Clustered localization of oligomeric Nef protein of human immunodeficiency virus type 1 on the cell surface

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Received 24 July 1996; revised version received 10 September 1996

Abstract We studied human immunodeficiency virus type 1 (HIV-1) Nef protein biochemically and histologically. HIV-1 Nef, derived from baculosystem and from cells infected with HIV-1, formed homomeric monomers, dimers, trimers, and further polymers. These oligomers were non-covalently associated. In cells infected with HIV-1, Nef molecules were clustered at the cell surface as well as cytoplasm. Our previous results have indicated that the Nef on the surface of cells infected with HIV-1 is cytotoxic against uninfected CD4+ T cells. Thus, it is very likely that the HIV-1-mediated cytotoxic reaction is due, at least in part, to the clustered localization of oligomeric Nef on the cell surface.

Key words: Human immunodeficiency virus type 1; Nef; Env

1. Introduction

The human immunodeficiency virus type 1 (HIV-1) *nef* gene, an accessory gene [1,2], is conserved among all strains of HIV-1, HIV-2 as well as simian immunodeficiency viruses (SIVs). This coding region is located at the 3′ end of HIV-1 genome, partially overlapping the 3′ long terminal repeat (LTR). Two types of Nef are known to be present, namely a myristoylated 27 kDa and an unmyristoylated 25 kDa form. Myristoylation is necessary for the association of Nef with the plasma membrane, whereas unmyristoylated Nef is present predominantly in the cytoplasmic cell fraction [3]. In tissue culture systems, HIV-1 Nef has been reported to have a positive effect upon HIV-1 replication [4,5] and to down-regulate cell-surface expression of CD4 receptors [6,7]. In vivo, Nef is required for efficient virus replication and for induction of immunosuppressive disorders in rhesus monkeys [8]. Nef protein is necessary for efficient viral replication also in SCID-Hu mice [9]. These reports suggest that Nef plays an important role in HIV-1 infection and AIDS pathogenesis. We have recently demonstrated that the carboxyl-terminal domain of HIV-1 Nef is expressed on the HIV-1-infected T cell surface and that this region has an affinity for uninfected CD4+ T cells [10]. In addition, we have shown that Nef is critical, at least in part, for cytolysis of CD4+ T cells (our unpublished results).

One report has shown that Nef molecules expressed by bacterial and eukaryotic systems form oligomers [11], but there is no information about the oligomerization of Nef in HIV-1-infected T cells. To clarify the mechanism of Nef-dependent cell killing, we studied the biochemical and histological properties of Nef. In this report we show that the Nef molecules form oligomers and cluster on the surface of HIV-1-infected CD4+ cells.

2. Materials and methods

2.1. Cells and antibodies

HeLa CD4+ cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum. The anti-Nef monoclonal antibody (mAb), E7, was purified as described previously [10]. The mAbs to HIV-1 Env gp120, and Gag p24 were purchased from Repligen Inc. 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 [10].

2.2. Purification of recombinant Nef protein

The entire Nef of HIV-1 NL43 was expressed in insect cells infected with the Acnef-0 baculorecombinant virus [11] and finally purified with anti-Nef mAb-conjugated Affi-Gel column (unpublished). The purified Nef (5 μg) in 5 μl of 100 mM Tris-HCl (pH 7.5) containing 50 mM NaCl was incubated at 37°C for 1 h in the presence or absence of 2 M urea. Then a non-treated sample and treated samples were applied on Superose 6 HR 10/30 for high performance liquid chromatography (HPLC).

2.3. Immunoprecipitation analysis of Nef protein

About 1×10^7 HeLa CD4 cells were absorbed with cell-free HIV-1 at an m.o.i. of approximately 1.0 per cell and were cultured for 4 days. After culture, the cells were harvested and washed with PBS three times, and resuspended in 300 μl of lysis buffer (0.05% NP-40, 150 mM NaCl, 0.1 mM PMSF in 100 mM Tris-HCl), and then incubated on ice for 10 min. Cell lysate was centrifuged at $12000 \times g$ for 10 min and the extract was recovered. The lysate was incubated with anti-Nef mAb, E7 or anti-purified Nef rabbit coupled with Affi-Gel 10 (50 μl of a 50% slurry) for 2 h on ice. After washing the gel, 20 μl of 0.1 M citrate-phosphate buffer (pH 2.5) was added to the beads, centrifuged, and the supernatant was stored. Aliquots (20 μl) of the eluted material were analyzed by SDS-PAGE, and separated material was stained with immunoblotting by anti-Nef rabbit IgG as described previously [10].

2.4. Indirect membrane immunofluorescence assay

Cells were incubated with appropriately diluted murine mAbs (5 μg/10 μl) for 30 min on ice. After washing with PBS containing 0.5% BSA, cells were incubated for 30 min on ice with FITC-conjugated anti-mouse μ and γ (Cappel).

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PII S0014-5793(96)01048-4

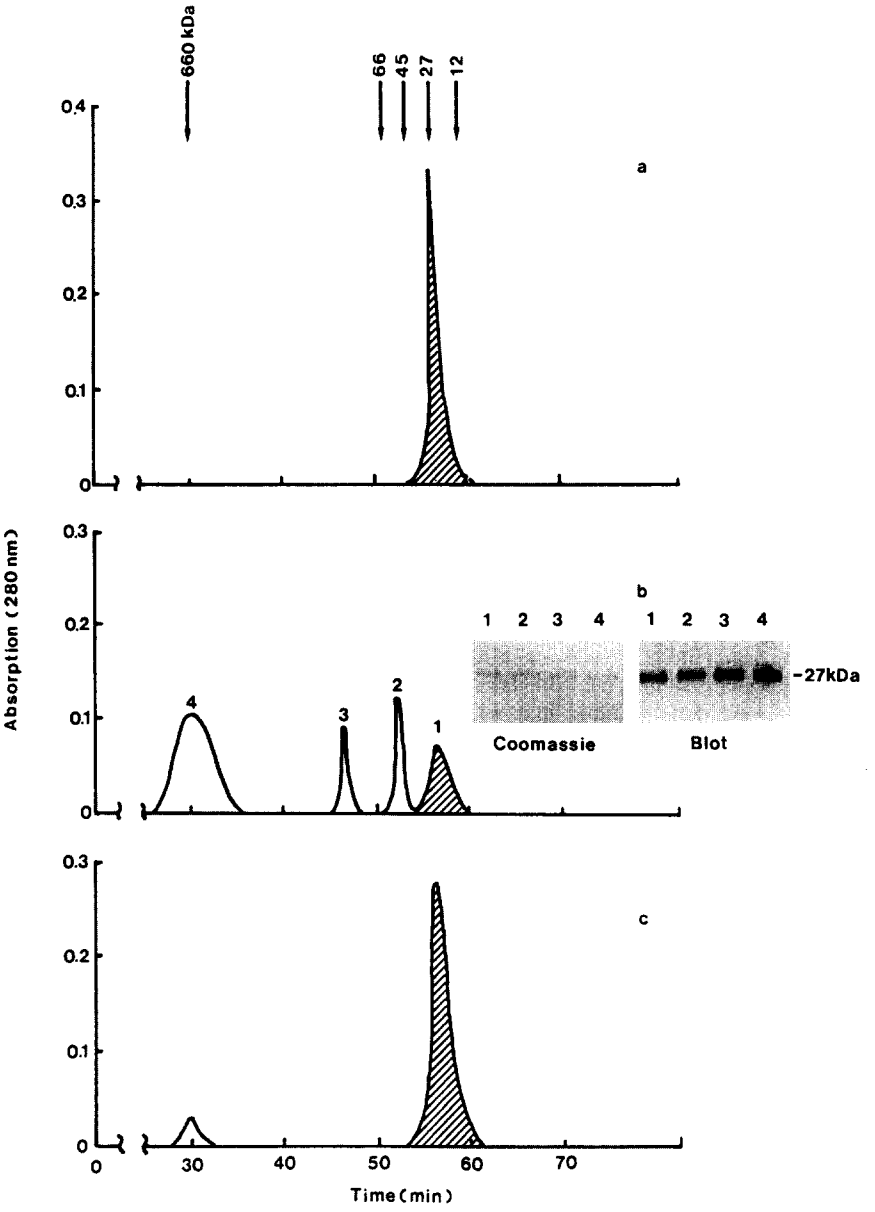

Fig. 1. Oligomerizing profile in Superose 6 chromatography of recombinant Nef protein. Purified recombinant Nef protein derived from baculosystem was applied to HPLC. Non-treated (a), pre-incubated (b) and urea-treated (c) Nef samples were separated by Superose gel filtration. The hatched peaks correspond to a molecular mass of 27 kDa. Buffer, 0.15 M NaCl-0.05 M Tris-HCl (pH 7.0). Flow rate, 0.4 ml/min. Marker proteins: thyroglobulin, bovine serum albumin, ovalbumin, and cytochrome *c* in molecular weight order. In (b), results obtained by SDS-PAGE analysis (with 2 M urea under reducing conditions) of samples from peaks 1, 2, 3, and 4 are also presented (Coomassie blue staining and immunoblot).

2.5. Electron microscopy

HIV-1-infected and uninfected cells were cultured for 48 h, then harvested. After washing, cells were treated with 0.05% Triton X-100 on ice for 10 min. Either treated or untreated cells were washed and were incubated with mAbs (5 μg/10 μl) for 30 min on ice. Cells were washed with PBS containing 0.1% BSA, and incubated for 30 min on ice with goat anti-mouse 10-nm gold conjugated Ab (E-Y Lab.) at a dilution of 1 in 50. After rinsing with PBS, cells were fixed in 2% glutaraldehyde followed by 2% osmium tetroxide, using 0.1 M phosphate buffer (pH 7.2) as described previously [12]. For scanning electron microscopy, infected or uninfected cells were labeled on ice with primary mAb, washed, stained with colloidal gold-conjugated secondary Abs, and fixed as mentioned above. Samples were assigned a code number and prepared for ultrastructural analysis of antigen distribution using a Hitachi S-9000 LVSEM.

3. Results

3.1. Oligomerization of HIV-1 Nef

Nef expressed by the baculovirus system was purified and applied to HPLC under non-reducing condition (Fig. 1). One peak corresponding to a molecular mass of 27 kDa was found with the sample on ice just before the application (Fig. 1a), but after incubation at 37°C for 1 h, three more peaks (approximately 55, 80 and 660 kDa) appeared (Fig. 1b). When further incubated at 37°C for 1 h in the presence of 2 M urea, 55 and 80 kDa peaks disappeared and the 27 kDa peak occupied about 95% of the whole protein content (Fig. 1c). To determine whether oligomerization of Nef occurs in cells in-

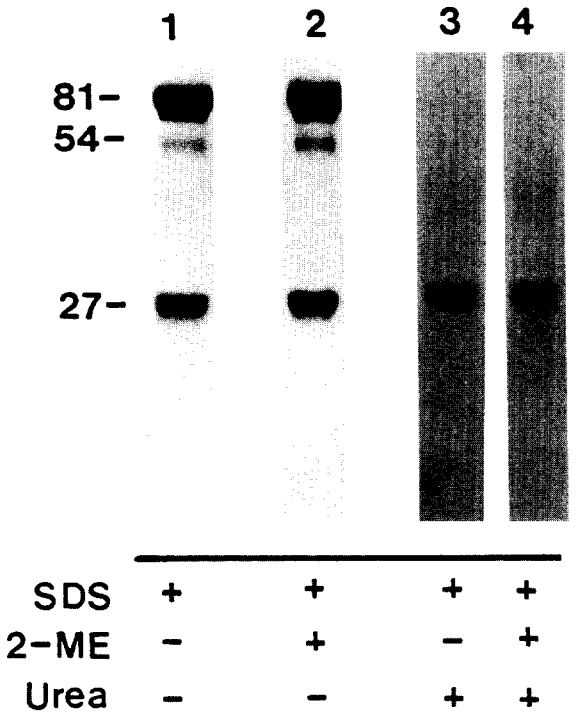

Fig. 2. Oligomerization of Nef protein in immunoprecipitation. Nef protein was precipitated with immunobeads from lysates of HIV-1-infected HeLa CD4 cells as described in Section 2. Precipitates were subjected to SDS-PAGE under reducing and non-reducing conditions and the proteins were immunostained by blotting. Using 2 M urea, monomerization of Nef protein was observed in lanes 3 and 4. ←

fected with HIV-1, cell lysates of HIV-1-infected HeLa CD4 were precipitated with anti-Nef immunobeads and subsequently analyzed by immunoblotting (Fig. 2). As shown in Fig. 2, the 27, 54 and 81 kDa Nef protein bands were observed under non-reducing (lane 1) and reducing condition (lane 2). Addition of 2 M urea resulted in the disappearance of 54 and 81 kDa bands (lanes 3 and 4). We used another reducing reagent dithiothreitol and then iodoacetamide for these experiments, but significant reducing effects could not be observed (data not shown). These results suggested that HIV-1 Nef protein forms oligomer without covalent disulfide bonds.

3.2. Clustered localization of HIV-1 Nef in infected cells

We previously showed that carboxyl-terminal portion of Nef is expressed at the surface of HIV-1-infected lymphocytes [10,13]. Using mAb E7 and membrane immunofluorescence, we compared the surface expression of Nef and Env proteins in Hela-CD4 cells to investigate oligomerization of Nef protein in HIV-1-infected cells morphologically (Fig. 3). Anti-Env

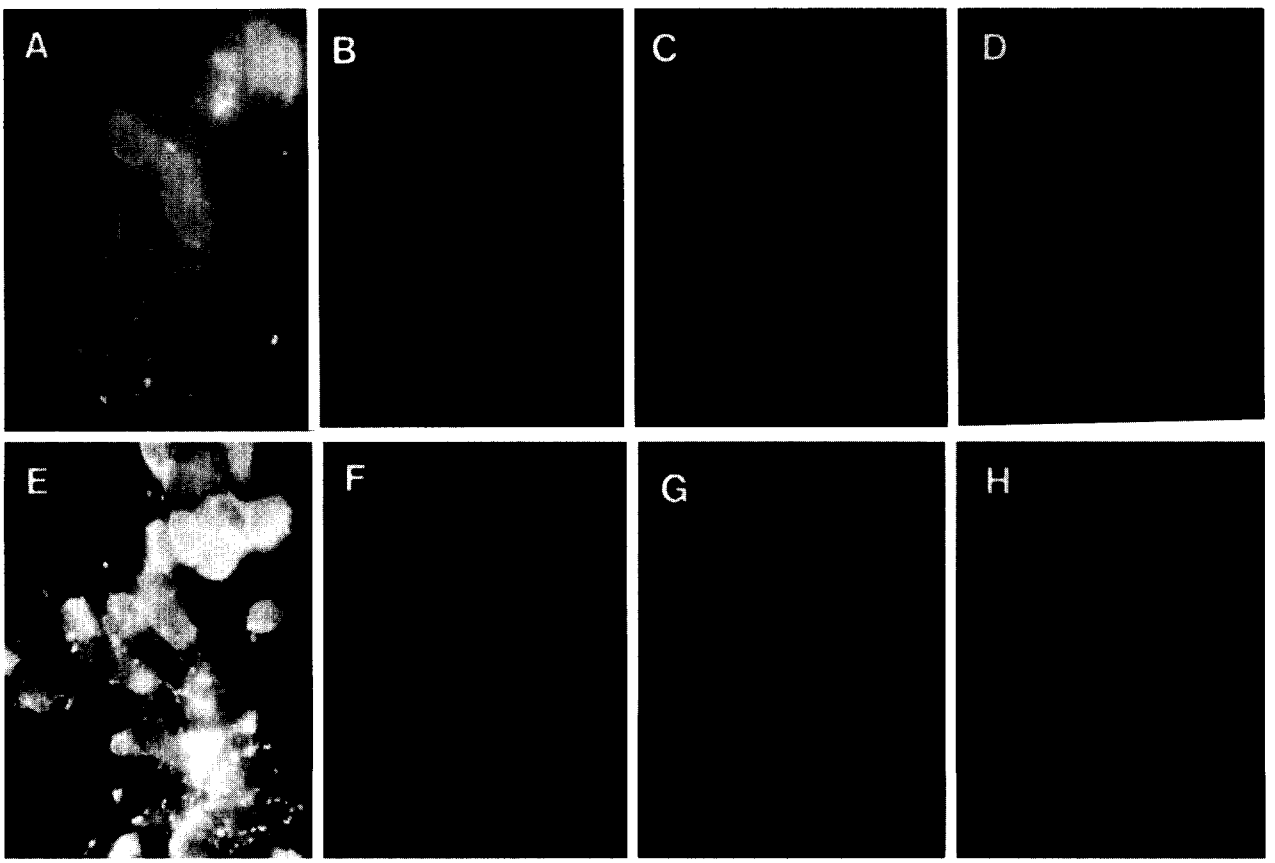

Fig. 3. Cell surface expression of Nef and Env proteins on HIV-1-infected cells visualized by membrane immunofluorescence. HeLa CD4 cells infected with HIV-1 (A, B, E and F) were stained with anti-Env gp120 mAb (A), anti-Nef mAb E7 (E), control mouse IgG (B) or control mouse IgM (F). As control, uninfected cells (C, D, G and H) were stained with anti-Env (C), anti-Nef (G), control mouse IgG (D) or control mouse IgM (H). Magnification: ×200.

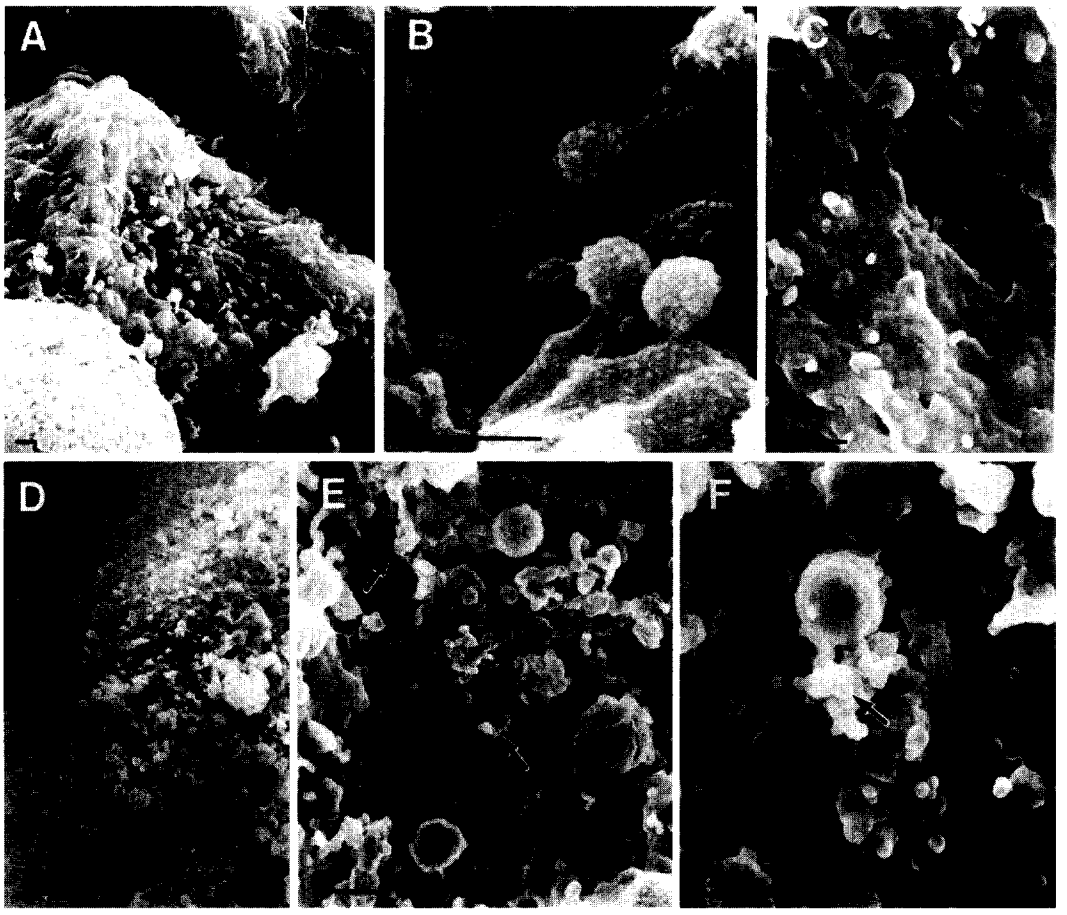

Fig. 4. Cell surface expression of Nef protein monitored by LVSEM electron microscopy. Numerous HIV-1 particles are typically found on infected HeLa CD4 cell line (A, B, D, E and F), but not on uninfected cell (C). At higher magnification, immunogold distribution is evident on cells expressing Env gp120 (A, B) and Nef (D–F). Note clusters of colloidal gold (typical examples are indicated by arrows in E and F) and non-clustered localization (B). Bars: 200 nm.

mAb stained HIV-1-infected cells entirely (Fig. 3A), but did not stain uninfected cells (Fig. 3C). Anti-Nef mAb also stained infected cells, but patches of staining were observed (Fig. 3E). No fluorescence was observed on uninfected cells (Fig. 3C,D,G,H), and on infected cells with normal mouse IgG and IgM (Fig. 3B,F). Next, morphological comparison of cell surface expression of Nef and Env was performed by scanning electron micrography (Fig. 4). Env protein was expressed on the cell surface and virus particles were observed but gold particles were rarely clustered (Fig. 4A,B), while frequent clustering of gold particles was found on the anti-Nef mAb reacting HIV-1-infected cells (Fig. 4D–F). A smooth surface was observed in control uninfected cells (Fig. 4C). These experiments showed different distribution patterns between Nef and Env molecules on the surface of HIV-1-infected cells. To know more about the cell surface and the intracellular distribution of Nef, thin sections of HIV-1-infected cells were examined by immunoelectron microscopy using anti-Nef mAb E7 and colloidal gold-conjugated anti-mouse μ Ab (Fig. 5). The gold particles were detected at the outer surfaces of the cell membrane (Fig. 5A,B) and the intracellular portion of HIV-1-infected cells (Fig. 5D,E) but not in uninfected cells at all (Fig. 5C,F). Nef antigen was clustered at budding sites (Fig. 5A), and the cell surface (Fig. 5B). Clustering of Nef was also found at the cytoplasmic space close to the plasma membrane (Fig. 5D,E).

4. Discussion

The present work shows that Nef, derived from baculosystem and from HIV-1-infected cells, forms a homomerically oligomerizing structure and that it clusters in HIV-1-infected cells. This is the first report that demonstrates the oligomerization and clustered localization of Nef in cells infected with HIV-1. The oligomerization of Nef was studied by HPLC and immunoprecipitation/immunoblotting techniques (Figs. 1 and 2). Intact Nef formed multimers and these were monomerized only by urea. From our data, this oligomeric structure of Nef molecules appeared not to be formed by disulfide bonds. The localization of Nef was investigated by indirect immunofluorescence assay and immunoelectron microscopic analysis (Figs. 3–5). All the results obtained by these techniques showed that Nef was clustered at the surface as well as the cytoplasm of cells infected with HIV-1.

In summary, oligomerization of Nef molecules by non-covalent binding and their clustered localization in HIV-1-infected cells may have an important role for Nef actions, such as up-regulation of virus replication, down-modulation of CD4, and finally cytotoxicity. To elucidate the mechanisms of these functions of Nef, further study needs to be carried out.

Acknowledgements: This work was supported in part by grants-in-aid

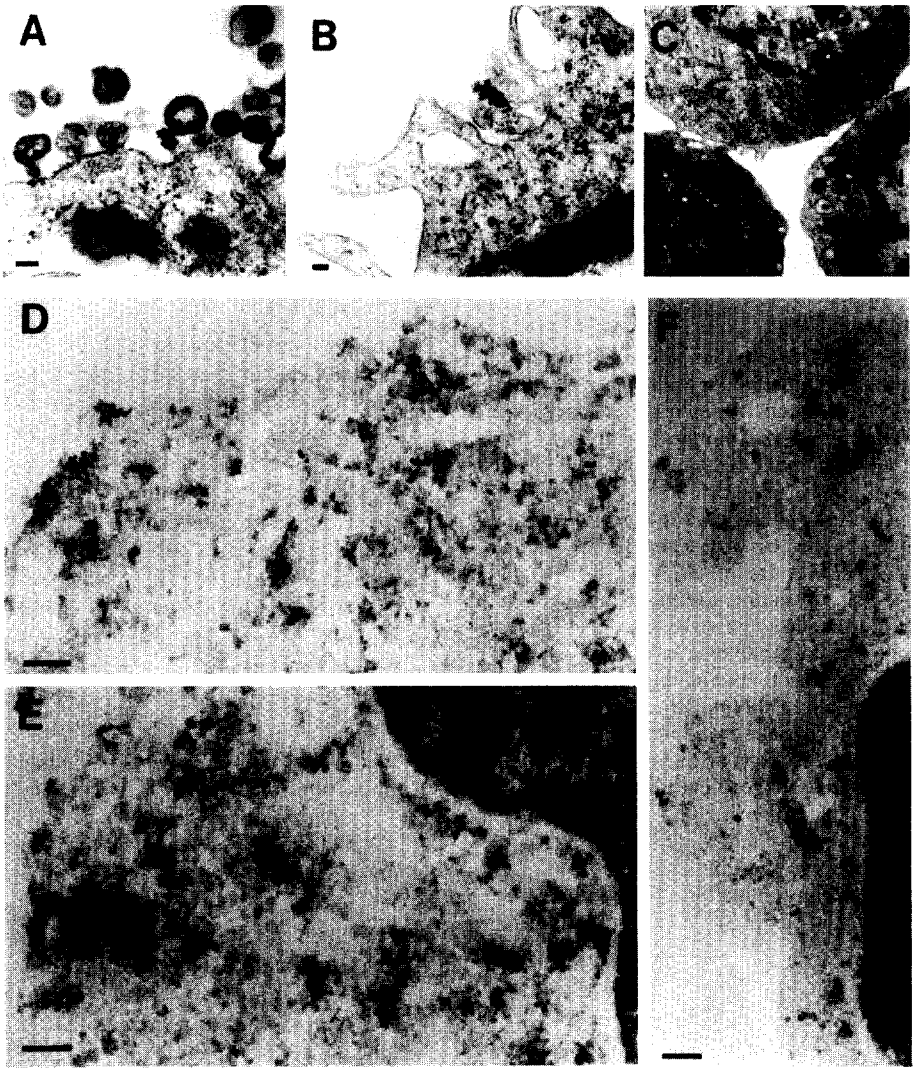

Fig. 5. Expression and oligomerization of Nef protein on cell surface and in cytoplasm as monitored by transmission electron micrography. Electron micrographs of human HeLa CD4 cell line infected with HIV-1 are shown. Cells, non-treated (A–C) and pre-treated with Triton X-100 (D–F), were labeled using anti-Nef mAb E7, followed by an anti-mouse μ-colloidal gold conjugate, and stained with uranyl acetate. Gold particles were not found in uninfected cells (C, F). Bars: 100 nm.

for AIDS research from the Ministry of Education, Science and Culture of Japan.

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