A Role for Natural Simian Immunodeficiency Virus and Human Immunodeficiency Virus Type 1 Nef Alleles in Lymphocyte Activation

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A T-lymphoid cell line termed 221 was derived from a rhesus monkey infected with herpesvirus saimiri. Growth of 221 cells was dependent on the addition of interleukin-2 (IL-2) to the culture medium. In the absence of IL-2, 221 cells arrested in G_0-G_1 but did not die. Simian immunodeficiency virus (SIV) replicated efficiently in IL-2-stimulated 221 cells whether or not the *nef* gene was present. In the absence of IL-2, *nef*-containing SIV replicated 8 to 100 times more efficiently in 221 cells than did the same virus lacking *nef*. *nef*-containing virus preferentially stimulated the production of IL-2 from 221 cells. HIV-1 *nef* and v-*ras* genes, but not the c-*ras* gene, were shown to substitute functionally for SIV *nef* when tested as recombinant viruses in this assay system. These results demonstrate a role for natural *nef* in causing lymphoid cell activation, and they provide a system for delineating the biochemical mechanisms responsible for this activation.

Several lines of evidence have previously suggested a role for the *nef* gene in causing lymphocyte activation. Transgenic mice that expressed human immunodeficiency virus type 1 (HIV-1) *nef* in the thymus displayed T-cell hyperactivation, CD4 downregulation, and arrested T-cell development (47). Expression of a CD8-*nef* chimera on the surface of Jurkat cells resulted in increased expression of activation markers, increased tyrosine phosphorylation, and increased activation of NF-κB (5). Simian immunodeficiency virus (SIV) isolate SIVpbj14 as well as a variant of SIVmac239 contain *nef* alleles that strongly activate lymphocytes in resting cultures of peripheral blood mononuclear cells (PBMC) (16, 17). These *nef* alleles contain tyrosines in a specific sequence that resembles immunoreceptor tyrosine-based activation motifs; the presence of this sequence in *nef* allows for high-level virus replication in lymphocytes without prior lectin stimulation and without addition of exogenous interleukin-2 (IL-2) (16, 17).

Since the tyrosine-containing *nef* alleles were derived from unusual SIV strains with unusual properties, it has been argued that they may be aberrations that do not reflect the properties of natural *nef*. Bolstering this view are reports that *nef* may actually inhibit lymphocyte activation (5, 12, 20, 22, 33, 39). The situation is further complicated by a large number of additional activities as well as a large number of cellular partners that have been associated with *nef* expression. These include CD4 downregulation (2, 7, 19, 34), major histocompatibility complex class I downregulation (46), infectivity enhancement (3, 36, 45), and association with β-COP (6), Src family kinases (17, 43), a serine/threonine kinase (44), a thioesterase (32), and a variety of other cellular proteins (22).

In this report, we describe a new assay system which demonstrates the lymphoid-activating properties of natural *nef* alleles of SIV and HIV-1. The presence of a *nef* gene in this assay system allows for much higher levels of virus replication, consistent with the phenotype associated with *nef* in SIV-infected monkeys (29) and HIV-infected humans (13, 30).

MATERIALS AND METHODS

Fluorescent-activated cell sorting (FACS). 221 cells were incubated with anti-CD4 fluorescein isothiocyanate-conjugated (OKT-4) and anti-CD8 phyco-erythrin-conjugated (51-1) monoclonal antibodies (American Type Culture Collection, Rockville, Md.). Cells were washed with phosphate-buffered saline and analyzed with a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, N.J.). Results were quantitated by using Cell Quest software (Becton Dickinson). Cell cycle analysis was performed by propidium iodide staining. A total of 5×10^6 221 cells were incubated in the presence of 10% IL-2 or in the absence of IL-2 for the indicated times. Cells were fixed with 95% ethanol, pelleted by centrifugation, and resuspended with 1 ml of a solution containing 50 µg of propidium iodide per ml, 0.6% Nonidet P-40, and 0.1% sodium citrate. The stained cells were analyzed by FACScan. The percentage of cells in different phases of the cell cycle was determined by using the ModFit cell cycle analysis program.

221 cell infections. 221 cells were grown in RPMI 1640 (Gibco BRL, Grand Island, N.Y.) medium with 25 mM HEPES buffer (Gibco BRL) supplemented with 10% IL-2 (60 to 90 U per ml; Hemagen, Waltham, Mass.) and 20% fetal calf serum (FCS; Gibco BRL). Every 3 to 4 days, the cells were pelleted by centrifugation at 1,000 rpm in a H6000 rotor in a RC3B centrifuge (DuPont Sorval, Newtown, Conn.) and resuspended in two to three times the original volume. For SIV infections, 10^6 cells were resuspended in 1 ml of medium with the indicated concentrations of FCS and IL-2 in a 48-well tissue culture plate (Corning CoStar, Cambridge, Mass.). Unless otherwise specified, 10 ng of SIVmac239 or a derivative virus was used for all infections of 221 cells.

Plasmid construction. All mutations described in this report were engineered by using the overlap extension technique (23). To maximize the translation of foreign genes expressed in the *nef* locus, the four ATGs in the region of SIVmac239 where the envelope and *nef* genes overlap were eliminated without altering the predicted Env amino acid sequence. Unique *Xba*I, *Eag*I, and *Sac*II restriction sites were then engineered immediately 3′ of the stop codon of the SIV envelope (base 9244 [41]) to create the vector pSIVΔnefXES. This vector contained a 189-bp deletion in the *nef* gene. *Xba*I and *Sac*II restriction sites were introduced at the 5′ and 3′ termini, respectively, of the v-*ras* gene. The sequence of the 5′ terminus of this DNA fragment was TCTAGAATGTAT. The A at the −3 position relative to the start codon is optimal for translation initiation (31), whereas the T at the +4 position is less than optimal but could not be mutated without affecting the amino acid sequence of *ras*. The engineered *ras* gene was inserted into the *Xba*I and *Sac*II sites of pSIVΔnefXES to produce the vector pSIVΔnef/v-ras. Amino acid 12 of the v-*ras* gene was mutated from an arginine to a glycine to create pSIVΔnef/c-ras. Unique *Avr*II and *Bam*HI restriction sites were introduced 120 bases 5′ of the NF-κB binding sites (41) in the 3′ long terminal repeat of pSIVΔnefXES to produce pSIVΔnefXESAB. *Xba*I and *Bam*HI restriction sites were engineered at the 5′ and 3′ termini of *nef* of HIV-1 NL4-3 and Rulda. The 5′ termini of these DNA fragments (TCTAGAACGCC
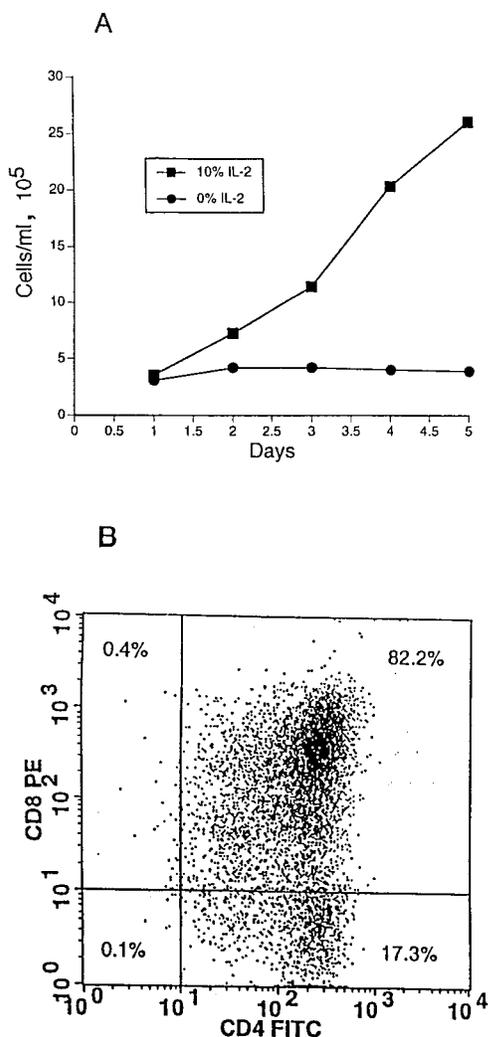
FIG. 1. Characteristics of the 221 cell line. (A) Growth of 221 cells in medium containing 20% FCS, either in the presence or in the absence of 10% IL-2. A 48-well tissue culture plate was seeded with 3×10^5 221 cells at the start of the experiment. (B) FACS analysis of 221 cells grown in 20% FCS and 10% IL-2. PE, phycoerythrin; FITC, fluorescein isothiocyanate.

ACC<u>ATG</u>G) were engineered to introduce a sequence optimal for translation initiation immediately surrounding the start codon (31). The HIV-1 *nef* sequences were engineered into the *Xba*I and *Bam*HI sites of pSIVΔnefXESAB to produce pSHIVnef/NL4-3 and pSHIVnef/Rulda. Thorough DNA sequence analysis was used to verify that the proper sequences for all mutants and recombinant clones were selected for study.

SIV replication assays. All stocks of SIV described in this report were generated by DEAE-dextran transfection (38) of cell line CEMx174. Levels of p27 viral protein produced from transfection or infection or in virus stocks were quantified by using an SIV core antigen kit (Coulter, Hialeah, Fla.).

IL-2 secretion. IL-2 secretion into the media of infected 221 cell cultures was detected and quantified by using an IL-2 quantification kit (Genzyme, Cambridge, Mass.).

RESULTS

Establishment of the 221 lymphoid cell line. Herpesvirus saimiri is a T-cell-transforming virus of New World primates (15). Strain C488 has been shown to possess a potent oncogene (28) and to exhibit transforming activity with human cells (8). Rhesus monkey 221-89 was infected with herpesvirus saimiri strain C488 by intravenous inoculation. By 15 days postinfection, the inoculated rhesus monkey developed a lymphoprolif-

erative disease, became moribund, and was euthanized. PBMC obtained on day 14 were cocultivated with owl monkey kidney cells, and herpesvirus saimiri was recovered. The lymphocytes were removed, washed with medium, and cultured in RPMI 1640 with 20% FCS and IL-2. The initial passages of 221 cells produced herpesvirus saimiri on the basis of cocultivation with OMK cells, but subsequent passages of the cells produced no detectable herpesvirus saimiri. Passaged 221 cells maintained herpesvirus saimiri genetic information, and the herpesvirus saimiri STP oncoprotein (28) and v-cyclin (27) were shown to be expressed by Western blot analysis (data not shown).

The growth of 221 cells is IL-2 dependent. In the presence of 10% IL-2 (60 to 90 U per ml; Hemagen) and 20% FCS in RPMI 1640, 221 cells exhibited a doubling time of approximately 32 h (Fig. 1A). In the absence of IL-2, with 20% FCS in RPMI 1640, little or no expansion of 221 cells occurred (Fig. 1A). Cell cycle analysis revealed that IL-2-starved 221 cells arrested in G_0-G_1 (Table 1). The 221 cell line can be maintained in a quiescent state without significant loss or gain of the numbers of cells for at least 10 weeks, the longest time period that we have examined. Staining with monoclonal antibodies and FACS analysis revealed that 99% of 221 cells expressed CD4 and 83% expressed CD8 on their surface (Fig. 1B). The double-positive phenotype is surprising because such cells are present at low frequency in peripheral blood. It seems likely that herpesvirus saimiri either altered the expression profile in cells upon transformation or selectively targeted this cell type.

***nef*-dependent virus replication and cellular activation.** SIVmac239 replicated in IL-2-stimulated 221 cells in a *nef*-independent fashion. *nef*+ and *nef*− SIVmac239 replicated indistinguishably in repeated experiments in 221 cells growing in the presence of IL-2 (Fig. 2A). This result is similar to numerous earlier experiments in which *nef*+ and *nef*− SIVmac239 replicated indistinguishably in CEMx174 cells and in lectin-stimulated rhesus PBMC cultures (21, 29). However, SIVmac239/nef-open replicated much more efficiently than SIVmac239Δnef in 221 cells in the absence of IL-2 stimulation (Fig. 2B). In repeated experiments performed by different investigators in the laboratory under identical or slightly different assay conditions, *nef*+ virus replicated to levels that were 8 to 100 times higher than those obtained with the matched *nef*− virus. The results shown in Fig. 2 were obtained with *nef*− virus that contained a 182-bp deletion in the nonoverlapping portion of the *nef* reading frame (29). However, the same impaired replication was observed with a point-mutated *nef*− virus that contained a TAA stop signal at the 92nd codon of the *nef* reading frame that was otherwise identical to SIVmac239/nef-open at all remaining base pairs (data not shown). The effect of *nef* was observed whether infection was initiated immediately after removal of cells from IL-2 or days or weeks after removal of cells from IL-2. The Ynef variant of SIVmac239 (16, 17) also replicated well in 221 cells in the absence of IL-2; in fact, the Ynef variant replicated to 10 to 50% higher levels than SIVmac239 (data not shown). The *nef*-dependent effects ob-

TABLE 1. Cell cycle analyses of 221 cells

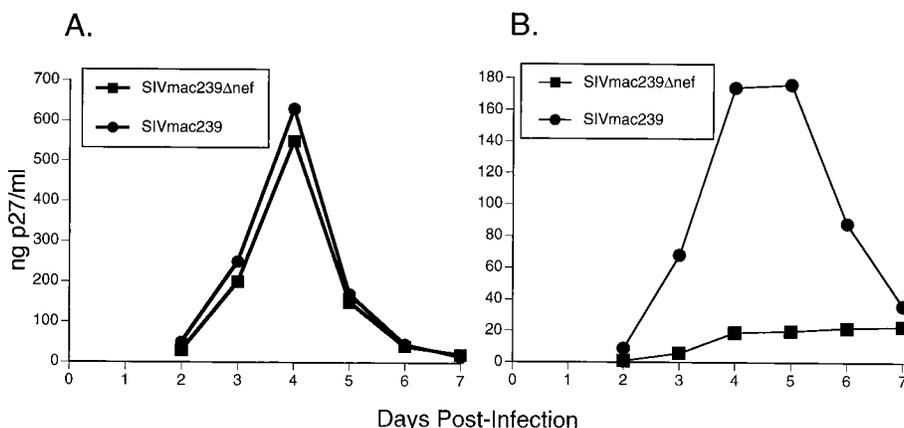| Cell condition	% of cells in:		
	G_0-G_1	G_2-M	S
+10% IL-2	64.09	7.42	28.49
No IL-2			
18 h	84.43	4.48	11.09
14 days	95.35	0.81	3.84
10 wk	94.08	1.19	4.73

A.

B.

FIG. 2. Permissivity of 221 cells for SIVmac replication. (A) Growth of *nef*+ and *nef*− viruses in 221 cells in medium containing 20% FCS and 10% IL-2. (B) Growth of *nef*+ and *nef*− viruses in 221 cells in medium containing 5% FCS in the absence of IL-2. For both panels, 221 cultures were infected with either SIVmac239/nef-open or SIVmac239Δnef containing 10 ng of p27. Virus production in the cell-free supernatant was monitored by quantitation of p27^{gag} antigen.

served in 221 cells do not appear to be peculiar to this particular cell line; we have obtained similar results with two additional herpesvirus saimiri-transformed rhesus monkey T-cell lines (data not shown). Thus, the presence of *nef* is able to substitute functionally for IL-2 stimulation in this assay.

In addition to IL-2, FCS is an essential cofactor for 221 cell growth. Unstimulated 221 cells were incubated in different concentrations of serum and infected with *nef*+ or *nef*− SIVmac239. In these experiments, the efficiency of virus replication decreased with decreasing serum concentration for both SIVmac239 and SIVmac239Δnef (Fig. 3). However, SIV with *nef* replicated much more efficiently at all serum concentrations tested. This was the case even in the complete absence of serum. The effects of *nef* in this assay appear to be most dramatic at the lowest serum concentrations.

It has been reported that *nef* may enhance the infectivity of HIV (3, 36, 45). Infectivity enhancement has been particularly evident in HeLa-CD4 cells. We thus investigated the effects of input dose on virus replication in 221 cells. Increasing the input dose of *nef*− virus from 1 to 50 ng did manage to increase the levels of replicating virus that were produced from 221 cells in the absence of IL-2 (Fig. 4). However, even with 50 ng of input *nef*− virus, the levels of SIV produced were still markedly reduced compared to a 1-ng input of the *nef*+ virus (Fig. 4). The infectivity titers for 221 cells for the viruses used in these

experiments were 2,440 50% tissue culture infective doses per ng of p27 of the *nef*+ virus and 920 50% tissue culture infective doses per ng of p27 of the *nef*− virus. Thus, the *nef*-dependent effects in 221 cells in the absence of IL-2 do not appear to relate to a simple infectivity enhancement.

IL-2 is secreted by CD4+ and CD8+ T cells in response to mitogens and natural forms of stimulation. Infection of 221

A

B

FIG. 4. Effect of dose of input inocula. (A) 221 cells in medium with 3% FCS and without IL-2 were infected with SIVmac239/nef-open (□) containing 1 ng of p27 or SIVmac239Δnef (■) containing 1 ng of p27. (B) 221 cells in media with 3% FCS and without IL-2 were infected with SIVmac239/nef-open (□) containing 1 ng of p27 or SIVmac239Δnef (■) containing 50 ng of p27. Virus production was monitored by quantitation of p27*gag* antigen in the cell-free supernatant. IL-2 in the cell-free supernatant was monitored by antigen capture. The line graphs show virus p27 production; the bar graphs show IL-2 production.
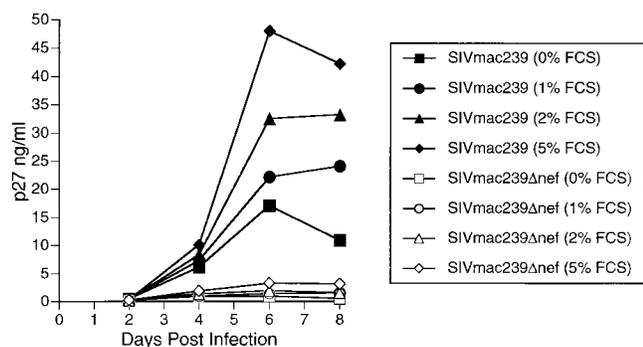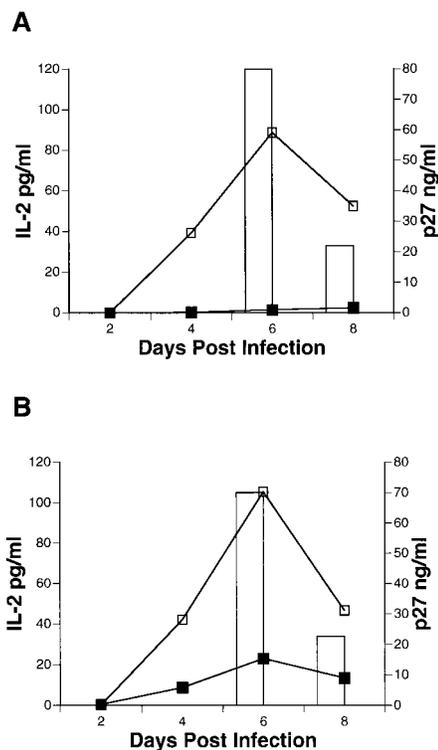
FIG. 3. Effects of FCS on virus replication. 221 cells were incubated in medium without IL-2 in the presence of various concentrations of FCS. Cells were infected with SIVmac239/nef-open (●) or SIVmac239Δnef (○) containing 10 ng of p27. Virus production was monitored by quantitation of p27*gag* antigen in the cell-free supernatant.
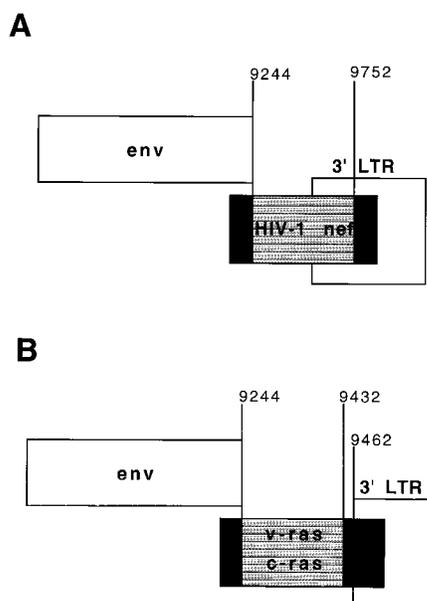
A

B

FIG. 5. Schematic representation of gene sequences in recombinant viruses. The black regions represent residual SIV *nef* sequences. Nucleotide numbers refer to the SIVmac239 sequence described by Regier and Desrosiers (41). In the SHIVnef construct, 509 bp of SIV *nef* sequence are deleted. In the *ras* constructs, 189 bp of SIV *nef* sequence are deleted. LTR, long terminal repeat.

cells with *nef*+ SIVmac239 stimulated the production of IL-2 that became detectable in the culture media (Fig. 4). Peak levels of IL-2 occurred around the time of maximal virus production (Fig. 4). 221 cells infected in parallel with SIVmac239Δnef did not secrete detectable levels of IL-2. In fact, levels of IL-2 were undetectable even with the 50-fold-higher inoculum of *nef*− SIVmac239 (Fig. 4).

Functional substitution for SIV *nef*. Recombinant SIVs that expressed foreign genes were constructed (Fig. 5). The recombinants were constructed in a way that optimized expression of the inserted gene in the *nef* locus (15a). ATGs in the region of *nef-env* overlap were mutated without altering the predicted Env amino acid sequence, and the initiating ATG of the inserted sequence to be expressed was placed immediately downstream of the *env* reading frame in a context appropriate for translational initiation (31). Oncogenic v-*ras* and nononcogenic c-*ras* (4), which differed only at amino acid position 12, were inserted immediately downstream of the envelope reading frame in the SIVmac239Δnef genome. In 221 cells in the absence of IL-2, the v-*ras* virus replicated as well as *nef*+ SIVmac239 (Fig. 6A). In contrast, the c-*ras* virus replicated poorly, just as *nef*− SIVmac239 (Fig. 6A). Thus, v-*ras*, but not c-*ras*, can functionally substitute for nef in the absence of IL-2 stimulation.

SIV *nef* sequences were also replaced with HIV-1 *nef* sequences. *nef* sequences from the infectious clone NL4-3 (1) were able to substitute functionally for SIV *nef* sequences in 221 cells in the absence of IL-2 (Fig. 6A and B). The NL4-3 *nef* allele did result in slightly slower replication in this assay than SIVmac239 with its own *nef* gene. However, a *nef* allele derived from a patient coded Rulda imparted replication kinetics similar to, or even slightly better than, those of SIVmac239/nef-open (Fig. 6B). Thus, natural HIV-1 alleles can substitute functionally for SIV *nef* in the 221 cell assay system.

We also measured the induction of IL-2 secretion by HIV/SIV *nef* (SHIVnef) recombinants. High levels of IL-2 secretion

were induced by SHIVnef (NL4-3) and SHIVnef (Rulda), comparable to the levels obtained with SIVmac239/nef-open (Fig. 6C). This contrasted with the levels obtained with SIVmac239Δnef, which again were below the limits of detection as in previous experiments.

DISCUSSION

The results described in this report define simple conditions under which *nef*+ SIV replicates 8 to 100 times more efficiently
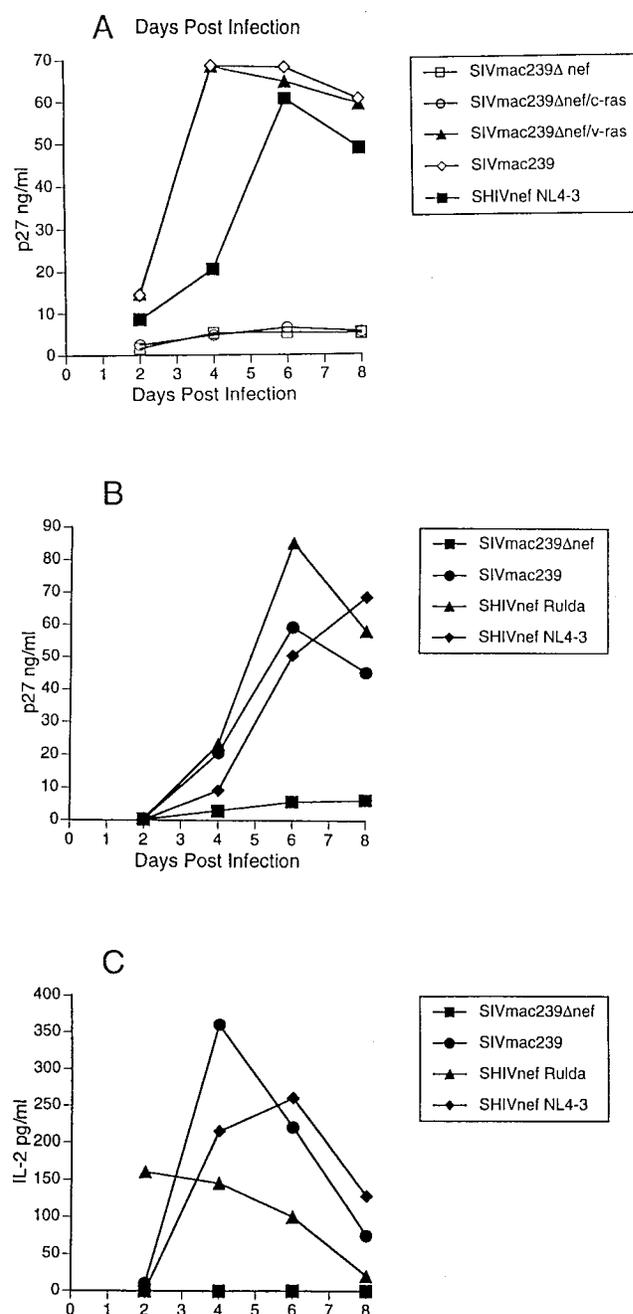

FIG. 6. Replication of recombinant SIV and stimulation of IL-2 release. 221 cells were infected with the indicated viruses containing 10 ng of p27. Cells were incubated in medium with 3% FCS and without IL-2. (A and B) Virus replication; (C) IL-2 production.

than *nef* − SIV. This large difference in virus replication contrasts markedly with a variety of other conditions described here and in previous reports for SIVmac239 (21, 29). While subtle differences have sometimes been noted in the replication of *nef*+ versus *nef*− HIV or SIV (14, 36, 48), such a dramatic effect of *nef* on replication has been noted previously only for Ynef-containing SIVs in unstimulated PBMC cultures (16, 17). The increased *nef*-dependent replication described here may be analogous to the positive effects of *nef* when unstimulated PBMC are infected and then subsequently stimulated several days later (36, 48). The difference in replication of 8- to 100-fold described here is similar in magnitude to that observed in the initial weeks after infection of rhesus monkeys with SIVmac239/nef-open versus SIVmac239Δnef (29).

Both direct and indirect evidence indicates that *nef*-dependent replication in 221 cells in the absence of IL-2 is due to activation of the cells. While virus replication was independent of *nef* in IL-2-stimulated cells, the presence of *nef* was able to substitute functionally for IL-2 stimulation when exogenously added IL-2 was omitted from the media. An oncogenic form of *ras* was in turn able to substitute for *nef* when expressed as a viral gene in the *nef* locus. Oncogenic *ras* is known to induce strong stimulatory signals in a wide variety of cells. Importantly, *nef*+ virus, but not *nef*− virus, induced high levels of IL-2 production from the 221 lymphoid cells. 221 cells resemble most or all normal lymphoid cells in that they do not produce IL-2 spontaneously but do so only upon stimulation. The need for cellular activation for optimal levels of virus replication in lymphoid cells has been well established (35). Lymphoid cell activation increases nucleotide pool sizes, can facilitate the replication of viral genetic information, can facilitate transport of proviral DNA to the nucleus, and increases the levels of transcription factors such as NF-κB important for virus expression (10, 18, 37).

It is important to note that SIVmac239 and the SIV recombinants that replicate well in 221 cells in the absence of IL-2 do not replicate well in unstimulated rhesus monkey PBMC. Thus, stimulatory signals that are provided by natural *nef* alleles appear to be sufficient for 221 cells, but they are not sufficient to allow appreciable levels of virus replication in unstimulated PBMC. The need for two or more signals for optimal lymphoid cell activation has been previously documented. One of the best-studied examples is T-cell receptor stimulation and costimulation provided by B7 on macrophages (24). It seems likely that herpesvirus saimiri provides signals that keep 221 cells from dying in the absence of stimulation and that allow 221 cells to respond strongly to a single stimulus. Herpesvirus saimiri is known to encode a number of proteins that could conceivably provide such a signal or signals. These include STP (25), an Lck-interacting protein (9, 26), a virus-encoded cyclin (27), a Bcl-2 homolog, and a superantigen homolog (49).

How does one reconcile these lymphocyte-activating properties of *nef* with reports that *nef* inhibits lymphocyte activation (5, 12, 20, 22, 33, 39)? There are previous examples that can be cited of signaling proteins that either stimulate or inhibit stimulation, depending on the level of expression, context, cell type, or conditions (11, 50). In this study, we have examined the activating properties of *nef* in a virus replication assay. In this system, *nef* is expressed in lymphoid cells at levels and in a context that is likely to be similar to in vivo conditions. These results, along with our earlier results with Ynef variants, strengthen the argument for the importance of *nef* in causing lymphocyte activation. This argument is complicated, however, by the large number of cellular partners and activities that have been associated with *nef* expression. A partial list of these

includes Src family kinases (43), β-COP (6), a serine-threonine kinase (44), thioesterase (32), p53 (22), CD4 downregulation (2, 7, 19, 34), major histocompatibility complex class I downregulation (46), and infectivity enhancement (3, 36, 45), in addition to lymphocyte activation and inhibition of lymphocyte activation. Additional studies, particularly those involving the analysis of mutants in rhesus monkeys, will be needed to determine which of these different activities may be intimately related and which may be critical for the striking importance of *nef* for virus replication in vivo.

Nef is not only a virion protein (40) but also an early gene product that is synthesized prior to the switch to structural gene product synthesis (42). As such, Nef could conceivably act to increase the state of cellular activation in association with the incoming virion and subsequently as an early gene product. Since most lymphoid cells in the body are not activated or only minimally activated, either or both strategies could facilitate viral replication in vivo. The 221 cell assay described in this report should prove useful in delineating the biochemical details of Nef-induced cellular activation.

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