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Mechanisms of Nonrandom Human Immunodeficiency Virus Type 1 Infection and Double Infection: Preference in Virus Entry Is Important but Is Not the Sole Factor

Jianbo Chen,[1] Que Dang,[1] Derya Unutmaz,[2] Vinay K. Pathak,[1] Frank Maldarelli,[1] Douglas Powell,[3] and Wei-Shau Hu[1]*

HIV Drug Resistance Program, National Cancer Institute,[1] and Data Management Services,[3] NCI-Frederick, Frederick, Maryland, Department of Microbiology and Immunology, Vanderbilt University, Nashville, Tennessee[2]

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We previously demonstrated that human immunodeficiency virus type 1 (HIV-1) infection is nonrandom and that double infection occurs more frequently than predicted from random events. To probe the possible mechanisms for nonrandom infection, we examined the role of HIV-1 entry pathways by using viruses pseudotyped with either CCR5-tropic HIV-1 Env or vesicular stomatitis virus G protein (VSV G). These two proteins use different receptors and entry pathways. We found that regardless of the protein used, double infection occurred more frequently than random events, indicating nonrandom HIV-1 infection in both entry pathways. However, the frequency of double infection differed significantly, depending on the envelope protein. In primary CD4$^+$ T cells, double infection occurred most frequently when both viruses had CCR5-tropic HIV-1 Env and least frequently when the two viruses had different envelopes. These results indicated that the preference in virus entry was a significant but not the only factor contributing to nonrandom double infection. Furthermore, we demonstrated that the CD4 expression level in primary T cells affects their susceptibility to CCR5-tropic HIV-1 infection but not VSV G-pseudotyped HIV-1 infection. We have also examined infection with two viruses pseudotyped with CCR5- or CXCR4-tropic HIV-1 Env and have found that double infection occurred more frequently than random events. These results indicate that coreceptor usage is not a barrier to recombination between the two virus populations. In our previous study, we also demonstrated nonrandom double infection via dendritic cell (DC)-mediated HIV-1 transmission. To test our hypothesis that multiple HIV-1 virions are transmitted during DC–T-cell contact, we used two populations of DCs, each capturing one vector virus, and added both DC populations to T cells. We observed a decreased frequency of double infection compared with experiments in which DCs captured both viruses simultaneously. Therefore, these results support our hypothesis that multiple virions are transmitted from DCs to T cells during cell-mediated HIV-1 transmission.

Human immunodeficiency virus type 1 (HIV-1) replicates with high genetic variation in the viral population, which contributes to the evasion of the host's immune system and the emergence of drug-resistant virus (3, 23). One of the major mechanisms that generates genetic variation is recombination (14, 28, 29). Like other retroviruses, HIV-1 packages two copies of viral RNA into one virion (8, 19); frequent recombination between the two copackaged RNAs occurs during reverse transcription (11). The copy choice and the dynamic copy choice models for recombination propose that reverse transcriptase can switch between the copackaged RNA molecules, use portions of each RNA as a template for DNA synthesis, and generate recombinant viral DNA (4, 12). Genetic analyses have shown that recombination occurs mainly during minus-strand DNA synthesis, which supports these models for recombination (2). In order for novel recombinants to be generated, the two RNAs packaged in the virion must be genetically different (heterozygous virions) (11). Only cells infected by more than one retrovirus (double infection) can produce heterozygous virions; therefore, how often double infection occurs affects the overall frequency of recombination.

* Corresponding author. Mailing address: HIV Drug Resistance Program, NCI-Frederick, P.O. Box B, Building 535, Room 336, Frederick, MD 21702. Phone: (301) 846-1250. Fax: (301) 846-6013. e-mail: whu@ncifcrf.gov.

In a previous report, we examined the randomness of HIV-1 infection and double infection (5). We used two HIV-1 vectors containing different marker genes to generate virus stocks pseudotyped with CCR5-tropic HIV-1 Env. These two virus stocks were mixed together and used to infect either primary activated CD4$^+$ T cells or a T-cell line, and the number of infected cells was scored by flow cytometry based on the marker gene expression. If double infection is random, then its frequency can be calculated from the frequencies of infection of the two virus stocks. However, in all experiments, regardless of the target cells used, we observed significantly more doubly infected cells than predicted from random events. These results indicated that HIV-1 infection and double infection are nonrandom. We proposed that variation in the infectibility of the target cells caused the nonrandom infection; however, the mechanisms responsible for the variation in infectivity remained unknown.

In addition to direct infection, HIV-1 can also be transmitted through a cell-mediated pathway. Dendritic cells (DCs) can capture HIV-1 particles and transmit the viruses to target cells (9, 10). In a previous study, we also examined the frequency of double infection by using primary human DCs and a cultured cell line, Thp-1/DC-SIGN, to capture HIV-1 (5). We observed that double infection via the cell-mediated pathway occurred more frequently than expected from random events regardless of the cells used to capture HIV-1. We hypothesized

that more than one virion was transmitted via the cell-mediated pathway, thereby causing nonrandom double infection.

In this study, we have explored the mechanisms that caused nonrandom double infection in direct and cell-mediated infection pathways. By examining the virus entry pathway in direct HIV-1 infection, we conclude that the entry pathway plays an important role in nonrandom double infection; however, other mechanisms also exist to cause nonrandom double infection. Furthermore, we have demonstrated that the CD4 expression level in primary T cells affects HIV-1 infection. We have also performed experiments to test our hypothesis that more than one virus is transmitted via cell-mediated HIV-1 infection, and our results support this hypothesis. Taken together, our results indicate that preference in the virus entry step is an important factor but is not the sole factor contributing to the observed nonrandom HIV-1 infection and double infection.

MATERIALS AND METHODS

Plasmids, cell lines, and primary cell isolation. The HIV-1-based vectors HDV-eGFP and HIV-IHSA have been described previously (32). Briefly, these vectors were derived from pNL4-3; encode Gag, Pol, Tat, and Rev; and contain inactivating deletions in *vif*, *vpr*, *vpu*, and *env*. Marker genes were inserted into *nef* in both vectors: HDV-eGFP encodes the green fluorescent protein (GFP), and HIV-IHSA contains an internal ribosomal entry site from encephalomyocarditis virus and the mouse heat-stable antigen gene (*HSA*). Plasmids pIIINL(AD8)env and pIIINL4env were kind gifts from Eric Freed (HIV Drug Resistance Program, National Cancer Institute). pIIINL(AD8)env expresses Env from the AD8 strain of HIV-1, which uses CCR5 as a coreceptor (i.e., CCR5-tropic), whereas pIIINL4env expresses Env from laboratory strain NL4-3, which uses CXCR4 as a coreceptor (i.e., CXCR4-tropic). Plasmid pHCMV-G expresses vesicular stomatitis virus G protein (VSV G) (35). Plasmid pCMVnef was a kind gift from David Rekosh (University of Virginia) (1).

293T cells are a human embryonic kidney cell line containing simian virus 40 large T antigen (7, 26). Thp-1/DC-SIGN cells are Thp-1 cells that express DC-specific ICAM-3 grabbing nonintegrin (DC-SIGN) on the cell surface (9); to avoid confusion, we used the name from the original publication, although a recent report indicates that these cells are Raji cells (33). Hut/CCR5 cells were derived from Hut78, a human T-cell line, and express CCR5 (34).

Primary $CD4^+$ T cells were isolated from peripheral blood mononuclear cells of healthy donors by use of an anti-CD4 monoclonal antibody conjugated to Dynabeads. This purification procedure generated greater than 99% purity of $CD4^+$ T cells as determined by flow cytometry analyses. Purified T cells were activated by phytohemagglutinin at 2 μg/ml or by cross-linking with plate-bound anti-CD3 antibody (OKT-3) and soluble anti-hCD28 antibody (BD PharMingen). These cells were maintained in medium containing an additional supplement of interleukin-2 (200 U/ml) for 6 to 8 days prior to infection.

Primary immature DCs were derived from primary blood mononuclear cells of healthy donors by use of anti-CD14 magnetic activated cell sorting beads (Miltenyl Biotec) and maintained in medium containing an additional supplement of granulocyte-macrophage colony-stimulating factor (50 ng/ml) and interleukin-4 (100 ng/ml) (25). Cells were used in experiments at 4 to 5 days postpurification.

Unless specified otherwise, all cells were maintained at 37°C with 5% CO_2 in medium supplemented with 10% fetal calf serum (HyClone Laboratories, Inc.), penicillin (50 U/ml) (Gibco), and streptomycin (50 μg/ml) (Gibco). Dulbecco's modified Eagle's medium was used for 293T cells, and RPMI medium was used for Thp-1, Thp-1/DC-SIGN, Hut/CCR5, primary T cells, and primary DCs. Puromycin (1 μg/ml) (Sigma) and G418 (500 μg/ml) (Gibco) were added to Hut/CCR5 cells to maintain the selection for CCR5 expression.

Transfections, infections, flow cytometry analyses, and cell sorting experiments. To generate vector-derived viruses, 293T cells were transiently transfected with HIV-1 vector and envelope-expressing plasmid at a 2:1 ratio by the calcium phosphate method (30), using the MBS mammalian transfection kit (Stratagene). For virus pseudotyped with CXCR4-tropic HIV-1 Env, 293T cells were transfected with HIV-1 vector, pIIINL4env, and pCMVnef at a 2:1:2 ratio. The supernatant was harvested 36 to 48 h later and clarified through a 0.45-μm-pore size filter to remove cellular debris.

Infections were performed in a 1-ml total volume in 24-well plates; for direct infection, 250 μl of each virus was added to 2.5×10^5 target cells. Cell-mediated infection was performed by adding 250 μl of virus to virus-capturing cells, incubating for 1 h at 37°C, and removing the unbound viruses by washing cells with medium. These virus-capturing cells were then incubated with target cells.

Cells were washed with phosphate-buffered saline at 72 h postinfection; phycoerythrin-conjugated anti-HSA antibody and allophycocyanin (APC)-conjugated anti-CD4 (Hut/CCR5) or anti-CD3 (primary T cells) antibody (BD PharMingen) was used to stain cells. The cells were then resuspended in 2% paraformaldehyde and analyzed by flow cytometry. In cell-mediated infection protocols, infections were measured only in $CD4^+$ (Hut/CCR5) or $CD3^+$ (primary T cells) cell populations. Flow cytometry analyses were performed on a FACSCalibur apparatus (BD Biosciences), and data were analyzed with FlowJo software (Tree Star).

For cell sorting experiments, peripheral blood mononuclear cells from healthy donors were activated with phytohemagglutinin for 2 days at 2 μg/ml, followed by further culturing in interleukin-2-containing medium for 7 days. Cells were stained with APC-conjugated anti-CD4 antibodies, and the desired cell populations were sorted by using FACSvantages (BD Biosciences). Sorted cell populations were cultured for an additional 3 days prior to virus infection experiments.

Odds ratio calculation and statistical analyses. Depending on HSA and GFP expression, target cells were divided into four phenotypes: HSA^+/GFP^-, HSA^+/GFP^+, HSA^-/GFP^-, and HSA^-/GFP^+; the numbers of cells that had these four phenotypes were designated *a*, *b*, *c*, and *d*, respectively. The odds ratio was calculated as the odds of HSA^+ cells also being GFP^+ versus the odds of HSA^- cells being GFP^+. The odds of HSA^+ cells being GFP^+ were $[b/(b + a)]/\{1 - [b/(b + a)]\} = b/a$, whereas the odds of HSA^- cells being GFP^+ were $[d/(c + d)]/\{1 - [d/(c + d)]\} = d/c$. Therefore, the odds ratio of a HSA^+ and HSA^- population being GFP^+ is $(b/a)/(d/c) = bc/ad$. The odds ratio of double infection could also be calculated by comparing the odds of HSA^+ observed in GFP^+ and GFP^- cell populations, or $(b/d)/(a/c)$. These two calculations yielded the same odds ratio mathematically: $(b/a)/(d/c) = bc/ad = (b/d)/(a/c)$. Pearson chi-square tests were used to analyze whether the odds ratios were significantly different from 1, which is expected from random double infection. The *P* value for statistical significance was set at 0.01.

Confidence intervals were used to determine whether different treatment groups within the same set of experiments yielded significantly different odds ratios. This determination is made by observing whether the confidence intervals of the odds ratios for two different treatment conditions are overlapping. If the two confidence intervals overlap, the two odds ratios are not statistically different; if the two confidence intervals do not overlap, the two odds ratios are significantly different. Overlapping indicates that the two confidence intervals have common points. Since the reported intervals are based on a confidence level of 99%, the implicit significance level for determining whether two odds ratios are significantly different is less than or equal to $1 - (0.99 \times 0.99) = 0.0199$.

RESULTS

Nonrandom double infection occurs with VSV G-pseudotyped HIV-1. It was previously demonstrated that concentrations of the receptor and coreceptor on the cell surface could influence the infectibility of the cells (15, 17, 27). Because only portions of the primary $CD4^+$ T cells express the CCR5 coreceptor, it remains possible that the presence of cells lacking the CCR5 coreceptor caused the observed nonrandom HIV-1 double infection in primary $CD4^+$ T cells. To test this possibility, we pseudotyped HIV-1 with VSV G, which has a wide host range and is considered pantropic, and examined the frequency of double infection in primary T cells.

Two previously described HIV-1 vectors, HDV-eGFP and HIV-IHSA, were used in these experiments. Both vectors contain all of the *cis*-acting elements essential for virus replication and encode Gag/Gag-Pol, Tat, and Rev. Additionally, each of the vectors expresses a marker gene located in *nef*, *GFP* for HDV-eGFP and *HSA* for HIV-IHSA. Viruses derived from HDV-eGFP and HIV-IHSA, which are referred to here as GFP virus and HSA virus, respectively, were generated separately by transfecting 293T cells with a HIV-1 vector plasmid and a VSV G-expressing plasmid. The two virus stocks were mixed together and used to infect activated $CD4^+$ primary T cells. These cells were processed later and analyzed by flow cy-

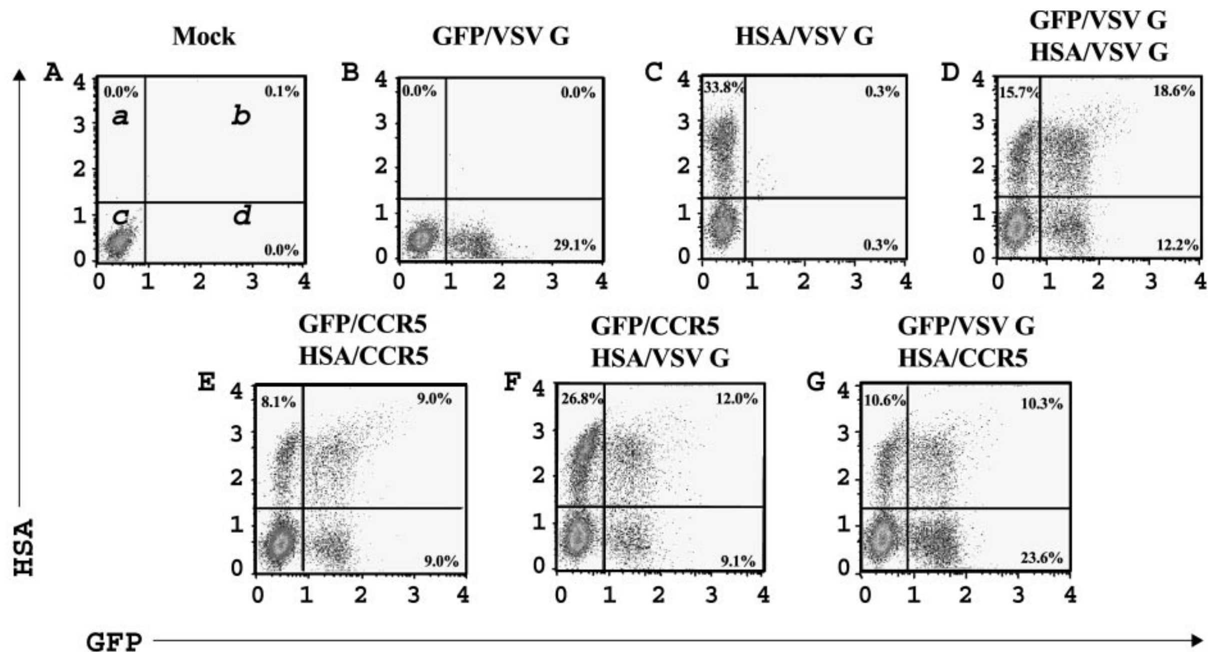

FIG. 1. Flow cytometry analyses of HIV-1 vector infections in primary CD4$^+$ T cells. (A) Mock-infected cells. (B) Cells infected with GFP virus pseudotyped with VSV G. (C) Cells infected with HSA virus pseudotyped with VSV G. (D) Cells infected with a mixture of GFP and HSA viruses, both pseudotyped with VSV G. (E) Cells infected with a mixture of GFP and HSA viruses, both pseudotyped with CCR5-tropic HIV-1 Env. (F) Cells infected with a mixture of GFP virus pseudotyped with CCR5-tropic HIV-1 Env and HSA virus pseudotyped with VSV G. (G) Cells infected with a mixture of GFP virus pseudotyped with VSV G and HSA virus pseudotyped with CCR5-tropic HIV-1 Env. The *x* and *y* axes represent GFP and HSA expression, respectively. Cells in quadrants a to d (labeled in panel A) have the phenotypes HSA$^+$/GFP$^-$, HSA$^+$/GFP$^+$, HSA$^-$/GFP$^-$, and HSA$^-$/GFP$^+$, respectively. The percentage of cells conferring each phenotype is indicated in its respective quadrant.

tometry; infected cells were identified based on GFP and HSA expression.

Representative flow cytometry analyses of primary CD4$^+$ T-cell infection are shown in Fig. 1A to D. In these analyses, the *x* and *y* axes represent GFP and HSA expression, respectively. Depending on the marker expression, cells can be in one of the four quadrants (labeled a to d in Fig. 1A). Quadrant a has cells that are positive for HSA but negative for GFP expression (HSA$^+$/GFP$^-$), b has HSA$^+$/GFP$^+$ cells, c has HSA$^-$/GFP$^-$ cells, and d has HSA$^-$/GFP$^+$ cells. As shown in Fig. 1A, very few GFP$^+$ or HSA$^+$ cells were detected in mock-infected samples; additionally, negligible numbers of GFP$^+$/HSA$^+$ cells were present in singly infected samples (Fig. 1B and C). In contrast, four different cell populations were observed in samples infected with a mixture of GFP and HSA viruses (Fig. 1D): 15.7% of the cells were HSA$^+$/GFP$^-$ (quadrant a), 18.6% of the cells were HSA$^+$/GFP$^+$ (quadrant b), 53.5% of the cells were HSA$^-$/GFP$^-$ (quadrant c), and 12.2% of the cells were HSA$^-$/GFP$^+$ (quadrant d). As previously described (15), the numbers of cells detected in these four quadrants were used to calculate the odds ratio of double infection by using the formula $(b/a)/(d/c)$, which measured the odds of HSA$^+$ cells being GFP$^+$ versus the odds of HSA$^-$ cells being GFP$^+$ (5) (see Materials and Methods). If double infection occurred randomly, then the proportion of GFP$^+$ cells would be the same in HSA$^+$ and HSA$^-$ populations, generating an odds ratio of 1. In contrast, if double infection occurred more frequently than expected from random events, then the HSA$^+$ population would have a higher proportion of GFP$^+$ cells than would the HSA$^-$ population, resulting in an odds ratio of greater than 1.

We calculated the odds ratio of double infection for the data shown in Fig. 1D; the numbers of cells in quadrants a to d were 2,911, 3,459, 9,931, and 2,265, respectively. This distribution yielded an odds ratio of 5.21, which was significantly greater than 1 ($P < 10^{-11}$). Data from five independent experiments using human primary CD4$^+$ T cells isolated from different donors are summarized in Table 1. The odds ratios of double infection in these experiments ranged from 2.26 to 6.10; all of them were significantly greater than 1 ($P < 10^{-11}$). Therefore, double infection occurred more frequently than expected from a random distribution for infection of CD4$^+$ primary T cells through the VSV G-mediated entry pathway. These results indicated that nonrandom double infection is not unique to viruses containing HIV-1 Env but also occurs in viruses containing the pantropic VSV G.

Effect of using different envelope proteins on randomness of HIV-1 double infection. Not only do VSV G and CCR5-tropic HIV-1 Env proteins use different cellular receptors, but viruses pseudotyped with these two envelopes also use different pathways for entry. Viruses pseudotyped with VSV G enter via endocytosis, whereas viruses pseudotyped with HIV-1 Env use direct fusion (6, 13). To examine the role of virus entry in nonrandom infection, we compared the distribution of GFP and HSA expression in target cells when the two virus stocks were pseudotyped with different proteins relative to that observed when the viruses had the same pseudotyped proteins. Representative analyses are shown in Fig. 1E to G. Cells in all experiments were infected with a mixture of GFP virus and HSA virus: for Fig. 1E, both viruses were pseudotyped with CCR5-tropic HIV-1 Env; for Fig. 1F, GFP and HSA viruses

TABLE 1. Double infection of CD4$^+$ primary T cells with CCR5-tropic HIV-1 Env- and VSV G-pseudotyped viruses

Expt	Viruses[a]	No. of cells[b]				Odds ratio[c]	99% confidence interval	
		H^+/G^-	H^+/G^+	H^-/G^-	H^-/G^+		Lower	Upper
1	R5/R5	1,525	1,676	13,776	1,687	8.97	8.02	10.05
	VG/VG	2,911	3,459	9,931	2,265	5.21	4.77	5.69
	R5/VG	4,748	2,133	9,235	1,613	2.57	2.33	2.83
	VG/R5	1,946	1,900	10,208	4,342	2.30	2.09	2.53
2	R5/R5	940	1,302	10,687	1,509	9.81	8.60	11.19
	VG/VG	1,180	628	12,197	1,064	6.10	5.24	7.10
	R5/VG	1,052	811	8,675	2,075	3.22	2.81	3.69
	VG/R5	1,882	829	9,336	1,244	3.31	2.89	3.78
3	R5/R5	600	648	17,452	1,248	15.10	12.81	17.80
	VG/VG	2,830	2,568	12,296	1,972	5.66	5.15	6.22
	R5/VG	4,537	1,216	13,142	1,013	3.48	3.09	3.91
	VG/R5	866	856	14,254	4,126	3.41	2.99	3.90
4	R5/R5	1,079	660	26,319	1,925	8.36	7.26	9.63
	VG/VG	5,197	4,246	14,273	5,171	2.26	2.11	2.41
	R5/VG	8,947	1,476	17,742	2,071	1.41	1.29	1.55
	VG/R5	1,572	1,085	17,781	9,054	1.36	1.22	1.51
5	R5/R5	969	1,048	20,370	2,032	10.84	9.52	12.34
	VG/VG	3,709	4,265	8,081	2,472	3.76	3.46	4.08
	R5/VG	10,155	1,438	8,651	815	1.50	1.33	1.69
	VG/R5	555	864	8,857	8,421	1.64	1.42	1.89

[a] R5, CCR5-tropic HIV-1 Env; VG, VSV G. The envelope used to pseudotype GFP virus is shown before the slash, and the envelope used to pseudotype HSA virus is shown after the slash.

[b] H^+/G^-, H^+/G^+, H^-/G^-, and H^-/G^+ correspond to quadrants a, b, c, and d, respectively, shown in the flow cytometry analyses in Fig. 1. G, GFP; H, HSA.

[c] All odds ratios were significantly greater than 1 ($P < 10^{-11}$).

were pseudotyped with CCR5-tropic HIV-1 Env and VSV G, respectively; and for Fig. 1G, the pseudotyped proteins were reversed (i.e., GFP and HSA viruses were pseudotyped with VSV G and CCR5-tropic HIV-1 Env, respectively).

Consistent with our previous findings, double infection in CD4$^+$ primary cells was nonrandom through the CCR5-tropic HIV-1-Env-mediated entry pathway (5) (Fig. 1E and Table 1). In the five sets of experiments summarized in Table 1, the odds ratios varied from 8.36 to 15.10, all of which were significantly greater than 1 ($P < 10^{-11}$).

When we used a mixture of GFP virus with CCR5-tropic HIV-1-Env and HSA virus with VSV G, the numbers of cells in quadrants a to d in Fig. 1F were 4,748, 2,133, 9,235, and 1613, respectively, generating an odds ratio of 2.57, which was significantly greater than 1 ($P < 10^{-11}$). Similar results were observed when the pseudotyped proteins were reversed; the odds ratio for the data shown in Fig. 1G was 2.30. Data from five independent sets of experiments revealed that cells infected with CCR5-tropic HIV-1 Env-containing GFP virus and VSV G-containing HSA virus had odds ratios of 1.41 to 3.48, whereas cells infected with VSV G-containing GFP virus and HIV-1 Env-containing HSA virus had odds ratios of 1.36 to 3.41 (Fig. 1F and G and Table 1). All of these odds ratios were significantly greater than 1. Not surprisingly, between the two experimental groups using viruses with different envelope proteins (Fig. 1F and G), the particular combination of virus and envelope protein did not significantly alter the odds ratio; similar odds ratios were generated whether GFP virus or HSA virus had CCR5-tropic HIV-1 Env.

Similar to the case in previous experiments, the odds ratio of double infection varied significantly among T cells derived from different donors. However, an obvious pattern emerged from these data when we compared different experimental groups in parallel experiments using cells from the same donors. In all experiments, the lowest double-infection odds ratios were observed when the two viruses used a combination of CCR5-tropic HIV-1 Env- and VSV G-mediated entry pathways, whereas the highest odds ratios were observed when both viruses used the CCR5-tropic HIV-1-Env-mediated entry pathway; the observed differences in odds ratios were consistently statistically significant (Table 1).

Effect of entry pathway on double infection when a cultured T-cell line is used as target cells. Primary CD4$^+$ T cells are known to have large variations in expression of genes that may affect the susceptibility to HIV-1 infection: not all of the cells express CCR5 receptors, and probably not all of the cells are infectible. Additionally, it is difficult to achieve a high multiplicity of infection in primary T cells. To further study the effect of entry pathways on double infection, we used a well-characterized cultured T-cell line, Hut/CCR5, as target cells. The data from three independent sets of infection are shown in Table 2. Consistent with our previous observations, double infection with two viruses both pseudotyped with CCR5-tropic HIV-1 Env was nonrandom, with the odds ratio varying between 2.00 and 2.71. However, double infection was also nonrandom when both viruses contained VSV G or when the two viruses contained different pseudotyped proteins. In these experiments, the odds ratios of double infection were similar among three experimental groups: both contained CCR5-tropic HIV-1 Env, or one virus contained VSV G and the other contained CCR5-tropic HIV-1 Env. The highest odds ratios were observed when both viruses had VSV G. Taken together, data from primary cells and cultured T cells revealed that in all experiments, even when different entry pathways were used, double infection was nonrandom. However, depending on the

TABLE 2. Double infection of Hut/CCR5 cells with CCR5-tropic HIV-1 Env- and VSV G-pseudotyped viruses

Expt	Viruses[a]	No. of cells[b]				Odds ratio[c]	99% confidence interval	
		H^+/G^-	H^+/G^+	H^-/G^-	H^-/G^+		Lower	Upper
1	R5/R5	5,923	6,628	6,571	2,709	2.71	2.52	2.93
	VG/VG	4,709	13,130	2,316	1,817	3.55	3.24	3.90
	R5/VG	9,427	8,511	2,539	983	2.33	2.10	2.59
	VG/R5	3,221	9,413	4,611	4,924	2.74	2.54	2.95
2	R5/R5	4,336	9,062	3,856	3,346	2.41	2.23	2.60
	VG/VG	4,671	6,393	7,591	2,245	4.63	4.27	5.01
	R5/VG	2,397	6,680	5,130	6,545	2.18	2.02	2.36
	VG/R5	8,849	5,343	5,095	1,510	2.04	1.87	2.22
3	R5/R5	4,410	3,754	8,824	3,765	2.00	1.85	2.15
	VG/VG	3,984	2,274	12,255	2,181	3.21	2.93	3.51
	R5/VG	2,940	2,388	11,204	4,247	2.14	1.97	2.33
	VG/R5	6,469	1,666	11,306	1,327	2.19	1.98	2.43

[a] See Table 1, footnote *a*.

[b] See Table 1, footnote *b*.

[c] All odds ratios were significantly greater than 1 ($P < 10^{-11}$).

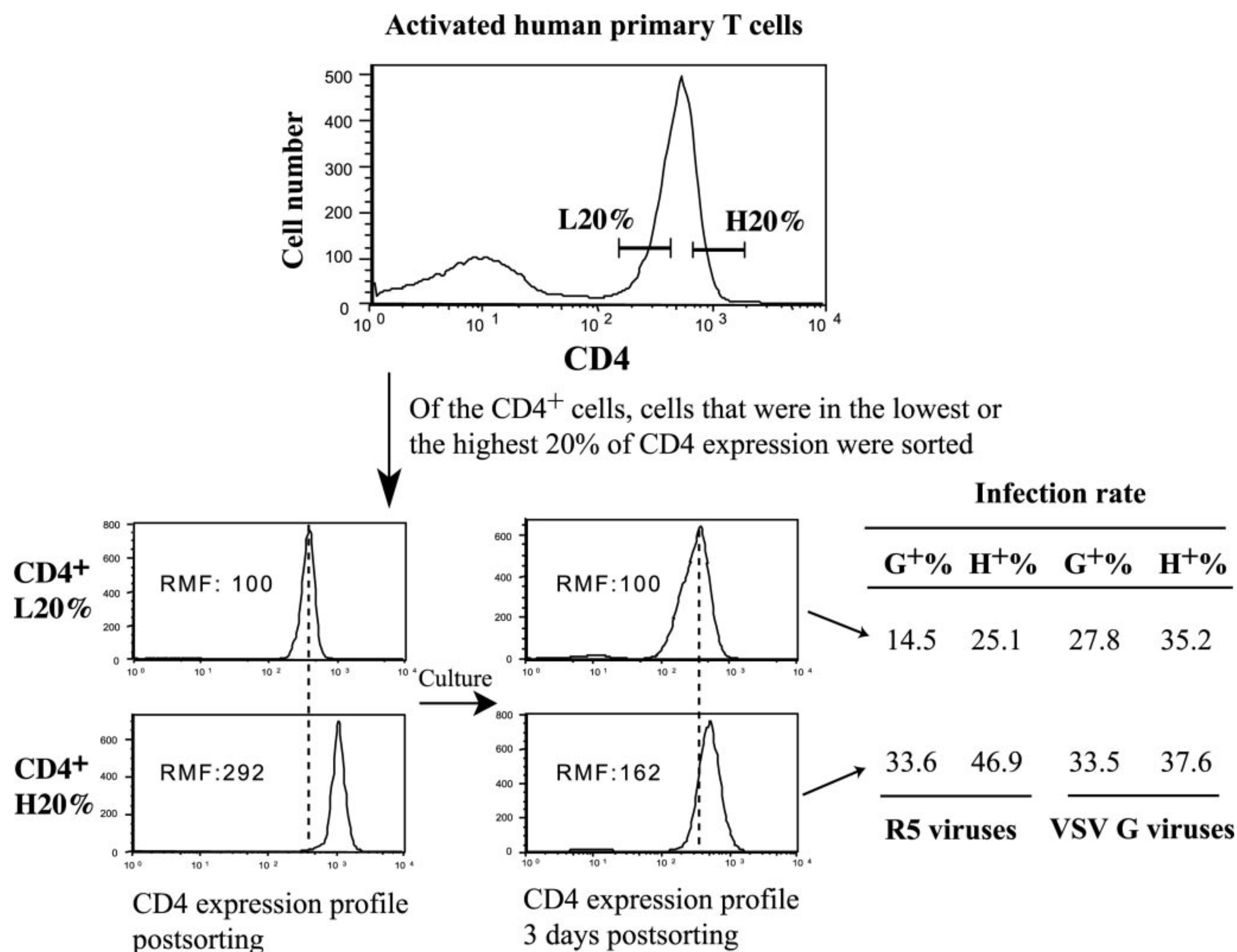

FIG. 2. Experimental protocol used to examine the effect of target cell CD4 expression on susceptibility to HIV-1 infection and representative results. In all panels, the *x* axis shows the CD4 expression level and the *y* axis shows the cell number. The relative mean fluorescence (RMF) of the low-CD4 cell subpopulation was set as 100. A set of representative results is summarized in the table. G^+% or H^+% indicates the percentage of target cells that are GFP^+ or HSA^+, respectively. To compare susceptibilities to virus infection, the same virus stocks were used to infect the two subpopulations of cells.

target cells and the envelopes used, different experimental groups had different ranges of odds ratios, indicating that the entry pathway played an important role in double infection.

Effects of CD4 expression in primary T-cell populations on HIV-1 infection. A critical part of HIV-1 entry is CD4-Env interaction. It was previously shown that HeLa cells engineered to have higher CD4 expression can be more susceptible to HIV-1 infection (15, 17, 27). However, higher CD4 expression does not always predict increased susceptibility to HIV-1 infection. For example, the level of CD4 expression did not correlate with susceptibility to HIV-1 infection in primary human macrophages (31). To directly address whether the level of CD4 expression in primary T cells affects their susceptibility to HIV-1 infection, we examined the frequency of HIV-1 infection in sorted primary T-cell subpopulations. The experimental design and data from a representative set of experiments are shown in Fig. 2. We stained activated primary T cells with APC-conjugated anti-CD4 antibodies and isolated two subpopulations of cells by cell sorting. Among $CD4^+$ cells, we isolated 20% of the cells that had either the highest or lowest CD4 expression and designated them the high- or low-CD4-expression subpopulation, respectively (Fig. 2, top panel). These cells were cultivated for an additional 3 days and were infected with viruses pseudotyped with CCR5-tropic HIV-1 Env. Further cultivation after cell sorting was performed to avoid the possible effect of the anti-CD4 antibody on HIV-1 infection. Flow cytometry analyses revealed that immediately after cell sorting, the CD4 expression of the high and low subpopulations was well separated, with a threefold difference in the relative mean fluorescence levels between the two subpopulations (Fig. 2, bottom two panels on the left). After 3 days of further cultivation, the CD4 distribution in cells within each subpopulation became more varied, and the two subpopulations contained an overlapping region. Nevertheless, the relative mean fluorescence level in the high-CD4 population was significantly higher than that in the low-CD4 population (Fig. 2, bottom two panels on the left). When infected with viruses containing CCR5-tropic-HIV-1-Env, 33.6 and 46.9% of the

TABLE 3. Double infection of primary T cell with viruses pseudotyped with CCR5- or CXCR4-tropic HIV-1 Env

Expt	Viruses[a]	No. of cells[b]				Odds ratio[c]	99% confidence interval	
		H^+/G^-	H^+/G^+	H^-/G^-	H^-/G^+		Lower	Upper
1	R5/R5	1,624	488	37,946	366	31.15	25.76	37.67
	X4/X4	2,093	208	52,630	369	14.17	11.25	17.86
	R5/X4	1,103	414	44,015	1,244	13.28	11.25	15.68
	X4/R5	3,625	177	51,966	232	10.94	8.42	14.20
2	R5/R5	2,700	1,193	31,128	640	21.49	18.75	24.64
	X4/X4	3,102	344	43,704	908	5.34	4.50	6.33
	R5/X4	1,920	755	37,379	2,850	5.16	4.57	5.82
	X4/R5	5,690	445	38,525	542	5.56	4.69	6.58
3	R5/R5	1,071	463	19,077	605	13.63	11.40	16.30
	X4/X4	991	159	22,875	758	4.84	3.81	6.16
	R5/X4	734	125	20,259	727	4.75	3.63	6.20
	X4/R5	1,598	201	18,958	578	4.13	3.31	5.15
4	R5/R5	512	227	18,046	255	31.38	24.14	40.79
	X4/X4	483	121	21,797	387	14.11	10.52	18.93
	R5/X4	434	114	22,738	439	13.61	10.09	18.34
	X4/R5	861	135	21,943	335	10.27	7.78	13.56

[a] See Table 1, footnote *a*. X4, CXCR4-tropic HIV-1 Env.
[b] See Table 1, footnote *b*.
[c] All odds ratios were significantly greater than 1 ($P < 10^{-11}$).

high-CD4-expressing cells were found to be infected by GFP and HSA viruses, respectively. In contrast, with the same stocks of viruses, 14.5 and 25.1% of the low-CD4-expressing cells were found to be infected by GFP and HSA viruses, respectively. These data indicated that CCR5-tropic HIV-1 infected the high-CD4-expressing cells more efficiently than the low-CD4-expressing cells (Fig. 2, table on the right, R5 viruses).

The different susceptibilities of the two subpopulations to HIV-1 infection are most likely caused by efficiency in virus entry. However, a previous report has described that HIV-1 postentry events were carried out significantly more efficiently in cells with higher CCR5 coreceptor density (24). To determine whether the higher efficiency of HIV-1 infection in high-CD4-expressing cells was caused by preference in the entry or postentry events, we infected the two subpopulations with VSV G-pseudotyped HIV-1. The high-CD4 cells and low-CD4 cells were infected at similar rates by GFP and HSA viruses pseudotyped with VSV G (Fig. 2, table on the right, VSV G viruses), indicating that cells with different levels of CD4 expression do not have significant differences in supporting HIV-1 replication at a postentry level. Similar results were also obtained in two independent sets of experiments. Taken together, these results indicate that primary T cells with high CD4 expression were more susceptible to HIV-1 infection because of more efficient virus entry.

Effect of coreceptor usage on HIV-1 double infection. Although HIV-1 can use many different coreceptors, the two major ones are CCR5 and CXCR4 (16). It is not known whether the use of different coreceptors can pose a barrier to the ability of the virus to recombine. If cells are less likely to be infected by a CCR5- and a CXCR4-tropic virus than by two CCR5-tropic or two CXCR4-tropic viruses, then coreceptor usage will present a barrier to the mixing of the two viral populations in individuals infected with both CCR5- and CXCR4-tropic viruses. To investigate the effect of coreceptor usage on HIV-1 double infection, we performed experiments using viruses with either CCR5- or CXCR4-tropic HIV-1 Env and primary $CD4^+$ T cells as targets of infection. Data from four independent sets of experiments with cells derived from different donors are shown in Table 3. We found that double infection also occurred more frequently than random events in CXCR4-tropic viruses, with odds ratios varying from 4.84 to 14.17, all of which were significantly greater than 1 ($P < 10^{-11}$). We also found that not only did double infection occur with both CCR5- and CXCR4-tropic viruses, it occurred more frequently than random events. Cells infected with CCR5-tropic GFP virus and CXCR4-tropic HSA virus had odds ratios of 4.75 to 13.61, whereas cells infected with CXCR4-tropic HSA virus and CCR5-tropic GFP virus had odds ratios of 4.13 to 10.94 (Table 3). All of these odds ratios were significantly greater than 1 ($P < 10^{-11}$). Similar to our previous observation for CCR5-tropic viruses, the absolute values of odds ratios varied greatly among cells from different donors. However, in all experiments, CCR5-tropic viruses yielded the highest odds

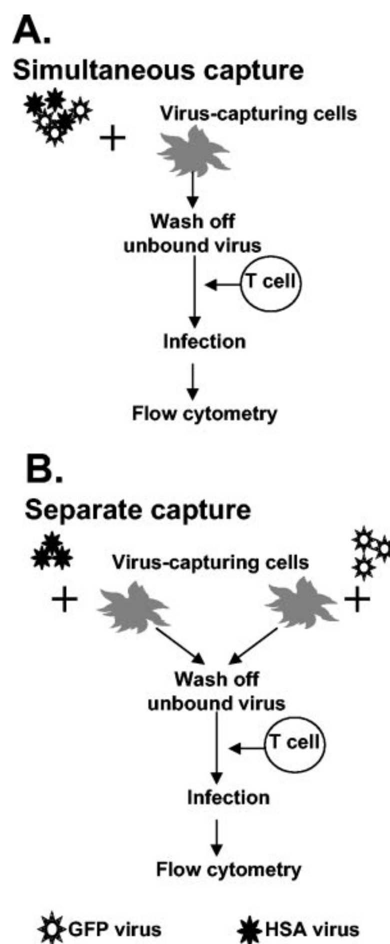

FIG. 3. Protocols used to examine the mechanisms of nonrandom double infection in the cell-mediated pathway. Virus-capturing cells were incubated either with both GFP and HSA viruses (simultaneous capture) or with one of the viruses (separate capture); after unbound viruses were removed by washing, target cells were added to allow infection to occur, and the results were analyzed by flow cytometry.

TABLE 4. Double infection of Hut/CCR5 cells through direct and cell-mediated (Thp-1/DC-SIGN) infection

Expt	Infection pathway[a]	No. of cells[b]				Odds ratio[c]	99% confidence interval	
		H^+/G^-	H^+/G^+	H^-/G^-	H^-/G^+		Lower	Upper
1	Direct (G and H)	2,297	10,540	2,737	5,893	2.13	1.96	2.32
	Thp (G and H)	973	2,432	1,518	1,234	3.07	2.68	3.53
	Thp (G)-Thp (H)	1,536	2,065	1,678	1,341	1.68	1.48	1.91
2	Direct (G and H)	4,269	11,391	6,468	9,077	1.90	1.79	2.02
	Thp (G and H)	5,099	6,979	22,081	8,421	3.59	3.39	3.80
	Thp (G)-Thp (H)	9,684	8,152	21,576	11,890	1.53	1.45	1.60
3	Direct (G and H)	3,025	7,740	4,853	6,630	1.87	1.74	2.02
	Thp (G and H)	2,147	3,364	8,171	3,230	3.96	3.63	4.33
	Thp (G)-Thp (H)	2,564	2,744	5,603	2,953	2.03	1.85	2.23

[a] Direct, direct infection; Thp, cell-mediated infection with Thp-1/DC-SIGN cells as virus- capturing cells; G, GFP virus; H, HSA virus.
[b] See Table 1, footnote *b*.
[c] All odds ratios were significantly greater than 1 ($P < 10^{-11}$).

ratios. CXCR4-tropic viruses and mixtures of CCR5- and CXCR4-tropic viruses generated similar odds ratios, but all of them were lower than those from two CCR5-tropic viruses.

Cell-mediated HIV-1 infection in Hut/CCR5 cells. Previously, we observed nonrandom double infection when HIV-1 was transmitted by the cell-mediated pathway (5). From these results we hypothesized that virus-capturing cells can capture and transmit multiple viruses to the target cells, thereby causing the high odds ratio and nonrandom infection. To test this hypothesis, we performed two groups of cell-mediated infections in parallel with viruses pseudotyped with CCR5-tropic HIV-1 envelope (Fig. 3). In the first group, a mixture of GFP virus and HSA virus was simultaneously added to the virus-capturing cells. In the second group, two sets of virus-capturing cells were used; one set was incubated with HSA virus, and the other set was incubated with GFP virus. After unbound viruses were washed off, both sets were mixed together and added to the target cells, and infection was later analyzed by flow cytometry.

We initially performed these experiments using Thp-1/DC-SIGN cells as virus-capturing cells and Hut/CCR5 as target cells. The results of three independent experiments are shown in Table 4. In all experiments, we observed significantly higher odds ratios when viruses were captured together than when viruses were captured separately, supporting our hypothesis that multiple virions were transmitted from Thp-1/DC-SIGN cells to target Hut/CCR5 cells. Consistent with our previous results, we also observed that when two viruses were captured together, cell-mediated infection exhibited higher odds ratios than those generated by direct infection.

DC-mediated HIV-1 infection in primary T cells. To further investigate the mechanisms of nonrandom HIV-1 double infection in cell-mediated pathways and to mimic conditions of infection in patients, we performed experiments by the aforementioned two protocols with primary human DCs as virus-capturing cells and primary human $CD4^+$ T cells as target cells. Data from four independent sets of experiments using viruses pseudotyped with CCR5-tropic HIV-1 envelope are summarized in Table 5. In all experiments, significantly higher odds ratios were observed when DCs simultaneously captured GFP virus and HSA virus than when different sets of DCs separately

TABLE 5. Double infection of primary $CD4^+$ T cells through primary DC-mediated and direct infection pathways

Expt	Infection pathway[a]	No. of cells[b]				Odds ratio[c]	99% confidence interval	
		H^+/G^-	H^+/G^+	H^-/G^-	H^-/G^+		Lower	Upper
1	Direct (G and H)	1,315	956	15,602	1,416	8.01	7.03	9.13
	DC (G and H)	842	800	12,557	1,103	10.82	9.30	12.58
	DC (G)-DC (H)	1,378	971	16,415	1,931	5.99	5.29	6.79
2	Direct (G and H)	600	648	17,452	1,248	15.10	12.81	17.80
	DC (G and H)	387	311	16,043	923	13.97	11.27	17.32
	DC (G)-DC (H)	481	250	16,248	1,026	8.23	6.62	10.23
3	Direct (G and H)	969	1,048	20,370	2,032	10.84	9.52	12.34
	DC (G and H)	625	1,024	23,095	1,638	23.10	19.95	26.75
	DC (G)-DC (H)	828	1,137	19,657	1,967	13.72	12.02	15.67
4	Direct (G and H)	984	308	45,179	1,216	11.63	9.67	13.98
	DC (G and H)	1,307	366	100,785	2,639	10.69	9.11	12.56
	DC (G)-DC (H)	2,075	293	119,304	3,435	4.90	4.15	5.80

[a] Direct, direct infection; DC, cell-mediated infection using human primary DCs as virus- capturing cells; G, GFP virus; H, HSA virus.
[b] See Table 1, footnote *b*.
[c] All odds ratios were significantly greater than 1 ($P < 10^{-11}$).

captured the two viruses. These results also support the capture and transmission of multiple virions from primary DCs to primary T cells, similar to the data for transmission from Thp-1/DC-SIGN to Hut/CCR5 cells. Although these data from cell-mediated infections were similar when cultured cell lines and primary cells were used, there were differences in the two cell systems. When cultured cell lines were used, the odds ratios for direct infection were always less than those for cell-mediated infection with the simultaneous capture protocol (Table 4) (5). In contrast, when primary cells were used, only two of the four independent experiments had significantly higher odds ratios for simultaneous cell-mediated infection than for direct infection. These data indicate that cell-mediated infection generates higher odds ratios than direct infection in the better-defined, more homogenous Hut/CCR5 and Thp-1/DC-SIGN cells but does not do so consistently in the primary T cells and DCs. This difference is presumably caused by the observed variation in the odds ratios of target primary $CD4^+$ T cells (Table 1, 3, and 5) and the virus-capturing primary DCs. Because each experiment used primary cells derived from different donors, it is possible that depending on the particular pools of DCs and T cells used in the experiments, cell-mediated infection may or may not generate a higher odds ratio than direct infection.

In summary, we have determined that virus entry plays an important role in nonrandom HIV-1 infection in both direct and cell-mediated infection pathways. However, double infection always occurs more frequently than expected from random events, indicating that factors other than virus entry also contribute to nonrandom HIV-1 double infection.

DISCUSSION

Nonrandom double infection observed in HIV-1 with different pseudotyped proteins. Previously, we demonstrated that HIV-1 double infection occurred at frequencies higher than expected from random events when the virions contained CCR5-tropic HIV-1 Env. In this study, we confirmed and extended results from the previous study to include virions pseudotyped with other proteins. Our results from the present study demonstrate that nonrandom double infection also occurred when viruses contained CXCR4-tropic HIV-1 Env or the pantropic VSV G. Although these results describe nonrandom double infection, they imply that HIV-1 infection is nonrandom. Therefore, these results indicate that nonrandom infection is a general phenomenon for HIV-1 and suggest that other viruses are also likely to exhibit nonrandom infection and double infection. Previously, we hypothesized that the heterogeneity in the infectibility of the cell population caused the observed nonrandom double infection. This hypothesis proposes that cells in the target cell population vary in their susceptibility to HIV-1 infection. Although the entire cell population is infected with the same dose of virus, highly infectible cells are more likely to be singly and doubly infected than less infectible cells, thereby resulting in the observed increased double infection. Results from our present study indicate that HIV-1 infection is nonrandom regardless of the pseudotyped protein and entry pathway, which lends further support to our hypothesis.

Virus entry plays an important role in nonrandom double infection. Retrovirus infection is a multistep process; variation in the cell population affecting any of the steps could influence the frequency of infection. Data from our study indicate that the entry pathway plays an important role in the nonrandom infection in the direct infection route, because altering the pseudotyped protein resulted in a different frequency of double infection. Although virions pseudotyped with any one of the three tested proteins yielded nonrandom double infection, the frequencies of double infection differed significantly when the virions were pseudotyped with specific proteins (Tables 1 to 3). Double infection occurred most frequently in primary cells when HIV-1 was pseudotyped with CCR5-using Env. Because the viruses used in different experimental groups were identical except for the envelope protein, the differences in double infection frequency had to originate from the virus entry pathway. Furthermore, double infection occurred least frequently in primary cells when one virus contained CCR5-tropic Env and the other contained VSV G. These results indicate that the entry pathways used by these two different pseudotyped viruses have different preferences in the cell population. Despite these data indicating the importance of virus entry in nonrandom double infection, other factors must also contribute to this phenomenon, which is supported by the fact that even when the two viruses used different entry pathways mediated by either HIV-1 Env or VSV G, double infection was still not random. It is likely that the physiological state of the cells affects various steps of virus infection and that multiple cellular factors and conditions affect the infectibility of target cells. For example, cells in one population could vary in the levels of deoxynucleoside triphosphate pools, thereby influencing the efficiency of reverse transcription; similarly, cells could also vary in the expression of host proteins that affect steps leading to integration, thereby influencing the efficiency of provirus formation. Further experiments are needed to probe the effects of these steps in nonrandom HIV-1 infection.

Mechanisms that generate the preference for viral entry in nonrandom double infection. The entry of HIV-1 into target cells is dictated by interactions between the viral envelope, CD4, and a coreceptor. Using engineered cell lines that express a defined amount of receptors and/or coreceptors, it has been shown that, within certain thresholds, HIV-1 entry is more efficient with cells expressing larger amounts of receptors (15, 17, 27). In this report, we have shown that activated T cells with higher CD4 expression were more susceptible to HIV-1 infection. To our knowledge, this is the first direct demonstration that the CD4 expression level in human primary T cells affects HIV-1 entry.

Although important, CD4 is unlikely to be the only factor that affects the efficiency of HIV-1 entry, because membrane fusion for HIV-1 infection is a cooperative process that involves multiple coreceptor molecules in addition to CD4 (18, 20). Other factors, such as coreceptor expression and the distributions of CD4 and coreceptor on the cell membrane, are likely to also play important roles in virus entry. T cells have considerable variation in receptor and coreceptor expression. The CD4 density on $CD4^+$ peripheral blood mononuclear cells is estimated to be than 10^5 molecules per cell (22); among $CD4^+$ cells, the CCR5 density varies from undetectable to 10^4 molecules per cell. The CXCR4 density in T cells varies depending on the subset of cells, but it can range from undetectable to 5×10^3 molecules per cell (21). These variations pro-

vide a basis for the heterogeneity of infectibility within the target cell population, thereby contributing to nonrandom double infection. Compared with CXCR4-tropic virus, CCR5-tropic HIV-1 generated more frequent double infection; we propose that this difference in the frequency of double infection reflects the higher heterogeneity in CCR5 expression than in CXCR4 expression on $CD4^+$ cells.

In our previous study, we demonstrated that in cell-mediated infection, double infection occurred at frequencies higher than expected from random events. In the present study, we found that the frequency of double infection increased significantly with a simultaneous capture compared to with a separate capture protocol in cell-mediated infection (Fig. 3). These data support our hypothesis that multiple viruses are captured and transmitted by each DC to T cells. Interestingly, we observed nonrandom double infection even with the separate capture protocol (Tables 4 and 5). There are several possible explanations for this observation: more than one DC can interact with each T cell, some of the viruses captured by a DC may be released and infect a T cell through direct infection or may be recaptured by another DC, or different DCs may exchange captured virions through an unknown mechanism. We have proposed that the heterogeneity of infectibility in the target cell population contributes to nonrandom infection. Because cell-mediated infection also involves cell surface molecules, it is very likely that variation in the target cell population also plays a role in nonrandom double infection via the cell-mediated infection pathway.

Implications for HIV-1 evolution and pathogenesis. HIV-1 exhibits rapid evolution during infection. At an early stage of infection, HIV-1 isolates tend to use CCR5 for viral entry. During later stages of infection, CXCR4-tropic viruses emerge and coexist with CCR5-tropic viruses; therefore, both types of viruses play important roles in HIV-1 infection and pathogenesis. We have now established that double infection occurs more frequently than expected from random events in both CXCR4- and CCR5-tropic HIV-1. Furthermore, double infection with the two viruses using different coreceptors can occur frequently. These results suggest that in infected individuals, frequent double infection can occur with CCR5- and/or CXCR4-tropic viruses, thereby generating opportunities for recombination to occur within viral populations.

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