Soluble Nef antigen of HIV-1 is cytotoxic for human CD4+ T cells

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Abstract We have previously shown that Nef-gene 10 fusion protein induces marked growth arrest of human primary CD4+ T cells. Here, in vitro cytostatic and cytotoxic activities of human immunodeficiency virus type 1 (HIV-1) Nef against CD4+ T cells were extensively investigated. Growth of human CD4+ cells was inhibited significantly just by the addition of purified full-length Nef to cultures. When Nef was cross-linked by anti-Nef antibodies, it became very cytocidal for CD4+ T cells. A high percentage of sera from HIV-1-infected individuals contained soluble Nef. Thus, soluble Nef in vivo may play an important role in immunodysfunction of CD4+ T lymphocytes in HIV-1 infection.

Key words: AIDS; HIV-1; Nef; CD4+ T cell; Cytotoxicity

1. Introduction

The human immunodeficiency virus type 1 (HIV-1) contains various accessory (*nef, vif, vpr* and *vpu*) [1] and regulatory (*tat* and *rev*) genes in addition to structural genes (*gag, pol* and *env*) which are common to all retroviruses [2–7]. One of the accessory genes designated *nef* is located at the 3′ end of the viral genome, partially overlapping the 3′ long terminal repeat (LTR) and conserved in virtually all strains of HIV-1, HIV-2, and simian immunodeficiency viruses (SIVs). Recent reports have demonstrated that Nef up-regulates viral replication both in tissue cultures [8] and in animal systems [9,10].

HIV-1 infection causes serious depletion of CD4+ T lymphocytes, that is essential for the development of AIDS. The basis for this drastic loss of T cells has not been elucidated as yet. It is unlikely that the observed depletion of T cells is a direct result of HIV-1 infection, since the cell population infected with the virus is so small [11–14]. Recently, several reports have indicated that HIV-1 gene products, such as Nef, Tat, and Env, can induce T cell death [15–18]. These findings endorse the contention that a certain signal of T cell death may be attributable to critical accumulation of HIV-1 proteins.

In this study, we demonstrate that the soluble form of Nef protein inhibits the proliferation of CD4+ T cells and that its cross-linking induces cytotoxic effects upon CD4+ T cells. We also show that soluble Nef was detected frequently in the sera from individuals infected with HIV-1.

2. Materials and methods

2.1. Cells

As CD4+ human T cell lines, Molt4 and Molt4 clone 8 were used. CD4+ and CD8+ T cells from peripheral blood mononuclear cells (PBMCs) were prepared by centrifugation on Ficoll-Hypaque, separation with nylon wool, and selective panning selection as described previously [16]. CD4+ T cells from lamina propria mononuclear cells (LPMCs) were a gift from Dr. M. Ito, Yamagata University School of Medicine. The T cells from PBMC and LPMC were cultured in Iscov's modified Dulbecco's medium (IMDM) containing 25 U/ml of rIL-2 and 10% heat-inactivated human AB serum (Gibco).

2.2. Antibodies (Abs) and sera

Mouse monoclonal antibodies (mAbs), OKT-4 (anti-CD4) and OKT-8 (anti-CD8), were purchased from Ortho Diagnostic Inc. Anti-Nef mAb (E7), for Nef antigen capture assay, was purified as described previously [16]. Hyperimmune anti-Nef serum was prepared by immunizing a specific pathogen-free rabbit with the purified Nef recombinant protein expressed by the baculovirus system as described previously [19]. HIV-1-seropositive sera were collected at the Sanjay Gandi Post-Graduate Institute for Medical Science Hospital in India as described previously [20].

2.3. Construction of the plasmids

The preparation of the cDNA encoding the entire Nef (Nef) (encoding nucleotides 8343–9192 of the HTLV-III genome) and N-terminal truncated Nef (t-Nef) (encoding nucleotides 8473–9192)-containing baculovectors has been described previously [19]. The Nef plasmids pGEM101 (Nef101) (encoding nucleotides 8473–9192) and pGEM123 (Nef123) (encoding nucleotides 8773–9192) were prepared as described previously [19]. The plasmid pGEM638 (Nef638) was constructed by cutting pGEM101 at the *Eco*RV-*Pvu*II sites (encoding nucleotides 8473–9192), end filling with the Klenow polymerase, and then re-ligation (Fig. 1).

2.4. Extraction and purification of recombinant Nef protein

Nef fusion proteins and gene 10 protein expressed in *E. coli*, and the truncated Nef protein expressed by the baculovirus system were purified as described previously [16]. After extraction with 7 M urea and dialysis against 20 mM Tris-HCl (pH 7.5), soluble proteins were added to a QAE Zetaprep disk. The fraction containing the Nef protein was further separated on a Sephadex G-200 chromatograph. Finally, the eluted Nef protein was purified on an Affi-Gel 10 column (Bio-Rad Lab., Richmond, CA) coupled to anti-Nef mAb. The entire Nef protein expressed by the baculovirus system was purified by the above procedures using buffer containing 0.1% Triton X. The Gag p55 protein expressed by the baculovirus system was extracted and purified as described previously [19]. The purity of each recombinant protein was determined by immunoblotting analysis and the purity of the isolated products was over 98%.

2.5. Proliferation assay

The level of [^{3}H]thymidine (TdR) incorporation was assayed as described previously [16]. Cells (1×10^5) were cultured with IMDM containing 10% heat-inactivated FBS or human AB serum in 96-well microplates (Becton Dickinson) in the presence of purified recombinant protein at a final concentration of 500 ng/ml for 3 days. The cells were pulse-labeled with 1 μCi of [^{3}H]TdR for 6 h before harvesting. The percentage inhibition of [^{3}H]TdR uptake by the cells was calculated as follows: percentage of inhibition = {1−(cpm of experimental group in the presence of recombinant protein/cpm of control group in the absence of recombinant protein)} × 100. The number of cpm of the control group in the absence of recombinant protein was 35 000 ± 1200.

2.6. Flow cytometry

Flow cytometric analysis was performed essentially as described previously [16]. Cells were incubated for 30 min on ice with FITC-

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conjugated OKT-4 and PE-conjugated OKT-8. After incubation and washing, cells were subjected to flow cytometric analysis by EPICS-XL.

2.7. Cytotoxic assay

Cells (1×10^5) were incubated for 1 h on ice with the purified Nef protein and control gene 10 protein in 20 μl of IMDM supplemented with 10% heat-inactivated FBS or human AB serum. After washing with the above medium, cells were cultured at 37°C in 0.1 ml of complete IMDM in 96-well plates previously coated at 4°C overnight with purified rabbit anti-Nef IgG (25 μg/ml). Cells were collected at day 3 or every day, and before cell harvesting at the end of the culture, propidium iodide (PI) (final concentration 5.0 μg/ml) was added to cultures. The uptake of the DNA intercalating dye into cells was analyzed by flow cytometry with a 590 long-pass glass filter. The percentage of specific cell death was calculated as follows: percentage of cell death = {experimental incorporation of PI (%)−spontaneous incorporation of PI (%)/100%−spontaneous incorporation of PI (%)}$\times$100. The average of the spontaneous PI uptake in control cultures was 5.1%.

2.8. Soluble Nef antigen assay

Soluble Nef antigen in sera was measured by sandwich-type capture ELISA. Wells of the microplate (Titertek Flow Lab., Irvine, UK) were coated with 100 μl of 1.0 μg/ml purified rabbit anti-Nef IgG overnight at 4°C. After incubation with blocking buffer (PBS containing 0.05% Tween 20 and 5% low-fat milk) at 37°C for 1 h, sample sera, culture supernatants from Molt4 clone 8 and purified entire Nef recombinant protein (as a standard) at 2-fold dilutions (100 μl) were added to wells in duplicate. The plates were incubated overnight at 4°C and then washed three times. After washing, 100 μl of 0.5 μg/ml purified anti-Nef mAb E7 were added and the plates were incubated at 37°C for 1 h. After washing, the reactive Abs were detected with a 100-fold dilution of biotin-labeled goat anti-mouse μ (Zymed Inc., South San Francisco, CA) for 1 h at room temperature. Finally, the plates were washed and incubated with streptavidin-conjugated alkaline phosphatase (1:100; Zymed) for 1 h at room temperature. This enzyme reaction was started by adding 0.25 mM *p*-nitrophenol phosphate. Absorbance was measured by ELISA reader at 405 nm and the concentration of soluble Nef protein in the samples was calculated by interpolation from the standard curve.

3. Results

3.1. Effect of Nef on the growth of T cells

We have previously shown that Nef-gene 10 fusion protein inhibits proliferation of CD4+ T lymphocytes from PBMC [16]. We extended this analysis using CD4+ T cells from PBMC and LPMC, and Molt4 T cell lines. After addition of the entire Nef protein expressed by the baculovirus system (Fig. 1), growth of CD4+ cells of various origins was monitored (Fig. 2). The cell growth of CD4+ T cells from PBMC and LPMC at day 3 was inhibited in the presence of Nef protein but not Gag p55 control protein. Similar results

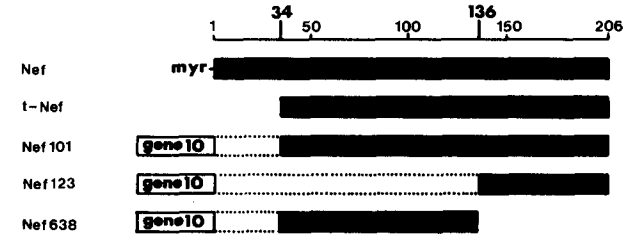

Fig. 1. Structures of various Nef proteins used in this study. The entire Nef (Nef) and N-terminal truncated Nef (t-Nef) were expressed by the baculovirus system. The truncated Nef-gene 10 fusion proteins (Nef101, Nef123 and Nef638) were expressed by *E. coli*. Each recombinant protein was purified as described in Section 2. Numbers at the top represent the amino acid residues of Nef.

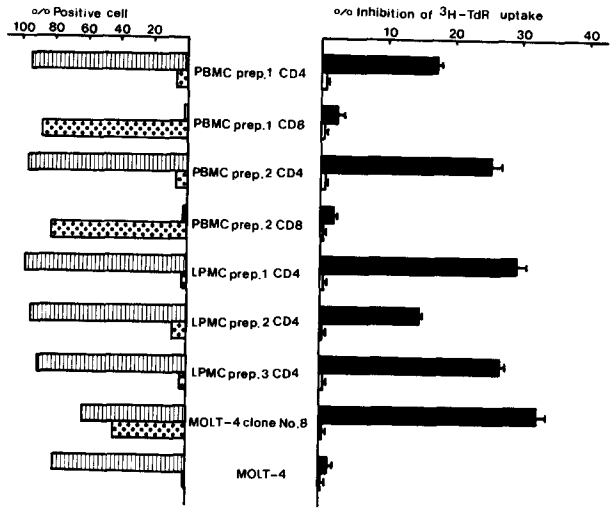

Fig. 2. Surface phenotype of T cells and suppression of CD4+ T cell proliferation by Nef. (Left) Phenotype of T cells from PBMC, LPMC and T cell lines was analyzed by flow cytometry with FITC-OKT-4 (hatched bars) and PE-OKT-8 (dotted bars). Percentages of positive cells were determined. (Right) Cells (1×10^5) were cultured for 3 days in complete medium containing entire Nef p27 (black) or Gag p55 (white) expressed by the baculosystem at a final concentration of 0.5 μg/ml. Before 6 h of the end of culture, cells were pulse labeled with [^{3}H]TdR. Percentage inhibition of [^{3}H]TdR uptake was calculated as described in Section 2.

were obtained with Molt4 clone 8 cells. In contrast, CD8+ T cells from PBMC, and Molt4 cells, which do not bind to the carboxyl-terminal domain of Nef [19], did not show a significant decrease in [^{3}H]TdR uptake. The growth inhibition of cells in the presence of Nef was dose-dependent and this effect was noted at a minimal concentration of 5 ng/ml of Nef (data not shown).

3.2. Cytotoxic effect of Nef on T cells

It is well known that cross-linkage of CD3 receptor on the cell surface causes activation of T cells as well as induction of T cell death. Recently, it has been reported that Nef-CD8 fusion protein expressed on the cell surface induces T cell death [15]. We, therefore, examined the cytotoxic activity of Nef cross-linked by anti-Nef Abs (Fig. 3). Death of CD4+ T cells from PBMC, as measured by incorporation of PI, by entire Nef and t-Nef was clearly observed at final concentrations of 10 ng/ml to 1 μg/ml at day 3 (Fig. 3A,B). Similar results were obtained in Molt4 clone 8 cells (Fig. 3C,D). Among various versions of Nef-gene 10 proteins (Fig. 1), Nef 101 and 123 fusion proteins were cytotoxic for CD4+ T cells from PBMC and Molt4 clone 8 cells, but Nef 638 protein were not at all (Fig. 3). The difference in cytotoxicity between t-Nef and Nef101 could be attributable to the chimeric structure of Nef101 (gene 10 protein and Nef). Cytotoxic activity of entire Nef protein was found in CD4+ T cells from LPMC, but not in Molt4 cells (data not shown). In addition, anti-Nef mAb E7, which recognizes Nef epitope located at amino acid residues 192–206 [19], blocked the cytotoxicity by Nef (data not shown).

3.3. Detection of soluble Nef antigen in sera

The level of soluble Nef in sera of HIV-1-infected individuals was monitored by antigen capture ELISA (Fig. 4). As control, culture supernatants of HIV-1-infected Molt4 clone 8

cells were used. The culture supernatants contained 0.5–5 ng/ml of soluble Nef antigen. 21 sera out of 32 of HIV-1 seropositive sera contained Nef at a concentration of 5–10 ng/ml, but Nef was not detected in 5 sera. These 5 sera contained high titers of anti-Nef antibodies (over 1:5000 by ELISA), whereas low levels of anti-Nef antibodies were found in the other sera [16]. In 28 control sera from healthy volunteers, soluble Nef antigen could not be found at all. These data indicate that effective Nef protein concentrations for T cell death could be reached in vivo.

4. Discussion

In this paper, we have described the cytostatic and cytolytic activities of soluble Nef antigen against CD4+ T cells. For cytotoxic activity of soluble Nef, it is essential that Nef binds to the CD4+ T cell surface and is cross-linked by anti-Nef Abs. We have previously found that the carboxyl-terminal region of Nef binds to CD4+ T cells derived from PBMC [19]. The carboxyl-terminal domain of Nef was also required for cytotoxic reaction against CD4+ T cells (Fig. 3). Although N-terminal truncated Nef (t-Nef) showed cytotoxic activity similar to that by entire Nef, Nef-gene 10 fusion proteins

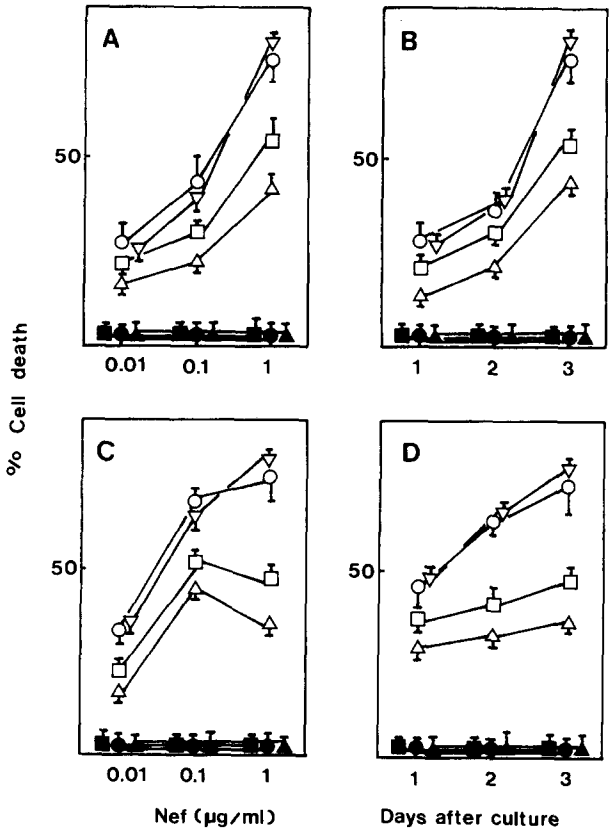

Fig. 3. Cytotoxic effect of Nef. CD4+ T cells from PBMC (A,B) and Molt4 clone 8 cells (C and D) were incubated for 1 h on ice with entire Nef (○), t-Nef (▽), Nef101 (□), Nef123 (△), Nef 638 (●), gene 10 (■) at the concentrations indicated. After incubation, cells were cultured for 3 days in the culture plates pre-coated with anti-Nef Ab (A,C). For kinetics, cells were incubated with 1 μg/ml of recombinant proteins, and cultured as indicated in the plates pre-coated with anti-Nef Ab (B,D). Before harvesting cells, PI was added and cells were analyzed by flow cytometry. Percentage of specific cell death was calculated as described in Section 2. (▲) Control cultures in plates pre-coated with anti-Nef Ab.

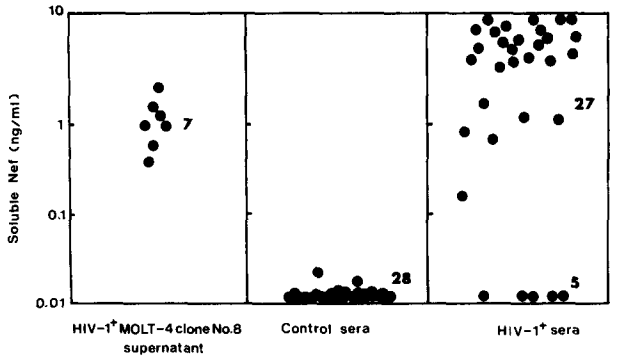

Fig. 4. Detection of soluble Nef. Supernatants of Molt4 clone 8 cells infected with HIV-1 (left), sera from healthy volunteers (center) and sera from individuals infected with HIV-1 (right) were examined for the presence of soluble Nef by antigen capture ELISA as described in Section 2. Recombinant entire Nef added to a control serum was used for the standard curve.

lacking the N-terminal portion of Nef (Nef101 and Nef123) were less effective (Fig. 3). These results suggest that for effective cytotoxicity for CD4+ T cells, not only the C-terminus but also the N-terminus of Nef is needed. However, since none of the synthetic peptides tested induced the cytotoxic activity, it is likely that a certain conformational structure of the Nef is important for this reaction.

We monitored the level of soluble Nef in sera of HIV-1-seropositive individuals (Fig. 4). A relatively high level of Nef, enough for cytotoxicity in vitro, was detected in many sera examined. As Nef was cytotoxic not only for PBMC-T but also for LPMC-T cells, it is conceivable that soluble Nef affects a process of specific loss of CD4+ T cells in HIV-1 positive individuals. Whether Nef contributes much to selective killing of T cells in HIV-1 infection needs to be determined.

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