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Regulatory protein Nef of Human Immunodeficiency Virus type 1 (HIV-1) is considered to be crucial for viral replication but its critical role in AIDS pathogenesis was first recognized when deletion of Nef was correlated with slow progression of infection. It was observed that some subjects infected with HIV-1 carrying naturally occurring Nef deletions became long-term nonprogressors.

Nef, a Negative Factor, is a 25-30 kD early phase myristoylated regulatory viral protein. Multiple and distinct functions have now been attributed to it which include down regulation of cell surface CD4 and MHC-1 receptors, modulation of cellular activation, influencing signal transduction pathways and enhancement of viral infectivity. It is also known to delay apoptosis of infected cells. More than 30 putative Nef targets and associated functions have already been identified. However, the molecular mechanisms and their contribution to pathogenesis are not fully elucidated. Most of the research findings discussing biological role of Nef are based on subtype B viruses predominant in the Western world while in India subtype C infection is dominant.
HIV-1 Subtype C infections have risen in numbers during the past 10 years and now constitute the predominant subtype worldwide. In India, over 5.28 million individuals are estimated to be infected with HIV and more than 80% of the strains tested belong to subtype C. However, characterization of subtype C isolates, particularly from India, is still lacking. Few researchers have reported that subtype B and subtype C viruses differ in viral fitness and infectivity in primary CD4+ T cells, macrophages and skin-derived human Langerhans cells. Genotypic differences in structural genes like env, pol and gag are also reported but data on regulatory gene, nef from Indian isolates is scarce. Hence, our present study aimed at critically evaluating Nef from Indian isolates with following objectives

1) To analyze genetic diversity in nef from HIV-1 Indian subtype C virus and evaluate its phylogenetic relationship with other major clades of HIV-1.

2) To study variations in Nef in context with rate of disease progression.

3) To study effect of clade-specific nef sequences on the viral fitness and growth characteristics.
4) To identify crucial domains in Nef influencing viral fitness using systematic site-directed mutagenesis.

Results from our study on nef characterization, broadly provide information about its genetic diversity and its influence on viral fitness.

**Genetic analysis of Indian HIV-1 nef: Subtyping, variability and implications**

The study population included five individuals at early seroconversion (SC) stage, five long-term survivors (LTS) and four subjects with normally progressive HIV-1 infection. LTS were asymptomatic for 8-10 years post infection without antiretroviral therapy. Seroconverters were viral antigenic positive (p24/gag) but with undetectable serum antibody response and were identified during a cohort study of individuals with high-risk behavior. The four symptomatic subjects having persistent opportunistic infections were selected at random as control group.

nef gene was cloned and sequenced from all the 14 study participants, and analyzed for genetic diversity and phylogeny. Attempts were also made to predict *in silico* putative HLA-1 binding epitope for Indian Nef sequences in context with common Indian HLAs.
Phylogenetically, Indian Nef sequences clustered away from African subtype C sequences, consensus C, ancestral C and representative sequences from other clades. The difference between Indian subtype C and African subtype C sequences, especially for the C terminal region, was as high as 25%. This genetic diversity reemphasized that Indian sequences tend to form a distinct subclade within the clade C, not only for structural proteins as reported previously, but also for a regulatory protein like Nef, thus suggesting a possibility of using regulatory gene sequences for viral subtyping studies.

Major functional domains in Nef were conserved in all sequences although we could identify few variations in juxtaposition to some of these functional domains, especially in seroconverter sequences. Our study provided no evidence of association between defective Nef and slow progression of disease in LTS.

HLA-1 binding epitope prediction software indicated that specific substitutions in predicted Nef epitopes, from seroconverters, can influence the affinity/strength of epitope binding between epitopes and HLA-motifs. This may eventually alter the outcome of immune response and would be useful in identifying critical Nef epitopes.
responsible for virus control and subsequent use of such epitopes in HIV-1 vaccine formulation for Indian population.

**Generation and characterization of HIV-1 clones chimeric for subtypes B and C nef**

The difference in ability of Nef from subtype B & C to influence viral fitness was evaluated by generating Nef (B/C and C/B) chimeras. To enable this construction, parental proviral clones pNL432 (subtype B) and pIndie-C1 (Indian subtype C) with distinct viral growth phenotype and having considerable sequence heterogeneity in nef were used to design cassette vector reporter systems, which precisely facilitated alteration in nef.

Using these reporter systems, chimeras with respect to the nef gene were constructed between subtypes B and C, and monitored for their replication kinetics and infectivity in stimulated human peripheral blood mononuclear cells. Our results showed no significant growth difference between the wild type and chimeric viruses. Although presence of Nef was crucial for viral replication, Nef from two different subtypes did not differ in their ability to influence *in vitro* viral infectivity in natural target cells. Nevertheless, this cassette vector
reporter system can be directly applied to analyze Nef from different categories of HIV infected patients.

**Generation and characterization of HIV-1 Nef mutants**

An attempt was made to identify the crucial functional domains in Nef which have capacity to alter its activity, and in-turn, viral infectivity. We constructed 55 mutant virus strains of proviral parental clone pNL432 with single or multiple point mutations in Nef. The mutants were designed based on analysis of sequence conservation across all major clades of HIV-1 and published structure-function reports.

The infectivity of the mutant strains in Nef dependent MAGI cells and replication kinetics in stimulated human PBMCs was studied. Nef expression levels were estimated for all the mutants using western blot analysis. Out of 55 mutants constructed, 14 mutants had markedly reduced infectivity in Magi cells. These mutants were further analyzed for growth kinetics in activated human PBMCs. Out of these 14 mutants, 11 mutants had impaired growth kinetics as compared to wild type strain.
Nef expression level was also monitored for all the mutants constructed. Out of 55 mutants, 12 mutants had substantially reduced Nef expression level. Five mutants with reduced Nef expression level also showed impaired infectivity and growth kinetics. This can be either ascribed to lower synthesis or enhanced degradation. No reports are available correlating Nef expression level and viral fitness and our results suggest this new possibility. We also report that amino acid V30, G119, P136, W141, L189A and H193 are crucial in Nef and have capacity to influence viral fitness.

The mutants constructed in the present study can be further characterized to assess the impact of substitutions in Nef on other Nef functions such as downregulation of CD4, CD28 and MHC-I on cell surface, signal transduction pathways etc. These results will further provide insight into biological relevance of nef gene.