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Characterization of Nef-Induced CD4 T Cell Proliferation

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Studies on Nef, a regulatory protein encoded by human immunodeficiency virus (HIV), suggest it plays an important role in HIV pathogenesis. Previously, we reported that Nef binds to class II MHC antigens and induces proliferation of human peripheral blood mononuclear cells (PBMC). Herein, we further characterize PBMC responses to Nef. Polyclonal antisera generated against Nef synthetic peptides blocked proliferation. Responses were T cell-specific and required antigen-presenting cells (APC). T cells responded in the presence of paraformaldehyde-inactivated APC, suggesting that Nef is presented in an unprocessed form. Nef-stimulated cells produced IL 2 and IFNγ, products of T helper-1 cells. Thus, Nef has superantigen properties in that it binds to MHC class II antigens, does not need processing to be presented by APC, and activates T cells, causing proliferation and production of the T helper 1 cytokines, IL 2 and IFNγ. The identification of an HIV protein that activates T cells is of considerable interest, given that HIV replicates in T cell blasts but not in quiescent cells.

There has been much speculation that a superantigen is associated with HIV infection. Theoretically, MHC class II on antigen-presenting cells (APC) and HIV superantigen would interact with T cells, resulting in the expansion of T cells bearing specific variable regions of the β chain (Vβ). Chronic activation of these T cells may lead to anergy and/or deletion. Evidence suggestive of a superantigen in HIV includes changes in the Vβ profiles of asymptomatic HIV$^+$ patients (1), as well as skewed Vβ usage by cells from asymptomatic patients upon stimulation *in vitro* with staphylococcal enterotoxins (2). Monozygotic twins discordant for HIV showed skewed Vβ profiles in HIV-infected individuals as compared to their uninfected twin, with perturbations in several Vβs (3). In another report, Vβ12$^+$ T cells, cultured in the presence of irradiated APC, supported enhanced replication of HIV compared to other Vβ subsets (4). Thus, several reports suggest the presence of an HIV superantigen may play a role in pathogenesis by augmenting a population of T cells, resulting in a reservoir for virus replication.

Candidate proteins for an HIV superantigen include regulatory gene products. One of these, Nef, is a myristylated protein that is mainly located in the cytoplasm of HIV-infected cells, but has also been shown to be associated with the plasma membrane (5, 6). Like the superantigen of the retrovirus mouse mammary tumor virus (7, 8), Nef is encoded by a gene in the 3′ long terminal repeat of the HIV genome. Although the role of Nef in HIV pathogenesis is not clearly understood, its importance was shown in a study where a live attenuated virus vaccine with a deletion in *nef* protected adult macaques against infection by pathogenic SIV (9).

We previously showed that Nef binds to MHC class II-bearing Raji cells at a site(s) involved in staphylococcal enterotoxin binding, and induces proliferation of human peripheral mononuclear cells (10). Upon Nef stimulation, increased CD4 T cell populations bearing HLA-DR and IL 2R were observed as well as expansion of T cells bearing specific Vβ regions of TCR (11). Nef stimulation required antigen presenting cells although Nef did not require processing in order to be presented. Most importantly, activation of PBMC by Nef was sufficient to render

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them capable of supporting HIV replication (11, 12). Herein we further characterize the Nef-induced response, and present evidence that Nef activates T cells to produce T helper 1 cytokines in a manner characteristic of superantigens.

MATERIALS AND METHODS

Nef protein. Nef protein was expressed and purified using a fusion protein and purification system. The HIV-1 nef gene (R & D Systems, Cambridge, MA) was amplified by PCR, using the following primer set:

5′ ATG GGT GGC AAG TGG TCA AAA AGT (+)

5′ GCC AAG CTT GAT GTC AGC AGT TCT (−)

After Klenow treatment, the amplified gene was inserted into the cloning site of the prokaryotic expression vector pMALTM-c2 (New England BioLabs, Beverley, MA). The fusion construct was engineered such that the N terminus of Nef was immediately downstream from the factor Xa cleavage site at the C terminus of maltose binding protein (MBP). *E. coli* strain TB1 was transformed with the vector containg mbp/nef and the cultured cells were sonicated. The clarified sonicates were loaded onto an amylose affinity column and the fusion protein was eluted using 10 mM maltose. After treatment with factor Xa (New England BioLabs, Beverley, MA), Nef protein was purified by column chromatography using hydroxyapatite, a second amylose affinity column, and an ion-exchange column (High-Q). Upon staining an SDS-PAGE gel with silver, a single band was found, and a corresponding band was detected by immunoblot with monoclonal anti-Nef antibody (Repligen, Cambridge, MA). MBP was also purified by this method.

Nef from two other sources was tested for proliferative activity. The following reagent was obtained through the AIDS Research and Reference Reagent Program, AIDS Program, NIAID, NIH: HIV-1 LAV Nef from the Division of AIDS, NIAID. The HIV-1 nef gene (LAV) used to produce this Nef preparation was isolated from pBENN 6 and cloned into the bacterial expression vector pPD-YN-61. The protein was produced in *E. coli* strain Sϕ930 and isolated as inclusion bodies. Nef protein was also obtained from Repligen (Cambridge, MA). In this case, the HIV-1 nef gene was cloned into the bacterial vector pD10, which adds a hexahistidine tag for protein purification using nickel chelate chromatography. The protein was produced in *E. coli* strain MC10611POL.1. All Nef preparations were negative for endotoxin as assessed by the limulus amoebocyte lysate assay.

Reagents. Staphylococcal enterotoxin A (SEA) was purchased from Toxin Technology (Sarasota, FL). Concanavalin A (Con A), anti-CD3, and anti-IgM were purchased from Sigma Chemical Co. (St. Louis, MO). Antibodies to Vβ3, Vβ17, and Vβ18 were obtained from Immunotech, Marseiiles, France. Antibodies to Vβ5.3, Vβ8, and Vβ12 were obtained from T cell Sciences, Inc., Cambridge, MA.

Proliferation assays. Proliferation studies on human peripheral blood mononuclear cells (PBMC) were performed as described (10−12). Peripheral blood from healthy adult blood bank donors was used in these studies and all donors were negative for cytomegalovirus, hepatitis B virus, and HIV. PBMC were isolated from peripheral blood using ficoll hypaque gradient centrifugation. After extensive washing, PBMC were plated into wells of microtiter plates at 2.8×10^6 cells/ml, followed by addition of superantigens and mitogens. Final volumes were adjusted to 150 μl/well with RPMI 1640 medium containing 5% fetal bovine serum (FBS) and penicillin/streptomycin. ^{3}H-thymidine (1μCi/well; Amersham Corporation, Indianapolis, IN) was added at 90 h and the cells were incubated for an additional 6 h prior to harvest onto filter paper. Radioactivity was quantified using a β scintillation counter.

Expansion of Vβ-specific T cells. Superantigenic activity of Nef was observed using anti-Vβ mAb in a novel detection method (T. Tanabe et al., manuscript in preparation). PBMC were cultured either in the presence of Nef (5 μg/ml) or with medium alone for 24 h. Following extensive washing, PBMC were plated into the wells of microtiter plates precoated with anti-Vβ antibodies (30 μl of a 10 μg/ml solution). At 66 h, PBMC cultures were pulsed with ^{3}H-thymidine (1 μCi/ml; Amersham, Arlington Heights, IL) and harvested 6 h later. Data are expressed as fold increase, and were calculated by dividing the CPM from Nef-stimulated cultures by the CPM from unstimulated cultures.

Synthetic Nef peptides and antibodies to Nef peptides. Nef peptides were synthesized with a Biosearch 9500AT automated peptide synthesizer using N-((-flurenyl)methoxycarbonyl chemistry (10). The sequences of the peptides can be found in Ref. 10. Polyclonal antibodies to Nef peptides were generated in rabbits. Anti-peptide antibodies were tested for their relative abilities to neutralize Nef mitogenic activity by incubating Nef with antibodies at 37°C for 1 h prior to addition of PBMC. Antibodies to both Nef(123-160) and Nef(182-206) showed strong reactivity to whole Nef protein, and these combined antisera had significant neutralizing activity. A mix of the appropriate preimmune sera was used as a negative control.

Cytokine production and assays. PBMC were plated at 10^6 cell/ml into wells of 24-well plates (Corning Corp., Corning, NY). Activators were added and final volumes were adjusted to 300 μl/well. Cells were cultured at 37°C over a 96 h period and samples (100 μl) were obtained at 24 h intervals. Wells were replenished with RPMI/5% FBS (100 μl). Culture supernatants were tested for IL 2 using IL 2-dependent HT-2 cells as described (10). IL 2 concentrations were determined using recombinant human IL 2 (Genzyme, Cambridge, MA) as a standard. Samples were tested

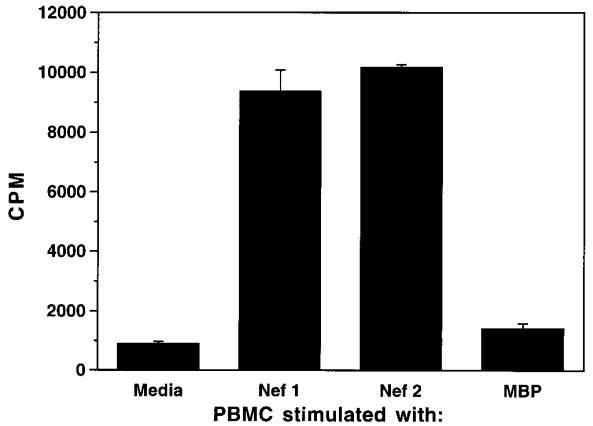

FIG. 1. Comparison of the mitogenic activities of recombinant Nef protein preparations, and the lack of proliferation in response to MBP. The proliferative activity of recombinant Nef protein produced and purified in our laboratory (Nef 1) was compared to Nef obtained commercially (Nef 2) and to the fusion partner MBP. MBP and both Nef preparations were used at 1 μg/ml. Data are from a representative experiment, performed in triplicate, and are expressed as mean ^{3}H-thymidine incorporation in CPM $\pm$ s.d.

for IFN on human WISH cells by a microplaque reduction method (13), using approximately 40 PFU of vesicular stomatitis virus per well. In our studies, 1 U/ml of IFN is defined as the concentration required to decrease PFU per well by 50%. IFN activity was typed by neutralization reactions with specific antisera as described (14).

RESULTS AND DISCUSSION

Nef protein produced and purified in our laboratory (referred to as Nef 1) was compared to purified Nef from Repligen (Nef 2), which was previously shown to induce proliferation of PBMC. Both recombinant Nef protein preparations induced similar levels of proliferation (Figure 1). Nef proteins from both sources were greater than 95% pure and neither preparation contained detectable levels of endotoxin. Thus, Nef proteins from both sources had similar proliferative activity, and the proliferation observed was significant. Nef from these two sources were subsequently used in these studies. The possibility was raised that the proliferative activity ascribed to Nef was due to a contaminant in our recombinant preparation. As a control, the proliferative activity of the Nef fusion partner, MBP, was also assessed. No proliferation was observed with MBP (Figure 1). Further, polyclonal antisera generated against Nef synthetic peptides were tested for neutralizing activity. As shown in Table 1, anti-Nef antibodies significantly reduced Nef-induced proliferative responses. Antibody neutralization of Nef activity occurred in a dose-dependent manner. These data confirm that the proliferative activity seen in these studies is due to Nef protein, and not to a vector-derived contaminant.

Nef was tested for the ability to induce proliferation of PBMC from a wide sampling of donors. Results are shown in Figure 2 of the proliferative responses of PBMC from a representative sample of ten donors. Nef induced significant proliferation in 90% of the donors tested. Variation in the Nef response was seen, similar to the responses to the potent staphylococcal enterotoxin, SEA. It is unlikely that these donors were previously sensitized to Nef, since they tested negative for HIV-1. Similar results were obtained using purified recombinant Nef protein preparations from two different sources, and which used different expression systems (see Materials and Methods). To date, PBMC from over 40 donors have been tested, with 85–90% of these donors reponding to Nef. The high number of donors that responded to Nef indicates Nef protein has similar mitogenic activity to SEA for PBMC from a wide sampling of donors.

A characteristic of superantigens is their ability to induce proliferation and activation of T

TABLE 1
Ability of Anti-Nef Peptide Antibodies to Block Proliferation Induced by Nef Protein

PBMC cultured in the presence of:	Antisera	^{3}H-Thymidine incorporation (Mean CPM ± SD)	p value
Nef	—	5525 ± 219	
Nef	Anti-Nef peptides	1291 ± 112	<0.001
Nef	Preimmune	5626 ± 444	
SEA	—	48952 ± 5207	
SEA	Anti-Nef peptides	50294 ± 4621	>0.15
SEA	Preimmune	51003 ± 5068	
—	Anti-Nef peptides	1361 ± 76	
—	Preimmune	1198 ± 123	
—	—	1151 ± 140	

Nef (3 μg/ml) and SEA (0.3 μg/ml) were incubated with anti-Nef peptide antibodies for 45 min prior to addition of PBMC. Anti-Nef peptides refers to a mixture of rabbit antisera to Nef (123–160) peptide and Nef (182–206) peptide, which were each used at a final dilution of 1:1000. Preimmune sera, which were obtained prior to immunization, were mixed and used at the same final dilution as the anti-Nef peptide sera. Data are from a representative experiment performed in triplicate.

cell subsets expressing specific Vβs. Nef-stimulated cultures were tested for specific Vβ expansion and the results of a representative experiment are shown in Table 2. Expansion of T cell subsets expressing Vβ3, Vβ5.3, and Vβ18 was observed consistently in Nef-stimulated PBMC cultures from several donors. These results have been verified using reverse transcriptase-polymerase chain reaction (RT-PCR), thereby confirming that Nef activates specific Vβ T cell subsets in a manner consistent with that of a superantigen.

We previously showed that a synthetic peptide corresponding to an internal Nef sequence, Nef(123-160), blocked the binding of Nef and SEA to Raji cells (10). It was important to determine if this peptide could also block proliferation induced by Nef and SEA. The results

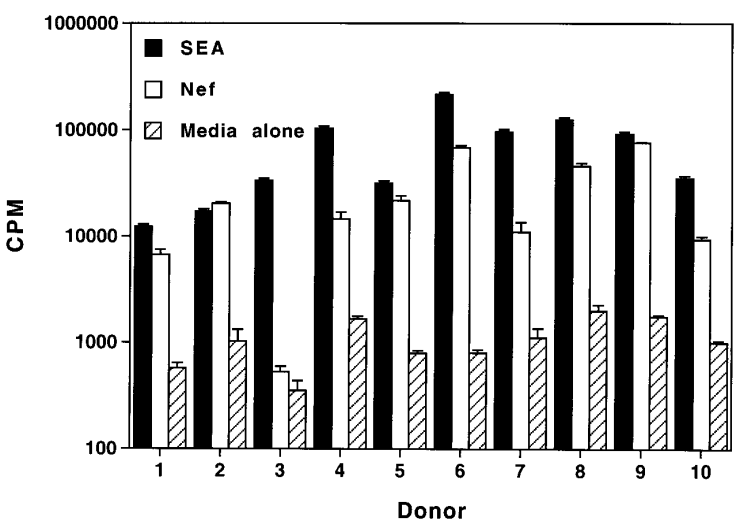

FIG. 2. Nef-induced proliferative responses of PBMC from a representative sampling of HIV-negative donors. PBMC of blood bank donors were tested for proliferation induced by SEA and Nef. Values are also shown for unstimulated cultures. SEA and Nef were both used at 300 ng/ml. Data are presented as mean ^{3}H-thymidine incorporation ± s.d.

TABLE 2
Expansion of Specific Vβ T Cell Subsets in Response to Nef

Vβ	Fold increase (Mean ± s.d.)
3	2.3 ± 0.1
5.3	3.2 ± 0.2
8	1.4 ± 0.1
12	1.3 ± 0.1
17	1.0 ± 0.1
18	4.2 ± 0.3

After 24 h stimulation with Nef (5 μg/ml) or with medium alone, PBMC were plated into the wells of microtiter plates precoated with anti-Vβ antibodies. Values for fold increase were calculated by dividing the CPM from Nef-stimulated cultures by the CPM from unstimulated cultures. Data are from a representative experiment performed in triplicate.

of this study are presented in Figure 3. Nef(123-160) blocked both Nef-induced and SEA induced proliferation, consistent with its ability to block binding of Nef and SEA to Raji cells. Further, the blocking was specific in that proliferation induced by the T cell mitogens Con A and anti-CD3, or the B cell mitogen anti-IgM, were not blocked by Nef(123-160). Nef(157-186), whose sequence slightly overlaps that of Nef(123-160), had no effect on the proliferative effects of either Nef, SEA, or Con A. These results confirm that the proliferative responses observed in PBMC cultures were specific for Nef, and were not due to a contaminant, and

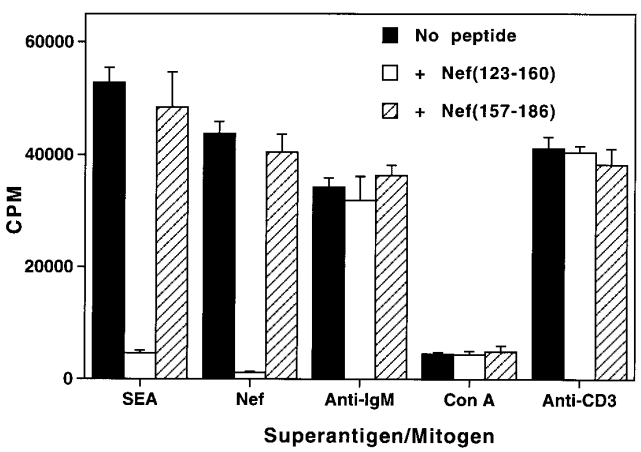

FIG. 3. Nef(123-160) specifically blocks proliferation of PBMC induced by Nef and SEA. PBMC cultures were stimulated for 96 h with mitogens in the absence of peptides or in the presence of either Nef(123-160) peptide or Nef(157-186) peptide. Data are from a representative experiment, performed in triplicate, and are expressed as mean ^{3}H-thymidine incorporation ± s.d. Nef and SEA were used at 300 ng/ml. Anti-IgM and anti-CD3 were used at 10 μg/ml. Peptides were used at a final concentration of 100 μM. The mean value for ^{3}H-thymidine incorporation by unstimulated cultures was 1005±37.

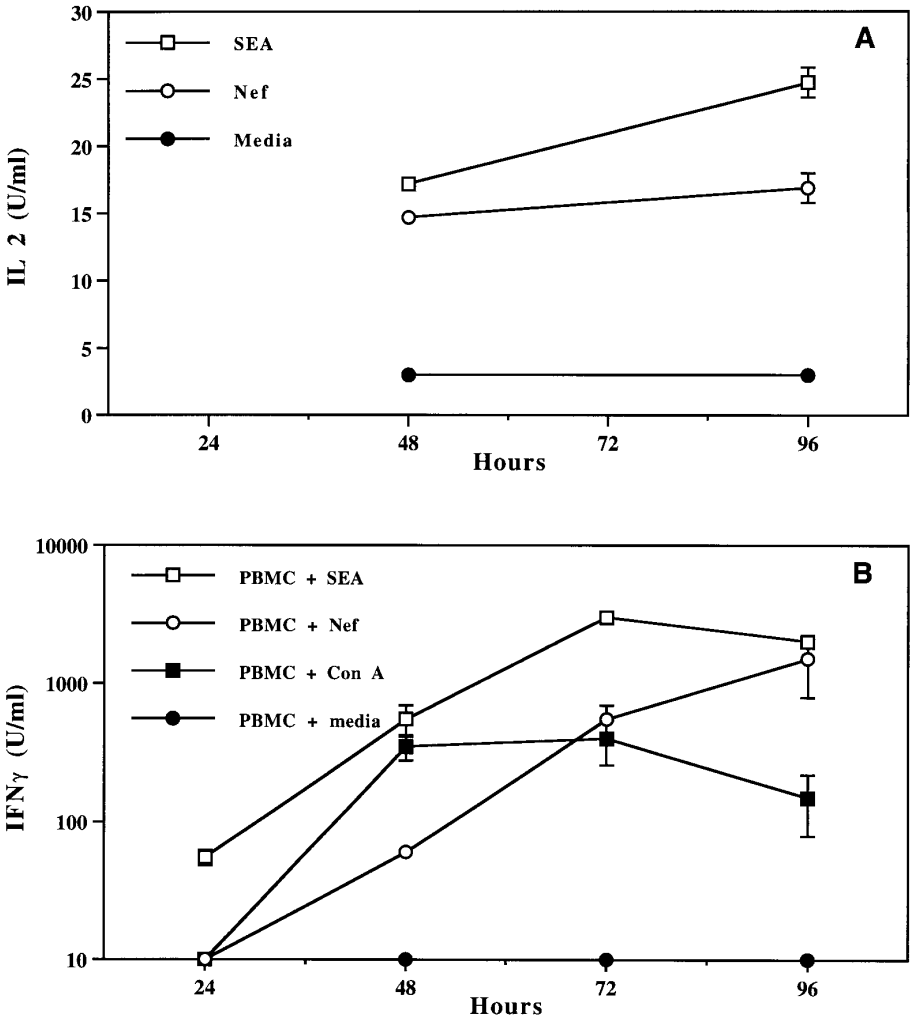

FIG. 4. Kinetics of IL 2 and IFNγ production induced by Nef. Data are from representative experiments performed in triplicate. Panel A: PBMC culture supernatants were tested for IL 2 after 48 and 96 h of stimulation with SEA (300 ng/ml) and Nef (500 ng/ml). Panel B: PBMC culture supernatants were tested for IFN production at 24, 48, 72, and 96 h after stimulation with Nef (1 μg/ml), SEA (100 ng/ml), or Con A (10 μg/ml), or were left unstimulated. All IFN activity produced in response to Nef was IFNγ, as determined by neutralization with specific antisera.

suggest that binding to MHC class II antigens is required for Nef proliferative activity. Binding of Nef to MHC class II occurs on APC, since Nef proliferative activity also requires presentation, but not processing, by APC. Thus, proliferative responses to Nef and SEA were specifically blocked by a peptide corresponding to the region on Nef that binds to class II antigens and which blocks binding of Nef and the superantigen SEA.

T cell activation by the staphylococcal superantigens results in the prodigious production of T cell cytokines, such as IL 2 and IFNγ. Because of the significant T cell proliferation induced by Nef, we determined if Nef induced T cell cytokines. The results of a representative experiment are presented in Figure 4 (Panel A). Consistent with proliferation data, Nef induced significant levels of IL 2, although IL 2 levels were lower than those induced by SEA. The question arose as to the ability of Nef to also induce another important T cell cytokine, IFNγ.

Nef induced high levels of IFN activity, with peak production occurring by 96 h (Figure 4, Panel B). IFN levels were lower than those induced by SEA, but significantly higher than those induced by Con A. The type of IFN activity induced by Nef was determined to be IFNγ by neutralization reactions with specific antisera. Thus, Nef induced high levels of IFNγ in cultures of PBMC, indicating that Nef activates T cells to produce the cytokines IL 2 and IFNγ, lymphokines that are products of activated T helper 1 cells.

The following evidence points to Nef, and not a vector-derived contaminant, as the inducer of the observed proliferation: 1) proliferation was observed using recombinant Nef from two different expression systems; 2) antibodies to synthetic Nef peptides neutralized the proliferative activity of Nef; 3) the Nef fusion partner, MBP, did not induce proliferation; and 4) a synthetic peptide, Nef(123-160), previously shown to block binding of superantigens to MHC class II, specifically blocked the Nef-induced proliferative responses.

The expansion of specific Vβ-bearing T cells was observed with Nef stimulation. Further, the production of the T helper 1 lymphokines IL 2 and IFNγ from Nef-induced cultures is evidence that Nef activates T helper 1 cells. Cytokine production is consistent with previous data on Nef-stimulated CD4 T cells, which expressed the activation marker HLA-DR and had increased expression of IL 2R (15). Thus, Nef binds to MHC class II antigens on APC, does not need processing to be presented by APC, and activates T cells in a Vβ-specific manner, causing proliferation and production of the T helper 1 cytokines, IL 2 and IFNγ.

It has been shown that HIV requires activated T cells in which to replicate. Specific antiviral immune responses may not be sufficient to activate large numbers of T cells. For this reason, the identification of an HIV protein that induces T cell proliferation is of considerable interest and may, in part, explain the role of Nef in HIV pathogenesis. We have shown that stimulation of PBMC with Nef is sufficient for subsequent infection by HIV (11), and suggest a model for the role of Nef in HIV pathogenesis. Activation of T cells results from interaction with Nef, either in a soluble form released from lysed cells or as a cell-associated complex with HLA-DR on the surface of infected cells. The latter scenario is more likely and, in fact, we have shown that Nef expressed on the surface of paraformaldehyde-inactivated infected PBMC can activate cells (11). These data strongly suggest that chronic activation results as a consequence of the expression of Nef on the surface of infected T cells, analogous to the cell-associated superantigen activity of MMTV (5, 6). T cell activation by Nef could result in a stable cellular reservoir for virus production as a result of continuous paracrine stimulation. In fact, hyperimmunization against Nef has been proposed as a means of reducing viral load, either prophylactically or therapeutically (15).

We feel that T cell stimulation due to Nef is not the only mechansim of polyclonal activation of CD4 T cells for HIV replication. Recent evidence points to a superantigen encoded by cytomegalovirus (CMV) that expands Vβ12-bearing T cells, thereby enhancing HIV replication in CMV-infected individuals (16). It is not surprising that we did not see expansion of Vβ12-bearing T cells in response to Nef, since the donors used in our studies were negative for CMV. This versatility in polyclonal CD4 T cell expansion via the endogenous superantigen Nef and exogenous superantigens such as that of CMV probably plays an important role in HIV pathogenesis. Clearly, the control of the mitogenic activity of these substances should help reduce the viral load in HIV-infected individuals.

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