Evidence of Nef Truncation in Human Immunodeficiency Virus Type 2 Infection

Human immunodeficiency virus (HIV)-2 differs from HIV-1 in its relative lower transmissibility and pathogenicity. To understand the virologic basis of these differences, the nef gene from HIV-2–seropositive persons was analyzed because of its importance for disease progression in the genetically related simian immunodeficiency virus (SIVmac). Proviral nef sequences from 60 HIV-2–infected persons were amplified from peripheral blood lymphocytes, and nef open-reading frames were screened by a transcription and translation assay for the presence of full-length (32- to 36-kDa) or truncated (<32 kDa) Nef proteins. Overall, 6 (10%) of 60 persons had truncated Nef proteins; of these, 5 were among the 36 asymptomatic subjects (13.9%) and only 1 was among the 24 symptomatic subjects (4.2%) (P = .23). The results of this study document the presence of defective nef genes in HIV-2 infections with a prevalence higher than that previously seen in HIV-1–infected cohorts of long-term nonprogressors or patients with AIDS.
gene in both symptomatic and asymptomatic HIV-2–infected persons. We determined the frequency of nef genes with defective reading frames in the study population and examined the association of a defective Nef protein with clinical status.

Materials and Methods

Study population. Peripheral blood mononuclear cells (PBMC) from 79 HIV-2–seropositive persons were collected in a cross-sectional manner and stored at −80°C. The study participants included 58 persons from the Ivory Coast (designated IC), of whom 38 were asymptomatic women followed in a perinatal transmission study and 20 were tuberculosis patients; 11 asymptomatic and 3 symptomatic persons residing in Spain (S); and 2 asymptomatic and 5 symptomatic persons residing in Portugal (P). Two persons from the Ivory Coast had repeat samples collected 6 months apart. The participants were classified as asymptomatic or having AIDS according to the 1994 World Health Organization expanded case definition for AIDS [16]. AIDS-defining diseases included tuberculosis for all asymptomatic patients from the Ivory Coast and other opportunistic infections (such as esophageal candidiasis and herpes zoster) for patients from Spain and Portugal. Participants were screened for HIV antibodies by HIV-1/HIV-2 mixed ELISA (Genelavia Mixt; Diagnostics Pasteur, Paris), and the viral type was determined by a synthetic peptide–based test (Pepti-LAV; Diagnostics Pasteur) and virus-specific Western blot (DuPont, Geneva, Switzerland) and virus-specific Western blot (DuPont, Geneva, Switzerland). Negative controls included using the Qiagen midi-prep system (Qiagen, Studio City, CA) to purify the viral DNA.

Sequence analysis of HIV-2 nef genes. nef genes that expressed truncated proteins by TT analysis were sequenced to determine the frequency of clones with truncated nef sequences. As described in Results, this TT procedure was validated on samples from the current study with known nef clones and was found to be a reliable and accurate alternative to TT analysis of cloned nef sequences.

Analysis of HIV-2 nef ORFs. To rapidly analyze the nef gene for ORF interruptions, a coupled transcription and translation (TT) assay was done to determine the size of the Nef protein that is translated from the nef gene [18]. We have previously described the application of this technique to ORF analysis and found that the TT assay accurately and reliably determined the presence or absence of premature stop codons in 47 SIV nef clones with known ORFs [18]. The TT-based approach obviates the need for the laborious sequence analysis in all samples. The proviral HIV-2 nef sequences were analyzed by two different TT protocols. The first protocol included cloning the nef PCR product and subsequent TT analysis of multiple clones to determine the frequency of quasi-species with truncated Nef proteins in each sample. PCR-amplified nef genes from 14 samples were cloned into the pT7blue plasmid vector, and positive recombinants were selected by colony PCR using the T7-tagged sense primer 2T7NEF (5′-CTTACAGCTCCTATTAGGATGGGKKCRASTGGWTCAAAG-AAG-3′; K = G or T, R = G or A, S = G or C, W = A or T) and the 2LTRRA primer. The PCR-amplified nef gene from HIV-2ST was used as a positive control template to generate a full-length Nef protein (~34 kDa). Eight microliters of the T7-tagged PCR product was used as a template for the in vitro TT assay (Promega, Madison, WI) using rabbit reticulocyte lysates in the presence of [35S]methionine (Amersham, Arlington Heights, IL). After incubation of the reaction mixture at 30°C for 2 h, 5 μL of the TT reaction was mixed with 20 μL of SDS sample buffer and boiled for 3 min to denature the proteins. Ten microliters of denatured protein and 15 μL of heat-denatured [14C]-labeled protein standards were electrophoresed in a 12% SDS-polyacrylamide gel, and the radiolabeled proteins were detected by fluorography after overnight exposure at −80°C [18].

The second TT protocol for ORF examination involved screening for Nef truncation by direct TT analysis of uncloned nef PCR products from a subset of samples (n = 46). Primers 2T7NEF and 2LTRRA were used in a third round of PCR to amplify proviral nef sequences from 2 μL of the nested PCR reactions amplified from PBMC. These reactions generate T7-tagged nef sequences that serve as templates in the TT reaction. PCR templates that expressed only truncated Nef proteins, or a mixture of truncated and full-length proteins, were selected and reanalyzed by the first TT protocol to quantitate the frequency of clones with truncated proteins. The second TT protocol allows a more rapid determination of nef ORF status directly from PBMC by obviating the need for cloning and screening of bacterial recombinants for full-length or truncated nef sequences. As described in Results, this TT procedure was validated on samples from the current study with known nef clones and was found to be a reliable and accurate alternative to TT analysis of cloned nef sequences.

Sequence analysis of HIV-2 nef genes. nef genes that expressed truncated proteins by TT analysis were sequenced to determine the mechanism of truncation. The nef genes were cloned into the pT7blue vector as described earlier. Plasmid DNA obtained by using the Qiagen midi-prep system (Qiagen, Studio City, CA) was sequenced in both directions following the manufacturer’s recommendations (Sequenase; United States Biochemical, Cleveland) [18]. The FASTA, MAP, PILEUP, and BESTFIT programs in the Wisconsin Genetics Computer Group software package on a UNIX computer system were used for sequence analysis.

Nucleotide sequence accession numbers. The GenBank accession numbers of the seven HIV-2 nef genes sequenced in this study are U76639–U76643 and U76645.
Table 1. Frequency of Nef truncation in HIV-2–infected persons.

<table>
<thead>
<tr>
<th>Country</th>
<th>Asymptomatic</th>
<th></th>
<th></th>
<th>AIDS</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Truncated Nef</td>
<td>Full-length Nef</td>
<td>n</td>
<td>Truncated Nef</td>
</tr>
<tr>
<td>Ivory Coast</td>
<td>28</td>
<td>4 (14)</td>
<td>24 (86)</td>
<td>16</td>
<td>1 (6)</td>
</tr>
<tr>
<td>Spain</td>
<td>6</td>
<td>1 (17)</td>
<td>5 (83)</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Portugal</td>
<td>2</td>
<td>0</td>
<td>2 (100)</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Subtotal</td>
<td>36</td>
<td>5 (13.9)</td>
<td>31 (86.1)</td>
<td>24</td>
<td>1 (4)</td>
</tr>
</tbody>
</table>

NOTE. Data are no. (%).

Results

Amplification of HIV-2 LTR and nef sequences. Of the 79 samples studied, 74 (94%) were positive for the LTR region and 60 (76%) were PCR-positive for nef sequences. Of the 19 samples negative for nef sequences, 15 (79%) were from asymptomatic persons and 4 (21%) were from symptomatic persons. Of the 5 persons with negative LTR PCR results, 4 were asymptomatic and 1 was symptomatic. The negative nef and LTR PCR results were seen despite the use of nested PCR with consensus primers, suggesting that the negative results may be due to extremely low provirus loads or sequence variation at the primer sites (or both).

Analysis of HIV-2 nef ORFs. Nef TT results for the asymptomatic and symptomatic patient groups are summarized in table 1. Overall, samples from 6 persons were identified in which all, or the majority of, Nef clones were truncated (6/60, 10%). Of these 6 samples, 5 were from the asymptomatic group (5/36, 13.9%) and 1 was from the symptomatic group (1/24, 4.2%). These data may suggest a relationship between Nef truncation and the asymptomatic state. However, this relationship was not found to be statistically significant because the sample size of the study population was small (odds ratio, 4.5; 95% confidence interval, 0.5–214; P = .23, two-tailed Fisher’s exact test). Of the 6 samples with truncated Nef, 3 were identified by using the TT protocol with cloned nef sequences and 3 were identified by using the direct TT analysis of the uncloned nef PCR products. The size of the full-length Nef proteins was found to range predominantly between ~32 and 34 kDa, although a few clones generated Nef proteins of ~36 kDa. In contrast, the sizes of the truncated (or faster-migrating) Nef proteins were found to be significantly <32 kDa. The difference in the size of truncated and full-length Nef allowed easy identification of truncated Nef proteins.

Table 2 shows the ORF analysis of cloned HIV-2 nef genes in 17 samples, including all 7 samples (1 person had 2 samples collected from two time points) with Nef truncation and 10 samples with full-length Nef. There were 5 samples from 4 persons from whom all Nef clones were found to be truncated. Three of these 4 persons (IC763012, IC763124, IC762993, S1084) were asymptomatic and 1 was symptomatic (IC03689). Seven persons had samples that showed a mixture of full-length and truncated proteins with varying proportions. Of these, 5 samples (IC763001, IC763000, IC763023, S139, S173) had a majority of clones expressing full-length Nef proteins, with truncated Nef proteins present in only 5%–29% of the clones examined from these samples. These 5 samples were considered to have full-length nef genes. In contrast, 2 samples (IC763006 and IC763115) were clearly different and had a higher proportion of clones with truncated Nef proteins (62% and 81%, respectively). Therefore, these samples were considered to have the majority of nef clones with truncated Nef proteins.

The use of TT analysis on uncloned nef sequences allowed a direct and rapid determination of the major nef quasispecies from the HIV-2–infected PBMC. This modified procedure was...
first validated on 14 PBMC samples in which nef quasispecies were previously characterized by TT analysis of nef clones. A comparison of representative TT results from cloned and uncloned nef genes from the same samples are shown in figures 1 and 2, respectively. In figure 1, lanes 1–9 are from subject IC763006, showing a mixture of full-length (34-kDa) and truncated (24-kDa) Nef clones, lanes 13–21 are from subject IC763017, with only full-length Nef proteins, and lanes 22–30 are from subject IC03689, with only truncated Nef proteins. Low-molecular-mass proteins (<16 kDa) were commonly observed traveling with the dye front (figure 1, lanes 1–12 and 22–30) and may represent labeled proteins that are translated from nonspecific internal initiation sites, as previously experienced when using TT analysis [18].

Lanes 1–4 in figure 2 show the TT results of uncloned nef sequences. PCR product from samples IC763006 (lane 1) and S173 (lane 2) showed a mixture of truncated (24-kDa) and full-length (34-kDa) Nef proteins, and samples IC763017 (lane 3) and IC03689 (lane 4) showed only full-length (34-kDa) or truncated (<14-kDa) Nef proteins, respectively. Concordant TT results were observed between the two methods, suggesting that direct screening by TT analysis of uncloned nef products is equivalent to screening of cloned products. Therefore, this assay is a reliable alternative to TT analysis of cloned nef genes and has the advantages of being rapid and less labor-intensive. Additional samples identified by this method as having only truncated Nef proteins are shown in lanes 7 and 12 of figure 2. These 2 samples are from patients IC763124 and IC763115.
Sequence abnormalities of HIV-2 nef genes with Nef truncation. Open boxes, normal sequences; blank space, deletion in sequence; stop sign, termination codon; A, area with G→A hypermutation; stippled boxes, polymerase chain reaction primer location in flanking sequences of HIV-2_D194 and HIV-2_UC1 subtype A and B reference strains, respectively.

and were further subjected to additional TT analysis of cloned nef genes and shown to have a majority of truncated Nef proteins (table 2). The remaining samples in figure 2 represent specimens that were all considered to have full-length Nef proteins.

Mechanism of Nef truncation. To determine the mechanism of protein truncation in the 7 samples with evidence of truncated Nef, nef clones were sequenced, and the deduced amino acid sequence was analyzed. Two mechanisms of Nef protein truncation were observed. First, Nef proteins from subjects IC763124, IC763012, and IC762993 were truncated from the introduction of premature stop codons in the nef ORF as a result of G→A hypermutation (figure 3). The rate of G→A hypermutation calculated for the region encompassing the nef gene amplified in patient samples IC762993 and IC763124 was 42.8% and 27.3%, respectively. The second cause of Nef truncation, observed in subjects IC03689, S1084, IC763006, and IC763115, was deletions in the nef gene. The gross deletions either caused frameshift mutations that introduced premature stop codons (e.g., sample IC03689) or kept the nef reading frame open but significantly shortened the size of the Nef protein (e.g., samples S1084, IC763115, and IC763006) (figure 3). Analysis of the nef-LTR overlap region showed that both the gross deletions and the hypermutations in the nef defective sequences did not affect the polypurine tract, the NF-κB binding site, or other sequence motifs thought to be important for transcription.

Discussion

Because of the overall genetic relatedness of HIV-2 to SIV, and the importance of intact nef genes to the development of AIDS in SIV infections, the nef gene of HIV-2 was selected in this study as a primary target for analysis as having a possible role in the pathogenesis of this virus [4, 7]. We have examined clones of nef genes from a large number of symptomatic and asymptomatic HIV-2 infections and screened for nef ORF interruptions by using TT analysis. In addition, we have determined by sequence analysis the mechanism of Nef truncations that were detected in some samples.
The data show that 10% of HIV-2 infections had predominantly Nef truncations and that there was a trend toward the association of Nef truncation with asymptomatic (13.8%) infection compared with symptomatic infection (4.2%). While the difference in frequency of Nef truncation between symptomatic and asymptomatic subjects was not statistically significant, the data, nevertheless, raise important observations. First, Nef truncation may be occurring more frequently in HIV-2 infection (10%) than in HIV-1 infection (<1%). Although the prevalence of Nef truncation in HIV-1 infections has been studied only in small cohorts and may not allow a good comparison with the findings from this study, the data from these cohorts suggest a low prevalence of HIV-1 Nef truncation in patients with various rates of disease progression [10–15]. Evidence of HIV-1 Nef truncation has been observed only in 7 long-term survivors [8, 9]. In contrast, studies of 13 long-term nonprogressors and 32 patients with AIDS showed evidence of intact HIV-1 nef genes in the majority (65%–100%) of nef clones or quasispecies [10–15].

Second, while nef studies in HIV-1 have mainly focused on preselected and well-characterized long-term survivors, the present study examined nef in HIV-2 from persons identified in a cross-sectional manner with no preselection for infections with long durations. In view of the data from this study documenting the presence of Nef truncation in HIV-2 infections, it may be of particular importance to determine if the prevalence of Nef truncation is higher in long-term asymptomatic infections with HIV-2. These studies may truly determine the role of nef in the long asymptomatic stage seen in a subset of HIV-2 infections [19]. Seroconversion dates were not available in our study population to analyze the association of Nef truncation with length of infection.

The mechanism of Nef truncation found in the 7 HIV-2 samples in the current study was due to the introduction of premature stop codons by G−A hypermutation in 3 samples and by gross deletions in 4 samples. Whereas others have reported that mutations, such as single-base substitutions and gross deletions in HIV-1 nef, were responsible for interruption of the nef ORF, this is the first known report of a G−A hypermutation mechanism of Nef truncation. One of these Nef truncations was observed to persist in a person from this study over a 6-month period, suggesting that repair of such mutations may not occur very easily. The full extent of G−A hypermutation in genes other than nef in the 3 HIV-2 samples is not known.

The nef deletions seen in the HIV-2−infected samples were not confined to the U3 region, as has been reported in vivo with the infectious nef-defective SIVMAC molecular clones and in the HIV-2nin2 molecular clone [20, 21]. For example, the sample from subject IC763115 had the majority of nef deleted in the 5′ region of the nef ORF. While repair of small deletions (up to 12 bp) in nef has been found to occur in experimental SIV infections of macaques and has been associated with restoration of virulence, the deletions seen in all 4 HIV-2 samples are much larger and may be more difficult to repair [22].

In summary, the present study provides new evidence on the presence of HIV-2 infections with Nef truncation. The high frequency of this Nef truncation raises important questions regarding the possible role of defective Nef in HIV-2−infected persons with long asymptomatic stages or delayed disease development and, therefore, argues for additional studies of nef in these infections. The laboratory tools used here to rapidly screen for Nef truncation may be useful for future studies in this field.

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References