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Inefficient Enhancement of Viral Infectivity and CD4 Downregulation by Human Immunodeficiency Virus Type 1 Nef from Japanese Long-Term Nonprogressors

Minoru Tobiume,[1,2] Mikako Takahoko,[1] Takeshi Yamada,[3] Masashi Tatsumi,[4] Aikichi Iwamoto,[3] and Michiyuki Matsuda[1]*

Department of Tumor Virology, Research Institute for Microbial Diseases, Osaka University, Osaka,[1] and Division of Infectious Diseases, Advanced Clinical Research Center, Institute of Medical Science, University of Tokyo,[3] and Department of Veterinary Science, National Institute of Infectious Diseases,[4] Tokyo, Japan, and Department of Microbiology and Immunology, Vanderbilt University Medical Center, Nashville, Tennessee[2]

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It has been reported that patients infected with *nef*-defective human immunodeficiency virus type 1 (HIV-1) do not progress to AIDS; however, mutations that abrogate Nef expression are not common in long-term nonprogressors (LTNPs). We postulated that Nef function might be impaired in LTNPs, irrespective of the presence or absence of detectable amino acid sequence anomalies. To challenge this hypothesis we compared in vitro function of *nef* alleles that were derived from three groups of Japanese patients: LTNPs, progressors, and asymptomatic carriers (ACs). The patient-derived *nef* alleles were subcloned into a *nef*-defective infectious HIV-1 molecular clone and an expression vector. We first examined Nef-dependent enhancement of infection in a single-round infectivity assay by the use of MAGNEF cells, in which Nef is required more strictly for the infection than in the parent MAGI cells. All *nef* alleles from LTNPs showed reduced enhancement in the infectivity of *nef*-defective HIV-1 mutants compared to the *nef* alleles of progressors or ACs. Second, we found that *nef* alleles from LTNPs were less efficient in CD4 downregulation than those of progressors or ACs. Third, all *nef* alleles from LTNPs, progressors, and ACs reduced the cell surface expression of major histocompatibility complex class I to a similar level. Last, there was no correlation between Hck-binding activity of Nef and clinical grouping. In conclusion, we detected inefficient enhancement of HIV-1 infectivity and CD4 downregulation by HIV-1 *nef* alleles of LTNPs. It awaits further study to conclude that these characteristics of *nef* alleles are the cause or the consequence of the long-term nonprogression after HIV-1 infection.

The median time to development of AIDS in most cohorts is approximately 10 years after initial exposure to the virus (36, 40). However, some human immunodeficiency virus type 1 (HIV-1)-infected persons remain clinically healthy without any drug treatment and show no decline in $CD4^+$ T-cell counts even though they have been seropositive for more than 10 years (5, 6, 15, 21, 29, 35, 43). The mechanism that generates the long-term nonprogressors (LTNPs) is under intense investigation, because a clue to vaccine development for HIV-1 may be underlie it.

Defects in the *nef* gene of HIV-1 have been linked to nonprogressive infection. Following an earlier case report of an LTNP carrying only *nef*-defective HIV-1 (28), an Australian cohort study has also shown that six LTNPs were infected with *nef*-deleted HIV-1 from a single blood donor (30). Supporting this view, rhesus monkeys inoculated experimentally with a *nef*-defective simian immunodeficiency virus have low viral loads and normal $CD4^+$ T-cell numbers (26). Furthermore, there are reports that document the presence of *nef*-defective HIV-1 genomes in LTNPs (14, 19, 32, 42). However, some reports argue against the role of *nef* in the establishment of long-term nonprogression because most of the *nef* alleles isolated from LTNPs were intact in the length of the coding region and in the tested biological function (9, 23, 24, 32, 34).

The in vitro function of *nef* can largely be summarized in four categories (for reviews see references 12, 13, 16, and 19): downregulation of cell surface CD4, downregulation of the class I major histocompatibility complex (MHC), stimulation of the signal transduction cascades, and enhancement of viral replication in specific cell types. More recently, an antiapoptotic effect of Nef has also been reported (46). Extensive mutagenic studies have revealed that these activities are genetically separable and mapped to different regions of Nef (reviewed in reference 19); however, it remains elusive as to which in vitro function is most critical for the in vivo pathogenicity of primate lentiviruses.

Among the many in vitro functions of Nef, the enhancement of viral infectivity appears to be associated most directly with the replication cycle of HIV-1; however, Nef is dispensable for viral replication under commonly used laboratory conditions (26). Thus, reporter cells including HeLa-CD4-LTR-βGal cells (MAGI) (27) are often preferred for analysis. In assays using reporter cells, the difference in infectivity between the *nef*-defective mutant and wild-type HIV is less than 10-fold in most cases (2, 3, 7, 11, 25). Considering a large statistical error in the infectivity assay, this difference seems to be subtle. We postulated that the previous failure in the detection of functional differences between the *nef* alleles from LTNPs and those from progressors might have been caused by this small requirement

* Corresponding author. Mailing address: Department of Tumor Virology, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565-0871, Japan. Phone: 81-6-6879-8316. Fax: 81-6-6879-8314. E-mail: matsudam@biken.osaka-u.ac.jp.

for Nef in many reporter cells. Based on the observation that the Nef-induced enhancement of HIV-1 infectivity correlates inversely with the amount of CD4 in the target cells (45), we have isolated a MAGI-derived cell line, MAGNEF, which requires *nef* more strictly than does the parent MAGI cell in the single-round infectivity assay (44). Here we used MAGNEF cells for the quantitative analysis of the enhancement of HIV-1 infectivity by *nef* genes isolated from patients with different clinical outcomes.

MATERIALS AND METHODS

Subjects. Characterization of the patients and isolation of HIV-1 proviral *nef* gene from genomic DNA of peripheral blood mononuclear cells (PBMCs) by nested PCR have been described previously (47, 48). Briefly, we studied 14 Japanese hemophiliacs who were infected with HIV-1 through contaminated blood products more than 10 years before sample collection. Five were LTNPs and maintained their $CD4^+$ cell count above 450/μl without antiretroviral therapy. Six were progressors with $CD4^+$ cell counts below 100/μl at the time of sample collection. In the present study, we included 5 asymptomatic carriers (ACs) who were infected with HIV-1 within 3 years of sample collection and maintained their $CD4^+$ cell count above 450/μl without antiretroviral therapy.

Isolation, cloning, and expression of the proviral *nef* gene. Primers used in nested PCR were set at highly conserved regions of *env* and the long terminal repeat as described previously (48). PCR products were cloned into pCR-blunt Topo II (Invitrogen) and were sequenced with a BigDye sequencing kit (PE Biosystems). To facilitate subcloning, the coding sequence of *nef* was amplified by PCR with primer sets containing *Sal*I and *Not*I sites in forward and reverse primers, respectively. A primer set used to amplify each *nef* gene was chosen so that the amino acid sequence of each Nef would not change after PCR amplification. After PCR amplification, the fragments were again subcloned into pCR-blunt Topo II and verified for the sequence. For Nef expression, we used pCAGGS-IRES-EGFP, which was derived from the pCAGGS eukaryotic expression vector (37) and contained the internal ribosome entry site (IRES) of encephalomyocarditis virus at the 3′ end of the cloning site, followed by the enhanced green fluorescent protein (EGFP) gene. The Nef-coding DNA fragments were cleaved out from the plasmids with *Sal*I and *Not*I and were subcloned into pCAGGS-IRES-EGFP cleaved with *Xho*I and *Not*I.

Plasmid. An infectious proviral clone of HIV-1, pNL4-3, and its *nef*-defective mutant, pNL-Xh, have been described previously (1). We introduced *Xho*I and *Not*I sites in pNL4-3 for insertion of the *nef* gene from patients as follows: DNA fragments extending from the 3′ region of *env* of NL4-3, nucleotides (nt) 8032 to 8785, were amplified by PCR with a forward primer, 5′-TGCTGTGCCTTGGA ATGCTAG-3′, and a reverse primer, 5′-GTCGACGCGGCCGCACGCGTCT CGAGTTATAGCAAAATCCTTTCCAAGCCC-3′. The underlined sequence contains recognition sites of *Sal*I, *Not*I, *Mlu*I, and *Xho*I from the 5′ end. The amplified DNA fragment was cloned into pCR-blunt Topo II, and the sequences were verified. From this plasmid, a DNA fragment of the carboxyl-terminal region of *env* was cleaved out by *Bam*HI and *Sal*I, and it was inserted into pNL4-3 cleaved with *Bam*HI at nt 8465 and *Xho*I at nt 8892. Therefore, in the resulting plasmid, named pNL-Not, the 5′ region of *nef* from nt 8786 to 8892 was replaced with the recognition sites of *Xho*I, *Mlu*I, and *Not*I. The *nef* alleles derived from patients and NL4-3 were cleaved with *Sal*I and *Not*I and inserted into pNL-Not cleaved with *Xho*I and *Not*I. As a positive control, we amplified the *nef* allele of pNL4-3 and inserted it into pNL-Not.

Cells and transfection. MAGIC5 and MAGIC5D cells were CCR5-expressing derivatives of HeLa-CD4-LTR-βGal (MAGI) cells (22, 27). The cell line named MAGNEF was obtained by limiting dilution of MAGIC5 cells. Compared with the parent MAGIC5 cells, MAGNEF cells expressed a lower level of CD4 and required Nef more strictly for HIV-1 infectivity (44). By using of a phycoerythrin fluorescence quantitation kit (Becton Dickinson), the numbers of cell surface CD4 were determined as 4×10^4 in human peripheral CD4-positive T cells, 5×10^3 in MAGIC5, 5×10^5 in MAGIC5D, and 1×10^3 in MAGNEF. MAGNEF and MAGIC5D cells were cultured in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum (FCS). 293T human embryonic kidney cells were a gift from Bruce J. Mayer at the University of Connecticut and were maintained in Dulbecco's modified Eagle's medium with 10% FCS. Plasmid DNAs were transfected into 293T cells by the calcium phosphate coprecipitation method or into MAGIC5D cells with Lipofectamine 2000 (Gibco-BRL).

MAGI assay. Infectious DNAs of HIV-1 were transfected into 293T cells by the calcium phosphate coprecipitation method. Forty-eight hours later, cultured supernatants were filtered through a 0.45-μm-pore-size filter (Whatman, Clifton, N.J.) and examined for their $p24^{gag}$ concentration by an HIV-1 p24 antigen enzyme-linked immunosorbent assay kit (ZeptoMatrix, Buffalo, N.Y.). We plated 10^4 MAGNEF cells on 96-well tissue culture plates and inoculated them with serially diluted virus stocks. Forty-eight hours after infection, cells were fixed and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactoside as described elsewhere (27). We chose wells in which the number of positive cells was about 50 for calculation of the infectivity of the virus stocks. Under this condition, the dilution of the viral stocks and the number of infected cells were in a linear range.

Flow cytometry analysis . MAGIC5D cells were transfected with pCAGGS-IRES-EGFP-derived vectors carrying the *nef* alleles of patients. Thirty-six hours after transfection cells were detached from culture dishes with EDTA treatment and were suspended in phosphate-buffered saline containing 1% FCS. After being stained with saturating amounts of phycoerythrin-conjugated Leu3A monoclonal antibody specific for CD4 (Becton Dickinson) or phycoerythrin-conjugated W6/32 monoclonal antibody specific for the assembled MHC-I heavy chain β_2-microglobulin complex (DAKO), cells were washed with phosphate-buffered saline and fixed with 1% formaldehyde. Fluorescence intensities of green fluorescent protein (GFP) and either CD4 or MHC-I were analyzed with a FACSCalibur flow cytometer (Becton Dickinson). After gating with GFP intensity, the geometric mean fluorescence intensity of CD4 or MHC-I was calculated with CELLQuest software (Becton Dickinson). The relative down-regulation efficiency was shown by the formula (intensity of NL4-3-Nef-expressing cells)/(intensity of cells to be evaluated).

Immunoblotting. Cells were lysed in lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100), clarified by centrifugation, separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, Mass.). We used a mixture of monoclonal antibodies against $p24^{gag}$ and $gp41^{env}$ for the detection of viral proteins. Nef on the filters was detected by a pool of monoclonal antibodies provided by K. Ikuta at Osaka University (38).

GST fusion protein and in vitro binding assay. The 5′ region of Hck coding the SH2 and SH3 domains was amplified by PCR with the following primers: forward primer, 5′-GGGCCCCCCGTCGACATGGGGTGCATGAAG-3′; reverse primer, 5′-CGCGGCCGCCCCAAGGCTTCTGGGCTTGGA-3′. The underlined sequences indicate *Sal*I and *Not*I restriction sites. The PCR product was cleaved with *Sal*I and *Not*I and subcloned in pGEX3X (Amersham-Pharmacia Biotech). The SH2 and SH3 domains of Hck were expressed as a fusion protein with glutathione *S*-transferase (GST) in transformed *Escherichia coli* and purified with glutathione-Sepharose beads as described previously (33). 293T cells were transfected with pCAGGS-IRES-EGFP-derived Nef expression vectors carrying the *nef* allele of each patient. Forty-eight hours after transfection, cells were lysed in lysis buffer, cleared by centrifugation, and incubated with equal amounts of the GST-Hck proteins bound to the glutathione beads. The binding reaction was carried out at 4°C for 12 h. After being washed six times with lysis buffer, bound proteins were separated on SDS–12.5% polyacrylamide gels and analyzed by being immunoblotted with a pool of anti-Nef monoclonal antibodies.

RESULTS

Nef-defective virus used in the assay. Our aim in this study was to examine whether there is any difference between the activities of Nef from LTNPs and from AIDS patients. For this goal we first constructed a pNL4-3-derived vector, named pNL-Not, which lacked the 5′ region of *nef* and contained restriction enzyme recognition sites for the insertion of *nef* alleles of patients (Fig. 1A). As a positive control we inserted the *nef* allele of NL4-3 into pNL-Not, generating pNL-Not-Nef. Plasmids encoding the wild-type NL4-3, a Nef-defective mutant of NL4-3 (NL-Xh), pNL-Not, and pNL-Not-Nef, were transfected to 293T cells to produce viruses. Expression of Nef and structural proteins was confirmed in cells transfected with pNL4-3 and pNL-Not-Nef but not pNL-Xh and pNL-Not (Fig. 1B). We used a pool of anti-Nef monoclonal antibodies, epitopes of which were distributed throughout Nef (38); however, we could not detect any protein in the cells transfected with pNL-Not, negating the expression of a truncated Nef protein from pNL-Not. Notably, we did not find any difference

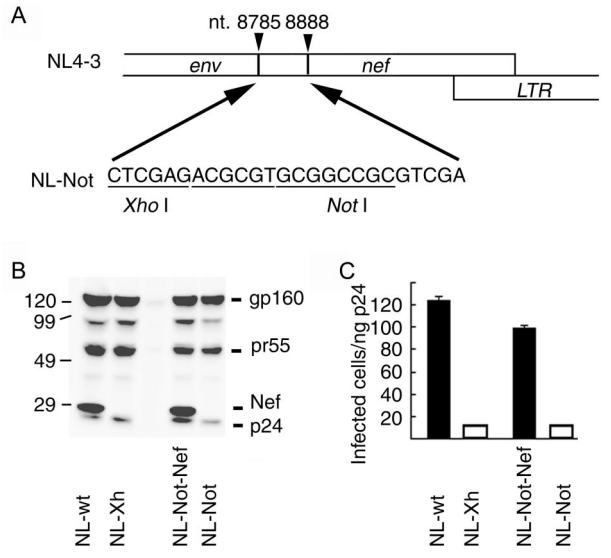

FIG. 1. Basic vector for the analysis of Nef enhancement of infectivity. (A) The 5′ region of *nef* of the HIV-1 NL4-3 strain was replaced with oligonucleotides containing *Xho*I and *Not*I restriction sites to generate NL-Not. Then *nef* allele of NL4-3 amplified by PCR was inserted into the *Xho*I/*Not*I site to generate NL-Not-Nef. (B) Wild-type NL4-3, *nef*-defective NL4-3 (NL-Xh), NL-Not, and NL-Not inserted with NL4-3 *nef* (NL-Not-Nef) were transfected into 293T cells for propagation of virus. 293T cells were lysed and analyzed by immunoblotting with a mixture of monoclonal antibodies against $p24^{gag}$, $gp41^{env}$, and Nef. Molecular standards are shown on the left. (C) Serially diluted virus stocks were inoculated into MAGNEF cells plated on 96-well dishes. Forty-eight hours later, infected cells were identified by being stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. Infectivity was normalized by the quantity of $p24^{gag}$ in the virus stocks. Experiments were done in triplicate and repeated three times. A representative result is shown with standard deviations.

in the expression level of $gp160^{env}$ and $pr55^{gag}$ among the cells transfected with Nef-positive or Nef-negative proviral DNA.

The infectivity of these viruses was examined by use of MAGNEF indicator cells (Fig. 1C). The infectivity of NL4-3 was about 10-fold higher than that of the Nef-defective NL4-3, NL-Xh. The infectivity of NL-Not-Nef was slightly lower than that of NL4-3; however, the Nef-dependent increase in the infectivity was obvious when we compared the infectivity of NL-Not with that of NL-Not-Nef. The result validated the use of NL-Not for quantifying the Nef enhancement of infectivity.

Analysis of *nef* alleles from patients. We analyzed four LTNPs (patients 1 [p1], p3, p4, and p6), six progressors (p13 to p18), and five ACs (p21, p22, p24, p25, and p26). The *nef* alleles were PCR amplified from PBMCs and cloned into plasmids as described previously (48). More than five independent clones were sequenced for each patient. When all of the analyzed sequences were identical (p13, p17, p18, p22, and p24), only one *nef* allele for each patient was further subcloned into pNL-Not and pCAGGS-IRES-EGFP. In the other cases, two representative clones were chosen for construction of expression vectors.

To search for any sequence motif that is characteristic for each of the three groups, we analyzed the *nef* sequences by creating a phylogenetic tree with the neighbor-joining method (Fig. 2). When two distinct sequences were found from any one patient, i.e., p1, p3, p4, p6, p14, p15, p16, p21, or p26, the two sequences formed a cluster, validating the same origin. The only exception was p25, whose two *nef* alleles clustered with either p21 or p26. This may suggest that patient 25 had suffered from repeated infection. Most importantly, *nef* alleles of LTNPs, progressors, and ACs did not form any clusters in the phylogenetic tree. We also created a phylogenetic tree by using the decoded amino acid sequences of the *nef* alleles and found that *nef* proteins of LTNPs, progressors, and ACs did not form any clusters. The amino acid sequences of the *nef* alleles and their alignment can be obtained from our web site (http://www-tv.biken.osaka-u.ac.jp/nef/).

Enhancement of viral infectivity by patient-derived *nef* alleles. pNL-Not-derived plasmids carrying the *nef* allele of patients were transfected into 293T cells for the preparation of viruses. Expression of Nef was confirmed by immunoblot analysis of the transfected 293T cells. All viruses produced Nef proteins of about 30 kDa (Fig. 3). We did not find any remarkable difference in the level of Nef from LTNPs, progressors, or ACs.

We analyzed the infectivity of these viruses by using MAGNEF indicator cells. As shown in Fig. 4, the enhancement of viral infectivity by the *nef* alleles of LTNPs was significantly lower than that by *nef* of NL4-3. The level of increase in infectivity was at most threefold by the *nef* alleles of LTNPs, whereas the *nef* alleles derived from progressors and ACs increased the infectivity at least fivefold. The difference between LTNPs and progressors or ACs was statistically significant by *t* test and Welch's test ($P < 0.01$).

Downregulation of cell surface expression of CD4 by *nef* alleles from patients. For the purpose of downregulation of cell surface expression of CD4 by *nef* alleles, *nef* alleles were inserted into the pCAGGS-IRES-EGFP vector and intro-

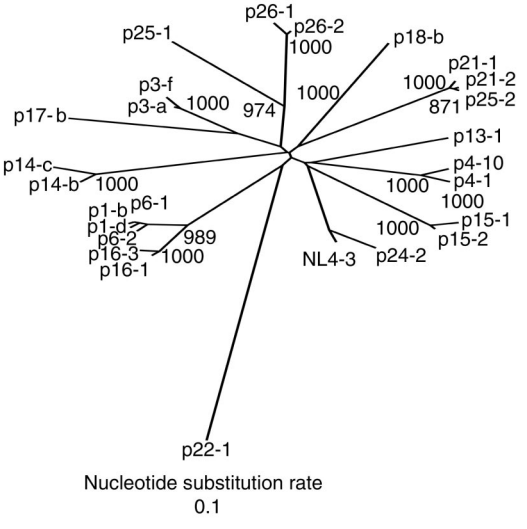

FIG. 2. Phylogenetic analysis of the nucleotide sequences of *nef* alleles from LTNPs, AIDS patients (AIDS), and asymptomatic carriers (ACs). The phylogenetic tree was constructed by the neighbor-joining method. Symbols before hyphens indicate patient codes, and numbers or letters after hyphens specify each *nef* clone. Values along the branches indicate the bootstrap probability. All branch lengths are drawn in accordance with their relative genetic distances.

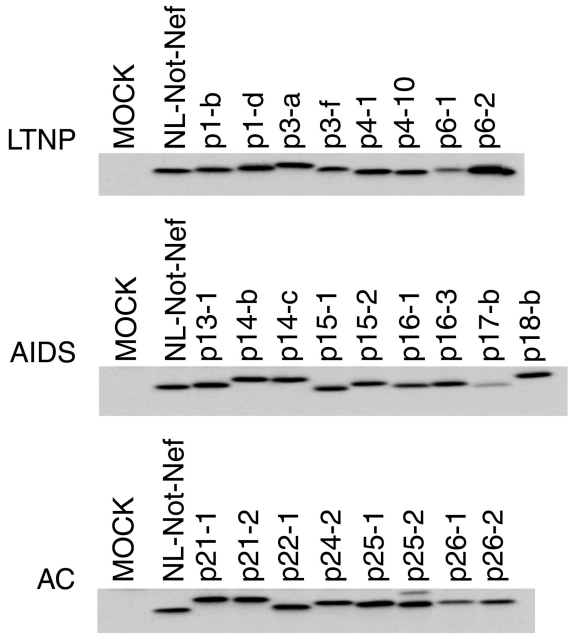

FIG. 3. Expression of Nef from recombinant HIV-1. The sequence-verified *nef* alleles from LTNPs, AIDS patients, and ACs were inserted into the *Xho*I/*Not*I site of pNL-Not. The infectious DNAs were transfected into 293T cells to propagate viruses. The cell lysates were analyzed by being immunoblotted with a pool of anti-Nef monoclonal antibodies.

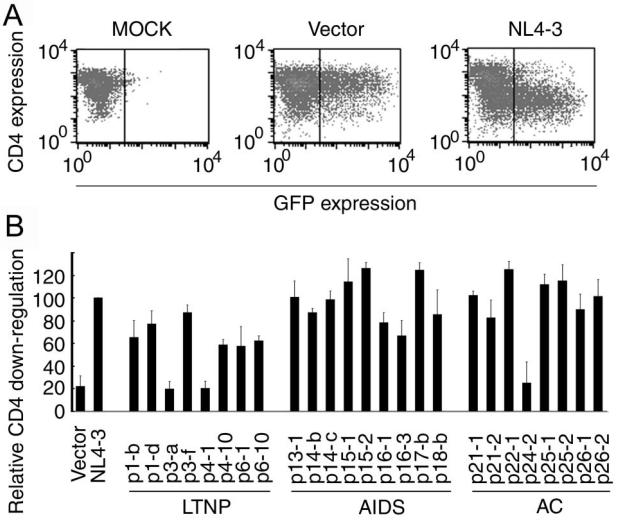

FIG. 5. Downregulation of cell surface CD4 by Nef derived from LTNPs, AIDS patients, and ACs. (A) MAGIC5D indicator cells were transfected with pCAGGS (MOCK), pCAGGS-IRES-EGFP (Vector), or pCAGGS-IRES-EGFP-4-3-Nef (NL4-3). After 36 h, cells were stained with phycoerythrin-conjugated anti-CD4 monoclonal antibody and were analyzed for fluorescence intensity by FACSCaliber. The vertical bars indicate the thresholds for GFP. (B) pCAGGS-IRES-EGFP (Vector), pCAGGS-IRES-EGFP-4-3Nef (NL4-3), or expression vectors for the *nef* alleles from patients as indicated at the bottom were transfected and analyzed as described for panel A. After gating for the EGFP-positive cells, the expression level of CD4 was determined as geometric mean fluorescence with CELLQuest software. Relative downregulation efficiency (%) is shown by the following equation: (mean fluorescence intensity of cells expressing NL4-3 Nef)/(mean fluorescence intensity of cells to be evaluated) × 100. Averages from three independent experiments are shown with standard deviations.

duced into MAGIC5D cells. In these plasmids, a single mRNA could produce both Nef and EGFP; the coding sequence of EGFP was placed downstream to the internal ribosomal entry site. Cells were detached from dishes, and the cell surface expression of CD4 was analyzed by flow cytometry. As shown in Fig. 5A, expression of Nef of NL4-3 markedly suppressed the cell surface expression of CD4.

Similarly, we examined the CD4 downregulation by the *nef* alleles from patients. In Fig. 5B, we show the downregulation efficiency as a ratio to that of NL4-3. Three of 7 *nef* alleles from LTNPs reduced the CD4 expression level as efficiently as the *nef* allele of NL4-3, 2 did so less efficiently, and the other 2 did not have any effect. Most of the *nef* alleles from progressors and ACs, except p24-2, reduced the CD4 level as efficiently as the *nef* allele of NL4-3. Thus, the *nef* alleles from LTNPs downregulate CD4 less efficiently than do the *nef* alleles of progressors and ACs. The difference was statistically significant by *t* test and Welch's test ($P < 0.05$).

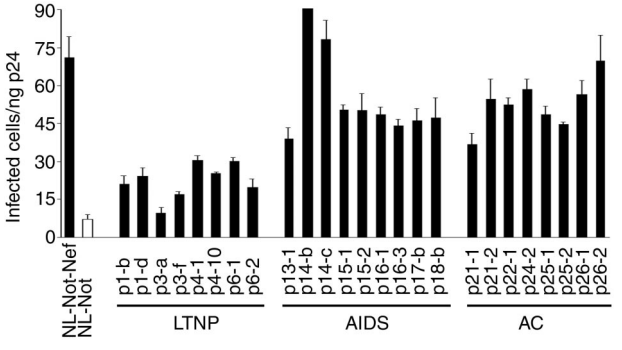

FIG. 4. Enhancement of viral infectivity by the *nef* alleles derived from LTNPs, AIDS patients, or ACs. The recombinant HIV-1 viruses carrying *nef* alleles of patients were prepared by transfection of infectious DNAs to 293T cells. Infectivity was examined as for Fig. 1. Three independent experiments were performed, and representative data with standard deviations are shown.

Downregulation of cell surface MHC-I by *nef* alleles from patients. We also examined the downregulation of MHC-I (Fig. 6). All *nef* alleles reduced the surface expression level of MHC-I, at least as efficiently as did NL4-3-derived *nef*. Most importantly, we did not observe any difference in the level of MHC-I downregulation among LTNPs, progressors, and ACs.

Binding of Nef to Hck. Lastly, we examined the binding of patient-derived Nef to Hck in vitro (Fig. 7). Nef proteins derived from patients were expressed in 293T cells and assayed for the binding to GST-tagged Hck, purified from *E. coli*. Nef proteins that showed reduced binding to Hck were p4-1, p16-1, p16-3, p18-b, p22-1, and p24-2. Thus, the ability of Nef to bind Hck was not correlated with patient clinical status.

DISCUSSION

Despite several reports of *nef* deletion in LTNPs (28, 30, 32, 42), deterioration of *nef* function is not regarded as a common feature among LTNPs, because the deletion of *nef* alleles is

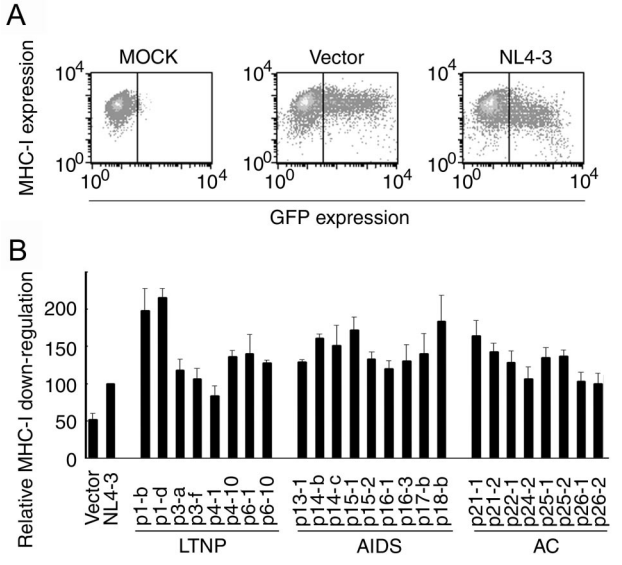

FIG. 6. Downregulation of cell surface MHC-I by Nef derived from LTNPs, AIDS patients, and ACs. (A) MAGIC5D indicator cells were transfected with pCAGGS (MOCK), pCAGGS-IRES-EGFP (Vector), or pCAGGS-IRES-EGFP-4-3Nef (NL4-3). After 36 h, cells were stained with phycoerythrin-conjugated anti-MHC-I monoclonal antibody and analyzed for fluorescence intensity by FACSCalibur. The vertical bars indicate the thresholds for EGFP. (B) pCAGGS-IRES-EGFP (Vector), pCAGGS-IRES-EGFP-4-3Nef (NL4-3), or expression vectors for the *nef* alleles from patients as indicated at the bottom were transfected and analyzed as described for panel A. After gating for EGFP-positive cells, the expression level of MHC-I was determined as geometric mean fluorescence with CELLQuest software. Relative downregulation efficiency (%) is shown by the following equation: (mean fluorescence intensity of cells expressing NL4-3 Nef)/(mean fluorescence intensity of cells to be evaluated) × 100. Averages from three independent experiments are shown with standard deviations.

found only in a minor fraction of LTNPs (9, 23, 34). Previously, we also found deletion of a *nef* allele only in one patient among seven Japanese LTNPs (48). In this study, we asked whether the other *nef* alleles without gross deletion differed from those of progressors or ACs in biological or biochemical activities.

The results of our analyses are summarized in Table 1. The *nef* alleles of LTNPs were inefficient in the enhancement of infectivity and in CD4 downregulation. However, these two activities were not always correlated in the *nef* alleles of progressors or ACs. For example, *nef* p24-2, which was derived from an AC, enhanced infectivity efficiently but downregulated CD4 only at a marginal level. Similarly, we could not find any correlation when we expanded the comparison to the four parameters. These findings are in agreement with previous reports proposing that the four *nef* functions are genetically separable and mapped to different regions of *nef* (reviewed in reference 19).

To the best of our knowledge, three groups have performed experiments similar to ours (8, 18, 23). Huang et al. replaced the *nef* allele of HIV-1 HXB2 with those of LTNPs (23). They did not find any difference in the enhancement of infectivity between the 10 recombinant viruses carrying LTNPs *nef* and the parent HXB2 virus by using MAGI cells. In our hands, the enhancement of HIV-1 infectivity by Nef is usually less than 10-fold in MAGI cells, which hampers the detection of subtle differences in Nef activity. Therefore, the use of MAGNEF cells, which express a lesser amount of CD4 than do MAGI cells (44), might have enabled us to detect the difference in Nef activity between LTNPs and progressors. Carl et al. constructed NL4-3-based recombinant viruses carrying *nef* alleles of a single LTNP (8). These viruses showed reduced infectivity both in a single-round infectivity assay with MAGI cells and in PBMCs. Geffin et al. also constructed NL4-3-based recombinant viruses carrying *nef* alleles of two pediatric LTNPs and examined their infectivity in herpesvirus saimiri-transformed primary human T cells (18). Again, the authors found that *nef* alleles of two LTNPs enhance HIV-1 infectivity less efficiently than the *nef* alleles of progressors. Although the number of the examined LTNPs is still small, these reports are in agreement with our finding that *nef* alleles of LTNPs enhanced HIV-1 infectivity less efficiently than did those of progressors and ACs in a single-round infectivity assay.

It has been reported that some *nef* alleles derived from LTNPs are defective in CD4 downregulation (7, 32). Mariani and coworkers reported that one patient among four LTNPs carried *nef* alleles that did not downregulate CD4 expression (32). Carl and coworkers reported a similar observation with *nef* alleles from one LTNP. Although we also found that Nef-induced CD4 downregulation was abrogated in two *nef* alleles from LTNPs (Fig. 5), three downregulated CD4 to a level comparable to that of the wild-type Nef. To reach a conclusion,

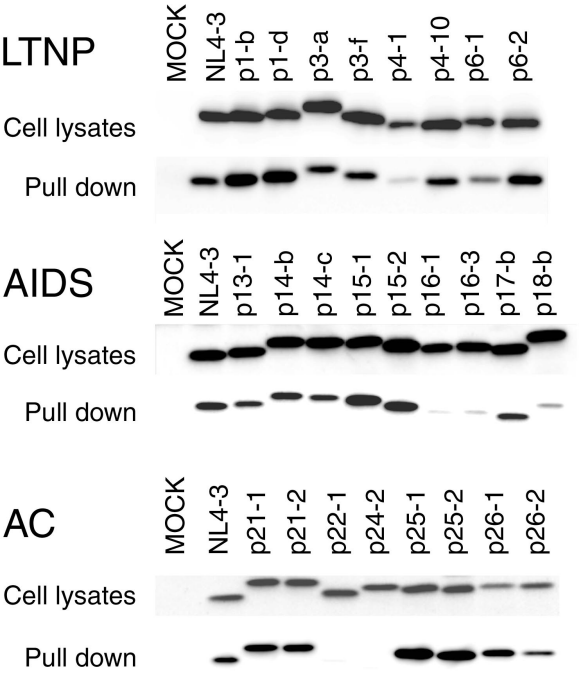

FIG. 7. Binding of Hck to the Nef proteins derived from LTNPs, AIDS patients, and ACs. 293T cells were transfected with expression plasmids encoding the *nef* alleles derived from patients. Two days after transfection, cells were lysed in lysis buffer, cleared by centrifugation, and incubated with GST-Hck SH2-SH3. Proteins bound to the GST fusion proteins were separated by SDS-polyacrylamide gel electrophoresis and were analyzed by being immunoblotted with a pool of anti-Nef monoclonal antibodies.

TABLE 1. Summary of the analyses

Group	Clone[a]	Enhancement of infectivity[b]	CD4 down-regulation[c]	MHC-I down-regulation[d]	Binding to Hck[e]
LTNPs	p1-b	+	+	++	++
	p1-d	+	+	++	++
	p3-a	−	−	++	+
	p3-f	+	++	+	+
	p4-1	+	−	+/−	+/−
	p4-10	+	+	++	+
	p6-1	+	+/−	++	+
	p6-2	+	+/−	++	++
AIDS	p13-1	++	++	++	+
	p14-b	+++	++	++	+
	p14-c	+++	++	++	+
	p15-1	++	++	++	++
	p15-2	++	+++	++	+
	p16-1	++	+	+	+/−
	p16-3	++	+	+	+/−
	p17-b	++	+++	++	+
	p18-b	++	++	++	+/−
ACs	p21-1	++	++	++	+
	p21-2	++	+	++	+
	p22-1	++	+++	+	−
	p24-2	++	+/−	+	−
	p25-1	++	++	++	++
	p25-2	++	+++	++	++
	p26-1	++	+	+	+
	p26-2	+++	++	+	+

[a] Numbers before hyphens indicate patient codes, and numbers or labels after hyphens specify the *nef* clone.
[b] Enhancement of infectivity is scored by the relative infectivity to NL-Not-Nef (Fig. 4): −, <30%; +, 30 to 60%; ++, 60 to 90%; +++, >90%.
[c] Downregulation of CD4 is scored by the relative efficiency to that by NL4-3 Nef (Fig. 5): −, <30%; +/−, 30 to 60%; +, 60 to 90%; ++, 90 to 120%; +++, >120%.
[d] Downregulation of MHC-I is scored by the relative efficiency to that by NL4-3 Nef (Fig. 6): −, <50%; +/−, 50 to 80%; +, 80 to 120%; ++, >120%.
[e] Binding affinity of Nef to Hck is scored from the data derived from Fig. 7.

we need to expand the number of LTNPs and to set up more quantitative assays.

The ability to downregulate cell surface MHC-I expression was retained in all *nef* alleles (Fig. 6). Carl and coworkers recently reported that *nef* alleles from progressors downregulate MHC-I less efficiently than those from ACs or LTNPs (7). The discrepancy between their results and ours may arise from the difference in cell types: we used MAGI-derived MAGIC5D cells, whereas Carl et al. used Jurkat T cells. Nevertheless, our data agree with respect to the lack of association of defective MHC-I downregulation with the establishment of LTNPs.

Binding to the SH3 domain of Hck is another function of Nef that has been associated with the Nef-dependent increase in HIV-1 infectivity (41); however, subsequent studies have shown that Nef binding to the SH3 domain-containing proteins is required for efficient downregulation of MHC-I (20, 31). Furthermore, it has been shown that dominant-negative mutants of Hck inhibit downregulation of MHC-I (10). In agreement with these previous reports, we found that the efficiency of Hck binding correlated with the level of MHC-I downregulation induced by each Nef (Table 1).

Our results of the sequence analysis of *nef* alleles from Japanese LTNPs were in complete agreement with those of previous studies denying the presence of a common sequence anomaly in LTNPs (9, 24, 34). We paid particular attention to the 9 sequence motifs in Nef that have been associated with biochemical or biological features (19); however, no mutation was commonly found in any patient group (www-tv.biken.osaka-u.ac.jp/nef/). Moreover, sequences from each group are found to be interdigitated in the phylogenetic tree (Fig. 2), confirming previous reports (9, 24, 34). Future development of a program that predicts the structure-function relationship of Nef may decipher this discrepancy; however, at present our infectivity assay that uses the patient *nef* gene will help to predict the prognosis of HIV-infected patients.

Nef is known to have many functions, and the number of functions is still increasing. For example, it has been recently shown that Nef binds to Pak to protect the infected cells from apoptosis (4, 17, 39, 46). Therefore, further efforts should be directed to seek a function of *nef* that correlates better with the clinical course. Finally, a positive correlation in vitro does not give evidence that the deteriorated Nef function causes the long-term nonprogression. Further study is required to examine whether these characteristics of *nef* alleles of LTNPs are the cause or the consequence of the long-term nonprogression after HIV-1 infection.

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