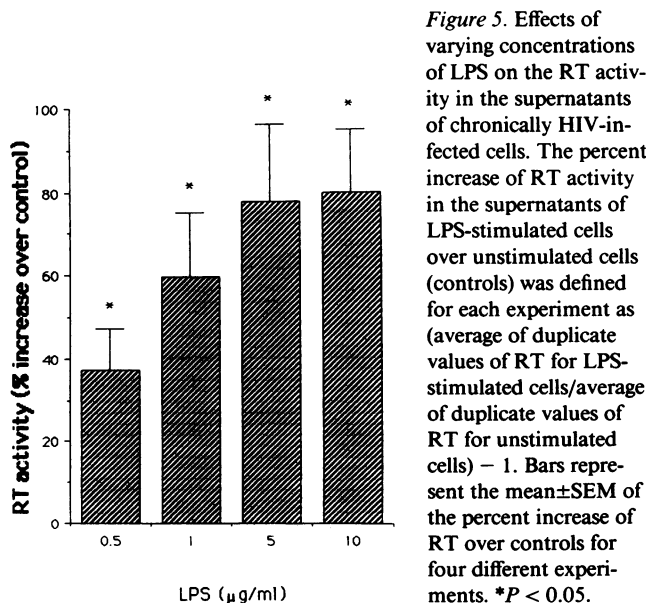
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**Figure 5.** Effects of varying concentrations of LPS on the RT activity in the supernatants of chronically HIV-infected cells. The percent increase of RT activity in the supernatants of LPS-stimulated cells over unstimulated cells (controls) was defined for each experiment as (average of duplicate values of RT for LPS-stimulated cells/average of duplicate values of RT for unstimulated cells) - 1. Bars represent the mean $\pm$ SEM of the percent increase of RT over controls for four different experiments. \* $P < 0.05$ .

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