Human immunodeficiency virus type 1 Nef protein on the cell surface is cytocidal for human CD4+ T cells

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Abstract We have previously shown that the carboxyl-terminal region of human immunodeficiency virus type 1 (HIV-1) Nef antigen present on the outer surface of virus-infected cells has affinity for uninfected T cells. Here, the in vitro cytotoxic potential of HIV-1 Nef on the T cell surface against CD4+ T cells was investigated in detail. Human T cells expressing Nef on the cell surface by transfection with non-infectious mutant HIV-1 proviruses were demonstrated to kill CD4+ T cells effeciently. Furthermore, it was shown that the carboxyl-terminal portion of Nef was cytotoxic for CD4+ T cells and that monoclonal antibody against the carboxyl-terminal region of Nef inhibited Nef induced-cytolysis. Thus, we concluded that Nef protein on CD4+ T cells may play an important role in the specific loss of CD4+ T lymphocytes during HIV-1 infection.

Key words: HIV-1; Nef; Cytotoxicity

1. Introduction

The human immunodeficiency virus type 1 (HIV-1) is a complex retrovirus that contains several auxiliary (*nef, vif, vpr,* and *vpu*) [1,2] and two regulatory (*tat* and *rev*) genes in addition to the structural genes common to all retroviruses (*gag, pol,* and *env*) [3–8]. Nef is encoded by an open reading frame located at the 3′ end of the viral genome, partially overlapping the 3′ long terminal repeat (LTR). Conservation of this coding region among all strains of HIV-1, HIV-2 as well as simian immunodeficiency viruses (SIV) suggests that Nef plays an important role in natural infection. The role of HIV-1 Nef in the virus life cycle in vitro has been controversial. Earlier reports described that Nef reduces the rate of HIV-1 replication and represses the HIV-1 LTR promoter activity [9–11]. However, these results were not confirmed by other investigators [12–14]. Recent studies demonstrated that Nef has a positive effect upon HIV-1 replication in T cells and acts at a very early stage of the replication cycle [14–18]. Consistent with this Nef-enhanced virion infectivity in vitro, Nef has been shown to be essential for efficient viral replication in SCID-Hu mice in vivo [19]. Furthermore, Nef from a macaque strain of SIV, SIVmac239, is required for efficient virus replication and for induction of immunosuppressive disorders in rhesus monkeys [20]. Apart from its functional role in virus replication, several biologically important activities of HIV-1 Nef, such as the down-modulation of CD4 expression, have been documented [21].

In this study, we demonstrated that the carboxyl-terminal domain of HIV-1 Nef on the cell surface specifically induces cytolysis of the CD4+ T cells.

2. Materials and methods

2.1. Cells

Human CD4+ T cell lines, Molt4 and Molt4 clone 8, were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 100 μg/ml of streptomycin and 100 IU/ml of penicillin.

2.2. Antibodies

Three murine monoclonal antibodies (mAb) to Nef, designated F1, E7, and E9, were used and the epitopes recognized by F1, E7, and E9 covered the amino acid sequences 148–157, 192–206, and 158–206 of Nef [22]. The mAbs to HIV-1 Env gp120 and Gag p24 were purchased from Repligen and Cellular Products Inc. The OKT4 conjugated with FITC, and OKT8 conjugated with PE were purchased from Ortho Diagnostic System.

2.3. Plasmid construction and protein expression

The mutant plasmid DNAs of HIV-1, designated pNL-Ac, pNL-Ps1, pNL-Ps2, pNL-Bl, pNL-Af1, pNL-ΔBM, pNL-Kp, pNL-St, pNL-Hi, and pNL-Xh have been previously described [23]. Plasmids, pLRNL (MuLV-based plasmid), pL001+ and pL001− also have been described previously [24]. The *Bam*HI fragments of sCD4 in pUC119 were cut with *Rsa*I, and the *Xho*I–*Hin*dIII fragments of *nef* in pCV-1 were inserted into the digested plasmid (pUCs-nef). Plasmid pL101 was constructed by ligating the *Bam*HI fragments of pUCs-nef plasmid into *Bam*HI site of pLRNL plasmid. Plasmid pL212, pL334, and pL453 were constructed by cutting at the *Xho*I–*Bgl*II (at nucleotide 8627), -*Eco*RV (at nucleotide 8694) and -*Eco*RV (at nucleotide 8773) sites of pL101 plasmid, end filling with the Klenow polymerase, and religation. The pL598 plasmid was constructed by cutting pL101 at *Eco*RV–*PVU*II sites, end filling with the Klenow polymerase, and then religation to generate a new stop codon 301 bp downstream of the *Xho*I site of the nef fragment (Fig. 4).

Molt4 cells (5×10^6) were transfected with HIV-1 mutant DNA or MuLV-based plasmid DNA (10 μg) mixed with 5 μl of lipofectin [25] and were cultured for 30 h at 37°C in 0.5 ml of RPMI-1640 medium containing 10% Nuserum IV (Becton Dickinson) in a 24-well culture dish. After the culture, 1 ml of FBS was overlaid with 0.5 ml of the harvested cell suspension and cells were centrifuged at $200\times g$ for 10 min. The supernatant containing dead cells was removed. The cells were washed 3 times and were used for cytotoxic assay.

2.4. Flow cytometry

Flow cytometry was performed essentially as described previously [22].

2.5. Cytotoxic assay

Cytolytic death of T cells was determined by the method of Slezak and Horan [26]. Cell surface of Molt4 clone 8 cells ($1–8\times10^6$) was labeled with a fluorechrome, PKH-26 (Zynaxis Cell Science Inc.). Effector cells (1×10^6) expressing recombinant HIV-1 proteins were prepared as described above. For assay of cytotoxicity inhibiting activity of mAbs to Nef, 2-fold dilutions of the mAbs were added to both cell mixture during the co-culture. The cells harvested were stained with 1×10^{-6} M propidium iodide (PI) and analyzed by flow cytometry. The effector cells were gated out by FS and SS,

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and incorporation of PI into PKH-26-labeled target cells was analyzed with use of two colored histograms. Filters used to select for different parameters were: PKH-26, 525 band pass filter; PI, 590 long-pass glass filter. The percentage cytotoxicity was calculated by the equation:

$$\%\text{lysis} = (\text{quadrant [Q]2/Q2} + \text{Q4 specific} - \text{Q2/Q2} + \text{Q4 spontaneous}) \times 100.$$

The Q4 presented as cells which are positive only for PKH-26 fluorescence and the Q2 contained dead target cells which are positive for PKH-26 and PI staining. The values of Q2 and Q4 'specific' were determined by the number of events in each sample. The Q2 and Q4 'spontaneous' were determined by the number of events in a control sample containing only target cells.

3. Results

3.1. Cytotoxicity of human T cells transfected with non-infectious HIV-1 mutants against CD4+ T cells

We examined whether human T cells expressing HIV-1 Nef on the cell surface kill CD4+ T cells. Non-infectious HIV-1 mutants with intact *nef* and various defects in the other genes (Fig. 1) were transfected into Molt4 cells as effector cells. Effector cells that were transfected with *gag* mutants (pNL-Ac and pNL-Ps1), *pol* mutants (pNL-Ps2, pNL-Bl and pNL-Af1), or *env* mutants (pNL-Kp, pNL-St and pNL-Hi) effectively killed CD4+ target cells within 8 h (Fig. 2). In contrast, effector cells transfected with *tat* mutant pNL-ΔBM did not show significant cytotoxicity to the target cells. In addition, cells transfected with the infectious mutant pNL-Xh carrying the inactive *nef* gene did not kill Molt4 clone 8 cells within 8 h. After 12 h of co-culture, however, 24.9 ± 0.5% of target cells died probably by the effect of Env.

To investigate whether the fluctuation of cytotoxic efficiency among the effector cells was due to the difference in the amount of Nef expressed on the cell surface, we examined the level of the effector cells for cell surface Nef by membrane immunofluorescence at the same time of cytotoxic assay (Fig. 3). Cell surfaces of Molt4 cells transfected with pNL-Ac, -Ps1, -Ps2, -Bl, -Af1, -Kp, -St, or -Hi were similarly stained with E7, strongly with E9, faintly with F1, and not at all with IgM control. The surface of cells transfected with pNL-Ac, -Ps1, -Ps2, -Bl or -Af1 were also stained with anti-Env mAb. Cells transfected with pNL-Ps2, pNL-Bl or pNL-Af1 produced Gag p24 protein that was observed by immunoblotting with anti-Gag p24 mAb (data not shown). However, when transfected with pNL-ΔBM, the cell surface was not stained with any of the mAbs and Gag p24 was not observed at all in the cells. None of pNL-Kp, -St or -Hi-transfected

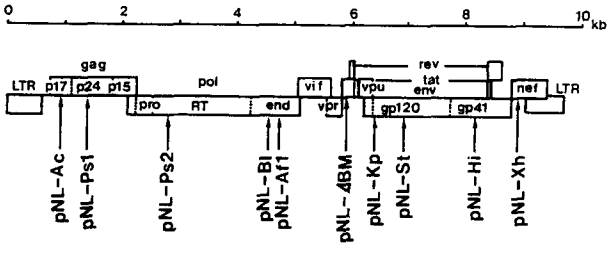

Fig. 1. Schematic representation of HIV-1 mutants used in this study. The sites of mutation within proviral genome of HIV-1 are indicated by arrows. Designations of the mutants are shown at the bottom. pro, protease; RT, reverse transcriptase; end, endonuclease.

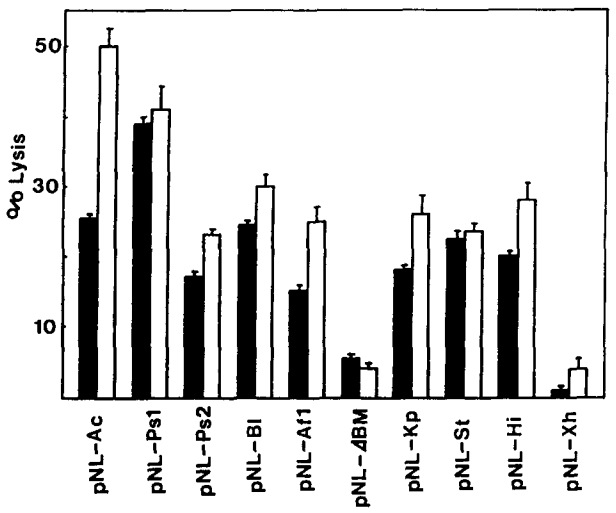

Fig. 2. Cytotoxic activity of Molt4 cells transfected with HIV-1 mutants against Molt4 clone 8 cells. Molt4 cells were transfected with HIV-1 mutants, and after 30 h, cytotoxicity was determined at 8 h post-co-culture at the effector to target Molt4 clone 8 cell ratio of 1:1 (□) and 5:1 (■). Percent lysis was determined by flow cytometric analysis. Data are expressed as the mean ± SE of three separate experiments.

cells were stained with anti-Env mAb but Gag p24 was found in the cells. These results showed that cell surface expression of the carboxyl-terminal domain of Nef, recognized by E7 and E9, was responsible for cytotoxicity to CD4+ T cells.

3.2. Cytotoxicity of carboxyl-terminal region of HIV-1 Nef to CD4+ cells

Plasmids, which can express full-length Nef or truncated Nef, were constructed (Fig. 4) and the surface expression of the full-length and truncated Nef forms in Molt4 cells was examined by flow cytometry with anti-Nef mAbs (Fig. 5). When pL001+ expressing full-length Nef (amino acid residues 1–206) and pL598 (aa 34–136) with a deletion in the carboxyl-terminal region were examined, transfected cells did not react with any anti-Nef mAbs. We previously observed that, without co-expression of Gag or Env, the full-length Nef was not expressed on the surface of cells (manuscript submitted). On the contrary, cells transfected with pL101 (aa 34–206), 212 (aa 84–206), 334 (aa 108–206), or 453 (aa 136–206), all of which can express signal peptide, were found to react with E7 and E9 strongly. F1 mAb stained the surface of cells transfected with pL101, 212, or 334, but did not react to cells transfected with pL453. These results, therefore, indicate that the carboxyl-terminus of Nef was expressed on Molt4 cells transfected with pL101, 212, 334 or 453.

Next, the carboxyl-terminus of Nef expressed on T cells was examined for cytotoxic effects against Molt4 clone 8 cells. As presented in Fig. 6, Molt4 clone 8 cells were effectively lysed by effector cells transfected with pL101, 212, 334, or 453. On the other hand, significant cell killing was not observed using effector T cells transfected with pLRNL, 001−, 001+, or 598. Anti-Nef mAb E7, which recognizes the carboxyl-terminal region of Nef, inhibited the cytotoxic activity of cells transfected with pL101 but control normal mouse IgM did not. These data also suggest that the C-terminus of Nef protein, especially amono acid residues 136–206, is important for

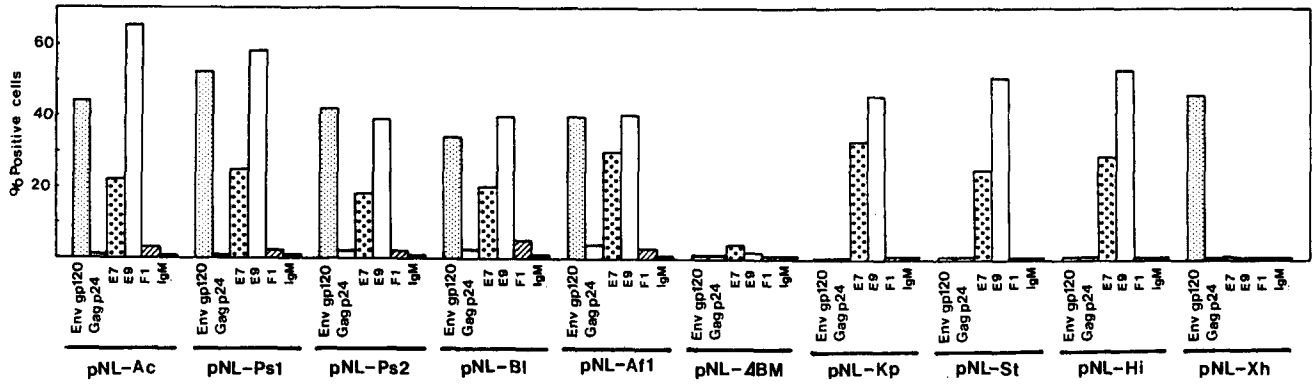

Fig. 3. Surface expression of Nef protein on CD4+ T cells. HIV-1 mutants were transfected into Molt4 cells and surface levels of Env, Gag, and Nef antigen were determined 30 h later by flow cytometric analysis. Cells were stained with anti-gp120 Env mAb, anti-p24 Gag mAb, anti-Nef mAbs (E7, E9, and F1), and normal mouse IgM. Antigenic epitopes recognized by each anti-Nef mAb were described in Section 2.

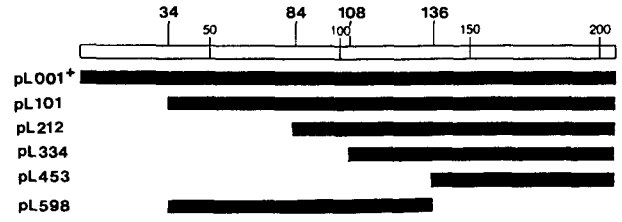

Fig. 4. Structures of MuLV-based plasmids encoding full-length myristoylated Nef (pL001) and signal peptides-fused, truncated version of Nef (pL101, 212, 334, 453, 598). Nef expressing plasmids were constructed as described in Section 2. The numbers on the top indicate the amino acid residues of Nef.

CD4+ T cell killing. The cell death observed here was apoptosis as judged by morphology (data not shown).

4. Discussion

Our present study showed that human T cells expressing Nef on the surface specifically caused cytolysis of the unifected cells (Figs. 2 and 6). We further examined the cytolytic activity of Nef using various cells. While CD4+ T cells prepared from peripheral blood mononuclear cells (PBMC) were lysed effectively by this cell surface Nef, CD8+ T cells from PBMCs and some of CD4+ T cell lines were not killed (manuscript submitted). Inhibition of the cytotoxic activity of effector cells by anti-Nef mAbs recognizing the carboxyl-terminal domain and retainment of cytotoxic activity of truncated Nef lacking amino-terminal region clearly show an important role of the carboxyl terminus of Nef for the cytotoxicity against CD4+ T cells. Soluble Nef, which lacks amino-terminal domain, did bind to uninfected CD4+ T cells and induced cell lysis upon cross-linking by anti-Nef antibodies (our unpublished results). It seems likely that the carboxyl-terminal structure (aa 136–206) of Nef is solely responsible for the cytotoxicity and Nef binding protein may present on certain population of CD4+ T cells. It has been shown that among HIV-1 strains isolated from long-term survivors of HIV-1-infected individuals, two stretches in the Nef are variable, one of which is located between amino acids 189 and 200 [27]. Deacon and his colleages [28] have reported that HIV-1 *nef* gene deletions from long-term survivors are concentrated on 3′ LTR U3 region corresponding to the carboxyl-terminal region of Nef. Since this position is overlapping the putative cytotoxic domain of Nef, mutations or truncations in this region might weaken the cytotoxic potential of Nef for uninfected CD4+ T cells.

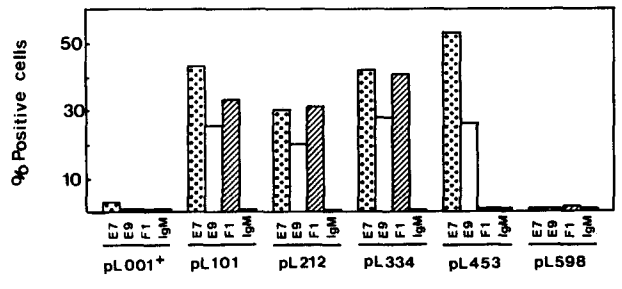

Fig. 5. Cell surface expression of truncated Nef version. Molt4 cells were transfected with MuLV-based plasmids (Fig. 4), cultured for 30 h, and the expression levels of C-terminus of Nef on the cell surface were determined by flow cytometric analysis using anti-Nef mAbs (E7, E9, and F1), and normal mouse IgM.

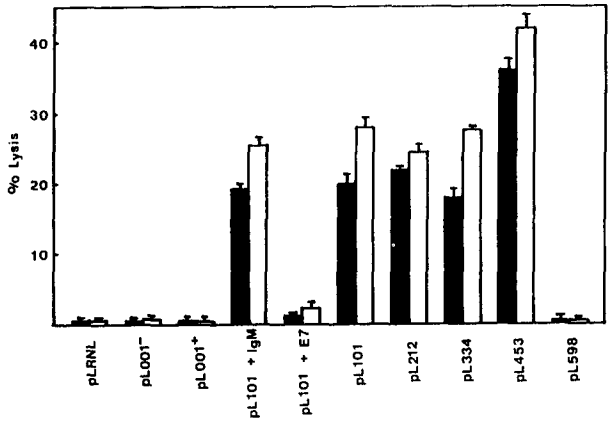

Fig. 6. Cytotoxicity of T cells expressing wild-type and signal sequence-bearing version of Nef. Molt4 T cells were transfected with wild-type or signal peptide-bearing Nef expression plasmids. Viable cells were adjusted at the beginning of cytotoxic assay, and transfected cells were co-cultured with Molt4 clone 8 target cells for 8 h at the effector to target ratio of 1:1 (□) and 5:1 (■). Cytolysis was determined by flow cytometric analysis. For inhibition of cytotoxic activity, anti-Nef mAb E7 (10 μg/ml) was mixed with the co-culture just before the start of assay. Normal mouse control IgM (100 μg/ml) was used as negative control.

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