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Replication and Pathogenicity of Human Immunodeficiency Virus Type 1 Accessory Gene Mutants in SCID-hu Mice

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The functional roles of the human immunodeficiency virus type 1 (HIV-1) accessory genes (*nef*, *vpr*, *vpu*, and *vif*) are as yet unclear. Using the SCID-hu model system, we have examined the infectivity, replicative capacity, and pathogenicity of strains of the molecular clone HIV-1_{NL4-3} that contain deletion mutations in these individual accessory genes. We determined that deletion of these genes had differential effects on both infectivity and pathogenicity. Deletion of *vpr* had little or no effect on viral infectivity, replication, and pathogenicity; however, deletion of *vpu* or *vif* had a significant effect on infectivity and moderate effects on pathogenicity. *nef*-minus strains were the most attenuated in this system, demonstrating significantly lower levels of infectivity and pathogenicity. However, deletion of these individual genes attenuated but did not abrogate the pathogenic properties of HIV-1. Mutant viruses still retained the ability to induce thymocyte depletion to various degrees if implants were infected with higher doses of virus or observed for longer periods of time. The relative contributions of these genes to in vivo pathogenic potential should be taken into consideration when one is contemplating a live attenuated vaccine for HIV-1.

The functional roles of the human immunodeficiency virus type 1 (HIV-1) accessory genes, *nef*, *vpr*, *vpu*, and *vif*, have not been definitively clarified. Conservation of these genes among lentiviruses argues for an important role in the viral life cycle. However, these genes can be deleted without abrogating viral replication in vitro. Studies designed to ascertain their functions have sometimes yielded disparate results, depending at least to some extent on the cell types and viral strains used. Interest in the functions of these genes has increased in recent years, as data from the simian immunodeficiency virus (SIV) system suggests critical roles in pathogenicity and the possible candidacy of accessory gene mutants as live attenuated vaccines.

Viral protein R (Vpr) is a *trans*-acting protein which is present in the virion (8, 30). Virion association suggests a role in the early stages of infection; however, its precise role in pathogenesis is unclear. Properties attributed to Vpr include facilitating nuclear import (21, 51), facilitating infection in macrophages and irradiated cell lines (19), induction of latent virus, differentiation of rhabomyosarcoma and other cell lines (32), induction of growth arrest (20, 26, 32, 41, 42), and prevention of the establishment of chronic infection (39, 42).

Viral protein U (Vpu) is a protein unique to HIV-1 and the SIV strain found in chimpanzees. It shares some structural and biochemical similarities with the influenza virus M2 protein. Vpu is a cytoplasmic protein that facilitates assembly and export of virions (49) and the degradation of CD4 in the endoplasmic reticulum (31, 40).

The viral infectivity factor (Vif) protein increases the efficiency of infection approximately 1,000-fold (13, 48); however, this effect is dramatically dependent on the cell type in which the virus is produced. *vif*-minus virus produced in some cells (HeLa, COS) is fully capable (permissive) of viral replication, but virus produced in other cells (H9, PBL) is severely impaired (restrictive) (7, 11, 14, 44). Certain CEM lines and other cells have been termed semipermissive, since *vif*-minus mutants can replicate to intermediate levels (14, 44, 48). Vif is a nonstructural protein, and it remains an area of debate as to how it increases infectivity without virion association. It appears that Vif is necessary for efficient reverse transcription (9, 45, 50) and proper packaging of the viral nucleoprotein core (23), as well as proper viral particle assembly (5).

Perhaps the most contentious of these accessory genes, *nef*, has been reported to have a negative, positive, or no effect on viral replication, depending on the in vitro system used. There is general agreement that Nef causes down-regulation of CD4 (15, 18); however, the in vivo significance of this phenomenon is uncertain. Recent work (35, 46) has determined that Nef is important for maximal replication in primary CD4 cells during cellular induction from a quiescent to an activated state. Nef also appears to be important for optimal infectivity (1, 7, 35). Other functions ascribed to Nef include suppression of the interleukin 2 gene (33) and inhibition of nuclear factor κB induction (37). A recent report also showed that a single mutation in SIV *nef* could cause activation of target cells (11).

SIV infection of rhesus macaques has been used as a model for the study of the contribution of the SIV accessory genes, *nef*, *vpr*, *vpx*, and *vif*, to viral pathogenesis. The most provocative studies have been those involving deletions of *nef*. Kestler et al. (27) found that although deletion of SIV *nef* had little or no effect on in vitro replication, infection with SIV *nef*-minus strains in vivo resulted in low-level viremia without development of disease. Moreover, several years later, these animals were protected against challenge with wild-type virus (10), suggesting that the *nef*-minus strain was functioning as a live attenuated vaccine. Lang et al. (29) found that point mutations in SIV *nef* and *vpr* rapidly reverted in vivo, suggesting a strong selective pressure for the functional form of these genes. This phenomenon has not been observed in vitro. More recently, Gibbs et al. (16) and Hoch et al. (22) have reported that deletion of the SIV *vpr* gene alone did not attentuate pathogenicity or progression to AIDS; however, deletion of both *vpr*

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and *vpx* severely attenuated viral replication, and the infected animals did not progress to AIDS (16).

We recently reported that deletion of HIV-1 *nef* markedly attenuated replication and pathogenicity in the SCID-hu mouse model but this deletion had no such effect in cultured phytohemagglutinin (PHA)-activated peripheral blood lymphocytes (24). These results as well as the previously mentioned experiments in rhesus macaques demonstrate the vital role of in vivo studies in elucidating the function and importance of HIV-1 accessory genes in the pathogenic process. The demonstration that infection with a *nef*-deleted virus protected against infection with wild-type SIV has led some researchers to advocate attenuated HIV-1 strains as vaccine candidates. However, prior to consideration of HIV-1 strains with deletions of the accessory genes as vaccines, it will be necessary to ascertain the pathogenic properties of these viruses in a relevant in vivo system that demonstrates pathology following infection with the human virus.

The SCID-hu mouse is a unique in vivo system which permits evaluation of HIV pathogenicity in the absence of a host immune response. Therefore, it is a valuable tool in the preclinical evaluation of the pathogenic potential of such mutated viruses. The SCID-hu mouse is constructed by the surgical implantation of human fetal liver and thymus under the murine kidney capsule of severe combined immunodeficient (SCID) mice (34, 36). These tissues form a conjoint (Thy/Liv) organ. Histologically, this organ resembles a normal human thymus wherein normal lymphocyte differentiation occurs for periods of up to 1 year (36). We and others (2, 4, 24, 28, 47) have demonstrated that infection of this organ with HIV-1 induces pathology similar to that described in infected humans (38, 43). In the SCID-hu system, HIV-1 causes depletion of CD4-bearing cells, and this depletion usually correlates with high viral load (2, 25). Thymocyte depletion in this dynamic system appears to be dependent on the ability of the virus to replicate more rapidly than thymocytes can be regenerated from precursor cells.

Our earlier studies indicated that HIV-1 strains containing deletions or frameshift mutations in *nef* were attenuated in their replicative and pathogenic properties in the SCID-hu mouse (24). These studies suggested that the efficacy of the *nef*-deleted SIV strain as a live attenuated vaccine may have been due to its inability to replicate rapidly in lymphoid tissues, thereby allowing the resulting host immune response to control the infection. In addition, these data indicated that the SCID-hu mouse could be used to assess the replicative and cytopathic properties of attenuated HIV strains in a functional and dynamic lymphoid organ.

In this study, we compare effects of deletions in the auxiliary genes *nef*, *vpr*, *vpu*, and *vif* on HIV-1 pathogenicity in SCID-hu mice. We report that deletions of individual accessory genes result in minimal to severe attenuation of the infectivity and pathogenicity of these mutant viruses. However, none of these deletions completely abrogated viral replication or cytopathicity in the SCID-hu system.

MATERIALS AND METHODS

Virus and cells. Viral accessory gene deletion mutants using the infectious molecular clone, HIV-1_{NL4-3}, were constructed in the laboratory of Ron Desrosiers and have been previously described in detail (17). Substantial portions of the accessory genes (115 to 212 bp) were deleted such that in vivo reversion could not occur. However, only the *vpu* mutant had its initiating methionine deleted, so other mutants could yield truncated N-terminal translation products. The *vpr* deletion mutant had a 115-bp deletion that resulted in a frameshift.

Virus stocks were prepared by cotransfection of the appropriate constructs into CEMx174 cells as previously described (6). Viral stocks were collected, filtered, and assayed for p24 content by enzyme-linked immunosorbent assay (ELISA) (Coulter; Hialeah, Fla.) on days 5, 6, and 7. Aliquots of the viral stocks were stored at −70°C. Viruses were titrated in parallel by fivefold limiting dilution in duplicate on human peripheral blood mononuclear cells (PBMC) from a single donor that had been stimulated for 3 days with PHA. Infectious units (IU) were standardized to HIV-1_{NL4-3} (wild type), in which 2.5 ng of p24 was equivalent to 100 IU. Normal human PBMC were obtained from leukopaks purchased from the American Red Cross. Peripheral blood lymphocytes were isolated by centrifugation over Ficoll-Hypaque and depleted of macrophages by adherence to plastic for 72 h. Growth kinetics of the different viral isolates were determined by infection with equal infectious units on human PHA-stimulated PBMC, followed by quantitation by ELISA specific for the viral p24 Gag protein. After infection of the Thy/Liv grafts, an aliquot of virus from the same vial used to infect the tissue was used to infect PHA-stimulated PBMC to confirm virus viability.

Construction, infection, and biopsies of SCID-hu mice. Severe combined immunodeficient C.B.-17 *scid/scid* (SCID) mice were originally obtained from K. Dorshkin and subsequently bred at the University of California, Los Angeles. All experimental animals were housed in a biosafety level 3 facility at the University of California, Los Angeles, and handled in accordance with institutional guidelines. All animals were anesthetized by intraperitoneal injection of a ketamine HCl-xylazine mixture (1 mg/10 g of body weight) prior to any invasive manipulation. At 6 to 8 weeks of age, ≈1-mm^3 pieces of human fetal thymus and liver were surgically implanted under the murine kidney capsule as previously described (2). Fetal tissue (Advanced Bioscience Resources, Alameda, Calif.) was obtained from donors ranging in gestational age from 16 to 24 weeks. Four to six months postimplantation, the grafts were infected with 100 or 1,000 IU in approximately 50-μl volumes by direct injection. Mock-infected implants were injected with media.

Since it is possible to obtain only limited numbers of reconstituted SCID-hu mice from a single donor, multiple donors were used for these experiments. To control for any variations in genetic backgrounds that might have had a bearing on the susceptibility of the target cells to HIV infection, mice transplanted with tissues from different donors were distributed randomly among the experimental groups that were infected with the various HIV-1 mutants. In addition, wild-type, mock, and at least two isogenic accessory gene mutants were inoculated into tissue from a single donor. In these experiments, we did not detect any obvious differences attributable to donor variation; however, the numbers of animals and donors were too small to for statistical analysis to be performed.

Wedge biopsies of Thy/Liv tissue were obtained at 3-week intervals after infection. Approximately one-fourth to one-third of the implant was removed at each biopsy. Human thymocytes were teased from the stromal elements, filtered through a screen (Cell strainer; Falcon, Franklin Lakes, N.J.), washed in phosphate-buffered saline, counted, and then aliquoted for subsequent PCR and flow cytometric analyses.

Quantitative PCR amplification. Single cells obtained from the biopsied implants were lysed in urea lysis buffer (4.7 M urea, 1.3% [wt/vol] sodium dodecyl sulfate, 0.23 M NaCl, 0.67 mM EDTA [pH 8.0], 6.7 mM Tris-HCl) and subjected to phenol-chloroform extraction followed by ethanol precipitation of nucleic acids. Alternatively, DNA was isolated by using a QIAamp Blood Kit (Qiagen, Chatsworth, Calif.) according to the manufacturer's protocol. Purified DNA was then subjected to quantitative PCR as previously described (2, 52, 53). Briefly, PCR amplifications were carried out for 25 cycles with ^{32}P end-labeled primers. The M667-AA55 primer pair, which is specific for the R/U5 region of the viral long terminal repeat, was used for detection of HIV-1 sequences (52, 53). The amount of human cellular DNA in each sample was quantified by PCR amplification using primers specific for the human β-globin gene (nucleotides 14 to 33 and 123 to 104). Standard curves for HIV-1 DNA consisted of linearized HIV-1_{JR-CSF} in normal human PBMC DNA (10 μg/ml) as a carrier. Standard curves for human β-globin were derived from 10-fold dilutions of normal PBMC DNA. Both the HIV-1 and β-globin standard curves were amplified in parallel with Thy/Liv samples. The PCR amplifications were carried out in 15 μl of low-salt PCR buffer (25 mM Tris [pH 8.0], 2 mM $MgCl_2$, 30 mM NaCl, 0.1 mg of bovine serum albumin per ml, 0.25 mM deoxynucleoside triphosphate). S/P high-purity water (Baxter Healthcare Corp., McGaw Park, Ill.) was used to bring the reaction volume to 25 μl. Following amplification, PCR products were resolved on a 6% polyacrylamide gel. Quantitation was achieved by extrapolation to the standard curves, using radioanalytic image analysis (Ambis, San Diego, Calif.). This method of DNA PCR can detect 10 proviral copies per microgram of genomic DNA. Values obtained from this assay never varied above 30% of the actual values in controlled experiments.

Confirmation of infection. To confirm infection of each implant with the appropriate deletion mutant, primer pairs flanking each deletion were used to amplify proviral sequences by PCR. Analysis was performed on DNA obtained 6 weeks postinfection. No contamination with wild-type sequences was ever detected. Primer sequences were as follows: *nef*, 5′-AGCAGTAGCTGAGGGG ACAGATAGG-3′ (sense) and 5′-AGCTCGATGTCAGCA-3′ (antisense); *vpr*, 5′-GGACGTATTAGTTAGTCCTAGGTGTGAATATCCAAGC-3′ (sense) and 5′-CGAGTAACGCCTATTCTGCTATGTCGAC-3′ (antisense); *vpu*, 5′-GCG GAGACAGCGACGAAGAGCTCATC-3′ (sense) and 5′-CCTCTGTATCATA TGCTTTAGCATCTGATGCAC-3′ (antisense); and *vif*, 5′-CAGATGGCAGG TGATGATTGTGTGGC-3′ (sense) and 5′-CACACCAGGACTAACTATACG TC-3′ (antisense).

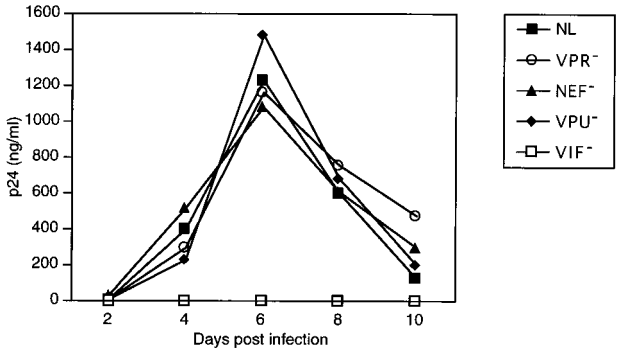

FIG. 1. In vitro replication of HIV-1_{NL4-3} and accessory gene mutants. PHA-stimulated PBMC (10^6) from a single donor were infected with $\approx 2.5 \times 10^3$ IU of the various viral strains. Viral replication was quantitated by ELISA for HIV-1 p24 antigen at the indicated days postinfection. Data are representative of three separate experiments.

Flow cytometric analysis of Thy/Liv cells. Thymocytes were stained with a phycoerythrin-conjugated mouse monoclonal antibody to human CD4 (Becton Dickinson, Mountain View, Calif.) and fluorescein isothiocyanate-conjugated mouse monoclonal antibody to human CD8 (Becton Dickinson) according to the manufacturer's instructions. Thymocytes were also stained with phycoerythrin- and fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin G1 as isotype controls. Data were acquired on a FACScan flow cytometer and analyzed with the Lysis II or Cell Quest program (Becton Dickinson). The live cell population was determined by gating on the forward versus side scatter plot of thymocytes derived from mock-infected implants. A total of 5,000 to 10,000 events was acquired, except in the case of severely depleted implants.

Statistical analyses. Comparisons of proportions between groups were made by using Fisher's exact test. This test is more appropriate than a chi-square test for small sample sizes and is asymptotically equivalent to a chi-square test for large samples. To test whether two distributions were the same, the Wilcoxon rank-sum test was used. This nonparametric test is valid over a wide range of distributional assumptions and is invariant with respect to monotonic transformations such as taking logarithms. All comparisons were for two-sided alternatives. The Kruskal-Wallis one-way analysis of variance was used for comparing three or more groups.

Infectivity was measured by the proportion of implants showing detectable HIV at the 3- and/or 6-week time points. Animals that had undetectable HIV 3 weeks postinfection and that died prior to the 6-week biopsy were excluded from the analysis, since infection could not be confirmed. Calculations for demonstrating viral replication and differences in pathogenicity were made by using only the animals that were positive for HIV DNA sequences at the 3- and/or 6-week time points. Pathogenicity was measured by the percentage of CD4 CD8 double-positive thymocytes at both time points. Viral replication was calculated by proviral copies per 100,000 cells.

RESULTS

In vitro replication of HIV-1_{NL4-3} and accessory gene mutants. Growth of the wild-type viral strain, HIV-1_{NL4-3}, was compared with growth of strains which had deletions in the individual accessory genes (*nef*, *vpr*, *vpu*, and *vif*) in PHA-activated PBMC. As had been previously established (17), all of the mutant viruses had kinetics similar to those of wild-type virus (Fig. 1). Replication of the *vif* deletion mutant was consistently and reproducibly lower than that of wild-type virus, in agreement with the findings of others (23, 50). CEMx174 cells used for production of these viral stocks are only intermediately permissive for *vif*-minus virus; therefore, infection with *vif*-minus mutants may have been somewhat less efficient.

In vivo replication of wild-type and accessory gene mutants. Thy/Liv implants of SCID-hu mice were inoculated with approximately 100 IU of wild-type and accessory gene mutant viruses. Sequential biopsies were obtained at 3-week intervals postinoculation, and biopsies were analyzed by flow cytometry for CD4 and CD8 and by quantitative DNA PCR for viral sequences. To assess viral replication kinetics, implants were first biopsied and assessed at 3 weeks postinoculation, since wild-type virus generally does not dramatically deplete thymocytes within this time frame. At the 3-week timepoint, none of the 14 implants injected with the *vif*-minus strain and only one of the 11 implants injected with the *nef*-minus strain had detectable proviral sequences (Fig. 2A). In contrast, 20 of 20 implants injected with wild-type virus, 10 of 11 implants injected with *vpr*-minus, and 5 of 11 implants injected with *vpu*-minus virus had detectable HIV-1 proviral sequences. The implants injected with wild-type virus had a median of 4,187 and a mean of 9,608 of proviral copies per 100,000 cell equivalents. Implants injected with the *vpr* mutant had a median of 789 and a mean of 2,807 proviral copies per 100,000 cell equivalents. The median HIV copy number per 100,000 cells of the productively injected *vpu*-minus implants was 895, with a mean of 2,347.

At 3 weeks postinoculation, implants injected with viruses containing deletions in the *vpu*, *vif*, and *nef* genes had flow cytometric profiles similar to those of mock-injected implants (Fig. 2B). However, 10 of 18 implants injected with wild-type virus and 1 of 9 implants injected with the *vpr*-minus strain showed depletion of CD4 CD8 double-positive cells, as defined by a decrease in this subset from normal levels of 70 to 90% to less than 40%. The distributions of the CD4 CD8 double-positive cells among injected implants at 3 weeks postinoculation were significantly different between the wild-type and *vpr*-minus groups ($P < 0.0009$).

Six weeks postinoculation, virus was detected in only 1 of 9 implants injected with *nef*-minus virus, 2 of 10 implants injected with *vpu*-minus virus, and 2 of 13 implants injected with *vif*-minus virus (Fig. 3A). Thus, apparent infectivity of these

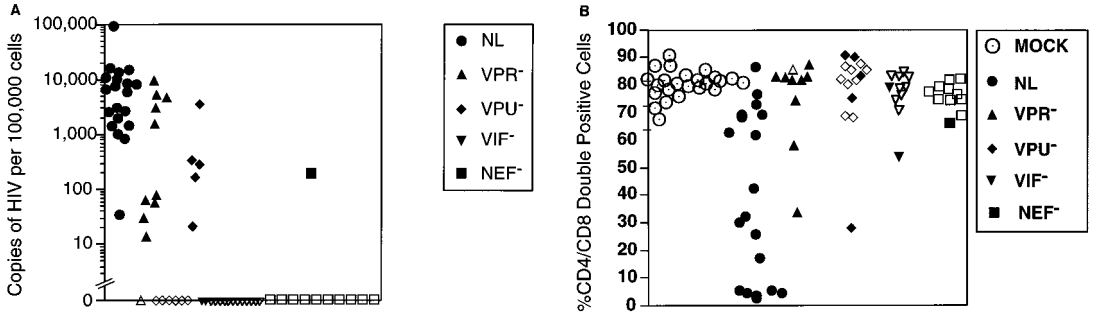

FIG. 2. Thymocyte depletion and proviral load in implants infected with 100 IU at 3 weeks postinfection. Each symbol represents a different implant infected with the corresponding viral strain as described for Fig. 1. Open symbols represent animals that did not have detectable proviral sequences at either the 3- or 6-week time point. The number of HIV-1 genomes in 10^5 human thymocytes, as determined by quantitative DNA PCR, is plotted in panel A. Points positioned on the baseline indicate undetectable proviral DNA sequences. The percentage of CD4 CD8 double-positive thymocytes was obtained by flow cytometry as described in Materials and Methods (B).

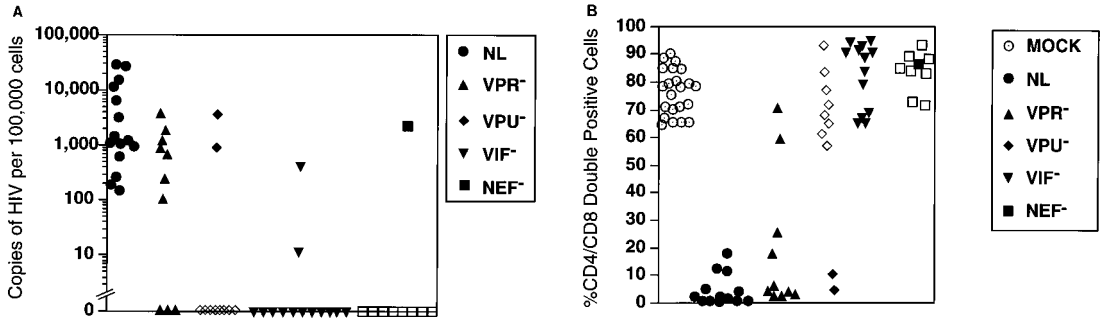

FIG. 3. Thymocyte depletion and proviral load in implants infected with 100 IU at 6 weeks postinfection. Symbols and methods are as described in the legend to Fig. 2.

viruses was significantly less than that of wild-type virus ($P < 0.0002$ for all viruses).

The median HIV copy number per 100,000 cell equivalents dropped to 1,173 (mean of 6,328) in the implants injected with wild-type virus, which is consistent with our previous studies and reflects depletion of the $CD4^+$ target cells and the subsequent loss of proviral DNA (2). The median HIV copy numbers per 100,000 cells of implants that were productively infected with the mutated strains were 2,173 for the implants infected with the *vpr*-minus strain, 2,276 for implants infected with the *vpu*-minus strain, 197 for implants infected with the *vif*-minus virus, and 2,160 for the only implant infected with the *nef*-minus virus. At the 6-week time point, all of the implants infected with wild-type virus (12 of 12) and almost all of the implants productively infected with the *vpr*-minus strain (8 of 9) had severely depleted CD4-bearing thymocyte subsets (Fig. 3B). The difference in pathogenic potential between wild-type and *vpr*-minus viruses was not significant at this time point ($P = 0.051$). However, the implant that had received *vpr*-minus virus and did not have detectable proviral DNA had a normal flow cytometric profile. The profiles of the implants injected with *vif* and *nef* mutants and 8 of 10 *vpu*-minus implants were identical to those of the mock-injected implants, reflecting the apparent lack of infection. Two implants productively infected with the *vpu*-minus strain showed severe depletion of thymocytes by this timepoint.

Implants were subsequently biopsied at 9 and 12 weeks postinoculation and analyzed (data not shown). However, even at these later time points, no additional implants injected with either *vpu*-minus (none of six) or *vif*-minus (none of eight) strains were found to have detectable proviral DNA. In contrast, two additional implants injected with the *nef*-minus strain had detectable provirus at later time points, suggesting slower growth of the virus that resulted in delayed detection. Significantly, in implants that were productively infected with the *vpu*- or *vif*-minus strain, virus replicated to high titers and caused depletion of CD4-bearing thymocytes at these later time points. One of the two implants infected with the *nef*-minus strain replicated to a high enough titer to cause depletion within this time frame.

In vivo effects of larger inocula of mutated viral strains. The results described above suggested that the lack of detectable *nef*-, *vpu*-, or *vif*-minus proviral DNA in the implants could be due either to extreme attenuation of replication or to decreased infectivity of these viruses. To determine if these results were due to differences in infectivity that had not been observed in the in vitro studies in PHA-stimulated PBMC, we performed a second series of experiments involving inoculation with 10-fold more virus. Approximately 1,000 IU (10×) of the various mutated viral strains was directly injected into the implants and compared with wild-type virus infections of the standard 100 IU (1×). At 3 weeks postinfection, all implants injected with 1× wild-type or 10× *vpr*-minus strain and eight of nine injected with 10× *vpu*-minus strain had detectable HIV proviral DNA; however, only 6 of the 15 implants injected with 10× *nef*-minus virus and two of the five implants injected with 10× *vif*-minus virus had detectable viral sequences (Fig. 4A). The flow cytometric profiles generally reflected the proviral load at this time point (Fig. 4B): implants with higher proviral loads demonstrated thymocyte depletion, with the exception of implants infected with the *nef*-minus strains, which demonstrated profiles similar to those of mock-injected implants. At 6 weeks postinoculation, 12 of 15 implants injected with 10× *nef*-minus virus had detectable HIV sequences, and levels of infectivity were not significantly different from that for 1× wild-type virus ($P = 0.066$) (Fig. 5A), but no additional im-

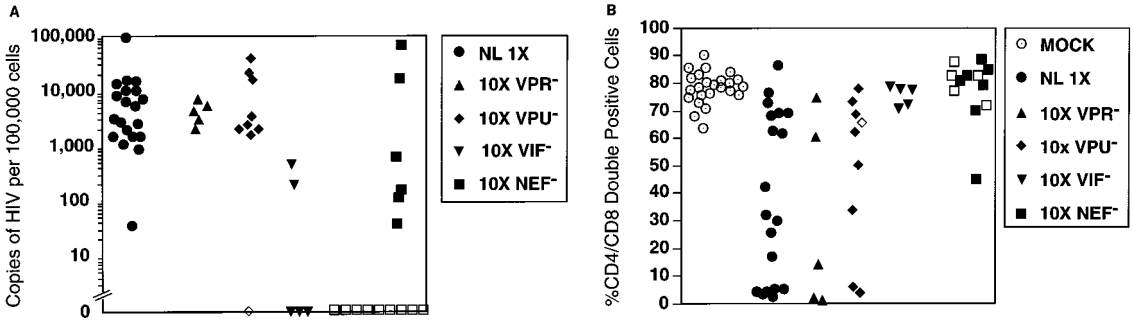

FIG. 4. Thymocyte depletion and proviral load in implants infected with 1,000 IU at 3 weeks postinfection. Symbols and methods as described in the legend to Fig. 2.

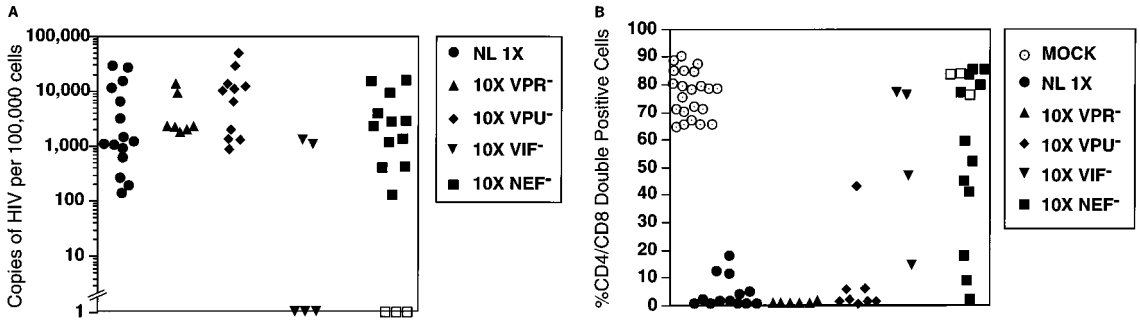

FIG. 5. Thymocyte depletion and proviral load in implants infected with 1,000 IU at 6 weeks postinfection. Symbols and methods are as described in the legend to Fig. 2.

plants (two of five) injected with 10× *vif*-minus had detectable viral sequences. Thus, the infectivity of the 10× *vif*-minus virus was statistically different from that of wild-type virus ($P = 0.0034$). Implants injected with *vif*-minus virus that had undetectable proviral sequences at 6 weeks postinfection remained negative for viral DNA even at 12 weeks postinfection (not shown).

All of the implants injected with the wild-type, 10× *vpr*-minus, or 10× *vpu*-minus strain had marked thymocyte depletion at the 6-week time point. No statistically significant differences in pathogenicity were seen among these three strains ($P > 0.4$) (Fig. 5A). However, only 3 of 12 implants productively infected with 10× *nef*-minus virus and one of two implants productively infected with 10× *vif*-minus virus were depleted (Fig. 4A). The difference in distribution of the CD4 CD8 double-positive cells in the implants injected with the 10× *nef*-minus virus at the 6-week time point was highly significant compared with implants injected with wild-type virus ($P = 0.0001$). By 9 weeks postinfection, only one additional implant injected with 10× *nef*-minus virus was depleted, but both implants productively infected with the 10× *vif*-minus strain were depleted (not shown). Thus, in contrast to 10× *nef*-minus virus, when implants were productively infected with the 10× *vif*-minus strain, replication to high proviral load and subsequent depletion did occur, suggesting that if the block to infectivity is overcome, human thymocytes are permissive for *vif*-minus virus, and thus this virus has pathogenic potential in vivo.

DISCUSSION

The SCID-hu model provides a system in which phenotypic differences not seen in traditional in vitro systems can be observed. Thus far, these differences recapitulate what has been observed in SIV infection of rhesus macaques. The SCID-hu Thy/Liv mouse has the additional advantage of not being immunologically competent, thereby allowing assessment of the intrinsic pathogenicity of HIV in the absence of a modulating host immune response. We used the SCID-hu system to examine the pathogenic potential of viral strains of HIV-1_{NL4-3} with deletions in the various accessory genes. In this system, deletion of these genes had differential effects on replication and pathogenicity. It appears that the contribution to pathogenicity by these genes is quantitatively different, with *vpr*-minus strains being almost as pathogenic as wild-type virus, *vpu*-minus and *vif*-minus strains being moderately less pathogenic, and *nef*-minus strains being significantly attenuated. Thymocyte depletion usually only occurs as a consequence of high levels of viral replication (25). Our previous study (24) reported that *nef* mutants had a decreased replicative and, hence, decreased pathogenic potential in SCID-hu mice. In the current study, we introduced 10-fold more of the mutant virus and seem to have overcome the inability of *nef*-minus virus to replicate to high levels in vivo. Indeed, the proviral loads were statistically indistinguishable from wild-type loads. In a previous study, we had calculated proviral load per remaining CD4 cell as an alternate method of measuring differences in replicative capacity (24); however, even when examined in this manner, there was no significant difference between wild-type virus and 10× *nef*-minus virus in our current study (data not shown). These results suggest that *nef*-minus viruses may be intrinsically less cytopathic for thymocytes than wild-type viruses. However, studies involving much larger numbers of animals will be required to definitively determine if proviral loads for 10× *nef* mutant viruses are actually equivalent to those of 1× wild-type virus. It appears that the attenuated natures of *vpu*- and *vif*-minus mutants are at least partially due to decreased abilities to productively infect Thy/Liv implants.

The relative lack of effect of the *vpr* deletion on thymic pathology is quite striking. Certain effects of Vpr, including nuclear localization and facilitation of infection, have been observed only in macrophages and irradiated cell lines. Since HIV-1_{NL4-3} is not a macrophage-tropic virus, and the SCID-hu system predominantly assesses replication in T-lineage cells, these factors may account for the lack of this type of effect. Other putative functions of Vpr, including cell cycle arrest and prevention of chronic infection and cellular differentiation, should be operative in lymphocytes. Our results suggest that they apparently have little relevance in the thymus. Similarly, deletion of *vpr* in SIV had no effect on progression to AIDS in rhesus macaques (16, 22), although deletion of both *vpr* and the related *vpx* markedly decreased pathogenicity (16). HIV-1 does not have a *vpx* gene, so a precise correlation with SIV is not possible.

It should be emphasized that in SCID-hu mice, deletion of these individual viral genes attenuated the pathogenic potential of HIV but did not abrogate it. If Thy/Liv implants were inoculated with 10-fold higher doses of virus or were analyzed at later time points, all of these mutated viral strains were capable of achieving high proviral loads and inducing pathology (i.e., thymocyte depletion). These data could help explain the recent finding that SIV containing deletions in *vpr*, *nef*, and the negative regulatory element caused disease in newborn but not adult monkeys (3). Our data suggest that in the absence of a complete immune response, deletion of accessory genes results in less virulent but not avirulent viral phenotype. It is hypothesized that the mature competent immune system of adult animals controls infection by an attenuated strain of SIV,

but newborn animals, which lack a fully mature immune system, cannot control viral replication and succumb to the pathogenic effects of the virus (3). Thus, as is true of other live vaccines, target populations must be carefully selected if an attenuated HIV vaccine is to be contemplated.

Our experiments have important implications for the development of a live attenuated vaccine strategy for HIV-1, as all of the mutated strains were capable of causing pathology to some extent. For safety considerations, an avirulent vaccine would be the ideal; however, other live viral vaccines (e.g., measles, mumps, vaccinia, and polio vaccines) in which the viruses were rendered significantly less virulent but not avirulent have been extremely successful. Smallpox has been eradicated from the planet, and polio has disappeared from the Western Hemisphere. However, these other attenuated viral vaccines show much less pathogenic potential than what we have observed in these HIV deletion mutants in the SCID-hu mouse. The differential effects on viral replication and pathogenicity that we have observed suggest that deletion of certain genes of HIV may be more advantageous than deletion of others. Further experiments to study the interactive effects of multiple auxiliary genes are important to establish an optimally attenuated strain and are currently in progress. Investigations of how these mutated HIV strains might behave in an immunocompetent host must await the advent of a more complete model system.

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