Phagocytosis of *Mycobacterium tuberculosis* modulates human immunodeficiency virus replication in human monocytic cells

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Macrophage activation resulting from phagocytosis has the potential to modulate human immunodeficiency virus (HIV) replication. We have determined the effects of phagocytosis of particulate stimuli on transcription and release of HIV. Using THP-1 and Mono Mac 6 human monocytic cell lines transfected with HIV long terminal repeat sequence chloramphenicol acetyltransferase (LTR CAT) constructs we have demonstrated that phagocytosis of *Mycobacterium tuberculosis* enhanced HIV-1 and -2 LTR CAT expression. However phagocytosis of zymosan or inert latex beads had little or no effect on CAT expression. Enhancement of HIV LTR CAT expression was dependent upon intact NF-κB binding sites and was independent of tumour necrosis factor alpha secretion. *M. tuberculosis* strains of different degrees of virulence induced similar levels of enhanced CAT expression. In contrast, phagocytosis of *M. tuberculosis* by HIV-1-infected THP-1 cells reduced supernatant reverse transcriptase (RT) activity without suppression of p24 antigen release. Phagocytosis of zymosan granules or latex particles did not alter released RT activity. However, phagocytosis of either *M. tuberculosis*, zymosan granules or latex particles by HIV-1-infected human peripheral blood monocyte-derived macrophages reduced supernatant RT activity. These data indicate that phagocytosis of *M. tuberculosis* may enhance HIV transcription in monocytic cells although it may reduce release of intact HIV.

Introduction

Monocytes and macrophages are an important target of human immunodeficiency virus (HIV) infection and may represent a principal viral reservoir in tissues (Gendelman et al., 1989). HIV replication has been detected in monocytes and macrophages from a variety of tissue sites including blood (Gendelman et al., 1988), brain (Koenig et al., 1986) and lung (Jeffrey et al., 1991), and blood monocytes have also been shown to harbour latent HIV (Mikovits et al., 1992). Activation of monocyte-derived cells might potentially activate or enhance HIV replication in such cells. Such activation may follow phagocytosis of another pathogen such as *Mycobacterium tuberculosis*. HIV replication in monocytes has previously been shown to be induced by binding of the transcriptional activator NF-κB to the enhancer region of the viral long terminal repeat sequence (LTR) and is dependent upon the state of cellular differentiation (Griffin et al., 1989). NF-κB consists of a family of heterodimeric protein subunits (Perkin et al., 1992) and different subunit combinations have distinct binding kinetics for the HIV-1 provirus and their release into the nucleus is inducer-specific (Vlach & Pitha, 1992). Induction of NF-κB binding activity has been demonstrated in a number of monocytic cells following stimuli such as bacterial lipopolysaccharide (LPS) (Pomerantz et al., 1990) and tumour necrosis factor alpha (TNF-α) (Osborn et al., 1989) which cause dissociation of NF-κB from the inhibitor IκB (Ghosh & Baltimore, 1990). Peripheral blood monocytes, adherent macrophages and the monocytic cell lines used in this study constitutively express nuclear NF-κB binding activity (Griffin et al., 1989). However the regulation of HIV transcription in monocytic cells can be latent, restricted or productive (Raziuddin et al., 1991). Phagocytosis could enhance HIV transcription by increasing levels of nuclear NF-κB, inducing combinations of NF-κB subunits with enhanced activating properties, or by inducing other transcriptional regulators (Cullen & Greene, 1989).

We have previously demonstrated that phagocytosis of *M. tuberculosis* or zymosan granules, particulate granules made from the cell wall of the yeast *Saccharomyces cerevisiae*, induces expression of NF-κB-responsive genes for TNF-α and interleukin-8 (Friedland et al., 1992). These experiments suggested that phagocytosis of either mycobacteria- or yeast-derived particles stimulates NF-κB translocation to the nucleus. In addition, intraperitoneal inoculation of transgenic mice,
carrying a transgene of the HIV LTR linked to the gene encoding chloramphenicol acetyltransferase (CAT), with *M. bovis* (BCG strain) increased CAT activity in peritoneal macrophages (Leonard et al., 1989). Thus phagocytosis of mycobacteria appears to activate the LTR of HIV as well as to increase NF-κB binding.

We have therefore investigated the hypothesis that phagocytosis may modulate HIV transcription, via activation of NF-κB, in human monocytic cells. This hypothesis was investigated in two human monocytic cell lines (THP-1 and Mono Mac 6) transfected with HIV LTR CAT constructs. In further experiments we have determined the effects of phagocytosis on viral release from HIV-infected THP-1 cells and human peripheral blood monocyte-derived macrophages (MDM). *M. tuberculosis* and zymosan granules were chosen as positive particulate stimuli since opportunistic infections with mycobacteria or yeast are common in HIV-infected individuals (Elliot et al., 1990; Barnes et al., 1991; Horsburgh, 1991: Centers for Disease Control. 1987). Modulation of HIV replication following phagocytosis of these opportunistic pathogens may influence clinical progression of HIV disease.

**Methods**

**Cell culture.** The human phagocytic monocyte cell line THP-1 (Tsuchiya et al., 1980) was maintained in RPMI-1640 medium supplemented with 5% fetal calf serum (FCS) (same batch used for all experiments), 2 mM-glutamine, 0.1 mg ampicillin per ml (non-antimycobacterial). The phenotypically more differentiated Mono Mac 6 cell line (gift of Dr H. W. L. Ziegler-Heitbrock, University of Munich, Munich, Germany: Ziegler-Heitbrock et al., 1988), was grown in RPMI-1640 medium supplemented with 10% FCS, 2 mM-glutamine, 0.1 mg non-essential amino acids per ml, 0.075% sodium bicarbonate and 0.1 mg ampicillin per ml. Both cell lines were cultured at 37 °C in 5% CO₂ and passaged every 4 to 5 days.

**Human peripheral blood monocyte isolation.** Human mononuclear cells were isolated from buffy coat preparations (National Blood Transfusion Service, London, U.K.) by density gradient separation (Histopaque-1077, Sigma). Platelets were depleted by four washes in PBS containing 3 mM-EDTA and RPMI-1640 with 5% CO₂ in elution buffer (1:1 ratio of PBS with 10 mM-EDTA and RPMI-1640 medium with 10% human serum). Cells were resuspended in RPMI-1640 medium supplemented with 10% human serum (serum from a single donation was used for all experiments), 5% FCS and cultured in six-well tissue culture plates (Becton Dickinson) at a final concentration of 10⁷ cells per 5 ml tissue culture medium per well.

**Cell stimulation.** Twenty-four hours after transfection, cells were stimulated with particulate or soluble stimuli. Phagocytic stimuli were H37-Rv, a virulent strain of *M. tuberculosis* (10 organisms per cell), H37-Ra, an avirulent strain of *M. tuberculosis* (10 organisms per cell) (Allen, 1969), and zymosan granules (1 mg per ml). LPS from *Escherichia coli* O127.B8 (1 mg per ml) was the soluble positive control and 3 μm diameter latex beads (10 beads per cell; Sigma) were used as the inert negative particulate control. Phagocytosis of *M. tuberculosis* by at least 65% of the THP-1 or Mono Mac 6 cells occurred within 30 min of mixing mycobacteria and monocyte cells without requirement for specific opsonization. Fewer than a further 10% of cells ingested mycobacteria over the following 24 h. Control experiments showed that the culture medium used to grow *M. tuberculosis* stocks had no effect on its own on HIV LTR CAT expression or release of supernatant reverse transcriptase (RT) levels. Phagocytosis of either zymosan or latex particles by at least 80% of the THP-1 or Mono Mac 6 cells occurred within 4 h of mixing with little increase over the following 24 h. We have previously demonstrated that phagocytosis of latex particles did not stimulate TNF-α production and secretion from monocytes (Friedland et al., 1992). In some experiments transfected cells were incubated in the presence of 0.1 mM monomeric antibody to human TNF-α (CB0006; Celltech). The ability of this antibody to neutralize released TNF-α was assessed using the previously described sensitive bioassay involving the WEHI 164 cell line (Espievik & Nissen-Meyer, 1986). A minimum of three independent experiments were carried out for each condition.

**CAT assay.** Cytoplasmic CAT activity was assayed 48 h after transfection of cells (Gorman et al., 1982). Samples were assayed in a 150 μl volume containing 0.5 μCi (18.5 kBq) [14C]chloramphenicol (Amersham), 4 μM-acyl coenzyme A (Sigma), 0.25 M-Tris–HCl pH 7.4, and cell lysate containing approximately 300 μg of protein. Protein concentrations were standardized spectrophotometrically using the method of Bradford (1976). Following separation by thin-layer
chromatography, percentage conversion of chloramphenicol to its acetylated forms was determined by liquid scintillation counting.

**HIV infection of monocytic cells.** THP-1 cells in log-phase growth were infected with the HIV-1<sub>DR</sub> strain. Cells (4 x 10<sup>6</sup>) were incubated in 1 ml of cell-free viral supernatant (6 x 10<sup>4</sup> TCID<sub>50</sub>) derived from Jurkat TAT cells (Rosen et al., 1986) for 2 h at 37 °C in 5% CO<sub>2</sub>. HIV-infected THP-1 cells were used in experiments after 10 days of subculture, when > 30% of cells were demonstrated to express p24 antigen by immunohistochemistry. MDM were infected with the monocytotropic HIV-1<sub>BaL</sub> strain (Gartner et al., 1986) 5 days after isolation of monocytes. Cells (8 x 10<sup>6</sup>) were incubated for 2 h in 1 ml of cell-free viral supernatant (1 x 10<sup>6</sup> TCID<sub>50</sub> derived from MDM cultures) in Teflon vials at 37 °C in 5% CO<sub>2</sub>. HIV-infected MDM were used in experiments after 7 days of subculture in Teflon vials, after which 25% of cells were demonstrated to express p24 antigen.

Immediately before experimentation, HIV-infected non-adherent cells were washed three times in PBS to remove all extracellular virus. Cell numbers adjusted to a density of 2 x 10<sup>6</sup> cells/ml culture medium. Such cells were incubated in six-well tissue culture plates and were exposed to soluble or particulate stimuli as previously described. Cell-free supernatants were assayed for RT activity or p24 levels 24, 48 and 96 h post-stimulation.

**HIV RT and p24 antigen assays.** RT activity was determined in 10 μl samples of cell-free tissue culture supernatant using [32P]dTTP incorporation (Potts, 1990) determined using a direct β plate reader (Canberra Packard). Viral p24 levels were measured in cell-free tissue culture supernatant using p24 antigen capture ELISA. Viral supernatants treated with 1% Empigen (Calbiochem) were added to enzyme immunoassay microtitre plates (Costar) coated with an affinity-purified sheep anti-HIV p24-gag antibody (Aalto Bioreagents). After washing, bound p24 was detected with a biotinylated anti-p24 monoclonal antibody, EH12E1 (Ferns et al., 1987; MRC, AIDS Reagent Project, Potters Bar, U.K.). The plates were washed and incubated with streptavidin–alkaline phosphatase (Boehringer Mannheim). After repeated washing p24 was detected by the addition of the substrate 5-nitrophenyl phosphate (Sigma). The release of p24 was not measured for MDM infected with HIV-1<sub>BaL</sub> because this viral p24 reacted poorly in this ELISA.

**Results**

**Phagocytosis and HIV transcription in THP-1 cells**

We determined the effects of phagocytosis of *M. tuberculosis* on HIV transcription using THP-1 cells transfected with either HIV-1 or HIV-2 LTR CAT (Fig. 1). HIV-1 LTR CAT transcription was enhanced in THP-1 cells following phagocytosis of *M. tuberculosis* (threefold) or exposure to LPS (27-fold). Phagocytosis of zymosan or latex particles by THP-1 cells resulted in little or no enhancement of CAT expression compared with unstimulated controls. Basal HIV-2 LTR CAT expression was lower than that of HIV-1 LTR CAT. However HIV-2 LTR CAT expression was also enhanced following phagocytosis of *M. tuberculosis* (33-fold) or exposure to LPS (17-fold). The fold increase of such enhancement was greater than that observed with HIV-1 LTR CAT, however the overall induction in terms of percentage acetylation was comparable. Phagocytosis of zymosan granules or latex particles produced a small enhancement in HIV-2 LTR CAT expression.

**Phagocytosis and HIV transcription in Mono Mac 6 cells**

In order to determine whether monocyte maturation influenced the effects of phagocytosis on HIV transcription we performed experiments with the more phenotypically mature cell line Mono Mac 6 (Fig. 2). HIV-1 LTR CAT expression following phagocytosis of...
M. tuberculosis by Mono Mac 6 cells produced greater enhancement of CAT expression (4.8-fold) than that measured in THP-1 cells (3.2-fold). Phagocytosis of zymosan granules also enhanced HIV-1 LTR CAT expression in Mono Mac 6 cells (fourfold), whereas LPS stimulation did not enhance basal CAT expression. HIV-2 LTR CAT was similarly enhanced in Mono Mac 6 cells following phagocytosis of M. tuberculosis (3.5-fold). However phagocytosis of zymosan granules produced a lower level of enhanced expression (1.7-fold).

**NF-κB dependence of phagocytosis-enhanced CAT expression**

Using HIV-1 and HIV-2 constructs with mutated NF-κB binding sites we next determined whether enhanced HIV LTR CAT expression following phagocytosis of M. tuberculosis or zymosan was dependent upon binding of NF-κB to the HIV LTR. Phagocytosis of M. tuberculosis or zymosan produced only background levels of CAT activity in THP-1 cells transfected with either ΔHIV-1 or ΔHIV-2 LTR CAT (Fig. 3). Similarly, only background levels of CAT activity were detected in Mono Mac 6 cells transfected with either of the mutated constructs (data not shown).

**Effects of anti-TNF-α on enhancement of HIV transcription**

To evaluate whether secreted TNF-α contributed to the enhancement of HIV LTR CAT expression demonstrated after phagocytosis of M. tuberculosis or zymosan, experiments were carried out in the presence of monoclonal antibody to TNF-α. Anti-TNF-α was present in excess and completely neutralized TNF bioactivity in culture supernatants assessed using the sensitive WEHI 164 cell line (data not shown). Phagocytosis of M. tuberculosis strains H37-Rv (virulent) or H37-Ra (avirulent) or of zymosan granules in the presence of anti-TNF-α did not attenuate the increase in CAT expression (Fig. 4). HIV LTR CAT expression in either THP-1 or Mono Mac 6 cells was not significantly affected by the virulence of the M. tuberculosis strain phagocytosed (data shown for THP-1 cells).

**Effects of phagocytosis on release of RT activity and p24 antigen from HIV-1-infected THP-1 cells**

These experiments investigated the effects of phagocytosis on viral release by measuring the release of RT activity and p24 antigen from washed HIV-infected THP-1 cells. In marked contrast to transcriptional data, phagocytosis of M. tuberculosis or LPS stimulation of HIV-1<sub>RF</sub>-infected THP-1 cells resulted in greater than 50% reduction in supernatant RT activity compared with unstimulated controls (Fig. 5). However phago-
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Fig. 5. Release of RT activity (a) and p24 antigen (b) from HIV-infected THP-1 cells. Immediately before experimentation THP-1 cells infected with HIV-1\textsuperscript{Ra} were washed in PBS to remove all extracellular virus. Such cells were resuspended in fresh medium and stimulated with different agents. Supernatants were analysed for RT activity and p24 antigen levels at 24, 48 and 96 h post-stimulation. Treatment regimes included no treatment (□), 10 µg LPS per ml (●), \textit{M. tuberculosis} strain H37-Rv (Δ), 10 mg zymosan granules per ml (▲) or latex particles (■). The graph illustrates the arithmetic mean and S.E.M. of a minimum of three independent experiments.

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Phagocytosis of zymosan granules or latex particles by THP-1 cells produced little difference in the release of RT activity compared with unstimulated controls. In contrast, there was a small increase in p24 antigen release following phagocytosis of \textit{M. tuberculosis}. Exposure to LPS or phagocytosis of zymosan or latex particles had no effect on release of p24 antigen (Fig. 5). None of these treatments significantly affected cell viability (as assessed by trypan blue exclusion) over the culture period.

Effects of phagocytosis on release of RT activity from HIV-1\textsuperscript{Ba}L-infected MDM

In addition to demonstrating that phagocytosis reduced release of RT activity from THP-1 cells, we determined the effects of such stimulation on RT release from washed HIV-1\textsuperscript{Ba}L-infected MDM. Stimulation of such cells with either \textit{M. tuberculosis} or LPS or zymosan or latex particles all resulted in 50% or greater reduction in released supernatant RT activity, 96 h post-stimulation, compared with unstimulated control cells (Fig. 6). Treatments did not significantly affect cell viability over the culture period.

Discussion

This study demonstrates that phagocytosis of \textit{M. tuberculosis} by human monocytic cells enhances both HIV-1 and -2 LTR CAT transcription. These data suggest that phagocytosis of \textit{M. tuberculosis} by monocytes expressing latent or restricted HIV replication has the potential to induce or enhance viral replication in vivo. Phagocytosis of zymosan granules also produced a
The small enhancement of HIV LTR CAT expression in THP-1 cells. However phagocytosis per se was not sufficient to enhance HIV LTR CAT transcription, as phagocytosis of inert latex particles had no effect on HIV-1 or -2 CAT expression. Thus phagocytosis of mycobacteria had a specific effect on HIV LTR CAT expression.

The pattern of enhanced CAT expression following phagocytosis or LPS stimulation was the same in THP-1 cells transfected with either HIV-1 or -2 constructs. However the fold increase in enhanced CAT expression was greater in cells transfected with HIV-2 LTR CAT. Similarly replication of HIV-2 strains in promonocytic U937 cells is known to be more efficient than HIV-1 replication (Innocenti & Seigneurin, 1990). The observed effect in monocytes is in contrast to a previous report that HIV-1 LTR CAT transcription in T lymphocytes is more responsive to T cell activation signals than HIV-2 LTR CAT (Tong-Starksen et al., 1990). Differences in fold increase of HIV-1 and -2 LTR CAT expression seen in this study were in part mediated by the lower level of constitutive HIV-2 LTR CAT expression in THP-1 cells, induced CAT expression being comparable with both HIV-1 and -2 constructs. In addition, levels of HIV-1 and -2 LTR CAT expression were similar in phenotypically more mature Mono Mac 6 cells suggesting such differences occur only in undifferentiated monocytes. Such differences in expression of HIV-2 LTR CAT expression may reflect a requirement for transcription factors expressed only after monocyte differentiation or activation (Markovitz et al., 1990).

Use of HIV LTR CAT constructs that had mutated NF-κB binding sites, demonstrated that in both cell lines enhanced CAT expression following phagocytosis of M. tuberculosis was dependent upon intact NF-κB binding sites. These data are supported by a previous study in which LPS enhancement of HIV-1 LTR CAT expression in human monocytic cells was dependent upon the presence of intact NF-κB sites (Pomerantz et al., 1990). However, requirement for intact NF-κB sites does not preclude the involvement of additional transcription factors in induction of enhanced CAT expression (Cullen & Greene, 1989).

We have previously shown that phagocytosis of either M. tuberculosis or zymosan granules, but not latex particles, induced TNF-α secretion (Friedland et al., 1992). Release of TNF-α had the potential to activate HIV transcription in vitro in an autocrine manner (Clouse et al., 1989). Using monoclonal antibody raised against TNF-α we were able to neutralize all detectable TNF-α that was released into the supernatant after phagocytosis of M. tuberculosis or zymosan granules by monocytic cells. It is possible that very low undetected levels of TNF-α or cytoplasmic TNF-α could have activated HIV LTR CAT expression. However the observation that the use of antibody raised against TNF-α in this study had no effect on HIV LTR CAT expression suggests that enhancement of HIV LTR CAT activity induced by phagocytosis of M. tuberculosis or zymosan granules was independent of TNF-α secretion. This finding is consistent with a previous report which showed that LPS stimulation of HIV-1 LTR CAT was independent of TNF-α secretion (Pomerantz et al., 1990).

Similar enhancement of HIV LTR CAT expression was observed following phagocytosis of M. tuberculosis strains of differing virulence in both cell lines. Such data suggest that phenotypic features common to M. tuberculosis other than determinants of virulence may be important in triggering enhanced LTR CAT expression. This may have important implications in vivo as such proteins or cell wall peptidoglycans may be common to other mycobacteria which are known to cause opportunistic infections in HIV-infected individuals such as the Mycobacterium avium intracellulare complex (Horsburgh, 1991). Interestingly, although M. tuberculosis strains H37-Rv and H37-Ra show markedly different virulence in rodents in vivo (Allen, 1969), we have previously demonstrated that they cause similar increases in cytokine expression in human monocytic cells (Friedland et al., 1992).

HIV release has been shown to relate inversely to the degree of macrophage stimulation (Nottet et al., 1993). In contrast to the enhanced HIV transcription observed, phagocytosis of M. tuberculosis or exposure to LPS reduced the release of supernatant RT activity from HIV-infected THP-1 cells. Similarly, phagocytosis of M. tuberculosis or zymosan or latex particles, or LPS stimulation, reduced release of RT activity from HIV-infected MDM. This suggests that phagocytosis per se is sufficient to reduce released supernatant RT activity from HIV-infected MDM over the time course of the experiments. The inability of zymosan or latex to reduce RT release from THP-1 cells suggests that reduction in RT release in response to such stimuli is dependent on monocyte differentiation. Thus although the decrease in RT activity induced by phagocytosis of M. tuberculosis is not specific, it shows more potency in its ability to down-regulate RT release from both monocytic cell lines and MDM.

Unlike the release of RT activity, p24 antigen release was not reduced following stimulation of HIV-infected THP-1 cells, a phenomenon previously observed after phagocytosis of M. tuberculosis in human peripheral blood monocytes (Meylan et al., 1992). RT activity has been shown to reflect the release of whole virus due to its tight association with the virus particle, whereas capsid (including p24 antigen) and envelope proteins may be shed as free (soluble) antigen (Fernie et al., 1991). Thus...
dissociation of released RT activity and p24 antigen may reflect disruption of viral assembly and release but not transcription and translation following stimulation. Such differences between RT activity and p24 release have been previously described (Fernie et al., 1991: Innocenti & Seigneurin, 1990; Gendelman et al., 1990). Furthermore, it has been demonstrated that release of superantigen RT activity can be reduced without suppression of HIV mRNAs or proteins in the persistently HIV-infected promonocytic cell line U1. Such data may reflect a redistribution of virions to intracytoplasmic vacuoles (Biswa et al., 1992). We have previously shown that activation with soluble stimuli of HIV-infected THP-1 cells, transfected with HIV-1 LTR CAT, reduces RT release without suppression of HIV LTR CAT expression (Shattuck et al., 1993). Thus measurement of HIV LTR CAT expression is useful in that it reflects control of HIV replication at the transcriptional level. However it cannot predict the effects of post-transcriptional events on HIV assembly and release. We suggest our data demonstrate that phagocytosis of \textit{M. tuberculosis} in adherent conditions may reduce the release of infectious virus without disrupting HIV transcription and translation. It remains to be determined whether such a reduction in the release of intact HIV reflects an accumulation of intracytoplasmic virions or a dysfunction of viral assembly.

In summary, phagocytosis of \textit{M. tuberculosis} by human monocytic cells induced enhancement of HIV transcription and may have important implications \textit{in vivo}. Such a secondary opportunistic pathogen might activate viral replication in mononuclear cells latently infected with HIV, hastening clinical progression to AIDS. We have also demonstrated that phagocytosis of \textit{M. tuberculosis} by monocytic cells with productive HIV infection reduced viral release in the short term. However in terms of HIV disease progression, reduction of HIV release from productively infected cells may be less important than activation of viral release from latently infected cells. In addition, if the observed reduction in RT activity reflected a retention of virions in intracytoplasmic vacuoles, this would increase the intracellular viral reservoir which could lead to later infection of susceptible cells.

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