JOURNAL OF VIROLOGY, Dec. 1996, p. 8355–8360 Vol. 70, No. 12
0022-538X/96/$04.00+0

Primary, Syncytium-Inducing Human Immunodeficiency Virus Type 1 Isolates Are Dual-Tropic and Most Can Use Either Lestr or CCR5 as Coreceptors for Virus Entry

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Received 1 July 1996/Accepted 13 August 1996

A panel of primary syncytium-inducing (SI) human immunodeficiency virus type 1 isolates that infected several CD4$^+$ T-cell lines, including MT-2 and C8166, were tested for infection of blood-derived macrophages. Infectivity titers for C8166 cells and macrophages demonstrated that primary SI strains infected macrophages much more efficiently than T-cell line-adapted HIV-1 strains such as LAI and RF. These primary SI strains were therefore dual-tropic. Nine biological clones of two SI strains, prepared by limiting dilution, had macrophage/C8166 infectivity ratios similar to those of their parental viruses, indicating that the dual-tropic phenotype was not due to a mixture of non-SI/macrophage-tropic and SI/T-cell tropic viruses. We tested whether the primary SI strains used either Lestr (fusin) or CCR5 as coreceptors. Infection of cat CCC/CD4 cells transiently expressing Lestr supported infection by T-cell line-adapted strains including LAI, whereas CCC/CD4 cells expressing CCR5 were sensitive to primary non-SI strains as well as to the molecularly cloned strains SF-162 and JR-CSF. Several primary SI strains, as well as the molecularly cloned dual-tropic viruses 89.6 and GUN-1, infected both Lestr$^+$ and CCR5$^+$ CCC/CD4 cells. Thus, these viruses can choose between Lestr and CCR5 for entry into cells. Interestingly, some dual-tropic primary SI strains that infected Lestr$^+$ cells failed to infect CCR5$^+$ cells, suggesting that these viruses may use an alternative coreceptor for infection of macrophages. Alternatively, CCR5 may be processed or presented differently on cat cells so that entry of some primary SI strains but not others is affected.

Human immunodeficiency virus type 1 (HIV-1) and HIV-2 strains enter cells by interacting with both CD4 and a coreceptor at the cell surface. Coreceptors are determinants of virus tropism so that viruses with distinct tropisms use different coreceptors during entry. Recently Lestr (also called fusin) (18, 20, 21, 24, 31), a member of a large family of G-protein-coupled receptors with seven-transmembrane domains including the more closely related CC and CXC chemokine receptors (4, 13, 30, 34–36), has been shown to be a coreceptor for T-cell line-adapted HIV-1 strains (19). Several studies have since shown that CCR5 acts as a coreceptor for macrophage-tropic non-syncytium-inducing (NSI) strains (1, 15, 17, 42), although at least some NSI strains can use either CCR3 or CCR5 (6). Furthermore, we and others have shown that individual virus strains can be promiscuous, using one of several compatible coreceptors (3, 16).

The majority of HIV-1 isolates from infected asymptomatic patients display an NSI phenotype and generally fail to infect most established CD4$^+$ T-cell lines (2). Primary HIV-1 strains that can infect and induce syncytia in T-cell lines can, however, be isolated from many patients with AIDS (14, 39). Little is known about how this property influences pathogenesis, transmission, or tropism in vivo. Individuals that yield such strains, however, are more likely to suffer a faster disease progression (14). In vitro, HIV-1 isolates passaged in and adapted to human CD4$^+$ T-cell lines are likely to evolve into syncytium-inducing (SI) strains even if they are NSI to begin with. Such adapted viruses usually infect primary macrophages inefficiently, although some that are more proficient for macrophage infection have been termed dual-tropic (12, 26, 37). It is less clear whether primary strains that can infect T-cell lines in vitro have concomitantly evolved a reduced tropism for macrophages. Valentin et al., however, showed that all primary isolates that they tested, as well as T-cell line-adapted LAI, infected macrophages at least to some extent (40). Here, we show that a panel of SI primary isolates infect primary human macrophages cultures more efficiently than T-cell line-adapted HIV-1 strains. Furthermore, several dual-tropic SI strains were able to use either Lestr or CCR5 as coreceptors, in contrast to T-cell line-adapted strains, which infected Lestr$^+$ cells but not CCR5$^+$ cells.

MATERIALS AND METHODS

Cells. Macrophages were prepared from blood monocytes by plastic adherence as previously described (37). The purity of macrophage preparations was tested at the time of infection. Over 99% of cells in macrophage preparations stained positive with an anti-CD14 monoclonal antibody (Sigma). Peripheral blood mononuclear cells (PBMCs) were stimulated for 2 days with phytohemagglutinin (0.5 μg/ml) and then cultured in medium with interleukin-2 (20 U/ml). CCC S+L− cells stably expressing recombinant human CD4 have been described previously (7, 25). CD4$^+$ human T-cell lines used were MT-2 cells (28), which are indicator cells for SI strains (22), and C8166 cells (9), which are used to propagate and titrate T-cell line-adapted HIV-1 strains.

Viruses. LAI (41) and RF (33) are T-cell line-adapted HIV-1 strains. GUN-1 is also T-cell line adapted but dual-tropic (26, 37). 89.6 is an HIV-1 strain that infects macrophages but can infect and induce syncytia in certain CD4$^+$ T-cell lines and is therefore also dual-tropic (12). SF-162 is an NSI/macrophage-tropic strain of HIV-1 (5), while JR-CSF is also NSI yet infects macrophages less efficiently

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TABLE 1. Infection of the C8166 T-cell line and primary cultures of PBMCs or macrophages by primary HIV-1 isolates

Virus strain	SI status	Infectivity titer ($TCID_{50}$/ml)			Ratio, macrophage infectivity/C8166 cell infectivity
		PBMCs	Macrophages	C8166 cells	
Primary					
2005	SI	NT[a]	4.6×10^4	1.3×10^5	0.4
2006	SI	1.3×10^3	4.6×10^3	1.3×10^2	35
2028	SI	6.3×10^4	4.4×10^3	2.9×10^3	1.5
2029	SI	6.3×10^3	5.3×10^3	2.9×10^4	0.2
2036	SI	6.3×10^3	6.4×10^2	2.9×10^4	0.02
2044	SI	3.0×10^4	6.9×10^3	6.3×10^4	0.1
2053	SI	6.3×10^3	3.0×10^1	2.9×10^2	0.1
2075	SI	1.3×10^3	1.5×10^3	1.3×10^3	1.2
2076	SI	3.0×10^5	7.4×10^4	1.3×10^5	0.6
3028	SI	1.3×10^3	2.1×10^3	2.9×10^2	7.2
M13	SI	2.0×10^3	8.5×10^2	1.3×10^2	6.5
89.6	SI	5.0×10^3	6.3×10^3	5.0×10^4	0.1
E80	NSI	2.0×10^4	2.5×10^4	$<1.3 \times 10^1$	$>1.9 \times 10^3$
M23	NSI	1.3×10^1	1.4×10^4	$<1.3 \times 10^1$	$>1.1 \times 10^3$
SL-2	NSI	3.0×10^2	1.0×10^3	$<1.3 \times 10^1$	>77
SL-3	NSI	2.9×10^1	5.0×10^2	$<1.3 \times 10^1$	>38
SL-4	NSI	1.3×10^1	5.0×10^2	$<1.3 \times 10^1$	>38
SF162	NSI	2.0×10^3	1.8×10^5	$<1.3 \times 10^1$	$>1.3 \times 10^4$
T-cell line adapted					
GUN-1	SI	NT	6.0×10^4	4.6×10^7	0.001
RF	SI	6.8×10^5	2.0×10^2	6.4×10^8	3.3×10^{-7}
LAI	SI	2.0×10^5	5.0×10^1	6.4×10^7	7.8×10^{-7}

[a] NT, not tested.

than SF-162 (23). Primary strains M13, M23, E80, SL-2, SL-3, and SL-4 were isolated at St. Mary's Hospital, London, England. SL-2, SL-3, and SL-4 were from asymptomatic patients from Thailand. M13, M23, and E80 were from British patients attending the St. Mary's Hospital STD clinic. Isolates 2005, 2006, 2028, 2029, 2036, 2044, 2053, 2075, 2076, and 3028 were made at Addenbrooke's Hospital, Cambridge, England, from patients whose $CD4^+$ blood cell counts were less than 190 cells mm^{-3} except for the patients from whom isolates 2076 and 3028 were derived; these individuals had CD4 counts of 400 and 328, respectively. Isolates 2005, 2029, and 2036 were from patients with AIDS. Isolates made in Cambridge were from patients registered in Birmingham (2006), Hull (2005 and 2075), London (2036, 2044, 2053, and 2076), and Portsmouth (2028 and 2029), England, and Uganda (3028). All isolates were of subtype B except for 3028, M13, M23, and E80, which were unclassified. Isolates were cultured from phytohemagglutinin–interleukin-2-stimulated PBMCs derived from the peripheral blood of infected individuals. Freshly cultured PBMCs from HIV-negative donors were added every 4 to 8 days. Supernatants were harvested twice weekly and screened for p24 antigen. Positive supernatants were aliquoted and stored in liquid nitrogen. All primary strains were minimally passaged in PBMCs to prepare virus stocks.

Biological clones of viruses were made by limiting dilution. Fivefold dilutions of virus supernatant were added to PBMC cultures on 96-well trays. Virus was rescued and propagated from $p24^+$ wells, of which less than 10% of wells per tray were positive. Plasmid DNA containing molecularly cloned virus was transfected directly into either PBMCs or C8166 cells by using Lipofectamine (Gibco BRL) as indicated by the manufacturer before preparation of virus stocks.

Infectivity assays. Macrophages and PBMCs were infected with appropriate HIV-1 strains 5 to 6 days after establishment as cultures. Macrophages were exposed to half-log dilutions of virus for 2 h, washed once, and cultured in RPMI 1640 containing 10% human serum and 5% fetal calf serum for 21 days. Each virus strain was tested for infection of three batches macrophages prepared from separate donors. PBMCs and C8166 cells were adhered to plastic with poly-L-lysine for 1 h, infected for 2 h, washed, and overlaid with growth medium containing low-viscosity (3 mg/ml) and high-viscosity (3 mg/ml) carboxymethylcellulose. C8166 cells were cultured for 7 days and PBMCs were cultured for 9 days before immunostaining for p24 antigen as previously described (8).

Transient expression of Lestr and CCR5 on CCC/CD4 cells. Three and a half micrograms of plasmid pcDNA3.1 plasmid (Invitrogen) containing cDNA clones of either Lestr and CCR5 was transfected into CCC/CD4 cat cells, using Lipofectamine as described above. A total of 7.5×10^4 transfected cells were challenged for virus infection 48 h later.

RESULTS

Infectivity of primary SI, NSI, and T-cell line-adapted HIV-1 strains for $CD4^+$ T-cell lines and for PBMC and macrophage cultures. Eleven primary SI and five NSI HIV-1 isolates were assayed for infection of the $CD4^+$ T-cell line C8166 as well as primary PBMC and macrophage cell cultures. T-cell line-adapted strains RF and LAI were tested for comparison, as was the T-cell line-passaged dual-tropic strain GUN-1. SF-162 and 89.6 were also included as well-characterized, molecularly cloned NSI/macrophage-tropic and SI/dual-tropic strains, respectively (5, 12). All primary SI and T-cell line-adapted SI strains replicated and induced syncytia in MT-2 cells, whereas none of the NSI strains did (data not shown). Table 1 shows virus infectivity titers (50% tissue culture infective doses [$TCID_{50}$]) for each cell type. Infectivity titers on primary macrophages were compared with those on C8166 cells and expressed as a ratio for each virus strain tested. T-cell line-adapted strains RF and LAI infected PBMCs and C8166 cells to high titers but did not to infect macrophages efficiently. Macrophage/C8166 infectivity ratios for these viruses are therefore very low (3.3×10^{-7} to 7.8×10^{-7}). All primary SI isolates infected PBMCs and C8166 cells and showed variation in the capacity to infect macrophages, as illustrated by their macrophage/C8166 infectivity ratios, which varied from 0.02 to 35. However, all ratios for primary SI viruses were several orders of magnitude higher than ratios for T-cell line-adapted viruses. Thus, primary SI strains are consistently more efficient for macrophage infection than T-cell line-adapted viruses. Some isolates, e.g., 2005 and 2076, infect both C8166 cells and macrophages efficiently and are thus dual-tropic, whereas others, e.g., 2053, infect macrophages only to levels similar to those of LAI (Table 1). Such strains, however, are more macrophage-tropic than LAI since they yield lower titers on PBMCs and C8166 cells. Each of the six NSI strains, including SF-162, replicated more efficiently in macrophages than in PBMCs. The differences between primary SI and T-cell line-adapted SI strains was also apparent when we plotted macrophage/PBMC infectivity ratios (Fig. 1). Such ratios for four of six primary NSI strains were highest, suggesting that these

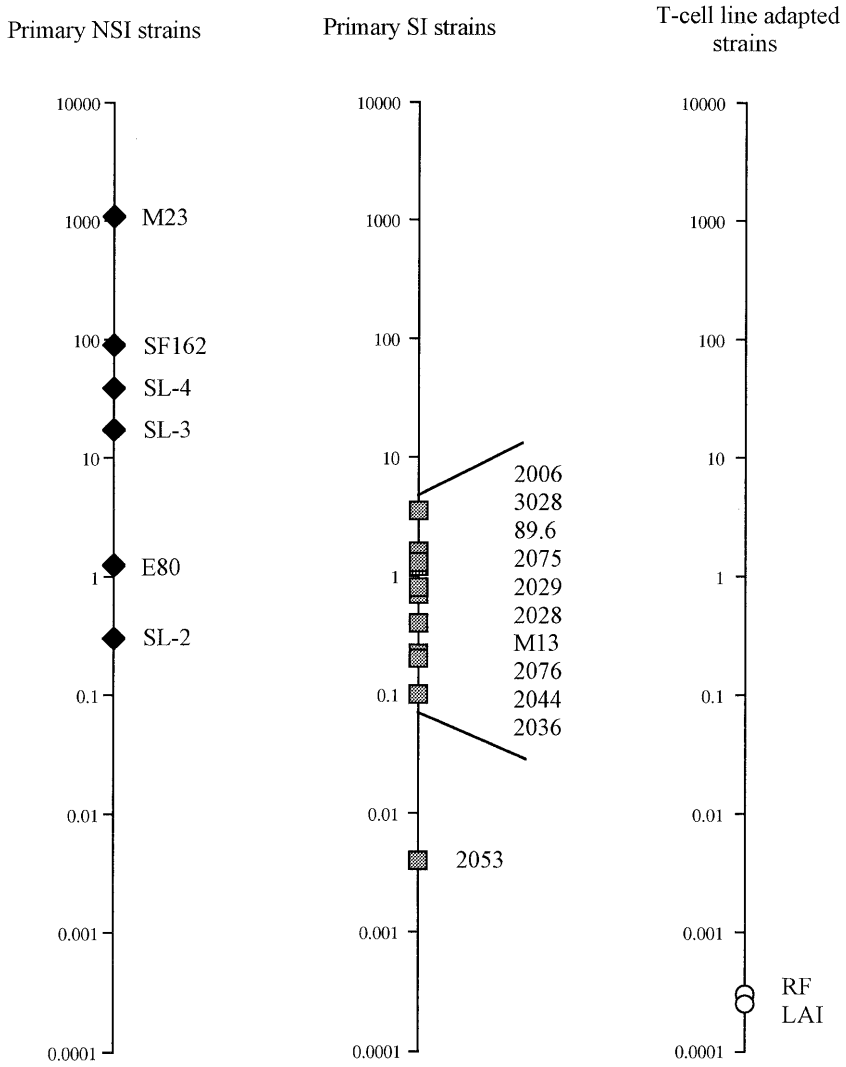

FIG. 1. Macrophage/PBMC infectivity ratios for primary and T-cell line-adapted HIV-1 strains. HIV-1 infectivity titers for primary macrophage and PBMC cultures (taken from Table 1) were used to calculate macrophage PBMC infectivity ratios, which are plotted for each HIV-1 isolate used.

strains were the most tropic for macrophages (however, see Discussion).

Biological and molecular clones of primary SI strains are dual-tropic. As the dual-tropism of some of the primary SI isolates could be due to a mixture of viruses with different phenotypes within a single isolate, two of the dual-tropic isolates were biologically cloned by limiting dilution (see Materials and Methods). Table 2 shows the titers for five clones of 2076 and four clones of 2005. In all cases, the cloned viruses retained the parental dual-tropic phenotype, strongly suggesting that their dual-tropic phenotype does not result from a mixture of macrophage-tropic NSI and T-cell tropic SI viruses. It therefore seems likely that the dual-tropic/SI phenotypes of 89.6 and GUN-1 viruses, which are derived from molecular DNA clones, are more representative of primary SI isolates than are T-cell line-adapted LAI and RF.

Coreceptors used by primary SI viruses. Recently Lestr (19) and the chemokine receptor CCR5 (1, 15, 17), members of the extensive family of seven-transmembrane, G-protein-coupled receptors, have been identified as coreceptors for SI, T-cell line-adapted HIV-1 strains and NSI, macrophage-tropic viruses, respectively. We tested whether either of these corecep-

TABLE 2. Infectivity of biological clones made from HIV-1 primary SI isolates

Virus isolate	Infectivity titer ($TCID_{50}$/ml)		Ratio, macrophage infectivity/C8166 cell infectivity
	Macrophages	C8166 cells	
2076			
Parental	7.4×10^4	1.3×10^5	0.6
Clones			
1	8.0×10^4	3.6×10^5	0.2
2	8.0×10^3	2.6×10^3	3.0
3	5.0×10^3	8.0×10^3	0.6
4	1.1×10^3	3.6×10^3	0.3
5	3.6×10^4	1.6×10^5	0.2
2005			
Parental	4.6×10^4	1.3×10^5	0.4
Clones			
1	1.6×10^3	5.0×10^4	0.03
2	1.1×10^3	3.6×10^3	0.3
3	2.6×10^2	1.6×10^3	0.16
4	1.1×10^3	8.0×10^3	0.14

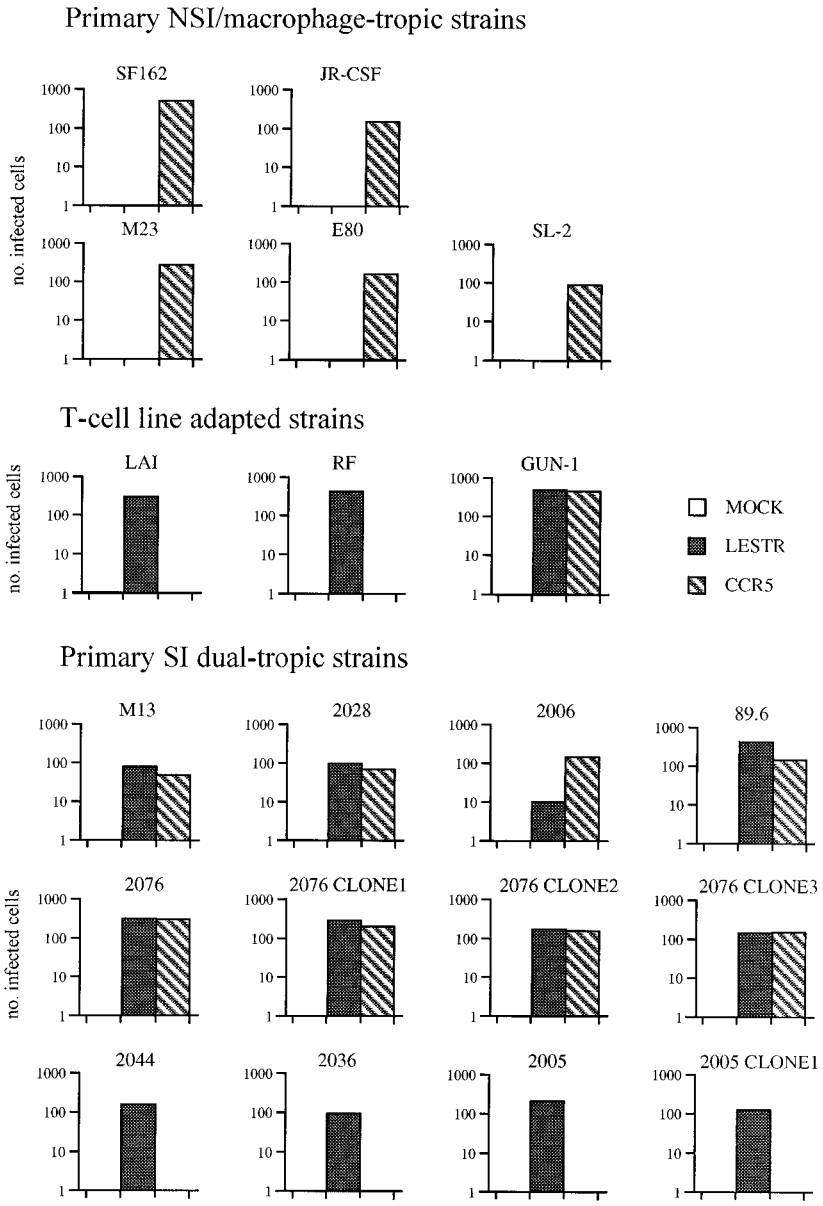

FIG. 2. Infection of CCC/CD4 cat cells expressing either Lestr or CCR5 by primary NSI and SI HIV-1 strains. Two hundred to 1,000 $TCID_{50}$ for PBMC or macrophages (whatever was highest) was added to 7.5×10^4 CCC/CD4 cells expressing either Lestr or CCR5. The number of infected cells, estimated following 4 days culture and immunostaining for p24, is shown for each transfectant. Untransfected CCC/CD4 cells were resistant to infection by all strains tested.

tors functioned for primary dual-tropic SI viruses. Lestr or CCR5, cloned into the expression plasmid pcDNA3.1, was transiently expressed in CCC/CD4 cells following transfection of plasmid DNA by Lipofectamine. CCC/CD4 cells are resistant to HIV-1 but permissive to entry and productive replication by several T-cell line-adapted HIV-2 strains (7, 25). Forty-eight hours after transfection, the CCC/CD4 cells were challenged with seven primary SI viruses and three primary NSI viruses as well as by standard T-cell line-adapted viruses and viruses derived from molecular clones (NSI strain JR-CSF [23], NSI/macrophage-tropic strain SF-162 [5], and the dual-tropic SI strains 89.6 [12] and GUN-1 [38]). After 4 days of incubation, cells were fixed and stained for p24 antigen as described in Materials and Methods. Figure 2 shows that T-cell line-adapted viruses LAI and RF infected only $Lestr^+$ CCC/CD4 cells, whereas primary NSI viruses M23, E80, SL-2, and SF-162 infected only $CCR5^+$ CCC/CD4 cells. In contrast, four of the primary dual-tropic SI viruses, 2006, M13, 2028, and 2076 (as well as three biological clones of 2076), infected both $Lestr^+$ and $CCR5^+$ CCC/CD4 cells. Furthermore, the two dual-tropic viruses derived from molecular DNA clones (GUN-1 and 89.6) also infected both $Lestr^+$ and $CCR5^+$ CCC/CD4 cells. Thus, these primary SI strains of HIV-1 are dual-tropic and can use either Lestr or CCR5 as coreceptors. Interestingly, three other primary SI strains (2044, 2036, and 2005), each of which infected macrophage cultures (Table 1), infected only $Lestr^+$ CCC/CD4 cells and not $CCR5^+$ CCC/CD4 cells.

DISCUSSION

HIV-1 strains that have been passaged extensively in $CD4^+$ T-cell lines, e.g., LAI and RF, usually infect primary macro-

phage cultures inefficiently (11, 37). Here, we tested whether macrophage infection by primary SI isolates was comparable to that of T-cell line-adapted strains, i.e., whether the SI phenotype correlated with inefficient infection of macrophages. Our results indicate that macrophage infection by primary SI strains is highly variable but that all strains tested infected macrophages more efficiently than the T-cell line-adapted strains LAI and RF. Macrophage infection by the six NSI strains tested was also highly variable (Table 1), yet in contrast to the SI strains, these viruses consistently infected macrophages more efficiently than PBMC cultures (Fig. 1). Our study therefore suggests that efficiency of macrophage replication follows the order primary NSI > primary SI >> T-cell line-adapted SI strains. Yet, it is likely that the titers for NSI/macrophage-tropic viruses recorded on PBMC cultures are artificially low as a result of inhibitory chemokines, e.g., RANTES, produced by lymphocytes (10, 17, 32), thus overestimating the macrophage-tropism of such strains.

The strain differences reported above are attributable at least in part to differences in coreceptor usage. We transiently expressed either Lestr or CCR5 on CCC/CD4 cat cells and tested for infection by representative NSI and SI primary strains. CCC/CD4 cells resist HIV-1 infection but are fully permissive to productive replication by several HIV-2 strains (25). Our results show clearly that dual-tropic viruses derived from DNA molecular clones as well as several primary SI viruses (M13, 2028, 2006, and 2076) use either Lestr or CCR5. In contrast, T-cell line-adapted HIV-1 strains infected $Lestr^+$ cells only and NSI strains infected $CCR5^+$ cells only, as previously reported (1, 15, 17, 19). Some primary SI strains (2005, 2036, and 2044) that infected macrophages (Table 1) infected $Lestr^+$ CCC/CD4 cells but not $CCR5^+$ CCC/CD4 cells. Presumably these strains use a coreceptor different than CCR5 for infection of macrophages. However, at this stage we cannot exclude the possibility that CCR5 is differently presented or processed on CCC/CD4 cells so that entry of some strains but not others is affected.

We were concerned that the dual-tropic phenotype of primary SI strains could be caused by a mixture of NSI/macrophage-tropic and SI/T-cell tropic strains in the original isolate. However, biological clones prepared from two primary SI strains each maintained a dual-tropic phenotype for macrophages and T-cell lines (Table 1). Clones derived from isolate 2076 infected both $Lestr^+$ and $CCR5^+$ CCC/CD4 cells, whereas clones derived from 2005 infected only $Lestr^+$ CCC/CD4 cells. Thus, each biological clone retained the phenotype of the parental virus. These observations, including infection of either $Lestr^+$ or $CCR5^+$ CCC/CD4 cells by the 2076 subclones and by the GUN-1 and 89.6 molecular clones, show that at least some HIV-1 strains can use more than one coreceptor and can choose between compatible coreceptors for entry. Indeed, recently we demonstrated that the HIV-2_{ROD} envelope glycoproteins could use a number of CC and CXC chemokine receptors to induce cell-cell fusion of $CD4^+$ cells, although only some supported efficient cell-free virus entry (3).

The difference between T-cell line-adapted strains and primary SI strains described above is likely to be caused by selection against CCR5 use by extensive in vitro passage in $Lestr^+$ $CCR5^-$ T-cell lines. Most $CD4^+$ T-cell lines used for in vitro passage of HIV resist infection by primary NSI strains and are thus likely to express lower levels of CCR5 or no CCR5 (35). Extensive passage of SI strains in such lines is therefore likely to select for more efficient use of Lestr against CCR5 for virus entry.

Our results show conclusively that Lestr can also act as a coreceptor for primary SI viruses. Every primary SI virus tested infected $Lestr^+$ CCC/CD4 cells. Lestr is thus likely to play an important role in HIV-1 pathogenesis and in the rapid decline of $CD4^+$ T-cells that occurs after emergence of SI strains in vivo. SI strains can be isolated from only about 50% of patients with AIDS, and it will therefore be interesting to investigate whether a change in coreceptor use might also occur for rapid/high NSI viruses prevalent in these patients (14).

The discovery of the nature of coreceptors for HIV-1 entry opens up the possibility of designing inhibitors to block their interaction with HIV envelope glycoproteins. RANTES, MIP-1α, and MIP-1β all bind to CCR5 and block entry of NSI/macrophage-tropic HIV-1 strains (10). Furthermore, lymphocytes from multiply exposed yet HIV-seronegative individuals produce high levels of these chemokines (32). RANTES, MIP-1α, and MIP-1β, however, are not specific for CCR5 and bind other chemokine receptors too (4, 13, 30, 34, 36). Recently, we reported that a monoclonal antibody (12G5) that recognizes Lestr but not other chemokine receptors blocked fusion of T-cell line-adapted HIV-1 strains with some $Lestr^+$ cells but not others (27). Either alternative unidentified coreceptors allowed escape from 12G5 or Lestr might be processed or presented differently to enable escape from 12G5 blocking. Whatever the reason, these observations indicate that design of inhibitors directed to specific chemokine or coreceptor molecules for HIV inhibition is a new challenge.

ACKNOWLEDGMENTS

We thank Aine McKnight, Laurent Picard, Simon Talbot, Sandra Potempa, Yasu Takeuchi (Chester Beatty Laboratories, London), Romke Bron, P. J. Klasse, Mark Marsh (LMCB, UCL, London), and Jim Hoxie (University of Pennsylvania, Philadelphia) for valuable discussion. We are grateful to Yasu Takeuchi (Chester Beatty Laboratories), and Nobu Shimizu and Hiroo Hoshino (both at the Gunma University Medical School, Japan), who kindly provided HIV-1 GUN-1, and to Ronald Collman (University of Pennsylvania, Philadelphia) for the 89.6 strain. Many of the primary HIV-1 isolates used here and provided by Addenbrooke's Hospital were made from blood samples collected by members of the UK HIV molecular epidemiology group: P. Dore (Kingston General Hospital, Hull), M. Johnson (Royal Free Hospital, London), M. Shamanesh (General Hospital, Birmingham), J. Tobin (St. Mary's Hospital, Portsmouth), and R. Young (Uganda Virus Research Institute, Entebbe, Uganda). We also thank the Medical Research Council (MRC) AIDS Reagent Project for supply of many reagents used in this study, which itself was supported by the MRC.

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