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Biological Characterization of nef in Long-Term Survivors of Human Immunodeficiency Virus Type 1 Infection

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We have previously shown that there were no gross deletions or obvious sequence abnormalities within nef of human immunodeficiency virus type 1 (HIV-1) in the 10 long-term survivors studied (Y. Huang, L. Zhang, and D. D. Ho, J. Virol. 69:93–100, 1995). Here we extend our study to examine these nef alleles in a functional context. Using a new technique, termed site-directed gene replacement, we have precisely replaced the nef of an infectious molecular clone, HIV-1BHXB2, with nef alleles derived from 10 long-term survivors as well as from a patient with AIDS. The replication properties of these chimeric viruses demonstrated that the nef allele derived from long-term survivors neither significantly increased nor decreased viral replication, compared with the nef allele of Nef+ HIV-1BHXB2 and that derived from a patient with AIDS. However, Nef− viruses always replicated faster than virus lacking nef. Moreover, single-cell infection analysis by the MAGI assay showed that these chimeric viruses, as well as Nef− HIV-1BHXB2, were more infectious than Nef− HIV-1BHXB2 was. Therefore, we conclude that the genotypic and phenotypic features of nef are not likely to account for the nonprogression of HIV-1 infection in the 10 cases studied, unless the function of the nef gene in vivo is not accurately reflected by the in vitro assays we used.

Following primary infection with human immunodeficiency virus type 1 (HIV-1), the majority of patients experience an extended asymptomatic period after seroconversion and prior to the development of AIDS, whereas some become immunosuppressed and develop opportunistic complications rapidly (1, 17). It has been estimated that the median time from the onset of HIV-1 infection to laboratory evidence of immunodeficiency is approximately 10 years (23). However, a small proportion of infected individuals remain clinically healthy and immunologically normal despite prolonged HIV-1 infection (2, 23). This group of patients constitutes approximately 5% of all HIV-1-infected individuals and has been termed long-term survivors or persons with long-term nonprogressive infection. Although the precise definition of long-term survivors of HIV-1 infection varies (23), these persons generally have been infected for over a decade, lack clinical symptoms, and maintain normal and stable CD4+ T-cell counts (8, 13, 15, 16, 23, 24).

Multiple host, viral, and environmental factors are believed to contribute to the spectrum of disease progression induced by HIV-1 infection (29). Host factors may result in differential susceptibility to viral infection and its pathogenic effects, whereas HIV-1 variation may account for differential virulence and disease course. Therefore, the sequelae of infection depend on the fine balance among these interactions. Although we have recently found strong HIV-1-neutralizing antibodies and virus-inhibitory CD8+ T-cell responses in our cohort of long-term survivors, the kinetics of viral replication in two subjects (patients 7 and 8) clearly show the presence of an attenuated strain of HIV-1 (3). Additionally, the importance of viral load and phenotype in influencing the rate of disease progression to AIDS has become more evident recently (6, 9, 20, 22, 26, 28). Two studies in particular strongly support the notion that viral characteristics play a critical role in long-term nonprogression. First, an epidemiological observation made by Learmont et al. (14, 15) showed that seven transfusion recipients from an HIV-1-infected individual have remained clinically well and immunologically stable despite a decade of infection. Likewise, the blood donor has remained healthy. The existence of this cluster of long-term survivors raises the possibility that an attenuated virus strain of HIV-1 was transmitted. Second, experiments carried out by Kestler et al. (11) showed that monkeys experimentally inoculated with simian immunodeficiency virus from which nef had been deleted exhibited no sign of disease and maintained low viral burdens with normal CD4+ T-cell counts. These characteristics are very similar to those seen in long-term survivors of HIV-1 infection, thereby prompting us to determine whether these long-term survivors are infected with viruses that are attenuated because of defects in Nef.

We previously demonstrated that most of the nef alleles derived from long-term survivors in our study were full length with an open reading frame (10), in contrast to one unique case reported by Kirchhoff et al. (13). At the protein level, there were no discernible differences between the Nef consensus sequences derived from our long-term survivors and those from patients with AIDS, although a substantial degree of sequence diversity was noticed. Here we examine the possibility that functional defects in Nef are responsible for the well-being of our long-term survivors.

To characterize nef functionally, we created chimeric viruses that contain nef derived from long-term survivors. Commonly, this process is accomplished by enzyme digestion and ligation (21). However, this technique is frequently hampered by a lack of appropriate recognition sites for restriction enzymes. Consequently, the reconstituted chimeras often contain undesired flanking sequences at both ends of the target gene, which could, in principle, alter the properties of the gene in an assay system. Here we report a new technique that allows the precise replacement of the nef of an infectious molecular clone, HIV-1BHXB2, with alleles derived from study subjects without limi-
FIG. 1. Schematic diagram of the site-directed gene replacement procedure. Phagemid single-stranded DNA containing the 3' half of the HIV-1 HXB2 genome (BamH1-Xbal fragment) in the pAlter-1 vector was prepared by a standard polyethylene glycol-phenol procedure (Promega). HIV-1 nef (approximately 650 bp in length) was amplified by PCR (94°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min) directly from a previously sequenced clone (10), with precise start and stop codons delineated by primers corresponding to individual nef sequences (see Fig. 2). Prior to its use in the mutagenesis reaction, the nef PCR product was treated with 10 to 12 U of either T4 or T7 polymerase (New England Biolabs) in 5 to 7 mM MgCl2 (by adding an appropriate amount of MgCl2 directly to the PCR product) to remove the 3’-deoxyadenosine overhang that had been added nonspecifically by Taq polymerase during amplification (5). The modified nef PCR product was then gel purified by GeneClean (Bio 101) and 5’ phosphorylated (50 mM Tris-HCl [pH 7.5], 10 mM MgCl2, 5 mM dithiothreitol, 0.1 mM spermidine, 1 mM ATP). Because of the inactivation of the ampicillin resistance gene in the single-stranded (ss) DNA template, the ampicillin repair oligonucleotide was added to restore the resistance phenotype; this subsequently served as a marker to screen mutants. This step was accomplished by annealing ampicillin repair primer to the ss DNA template simultaneously with the modified nef PCR product at 95°C for 5 min, with subsequent gradual cooling to room temperature. Subsequent steps, including mutant-strand synthesis and ligation, transformation of competent bacteria, and preparation of plasmid DNA, were carried out according to the manufacturer’s (Promega) instructions.
tions imposed by the need for restriction enzyme sites. This method, which we term site-directed gene replacement, relies primarily on the use of in vitro mutagenesis and PCR amplification techniques. The key feature of this method is that instead of short oligonucleotides, a denatured PCR product (confined to the exact region of interest by primers) is used as an initiation primer to perform mutant-strand synthesis and ligation, thereby controlling gene replacement to the level of a single nucleotide.

As illustrated in Fig. 1, modified PCR amplicons containing nef were denatured and annealed to the single-stranded DNA template containing the nef gene of the HIV-1 HXB2 BamHI-XbaI fragment. Following mutant-strand synthesis and ligation, the mutated plasmids were transformed into competent bacteria. Screening for mutants with the nef sequence was carried out by direct double-stranded DNA sequencing with a Sequenase kit (United States Biochemical Corp.). Under these conditions, the gene replacement efficiency was approximately 23%. The proviral chimeric construct was generated by transferring the mutated BamHI-XbaI fragment of the HIV-1 HXB2 genome, which contained the patient's nef allele, into the remaining HIV-1 HXB2 background in the pBluescript vector. As controls, the Nef+ HIV-1 used in this study was a modification of the HXB2 molecular clone in which the premature stop codon at position 118 was opened by site-directed mutagenesis (stop → Trp), whereas Nef+ HIV-1 was constructed by replacing the start codon of HXB2 nef with a stop codon (M → stop) (Fig. 2). In order to generate infectious viruses, 20 μg of each plasmid construct was transfected into cells of the 293 line by calcium phosphate precipitation (21). Two days posttransfection, virus-containing supernatants were harvested, filtered through a 0.45-μm-pore-size filter, aliquoted, and frozen at −70°C. The p24 concentrations in supernatants as determined by an antigen capture assay (Abbott Laboratories) were relatively high, ranging from 600 to 2,000 ng/ml. The numbers of virion-associated RNAs in supernatants were also determined by a quantitative, branched DNA signal amplification assay (Chiron Corp.). The amount of each virus used in subsequent experiments was normalized by these two parameters; the inocula were approximately 5 ng of p24 antigen or about 1.7 × 10^6 HIV-1 RNA copies.

Although the precise function of nef is unknown, recent studies have shown a positive effect of nef on the rate of viral replication in primary peripheral blood mononuclear cells (PBMC) (7, 18, 27, 30) as well as in certain T-cell lines (4). To examine whether nef alleles derived from long-term survivors were capable of enhancing HIV-1 replication in vitro, we chose the predominant nef sequence from each of 10 long-term survivors (Fig. 2) and introduced these sequences into the infectious molecular clone HIV-1 HXB2. A nef gene derived from a patient with AIDS was also introduced into HXB2 as a control. We reasoned that the predominant sequence is more representative of a viral population in vivo than minor variants are. The replication kinetics of chimeric viruses were tested in phytohemagglutinin-stimulated PBMC and in initially resting PBMC that were activated by phytohemagglutinin 2 days postinfection. As shown in Fig. 3, the replication kinetics of viruses in initially resting PBMC were slightly slower than those in mitogen-pretreated PBMC. In both cases, Nef+ HIV-1 HXB2 replicated more efficiently than Nef− HIV-1 HXB2 did. The difference was more profound (approximately 100-fold) in initially resting PBMC cultures (Fig. 3B), which is

![Multiple sequence alignment and comparison of Nef protein sequences that were functionally characterized.](http://jvi.asm.org/pdfs/notes/8144_02.jpg)
To further characterize the biological properties of nef alleles derived from long-term survivors, we utilized the single-cell infection assay (MAGI assay) developed by Kimpton and Emerman (12). In this assay, the infection of HeLa-CD4-LTR-β-gal cells by HIV-1 induces Tat-dependent, long terminal repeat-driven β-galactosidase expression, thus causing infected cells to stain blue in the presence of X-Gal. This substrate was used in the single-cell level. As in replication kinetics assays, 5 ng of p24 per well of viral supernatant was used to initiate each infection. In accordance with previous studies (18), we found that Nef+ HIV-1HXB2 is approximately 10-fold more infectious than Nef– HIV-1HXB2 in this assay (Fig. 4). The infectivities of chimeric viruses containing nef alleles derived from long-term survivors were comparable to that of Nef+ HIV-1HXB2, all of these viruses infected more cells than Nef– HIV-1HXB2 did, ranging from 7.2-fold for nef from patient 4 to 14-fold for nef from patient 9. Similarly, a ninefold enhancement in infectivity was observed for a chimeric virus containing a nef allele from a patient with AIDS. These results suggest that nef alleles derived from long-term survivors are not inherently defective and can facilitate infection events leading to Tat production in the first round of infection.

By comparing the individual consensus Nef sequences of our study subjects with the consensus Nef sequence obtained by Shugars et al. (25) from patients with AIDS, we have previously shown that the sequences of proposed functional regions are highly conserved in most cases (10) (Fig. 2). A few changes were observed in specific regions; for instance, the consensus sequence of nef from patient 7 was found to have a glycine-to-serine change in the second position of the N-terminal myristylation signal sequence (GGXXS) (Fig. 2). Changes from lysine to glutamine in the polyuracil tract were also observed in nef from the same subject. However, the (PXXP) repeat sequence which resembles an SH3-binding site (19) is invariant. Another well-conserved (PXXP) SH3-binding motif close consistent with previous reports (18, 27). A comparison of the growth kinetics of the chimeric viruses carrying nef alleles derived from long-term survivors with that of the virus containing nef derived from a patient with AIDS revealed no obvious differences (Fig. 3). All chimeric viruses replicated with kinetics virtually identical to that of the parental Nef+ HIV-1HXB2. These results indicate that nef alleles derived from our long-term survivors can enhance viral replication in PBMC in vitro.
to the C terminus was found to be replaced by PXQX in nef from patient 5. Nevertheless, these changes do not seem to have a measurable impact on nef-mediated viral replication and infectivity.

In summary, we have developed a site-directed gene replacement technique which allowed us to replace the nef gene of an infectious molecular clone, HIV-1HXB2, with nef alleles derived from 10 long-term survivors and from a patient with AIDS. We chose one predominant sequence from each subject and biologically characterized these nef alleles in the context of infectious virus. Our results demonstrated that the replication competencies of chimeric viruses containing nef alleles derived from long-term survivors were comparable to that of Nef+ HIV-1HXB2. Moreover, these chimeric viruses were always more infectious than Nef− HIV-1HXB2 in a single-cell infection assay. Thus, we conclude that the genotypic and phenotypic features of nef are not likely to account for the non-progression of HIV-1 infection in the 10 cases we studied, unless the function of the nef gene in vivo is not accurately reflected by the in vitro assays we used.

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