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Vpr and Vpu Are Important for Efficient Human Immunodeficiency Virus Type 1 Replication and CD4^{+} T-Cell Depletion in Human Lymphoid Tissue Ex Vivo

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The relevance of the accessory *vpr*, *vpu*, and *nef* genes for human immunodeficiency virus type 1 (HIV-1) replication in human lymphoid tissue (HLT), the major site of viral replication in vivo, is largely unknown. Here, we show that an individual deletion of *nef*, *vpr*, or *vpu* significantly decreases HIV-1 replication and prevents CD4^{+} T-cell depletion in ex vivo HLT. However, only combined defects in all three accessory genes entirely disrupt the replicative capacity of HIV-1. Our results demonstrate that *nef*, *vpr*, and *vpu* are all essential for efficient viral spread in HLT, suggesting an important role in AIDS pathogenesis.

Human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus (SIV) have several genes that are not absolutely required for viral spread in cell lines (7, 46) and are, therefore, called accessory genes. However, subsequent studies have indicated that these genes might play important roles in infected hosts in vivo, as well as in primary cells (reviewed in references 7 and 46). HIV-1 encodes Vif, Vpr, Vpu, and Nef. In contrast, HIV-2 and SIVmac do not encode Vpu but rather another late protein, Vpx (51). Vif suppresses the antiretroviral cellular enzyme APOBEC3G (36, 42, 48) and is essential for efficient viral replication in primary cells and in vivo (12). The in vivo importance and exact functions of the other accessory proteins are less clear, although it has been established that they modulate multiple host cell processes (reviewed in references 7 and 46). For example, Nef downregulates CD4 (2, 19, 37, 45), enhances virion infectivity (9, 37), alters T-cell activation (4, 13, 43), and interferes with major histocompatibility complex antigen presentation (26, 41, 50). Vpr is a virion-associated protein (10) which induces G_2/M arrest (18, 40) and plays a role in the nuclear transport of the preintegration complex in nondividing cells (39). Vpr also enhances infection of macrophages (14), activates HIV transcription (1, 17), and induces apoptosis (38, 44, 47). Vpu promotes virion release (49, 54) by counteracting host restriction factors (30, 52), downregulates CD4 during the late stages of HIV-1 infection (34, 53), and inhibits NF-κB activation (3).

Studies using the SIVmac model have demonstrated that *nef* is important for efficient replication in vivo and for disease progression (31). The contribution of the other accessory genes to SIV or HIV-1 virulence is less clear. Deletion of *vpr* neither attenuates SIV replication nor prevents disease progression in infected monkeys (20). Results obtained with chimeric simian-human immunodeficiency viruses suggest that *vpu* might contribute to viral pathogenesis (35). The role of the accessory genes in HIV-1 pathogenesis is even less clear than their role in SIVmac pathogenesis. The importance of Nef in HIV infection of humans has been confirmed in several long-term nonprogressors (11, 32). Also, it has been suggested that sequence variations in Vpr and Vpu might be associated with nonprogressive HIV-1 infection (5, 44).

Understanding the role of the accessory HIV-1 proteins in AIDS in humans requires adequate experimental systems. In vivo, critical events in HIV disease occur in lymphoid tissues (15, 16). It has been shown that Nef, concordantly with its important role in vivo (11, 31, 32), enhances HIV-1 replication in human lymphoid tissue ex vivo (24). This system supports productive HIV-1 infection without exogenous stimulation (22, 23) and provides a useful model for studying the importance of the accessory genes for HIV-1 replication in infected human individuals. In the present study, we used this system to investigate the role of accessory genes by infecting blocks of human tonsillar tissue with HIV-1 mutants containing single or combined deletions of *vpu*, *vpr*, and *nef* and evaluated virus replication and CD4^{+}-T-cell depletion.

HIV-1 NL4-3 *vpr* and *vpu* deletion mutants (21) were kindly provided by Ronald C. Desrosiers through the AIDS Research and Reference Reagent Program. Full-length proviral pBRNL4-3 variants with single and combined deletions of *vpr*, *vpu*, and *nef* were generated with standard cloning techniques. Briefly, to generate the *vpr*-defective forms, the *gag-pol-vif-vpr* region of p210-19 containing a deletion in *vpr* (21) was inserted into full-length *nef*-open and *nef*-defective HIV-1 NL4-3 proviral clones (8) by using the NarI and EcoRI sites located in the 5′ long terminal repeat and the *vpr* gene. Similarly, a *vpu*-deleted EcoRI-NheI restriction fragment derived from p210-13 (21) was cloned into the NL4-3 proviral constructs to obtain *vpu*-deleted HIV-1 mutants. Finally, *vpr*-deleted NarI-EcoRI restriction fragments were inserted into the *vpu*- and/or *nef*-

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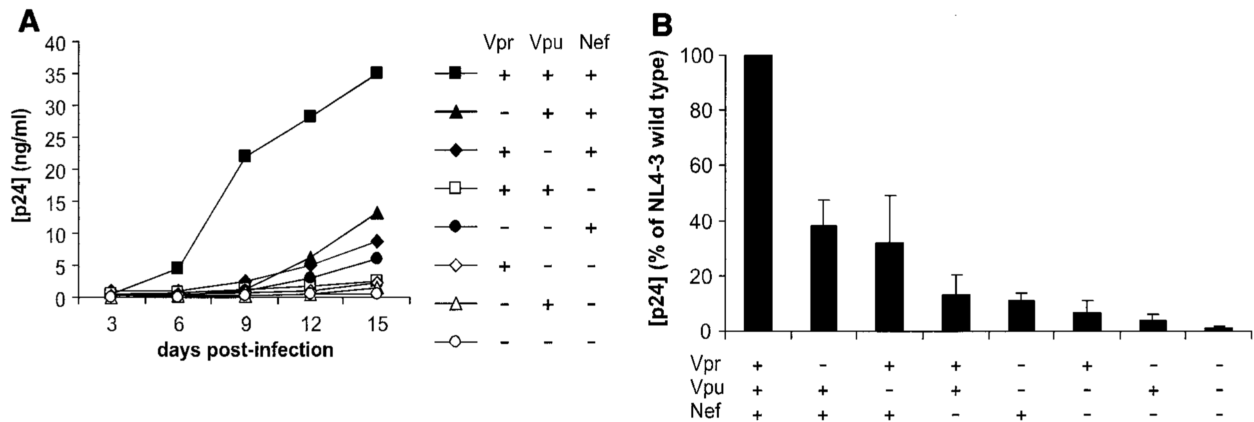


FIG. 1. Replication of HIV-1 variants in human lymphoid tissue ex vivo. For each of the indicated HIV-1 variants, 27 tissue blocks were inoculated with 100 ng of p24 and medium was collected every 3 days. (A) Representative replication kinetics of wild-type NL4-3 and deletion mutants. (B) Average production of virus. Matched tissues from 13 donors were inoculated with the wild-type virus or with accessory gene-deleted mutants as indicated, and for each condition cumulative production of p24 by 27 tissue blocks over 15 days was measured. Presented are means ± standard errors of the means of these values expressed as percentages of those measured in cultures infected with the wild-type virus.

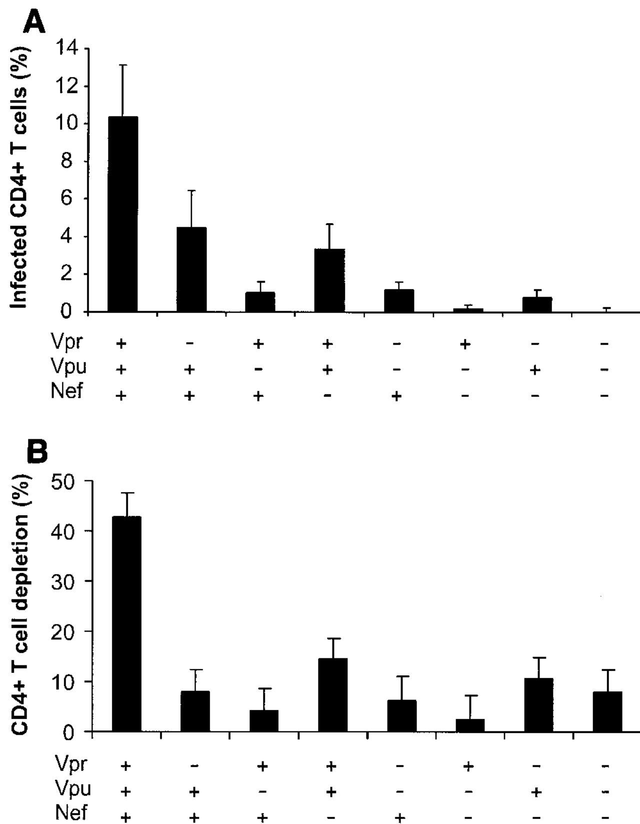
defective proviral constructs to obtain HIV-1 variants with combined defects in the accessory genes.

Human tonsils removed during routine tonsillectomies were dissected, set up in culture at the air-liquid interface, and infected as described earlier (23). Briefly, for testing of each virus in tissue from one donor, 100 ng of p24 was applied to each of 27 tissue blocks. For each HIV-1 variant, the experiment was repeated *n* times (each time with tissues from a different donor). Viral production was evaluated by measurement of the p24 core antigen released into the pooled medium bathing all 27 blocks by HIV-1 p24 enzyme-linked immunosorbent assay (Coulter, Miami, Fla.). In agreement with earlier observations for various HIV-1 isolates (23, 29), p24 production was first noted on day 6 postinoculation, and virus replication increased until the end of the experiment on day 12 or 15 (Fig. 1A).

A single deletion of any of the accessory genes *nef*, *vpr*, and *vpu* decreased viral replication (Fig. 1A). On average, deletion of *nef* decreased the ability of HIV-1 NL4-3 to replicate in ex vivo-infected human lymphoid tissues to 13% ± 7% ($n = 11$, $P = 0.001$) of that of the wild-type parental virus and deletion of *vpr* and *vpu* decreased it to 38% ± 9% ($n = 14$, $P = 0.003$) and 32% ± 16% ($n = 14$, $P = 0.002$) of that of the wild-type virus, respectively (Fig. 1B). Combined deletion of *vpr* and *vpu* impaired HIV-1 replication more severely and reduced cumulative p24 production to 11% ± 3% ($n = 12$, $P = 0.002$) of that of the wild-type virus. Similarly, additional deletion of either *vpr* or *vpu* further attenuated replication of the *nef*-deleted HIV-1 variant (Fig. 1B). Thus, although *vpr* and *vpu* seem to be less critical than *nef*, both clearly contribute to efficient viral replication in ex vivo-infected human lymphoid tissue. Nevertheless, only the combined deletion of all three accessory genes completely disrupted HIV-1 replication (Fig. 1B).

We evaluated the number of productively infected $CD4^+$ T cells by flow cytometry of cells mechanically isolated from control and infected-tissue blocks and stained for CD3, CD8, CD4, and p24 (23, 27). To account for CD4 downregulation by viral infection, productively infected $CD4^+$ T cells were defined as $CD3^+$ $CD8^-$ $p24^+$. The numbers of HIV-1-infected cells in tissues infected with all tested mutants were significantly diminished, relative to those in tissues infected with wild-type virus ($P < 0.008$) (Fig. 2A). The progressive loss of $CD4^+$ T lymphocytes is a major characteristic of HIV-1 infection and AIDS. We evaluated this loss in tissues infected by wild-type virus and accessory gene-deleted HIV-1 variants using flow cytometry. To normalize for differences in tissue block size and cellularity and to account for CD4 downregulation by viral infection, $CD4^+$-T-cell depletion was expressed as the ratio of the number of $CD8^-$ T cells to the number of $CD8^+$ T cells, the amount of which was not changed by HIV infection (23, 27). Consistent with findings in a previous study, wild-type NL4-3 HIV-1 depleted ex vivo-infected tissues of 40 to 50% of these cells within 12 days of infection. In contrast, the deletion mutants caused less $CD4^+$-T-cell depletion (a maximum of about 15%) (Fig. 2B). There was a strong correlation ($R^2 = 0.88$, $P = 0.0006$) between the number of productively infected $CD4^+$ T cells and the level of their depletion (Fig. 2C). Although deletion of either of the accessory genes significantly decreased the efficiency of mutant replication, the number of infected cells, and therefore $CD4^+$-T-cell depletion, from levels observed with the wild-type virus, donor-to-donor variability did not allow the ranking of different mutants in terms of these parameters.

We have previously shown that the loss of $CD4^+$ T cells in ex vivo human lymphoid tissue results mainly from the death of HIV-1-infected cells (27, 28). Thus, although molecular mechanisms for low viral infectivity and virus production may be different for different mutants, they all result in a lower number of infected cells and hence less $CD4^+$-T-cell depletion in infected tissues. However, more experiments need to be done to clarify whether the accessory genes might also play a direct role in cell killing, for example, through the proapoptotic activity reported for Vpr (38, 44, 47). We found that in 3 out of 13 experiments the *vpr*-deleted virus replicated with an efficiency similar to that of wild-type HIV-1 but caused no significant $CD4^+$-T-cell depletion (1.3% ± 1.3% in tissues infected with *vpr*-deleted HIV-1 versus 36% ± 10% in tissues infected with wild-type virus). In any case, our data indicate that intact



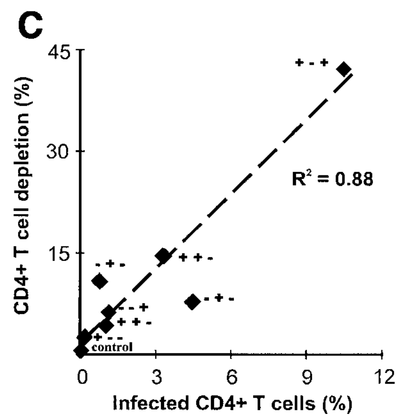


FIG. 2. $CD4^+$-T-cell depletion in human lymphoid tissue infected ex vivo with HIV-1 variants. (A) Percentages of infected cells; (B) loss of $CD4^+$ T cells in human lymphoid tissue infected ex vivo with HIV-1. Productively infected $CD4^+$ T cells were defined as $CD3^+$ $CD8^-$ $p24^+$, as described in the text. To evaluate $CD4^+$-T-cell depletion, cells were mechanically isolated from control and infected matched tissues (27 pooled blocks for each variant) on day 12 postinfection, stained for CD3, CD4, CD8, and p24, and analyzed with flow cytometry. Depletion is expressed as 100% minus the percentage of $CD4^+$ T cells that remained in the tissue after 12 days of infection, evaluated as described earlier (23, 29). Presented are average depletion values ± standard errors of the means for tissues from 4 to 12 donors. (C) Correlation between depletion and virus infection of $CD4^+$ T cells in ex vivo-infected human lymphoid cultures. Accessory gene deletions are indicated in the following order: Vpr, Vpu, Nef.

vpu, *vpr*, and *nef* genes are critical for the loss of $CD4^+$ T lymphocytes, resulting in immunodeficiency associated with AIDS.

Many of the suggested mechanisms of facilitation of HIV-1 infection by accessory genes are related to their involvement in cell activation. For example, Vpr has been shown to potentiate Nef-induced activation of NFAT (33). We have previously shown, and we confirm in the present study (Fig. 3), that Nef enhances HIV-1 responsiveness to interleukin-2 (IL-2) in human lymphoid tissue ex vivo (24). It is unclear whether Vpu or Vpr might also facilitate cell activation by autocrine and paracrine cytokine production. Therefore, we tested in matched tissues from seven donors whether Vpu and Vpr change the sensitivity of the system to IL-2. Our results demonstrate that IL-2 stimulates the replication of both wild-type NL4-3 and its mutants (Fig. 3A). On average, however, exog-

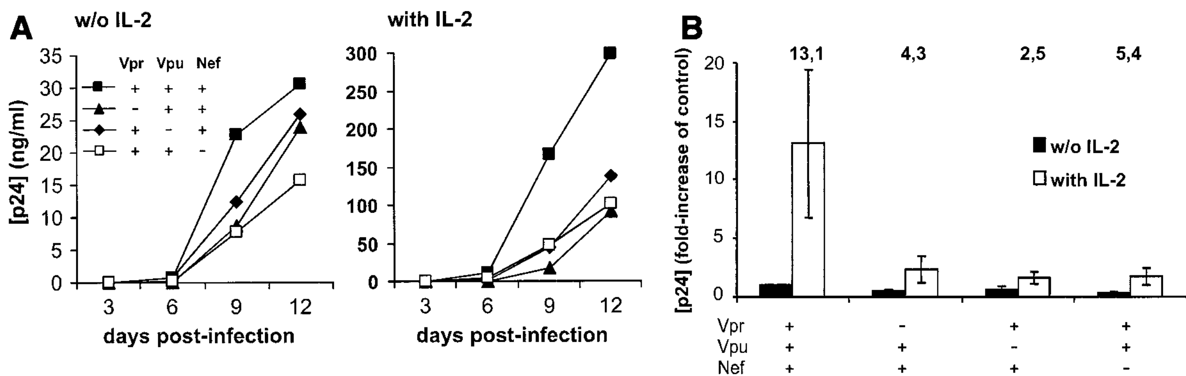


FIG. 3. Effect of exogenous IL-2 on replication of HIV-1 variants in human lymphoid tissue ex vivo. Matched infected tissues were inoculated with HIV-1 variants and cultured without or with IL-2 (50 U/ml). For each condition and each donor tissue, 27 tissue blocks were inoculated. (A) Representative time course of p24 production in unstimulated and IL-2-stimulated tissues inoculated with wild-type virus or HIV-1 variants with accessory gene deletions. (B) Cumulative viral production over 12 days of infection by wild-type virus or HIV-1 variants with accessory gene deletions. Presented are means ± standard errors of the means of the fold increases of p24 production (relative to the replication of the wild-type virus in the absence of IL-2) in tissues from seven to nine donors inoculated ex vivo with the indicated HIV-1 variants. The numbers above each bar give the fold increase of virus production in the presence of IL-2 relative to production of the respective HIV-1 mutants in the absence of IL-2.

enous IL-2 increased NL4-3 wild-type virus production about 13-fold, whereas production by the *vpr* and *vpu* deletion mutants was enhanced only 2.5- to 5.4-fold (Fig. 3B). Consequently, the difference between the replication rate of the wild-type HIV-1 and those of *vpr*- or *vpu*-deleted variants becomes more dramatic in IL-2-stimulated tissues: without IL-2, this difference was approximately two- to threefold, whereas in the presence of IL-2 it increased to five- to eightfold (Fig. 3B). Thus, accessory gene-deleted HIV-1 variants are less sensitive to stimulation by cytokines.

To investigate mechanisms by which accessory genes might affect viral spread in ex vivo-infected human lymphoid tissue, we measured the production of various cytokines known to affect HIV-1 replication. However, we did not detect any significant effect of *nef*, *vpr*, or *vpu* on the levels of macrophage inflammatory protein 1α (MIP-1α), MIP-1β, stromal cell-derived factor 1, RANTES, inducible protein 10, IL-1α, IL-1β, tumor necrosis factor alpha, IL-15, and IL-16 (data not shown). Thus, it remains to be clarified whether the described effects of mutant viruses occur at transcriptional or posttranscriptional levels. Previous data suggest, however, that accessory genes might contribute to efficient replication in human lymphoid tissue ex vivo by various mechanisms. We have demonstrated that the ability of Nef to enhance HIV-1 replication in ex vivo-infected human lymphoid tissue correlates with its functional activity in CD4 downmodulation (25). This Nef function might be critical for the production of fully infectious viral particles from $CD4^+$ T cells (6). Vpu might enhance viral spread by both the same and different mechanisms because it downmodulates CD4 (34, 53) but it also increases the release of virus particles (49, 54). Finally, Vpr is known to play a role in virus transcription, cell proliferation, and apoptosis. Most likely, the multifunctional *nef*, *vpu*, and *vpr* genes enhance HIV-1 replication in ex vivo-infected human lymphoid tissue by several mechanisms. A major finding of our study is that the levels of viral replication and the numbers of infected cells are low in human lymphoid tissue infected with the *vpu*-, *vpr*-, or *nef*-defective viruses.

In conclusion, all three accessory genes, *nef*, *vpr*, and *vpu*, are important for efficient replication and $CD4^+$-T-cell depletion in ex vivo-infected human lymphoid tissues. Some effects of these genes may be related to cell activation. Most importantly, our data suggest that, like Nef, Vpr and Vpu are relevant for efficient viral infection and for CD4 T-cell depletion in HIV-1-infected individuals.

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