The human immunodeficiency virus type 1 regulatory protein Tat inhibits interferon-induced iNos activity in a murine macrophage cell line

C. Howard Barton,1 Thelma E. Biggs,1 Trevor R. Mee1 and Derek A. Mann2

1 Department of Biochemistry, University of Southampton, Southampton SO16 7PX, UK
2 University Clinical Biochemistry Department, University of Southampton Medical School, Southampton General Hospital, Southampton SO16 6YD, UK

Human immunodeficiency virus type 1 (HIV-1) infection is frequently associated with concurrent infection by opportunistic pathogens, against which production of nitric oxide by host macrophages provides a first line of defence. We have investigated whether regulatory HIV-1 proteins, such as Tat, can modulate the activity of the inducible nitric oxide synthase (iNos) gene when expressed in stable transfectant lines of RAW264.7 cells. A bioassay for Tat, based on transactivation of an HIV-1 LTR–CAT reporter gene, allowed selection of Tat-expressing cells. Parental and Tat-expressing macrophages accumulated identical levels of nitrite following lipopolysaccharide (LPS) stimulation. Interferon γ (IFN-γ) stimulation, however, resulted in reduced levels of nitrite accumulation as a direct consequence of Tat expression. Conditioned media from Tat-expressing cells reduced the level of nitrite accumulation in parental cells following IFN-γ stimulation but not stimulation with LPS. These results implicate HIV-1 Tat as a modulator of the IFN-γ-specific signal transduction pathways leading to iNos expression.

The monocytic and macrophage cell lineage constitutes a major cellular reservoir for the human immunodeficiency virus (HIV) and plays an important role in AIDS pathogenesis (Mosier & Sieburg, 1994). Previous studies have demonstrated abnormalities in macrophage–monocyte function following HIV infection, including alterations in cytokine and cell surface marker gene expression (Denis & Ghadirian, 1994; Kent et al., 1994), reduced phagocytosis and killing of a number of pathogens (Crowe et al., 1994; Biggs et al., 1995) or increased susceptibility to infection by others (Crowe et al., 1992) and impaired accessory cell function (Twigg et al., 1994). In this study, we investigated the possibility that the HIV-1 regulatory protein Tat may be able to influence directly the programme of gene expression necessary for and resulting in macrophage activation. HIV-1 Tat is a small nuclear protein essential for productive virus infection. Tat stimulates increased synthesis of full-length virus transcripts by binding to the transactivator response (TAR) element at the 5' end of all HIV-1 mRNAs (Jones & Peterlin, 1994). Several laboratories have now demonstrated that Tat can also regulate cellular gene expression by TAR-independent mechanisms (Biswas et al., 1995); these reports include studies on monocyte–macrophages in which both transforming growth factor (TGF) and interleukin 6 production are modulated by Tat (Zauli et al., 1992; Iwamoto et al., 1994).

We chose to examine the effects of Tat on the inducible nitric oxide synthase gene (iNos), since nitric oxide has been proposed as a principal mediator of macrophage anti-microbial and tumoricidal activity (Green et al., 1991). Nitric oxide is the product of the L-arginine oxidative deaminase pathway (Hibbs et al., 1987) catalysed by iNos following direct cell stimulation with either lipopolysaccharide (LPS) or interferon γ (IFN-γ).

To investigate the role of Tat on macrophage function, the murine macrophage BALB/c-derived RAW264.7 cell line was transfected by electroporation (900 μF, 750 V/cm) with pC63.4.1 (10 μg; donated by J. Karn, MRC Laboratory of Molecular Biology, Cambridge, UK). pC63.4.1 is a Moloney murine leukaemia virus vector incorporating the HIV-1 tat gene, transcribed from the virus long terminal repeat (LTR), and a neomycin phosphotransferase gene from the simian virus 40 early promoter (Dingwall et al., 1989). Post-electroporation, cells were placed in Dulbecco's modified Eagle's medium containing 10% fetal calf serum to recover for 48 h. Media including antibiotic (G418 at 1 mg/ml) were replenished every 2–3 days for the first week and transfectants selected over the following 2–3 weeks. Colonies were then cloned by limiting dilution into microwell plates and expanded.

Tat expression in RAW264.7 cell clones was assessed by determining the ability of transfectants to transactivate the
HIV-1 LTR using an LTR–CAT reporter construct pD5.3.3 (kindly provided by J. Karn). In screening for bioactive Tat, stable G418-resistant clones and parental cells were transiently transfected (conditions as described above) with DNA (10 μg) comprising pD5.3.3 (2 μg) and pUC12 (8 μg). Parental cells were also transfected transiently with Tat by substituting 5 μg of pUC12 with pC63.4.1 (Fig. 1). Transiently transfected cells were cultured for 48 h, harvested, and lysates were prepared and normalized for total cell protein and assayed for CAT using standard procedures. Parental cells showed little conversion of 14C-chloramphenicol (5.6%) in the absence of Tat (P–) and following transient co-transfection with tat (P+) the conversion (46.5%) increased by a factor of 8.5. Stable lines T1, T21, T22, T29, and T31 exhibited 15, 17, 9, 4 and 27% conversion, respectively, of chloramphenicol to the acetylated derivative and lines T21, T22, and T31 showed greater conversion than parental cells without tat (P–). (b) Tat–TAR interaction is necessary for Tat-dependent LTR transactivation. Activities of a wild-type LTR (pD5) and a construct lacking the U-rich bulge in TAR (pC120) linked to CAT reporters were determined by transient transfection in line T31. Parental cells transiently transfected the mutant construct (ΔU LTR) exhibited little conversion either in the presence (P+) or absence (P–) of tat (0.3% and 0.5% respectively, data not shown). T31 cells exhibits strong activity (14% conversion) following transfection with the wild-type HIV-1 LTR (wt LTR), but little or no conversion with ΔU LTR (0.6%).

Fig. 1. (a) Analysis of bioactive Tat in stable RAW264.7 transfectants and transiently transfected parental cells. Bioactive Tat was identified by its ability to transactivate a transiently transfected reporter gene (CAT) under the control of the HIV-1 LTR (pD5). Tat expression, driven by pC63.4.1, was either via transient co-transfection (P+) or from drug selected stable cell lines (T1, T21, T22, T29, T31). In all transfections total plasmid DNA was normalized with pUC12. Following transfection, cell lysates were prepared and normalized for total cell protein and assayed for CAT using standard procedures. Parental cells showed little conversion of 14C-chloramphenicol (5.6%) in the absence of Tat (P–) and following transient co-transfection with tat (P+) the conversion (46.5%) increased by a factor of 8.5. Stable lines T1, T21, T22, T29, and T31 exhibited 15, 17, 9, 4 and 27% conversion, respectively, of chloramphenicol to the acetylated derivative and lines T21, T22, and T31 showed greater conversion than parental cells without tat (P–). (b) Tat–TAR interaction is necessary for Tat-dependent LTR transactivation. Activities of a wild-type LTR (pD5) and a construct lacking the U-rich bulge in TAR (pC120) linked to CAT reporters were determined by transient transfection in line T31. Parental cells transiently transfected the mutant construct (ΔU LTR) exhibited little conversion either in the presence (P+) or absence (P–) of tat (0.3% and 0.5% respectively, data not shown). T31 cells exhibits strong activity (14% conversion) following transfection with the wild-type HIV-1 LTR (wt LTR), but little or no conversion with ΔU LTR (0.6%).

Analysis of bioactive Tat in stable RAW264.7 transfectants and transiently transfected parental cells. Bioactive Tat was identified by its ability to transactivate a transiently transfected reporter gene (CAT) under the control of the HIV-1 LTR (pD5). Tat expression, driven by pC63.4.1, was either via transient co-transfection (P+) or from drug selected stable cell lines (T1, T21, T22, T29, T31). In all transfections total plasmid DNA was normalized with pUC12. Following transfection, cell lysates were prepared and normalized for total cell protein and assayed for CAT using standard procedures. Parental cells showed little conversion of 14C-chloramphenicol (5.6%) in the absence of Tat (P–) and following transient co-transfection with tat (P+) the conversion (46.5%) increased by a factor of 8.5. Stable lines T1, T21, T22, T29, and T31 exhibited 15, 17, 9, 4 and 27% conversion, respectively, of chloramphenicol to the acetylated derivative and lines T21, T22, and T31 showed greater conversion than parental cells without tat (P–). (b) Tat–TAR interaction is necessary for Tat-dependent LTR transactivation. Activities of a wild-type LTR (pD5) and a construct lacking the U-rich bulge in TAR (pC120) linked to CAT reporters were determined by transient transfection in line T31. Parental cells transiently transfected the mutant construct (ΔU LTR) exhibited little conversion either in the presence (P+) or absence (P–) of tat (0.3% and 0.5% respectively, data not shown). T31 cells exhibits strong activity (14% conversion) following transfection with the wild-type HIV-1 LTR (wt LTR), but little or no conversion with ΔU LTR (0.6%).
for 48 h with 100 ng/ml LPS accumulated identical levels of nitrite. Stimulation of parental and T31 lines with IFN-γ also resulted in increased accumulation of nitrite over the 48 h time-course compared with untreated cultures; however, parental cells produced substantially more nitrite than cultures of the T31 line. In four independent experiments undertaken by three independent researchers the magnitude of this difference ranged from 4.9- to 1.5-fold. This phenomenon was consistent and may be of importance in pathogenesis during periods of rapid virus reproduction. Conditioned media collected from bioactive Tat-expressing cells can inhibit IFN-γ signalling for nitrite accumulation in trans. Conditioned media from T31 (hatched) or parental cells (open) were used for culturing parental cells in the presence of IFN-γ and/or LPS for 48 h at the concentrations given in (a). Parental cells in either media failed to produce nitrite without additional activation. For LPS, cells accumulated 18.4 μM- and 14.9 μM-nitrite (1.2-fold difference, P < 0.01), whereas following stimulation with IFN-γ, nitrite accumulation of 29.6 and 10.0 μM (3.0-fold reduction, P < 0.001) was observed, and for IFN-γ and LPS in combination, 85.8 and 25.6 μM (3.4-fold reduction P < 0.001) was determined for parental- and T31-conditioned media, respectively.

Fig. 2. (a) Tat suppresses IFN-γ-, but not LPS-induced nitrite production. To examine the effects of Tat on the accumulation of nitrite, parental and T31 cells were cultured in the presence of IFN-γ and/or LPS for 48 h. Both parental RAW264.7 (open) and T31 cells (hatched) accumulated little or no nitrite without activation, as detected by the Griess reagent. Following exposure to LPS (100 ng/ml) for 48 h, parental and T31 cells accumulated 40.2 and 39.6 μM-nitrite, respectively, which were not statistically different. Upon exposure to IFN-γ (100 U/ml) parental cells accumulated 69 μM- and T31 cells 18.9 μM-nitrite, representing a 3.7-fold greater level in parental cells (P < 0.001). Following treatment with both agents, nitrite levels of 144 μM and 80.5 μM were observed in parental and T31 cells, representing a 1.8-fold greater level in parental cells (P < 0.001). (b) Conditioned media from bioactive Tat-expressing cells can inhibit IFN-γ signalling for nitrite accumulation in trans. Conditioned media from T31 (hatched) or parental cells (open) were used for culturing parental cells in the presence of IFN-γ and/or LPS for 48 h at the concentrations given in (a). Parental cells in either media failed to produce nitrite without additional activation. For LPS, cells accumulated 18.4 μM- and 14.9 μM-nitrite (1.2-fold difference, P < 0.01), whereas following stimulation with IFN-γ, nitrite accumulation of 29.6 and 10.0 μM (3.0-fold reduction, P < 0.001) was observed, and for IFN-γ and LPS in combination, 85.8 and 25.6 μM (3.4-fold reduction P < 0.001) was determined for parental- and T31-conditioned media, respectively.
for cells in control conditioned media ($P < 0.001$ for both). The level of reduction of IFN-$\gamma$-induced nitrite accumulation compared well with results obtained from parental and T31 cells. One possible explanation for these observations is that T31 conditioned media contains bioactive Tat protein which can enter and inhibit IFN-$\gamma$ signalling pathways in parental cells. Alternatively, Tat expression in T31 cells might promote expression of an inhibitory cytokine capable of modulating IFN-$\gamma$ responses. There is evidence supporting the second hypothesis, since IFN-$\gamma$-induced $i$Nos gene expression and nitrite production have been reported to be negatively regulated by TGF-$\beta$ (Vodovotz et al., 1993) and corresponding increases in TGF-$\beta$ levels have been observed in marrow macrophages following exposure to Tat (Zauli et al., 1992). The mechanism underlying Tat inhibition of IFN-$\gamma$-induced nitrite production in RAW264.7 cells has not been investigated and we have still to determine if this effect is specific to the $i$Nos gene or reflects a general inhibition of IFN-$\gamma$ signalling in the macrophage line. Studies on the murine $i$Nos gene have shown that the inducible region of its promoter can be divided into separate LPS- and IFN-$\gamma$-responsive elements (Xie et al., 1993), which account for the ability of LPS and IFN-$\gamma$ to synergise for the induction of nitrite production in murine macrophages. Tat might be inhibiting a component of the macrophage IFN-$\gamma$ signalling pathway or alternatively may be acting on a downstream target of this pathway that is specific to the $i$Nos gene. Studies are currently in progress to ascertain which of these two mechanisms is operating in Tat-expressing RAW264.7 cells and to determine if similar effects can be observed in human macrophages. In another study, Bukrinsky et al. (1995) report that infection of human monocytes by HIV-1 both primes and induces NOS II (human homologue of murine $i$Nos) expression which in early infection can be boosted by LPS and tumour necrosis factor $\alpha$. The differences observed with the results obtained from our study may reflect species or cell type heterogeneity in the regulation of the $i$Nos/NOS II gene, but could also be explained by effects due to the presence of other virus proteins. Nevertheless, the diminished signalling by IFN-$\gamma$ for $i$Nos expression in a murine macrophage by Tat warrants further investigation in order to determine if it is a phenomenon specific for $i$Nos or a more general effect on IFN-$\gamma$-responsive genes.

This work was supported by grants from the UK MRC (Grant number G9402974) to D.A.M., the Wessex Medical Trust (to C.H.B.) and the University of Southampton Faculty of Medicine (to D.A.M.). T.R.M. was the recipient of a Nuffield Undergraduate research bursary and T.E.B. is a BBSRC research student.

References


Received 31 January 1996; Accepted 17 April 1996