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Human Immunodeficiency Virus Type 1 IIIB Selected for Replication In Vivo Exhibits Increased Envelope Glycoproteins in Virions without Alteration in Coreceptor Usage: Separation of In Vivo Replication from Macrophage Tropism†

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Analysis of viral replication and pathogenicity after in vivo selection of human immunodeficiency virus type 1 (HIV-1) attenuated in vitro will help to define the functions involved in replication and pathogenesis in vivo. Using the SCID-hu Thy/Liv mouse and human fetal thymus organ culture as in vivo models, we previously defined HIV-1 *env* determinants (HXB2/LW) which were reverted for replication in vivo (L. Su et al., Virology 227:46–52, 1997). In this study, we examined the replication of four highly related HIV-1 clones directly derived from Lai/IIIB or after selection in vivo to investigate the envelope gp120 determinants associated with replication in macrophages and in the thymus models in vivo. The LW/C clone derived from the IIIB-infected laboratory worker and HXB2/LW both efficiently infected monocyte-derived macrophages (MDM) and the human thymus. Although the laboratory worker (LW) isolates showed altered tropism from IIIB, they still predominantly used CXCR4 as coreceptors for infecting peripheral blood mononuclear cells, macrophages, and the thymus. Interestingly, a single amino acid mutation in the V3 loop associated with resistance to neutralizing antibodies was also essential for the replication activity of the LW virus in the thymus models but not for its activity in infecting MDM. The LW virions were equally sensitive to a CXCR4 antagonist. We further demonstrated that the LW HIV-1 isolate selected in vivo produced more infectious viral particles that contained higher levels of the Env protein gp120. Thus, selection of the laboratory-attenuated Lai/IIIB isolate in vivo leads to altered tropism but not coreceptor usage of the virus. The acquired replication activity in vivo is correlated with an early A-to-T mutation in the V3 loop and increased virion association of HIV-1 Env gp120, but it is genetically separable from the acquired replication activity in macrophages.

Human immunodeficiency virus type 1 (HIV-1) diseases (AIDS) are associated with high levels of HIV-1 replication and loss of $CD4^+$ T lymphocytes. HIV-1 can infect diverse cell types, including $CD4^+$ T cells, macrophages, dendritic cells, Langerhans cells, and hematopoietic progenitor cells (14, 26, 30, 39). However, the HIV-1 isolates employed in many studies have been expanded and maintained in immortalized human T-cell lines or phytohemagglutinin (PHA)-activated peripheral blood mononuclear cells (PBMCs) in vitro. The different selective pressures in vitro may have led to the generation of HIV-1 variants with attenuated replication and pathogenicity in vivo. Many laboratory-adapted isolates of HIV-1 accumulate mutations in gene functions such as *env*, *vpr*, *vpu*, and *nef* (37). A good example of such adaptation in vitro is Lai/IIIB (HTLV-IIIB [9]). Initially derived from a patient blood sample and cultured in MT2/B cells, Lai/IIIB stock was prepared by infecting the human T-leukemia cell line, H9, with infected M2T/B cell supernatant. Subsequent analyses of the genome from the Lai/IIIB isolate showed that multiple changes accumulated during expansion in vitro (37). For example, the HXB2 genome cloned from Lai/IIIB carries mutations that lead to premature termination of three of the nine open reading frames (ORFs): *vpr*, *vpu*, and *nef*. Many other subtle mutations may have also accumulated. These mutations do not usually affect HIV-1 replication in vitro under specific culture conditions, though some of them may enhance viral replication in certain cell lines. It was recently reported that laboratory-adapted HIV-1 isolates have accumulated mutations that contribute to attenuated HIV replication and pathogenesis in vivo (12).

Comparison of HIV-1 isolates attenuated in vitro with pathogenic revertants selected in vivo will help to identify important viral determinants for replication and pathogenesis in vivo. One successful example is found in the simian-human immunodeficiency virus (SHIV) *env* chimeric genome adapted in monkeys. SHIV variants with enhanced replication and pathogenicity have been isolated from monkeys infected with SHIV recombinant viruses (19). Mutations in the HIV *env* genes have been identified which contribute to enhanced replication in monkeys. Interestingly, *env* determinants have also been defined that specifically contribute to CD4 T-cell depletion (i.e., pathogenicity), but not replication, in monkeys (13).

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† Dedicated to the memory of Eric D. Miller.

Therefore, unique *env* determinants have intrinsic replication or pathogenic activity in monkeys.

The Lai/IIIB isolate and its infectious molecular clones (e.g., HXB2) infect T-cell lines such as H9 as well as PBMCs in vitro but are replication defective in vivo (15, 35, 40). When a laboratory worker was accidentally infected by Lai/IIIB, infectious virus was isolated from plasma by infection of primary PBMCs with macrophage tropism but not by infection of T-cell lines (21, 44). We have previously employed the SCID-hu Thy/Liv mouse as an in vivo model (29, 31) to study the replication of HXB2 and of HXB2-recombinant viruses containing HIV-1 fragments isolated from the infected laboratory worker (40). Like Lai/IIIB, HXB2 failed to replicate in the Thy/Liv organ or in the human fetal thymus organ culture (HF-TOC) models (8, 40). Replacement of an HXB2 subgenomic fragment carrying the *env* ORF with the corresponding fragment from the laboratory worker (LW) isolate (LW12.3) generated a recombinant virus (HXB2/LW) which replicated in SCID-hu Thy/Liv mice and in the HF-TOC model (22, 40). The specific in vivo replication determinants were mapped to the V1-V3 region of the HXB2/LW *env* gene (40). Therefore, the attenuated Lai/IIIB isolate acquired in vivo replication activity by mutational reversion of the *env* gene in the infected laboratory worker. In addition, multiple mutations in the *env* and *nef* genes that lead to reduced pathogenicity have been implicated (12).

We further showed here that the infectious LW/C clone derived from the IIIB-infected laboratory worker (28), like HXB2/LW, replicated to high levels in the SCID-hu Thy/Liv mouse and HF-TOC models. As expected, both LW/C and HXB2/LW efficiently infected monocyte-derived macrophages (MDM). Although the LW isolates showed altered tropism from IIIB, they still only used CXCR4 as coreceptors for infecting PBMCs, macrophages, and the thymus. Interestingly, a single amino acid mutation in the V3 loop associated with resistance to neutralizing antibodies was also essential for the replication activity of the LW/C clone in the thymus models. The single mutation back to IIIB (LW/CIIIB) did not affect its activity to infect MDM but impaired its replication in the thymus. We further demonstrated that the LW HIV-1 isolate selected in vivo produced more infectious viral particles that contained higher levels of the Env gp120 protein. Thus, selection of the laboratory-attenuated Lai/IIIB isolate in vivo leads to altered tropism but not coreceptor usage of the virus. The acquired replication activity in vivo is correlated with increased virion association of HIV-1 Env gp120 but not with the replication activity in MDM.

MATERIALS AND METHODS

HIV-1 replication in PBMCs and viral supernatant production. Equal amounts of proviral DNA were transfected into 293T cells and supernatant was used to infect PHA-activated PBMCs as described previously (22, 39). Supernatant was collected and the titer was determined by using a multinuclear activation of galactosidase inhibitor (MAGI) assay, performed as previously described using U-373-MAGI-CXCR4$_{CEM}$ glioblastoma cells (42). The supernatants with titers greater than 5×10^4 infectious units (IU)/ml were stored as viral stock for infection. HXB2 and HXB2/LW have been described previously (40). LW/C and LW/CIIIB have also been reported elsewhere (28).

HIV-1 replication in MDM. MDM were isolated from peripheral blood of HIV-seronegative donors as previously described (34). Approximately 5×10^5 monocytes were allowed to differentiate for 7 days in culture medium (RPMI with 5% donor serum, 50 μg of streptomycin/ml, 50 U of penicillin G [Gibco BRL]/ml), in 6-well tissue culture plates. The monolayers were then infected for 1 h at 37°C with 500 μl of virus stock (≈5×10^4 IU/ml) or medium from mock-infected PBMCs, washed three times with phosphate-buffered saline (PBS), and then cultured for an additional 15 days. Virus replication was detected by reverse transcriptase (RT) assays of culture medium aliquots removed every 3 days prior to changing the medium.

HIV-1 Env-mediated cell fusion with MDM and U87 cells. Env-mediated fusion was tested in a cell-cell fusion assay employing primary macrophage targets as described previously (16). Effector 293T cells were infected with recombinant vaccinia virus vP11T7 gene1, which expresses the T7 polymerase, and then cotransfected with plasmids carrying *env* under control of the T7 promoter and a luciferase reporter gene under control of the SP6 promoter. One-week-old MDM cultures were infected with recombinant vaccinia virus vSIMBE/L, which expresses the SP6 RNA polymerase under control of a synthetic vaccinia virus early-late promoter. After incubation overnight at 32°C in the presence of rifampin (100 μg/ml), Env-expressing 293T cells were mixed with macrophages and incubated for 6 h in the presence of rifampin and AraC (0.1 μM). Cells were lysed 6 h later for measurement of luciferase expression, which results from fusion, content mixing, and SP6 polymerase transactivation of the luciferase reporter gene. In parallel, U87 cells stably expressing CD4 alone or in conjunction with CCR5 or CXCR4 (provided by D. Littman through the National Institutes of Health [NIH] AIDS Reagent Program [6]) were used as targets for fusion under identical conditions. Env from X4 strain UG024, which utilizes CXCR4 only for entry into macrophages (45), was analyzed as a control.

Infection of SCID-hu Thy/Liv mice or HF-TOC. Animal transplantation procedures for SCID-hu Thy/Liv construction have been described previously (33). Infection of SCID-Thy/Liv mice was performed as previously described (39). Briefly, SCID-hu Thy/Liv mice were infected with supernatant collected from PHA-activated PBMCs containing no HIV-1 (mock) or 4×10^4 IU of HIV-1/ml. Fifty microliters (≈2000 IU) was injected into each thymus graft. Biopsies were removed from Thy/Liv organs at indicated times and thymocytes were analyzed for p24 levels and proviral DNA.

The TOC procedures were modified from those described previously (8). Briefly, human fetal thymuses (19 to 24 gestational weeks) were dissected into ≈2-mm^3 fragments containing at least 3 to 5 intact thymic lobules under a dissecting microscope. These fragments were transferred onto sterile organ culture membranes (Millipore) floating on media (RPMI with 10% fetal calf serum, 50 μg of streptomycin/ml, 50 U of penicillin G/ml, 1× MEM vitamin solution [Gibco/BRL], 1× insulin-transferrin-sodium selenite medium supplement [Sigma]), in 6-well tissue culture plates. An equal amount of virus (≈800 IU) in 20 μl of supernatant from infected PHA-stimulated PBMCs or control supernatant from mock-infected PHA-stimulated human PBMCs was added to each fragment. The fragments were then cultured at 37°C in 5% CO_2 for 10 to 12 days with daily changes of culture media. Thymocytes were teased out of the fragments and analyzed as described above.

Viral replication assays. Measurement of p24 production (picograms per 10^6 thymocytes) was performed using a Vironostica p24 enzyme-linked immunosorbent assay (ELISA) kit (Organon Teknika Corp., Durham, N.C.) with cell lysates in PBS–1% Triton X-100. RT assays to measure virus replication were performed.

Flow cytometric analyses. Thymocytes isolated from SCID-hu Thy/Liv organs or HF-TOC fragments were stained with PE-CD4 and TC-CD8 (Caltag) in PBS–2% fetal bovine serum, washed, and resuspended in PBS–1% formaldehyde as previously described (22, 39).

U-373-MAGI assays. MAGI assays were performed as previously described using U-373-MAGI-CCR5E and U-373-MAGI-CXCR4$_{CEM}$ glioblastoma cells (42). Briefly, 24 h prior to infection, each of the MAGI cell lines were seeded into 48-well culture plates (2×10^4 cells/well). Virus stocks amplified in PHA-activated PBMCs as described below were diluted to approximately 10^4 IU/ml, and 100 μl was used to infect duplicate wells of each of the three cell lines for 2 h at 37°C. At 48 h postinfection (hpi) the cells were stained, and the number of blue foci in each well was counted. To analyze the sensitivity of HXB2 or HXB2/LW to AMD3100 (11; kindly provided by J. Moore, Cornell Medical School, New York, N.Y.), medium containing 0.15 to 15 ng of AMD3100/ml was added to the virus stock prior to infecting the U-373-MAGI-CXCR4$_{CEM}$ cells.

AMD3100 inhibition of CXCR4 entry in PBMCs and HF-TOC. AMD3100 was added to the virus supernatant at a concentration of 100 ng/ml prior to infection of the PHA-activated PBMCs, to inhibit CXCR4-mediated viral entry (11, 25). Virus replication was detected by RT assays of culture medium aliquots removed every 3 days. To inhibit CXCR4-mediated virus entry in HF-TOC assays, AMD3100 (100 ng/ml) was added to fresh HF-TOC medium every day. Thymocytes were analyzed for viral replication by p24 ELISA as described above.

Fusogenicity assay. pcENV-NL4 is a pcDNA3-based vector containing the *Eco*RI-*Xho*I fragment of the NL4-3 *env* gene (H. Zhang and L. Su, unpublished

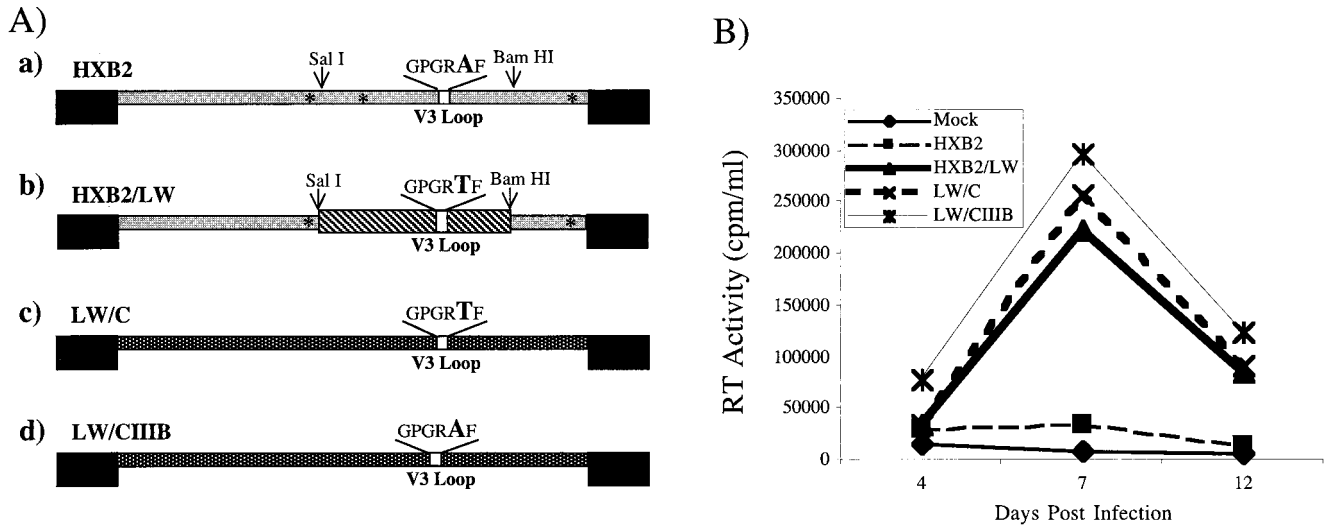

FIG. 1. Replication of LW clones in MDM. (A) Genomic organization of HXB2 and LW-derived HIV-1 clones. In panel a, the HXB2 HIV-1 genome has nonsense mutations within the *vpr*, *vpu*, and *nef* ORFs, denoted by asterisks. A six-amino-acid sequence within the V3 loop is highlighted (white segment). In panel b, HXB2/LW was created by replacing the *Sal*I-*Bam*HI fragment from LW12.3 (striped segment) into an HXB2 background. The V3 loop is in white, with the amino acid change to Thr (T) in bold. In panel c, LW/C is a full-length macrophage-tropic clone derived from the LW isolate LW12.3. LW/C contains repaired *vif*, *vpr*, and *nef* accessory ORFs (28). In panel d, LW/C IIIB is identical to LW/C except for the T-to-A (back to HXB2) change at the V3 loop. (B) Replication of the LW clones in MDM. Monocytes were cultured and infected with each HIV-1 stock. HIV-1 replication was monitored by measuring virion-associated RT activity (^{3}H activity, in counts per minute per milliliter) in the culture medium. Experiments were repeated with independent PBMC donors with similar results.

data). The *Eco*RI-*Xho*I fragment of HXB2/LW or HXB2 was cloned into pcDNA3 to produce pcENV-LW or pcENV-HXB2 plasmid constructs. 293T cells were cotransfected with a plasmid expressing the HIV-1 Tat gene and the *env* expression vector using Effectene Transfection reagent (Qiagen). At 24 h posttransfection (hpt), the Jurkat cells stably transfected with the HIV-1 long terminal repeat (LTR) luciferase gene (1G5, provided by the NIH AIDS Research and Reference Reagent Program) were cocultured with the transfected 293T cells. Tat-induced luciferase expression in 1G5 cells was measured at 24 h postcoculture (48 hpt).

Heat-stability analysis of HIV-1 stocks. Equal numbers of HXB2 or HXB2/LW infectious units, as determined on MAGI cells, were incubated at the indicated temperatures for 10 min, and the remaining infectious units were determined on U-373-MAGI-CXCR4 indicator cells as described above. Triplicate samples were included in each experiment.

Western blot analysis. Transfected 293T cells were lysed 48 hpt in 0.5% NP-40 lysis buffer as described elsewhere (22). To detect viral proteins in virions, supernatants containing viruses were centrifuged for 2 h at 4°C in a standard 20% sucrose cushion as described previously (1). Virus pellets were lysed in 1% Triton X-100 and analyzed by standard Western blotting with HIV-positive patient sera (provided by the NIH AIDS Research and Reference Reagent Program).

RESULTS

T-cell-line-attenuated HIV-1 isolates acquired activity in vivo to infect macrophages and to replicate in the human thymus models. Four highly related HIV-1 clones derived from Lai/IIIB were used to investigate the viral envelope gp120 determinants associated with replication in macrophages and in the in vivo human thymus models (Fig. 1A). HXB2 is an infectious HIV-1 molecular clone of Lai/IIIB with mutations that lead to premature termination in the ORFs of three accessory proteins, Vpr, Vpu, and Nef. Like Lai/IIIB, HXB2 replicates efficiently in T-cell lines in vitro but lacks the ability to replicate in vivo (8, 40). The other three clones contain HIV-1 sequences from the laboratory worker who was accidentally infected with Lai/IIIB. Previous reports show that a recombinant virus that contains the *env* gene from LW in an HXB2 background, HXB2/LW, in contrast to Lai/IIIB or HXB2, replicates efficiently in human thymus models. The V3 loop regions between these two viruses differ in six amino acids, including the alanine (A)-to-threonine (T) change at the tip of the V3 loop (36, 40). The A-to-T change is known to be responsible for escape from certain neutralizing monoclonal antibodies specific to the HXB2 (IIIB) envelope. In this study we also used two additional viruses, LW/C and LW/C IIIB, that are derived from LW and are macrophage tropic (28). LW/C is a viral clone that was derived from virus isolated from the infected laboratory worker, with repaired *vif*, *vpr*, and *nef* ORFs. LW/CIIIB is identical to LW/C except for the T-to-A (back to IIIB) change in the V3 loop region.

As expected, both LW/C and HXB2/LW efficiently infected MDM. LW/CIIIB, with the T-to-A back mutation, also replicated in MDM, and HXB2 failed to replicate in MDM (Fig. 1B). Therefore, the A-to-T mutation selected early in vivo in the laboratory worker was not required for the acquired replication activity of LW/C in macrophages. It may contribute to other important activities for in vivo replication.

A single amino acid change in the V3 loop region, but not MDM tropism, contributed to HIV-1 replication in the human thymus. Replication of HXB2 and the LW viruses was analyzed in the HF-TOC and SCID-hu Thy/Liv mouse model systems. In HF-TOC, HXB2 exhibited no significant replication, whereas HXB2/LW infection resulted in 1,000-fold-higher levels of viral replication, similar to those of NL4-3 and JR-CSF (Fig. 2A). This is consistent with our previous report (40). LW/C, a macrophage-tropic LW clone, was also observed to replicate to high levels in HF-TOC, approximately 100-fold higher than that for HXB2. In contrast, LW/CIIIB, which differs by one amino acid from LW/C (Fig. 1A), exhibited no

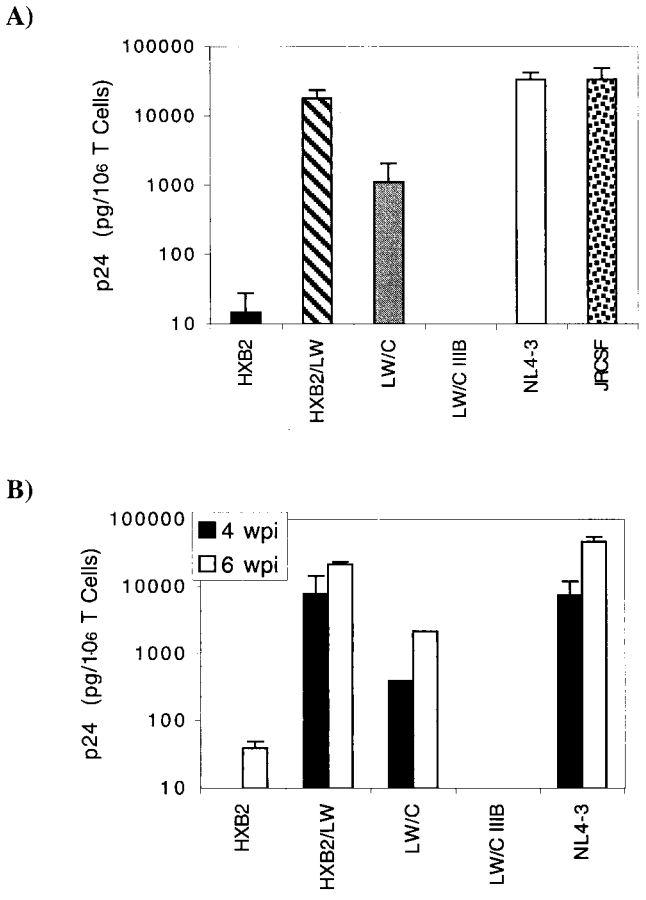

FIG. 2. Replication of LW clones in the human thymus. (A) Replication of LW clones in HF-TOC. Shown is the level of p24 present in HF-TOC at 10 to 12 days postinfection (dpi), with HXB2, HXB2/LW, LW/C, LW/CIIIB, NL4-3, and JRCSF. Error bars are standard deviations based on three or more independent experiments. (B) Replication of LW clones in SCID-hu Thy/Liv mice. Shown is the level of p24 present in the SCID-hu Thy-Liv organ at 4 and 6 weeks postinfection (wpi) with HXB2, HXB2/LW, LW/C, LW/CIIIB, and NL4-3. Error bars represent standard deviations.

significant replication in the thymus. The SCID-hu Thy/Liv mouse model was also used to confirm that HXB2 showed no significant replication, whereas HXB2/LW replicated to high levels (40). For LW/C, replication was also readily detectable, whereas LW/CIIIB replication was undetectable (Fig. 2B and data not shown). Therefore, the replication of each virus in the Thy/Liv organ is similar to that in the HF-TOC model. Fluorescence-activated cell sorter analyses of T cells from thymus organs infected with HXB2/LW and LW/C, but not HXB2 and LW/CIIIB, showed upregulation of major histocompatibility complex class I in the $CD4^+CD8^+$ double-positive thymocytes from both the HF-TOC and SCID-hu Thy/Liv mice (data not shown). This is consistent with previous findings that showed that productive HIV-1 infection in the thymus induces upregulation of major histocompatibility complex class I antigens upon infection (22). These data suggest that the A-to-T conversion in the V3 loop of LW/C contributes to the acquired replication in the thymus in vivo but not to its replication in MDM (Fig. 1B).

The in vivo-selected HIV-1 clones still used CXCR4 as coreceptor for entry. To address the observation of differential cell tropism of the highly related viruses, we first looked at coreceptor usage by each virus. We hypothesized that the ability to replicate in MDM and in the thymus could be due to a change from using the CXCR4 (IIIB) coreceptor to the CCR5 coreceptor (5). To test this hypothesis, we analyzed each virus on MAGI cells expressing CD4 and either the CCR5 or CXCR4 coreceptor (42). All three LW viruses, as well as HXB2, used the CXCR4 (not CCR5) coreceptor for entry (Fig. 3A). This indicated that the change in tropism was not due to a change in coreceptor preference. As a positive control for CCR5 entry in this system, we used the CCR5-tropic JRCSF virus to infect the CCR5-expressing MAGI cells. JRCSF efficiently utilized the CCR5 coreceptor and not the CXCR4 coreceptor for entry (Fig. 3A).

In order to determine if CXCR4 was the only coreceptor used in vivo and in primary PBMCs, we used a specific inhibitor of CXCR4-tropic HIV-1, the bicyclam AMD3100, to specifically block virus entry via this coreceptor (11, 25). We analyzed the effect of AMD3100 on viral replication in both PBMCs and HF-TOC. NL4-3, HXB2, and HXB2/LW were all efficiently inhibited by AMD3100 in PBMCs, whereas JRCSF was not (Fig. 3B). In the HF-TOC assay, NL4-3 was used as a positive CXCR4 control due to the lack of HXB2 replication in the thymus. AMD3100 was able to efficiently inhibit both NL4-3 and HXB2/LW replication but not replication of the CCR5-utilizing virus JRCSF (Fig. 3C). Since CXCR4 is the only HIV-1 entry coreceptor that is sensitive to AMD3100, this result indicated that HXB2/LW used predominantly CXCR4 as coreceptor in the thymus. In addition, we showed that the HXB2/LW env protein used CXCR4, but not CCR5, coreceptor to fuse with MDM and macrophage cell lines, and its fusion with MDM was efficiently inhibited by AMD3100 (Fig. 3D). Further supporting the notion that LW uses CXCR4 and not CCR5 for entry into macrophages, there was no difference in the overall level of fusion, compared with wild-type macrophages, when CCR5-negative macrophages obtained from a donor homozygous for the Δ32 deletion allele were used as targets (data not shown). These data suggest that, like Lai/IIIB and HXB2, the LW virus also predominantly used CXCR4 as coreceptor for entry in the thymus, macrophages, and PBMCs.

LW and HXB2 Env proteins showed similar affinity for CXCR4 and fusogenicity. To further investigate the different replication activity exhibited by HXB2/LW and HXB2 in the thymus in vivo, we analyzed the efficiency of these two viral envelopes to utilize CXCR4 in competing concentrations of AMD3100. Virus (HXB2 or HXB2/LW) was incubated with various concentrations of AMD3100 and allowed to infect MAGI-CXCR4 cells. The induction of β-galactosidase activity by HIV-1 infection was quantitated as a measure of fusion and viral entry. As shown in Fig. 4A, the dose of AMD3100 required to inhibit HXB2 and HXB2/LW was the same. These data suggest that the HXB2 and LW Env proteins had a similar affinity for CXCR4 and interacted similarly with CXCR4 for entry.

To test if the LW Env protein had increased fusion activity, we performed the fusogenicity assay by coculturing a Jurkat T-cell line stably expressing the luciferase reporter gene under control of the HIV-1 LTR with 293T cells transfected with

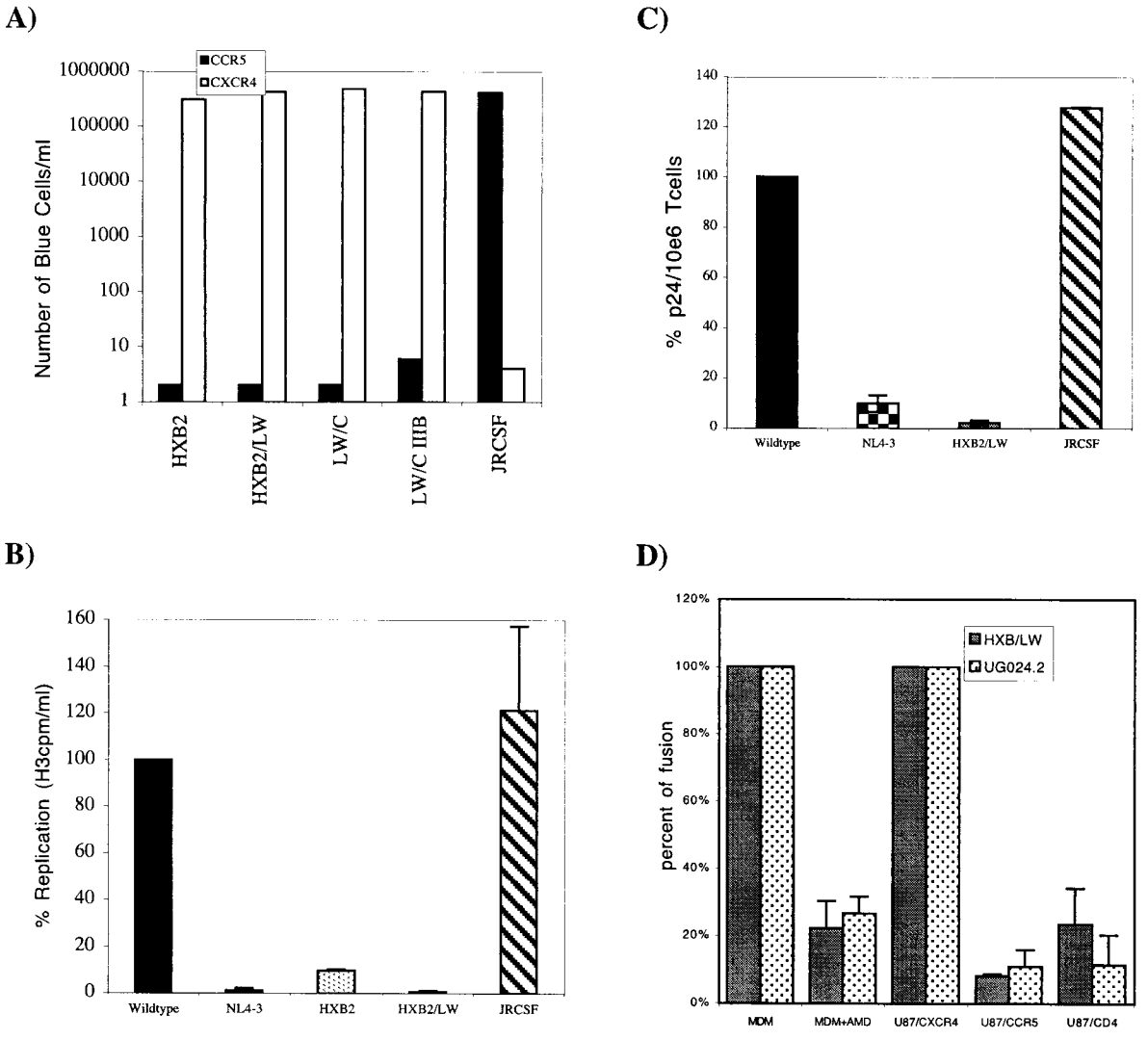

FIG. 3. HXB2/LW, LW/C, and LW/CIIIB utilize the same coreceptor (CXCR4) as HXB2. (A) The LW viruses used CXCR4 but not CCR5 as coreceptor for entry. HIV-1 viral supernatants were used to infect the CXCR4- or CCR5-MAGI cells. Each virus was scored for the number of blue cells per milliliter for both cell lines. HXB2, HXB2/LW, LW/C, and LW/CIIIB utilized CXCR4 (gray) as the primary coreceptor. JRCSF, a macrophage-tropic virus, utilized CCR5 (black) for entry. (B) HXB2/LW only utilized CXCR4 for entry into PBMCs. The bicyclam AMD3100, a specific inhibitor of HIV-1 using the CXCR4 coreceptor, was added (15 ng/ml) to the media for the PBMC culture. Shown is AMD3100 inhibition in PBMC cultures at 9 dpi. HIV-1 replication for each virus in the absence of AMD3100 is normalized to 100% (wild-type; black bar). Error bars represent standard deviations. Three independent experiments were performed with similar results. JRCSF was used as a CCR5-tropic HIV-1 resistant to AMD3100 inhibition. (C) HXB2/LW only used CXCR4 as a coreceptor for infection in the human thymus. Shown is the relative replication (based on picograms of p24 per 10^6 T cells) of each virus with AMD3100 in HF-TOC. HIV-1 replication for each virus in the absence of AMD3100 is normalized to 100% (wild-type; black bar). Relative replication of NL4-3 (checked bar), HXB2/LW (gray bar), and JRCSF (striped bar) in the presence of AMD3100 is presented. Error bars represent standard deviations. Three independent experiments were performed with similar results. JRCSF was used as a CCR5-tropic HIV-1 resistant to AMD3100 inhibition. (D) HXB2/LW Env used only CXCR4 as a coreceptor for infecting macrophages. *env* genes from HX/LW or the CXCR4-dependent macrophage-tropic strain UG024 were expressed in 293T cells and tested for the ability to mediate fusion with primary human MDM or with U87 cells expressing CD4 alone or CD4 in conjunction with CCR5 or CXCR4. Macrophages were pretreated for 1 h with or without the CXCR4 antagonist AMD3100 (10 μg/ml). Data are expressed as the percentage of fusion relative to untreated MDM or relative to U87/CD4/CXCR4 cells. Error bars are the standard deviations of three independent experiments.

HIV-1 Tat and the *env* gene from HXB2, NL4-3, or HXB2/LW (Fig. 4B). The three HIV-1 Env proteins mediated similar levels of fusion with the Jurkat T cells, as indicated by the enhanced luciferase activity mediated by HIV-1 Tat. Therefore, the LW Env protein showed no increased fusogenicity over the HXB2 Env.

HXB2/LW produced more infectious virions with increased levels of glycoproteins. To analyze the quality of the LW virions, HIV-1 viral supernatants of HXB2 or HXB2/LW were analyzed by RT (total virions) and infectious unit (infectious virions) assays. Infection of PHA-activated PBMCs with HXB2 and HXB2/LW yielded comparable RT activity (total virions)

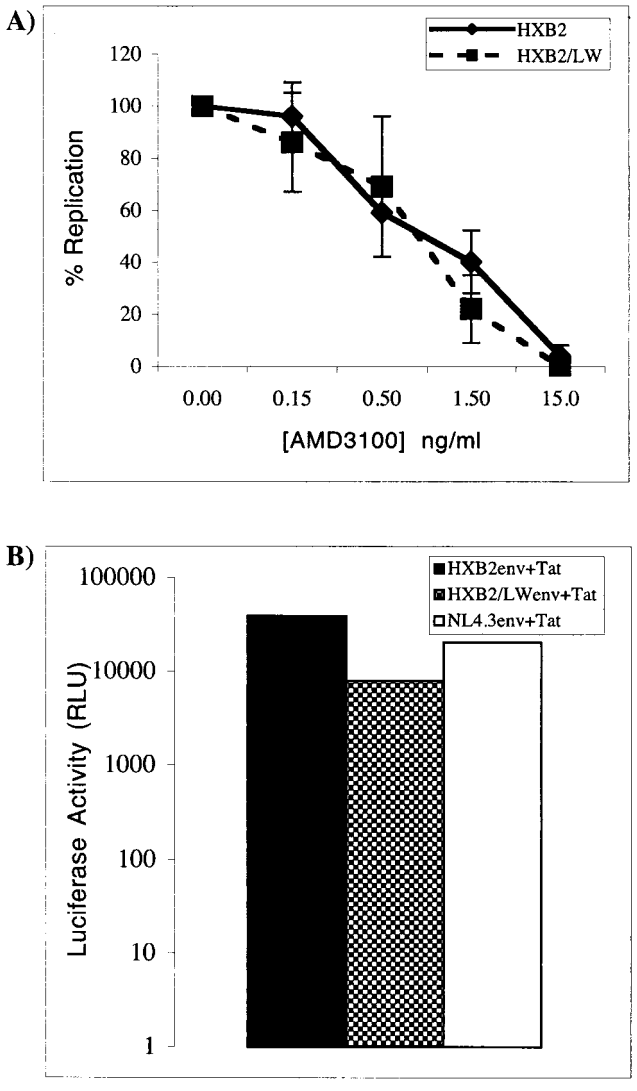

FIG. 4. HXB2/LW Env shows normal affinity for CXCR4 coreceptor and fusion activity. (A) Serial dilutions of AMD3100 were used to determine the inhibitory dose for HXB2 (black line) and HXB2/LW (dashed line) to infect CXCR4-MAGI cells. Results are indicated as the percentage of replication compared to replication in the absence of AMD3100. Error bars represent standard deviations. (B) The HXB2 env and LW env showed similar fusogenic activity. The IG5 Jurkat cell line with the LTR-luciferase gene was cocultured with 293T cells transfected with HIV-1 Tat and one of the HIV-1 *env* genes (HXB2, HXB2/LW, or NL4-3). Tat-mediated LTR activation after fusion was measured by luciferase activity (in relative light units [RLU]). Three independent experiments were performed with similar results.

in culture supernatants, suggesting that the LW Env did not affect expression, assembly, and release of total viral particles (data not shown). However, HXB2 viral stocks contained only about 10 to 15% of the infectious units of HXB2/LW viral stocks with the same amount of total virions ($P < 0.01$), indicating that HXB2/LW produced more infectious viral particles than HXB2 (Fig. 5A). These data indicate that the HXB2/LW virus enhanced assembly or stability of infectious virions.

To examine viral proteins in the virions, pelleted HXB2 or HXB2/LW virions were analyzed by Western blotting using human anti-HIV serum. The relative level of gp41, gp120, or p24 was measured by densitometry (Fig. 5B and Table 1). Relative to p24 levels, HXB2/LW virions contained approximately threefold more gp120 than HXB2 virions, although the gp41 levels relative to p24 were not significantly different (Table 1). Comparable levels of cell-associated gp41 and gp120 (relative to levels of p24/p25) were synthesized in transfected or infected cells (data not shown), indicating that HXB2/LW did not significantly affect the synthesis and proteolytic cleavage of the Env precursor. Interestingly, the single A-to-T mutation in LW/C contributed to the increased virion association of gp120, because LW/C showed a similar increase of gp120 in the virions in comparison to LW/CIIIB, which differs by one amino acid (T to A) at the V3 loop (Fig. 5 and Table 1). These results collectively suggest that the LW Env protein acquired mutations that lead to enhanced gp120 association with virions, probably through stabilization of the gp120-gp41 interaction.

DISCUSSION

From a laboratory worker accidentally exposed to a T-cell-line-adapted HIV-1 isolate, Lai/IIIB, viruses have been recovered that have reverted to replicating in macrophages and in the human thymus in vivo (40, 44). Utilizing the SCID-hu Thy/Liv mouse and HF-TOC as in vivo models for HIV-1 replication, we demonstrated that HIV-1 selected in the laboratory worker acquired activity for replication in vivo and for replication in the macrophages. In addition, the macrophage tropism was genetically separable from the replication activity in the human thymus. A single mutation in the V3 loop of the LW Env protein impaired the replication activity of the LW/C clone in the human thymus without affecting its replication activity in macrophages. We demonstrated that HIV-1 selected in the laboratory worker acquired altered tropism without a change in coreceptor usage. We further demonstrated that the LW virus produced more infectious virions than the LAI/IIIB clone HXB2, and more HIV-1 gp120 was associated with LW virions than HXB2 virions.

The HF-TOC and the SCID-hu Thy/Liv mouse have served as useful models for the analysis of normal thymocyte differentiation (23, 24, 31, 41, 43) and HIV-1 pathogenesis in vivo (8, 22, 40). As judged by the parameters of viral replication, tropism, cytopathic effects, and in vivo antiviral effects, these models both appear to faithfully reproduce the expected attributes of HIV-1 infection in vivo (2, 3, 7, 17, 18, 32, 38, 39). For example, multiple cell types are maintained in both thymus models, and thymocyte subsets are present at normal proportions, with quiescence phenotypes similar to the human thymus in vivo. Most importantly, laboratory-attenuated HIV-1 isolates like LAI/IIIB and HXB2 fail to replicate in the SCID-hu Thy/Liv mouse or in HF-TOC (8, 22, 40). HIV-1 determinants specifically involved in replication and pathogenesis in vivo have been defined using both thymus models (12, 40). Therefore, both SCID-hu Thy/Liv mice and HF-TOC are relevant models of HIV-1 replication in the thymus in vivo.

Thymocytes are clearly the primary cells infected by HXB2/LW in HF-TOC or SCID-hu Thy/Liv mice. First, HIV-1 replication was detected in thymocytes by both thymocyte-associated p24 assay and immunohistochemistry (Fig. 2 and reference 12). Second, monocytes or macrophages comprise a

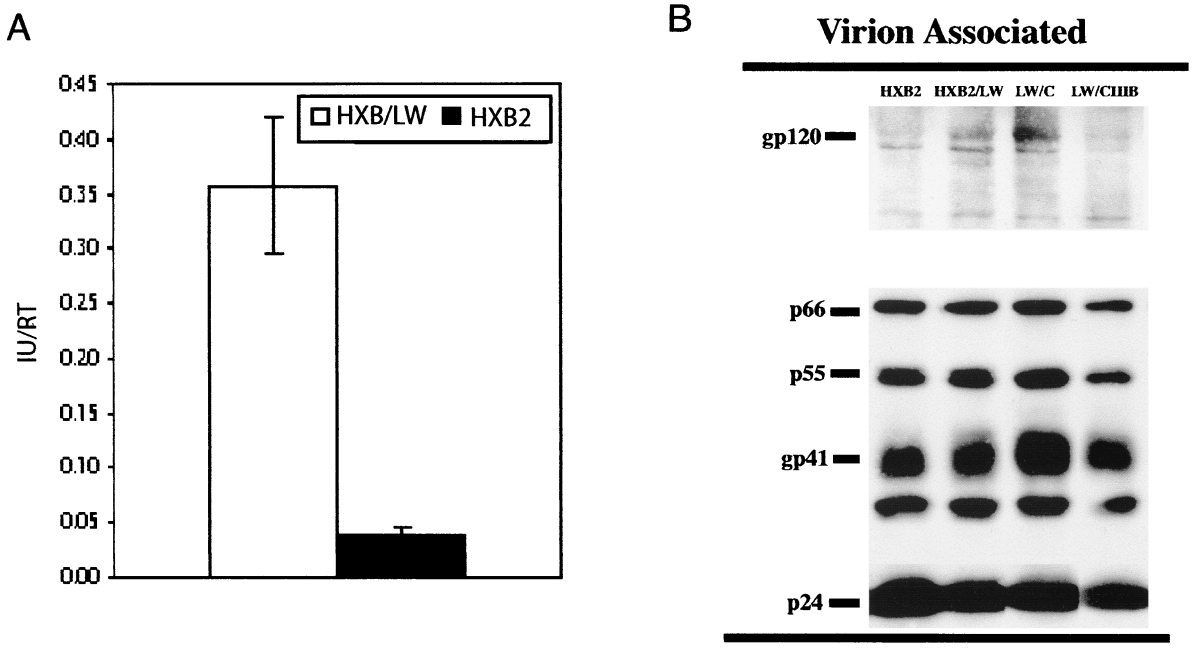

FIG. 5. HXB2/LW produced more infectious virions with higher levels of Env gp120. (A) The HXB2/LW viral stock contained higher fractions of infectious virions. The infectious virions and total virions from HXB2 or HXB2/LW viral stocks prepared from PBMCs were measured. The relative infectious virions (IU/RT) are presented. Standard deviations are shown as error bars. Three independent experiments were analyzed with similar results. (B) The HXB2/LW and LW/C virions contained higher levels of gp120. Pelleted virions were analyzed by Western blotting with HIV-positive human patient sera. The gp120, gp41, and major gag proteins are indicated. The level of gp120 or gp41for each virus relative to p24 capsid protein was calculated (gp120/p24 or gp41/p24; Table 1).

minute cell population in the thymus (31, 33). Third, a point mutation that did not affect the replication of LW/C in MDM significantly impaired its replication in HF-TOC or SCID-hu Thy/Liv mice (Fig. 2). Fourth, the same coreceptor (CXCR4) that is expressed by most thymocytes is predominantly used by both HXB2 and the LW virus (Fig. 3).

Cytokines that can enhance HIV-1 replication have been shown to be upregulated in human thymus organs productively infected with HIV-1 (reference 22 and unpublished observations). One likely mechanism of enhanced replication by the LW virus is that HXB2/LW or LW/C infected certain target cells (thymocytes or macrophages) to sensitize or enhance the ability of thymocytes to support HIV-1 replication. Identification of those "initiator cells" will be of importance in understanding the enhanced replication of HXB2/LW. Alternatively, the increased LW env gp120 in the LW virions may enhance the infectivity or transmission of HXB2/LW to infect human thymocytes. The low level of replication of HXB2 in the Thy/Liv organ at late times postinfection (Fig. 2 and reference 40) suggests that HXB2 can infect target cells in the Thy/Liv organ. However, it either replicates or transmits very slowly in the target cells, probably due to reduced levels of gp120 in the HXB2 virions. It is also possible that HXB2 at late times may have acquired mutations that contribute to enhanced replication. It will be of interest to analyze the HXB2 genome from late times postinfection for possible accumulation of specific mutations (e.g., in *nef* and/or *env* V3 loop regions).

TABLE 1. Summary of HIV gp120/p24 and gp41/p24 ratios relative to those for HXB2

Expt no.	Proteins[a]	Protein ratio			
		HXB2[b]	HXB2/LW	LW/C	LW/CIIIB
1	gp120 and p24	1.0	2.0	ND[c]	ND
	gp41 and p24	1.0	1.0	ND	ND
2	gp120 and p24	1.0	3.3	ND	ND
	gp41 and p24	1.0	1.3	ND	ND
3	gp120 and p24	1.0	2.8	6.4	1.4
	gp41 and p24	1.0	1.2	1.3	1.4

[a] The level of each protein was calculated by densitometer (see Fig. 5B).
[b] Ratios of HXB2 gp120/p24 and gp41/p24 were set at 1.0.
[c] ND, not done.

In other lentiviruses, single point mutations in *nef* have been shown to convert an attenuated virus to a pathogenic one with an enhanced replication activity in vivo (20). The present study documents the in vivo selection of a unique structural determinant in HIV-1 that appears to be necessary for infectivity in the thymus organ in vivo, but not in MDM, PBMCs, or in immortalized T-cell lines in vitro. The relevant change does not affect the *nef* gene, previously implicated for infectivity of simian immunodeficiency virus in rhesus macaques (20), or of HIV-1 in SCID-hu mice (17). Thus, novel features of the V3 region of *env* that are necessary for infection of natural target cells in vivo are revealed by analyses of HIV-1 isolates in the human thymus organ models. In a separate study, we showed that HXB2/LW replicated efficiently in the thymus models with no significant pathogenicity or thymocyte depletion (12). Therefore, other mutations specifically defective for pathogenicity in the LW genome are implicated.

It has been reported that *env* mutations in an in vivo-passaged SHIV are associated with increased resistance to neu-

tralizing antibodies (13). With viruses isolated 4 years after the isolation of LW/C from the infected laboratory worker, a recent report suggests that *env* mutations in the in vivo revertants are involved in increased resistance to neutralizing antibodies and in AIDS development (4). Interestingly, the A-to-T mutation in the *env* V3 loop common to LW/C, HXB2/LW (40), and other isolates from the infected laboratory worker (27, 44) is also associated with escape from antibody neutralization (10). Neutralizing antibodies are probably not involved in the SCID-hu Thy/Liv mouse or HF-TOC models. Therefore, the loss of replication of LW/CIIIB in the thymus models suggests that the A-to-T mutation selected in vivo may play an important role in the reversion of infectivity in vivo, independent of its possible escape from antibody neutralization. Further analyses of these recombinant viruses in different cells such as MDM and PBMCs in vitro and in the thymus models in vivo will shed light on our understanding of HIV-1 attenuation in vitro and reversion in vivo.

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