Regulated expression vectors demonstrate cell-type-specific sensitivity to human immunodeficiency virus type 1 Nef-induced cytostasis

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The nef gene product of both human and simian immunodeficiency viruses is critically important for virus replication and disease progression in vivo. However, the precise biological function of Nef remains poorly characterized in vitro, with previous reports suggesting that Nef might be either cytotoxic or cytostatic. As a result of difficulties encountered by several groups in establishing cell lines constitutively expressing Nef, we have developed two inducible systems resulting in stable Nef expression in various mammalian cell lines. Tetracycline-regulated Nef expression was achieved in HeLa cells but could not be established in human T cell lines. Jurkat E6-1 T cell and RAW264.7 murine macrophage cell lines expressing a regulated nef gene were generated using a system in which Nef expression was controlled by a mutated version of the heavy metal-inducible human metallothionein IIA promoter. Induction of high levels of Nef expression in HeLa–Nef and Jurkat–Nef cells resulted in a moderate (2-fold) and a dramatic (10-fold) retardation of cell growth respectively, supporting the contention that Nef may be a cytotoxic or cytostatic factor. This property was also observed at low basal levels of Nef expression in RAW264.7–Nef macrophage clones (5-fold reduction in growth) and was associated with an altered morphological phenotype suggesting that different cell types may be more susceptible to the cytostatic activity of Nef. The regulated Nef-expression systems provide tools for investigating the molecular basis of Nef function, including Nef-mediated cytopathogenicity, CD4 down-regulation and enhancement of virus infectivity.

Introduction

A unique genetic feature of the primate immunodeficiency viruses (HIV-1, HIV-2 and SIV) is the presence of an open reading frame for the nef gene located 3’ of the env gene and partially overlapping the U3 region of the 3’ LTR. The product of the HIV-1 nef gene is a 206 amino acid, N-myristoylated protein of apparent molecular mass 27–30 kDa that is predominantly associated with cellular membranes (Allan et al., 1985). Nef is expressed by a large proportion of the multiply-spliced 2 kb class of viral mRNAs transcribed during the regulatory phase of virus replication. This abundance suggests that Nef may play a vital regulatory role in the early stages of virus replication. However, functional studies initially demonstrated that HIV-1 Nef was either dispensable for virus replication in culture or exerted a modest inhibitory effect that reflected a transcriptional repression of the HIV-1 LTR (Ahmad & Venkatesan, 1988; Luciw et al., 1987; Niederman et al., 1989; Terwilliger et al., 1986). Subsequent work failed to confirm these early data (Benson et al., 1993; Chowers et al., 1994; Hammes et al., 1989; Kim et al., 1989) and by contrast there is...
now a growing consensus that HIV-1 Nef can significantly enhance virus replication (Chowers et al., 1994; Miller et al., 1994, 1995; Spina et al., 1994). The specific regulatory role of Nef in the virus life-cycle remains poorly defined, although recent work implicates Nef as a potential regulator of virus reverse transcription (Aiken & Trono, 1995; Schwartz et al., 1995).

The reaessment of the role of HIV-1 Nef as an essential positive factor for virus replication has been coincident with in vivo studies showing that an intact nef gene is critical for achieving high virus loads and for the development of AIDS in rhesus monkeys infected with the pathogenic SIVmac239 clone (Kestler et al., 1991). A similar requirement for maintenance of a high virus load has also been demonstrated for HIV-1 using the severe combined immunodeficiency (SCID) mouse transplanted with human foetal liver and thymus tissue (SCID–Hu mouse) model (Jamieson et al., 1994). More recently, the presence of defective nef and 3’ LTR sequences in a cohort of long-term non-progressing HIV-1-infected patients who had received a blood transfusion from the same HIV-1-positive donor was described (Deacon et al., 1995). Taken together, these in vivo observations indicate a potential for Nef to play an important role in AIDS pathogenesis.

Apart from the enhancement of virus replication alluded to above, there are a number of other potential in vitro correlates for the critical role played by Nef in pathogenesis. One function of Nef is to mediate down-regulation of cell surface CD4, the major virus receptor (Anderson et al., 1993; Garcia & Miller, 1991; Guy et al., 1987; Mariani & Skowronski, 1993). This function requires N-terminal myristoylation (Aiken et al., 1994; Mariani & Skowronski, 1993), is conserved between HIV-1 and SIV (Benson et al., 1993) and is characterized by an increase in the rates of internalization and lysosomal degradation of CD4 (Aiken et al., 1994; Rhee & Marsh, 1994b; Schwartz, 1995a). The precise mechanism for down-regulation of CD4 is still to be determined but involves specific amino acid sequences in the cytoplasmic tail of CD4 (Aiken et al., 1994; Garcia et al., 1993) and may also involve direct binding between Nef and the CD4 cytoplasmic domain (Greenway et al., 1995; Harris & Neil, 1994). Several investigators have also reported dramatic effects of Nef on T cell activation both when introduced into T cell lines or when expressed in transgenic mice (Bandres & Ratner, 1994; Baur et al., 1994; Brady et al., 1993; Collette et al., 1996a; Greenway et al., 1995; Luria et al., 1991; Niederman et al., 1992, 1993; Rhee & Marsh, 1994a; Skowronski et al., 1993). Unfortunately, much of the data are contradictory and fail to define a clear role. Lastly, there are several reports that nef genes can be either cytotoxic or cytoplast when expressed in transfected cell lines (Baur et al., 1994; Murphy et al., 1993; Ryan-Graham & Peden, 1995; Skowronski & Mariani, 1995).

Much of the controversy that has arisen concerning Nef function may simply be due to intrinsic differences in the experimental systems used by different investigators, but it may also result from the use of different nef alleles which can vary significantly, both in terms of primary amino acid sequence (Harris et al., 1992; Shugars et al., 1993) and function (Luria et al., 1991). Furthermore, the cytotoxic/cytostatic activity of Nef, coupled with the unstable nature of transfected nef genes (Baur et al., 1994; Skowronski & Mariani, 1995) may provide another major source of experimental variation. Previous attempts both by ourselves (M. P. G. Harris & D. A. Mann, unpublished) and others (Baur et al., 1994; Murphy et al., 1993; Ryan-Graham & Peden, 1995) to produce and maintain stable mammalian cell lines using constitutive expression vectors for Nef have failed. While some groups have managed to generate T cell lines expressing Nef using retroviral and plasmid vectors (Garcia & Miller, 1991; Luria et al., 1991; Niederman et al., 1992; Anderson et al., 1993; Aiken et al., 1994; Schwartz et al., 1995), the long-term stability of these lines is in doubt (Skowronski & Mariani, 1995).

We have attempted to overcome these problems by developing regulated Nef expression vectors producing low basal levels of expression that can be readily and reproducibly elevated to significantly higher levels in a titratable manner. Two different regulated vector systems have been developed. The first is based on the tetracycline repressor system described by Gossen & Bujard (1992). The second utilizes a version of the human metallothionein IIa (MT) promoter carrying a mutation in the upstream AP-1 binding site which provides reduced basal levels of transcription that are still inducible with sub-toxic levels of heavy metal ions (Makarov et al., 1994). The ability of these two vector systems to provide stable long-term Nef expression is compared in three mammalian cell lines (HeLa, human T cell lines and murine macrophage). Furthermore, we demonstrate that Nef expression is associated with reduced growth in vitro and that the level of growth retardation is dependent upon both the level of Nef expression and the cell lineage involved.

**Methods**

**Construction of Nef expression vectors.** To allow constitutive expression of the tetrar-VP16 transactivator from the RSV promoter, plasmid pREP4.tTA was constructed by subcloning an EcoRI–BanHI fragment containing the tTA coding sequence from pUHD15-1 (Gossen & Bujard, 1992) into pBluescript and then excising as a HindIII–BanHI fragment for insertion into HindIII–BanHI-digested pREP4 (Invitrogen). The tetracycline-responsive nef plasmid was constructed by initially cloning the BH10 nef gene (Harris et al., 1992) into pUHD10-3 as a SacI–EcoRI fragment and subsequently excising a 1·1 kb XbaI–Xhol fragment containing both the entire tetO-hCMV promoter (CMV) and nef coding sequences by partial digest and cloning into pREP9.DBA-X (a modification of pREP9 in which the RSV promoter was deleted and the BanHI site converted to an XhoI site).

A constitutive Nef expression vector was produced by insertion of the nef gene of the HIV-1 BH10 isolate (Harris et al., 1992) between the BanHI and XhoI sites of pcDNA3 (Invitrogen) to generate plasmid pDMNeF1.1. An inducible vector allowing expression of cloned genes under the control of a modified human MT promoter (Makarov et al.,
Regulated Nef expression

Fig. 1. Tetracycline-responsive Nef expression in HeLa cells. (a) pREP4.tTA and pREP9ΔCMVtNef were constructed as described in Methods. (b) A representative stable transfected HeLa–Nef clone exposed for 24 h to various levels of tetracycline. Cytoplasmic extracts (10 µg protein) were prepared and analysed for Nef expression by immunoblotting with a sheep polyclonal anti-Nef serum. Nef expression was measured with the Phorelix software package and is presented graphically in arbitrary units. Expression was totally repressed down to tetracycline concentrations of 50 ng/ml, partially repressed (25% of maximal expression level) at 10 ng/ml and fully switched on at 1 ng/ml.

1994) was prepared by inserting the MT promoter as a HindIII–BamHI fragment from pMTΔ302CAT into pcDNA3. The cytomegalovirus (CMV) promoter of this intermediate was then excised as a BglII–HindIII fragment, the termini filled in using Vent polymerase (New England Biolabs) and re-ligated to form pMTcDNA3. The BH10 nef gene was amplified using oligonucleotide primers incorporating 5′ BamHI (5′ AGTCAGGATCTCTCTTTAGATGGGTGGC 3′) and 3′ Apal (5′ AGTCAGGGCCCAATTCTACAGTTTCT 3′) cleavage sites. The resulting PCR product was digested with BamHI and Apal and cloned into pMTcDNA3 to yield pSCNef51.

**Propagation and selection of stable cell lines.** Cos-1, HeLa and RAW264.7 cells were maintained in DMEM containing 10% (v/v) foetal calf serum (FCS) (Life Technologies). Jurkat E6-1 cells were grown in RPMI 1640 supplemented with 10% FCS. All cell lines were cultured at 37 °C in a 5% CO₂ atmosphere. HeLa cells were co-transfected with pREP4.tTA and pREP9 nef using the calcium phosphate co-precipitation method. Transfected cells were cultured for 2 days, then split in a 1:10 ratio into selection medium (growth medium containing 400 µg/ml hygromycin B, 500 µg/ml G418 and 1 µg/ml tetracycline); resistant colonies isolated using cloning rings were then expanded. Following initial selection, the concentrations of hygromycin B and G418 were halved for routine passage and, unless otherwise stated, the concentrations of tetracycline were maintained at 1 µg/ml throughout.

Jurkat T cells were transfected with 10 µg of plasmid DNA by electroporation (10⁷ cells pulsed at 250 V/960 µF) and allowed to recover at 37 °C for 48 h prior to dispensing 150 µl (10⁶ cells) in each well of a 96-well microtitre plate. Approximately 10% of the wells generated G418-resistant colonies which were expanded and recloned. Electroporated RAW264.7 cells (10⁷ cells pulsed at 300 V/960 µF) were also allowed a 48 h period of recovery before addition of G418 (1 mg/ml) and selection for a period of 2–3 weeks. G418-resistant RAW264.7 cells were cloned by limiting dilution in 96-well microtitre plates and expanded.

**Western blot analysis of Nef and CD4 expression.** Nef-expressing G418-resistant clones and transiently transfected Cos-1 cells were identified by Western blotting. For Cos-1, Jurkat and RAW264.7 cells, cultures were washed with PBS, lysed and adjusted to 2 mg/ml
Fig. 2. Nef function in HeLa cells. (a) Human CD4 was expressed in HeLa–Nef and control cells by the vaccinia–T7 transient expression system. Cell-surface levels of CD4 were monitored by FACS analysis using OKT4 followed by goat anti-mouse IgG–FITC conjugate. The mean fluorescence values were: 1 (HeLa 4tTA+tetracycline), 56 ± 4; 2 (HeLa 4tTA–tetracycline), 59 ± 2; 3 (HeLa 4/9Nef+tetracycline), 17 ± 3; 4 (HeLa 4/9Nef–tetracycline), 14 ± 0. Total cellular expression of CD4 and Nef was...
total protein in 125 mM Tris–HCl pH 6.8, 4% (v/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol and 10 mg/ml bromophenol blue. Cytoplasmic extracts were prepared from HeLa cells harvested by scraping and washing in PBS followed by lysis in 10 mM PIPES–NaOH pH 7.2, 120 mM KCl, 30 mM NaCl, 5 mM MgCl$_2$, 1% (v/v) Triton X-100, 10% (v/v) glycerol containing protease inhibitors [0.2 mM AEBSF (Calbiochem), 1 µg/ml leupeptin, 2 µg/ml aprotinin and 1 µg/ml peptatin A]. HeLa cell lysates (10 µg), 40 µg T cell extracts and 20 µg of the macrophage and Cos-1 cell extracts were separated by SDS–PAGE, transferred onto PVDF membrane and probed with a sheep polyclonal anti-Nef serum (1:1000) followed by donkey anti-sheep HRP conjugate (Sigma). The ECL system (Amersham) was used for detection. Western blots for CD4 in transfected cells were probed with a sheep polyclonal anti-CD4 serum as previously described (Harris & Neil, 1994).

- **Assay for down-regulation of cell surface CD4.** HeLa cells (5 × 10$^5$) were infected with vTF7-3 in serum-free OptiMEM medium and transfected with 5 µg pSG5.CD4 at 2 h post-infection by lipofection (LipofectACE, Gibco). Cells were harvested at 24 h post-infection and processed for CD4 FACs analysis as follows. Cells were washed twice in PBS containing 1% BSA (PBSB) and incubated with 100 µl anti-CD4 MAb (OKT4, 2.5 µg/ml) for 20 min at 4 °C. After washing twice with PBSB, cells were incubated with goat anti-mouse IgG–FITC conjugate (Sigma) diluted 1/20 in PBSB for 30 min at 4 °C. Excess antibody was removed by washing twice with PBSB and the FITC label detected using an EPICS ELITE flow cytometer.

- **Infectivity assay for trans-complementation of nef-defective virus.** Both HXB2 and pLCW plasmids (5 µg) were transfected into HeLa and HeLa–Nef cells using lipofectamine (Life Technologies). The DNA–cell mixtures were incubated at 37 °C for 6 h, washed and fed with medium with or without increasing concentrations of tetracycline. After 48 h the cells were monitored for their production of soluble (McKeating et al., 1993) and intracellular p24 antigen. As a control to determine transfection efficiency cells were fixed with methanol–acetone (1:1, stored at −200 °C) and incubated with MAb specific for p24 antigen (MRC AIDS Directed Programme Reagent Repository). Bound antibodies were detected with a β-galactosidase-conjugated anti-mouse Ig and X-Gal as described previously (Clapham et al., 1992). Equivalent amounts of extracellular virus (500 pg of p24 antigen) were allowed to infect HeLa–CD4 LTR-LacZ cells (Clavel & Charneau, 1994). After 24 h the cells were washed, incubated with X-Gal and blue foci counted.

- **Analysis of the growth kinetics of the Nef-expressing cell lines.** HeLa cell proliferation was determined using the MTT assay (Mosmann, 1983). One thousand cells were seeded per well (100 µl) of a 96-well microtitre plate and, at daily intervals, 10 µl MTT (Sigma, 5 mg/ml in PBS) was added and incubated at 37 °C for 4 h followed by 100 µl of acidified propan-2-ol. The contents of each well were thoroughly mixed to solubilize the converted dye and the absorbance at 570 nm was determined. Cell growth was assayed by culturing cells at a density of 3-5 × 10$^5$/ml in a total volume of 50 ml and removing three 1 ml samples per time-point for viable cell number determinations by trypan blue exclusion. Growth of RAW264.7 macrophages was determined by seeding cells at a density of 10⁶ per well of a microtitre plate. At each time-point, three wells for each cell type were stained with crystal violet and cell numbers determined against a standard curve for RAW264.7 cell density.

## Results

### Functional analysis of tetracycline-responsive Nef expression in HeLa cells

The tetracycline-responsive expression system described by Gossen & Bujard (1992) failed to generate stable HeLa clones with detectable Nef expression. This possibly reflected low copy number of the vectors or may have been due to loss of the integrated nef gene during selection (Murphy et al., 1993). To overcome this potential problem a modified tetracycline-responsive system was developed in which the regulatory elements of the original vectors were inserted into episomal vectors of the pREP series (Fig. 1a). The modified vectors generated HeLa cells exhibiting a basal level of Nef expression that could be selected and maintained in the presence of tetracycline (1 µg/ml). Higher levels of Nef expression were achieved by lowering the concentration of tetracycline in the culture medium. Doses between 0 and 10 ng/ml provided measurable levels of protein expression with a maximal 30-fold induction observed in the cells cultured in the absence of the antibiotic (Fig. 1b).

To confirm that the transfected BH10 nef gene was functional, HeLa–Nef cells were tested for ability to down-modulate cell-surface CD4 expression. The vaccinia virus–T7 RNA polymerase system was used to express CD4 in HeLa–Nef and control cells (Fig. 2a) cultured in the presence and absence of tetracycline. Immunoblotting of cytoplasmic lysates demonstrated that total CD4 levels were broadly similar in all four transfections; however, FACS analysis showed that levels of surface CD4 were reduced by 4-fold in the Nef-expressing cells. Interestingly, the induction of high-level Nef expression by removal of tetracycline failed to elicit extensive down-regulation of CD4 suggesting that this function may require relatively low levels of Nef expression. In control experiments, feline CD9 expressed by vaccinia–T7 showed no differences in cell-surface expression (data not shown), confirming that Nef-induced down-regulation was specific for CD4.

HeLa–Nef cells were also tested for their ability to trans-complement the infectivity of the HXB2 infectious clone monitored using either a sheep polyclonal anti-CD4 antiserum (obtained from the MRC AIDS Directed Programme Reagent Repository; Dr M. Page.) or a sheep polyclonal Nef antiserum. (b) A Nef HXB2 clone was transfected into HeLa–Nef cells growing in the presence of 0–5000 ng/ml tetracycline. The ability of the transfected BH10 nef gene to trans-complement infectivity of Nef HXB2 was then tested by infecting HeLa–CD4 LTR-LacZ cells with equivalent amounts (500 pg of p24 antigen) of extracellular virus collected from transfected HeLa–Nef cells. Infectivity data are presented as number of blue foci counted/100 pg of virion p24 antigen. (c) One thousand cells were seeded per well of a 96-well plate in the presence and absence of tetracycline and growth was analysed each day by the MTT assay. Each point represents the mean of three identical wells.
Fig. 3. Heavy metal-responsive Nef expression. (a) The constitutive Nef expression vector pDMNef1.1 and the heavy metal-responsive Nef expression vector pSCNef51 were constructed as described in Methods. (b) Western blot analysis of total cell extracts (20 µg) prepared from Cos-1 cells transiently transfected with either PUC12 DNA (control, lane 1), pDMNef1.1 (lane 2) or pSCNef51 (lanes 3–8). ZnCl₂ was added 24 h after transfection and all cell cultures were harvested after a further 24 h incubation. Induction of increased expression from pSCNef51 was apparent at 50 µM ZnCl₂ (lane 6). (c) Western blot analysis of Nef expression in a representative stable transfected Jurkat E6-1–Nef clone (51–31). Cultures were harvested after 24 h incubation in control medium (lane 1), media containing various concentrations of ZnSO₄ (lanes 2–5), or medium containing 150 µM zinc chloride. Induction was apparent at 100 µM zinc and there was no difference between the level of expression induced by ZnCl₂ or ZnSO₄.

carrying a defective nef gene (Myers et al., 1995). HXB2 was transfected into HeLa and HeLa–Nef cells in the presence of increasing concentrations of tetracycline (Fig. 2b). The resultant extracellular virus was tested for its ability to replicate in HeLa–CD4 LTR-LacZ cells: a clear dose-dependent relationship was observed between concentration of tetracycline in the culture medium and virus infectivity (Fig. 2b). Virus recovered from the transfected HeLa cells infected HeLa–CD4 LTR-LacZ cells resulting in 18–20 infected foci, a level comparable to that observed from the HeLa–Nef cells in the presence of 0.5 µg/ml of tetracycline (data not shown). Maximal trans-complementation (3–4-fold) was achieved at a minimum tetracycline dose of 10 µg/ml, the concentration at which we also observed an induction of elevated Nef expression (Fig. 1).

**Effect of elevated Nef expression on HeLa cell growth**

HeLa–Nef cells in the presence or absence of tetracycline were compared for their ability to replicate. MTT assays showed that induction of maximal levels of Nef reduced the growth rate of HeLa–Nef cells by 2-fold (Fig. 2c). By contrast, removal of tetracycline from HeLa cells transfected with a control vector had no detectable effect on growth rate.

The tetracycline-responsive expression system was subsequently used in attempts to produce similarly regulated Nef expression in human T cell lines. Jurkat and SupT1 T cell lines expressing Nef were generated but they grew very slowly displaying constitutive Nef expression. These observations suggest that the tetracycline-responsive vectors are not suitable for generating regulated expression of Nef in cells of lymphocyte lineage. High basal gene expression and unresponsiveness to tetracycline have been reported for BHK and Vero cells suggesting that this may not be a T cell-specific effect (Ackland-Berglund & Leib, 1995).

**Heavy metal-responsive Nef expression in Jurkat E6-1 cells**

An alternative regulated gene expression system was sought as a result of the inability of the tetracycline-responsive vectors to produce regulated Nef expression in T cells. The use of a mutation in the upstream AP-1 binding site of the human MT promoter–CAT reporter vector (pMT302-CAT) to generate tightly regulated expression of CAT was recently described (Makarov et al., 1994). CAT remained inducible by addition of heavy metals or phorbol esters to culture medium. A construct containing the BH10 nef gene under the control of this modified MT promoter (pSCNef51) (Fig. 3) was produced and tested for its ability to express Nef in response to heavy metal treatment by transient transfection into Cos-1 cells (Fig. 3b). As a control, nef was cloned into pcDNA3 under the regulation of the constitutive CMV promoter (pDMNef1.1). In the absence of exogenously added heavy metals this construct directed high levels of Nef expression (lane 2); in contrast, pSCNef51 expressed low levels of Nef (lane 3). Incubation of
Table 1. Effect of Nef on growth and viability of Jurkat E6-1 cells

V, number of viable cells excluding trypan blue; NV, number of non-viable cells stained with trypan blue.

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pSCNef51-transfected cells with ZnCl₂ (12.5–200 µM; lanes 4–8) for 24 h generated levels of Nef expression that were similar to levels generated by pDMNef1.1. These data demonstrate the low basal activity of the mutated MT promoter and the inducibility of pSCNef51.

pSCNef51 was subsequently used to generate stable transfectants of Jurkat E6-1 cells. From an initial screen of 19 G418-resistant clones, a total of 12 displayed regulated Nef expression in response to ZnCl₂. Expression data for a representative clone are shown in Fig. 3(c). Very low, but detectable levels of Nef were observed in most clones; these could be elevated by addition of micromolar concentrations of zinc. Induction was observed at 100 µM and was optimal following a 24 h incubation of cells with 150 µM of either the chloride or sulphate salt of zinc. Extended culture of two different Nef–Jurkat lines, 51-6 and 51-31, over a period of several weeks in the absence of G418, was achieved without any appreciable loss of basal or induced Nef expression. Thus, the low levels of basal Nef expression provided by the uninduced pSCNef51 vector allow selection and maintenance of expressing T cell lines and do not exert any of the negative selective pressure reported for constitutive vectors (Murphy et al., 1993; Skowronski & Mariani, 1995).

Inhibition of Jurkat cell growth by high levels of Nef

Under standard culture conditions, growth rates of Jurkat–Nef clones were similar to that of untransfected parental cells over a period of several weeks indicating that low levels of Nef can be tolerated (data not shown). The effect of elevated Nef expression on cell growth was determined by culturing Jurkat–Nef cells (51-31) and the control vector cell line (MT-11) in the presence of ZnCl₂ (100 µM) over a period of 7 days. The number of viable MT-11 cells doubled each day of culture during the period 0–4 days with a steady increase in the ratio of viable (V) to non-viable (NV) cells from approximately 5:1 to 11:1 (Table 1). By 7 days in culture the cells had undergone a 34-fold increase in the total number of viable cells (Fig. 4). Under similar culture conditions, the number of viable Nef-expressing (51-31) cells was constant for up to 4 days and by 7 days had only undergone a 3-fold increase (Fig. 4). Numbers of viable and non-viable cells in the culture remained approximately equal (Table 1). This effect was reproducible over several independent experiments and similar restricted growth was observed in an independent clone, 51-6 (data not shown). Baur et al. (1994) recently reported that cell surface-associated Nef induced a state of cell activation that leads to increased rates of apoptosis in T cell lines. However, we have failed to detect any DNA fragmentation or increased DNase activity in Jurkat–Nef cells induced for maximal levels of Nef expression. Alternatively, the reduced Jurkat cell growth rate may be due to cytostatic effects of Nef (Murphy et al., 1993).

In support of this explanation, the numbers of non-viable cells in cultures of induced Jurkat and control cells were similar each day suggesting that there are no higher death rates in the induced cultures (Table 1). In addition, when zinc-treated Jurkat cells are washed and the culture continued in the absence of zinc, the cells proliferate at the normal rate observed in the absence of Nef (S. J. Cooke & D. A. Mann, unpublished observation).

Stable Nef-expressing RAW264.7 macrophages

Earlier studies demonstrated that clones of the murine macrophage cell line RAW264.7 carrying stable-integrated constitutive Nef expression vectors could not be maintained...
due to an uncharacterized cytostatic property of the nef gene (Murphy et al., 1993). We therefore used pSCNef51 to investigate if RAW264.7 lines expressing a regulated nef gene could be produced and maintained. An initial screen of G418-resistant cells identified a number of clones expressing Nef. Expression was regulated by culturing these cells in the presence of 10 μM CdCl₂, shown to be a more effective inducing agent than ZnCl₂ for RAW264.7 cells (Fig. 5a). The low basal level of Nef observed in RAW-N7 cells, common to all clones, increased 100-fold after a 24 h incubation time with CdCl₂.

RAW264.7–Nef clones could be cultured to high densities and displayed stable basal and inducible expression of Nef over several months in culture; however, the growth kinetics of RAW264.7–Nef cells appeared to be reduced relative to that of the parental cells even in the absence of exogenously added CdCl₂ (Fig. 5b). Proliferation of parental cells resulted in a 100-fold increase in cell density by 4 days. In contrast, RAW264.7–Nef clones (N5, N7 and N8) displayed an extended lag phase of growth and only increased cell density 20-fold by 4 days. Similar experiments with cultures in the presence of CdCl₂ showed some reduction in growth of parental cells and a complete cessation of proliferation of the RAW264.7–Nef clones (data not shown). Morphological examination of RAW264.7–Nef cells (Fig. 6c) revealed a distinct appearance characterized by the presence of processes on a greater proportion of cells relative to those of parental cultures (Fig. 6a). Both the extent of process outgrowth and the number of cells bearing processes appeared to increase in RAW264.7–Nef cells incubated for 48 h in the presence of 10 μM CdCl₂ (Fig. 6d). By contrast, similar treatment of parental cultures had no appreciable effect on cell morphology (Fig. 6b).

Discussion

Previous reports have suggested that Nef may be either cytotoxic or cytostatic (Baur et al., 1994; Greenway et al., 1995; Murphy et al., 1993). Two studies were mainly anecdotal, describing an inability to either generate (Baur et al., 1994) or maintain Nef-expressing cell lines (Murphy et al., 1993). In a third study, recombinant GST–Nef fusion protein introduced into PHA-activated PBMCs by electroporation caused a moderate (2-fold) reduction in cell proliferation. This latter study did not address whether there is any dose-dependency for this effect and did not investigate the possibility of Nef-induced apoptosis described by another group (Baur et al., 1994). In the present paper, we use two independent regulated expression systems to demonstrate that Nef expression is associated with reduced cell growth. Moreover, we show that this effect is both cell-type and dose dependent, whereby cells of the monocytic lineage appear more sensitive to the presence of Nef.

A modified tetracycline-regulated system using episomal vectors was successful in producing HeLa–Nef cells expressing basal levels of functional Nef capable of down-modulating levels of cell surface CD4. Expression of Nef could be increased 30-fold by simply reducing the concentration of tetracycline in the culture medium. This induction did not enhance down-
Regulated Nef expression

Regulated Nef expression

Regulated Nef expression

Regulated Nef expression

Regulated Nef expression

Fig. 6. Effect of Nef expression on the morphology of RAW264.7 cells. Each micrograph shows a representative field from 48 h cultures of parental cells growing in the absence (a) or presence (b) of CdCl₂ and RAW-N7 cells growing in the absence (c) or presence (d) of CdCl₂. Note the appearance of cellular outgrowths on RAW-N7 cells and the increased length of outgrowths in the presence of CdCl₂. The scale bar represents 50 µm.

regulation of CD4 but did significantly enhance the infectivity of a ∆nef virus indicating that these two functions of Nef may require different thresholds of Nef expression. Induction of maximal Nef expression was also associated with a moderate 2-fold reduction in cell growth. However, HeLa–Nef clones maintained inducible Nef expression over extended passage histories, even when cultured continuously in the absence of tetracycline. In contrast, stable cell lines expressing Nef constitutively from integrated retroviral vectors rapidly lose expression of Nef during passage (Skowronska & Mariani, 1995; M. P. G. Harris, unpublished observation). This latter effect may reflect the susceptibility of integrated nef genes to selective removal from the cellular genome (Murphy et al., 1993). We were unable to use the tetracycline-regulated system for production of viable T cell lines expressing Nef. The level of Nef expression in T cell lines was roughly equivalent to that observed in the induced HeLa–Nef cells; however, T cell lines proved impossible to expand into large-scale cultures suggesting that they were more susceptible to the cytostatic or cytotoxic effects of Nef.

Use of an alternative heavy metal-regulated system provided stable and inducible expression of Nef in T cell lines and resulted in an approximate 10-fold reduction of cell growth relative to that seen with control cells. This effect was not associated with increased rates of cell death and we could not find any evidence of the Nef-induced apoptosis described elsewhere (Baur et al., 1994). Since the ratio of viable to non-viable cells in the induced Nef–Jurkat lines remained unchanged we conclude that the effect resembles the cytostatic property reported by Murphy et al. (1993). This latter study described the use of constitutive expression vectors to produce clones of RAW264.7 macrophages that ceased to proliferate once reaching a moderate size. However, neither Nef protein nor mRNA expression was demonstrated in the clones; the authors were only able to show that loss of the nef gene correlated with escape from Nef-induced cytostasis. In the current study, we have shown that low basal levels of Nef protein expression provided by the heavy metal-inducible vector pSCNef51 can be tolerated by RAW264.7 cells since the vector was used to select and maintain several Nef-expressing lines: hence nef DNA sequences are not directly toxic to RAW264.7 cells. Although our Nef–RAW264.7 cell lines could be grown to high densities, their rates of proliferation were markedly (5-fold) lower than those of control cells. This effect was observed
in the absence of exogenously added heavy metals suggesting that RAW264.7 cells are particularly sensitive to Nef. This sensitivity may be related to the ability of Nef to form high-affinity interactions in vitro with the macrophage-specific tyrosine kinase Hck, which is a component of the macrophage activation signalling pathway (Saksela et al., 1995). However, at least one interaction between Nef and a member of the Src protein family (the T cell-specific tyrosine kinase, p56(lck)) has been reported in a cellular assay (Collette et al., 1996). These types of interactions may potentially alter the activation state of a Nef-expressing cell by modulating functions such as proliferation. For example, activation of RAW264.7 cells by LPS results in increased adhesion to plastic and decreased proliferation (Raschke et al., 1978). Intriguingly, we have observed a similar albeit less dramatic phenotype in Nef–RAW264.7 cells; furthermore, we have shown that this phenotype is enhanced in Nef-expressing cells induced with heavy metals. We suggest that the slower growth rate of Nef–RAW264.7 cultures may be due to these cells existing in a partial state of activation, possibly arising from a functional interaction between Nef and a macrophage signalling molecule, of which Hck is a strong candidate. The underlying molecular events responsible for effects of high Nef expression on T cell growth are also unknown but may be due to a similar activation phenomenon (via an interaction with p56(lck)). Indeed, several groups have described a dramatic influence of Nef on T cell signalling including the inhibition of transcription factors (AP-1 and NF-κB) which play a key role in growth and differentiation (Niederman et al., 1992; Niederman et al., 1993).

In vivo studies with both HIV-1 (Jamieson et al., 1994) and SIV (Du et al., 1995; Kestler et al., 1991) indicate reduced infectivity and cytopathicity of nef-deleted viruses. A recent report has shown that whereas the poor infectivity of nef-deleted HIV-1 can be compensated by challenging SCID–Hu mice with high doses of virus, the cytopathic potential of this challenge is still greatly reduced (Aldrovandi & Zack, 1996). Nef may therefore possess an intrinsic cytopathogenicity that could be a contributory factor in the development of AIDS. This cytopathogenicity may be related to the ability of Nef to modulate cell proliferation and activation. The cell lines described in the current study will provide important reagents for further investigations of this aspect of Nef function. One potential criticism of the cell lines used for such work is whether the levels of Nef we observe are physiologically relevant. While we have not directly addressed this issue, unpublished work from another laboratory indicates that the levels of expression achieved with a CMV promoter-driven construct in Jurkat cells are similar to those measured in HIV-1-infected PBMCs (Y. Collette, personal communication). Transient transfection of Cos-1 cells with plasmid pSCNe51 leads to levels of Nef equivalent to those produced by the pDMNef1.1 vector. Thus it is likely that levels of Nef expressed in the Jurkat E6-1 and RAW264.7 cells are physiologically relevant.

Skowronski & Mariani (1995) reported that cells expressing Nef from integrated retroviral constructs are unstable, with reduced Nef expression and increased cell-surface CD4 expression over time in culture. We have also observed this instability and suggest that the regulated vector systems described here allow cells to be cultured continuously with negligible loss of Nef expression. Furthermore, the ability of these vectors to provide regulated expression allows detailed analysis of dose dependency and kinetics of Nef function(s). Consequently, these regulated vectors represent a significant advance from the use of constitutive and retroviral vectors for generating cell lines expressing Nef. It is also anticipated that both the tetracycline- and heavy metal-inducible vector systems will provide useful vehicles for achieving regulated expression of other cytotoxic proteins.

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References


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