The Heptad Repeat 2 Domain Is a Major Determinant for Enhanced Human Immunodeficiency Virus Type 1 (HIV-1) Fusion and Pathogenicity of a Highly Pathogenic HIV-1 Env[▽]

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Human immunodeficiency virus type 1 (HIV-1)-mediated depletion of CD4⁺ lymphocytes in an infected individual is the hallmark of progression to AIDS. However, the mechanism for this depletion remains unclear. To identify mechanisms of HIV-1-mediated CD4 T-cell death, two similar viral isolates obtained from a rapid progressor patient with significantly different pathogenic phenotypes were studied. One isolate (R3A) demonstrates enhanced pathogenesis in both in vivo models and relevant ex vivo lymphoid organ model systems compared to another isolate, R3B. The pathogenic determinants were previously mapped to the V5-gp41 envelope region, correlating functionally with enhanced fusion activity and elevated CXCR4 binding affinity. To further elucidate specific differences between R3A and R3B within the V5-gp41 domains that enhance CD4 depletion, R3A-R3B chimeras to study the V5-gp41 region were developed. Our data demonstrate that six residues in the ectodomain of R3A provide the major determinant for both enhanced Env-cell fusion and pathogenicity. Furthermore, three amino acid differences in the heptad repeat 2 (HR-2) domain of R3A determined its fusion activity and significantly elevated its pathogenic activity. The chimeric viruses with enhanced fusion activity, but not elevated CXCR4 affinity, correlated with high pathogenicity in the thymus organ. We conclude that the functional domain of a highly pathogenic HIV-1 Env is determined by mutations in the HR-2 region that contribute to enhanced fusion and CD4 T-cell depletion.

Human immunodeficiency virus type 1 (HIV-1) is the causative agent for AIDS, which is characterized by a dramatic loss of CD4⁺ lymphocytes and impairment of the immune system against invading pathogens (13, 21, 22). Though much has been determined regarding interactions between HIV-1 virus and CD4⁺ target cells, the mechanisms by which the HIV-1 virus depletes CD4⁺ lymphocytes remain incompletely understood. Various studies have demonstrated that in an HIV-infected host, both infected and uninfected cells are prone to destruction, albeit by different pathways (15, 18, 29). Recently, our group and others have shown that while binding of CD4 and chemokine receptors contribute to syncytium formation in vitro, viral membrane fusion by the envelope glycoprotein plays an important role in depletion of both uninfected and infected cells by HIV-1 and simian-human immunodeficiency virus in vivo (1, 11, 12, 26, 29).

HIV-1 entry into a cell is mediated by a multistep process that begins with high-affinity binding between viral envelope (gp120) and the cellular CD4 receptor (9, 14, 16). This binding causes a conformational change in the viral envelope, allowing for subsequent coreceptor binding (mainly CCR5 or CXCR4). Upon coreceptor binding, another conformational change is thought to take place that allows gp41 to engage the cell to form a fusion complex. Envelope proteins have been demon-

strated to exist as a trimer, allowing for three gp41s to form a fusion assembly through noncovalent interactions. This fusion assembly is determined to exist in a six-helix bundle formation as the fusion event takes place, allowing for the virion to fuse to the host cell (5, 24).

The envelope glycoprotein (Env) of HIV plays a significant role in viral pathogenesis, as seen in several in vitro and in vivo models of infection. The Env functions to mediate virus entry of cells and is also a major target for immune responses (31, 39). While the envelope initially forms as a precursor protein (gp160), subsequent cleavage by a cellular protease yields the surface subunit gp120 and the transmembrane gp41 although the gp120 and gp41 interact noncovalently (36). The gp120 protein is comprised of five variable (V1 to V5) and five conserved constant (C1 to C5) domains and binds CD4 and the coreceptors. The gp41 protein is comprised of an amino-terminal fusion domain and two heptad repeats (HR-1 and HR-2) in the ectodomain (extracellular domain), a single transmembrane domain, and a cytoplasmic tail (intracellular domain) (8, 10, 36, 37). Due to the discovery of fusion inhibitor peptides such as C34 (23, 24) and T20 (38), much is now known about the fusion complex formed by the HIV-1 fusion domain. Similar to other viral envelopes that carry a type 1 fusion complex (such as influenza and corona viruses), the ectodomain of HIV-1 Env carries two HRs that form a coiled-coiled structure. In order for HIV-cell fusion to occur, the HR-1 domains of the trimeric Env protein must interact with the cell surface. Following this initial interaction, HR-2 domains are thought to intertwine over the HR-1 coils to form a stable six-helix bundle, which represents the gp41 core structure. X-ray crystallographic studies show that the six-helix bundle core consists of

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the HR-1 and HR-2 peptides bound in an antiparallel manner. This structure brings the fusion peptide to the target cell membrane, allowing for the formation of a fusion pore and the entry of virions into the cell.

HIV-1 Env expressed on the surface of infected cells can induce cell-cell fusion with adjacent uninfected cells to form multinucleated syncytia and single cell lysis in cell culture and apoptosis in primary cells. Various models (both *ex vivo* and *in vivo*) have been utilized to study HIV-1-induced depletion of CD4⁺ lymphocytes. Models such as SCID-human thymus-liver (SCID-hu thy/liv), tonsil histoculture, and human fetal thymus organ culture (HFTOC) have demonstrated significant use in the study of acute infection and pathogenesis in the appropriate lymphoid organ microenvironment as they retain the organ structure and do not require exogenous stimulation for productive viral infection to occur (2, 20, 28, 32). More importantly, tissue culture-adapted HIV-1 isolates such as HXB2 fail to replicate in the SCID-hu thy/liv or HFTOC models (30, 33). Organ models such as the SCID-hu thy/liv and HFTOC thus more accurately demonstrate infection, replication, and pathogenicity of primary HIV-1 strains.

Here, HFTOC is used to investigate mechanisms by which an HIV-1 virus with a highly pathogenic viral Env is able to deplete CD4⁺ lymphocytes. Two viral isolates obtained from rapid progressor patient 3 of the ALIVE cohort (40) show significant sequence homology, particularly in the Env region, while they carry stark differences in pathogenic ability (26, 27). One isolate (denoted R3A) was found to demonstrate enhanced fusion in cell-cell fusion assays as well as enhanced pathogenesis in relevant *ex-vivo/in vivo* organ model systems compared to another isolate, R3B. To define the pathogenic determinants that differentiate R3A from R3B, this study demonstrates that the enhanced fusogenicity of R3A (governed by the ectodomain of the gp41), but not the elevated CXCR4 binding affinity, confers the pathogenic phenotype in HFTOC. We further demonstrate that three amino acid differences in the HR-2 domain allow for this enhanced fusion for R3A Env, defining a possible mechanism for a pathogenic HIV-1 envelope.

MATERIALS AND METHODS

Cells. A293T and Magi-CXCR4 cells (obtained from the NIH AIDS Research and Reference Reagent Program) were cultured in Dulbecco's modified Eagle medium supplemented with 10% (vol/vol) bovine calf serum (Sigma Chemical, St. Louis, MO) and 100 µg/ml of penicillin and streptomycin. SupT1 and BC7 cells (catalog no. 100 and 11434, respectively; NIH AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases [NIAID], NIH, from James Hoxie) were cultured in RPMI 1640 medium supplemented with 10% (vol/vol) bovine calf serum and 100 µg/ml penicillin and streptomycin. HOS-CXCR4 cells (HOS-CD4-Fusin; obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, from Nathaniel Landau [3]) were stably transfected to express long terminal repeat (LTR)-driven luciferase and were cultured in Dulbecco's modified Eagle medium supplemented with 10% (vol/vol) bovine calf serum and 100 µg/ml penicillin and streptomycin. All virus isolates were prepared and stored in Iscove's modified Dulbecco's medium supplemented with 10% (vol/vol) bovine calf serum and 100 µg/ml penicillin and streptomycin. Peripheral blood mononuclear cells (PBMCs) were purified from the blood of healthy HIV-1-negative donors by Ficoll-plaque density gradient centrifugation and cultured in Iscove's modified Dulbecco's medium supplemented with 10% bovine calf serum and 100 µg/ml penicillin and streptomycin. PBMCs were stimulated with 5 µg/ml of phytohemagglutinin (Sigma) and 20 units/ml recombinant interleukin-2 (Sigma) for 3 days and then cultured in 20 units/ml recombinant interleukin-2.

Virus isolates and drugs. Isolation of Env from primary isolates and cloning of NL4-R3A and NL4-R3B have been previously described (27). Peptide C34 was synthesized by the MicroChemistry Laboratory of the New York Blood Center (kindly provided by S. Jiang). C34 was reconstituted in phosphate-buffered saline at a concentration of 1 mg/ml.

Chimeric envelope construction. R3A and R3B envelope chimeras were developed in a retroviral vector (high-titer, stem cell, pGK-GFP [HSPG]) (6, 27) using an overlap PCR strategy with the following primers: V3F, GTAACCTTA GGACCAGGCAGAG; V5F, GCTGTGTTCTTGGGTTCTTGG; V5R, CCA AGAACCAAGGAACACAGC; gp41F, CACCATTATCGTTCCAGACCCG; gp41R, CGGGTCTGGAACGATAATGGTG, and HSPGR, CTAAGCGCA TGCTCCAGACTG. Chimeric Env regions were cloned back into the HSPG vector expressing Env and verified by both restriction digestion and sequence analysis. A half-virus strategy was utilized to clone the chimeric Env proteins into the full-length NL4.3 backbone, and progeny virus was developed by transfection into A293T cells. Site-directed mutagenesis was performed to create single amino acid changes in the ectodomain of R3A, and these were verified by sequence analysis.

Virus production. Vesicular stomatitis virus G protein pseudotyped retrovirus was produced by calcium phosphate cotransfection of A293T cells with vesicular stomatitis virus G protein, Gag/Pol, and HSPG retroviral DNA as previously described (4, 27). The HSPG retroviral construct contains green fluorescent protein under the control of the phospho-glucose kinase promoter (after transfection) or the murine stem cell virus LTR (after transduction). Viral supernatant was harvested at 48 and 72 h posttransfection, clarified by low-speed centrifugation, aliquoted, and frozen at -70, as previously mentioned. Infectious virus for R3A and R3B chimeras was derived as follows: chimeric Env proteins were cloned into p83.10 plasmid containing a partial NL4 genome (EcoRI to XhoI) and cotransfected into A293T cells with the p83.2 plasmid containing the remaining NL4.3 genome. Supernatant was harvested at 48 h posttransfection and cocultured with stimulated PBMCs. Pseudotyped NL4-luciferase viruses (pNL4-3.Luc.R-E; obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, from Nathaniel Landau) (7, 17) expressing chimeric Env proteins were generated by cotransfection of pNL4-Luc and HSPG Env proteins into A293T cells as mentioned previously. Viral supernatants were harvested daily and were titered for expression of Gag by p24 enzyme-linked immunosorbent assay (ELISA).

Virus quantitation. Gag was detected in virus stocks as well as supernatant from HFTOC infections using a p24 ELISA kit (AIDS Vaccine Program, NIH). Viruses were quantified for the number of infectious units/ml by infection of Magi (CXCR4) cells, as we have reported previously (27, 30, 33).

HFTOC. The procedure for infection and culture of human fetal thymus has been previously described (26–28). In brief, human fetal thymuses (19 to 22 weeks of gestation) were dissected into ~2-mm² fragments, using a dissecting microscope to retain the lobe structure for each fragment.

Four fragments placed on organotypic culture membranes (Millipore) and underlaid with HFTOC medium (previously described) were plated in six-well tissue culture plates. Thymic fragments were infected with equivalent amounts of virus (100 to 800 IU) in 15 µl per fragment. Viral and mock supernatants were derived from the same donor sample per experiment.

Thymic fragments were cultured at 37°C in 5% CO₂ for the length of each experiment. On the experiment harvest day, thymic fragments were disassociated in 350 µl of phosphate-buffered saline with 2% fetal bovine serum using pestles (Bellco Co.) for fluorescence-activated cell sorting (FACS) analysis.

FACS analysis. Thymocytes (and other cells) were stained with CD4-phycoerythrin and CD8-fluorescein isothiocyanate antibodies (Caltag) for surface staining. Cell viability was assessed using staining for 7-aminoactinomycin D. Analysis was performed using Summit software, as previously reported.

Virus-cell fusion analysis. NL4-luciferase pseudotyped virus expressing R3A, R3B, and chimeric Envs was generated by transfection of 293T cells. Viruses were titered by Magi assay, and SupT1 cells were infected by spinoculation and then cultured for 48 h. At endpoint, cells were harvested and lysed for luciferase analysis. Luciferase was measured using luciferase assay buffer reagents (Promega) and a Fluorstar luminometer.

Cell fusion analysis. BC7 cells were transduced with retrovirus to express HIV Env, which was assessed for relative expression by FACS analysis by staining with 2G12 (HIV-1 gp120 monoclonal antibody at 10 µg/ml; obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, from Hermann Kattinger). Cells were then cocultured with HOS/LTR-luciferase cells for 48 h, at which time cells were pelleted and lysed for luciferase activity. To measure the rate of fusion of various Env-expressing BC7 cells to HOS/LTR-luciferase cells, C34 was added to wells (10 µg/ml) to inhibit fusion at

various time points, and cells were then pelleted and lysed at 48 h for luciferase activity.

Statistical analysis. Both a Student's *t* test and nonparametric Mann-Whitney tests were performed to determine statistical significance of data. All analysis was performed using GraphPad Prism software.

RESULTS

The enhanced pathogenicity of R3A envelope is determined by mutations in the gp41 ectodomain. We have previously demonstrated that the R3A Env is significantly more pathogenic than R3B Env in the thymus organ models. The enhanced pathogenicity of R3A Env is correlated with high CXCR4 binding and more fusogenic activity than R3B (26–28). Specifically, chimeric viruses containing the V5 and gp41 domains of R3A in the R3B envelope background demonstrated an elevated pathogenic phenotype of CD4⁺ lymphocyte depletion compared to the parental NL4-R3B virus. Five differences exist in the V5 domains of the R3A and R3B envelope while 10 amino acid differences exist in the gp41 regions, with six differences in the ectodomain and four differences in the cytoplasmic tail of the R3A Env. In order to determine which amino acid differences in these domains of the R3A envelope contribute to pathogenesis, chimeric R3A and R3B Envs were created (Fig. 1A). One chimera (denoted R3A/B-gp41) carried the entire gp120 of R3A with the R3B gp41. The second chimera (R3A/B-cytoplasmic tail) retained the R3A gp120 and the ectodomain of gp41, while carrying the gp41 cytoplasmic tail of R3B. The third chimera (R3B/A-ectodomain) carried the R3A ectodomain in the R3B Env. Upon transfection of Env-expressing plasmids into A293T cells, Western blot analysis was performed to show that all HIV-1 recombinant Env proteins were similarly expressed and processed (data not shown). To further show surface expression of HIV Envs, surface expression on A293T cells was assessed using FACS analysis. Similar surface levels of all recombinant Env genes were detected by staining with the 2G12 antibody (Fig. 1B), which binds the glycosylated gp120 (34).

To determine whether specific regions of R3A play a role in CD4⁺ cell depletion, recombinant HIV-1 with the R3A/R3B chimeric Envs in the NL4.3 backbone were produced and studied. Recombinant viruses have similar replication and CD4 depletion characteristics in phytohemagglutinin-activated peripheral blood lymphocytes in vitro. Infection of activated PBMCs demonstrated that the viruses were equivalently infectious, as assessed by p24 Gag protein (data not shown). We found that there was no difference in virus production levels between the recombinant viruses, as we have previously reported with R3A and R3B in activated PBMCs (28). CD4⁺ lymphocyte depletion was assessed by FACS analysis over several time points, and depletion was also found to be equivalent by parental strains and chimeras over time (Fig. 1C).

In human lymphoid organs R3A shows elevated pathogenic activity, which has been mapped in the gp120 V5 and the gp41 regions (28). In order to further dissect the determinants within the V5-gp41 region that impact CD4⁺ cell depletion by R3A Env, the R3A/R3B chimeric viruses were used to infect human fetal thymus fragments (HFTOC). At various time points after infection, the viral p24 level in the HFTOC supernatant was measured using an ELISA for p24 antigen. Virus replication over 11 days of HFTOC infection was found to be

different (Fig. 2A) in that NL4-R3A and viruses possessing the R3A gp41 ectodomain replicated to higher levels than NL4-R3B and viruses carrying the R3B gp41 ectodomain (R3A/B-gp41). On day 11 postinfection, fragments were harvested, and thymocyte cells were counted and analyzed by FACS analysis. Upon infection of HFTOC, a significant difference in CD4⁺ thymocyte depletion levels between R3A and R3B was detected (Fig. 2B). Thus, NL4-R3A depleted CD4⁺ lymphocytes by 80% while NL4-R3B depleted cells by only 40%. The relative depletion of CD4⁺ thymocytes by NL4-R3A/B-gp41 was significantly lower than that by NL4-R3A but not different from NL4-R3B. Both the chimera NL4-R3A/B-cytoplasmic tail and R3B/A-ectodomain depleted CD4⁺ cells to levels similar depletion by NL4-R3A, and both were significantly more pathogenic than NL4-R3B. Therefore, enhanced replication and CD4⁺ thymocyte depletion of R3A in the HFTOC model were determined by the six amino acid differences between R3A and R3B in the ectodomain of gp41.

The HR-2 of R3A gp41 determines the enhanced fusion ability of R3A. The ectodomain of HIV-1 gp41 encodes the fusion domain and two HRs, thereby allowing the virus to enter the host cell (5, 24, 25). It has been previously demonstrated that R3A Env confers enhanced fusion ability to cells compared to R3B Env (26, 27). The R3A and R3B Env genes encode the same fusion peptide in gp41. Thus, other determinants in the ectodomain of R3A Env must be important. The ectodomain also contains the N-terminal HR(HR-1), a miniloop region, and the C-terminal HR (HR-2). From sequence analysis (using HXB2 for consensus) (28), two amino acid changes (Q543R and Q564H) exist in HR-1, three exist in HR-2 (S644N, N651I, and E665K), and one occurs in the cysteine miniloop region between the two HR domains (H620D). It is possible that changes in an ectodomain could alter the interaction either between the HR-1 domain with the HR-2 domain or between the HR-1 domains in the coil-coil formation, leading to either enhanced or reduced fusion. A modeling diagram was constructed over the known crystal structure for the ectodomain of HIV-1 gp41 (5) carrying the individual amino acid differences between the R3A and R3B Envs (Fig. 3A). The crystal structure for the thermodynamically stable HIV-1 viral fusion protein contains 36 amino acids of HR-1 and 34 amino acids of HR-2 (25). Only three of the R3A/R3B differences exist within the known structure. As reported, C34 helices pack outside the N36 helices in an antiparallel fashion, and our mapping suggests that the changes that map to the N- and C-terminal HRs are unlikely to contribute to a direct HR-1–HR-2 interaction that could lead to an altered fusion phenotype. However, one upstream difference in HR-1 (Q543R), as well as one downstream difference in HR-2 (E665K), could not be mapped on the known structure. Further, the difference that maps to the cysteine miniloop region between the HRs also cannot be mapped (H620D) as this region does not exist within the crystal structure. Despite the mentioned limitations presented by the model, amino acid changes were inferred to exist on the outer regions of the helices, suggesting that an interaction due to mutation was not likely. Rather, individual changes may have conferred the observed fusion ability.

Site-directed mutagenesis of each amino acid site in the R3A HR-1/HR-2 region was performed to determine if substituting individual R3B changes in the R3A background

FIG. 2. The R3A ectodomain determines its higher pathogenic activity in vivo. HIV-1 viruses encoding the R3A, R3B, or the chimeric Env genes were used to infect HFTOC. (A) HIV-1 replication was measured from supernatants harvested from HFTOC at 7, 9, and 11 days postinfection. Shown are combined data at days 7 to 9 postinfection. (B) Relative HIV-1 pathogenesis (depletion of total CD4⁺ thymocytes) in HFTOC was assessed by FACS analysis, with the mock (M) sample set at 100%. Data demonstrate combined results from six independent experiments. Error bars represent standard deviations. *, $P < 0.05$ relative to mock; **, $P < 0.01$ relative to R3B. Cyto T, cytoplasmic tail; Ecto, ectodomain.

would impact the Env's ability to fuse (Fig. 3B). A cell-cell fusion assay was utilized to determine whether single changes in the R3A extracellular domain would affect the high-fusogenic phenotype. Retroviral vectors that express the various HIV-1 Envs and Tat were used to transduce BC7 cells. Upon coculture with HOS/LTR-luciferase cells expressing the necessary receptors CD4 and CXCR4, fusion from Env-cell fusion was quantified by luciferase activity due to Tat activation of LTR-luciferase. Our results show that R3A Env promoted levels of fusion on average fivefold higher than R3B Env, and Envs with single point mutations had similar levels of fusion to the parental R3A Env. We concluded that individual amino acid changes in the ectodomain did not affect the fusion ability of R3A Env (Fig. 4A).

We have previously reported that both R3A and R3B Envs were equivalently sensitive to fusion inhibitor T20 (26) or C34 (data not shown). As T20 or C34 is derived from the HR-2

sequence and binds to the HIV-1 Env HR-1 to inhibit fusion, we thus first focused on whether changes in the R3A HR-2 domain could alter fusion ability. To test the hypothesis that the R3A HR-2 domain contributes to enhanced fusion activity, chimeras were produced by exchanging the HR-2 domains between R3A and R3B (Fig. 3B). Interestingly, the R3A chimera containing the R3B HR-2 significantly reduced fusion ability while the R3B chimera containing the R3A HR-2 demonstrated a higher fusion function (Fig. 4B).

To determine whether the exchange of HR-2 domains would affect virion-mediated fusion or virus entry into host cells, pseudotyped viruses with HIV-1 Envs and NL4-luciferase were generated and used to transduce SupT1 cells (Fig. 4C). The data show that the increased entry of R3A is significantly attributed to the HR-2 domain as viruses carrying the R3A HR-2 domain demonstrate enhanced ability to enter host cells

FIG. 1. R3A, R3B, and chimeric recombinants. (A) A schematic diagram of R3A and R3B Envs and their gp41 chimeric recombinants. Six amino acid differences exist between R3A and R3B within the ectodomain (Ecto), and four exist within the cytoplasmic tail (Cyto T) domain. (B) Cell surface expression of Env proteins is equivalent between R3A, R3B, and the chimeric Env. Staining with 2G12 monoclonal antibody was performed for FACS analysis of 293T cells transfected with Env-expressing vectors. Relative expression levels are presented as mean fluorescence intensity (MFI). (C) R3A, R3B, and their HIV-1 recombinants show similar infection and CD4 T-cell depletion levels in activated PBMCs. Activated PBMCs were infected with HIV-1 stocks with parental or chimeric R3 Env genes. Relative CD4 T-cell depletion (with mock samples as 100%) is shown at 6, 9, and 12 days postinfection. Error bars represent standard deviations from triplicate samples. d, day; PE, phycoerythrin.

FIG. 3. Ectodomain recombinants of R3A and R3B. (A) A ribbon diagram depicts the changes seen between R3A and R3B in the ectodomain. Only three of the six differences between R3A and R3B can be modeled upon the known crystal structure. These changes do not seem to change direct interactions between the HR-1 and HR-2 domains. (B) The HR-2 domains were swapped between R3A and R3B Env genes. Individual amino acids in the R3A Env gene were also changed to R3B residues using site-directed mutagenesis. Ecto, ectodomain; Cyto T, cytoplasmic tail; TM, transmembrane.

while viruses expressing R3B HR-2 demonstrate low entry ability.

It is possible that changes in HR-2 provide an enhanced rate of fusion for the R3A Env and, hence, more efficient entry. To determine this possibility, a time course to measure the rate of fusion of HIV-1 Envs was performed. BC7 cells expressing HIV-1 Envs were first assessed for equal HIV-1 Env surface expression by FACS analysis with the 2G12 anti-Env monoclo-

FIG. 4. The HR-2 domain of R3A determines its enhanced fusion activity. BC7 cells expressing various HIV-1 Tat and Env genes were cocultured with cells expressing CD4, CXCR4, and LTR-luciferase. Relative fusion is measured by the activation of HIV-LTR-luciferase (measured by relative light units [RLU]) by Tat in the fused cells. (A) Individual amino acid changes in the R3A Env gene do not significantly affect the fusion phenotype of R3A. Site-directed mutations of the R3A ectodomain carrying R3B changes were assayed, and individual changes were not seen to confer an R3B-like phenotype. (B) Swapping of the HR-2 domain completely exchanged the fusion phenotype in the cell-cell fusion assay. (C) Swapping of the HR-2 domain also exchanges the HIV-1 virus-cell fusion phenotype. NL4-luciferase pseudotyped with various R3 Env proteins was used to infect SupT1 cells. Luciferase expression (RLU) was measured at 48 h postinfection. Error bars represent samples in triplicates.

nal antibody (Fig. 5A). Western blot analysis was also performed to show that all HIV-1 recombinant Env proteins were similarly expressed and processed in transfected cells (data not shown). Equivalent numbers of Env/Tat-expressing cells were cocultured with HOS/LTR-luciferase reporter cells on ice and

then placed at 37°C. Fusion inhibitor C34 was added at 0, 6, and 12 h to inhibit fusion. After 48 h, cells were lysed, and luciferase was measured to assess fusion. BC7 cells expressing R3A Env demonstrated an enhanced fusion activity constitutively over time compared to R3B-expressing cells, and this enhanced fusion ability was correlated with the HR-2 domain of R3A (Fig. 5B and data not shown). However, relative inhibition of fusion of Envs encoding the R3A HR-2 was more dependent on the early addition of C34 than the R3B Env. Thus, C34 added at 6 h postinitiation of fusion inhibited only 70% of fusion mediated by R3A or R3B/A-HR-2 but still completely inhibited R3B-mediated fusion. When added at 12 h postfusion initiation, C34 inhibited 40 to 50% of fusion by R3A or R3B/A-HR-2 but still inhibited 80% of R3B-mediated fusion. The data suggest that the R3A Env, through its unique HR-2 domain, fuses with target cells more rapidly.

Our previous studies suggest that the enhanced CXCR4 binding affinity could be a determinant for pathogenicity of R3A (26). As the R3A HR-2 was determined to be the major contributor for fusogenicity, the domain's role in CXCR4 binding affinity was analyzed. Analysis of CXCR4 binding affinity was performed, and the R3B/A-ectodomain recombinant showed intermediate resistance to the coreceptor antagonist AMD3100 compared to resistance shown by R3A and R3B (Fig. 6). Further, R3A/B-HR-2 and R3B/A-HR-2 showed CXCR4 affinity similar to that of their parent viruses R3A and R3B, respectively. As it has been shown that swapping of the HR-2 domain also exchanges fusion activity of Env, there is no clear correlation between CXCR4 binding affinity and enhanced fusogenicity.

The HR-2 domain of the R3A gp41 plays a significant role in the enhanced pathogenic ability of R3A in the HFTOC model. To address whether the R3A HR-2 also contributes to elevated CD4⁺ cell depletion in lymphoid organs, full-length HIV-1 viruses were generated that expressed chimeric HR-2 Envs to compare against NL4-R3A and NL4-R3B. As previously reported with R3A and R3B in activated PBMCs (26), similar replication and CD4 depletion activities were observed with all HIV-1 recombinants in activated PBMCs (data not shown). HFTOC was infected with parent and chimeric viruses, and both virus replication and CD4⁺ cell depletion were assessed. Enhanced virus replication was seen by NL4-R3A over NL4-R3B, as observed previously (26). Interestingly, both R3A/B-HR-2 and R3B/A-HR-2 chimeric viruses showed higher levels of replication than R3B (Fig. 7A). Consistent with the fusion activity, chimeric viruses with the R3A HR-2 domain were found to deplete CD4 thymocytes more efficiently than viruses with the R3B HR-2 domain (Fig. 7B). By Day 11 postinfection, NL4-R3A was found to deplete CD4⁺ cells by 90%, while NL4-R3B or NL4-R3A/B-HR-2 depleted cells by only ~30 to 40%. Interestingly, NL4-R3B/A-HR-2 showed an intermediate level of CD4⁺ cell depletion and was significantly more pathogenic than NL4-R3B but less pathogenic than NL4-R3A. These findings suggest that the R3A HR-2 contributes to the enhanced CD4⁺ cell depletion by R3A in the thymus organ, but additional determinants in the R3A ectodomain also play a role in enhanced pathogenesis. The entire ectodomain may function in concert to enhance HIV infection and pathogenesis, as seen with NL4-R3A in the HFTOC model. In addition,

FIG. 5. The R3A HR-2 domain confers accelerated fusion activity. (A) Equivalent expression of R3A, R3B, and their HR-2 chimeric recombinant Env proteins on the surface of transduced BC7 cells. Surface expression of HIV Env on transduced BC7 cells was assessed by 2G12 staining and FACS analysis. Relative Env expression levels are presented as mean fluorescence intensity. (B) Recombinant Env genes encoding the R3A HR-2 demonstrated accelerated fusion. Cells expressing various HIV Env genes were mixed with the fusion reporter cell line. Fusion inhibitor C34 was added at the indicated time after coculture at 37°C. Inhibition of fusion of each recombinant Env by C34 when added at time point 0 is calculated as 100% inhibition. Relative inhibition by C34 added at 6 and 12 h is shown. Data are representative of two independent experiments, and error bars represent standard deviations derived from duplicate samples.

the fusion activity, but not the CXCR4 affinity, is correlated with the enhanced pathogenicity of R3A in lymphoid organs.

DISCUSSION

The virologic mechanisms by which human lymphoid organs undergo CD4⁺ cell depletion during HIV-1 infection remain incompletely characterized. We have previously reported that HIV-1-induced fusion is the major contributor to pathogenesis of both infected and uninfected cells in human lymphoid or-

gans (26–28). In this report, we have investigated the virologic determinants that contribute to the elevated fusogenicity and pathogenicity of the R3A envelope. We discovered that the gp41 ectodomain of R3A Env determines its high levels of pathogenic and fusion activity. As this domain carries six amino acid differences between the R3A and R3B Env, we further determined that the three mutations in the HR-2 domain of R3A Env play a major role in its elevated fusion activity and partially contribute to the high pathogenic activity in the thymus organ model.

As a result of comparing Env chimeras of a highly pathogenic virus with a less pathogenic virus, six amino acid differences within the ectodomain were mapped to confer the enhanced fusion and pathogenic ability of R3A. Individual site-directed mutants did not impact the enhanced fusion phenotype of R3A Env (Fig. 4). Thus, a functional determinant consisting of redundant or complementary residues in the R3A ectodomain or HR-2 is implicated in contributing to the enhanced fusogenic activity.

R3A and R3B Envs were similarly sensitive to fusion inhibition by T20 or C34 (26). As T20 or C34 is an HR-2 homolog which functions to bind to HR-1 and inhibit fusion, the changes in the R3A HR-1 region were probably not involved in the elevated fusion activity. Consistent with the prediction, the three amino acid differences in the HR-2 domain of R3A were necessary and sufficient to confer the enhanced fusion ability of R3A Env. Three functional domains have been reported within the HR-2 of the gp41 ectodomain: an HR-1 binding domain (residues 628 to 666), a pocket binding domain (628 to 635), and a lipid binding domain (666 to 673) (19). Interestingly, all three differences in the HR-2s of R3A and R3B Envs are

FIG. 6. The enhanced CXCR4 binding affinity of R3A Env is not correlated with enhanced fusion activity. Resistance of each HIV-1 Env gene to the antagonist AMD3100 (200 nM) relative to R3A was determined. Error bars represent standard deviations derived from triplicate samples. *, $P < 0.05$; **, $P < 0.01$.

FIG. 7. The R3A HR-2 domain contributes significantly to its enhanced pathogenesis in human lymphoid organs. R3A, R3B, and the chimeric recombinants were used to infect HFTOC. (A) Viral replication was measured by p24 ELISA at 7, 9, and 11 days postinfection. Shown are data from days 7 to 9 postinfection. (B) CD4⁺ thymocyte depletion was measured by FACS analysis at 11 days postinfection of HFTOC with NL4-R3A, R3B, and chimeras. Error bars represent standard deviations from samples in triplicate, and two independent experiments were performed with similar results. *, $P < 0.05$ in comparison to R3B; **, $P < 0.001$ versus R3B.

encoded within the HR-1 binding domain. The HR-1 binding domain of R3A Env is thus a major determinant of enhanced fusion activity.

In addition to the elevated fusion activity associated with the R3A Env, HIV-1 recombinants containing the R3A ectodomain depleted thymocytes equivalently to parental NL4-R3A, suggesting that enhanced fusion may contribute significantly to pathogenesis. Because increased CXCR4 binding efficiency was previously suggested also as a determinant for thymic pathogenicity (26), the R3B/A-ectodomain Env was assayed

for relative CXCR4 binding efficiency (Fig. 6). As was seen with other chimeric Envs (expressed in pseudotyped virus) (26), an intermediate level of CXCR4 binding efficiency was observed. This intermediate phenotype was shared with chimeric Envs that demonstrated significantly less thymic pathogenicity, suggesting that coreceptor binding efficiency and sensitivity to coreceptor antagonist AMD3100 do not directly correlate with the observed pathogenesis of the Env.

It is thought that the HR-2 domain functions to “zip” over the HR-1 domain after the initial step of Env-cell fusion, so the amino acid changes seen in the R3A HR-2 may allow a more rapid ability to fuse the envelope to the cell, providing for significantly enhanced ability for the Env to enter the cell as well. It is possible that changes present in NL4-R3A provide for an enhanced rate of fusion of the Env, which allows for enhanced pathogenesis compared to NL4-R3B. To provide various snapshots of fusion over time, fusion inhibitor C34 was used to inhibit fusion at various time points. Envs expressing R3A HR-2 showed enhanced and accelerated fusion compared to Envs expressing R3B HR-2. Envs expressing the R3A HR-2 provided more robust fusion ability, with a significant difference in the slope of kinetics of the R3A and R3B Envs.

We demonstrate that HR-2 is an important contributor for both fusion and pathogenic activity in a relevant human lymphoid organ model. All three of the mutations in the R3A HR-2 region lead to changes in hydrophobicity and charge. Specifically, the R3A HR-2 carries changes to serine, asparagine, and glutamic acid, which are all hydrophilic. The hydrophilic nature of this region may shed light on the highly fusogenic ability of the R3A HR-2. Also, relative to the sequence of R3B, in R3A the H to D at position 620 removes a partially positive charge and replaces it with a negative charge, and E to K at position 665 removes a negative charge and replaces it with a positive charge. The HR-2 differences provide various changes that may affect the conformation of the six-helix bundle such that R3A HR-2 may interact differently with the viral membrane than R3B HR-2. We analyzed HIV-1 sequences in the Los Alamos National Laboratory HIV Sequence Database for their sequence comparison with R3A and R3B. Of approximately 500 clade B Envs, the Asp-651 in R3A Env is the consensus whereas the Ile-651 in R3B is not reported in other subtype B HIV-1 sequences. Interestingly, the Lys-665 in R3B is the consensus, but Glu-665 of R3A is not detected in the other subtype B HIV-1 strains. Amino acid 665 is the first position of the membrane-proximal external region, which is known to be a relatively conserved region that plays a distinct role in fusion and is a target for antiviral drugs (35). It is possible that this R3A mutation, in combination with other mutations in the HR-2 region, may contribute to an enhanced fusogenic phenotype.

Several groups have reported that HIV-1 Env-mediated fusion is the primary determining factor in CD4 T-cell loss (4, 11, 12). An enhancement of the ability of the Env to fuse would lead to an increase in the efficiency of new infections at the cost of higher amounts of cell death. Indeed, we have reported the enhanced pathogenicity of a highly fusogenic virus in the fetal thymus organ model. Further, we demonstrate that enhanced fusion by R3A is directly due to the HR-2 domain. The data from these experiments suggest that

the entire structural change is necessary for R3A's HR-2-mediated effect. Interestingly, pathogenesis conferred by the R3A ectodomain is not strictly due to the changes seen in the HR-2, so other changes observed in the ectodomain including HR-1 may contribute to the overall pathogenesis of R3A. Future experiments will be performed to determine interactions that may exist between the HR-1 and HR-2 domains that may provide this enhanced fusion-mediated pathogenesis observed with the R3A ectodomain.

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