JOURNAL OF VIROLOGY, Apr. 2010, p. 4100–4104 Vol. 84, No. 8
0022-538X/10/$12.00 doi:10.1128/JVI.02068-09

Donor and Recipient Envs from Heterosexual Human Immunodeficiency Virus Subtype C Transmission Pairs Require High Receptor Levels for Entry▽

Melissa Alexander,[2] Rebecca Lynch,[2] Joseph Mulenga,[3,4] Susan Allen,[1] Cynthia A. Derdeyn,[1,2] and Eric Hunter[1,2]*

Department of Pathology and Laboratory Medicine, Emory University, Atlanta, Georgia[1]; Emory Vaccine Center at Yerkes National Primate Research Center, Emory University, Atlanta, Georgia[2]; Zambia-Emory HIV Research Project (ZEHRP), Lusaka, Zambia[3]; and Zambia Blood Transfusion Service, Lusaka, Zambia[4]

Received 30 September 2009/Accepted 31 January 2010

Compact, glycan-restricted envelope (Env) glycoproteins are selected during heterosexual transmission of subtype C HIV-1. Donor and recipient glycoproteins (Envs) from six transmission pairs were evaluated for entry into HeLa cells expressing different levels of CD4 and CCR5. Donor and recipient Envs demonstrated efficient entry into cells expressing high levels of CD4 and CCR5, and entry declined as CCR5 levels decreased. Infectivity for all Envs was severely impaired in cells expressing low levels of CD4, even at the highest CCR5 levels. In 5/6 pairs, there was no significant difference in efficiency of receptor utilization between the donor and recipient Envs in these HeLa-derived cell lines. Thus, HIV-1 transmission does not appear to select for viruses that can preferentially utilize low levels of entry receptors.

Previously, our laboratory demonstrated that a severe genetic bottleneck selects for viruses with compact, glycan-restricted envelope (Env) glycoproteins during heterosexual transmission of subtype C HIV-1 (4). A similar bottleneck has since been demonstrated in other subtypes (3, 7, 12, 16). In our studies, chronically infected donor and acutely infected recipient Envs differed primarily in the first, second, and fourth variable loops (V1/V2 and V4), regions previously demonstrated to affect the structure and function of the envelope as well as binding and entering of target cells (1, 10, 11, 17). These biological properties could be important for transmission across a mucosal surface, which has been shown to play a critical but uncharacterized role in the restriction of entry (7). One level of this restriction could be entry into potential target cells expressing limiting amounts of receptors compared to that of T lymphocytes (6, 13, 15). One of several possible target populations is thought to be tissue macrophages. However, a recent study has demonstrated equivalent macrophage infections among these donor and recipient Envs (9). Studies presented here were designed to further investigate whether differences in transmissibility between the donor and recipient Envs can be related to their abilities to interact with the CD4 receptor and CCR5 coreceptor during the viral entry process.

For experiments described in this study, pseudotyped virus was generated by cotransfection of pcDNA3.1 or pCR3.1 expression plasmids containing individual *env* sequences from six donor-recipient pairs along with an HXB2 *env*$^{-}$*luc*$^{+}$ proviral plasmid into 293T cells. These six pairs included four pairs in which transmission occurred from female to male (pairs 153, 221, 135, and 53) and two pairs in which transmission occurred from male to female (pairs 205 and 109). A more detailed characterization of the transmission pairs has been described previously by Derdeyn et al. (4) (pairs 53, 109, 135, and 153) and Haaland et al. (7) (pairs 205 and 215). For a majority of the transmission pairs, we analyzed five independent donor and recipient *env* clones. Donor *env* clones were selected to reflect a broad representation of the donor phylogenetic tree and V1-V4 region length (see Fig. 1). Recipient clones were generally homogeneous, differing in only a limited number of amino acids (see below). In preparation for transfection, a total of 1×10^5 293T cells were seeded in 1 ml Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% penicillin and streptomycin (DMEM-FBS-Pen-Strep) in 24-well tissue culture-treated plates. The following day, transfection was performed using 1,200 ng HXB2 backbone, 600 ng donor or recipient *env* plasmid, and 5.4 μl Fugene 6 according to the manufacturer's instructions. Transfected cells were then incubated in tissue culture at 37°C for 2.5 days, after which supernatant was harvested and filtered through 0.45-μm filters. The day prior to virus harvesting, 8×10^3 target cells for each of the eight HeLa cell lines (described below) were seeded into 96-well tissue culture-treated plates. Infection was performed by aspiration of media from the target cells, followed by the addition of 25 μl 80 μg/ml DEAE in DMEM with 1% FBS along with 25 μl of filtered 293T cell supernatant. Target cells and virus containing supernatant were then incubated for 2 h at 37°C, after which 200 μl DMEM-FBS-Pen-Strep was added. Seventy-two hours later, supernatant was aspirated from target cells, 50 μl 1× reporter lysis buffer was added to the cells, and three rounds of freeze-thaw (−80°C/25°C) were performed. Luciferase substrate (100 μl; Promega, Madison, WI) was added to 40 μl of each cell lysate, and light emission was quantified using a Synergy multidetector microplate reader (Biotek, Winooski, VT). Target HeLa cells pre-

* Corresponding author. Mailing address: Emory Vaccine Center, Yerkes National Primate Research Center, Emory University, 954 Gatewood Rd., Atlanta, GA 30329. Phone: (404) 727-1970. Fax: (404) 727-9316. E-mail: ehunte4@emory.edu.

▽ Published ahead of print on 10 February 2010.

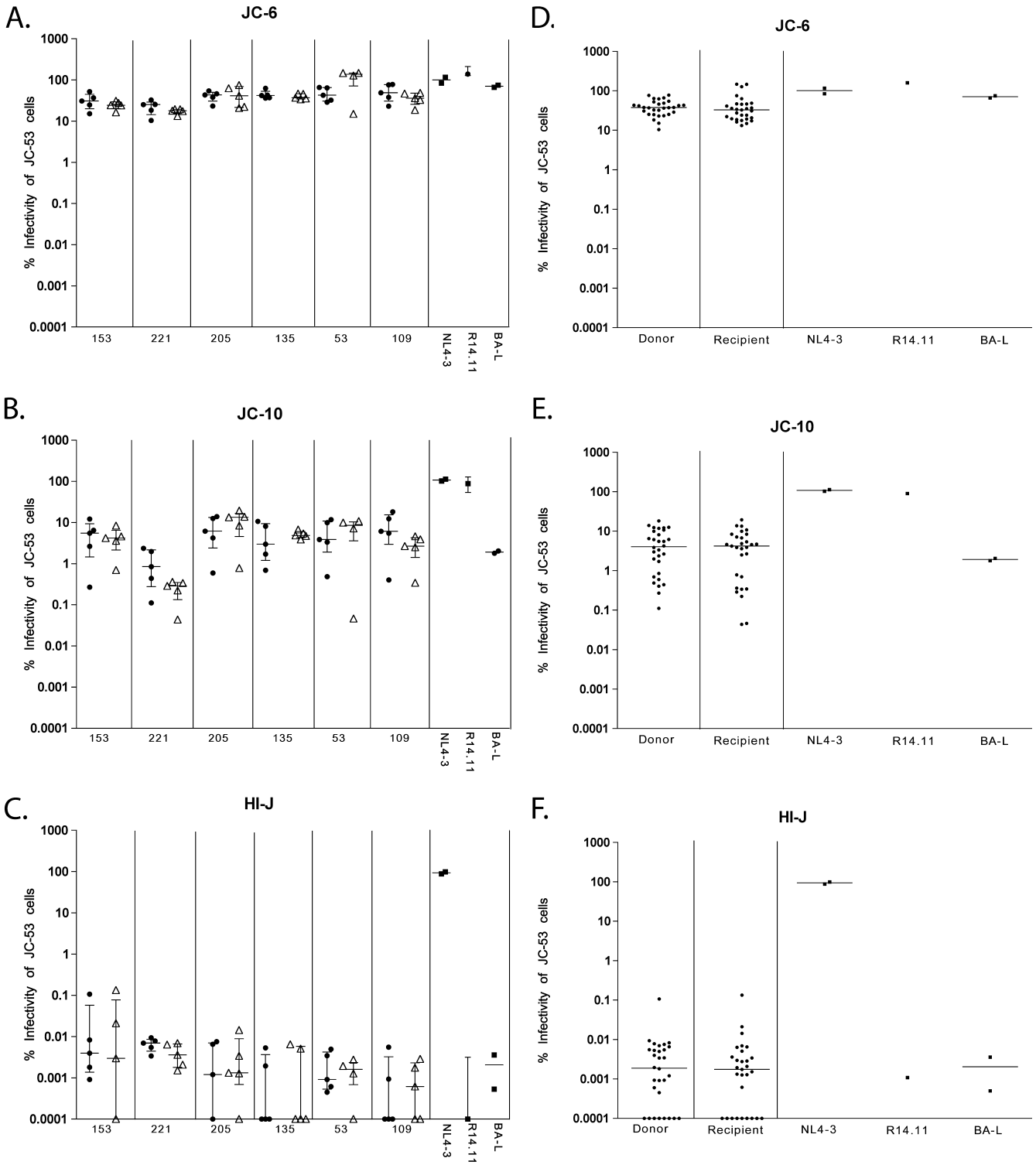

FIG. 1. High-CD4-expressing cell lines. Panels present results from cells expressing high levels of CD4 with decreasing levels of CCR5 (JC-6 > JC-10 > HI-J). (A to C) Infectivity for donor (closed circles) and recipient (open triangles) Envs for each transmission pair (indicated on the *x* axis) were plotted as a percentage of infectivity in JC-53 cells, which express high levels of CD4 and CCR5. Each symbol represents the mean of the results for three independent experiments performed in duplicate. The horizontal line for each set indicates the median with the interquartile range. (D to F) All donor and all recipient Envs were combined and plotted as in panels A to C. A horizontal line represents the median for each group. Controls for all panels included results from two NL4-3 Env clones, two BA-L clones, and one CCR5-dependent control Env clone, R14.11. The range of V1-V4 loop lengths for each donor:recipient pair was as follows: ZM53, 269 to 292:268; ZM109, 265 to 286:266; ZM135, 285 to 290:278; ZM153, 269 to 283:278; ZM205, 265 to 284:277; ZM215, 285 to 308:288.

viously described express either low (1.0×10^4 molecules/cell; R lines) or high (4×10^5 molecules/cell; J lines) levels of CD4 with four different levels of CCR5 molecules expressed per cell (HI-R, 0; RC-30, 2.4×10^3; RC-55, 2.1×10^4; RC-49, 8.5×10^4; HI-J, 0; JC-10, 2×10^3; JC-6, 2.7×10^4; and JC-53, 1.3×10^5) (15). For each Env-pseudotyped virus, the percentage of JC-53 infectivity was calculated by dividing the luminescence of that cell line by the luminescence in JC-53 cells.

We initially evaluated the donor and recipient Envs for infectivity in the J-cell lines (Fig. 1), which express high CD4 levels approximating those of T lymphocytes. Expressed as a percentage of JC-53 activity, the virus with CXCR4-tropic NL4.3 Env infected JC-6, JC-10, and HI-J cells approximately equally (100.4%, 107.7%, and 93.32% of JC-53 activity, respectively). The previously described subtype B macrophage-tropic BA-L Env demonstrated a pronounced dependence on CCR5 levels, decreasing from 70.47% of JC-53 activity in JC-6 cells to 1.921% in JC-10 and 0.002% in HI-J cells. We also tested another subtype B R5-tropic Env, R14.11 (8), which displayed a much-reduced dependence on CCR5, maintaining almost 90% of JC-53 activity in JC-10 cells, which express 50-fold fewer CCR5 molecules. This activity decreased to 0.0001% in HI-J cells, which express no CCR5 molecules. In contrast, infection of these cell lines by the donor and recipient Envs as a whole mimicked the BA-L Env and declined from a median range of 18 to 137% in JC-6 cells to a range of 0.8 to 13.1% in JC-10 cells and 0.0001 to 0.007% in HI-J cells. Nonparametric Mann-Whitney *t* test analyses of medians revealed that for all six pairs in JC-6, JC-10, and HI-J cells, there was no significant difference in infection between the donor and recipient Envs. Importantly, when medians from all donor Envs and all recipient Envs were grouped and compared by both Mann-Whitney and two-way analysis of variance (ANOVA), no significant difference in infection was observed between the donor and recipient groups in any of the J-cell lines (Fig. 1D to F).

We next evaluated infection of the R-cell lines that express various levels of CCR5 and a 10-fold lower level of CD4, which approximates levels on macrophages. Infection of these cells, relative to that of JC-53 cells, was drastically diminished for Envs from all six pairs and decreased further with decreasing levels of CCR5 (Fig. 2A to D). Medians ranged from 0.015 to 3.1% in RC-49 cells, 0.002 to 1.75% in RC-55 cells, 0.01 to 1.4% in RC-30 cells, and 0.0001 to 0.0038% in HI-R cells. For 5/6 transmission pairs, there was no statistically significant difference in infection between donor and recipient Envs in any of the R-cell lines. For one pair, transmission pair 53, recipient Envs more efficiently infected RC-49 cells ($P = 0.0159$), as well as RC-55 cells ($P = 0.0317$), RC-30 cells ($P = 0.0317$), and HI-R cells ($P = 0.0159$). Both BA-L and R14.11 Envs were able to infect R-cell lines better than a majority of transmission pair Envs, demonstrating 2.5% and 1.3% JC-53 activity, respectively, in RC-49 cells, which express the highest level of CCR5. They also both displayed similarly reduced infection efficiencies of 0.6% and <0.005% in RC-30 and HI-R cells, respectively. In contrast, NL4.3 Env exhibited infection efficiencies that varied inversely to CCR5 expression levels (5.4% in RC-49 cells, 7.2% in RC-55 cells, 19.5% in RC-30 cells, and 26.4% in HI-R cells), ruling out a general reduction in infectivity as CCR5 levels decreased. The 4-fold difference in infection efficiency between RC-49 and HI-R cells is consistent with a 6-fold difference in surface expression of CXCR4 between these two cell lines (our unpublished data). When donor Envs and recipient Envs from all transmission pairs were combined and analyzed by both Mann-Whitney and two-way ANOVA, there was again no significant difference in infection between medians of the two groups in any of the R-cell lines (Fig. 2E to H).

Results from this study demonstrate that in this subtype C-infected cohort of heterosexual couples, in which recent transmission from one partner to the other has occurred, donor and recipient Envs display a strict dependence on CCR5 levels as well as a requirement for high levels of CD4 receptor. Thus, although the majority of subtype C Envs has been shown to use CCR5 and rarely switch to CXCR4 tropism (2, 14, 18), this is the first study to evaluate the ability of primary subtype C Envs from chronically infected individuals and their acutely infected partners to utilize different levels of receptor and coreceptor. The requirement for high CCR5 levels by subtype C Envs is consistent with reports that infectivity by macrophage-tropic subtype B primary isolates are highly dependent on this coreceptor (20). Previous studies of the cell lines used in this report have also demonstrated a strict dependence on CCR5 expression by subtype B isolates from acutely infected individuals (15). However, in a study of several subtype B primary isolates, including BA-L, high levels of either CCR5 or CD4 could compensate for low levels of the other, and maximal infectivity was observed in JC-10 cells that express only trace levels of CCR5 (15). This was not observed for the subtype C Envs or BA-L in the current analysis. The differences in infection of JC-10 cells observed for BA-L in the current study and the prior report likely reflect the presence of multiple genetic variants in the primary HIV BA-L isolate used in the latter, versus the Env clone used in this study. Indeed, when previously described cloned Envs (BBB, NNB, NBB) were tested in a replication-competent green fluorescent protein (GFP) reporter system for entry into the cell lines used in this study, the infectivities were consistent with the prior report (data not shown) (19). Using V1-V5 chimeric replication-competent subtype A viruses, Etemad et al. (5) observed reduced replication in JC-10 cells for those chimeras containing the acute virus fragment relative to that for those containing fragments from chronically infected partners, although both replicated on these cells less efficiently than on JC-53 cells. Thus, it is possible that viruses from subtype A transmission pairs differ in their CCR5 requirements from those we have observed for subtype C Envs.

Results from the high CD4 J-cell lines revealed a fairly uniform infectivity among the Envs when both CD4 and CCR5 are abundant. As receptor and coreceptor levels are decreased, the infectivity profile becomes significantly more heterogeneous, likely reflecting subtle differences in the ability of individual Envs to interact with the target cell. This is consistent with a recent report showing a similarly heterogeneous infection profile in macrophages with these same Envs (9). While the infectivity profile of recipient Env clones would be predicted to be relatively homogeneous due to the small number of amino acid differences between these clones, there was an occasional outlier detected among the recipients within a given pair. Sequencing analyses of two sets of recipient sequences with clear outliers in entry phenotypes revealed nonsynony-

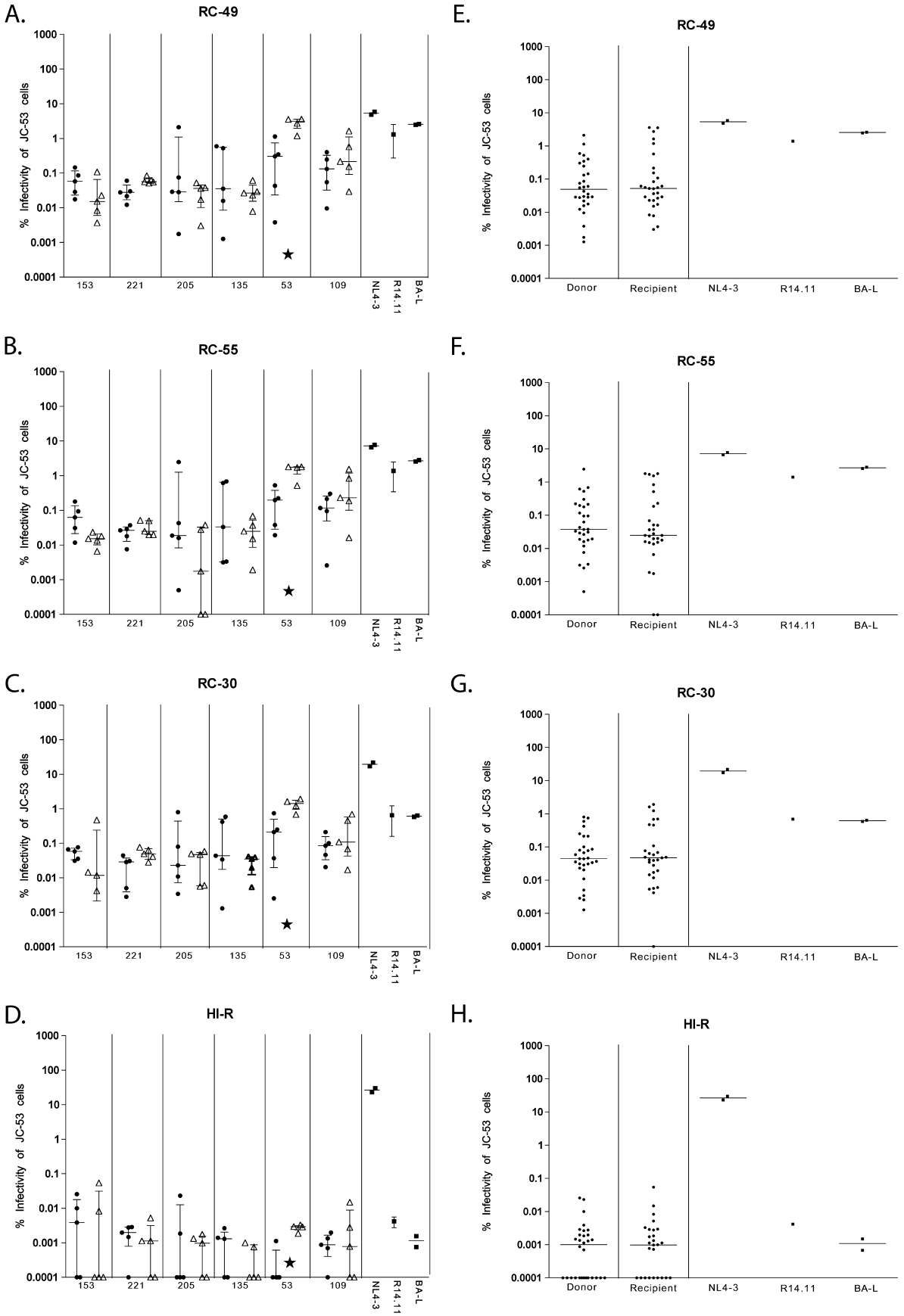

FIG. 2. Low-CD4-expressing cells. Panels present results from cells expressing decreasing levels of CCR5 (RC-49 > RC-55 > RC-30 > HI-R). Experiments are plotted as described in the legend for Fig. 1. A star indicates statistical significant difference ($P < 0.05$) between the medians of the donor and recipient Envs.

mous nucleotide changes unique to the outlier clone in each case that could be responsible for reduced infectivity; in pair 205, amino acid changes were present in the C1 and V2 loop region, and in pair 53, changes were observed in the bridging sheet of gp120 and the MPER region of gp41 (data not shown).

In our initial studies of subtype C transmission pairs, where we observed that Envs with more-compact variable loops were more frequently found in recipient partners, we suggested that this could reflect differential usage of receptors and/or coreceptors on key target cells early in infection. The current findings using epithelial cells expressing different levels of these molecules, presented here, are not consistent with this hypothesis and argue that the virus emerging from the genetic bottleneck is not selected for based on a unique pattern of CD4/CCR5 receptor utilization on target cells of the genital mucosa. Instead it appears that these viruses, like those replicating in the chronically infected partner, are effectively adapted to growth in CCR5-expressing, mucosal $CD4^+$ T cells.

This work was supported by NIH grant R01-AI51231 to E.H., NIH grant R01-AI58706 to C.A.D., and NIH Ruth L. Kirchstein National Research Award F32-AI067079 to M.A.

We thank Ling Yue for assisting in DNA plasmid purifications and Tianwei Yu for assistance with statistical analyses. We thank David Kabat and Emily Platt (Oregon Health Sciences University) for the R- and J-cell lines as well as experimental advice; Rachel LaCasse and Bruce Chesebro (Rocky Mountain Laboratories NIH/NIAID) for the NNB, NBB, and BBB expression plasmids; and Ronald Collman (University of Pennsylvania) for kindly providing the BA-L Env expression and HXB2-*luc*$^+$*env*$^-$ proviral vectors.

REFERENCES

1. **Cao, J., N. Sullivan, E. Desjardin, C. Parolin, J. Robinson, R. Wyatt, and J. Sodroski.** 1997. Replication and neutralization of human immunodeficiency virus type 1 lacking the V1 and V2 variable loops of the gp120 envelope glycoprotein. J. Virol. **71:**9808–9812.
2. **Cecilia, D., S. S. Kulkarni, S. P. Tripathy, R. R. Gangakhedkar, R. S. Paranjape, and D. A. Gadkari.** 2000. Absence of coreceptor switch with disease progression in human immunodeficiency virus infections in India. Virology **271:**253–258.
3. **Chohan, B., D. Lang, M. Sagar, B. Korber, L. Lavreys, B. Richardson, and J. Overbaugh.** 2005. Selection for human immunodeficiency virus type 1 envelope glycosylation variants with shorter V1-V2 loop sequences occurs during transmission of certain genetic subtypes and may impact viral RNA levels. J. Virol. **79:**6528–6531.
4. **Derdeyn, C. A., J. M. Decker, F. Bibollet-Ruche, J. L. Mokili, M. Muldoon, S. A. Denham, M. L. Heil, F. Kasolo, R. Musonda, B. H. Hahn, G. M. Shaw, B. T. Korber, S. Allen, and E. Hunter.** 2004. Envelope-constrained neutralization-sensitive HIV-1 after heterosexual transmission. Science **303:**2019–2022.
5. **Etemad, B., A. Fellows, B. Kwambana, A. Kamat, Y. Feng, S. Lee, and M. Sagar.** 2009. Human immunodeficiency virus type 1 V1-to-V5 envelope variants from the chronic phase of infection use CCR5 and fuse more efficiently than those from early after infection. J. Virol. **83:**9694–9708.
6. **Granelli-Piperno, A., B. Moser, M. Pope, D. Chen, Y. Wei, F. Isdell, U. O'Doherty, W. Paxton, R. Koup, S. Mojsov, N. Bhardwaj, I. Clark-Lewis, M. Baggiolini, and R. M. Steinman.** 1996. Efficient interaction of HIV-1 with purified dendritic cells via multiple chemokine coreceptors. J. Exp. Med. **184:**2433–2438.
7. **Haaland, R. E., P. A. Hawkins, J. Salazar-Gonzalez, A. Johnson, A. Tichacek, E. Karita, O. Manigart, J. Mulenga, B. F. Keele, G. M. Shaw, B. H. Hahn, S. A. Allen, C. A. Derdeyn, and E. Hunter.** 2009. Inflammatory genital infections mitigate a severe genetic bottleneck in heterosexual transmission of subtype A and C HIV-1. PLoS Pathog. **5:**e1000274.
8. **Heil, M. L., J. M. Decker, J. N. Sfakianos, G. M. Shaw, E. Hunter, and C. A. Derdeyn.** 2004. Determinants of human immunodeficiency virus type 1 baseline susceptibility to the fusion inhibitors enfuvirtide and T-649 reside outside the peptide interaction site. J. Virol. **78:**7582–7589.
9. **Isaacman-Beck, J., E. A. Hermann, Y. Yi, S. J. Ratcliffe, J. Mulenga, S. Allen, E. Hunter, C. A. Derdeyn, and R. G. Collman.** 2009. Heterosexual transmission of human immunodeficiency virus type 1 subtype C: macrophage tropism, alternative coreceptor use, and the molecular anatomy of CCR5 utilization. J. Virol. **83:**8208–8220.
10. **Kolchinsky, P., E. Kiprilov, P. Bartley, R. Rubinstein, and J. Sodroski.** 2001. Loss of a single N-linked glycan allows CD4-independent human immunodeficiency virus type 1 infection by altering the position of the gp120 V1/V2 variable loops. J. Virol. **75:**3435–3443.
11. **Kolchinsky, P., E. Kiprilov, and J. Sodroski.** 2001. Increased neutralization sensitivity of CD4-independent human immunodeficiency virus variants. J. Virol. **75:**2041–2050.
12. **Liu, Y., M. E. Curlin, K. Diem, H. Zhao, A. K. Ghosh, H. Zhu, A. S. Woodward, J. Maenza, C. E. Stevens, J. Stekler, A. C. Collier, I. Genowati, W. Deng, R. Zioni, L. Corey, T. Zhu, and J. I. Mullins.** 2008. Env length and N-linked glycosylation following transmission of human immunodeficiency virus type 1 subtype B viruses. Virology **374:**229–233.
13. **Ly, A., and L. Stamatatos.** 2000. V2 loop glycosylation of the human immunodeficiency virus type 1 SF162 envelope facilitates interaction of this protein with CD4 and CCR5 receptors and protects the virus from neutralization by anti-V3 loop and anti-CD4 binding site antibodies. J. Virol. **74:**6769–6776.
14. **Lynch, R. M., T. Shen, S. Gnanakaran, and C. A. Derdeyn.** 2009. Appreciating HIV type 1 diversity: subtype differences in Env. AIDS Res. Hum. Retroviruses **25:**237–248.
15. **Platt, E. J., K. Wehrly, S. E. Kuhmann, B. Chesebro, and D. Kabat.** 1998. Effects of CCR5 and CD4 cell surface concentrations on infections by macrophagetropic isolates of human immunodeficiency virus type 1. J. Virol. **72:**2855–2864.
16. **Sagar, M., O. Laeyendecker, S. Lee, J. Gamiel, M. J. Wawer, R. H. Gray, D. Serwadda, N. K. Sewankambo, J. C. Shepherd, J. Toma, W. Huang, and T. C. Quinn.** 2009. Selection of HIV variants with signature genotypic characteristics during heterosexual transmission. J. Infect. Dis. **199:**580–589.
17. **Stamatatos, L., M. Wiskerchen, and C. Cheng-Mayer.** 1998. Effect of major deletions in the V1 and V2 loops of a macrophage-tropic HIV type 1 isolate on viral envelope structure, cell entry, and replication. AIDS Res. Hum Retroviruses **14:**1129–1139.
18. **Tscherning, C., A. Alaeus, R. Fredriksson, A. Bjorndal, H. Deng, D. R. Littman, E. M. Fenyo, and J. Albert.** 1998. Differences in chemokine coreceptor usage between genetic subtypes of HIV-1. Virology **241:**181–188.
19. **Walter, B. L., K. Wehrly, R. Swanstrom, E. Platt, D. Kabat, and B. Chesebro.** 2005. Role of low CD4 levels in the influence of human immunodeficiency virus type 1 envelope V1 and V2 regions on entry and spread in macrophages. J. Virol. **79:**4828–4837.
20. **Wu, L., W. A. Paxton, N. Kassam, N. Ruffing, J. B. Rottman, N. Sullivan, H. Choe, J. Sodroski, W. Newman, R. A. Koup, and C. R. Mackay.** 1997. CCR5 levels and expression pattern correlate with infectability by macrophagetropic HIV-1, in vitro. J. Exp. Med. **185:**1681–1691.