

# Nef Enhances Human Immunodeficiency Virus Replication and Responsiveness to Interleukin-2 in Human Lymphoid Tissue Ex Vivo

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**The *nef* gene is important for the pathogenicity associated with simian immunodeficiency virus infection in rhesus monkeys and with human immunodeficiency virus type 1 (HIV-1) infection in humans. The mechanisms by which *nef* contributes to pathogenesis in vivo remain unclear. We investigated the contribution of *nef* to HIV-1 replication in human lymphoid tissue ex vivo by studying infection with parental HIV-1 strain NL4-3 and with a *nef* mutant ( $\Delta$ *nef*NL4-3). In human tonsillar histocultures, NL4-3 replicated to higher levels than  $\Delta$ *nef*NL4-3 did. Increased virus production with NL4-3 infection was associated with increased numbers of productively infected cells and greater loss of CD4<sup>+</sup> T cells over time. While the numbers of productively infected T cells were increased in the presence of *nef*, the levels of viral expression and production per infected T cell were similar whether the *nef* gene was present or not. Exogenous interleukin-2 (IL-2) increased HIV-1 production in NL4-3-infected tissue in a dose-dependent manner. In contrast,  $\Delta$ *nef*NL4-3 production was enhanced only marginally by IL-2. Thus, Nef can facilitate HIV-1 replication in human lymphoid tissue ex vivo by increasing the numbers of productively infected cells and by increasing the responsiveness to IL-2 stimulation.**

The product of the primate lentivirus accessory gene *nef* is important for viral pathogenesis, although its exact role remains unclear. Upon experimental inoculation,  $\Delta$ *nef* simian immunodeficiency virus (SIV) has displayed markedly attenuated in vivo viral replication in macaques (20), as did  $\Delta$ *nef* human immunodeficiency virus type 1 (HIV-1) in SCID-hu PBL mice (3, 18). Human subjects infected naturally or through blood transfusion with  $\Delta$ *nef* HIV-1 isolates have shown low viral loads and prolonged disease-free survival (8, 22).

However, the exact mechanisms by which Nef contributes to pathogenesis remain unclear. The inherent complexity of in vivo models makes identification of the critical stage(s) of HIV pathogenesis that requires *nef* product difficult and highlights the need to develop an in vitro model in which the properties of Nef can be evaluated in a better-defined system. Experiments with cell lines and isolated blood lymphocytes demonstrate that Nef may play a significant role at different stages of virus replication and may also affect the infected cells themselves (2, 26, 28, 29). The effects of Nef depend on cell activation status, and Nef may also affect this status (2, 4, 15, 26, 29, 32).

Recently, we developed a system for the culture of human

lymphoid tissue which supports productive infection with various HIV-1 isolates without any requirement for exogenous activation or stimulation (13, 14). Here, we used this system to study the contribution of Nef to HIV-1 replication and CD4<sup>+</sup> T-cell depletion in the context of ex vivo infection in a setting that maintains the mixed cell populations and tissue cytoarchitecture that characterize human lymphoid tissue in vivo.

## MATERIALS AND METHODS

**Viruses.** A parental virus stock and one stock of  $\Delta$ *nef* mutant virus were obtained by transfection of COS-7 cells with pNL43 and pNL43 $\Delta$ *nef* (kindly provided by D. Richman and described previously [1, 29]). A second stock of  $\Delta$ *nef*NL4-3 was obtained by transfection of CEMx174 cells with p83-10 as described previously (12). The results of experiments with these two  $\Delta$ *nef* viruses were similar and are described together below. The infectivity of NL4-3 and  $\Delta$ *nef*NL4-3 viral stocks was measured by terminal dilution in quadruplicate, using half-log dilutions. Infection was assessed by p24 antigen production after 15 days, and the 50% tissue culture infective dose was computed as described previously (19). The infectivity of NL4-3 and  $\Delta$ *nef*NL4-3 stocks was comparable overall in conventional in vitro culture systems, with the two viruses giving indistinguishable results when subjected to titer determination on phytohemagglutinin-activated primary lymphocytes propagated with interleukin-2 (IL-2) and showing approximately a fourfold-higher ratio of 50% tissue culture infective dose per picogram of p24 for NL4-3 than for  $\Delta$ *nef*NL4-3 when subjected to titer determination on the transformed T-cell line CEMx174. While these results support the approximately comparable infectivity of the NL4-3 and  $\Delta$ *nef*NL4-3 virus stocks, neither of these in vitro titer determination systems is directly relevant to the ex vivo lymphoid tissues described below.

**HIV infection of human lymphoid tissue ex vivo.** Human tonsillar tissue removed during routine tonsillectomy and not required for clinical purposes was received within 5 h of excision. The tonsils were washed thoroughly with medium containing antibiotics and then sectioned into 2- to 3-mm blocks. These tissue blocks were placed on top of collagen sponge gels in the culture medium at the air-liquid interface and infected as described previously (13, 14). In a typical experiment, 3 to 5  $\mu$ l of clarified virus-containing medium was applied to the top of each tissue block. To normalize infections of histocultures, we inoculated

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replicate tissue blocks from the same donor with equal amounts of either parental NL4-3 or  $\Delta$ nefNL4-3, based on p24 content. Productive HIV infection was assessed by measuring p24 in the culture medium by an HIV-1 p24 antigen enzyme-linked immunosorbent assay (ELISA; Cellular Products, Buffalo, N.Y., and AIDS Vaccine Program, National Cancer Institute, Frederick, Md.): specifically, the concentration of p24 accumulated in 3 ml of culture medium bathing six tissue blocks during the 3 days between the successive medium changes was used as a measure of virus replication. Some p24 may come from the lysis of productively infected cells, since samples were not filtered to remove cellular debris, but this contribution seems to be minor since the same kinetics of p24 release is observed with HIV-1 isolates that deplete CD4<sup>+</sup> T cells only mildly (14). In addition, the concentration of cytokines in the medium was measured by ELISA (R&D Systems, Minneapolis, Minn.).

Flow cytometry was performed on cells mechanically isolated from control and infected tissue blocks (14). Depletion of CD4<sup>+</sup> T cells was assessed as described previously (14) and expressed as a ratio of CD4<sup>+</sup> to CD8<sup>+</sup> T cells. For determination of the CD4<sup>+</sup>/CD8<sup>+</sup>-T-cell ratio, cells were stained for surface markers by using anti-CD3 peridin chlorophyl protein (PerCP), anti-CD4-fluorescein isothiocyanate (FITC), and anti-CD8 phycoerythrin (PE) with the Tritest kit (Becton Dickinson, San Jose, Calif.). For immunophenotyping of productively HIV-infected cells, the following monoclonal antibodies were used, in combination with the anti-p24 antibody KC57 RD1 (Coulter, Miami, Fla.): anti-CD3 PerCP, HLA-DR allophycocyanin (APC), anti-CD68 FITC, and anti-CD25 APC (Caltag, Burlingame, Calif.) and anti-CD69 FITC (Pharmingen, San Diego, Calif.). The cells were stained for the cell surface antigens, fixed and permeabilized with Cytotfix-Cytoperm (Pharmingen), and stained for the intracellular marker.

**Quantification of HIV-1 DNA.** Single-cell suspensions were prepared from infected tonsil cultures, and after being washed, dry cell pellets were cryopreserved at -70°C until needed for processing and analysis. After lysis, total DNA was extracted (PureGene kit; Gentra Systems, Minneapolis, Minn.). HIV-1 *gag* DNA, indicative of completion of first-strand DNA synthesis, was quantified by a real-time PCR assay on an ABI Prism 7700 sequence detection system. A detailed description of this instrument and its use for real-time quantitative PCR applications, including quantitation of retroviral sequences, is presented elsewhere (17, 31). For the present assays (31a), the following reagents were used: Gag, forward primer 5'-GtC ATC AIG CAG CCA TGC AAA T-3' (1366 to 1387), reverse primer 5'-CAT iCT ATT TGT TCI TGA AGG GTA CTA G-3' (1507 to 1480), probe 5'-(R)TCA ATG AGG AAG CTG CAG AAT GGG AT(Q)-3' (1402 to 1427) (based on the reference sequence for HIV-1, isolate HXB2, GenBank accession no. K03455), where R indicates the reporter fluorochrome (6-carboxy-fluorescein [FAM]), and Q indicates the quencher dye 6-carboxy-tetramethyl-rhodamine (TAMRA) conjugated through a linker arm nucleotide (33). (The fluorescent probe for HIV-1 was obtained from DNA Sciences, Inc., San Diego, Calif.). Each specimen was also analyzed for a unique sequence from the coding region for porphobilinogen deaminase (PBGD) (13, 23), using a fluorescent probe purchased from the Applied Biosystems Division of Perkin-Elmer (Foster City, Calif.). Since this sequence is present at two copies per diploid cell and there are no pseudogene sequences, quantitative analysis for this sequence in a given specimen provides an internal control, allowing normalization of HIV sequences relative to the number of amplifiable diploid genome equivalents of DNA present in the specimen. The average interassay coefficient of variation for the real-time PCR assays for HIV-1 *gag* and strong stop and PBGD DNA was <15%, with a threshold sensitivity of 3 DNA copy equivalents per reaction.

**Experimental analysis.** Data obtained with tissue from one donor constitutes the results of one experiment. Both viral replication and the ratios of cells of various leukocyte subsets varied from tissue to tissue (14). To normalize for such variation, for each experiment we compared parental NL4-3 and  $\Delta$ nefNL4-3 replication in replicate histocultures obtained from the same individual donor. CD4<sup>+</sup>-T-cell depletion in tissues from the same donor infected with either NL4-3 or  $\Delta$ nefNL4-3 was also compared. To average the results of different experiments and to analyze them statistically, we normalized the data on  $\Delta$ nefNL4-3 replication as the percentage of NL4-3 replication at the maximum of viral production.

## RESULTS

**$\Delta$ nefNL4-3 replicates to a lower level in human lymphoid tissue ex vivo than does the parental NL4-3.** In experiments with tissues from 14 different donors, NL4-3 exhibited replication kinetics similar to those described previously for other laboratory strains and primary isolates in ex vivo lymphoid tissues (14). p24 first became detectable in the medium around day 6 postinfection. In some experiments, viral replication continued to increase through the entire period of tissue culture up to day 15 (Fig. 1a). In other experiments, a peak of viral replication was evident before day 15 (results not shown). The average concentration of p24 reached  $17 \pm 3$  ng/ml during the last 3 days of infection with the parental NL4-3 isolate.

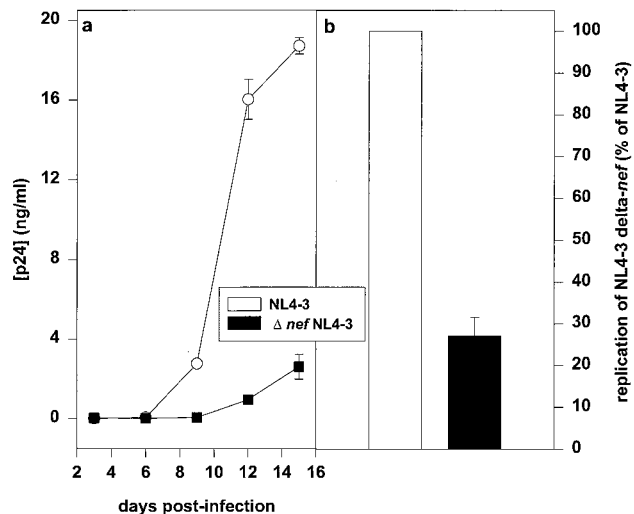


FIG. 1. Replication of parental NL4-3 and  $\Delta$ nefNL4-3 in human tonsillar tissue ex vivo. (a) Typical kinetics of p24 accumulation in the culture medium over a 3-day period between successive medium changes (mean and standard error of the mean of 12 pooled tissue blocks from an individual donor). (b) Comparison of the amount of p24 in culture media of tissues infected with  $\Delta$ nefNL4-3 and parental NL4-3 at the peak of infection (mean and standard error of the mean of tissues from 14 different donors). Peak measurements were obtained on days 12 to 15 postinfection.

The two  $\Delta$ nefNL4-3 variants used in this study replicated similarly. In all experiments, there was approximately a 3-day delay in the time when replication of  $\Delta$ nefNL4-3 became detectable relative to replication of NL4-3. Starting from this time point and through the entire experiment,  $\Delta$ nefNL4-3 replicated to a significantly lower level in matched tissue blocks than did the parental NL4-3 variant. On average, maximum p24 levels in  $\Delta$ nefNL4-3-infected cultures were  $73 \pm 8\%$  ( $n = 14$ ) lower than in cultures infected with the parental NL4-3 (Fig. 1b). The actual difference between levels of production of parental NL4-3 and  $\Delta$ nefNL4-3 at the maximum of viral replication varied from tissue to tissue by a factor of between 2 and 25. In the experiment in which both  $\Delta$ nefNL4-3 and parental NL4-3 replication reached a peak,  $\Delta$ nefNL4-3 replication remained 2.8-fold lower than that of parental NL4-3 at the peak of infection. Thus, in  $\Delta$ nefHIV-infected cultures, there appears to be both a delay in productive infection and an impairment of the ability to replicate.

Under the standard infection protocol, the difference between replication of NL4-3 and  $\Delta$ nefNL4-3 variants did not correlate with the absolute level of parental virus replication. However, the difference in replication between virus variants could be magnified: when matched tissue blocks were inoculated with 40-fold less NL4-3 or  $\Delta$ nefNL4-3 than usual, no productive infection was detected in  $\Delta$ nefNL4-3-inoculated tissues whereas viral production in the NL4-3-infected tissues reached 3.5 ng of p24 per ml.

**Fewer CD4<sup>+</sup> T cells are depleted in  $\Delta$ nefNL4-3-infected than in parental NL4-3-infected tissues.** Similar to other T-cell- and CXCR4-tropic HIV-1 isolates (14), NL4-3 infection of human lymphoid tissue ex vivo resulted in the depletion of CD4<sup>+</sup> T cells. The extent of CD4<sup>+</sup>-T-cell depletion varied from donor to donor. On days 13 to 15 postinfection, the level of CD4<sup>+</sup> T cells remaining in NL4-3-infected tissue had dropped to  $31\% \pm 7\%$  ( $n = 11$ ) of that in matched uninfected control cultures (Fig. 2). In tissues infected with  $\Delta$ nefNL4-3, the depletion of CD4<sup>+</sup> T cells was milder: the CD4<sup>+</sup> T-cell

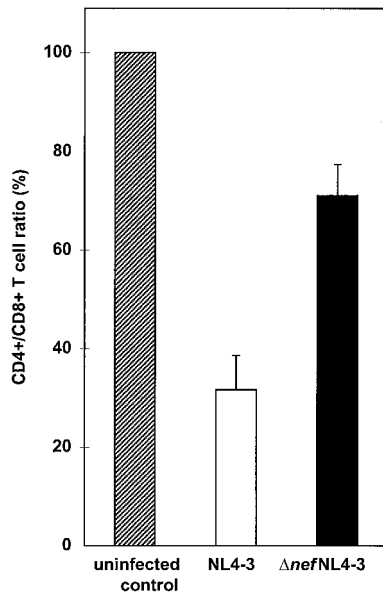


FIG. 2. CD4<sup>+</sup> T-cell depletion in NL4-3- and ΔnefNL4-3-infected human lymphoid tissue ex vivo. The numbers of CD4<sup>+</sup> and CD8<sup>+</sup> T cells were assessed by flow cytometry on matched uninfected control tissues and tissues infected with parental NL4-3 or ΔnefNL4-3 (mean and standard error of the mean of tissues from 11 different donors). To normalize for variations in the size of the blocks and in their cellularity, the results were shown as CD4<sup>+</sup>/CD8<sup>+</sup> ratios expressed as a percentage of the uninfected control.

level was 71% ± 6% ( $n = 11$ ) of that in matched uninfected control cultures (Fig. 2). The decrease in the number of CD3<sup>+</sup> CD4<sup>+</sup> lymphocytes in HIV-1-infected tissue was not accompanied by an increase in the number of CD3<sup>+</sup> CD4<sup>-</sup> lymphocytes. Thus, the flow cytometry data reflect the actual depletion of CD4<sup>+</sup> T cells rather than down-regulation of CD4<sup>+</sup> expression or epitope masking by gp120.

To determine whether the difference in CD4<sup>+</sup> T-cell depletion between NL4-3- and ΔnefNL4-3-infected tissues was due merely to the difference in the level of viral replication, we adjusted the replication of parental NL4-3 to that of ΔnefNL4-3 by decreasing the amount of parental NL4-3 used to inoculate the histocultures. When the amount of NL4-3 used for inoculation was 1/20 of that of ΔnefNL4-3, the replication curves for the two viruses matched each other (Fig. 3a), and the extent of CD4<sup>+</sup> T-cell depletion was similar: 88 and 84% of CD4<sup>+</sup> T cells remained in the tissue, respectively (Fig. 3b). Thus, the difference in CD4<sup>+</sup>-T-cell depletion between tissues infected with equal amounts of either parental NL4-3 or ΔnefNL4-3 is a consequence of the difference in replication level between these two virus variants.

**There are fewer infected cells in tissues infected with ΔnefNL4-3 than in those infected with parental NL4-3.** To determine whether the lower level of viral replication in ΔnefNL4-3-infected tissues was due to fewer infected cells or to lower virus production from each productively infected cell, we evaluated the number of infected and productively infected cells. We used flow cytometry to estimate the number of p24<sup>+</sup> cells in tissues from three donors infected separately with NL4-3 and ΔnefNL4-3. There were more p24<sup>+</sup> cells in tissues infected with the parental virus NL4-3 than in matched tissues infected with the ΔnefNL4-3: on average, 6% ± 1% ( $n = 3$ ) of T cells isolated from NL4-3-infected tissues were p24<sup>+</sup> on days 10 to 13 postinfection, while 3% ± 1% ( $n = 3$ ) of all T lymphocytes isolated from ΔnefNL4-3 infected tissues were

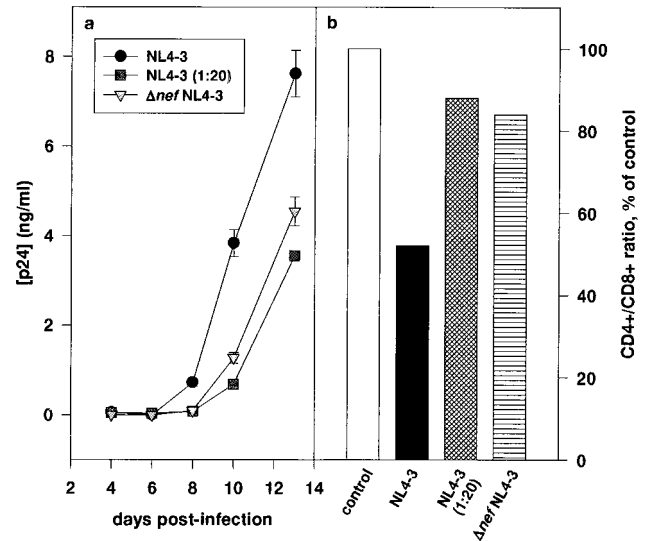


FIG. 3. Relation between CD4<sup>+</sup> T-cell depletion and viral replication in NL4-3- and ΔnefNL4-3-infected human lymphoid tissue ex vivo. Tissue blocks were infected with the standard amount of parental NL4-3 or ΔnefNL4-3 (9 pg/block) or with a 1:20 dilution of parental NL4-3. (a) Concentration of p24 in the culture medium from infected tissue (mean and standard error of the mean of 12 pooled tissue blocks from an individual donor; representative of experiments with tissue from three donors). (b) CD4<sup>+</sup>/CD8<sup>+</sup> ratios in the tissue on day 13 postinfection (pooled tissue blocks from an individual donor).

p24<sup>+</sup>. In these specimens, the number of p24<sup>+</sup> macrophages (CD68<sup>+</sup> HLA-DR<sup>+</sup>) was too small for statistically reliable measurements. Thus, fewer productively infected T cells were found in ΔnefNL4-3-infected tissues than in NL4-3-infected tissues.

We estimated the average productivity of infected cells by comparing the number of p24<sup>+</sup> cells in the infected tissues with the amount of p24 in culture medium. Figure 4 demonstrates that the amount of p24 produced by NL4-3- and by ΔnefNL4-3-infected tissue in five experiments is proportional to the number of p24<sup>+</sup> cells in the same tissues (coefficient = 0.98; the 95% confidence interval for the intercept is not significantly different from 0;  $P = 0.5$ ). Thus, for a given human tonsillar tissue ex vivo, the average virus production per infected cell is similar whether the tissue is infected with ΔnefNL4-3 or with parental NL4-3. However, this conclusion is based on comparison of a "snapshot" of a number of p24<sup>+</sup> T lymphocytes with the amount of p24 accumulated in the medium in a few rounds of infection over a 3-day period. Moreover, both integral viral particles and free protein are accumulated in the medium. We tested the above conclusion by measuring the fluorescence of tissue T cells stained with anti-p24 antibodies. The average fluorescence intensity of p24<sup>+</sup> T cells in NL4-3- and ΔnefNL4-3-infected tissues was similar: 158 ± 30, and 145 ± 13 arbitrary fluorescence units, respectively ( $n = 4$ ). This is another indication that the productivities of NL4-3- and of ΔnefNL4-3-infected cells are similar.

We also compared the extent of infection in cultures infected with parental and mutant viruses by quantification of HIV DNA levels. The HIV-1 *gag* DNA content on days 9 to 12 after infection with NL4-3 ranged from 18,000 to 81,000 copy equivalents per 10<sup>5</sup> diploid genome equivalents. In tissues from all four tested donors, there were lower levels of HIV DNA in ΔnefNL4-3-infected tissue than in NL4-3-infected tissue. This difference varied, however, from tissue to tissue and seemed to depend on the extent of CD4<sup>+</sup> T-cell depletion at the time of

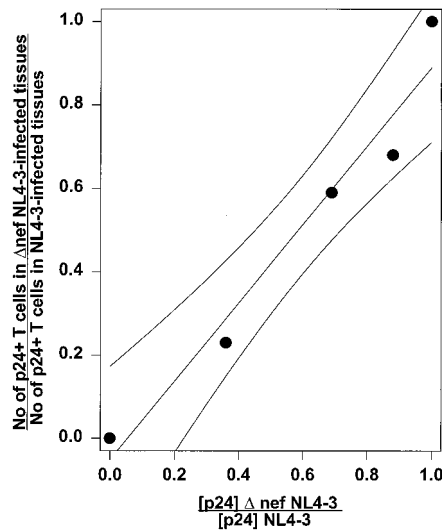


FIG. 4. Correlation of the number of productively infected cells and the amount of virus produced in NL4-3- and  $\Delta nef$ NL4-3-infected human lymphoid tissue ex vivo. The ratio of the number of p24<sup>+</sup> cells in the tissue infected by either of the two HIV-1 variants is plotted against the ratio of the amount of p24 in the culture medium from these tissues. The number of p24<sup>+</sup> cells was assessed by flow cytometry. The concentration of p24 in the culture medium was assessed by ELISA. Each data point represents tissue from one donor infected in parallel by either parental NL4-3 or  $\Delta nef$ NL4-3.

measurement. Thus, for two experiments evaluated at a time when approximately half of the CD4<sup>+</sup> T cells had been depleted in the culture infected with NL4-3 but there was almost no detectable CD4<sup>+</sup>-T-cell loss in the culture infected with  $\Delta nef$ NL4-3, the relative abundance of HIV-1 gag copies in the  $\Delta nef$ NL4-3-infected cultures was 39 and 20% of that of the parental virus. In two other experiments, in which there was virtually no CD4<sup>+</sup>-T-cell loss in the cultures infected with either virus when the cultures were evaluated on day 9, the

relative abundance of HIV-1 gag sequences per 10<sup>5</sup> diploid genome equivalents in  $\Delta nef$ NL4-3-infected tissues was 1.4 and 0.7% of that in tissues infected by the parental virus.

**Parental NL4-3 and  $\Delta nef$ NL4-3 infections are differently affected by tissue activation.** To determine whether there were differential effects on the cell activation status in  $\Delta nef$ NL4-3- and NL4-3-infected lymphoid tissues, we compared the amount of cytokines secreted into the medium. Measurements of IL-2, IL-4, and IL-6 did not reveal any consistent difference between uninfected tissues and tissues infected with either of the virus variants, nor were any differences found in the amount of the CC chemokines, MIP-1 $\alpha$ , MIP-1 $\beta$ , or RANTES, secreted into the medium by these tissues (data not shown).

Flow cytometry also revealed no difference between parental NL4-3- and  $\Delta nef$ NL4-3-infected tissues in the numbers of cells expressing the activation markers CD25, CD69, or HLA-DR. Also, no consistent difference in the frequency of cells expressing these markers was found for p24<sup>+</sup> T cells isolated from tissues infected with NL4-3 or  $\Delta nef$ NL4-3 viruses (Fig. 5).

To examine the effects of exogenous stimulation on NL4-3 and  $\Delta nef$ NL4-3 replication, we treated the cultures with IL-2. The cytokine was added simultaneously with virus and was present throughout the entire experiment. Although human lymphoid tissue ex vivo does not require exogenous stimulation to support efficient productive HIV infection (13, 14), IL-2 at 10, 20, or 50 U/ml did increase virus production in the NL4-3-infected cultures in a dose-dependent manner (Fig. 6).

The effect of IL-2 was much less pronounced in  $\Delta nef$ NL4-3-infected tissues than in NL4-3-infected tissues. In all eight experiments, viral replication was stimulated only slightly or not at all by 10 to 20 U of IL-2 per ml. IL-2 at 50 U/ml increased the replication of  $\Delta nef$ NL4-3 but much less than that of parental NL4-3: at the peak of infection, the mean p24 levels in  $\Delta nef$ NL4-3-infected tissues stimulated with 50 U of IL-2 per ml were lower by a factor of 7 than were those in similarly stimulated NL4-3-infected tissues (Fig. 6c). In a given experiment, the difference between the responsiveness of NL4-3 and  $\Delta nef$ NL4-3 replication to IL-2 was evident during both early

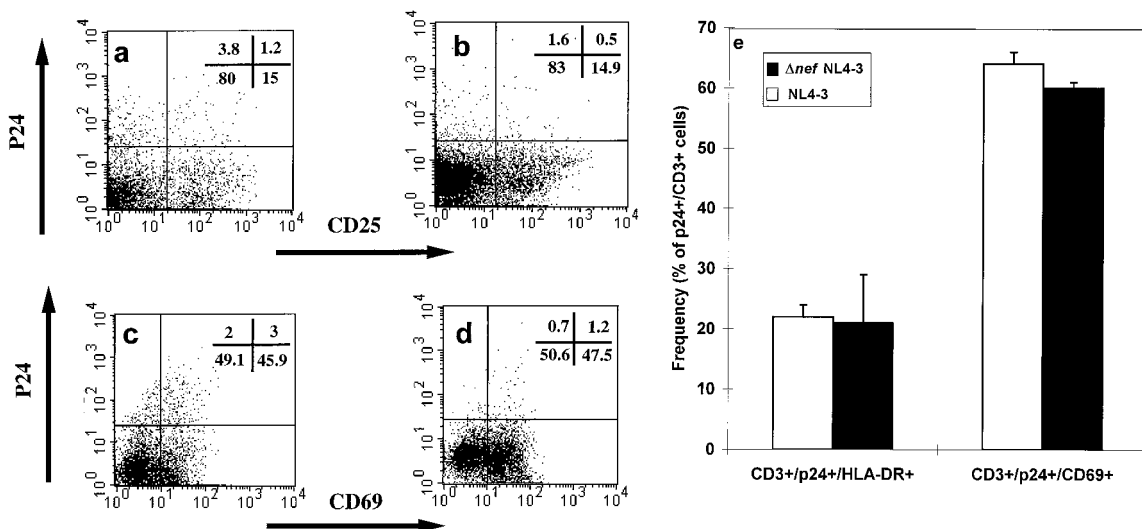


FIG. 5. Activated T lymphocytes in NL4-3- or  $\Delta nef$ NL4-3-infected human tonsillar tissue ex vivo. The results were obtained by flow cytometry. (a to d) Frequency of CD25<sup>+</sup> (a and b) and CD69<sup>+</sup> (c and d) in p24<sup>+</sup> and p24<sup>-</sup> T lymphocytes isolated from NL4-3-infected tissue (a and c) and  $\Delta nef$ NL4-3-infected tissue (b and d). The results are given as pooled data on 12 tissue blocks from one donor and are representative of experiments with tissue from three donors. Numbers represent the frequency of events in each quadrant. The ratio of activated to nonactivated cells among p24<sup>+</sup> cells is similar in NL4-3- and  $\Delta nef$ NL4-3-infected tissues; the same is true for p24<sup>-</sup> cells. (e) Frequency (mean and standard error of the mean) of CD3<sup>+</sup>/HLA-DR<sup>+</sup> and of CD3<sup>+</sup>/CD69<sup>+</sup> in tissue lymphocytes infected with parental NL4-3 or  $\Delta nef$ NL4-3 ( $n = 3$ ).



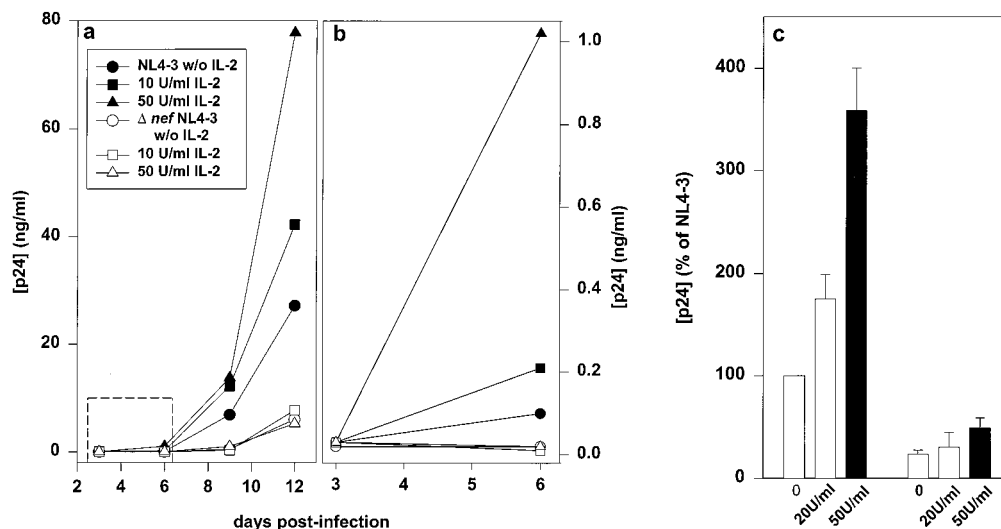


FIG. 6. Effect of exogenous IL-2 on viral replication in NL4-3- or  $\Delta$ nefNL4-3-infected human tonsillar tissue ex vivo. (a and b) Virus production as evaluated by the concentration of p24 in the medium at various times postinfection. Data on the culture media of 12 blocks of tissue from one donor are pooled for each time point and for every condition and are representative of experiments with tissue from three donors. Panel b is an enlarged detail of the dashed rectangle in panel a, to demonstrate the effect of IL-2 on NL4-3 at low level of viral replication. (c) Average concentration of p24 in the culture media of IL-2-stimulated tissues infected with parental NL4-3 or  $\Delta$ nefNL4-3 relative to the NL4-3-infected unstimulated control. Each column represents the mean and standard error of the mean of experiments with tissue from 5 to 11 donors.

culture when replication was low and later, during maximum virus replication (Fig. 6a and b). Moreover, we selected experiments in which the replication of parental NL4-3 was low (2 to 4 ng of p24 per ml) but still showed significant enhancement by IL-2. The results were compared to those of other experiments, where  $\Delta$ nefNL4-3 replicated at an overlapping level (4 to 9 ng of p24 per ml) yet without such an enhancement by IL-2. Thus, the different effects of IL-2 on  $\Delta$ nefNL4-3 and parental NL4-3 do not depend on the level of viral replication. In experiments in which we continued to culture the infected tissue beyond day 12, at later time points the responsiveness of parental virus stimulation to IL-2 (20 to 50 U/ml) was still  $5.4 \pm 1.6$ -fold higher ( $n = 3$ ) than that of  $\Delta$ nefNL4-3. Thus, exogenous IL-2 did not restore the impaired replication of  $\Delta$ nefNL4-3 in human lymphoid tissue even to one-half of that of NL4-3 in unstimulated tissue.

## DISCUSSION

The experiments described in this report demonstrate the enhancing effect of Nef in ex vivo HIV replication in human lymphoid tissue that maintains its original complexity of cell populations and cytoarchitecture (13, 14). In human tonsillar histocultures, NL4-3 replicates more efficiently than  $\Delta$ nefNL4-3 does. Increased virus production with NL4-3 infection relative to  $\Delta$ nefNL4-3 infection was associated with a rise in the frequency of productively infected cells and with greater loss of CD4<sup>+</sup> T cells over time. While there were fewer productively infected T cells in  $\Delta$ nefNL4-3-infected tissues than in NL4-3-infected tissues, the levels of viral production per infected T cell were similar whether the *nef* gene was present or not. At all levels of viral replication, exogenous IL-2 increases HIV-1 production in NL4-3-infected tissue in a dose-dependent manner. In contrast,  $\Delta$ nefNL4-3 production is only marginally enhanced by IL-2.

In view of the variety of the effects attributed to Nef, it is possible that it facilitates various stages of HIV infection to ultimately result in the observed increase in the number of p24<sup>+</sup> CD4<sup>+</sup> T cells. In particular, the presence of Nef could

increase the probability of a particular CD4<sup>+</sup> T cell being infected or/and the probability of an infected cell to become productively infected. This may result in a delay in the onset of  $\Delta$ nefNL4-3 replication and in the lower replication rates actually observed in our experiments. A Nef-dependent increase in HIV infectivity (6, 23, 29) and a Nef-dependent upregulation of virus production have both been reported for cultures of isolated cells (21, 26, 29). Our results show that similar phenomena occur in integral lymphoid tissue: both the number of HIV DNA copies per cellular genome and the number of p24-positive T cells per tissue block are larger in lymphoid tissue cultures infected with NL4-3 than in matched cultures infected with  $\Delta$ nefNL4-3.

The positive effect of Nef on HIV infection was reported to be related to the ability of this viral protein to activate cells (9, 10). Since it is well documented that efficient HIV replication in lymphocytes requires activation of the cells (25, 30, 33), Nef-mediated cell activation should increase the likelihood of a cell becoming productively infected with HIV. Cell activation augments the efficiency of reverse transcription, increases the intracellular pool of nucleotides, facilitates proviral DNA transport to the nucleus, and raises the level of transcription factors necessary for efficient virus expression (5, 11, 27). Whatever the dominant mechanism for Nef-mediated cell activation, Nef could turn a low-virus-expressing cell, a nonexpressing cell, or even a cell refractory to infection into an efficient HIV producer. This is what appears to be happening in our experiments with NL4-3-infected histocultures of human lymphoid tissue: the number of HIV-producing cells was significantly larger than in tissues infected with  $\Delta$ nefNL4-3.

A priori, Nef could enhance viral production by individual cells without increasing their number. However, our results do not support this. Although the multiple rounds of infection by the day of analysis may complicate the interpretation of the data, these results, corroborated by measurement of fluorescence of tissue T cells stained with anti-p24 antibodies, strongly indicate that the viral production by an individual cell is similar whether it is infected by parental or  $\Delta$ nef HIV-1. Thus, Nef

enhances HIV replication at the tissue level by increasing the number of productively infected T cells. However, once the viral replication machine is on, Nef does not seem to play any significant role in determining the level of cell productivity. Nor have we observed any direct effect of Nef on CD4<sup>+</sup> T-cell depletion, independent of a general enhancement of the level of infection: in both NL4-3- and  $\Delta$ nefNL4-3-infected tissue, the extent of depletion of CD4<sup>+</sup> T cells was proportional to the number of productively infected T cells. Thus, it seems that both Nef-dependent stimulation of HIV replication in human lymphoid tissue and the Nef-dependent high rate of CD4<sup>+</sup> T-cell loss in these tissues are direct consequences of a Nef-dependent increase in the number of productively HIV-infected cells.

We did not find any difference in the apparent level of T-cell activation, as assessed by expression of CD25, CD69, and HLA-DR (three surface markers commonly used to evaluate cell activation status), between tissues infected with NL4-3 and those infected with  $\Delta$ nefNL4-3 or between tissue T cells productively infected with NL4-3 or with  $\Delta$ nefNL4-3. However, analysis of other activation markers as well as evaluation of the efficiency of various steps involved in HIV replication may reveal more subtle differences between tissue cells infected *ex vivo* with parental virus and those infected with  $\Delta$ nef mutant virus. Moreover, to acquire enough p24<sup>+</sup> T cells for analysis, these measurements were made at the late phase of HIV infection, whereas Nef-mediated activation is more likely to be critical at the early stage in the establishment of HIV infection. An assessment of the full range of various intracellular mechanisms by which Nef may facilitate HIV infection is beyond the scope of this study, which was designed to reveal the effects of Nef at both the tissue and cellular levels.

Although *ex vivo* tissue seems to reproduce *in vivo* conditions more faithfully than do isolated cells, some of the key cytokines, such as IL-2, are not present in the culture medium in significant quantities (24). We added up to 20 U of IL-2 per ml to the cultures infected with NL4-3 or  $\Delta$ nefNL4-3 and found that without *nef*, production of HIV-1 by infected tissue was almost nonresponsive to IL-2 stimulation. At higher concentrations of IL-2, production of  $\Delta$ nefNL4-3 was increased but much less so than that of NL4-3. It remains to be tested whether Nef affects the functionality of the IL-2 receptor or events downstream of IL-2 interaction with the cell surface. At least CD25 itself was expressed equally well on T cells from both  $\Delta$ nefNL4-3- and NL4-3-infected tissues based on flow cytometry evaluation.

Current models of T-cell activation posit that efficient activation is triggered by engagement of the antigen-specific T-cell receptor but also requires a second signal, typically provided by ligation of costimulatory molecules such as CD28 by accessory molecules (CD80, CD86) on antigen-presenting cells (reviewed in reference 7). However, various different interactions, acting at various stages in the activation process, from cell surface receptor engagement to downstream signaling, can affect activation. One of the notable features of the *ex vivo* human tonsillar system is the lack of any requirement for exogenous activating stimuli to achieve productive HIV-1 infection (13, 14). The virus itself and an authentic lymphoid tissue milieu appear to provide the necessary level of activation for productive infection. If Nef is capable of providing an activating stimulus, this might explain the failure of exogenous IL-2 to increase  $\Delta$ nefNL4-3 replication, due to the absence of an effective activating signal.

These results are somewhat similar to those obtained by Alexander et al. (4) in experiments with isolated T cells from rhesus monkeys. The cells in that experiment were immortal-

ized by infection with herpesvirus saimiri, and exogenous IL-2 allowed them to grow. Under these conditions, SIV replicated in the cells, whether or not the *nef* gene was present. However, without exogenous IL-2, *nef* was required to sustain a high rate of virus replication. In contrast, in our experiments, IL-2 was unable to rescue low-level  $\Delta$ nef HIV infection in lymphoid tissues *ex vivo*. The results obtained in both experimental systems are consistent with the hypothesis that *nef* is able to provide one of the activating signals necessary for a high level of productive infection.

By extrapolating our results with tissue explants to the whole organism, we suppose that *in vivo*, where IL-2 or other signaling molecules are readily available, the presence of *nef* may facilitate HIV or SIV replication by increasing the number of productively infected T cells, which in turn can lead to rapid T-cell depletion and disease progression. Without *nef*, the pathologic development either becomes slow or is halted (8, 20, 22).

Unlike peripheral blood mononuclear cells, human lymphoid histocultures do not require exogenous stimulation for efficient HIV replication. The natural low level of endogenous cell activation in lymphoid tissues *ex vivo* allows one to detect the Nef-mediated activation of HIV replication, as was seen in other "natural" systems: HIV-infected humans (8, 22), SIV-infected monkeys (20), and HIV-infected SCID *hu* mice (3, 16, 18). *Ex vivo* human lymphoid tissue provides a system for delineating the mechanism by which Nef engages more tissue T cells in virus infection and for defining the role of various cytokines, cellular activation, and intercellular interactions in this process.

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