PBLs (1×10^6) were added to NP-2/CD4/GPCR cells and cultured for 1 week. The coculture was continued with cell passage at 1 : 3 dilution every 3 days. NP-2/CD4/GPCR cells infected with HIV-1 were detected by indirect immunofluorescence assay (IFA) using sera of HIV-1-positive patients as the first antibody. Infection of these NP-2/CD4/GPCR cells with HIV-1 was checked at every cell passage. Supernatants of cells determined to be infected with HIV-1 by IFA were collected and stored at −80°C as preparations of primary HIV-1 isolates. Reverse transcriptase activities of the supernatants were measured [27]. HIV-1 proviral DNA was detected by PCR using *gag* primers as follows: GAGN, 5′-AGTGGGGGGAC-ATCAAGCAGCCATGC-3′ (sense, 570th–595th in *gag* gene of NL4-3 strain) (U26942); GAGR, 5′-TTTGG-TCCTTGTCTTATGTCCAGAAT-3′ (antisense, 855th–869th). PBLs were obtained from HIV-1-positive patients after obtaining their informed consent.

Infection assay

NP-2/CD4/GPCR cells (5×10^4) were seeded into 24-well culture plates 24 h prior to viral inoculation. These cells were exposed to an amount of primary HIV-1 isolates corresponding to 1×10^4 counts per minute (cpm) of reverse transcriptase activity. After a 2-h incubation, the cells were washed three times with E-MEM containing 10% FCS to remove uninfected HIV-1 and then cultured in 500 μl of fresh medium at 37°C. The cells were passaged every 2 days and cells infected with HIV-1 were detected by IFA.

Amino acid sequence analyses of the V3 domain

DNA fragments of the *env* gene coding the V3 region of HIV-1 proviruses were amplified by PCR using cellular DNA extracted from PBLs and NP-2/CD4/GPCR cells infected with HIV-1. PCR primers with nucleotide sequences in C2 and C3 regions of the *env* gene (Sigma-Aldrich K. K.) were constructed according to the HIV-1 NL4-3 strain as follows: HIVV3SEQC2N, 5′-CAATGCTAAAACCATAATAGTAC-3′ (831st–853rd) and HIVV3SEQC3R, 5′-GTAGAAAAATTCCCCTC-CACAAT-3′ (1124th–1146th). Amplified DNA fragments were cloned into the TA-cloning plasmid pDrive and their nucleotide sequences were determined.

Ethics committee approval

The present study was performed with the approval of the Ethics Committee of Gunma University Graduate School of Medicine.

Results

G protein-coupled receptor expression in NP-2/CD4/G protein-coupled receptor cells

Most HIV-1 coreceptors and CKRs have tyrosines in their extracellular amino-terminal regions (nontranslated regions; NTRs) [28]; however, coreceptor activity was not detected in CCR4, CCR6, CCR7, CCR9B, CCR10, CCR11, XCR1, and DARC. To clarify the usage spectrum of GPCRs, including these CKRs by HIV-1 populations *in vivo*, we made NP-2/CD4 cells expressing each of 21 CKRs or two GPCRs, FPRL1 or GPR1 (i.e. NP-2/CD4/GPCR cells) in the same protocol. The expression of each GPCR in NP-2/CD4/GPCR cells was confirmed by RT-PCR (Fig. 1a). None of the mRNA of GPCRs examined in the present study could be detected in the parental cell line, NP-2/CD4 (Fig. 1b).

Detection of HIV-1 proviral DNA in peripheral blood lymphocytes

PBLs were isolated from whole blood samples of 17 HIV-1-positive patients. The patients consisted of eight individuals before highly-active antiretroviral therapy (HAART), four interrupted HAART, and five on HAART (Table 1). The copy numbers of HIV-1 RNA ranged from 71 to 151 000 per ml of plasma. The numbers of CD4-positive T cell ranged from five to 522 per ml. The *gag* gene DNA was detected in PBLs of all patients by PCR (Fig. 1c). Intensities of DNA bands were approximately correlated with the copy numbers of HIV-1 RNA in the plasma of each blood sample. These results indicate that all PBLs contained cells infected with HIV-1.

HIV-1 isolation from peripheral blood lymphocytes

We cocultured NP-2/CD4/GPCR cells with HIV-1-positive PBLs for 4 weeks. As shown in Fig. 2, HIV-1-positive cells were clearly detected by IFA in NP-2/CD4/CCR5 cells cocultured with 05JAGU-#01, #04, #07, #08, #11, #12, #14, #15, and #16 PBLs; in NP-2/CD4/CXCR4 cells with 05JAGU-#02, #07, #11, #12, and #15 PBLs; in NP-2/CD4/FPRL1 cells with 05JAGU-#06 and #15 PBLs; and in NP-2/CD4/GPR1 cells with 05JAGU-#05, #06, #07, #11, and #15 PBLs; however, the ratios of HIV-1-positive cells were less than 10% in NP-2/CD4/CXCR4 cells with 05JAGU-#02 PBLs; NP-2/CD4/FPRL1 cells with 05JAGU-#06; and in NP-2/CD4/GPR1 cells with 05JAGU-#05 and 05JAGU-#07 PBLs.

Levels of reverse transcriptase activities in coculture supernatants were approximately correlated with the levels of HIV-1-antigen-positive cells by IFA (Table 1). As expected, reverse transcriptase activities could not be detected in the supernatant of 05JAGU-#06 sample, in which efficient HIV-1 propagation could not be detected during the 4-week coculture. Higher reverse transcriptase activities were detected in the supernatants of NP-2/CD4/CCR5 cells cocultured with 05JAGU-#01, #07, #08, #11, and #12 PBLs; NP-2/CD4/CXCR4 cells with 05JAGU-#11, #12, and #15 PBLs; and NP-2/CD4/FPRL1 cells with 05JAGU-#15 PBLs. These supernatants were recovered as primary HIV-1 isolates.

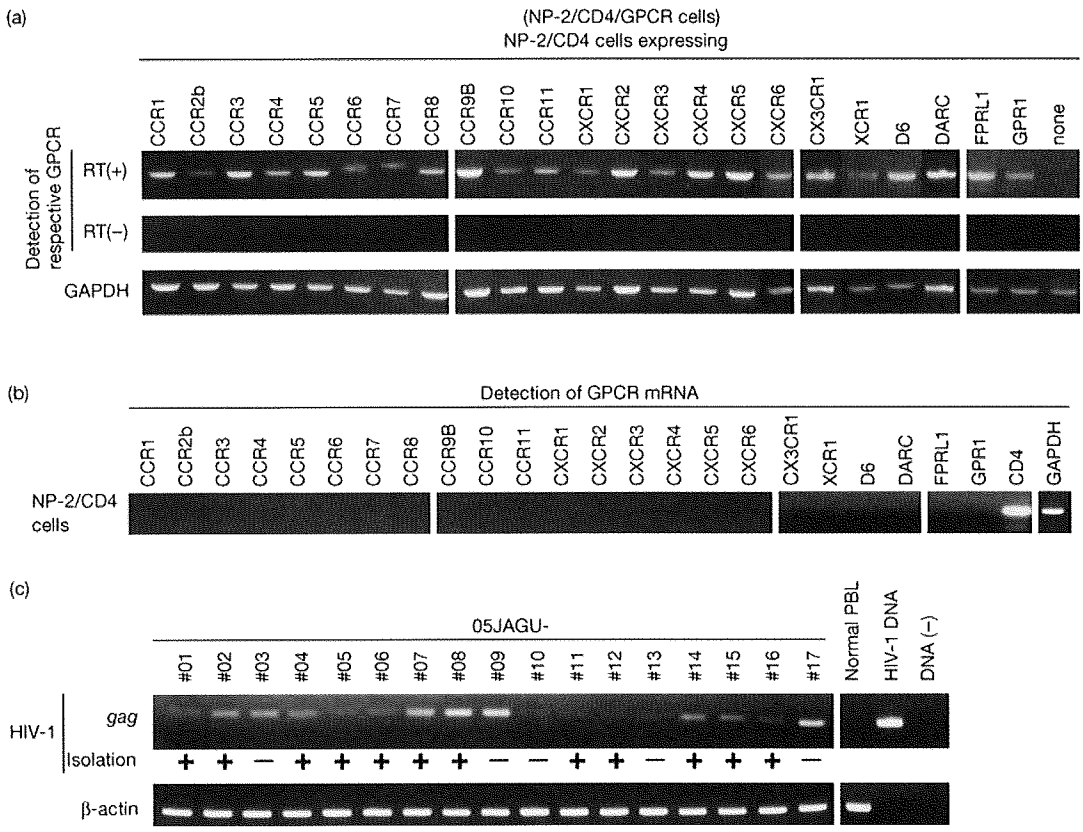

Fig. 1. Detection of G protein-coupled receptor mRNA expression in NP-2/CD4/G protein-coupled receptor cells and HIV-1 proviral DNA in HIV-1-positive peripheral blood lymphocytes. (a) The expression of respective G protein-coupled receptors (GPCRs) in NP-2/CD4 cells expressing each one of 23 GPCRs (NP-2/CD4/GPCR cells) was detected by reverse transcriptase-PCR. RNA preparations from NP-2/CD4/GPCR cells were applied to the same PCR to check for DNA contamination. (b) The mRNA expression of 23 GPCRs was examined in NP-2/CD4 cells as a control. Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) or CD4 mRNA was also detected by reverse transcriptase-PCR. (c) HIV-1 proviral DNA was detected by PCR amplifying the *gag* gene. Total cellular DNA was extracted from an equal amount of peripheral blood lymphocytes (PBLs) and used as a template. DNA fragments of 299 nucleotides were amplified if PBLs had been infected with HIV-1. Results of HIV-1 isolation by coculture shown in Fig. 2 are also indicated: +, isolated; −, failed. Amplified DNA was electrophoresed by 1% (w/v) agarose gel electrophoresis.

HIV-1 infection was not detected in NP-2/CD4/GPCR cells cocultured with 05JAGU-#03, #09, #10, #13, and #17 PBLs. Thus, HIV-1 isolation was performed in 11 of 17 PBLs.

Coreceptor usage of primary HIV-1 isolates

Next, we examined the coreceptor usage of primary HIV-1 isolates obtained by coculture. As shown in Table 2, the primary HIV-1 isolate produced by NP-2/CD4/CCR5 cells cocultured with 05JAGU-#14 PBL (i.e. HIV-#14/CCR5) used CXCR4 in addition to CCR5. Similarly, HIV-1 produced by NP-2/CD4/CCR5 cells with 05JAGU-#01 PBL (i.e. HIV-#01/CCR5) could use CCR3 and CXCR4 as well as CCR5. Interestingly, HIV-1 from 05JAGU-#11 PBL showed much broader GPCR use than those detected in coculture. HIV-1 produced by NP-2/CD4/CCR5 cells cocultured with 05JAGU-#11 PBL (i.e. HIV-#11/CCR5) used CCR1, CCR3, CCR5, CCR8, CXCR4, D6, and FPRL1. HIV-1 produced by NP-2/CD4/CXCR4 cells or NP-2/CD4/GPR1 cells (i.e. HIV-#11/CXCR4 or HIV-#11/GPR1) used CCR1, CCR3, CCR5, CCR8, CXCR4, FPRL1, and GPR1, although a few differences were detected in D6 and GPR1 usage between HIV-#11/CCR5 and -#11/CXCR4 isolates, suggesting that the populations of these isolates are different. The primary HIV-1 isolate produced by NP-2/CD4/CCR5 cells with 05JAGU-#12 PBL (i.e. HIV-#12/CCR5) used CCR3, CCR5, CCR8, CCR10, CXCR4, and D6. Similarly, HIV-1 produced by NP-2/CD4/CXCR4 cells (HIV-#12/CXCR4) could use CCR3, CCR5, CCR8, CCR9B, CCR10, XCR1, and FPRL1. Similarly to the HIV-1 from 05JAGU-#11 PBL, a few differences were detected in CCR9B, XCR1, and FPRL1 usage between HIV-#12/CCR5 and HIV-#12/CXCR4 isolates. HIV-1 was produced by NP-2/CD4/CXCR4 cells cocultured with 05JAGU-#15 PBL (i.e. HIV-#15/CXCR4) could use CCR1, CCR3, CCR5, CCR9B, XCR1 and FPRL1. Thus, the primary HIV-1 isolates unexpectedly showed broader GPCR usage than those

Table 1. Features of HIV-1-positive blood and HIV-1 isolation by coculture.

PBL No. 05JAGU-	HIV-1 RNA (copies/ml)	CD4(+) T cells (cells/μl)	AIDS-related disease	HAART[a,b]	HIV-1 isolation (NP-2/CD4/GPCR)	RT activity[c] (cpm/ml)
#01	151 000	393	–[a]	NT[b]	CCR5	250 122
#02	35 600	489	–	NT	CXCR4	58 360
#03	26 500	438	–	NT		
#04	6180	393	–	NT	CCR5	61 804
#05	1890	206	–	NT	GPR1	9817
#06	379	477	–	NT	GPR1	240
					FPRL1	60
#07	73 200	9	esophagus candidiasis	NT	CCR5	197 574
					CXCR4	82 155
					GPR1	110 595
#08	17 400	5	pneumocystis pneumonia	NT	CCR5	376 042
#09	29 700	132	pneumocystis pneumonia	3TC/EFV/TDF		
#10	3550	361	pneumocystis pneumonia	3TC/ABC/ATV		
#11	2560	148	pneumocystis pneumonia	3TC/EFV/TDF	CCR5	160 391
					CXCR4	101 593
					GPR1	97 666
#12	999	160	–	3TC/ATV/TDF	CCR5	117 503
					CXCR4	113 710
#13	71	367	–	3TC/AZT//NFV		
#14	90 600	350	–	interrupted	CCR5	26 850
#15	83 200	522	–	interrupted	CCR5	57 403
					CXCR4	463 062
					GPR1	123 118
					FPRL1	29 670
#16	27 100	234	–	interrupted	CCR5	144 175
#17	5950	292	–	interrupted		

3TC, lamivudine; ABC, ziagen; ATV, atazanavir; AZT, zidovudine; cpm, counts per minute; EFV, efavirentz; GPCR, G protein-coupled receptor; PBL, peripheral blood lymphocyte; NT, non-treatment; RT, reverse transcriptase; TDF, tenofovir.
[a]'–', a subject free from AIDS-related disease.
[b]NT, PBL was obtained before highly-active antiretroviral therapy (HAART).
[c]cpm/ml of culture supernatant.

observed in coculture (Table 2). We found that CCR9B, CCR10, and XCR1 work as novel coreceptors for primary HIV-1 isolates.

Amino acid sequence of the V3 domain of primary HIV-1 isolates

A critical domain of HIV-1 interacting with coreceptors is the V3 loop located on gp120 of Env protein [29]. We determined amino acid sequences of the V3 region for primary HIV-1 isolates derived from 05JAGU-#11 PBL. As shown in Table 3, HIV-1 provirus in NP-2/CD4/CCR5 cells cocultured with PBL showed two kinds of amino acid sequences. A leucine/isoleucine substitution at the 53rd amino acid position in the C3 region according to the numbering in Table 3 was detected. These two kinds of amino acid sequences were commonly observed in NP-2/CD4/CCR1, NP-2/CD4/CCR3, NP-2/CD4/CCR5, NP-2/CD4/CCR8, and NP-2/CD4/D6 cells infected with the primary HIV-1 isolate produced by NP-2/CD4/CCR5 cells (i.e. HIV-#11/CCR5).

HIV-1 provirus in NP-2/CD4/CXCR4 cells cocultured with 05JAGU-#11 PBL showed a different amino acid sequence from those in NP-2/CD4/CCR5: lysine at the 26th position was substituted with arginine. HIV-1 provirus in NP-2/CD4/GPR1 cells cocultured with 05JAGU-#11 PBL had the V3 sequence with an additional substitution to that in NP-2/CD4/CXCR4 cells: alanine at the 39th position was also substituted with threonine. These amino acid sequences in NP-2/CD4/CXCR4 and NP-2/CD4/GPR1 cells were also detected when these cells were infected with HIV-#11/CCR5 isolate. These results suggest that lysine/arginine substitution at the 26th position and alanine/threonine substitution at the 39th position in addition to isoleucine/leucine substitution at the 53rd position may be responsible for HIV-#11/CCR5 isolate to obtain the ability to use CXCR4 or GPR1 in addition to CCR5. CXCR4 or GPR1 use by HIV-#11/CCR5 isolate was not exclusive to its CCR5 use. HIV-#11/CCR5 isolate probably contained HIV-1 subpopulations that can use CXCR4 or GPR1 in addition to CCR5.

DNA fragments of the V3 region of HIV-1 provirus in 05JAGU-#05 PBL and NP-2/CD4/GPR1 cells cocultured with PBL could be amplified by PCR and the nucleotide sequences were determined. Their deduced amino acid sequences were identical and, interestingly, one amino acid was longer than that of the NL4-3 strain (Table 3) [30]. No DNA fragments of the V3 region could be amplified, not only in 05JAGU-#06 PBL, but also in NP-2/CD4/FPRL1 and NP-2/CD4/GPR1 cells cocultured with PBL.

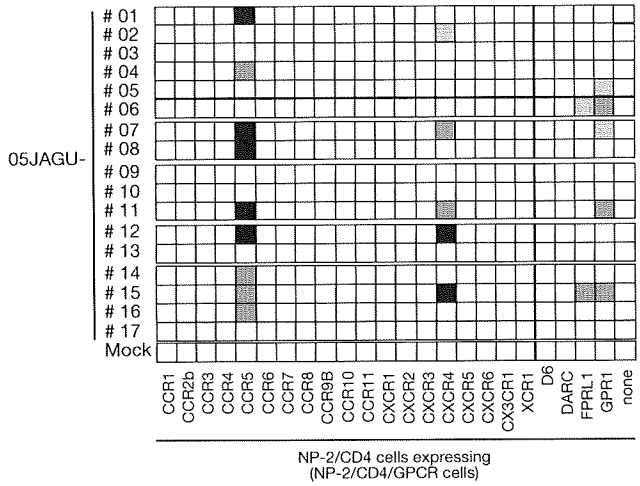

Fig. 2. Summary of HIV-1 isolation by coculture of NP-2/CD4/G protein-coupled receptor cells with peripheral blood lymphocytes of HIV-1-positive patients. NP-2/CD4/G protein-coupled receptor (GPCR) cells were cocultured with peripheral blood lymphocytes (PBLs) of HIV-1-positive patients. NP-2/CD4/GPCR cells infected with HIV-1 were detected by immunofluorescence assay (IFA). IFA was performed every 3 days and the ratios of HIV-1-positive cells were determined. The ratios of NP-2/CD4/GPCR cells that became HIV-1-antigen positive during 4-week coculture are shown as follows: black closed box ■, >50%; gray box ▩, 10–49%; pale gray box ▨, 1–9%; and opened box □, <1%. The PBL from which HIV-1 was isolated in coculture and the GPCR used by HIV-1 as a coreceptor is indicated in bold.

Discussion

The involvement of HIV-1 infection assisted by coreceptors other than CCR5 and CXCR4 in AIDS pathogenesis has hardly been elucidated, even though HIV-1 populations that can use them certainly exist in PBLs of HIV-1-positive patients [31]. These populations are thought to be extremely small in whole copies of HIV-1 *in vivo* [20,31]; however, the small size of the HIV-1 population does not necessarily link to the conclusion that they are less important in HIV-1 infection *in vivo*. To elucidate the usage spectrum of various GPCRs, including CCR5 and CXCR4 as coreceptors by primary HIV-1 isolates, will help to define the generation and roles of HIV-1 infection mediated by them in AIDS pathogenesis.

We established NP-2/CD4/GPCR cells using the same protocol to minimize differences in protein expression levels among GPCRs, because only a few kinds of antibodies for GPCRs were available. Abundant expression of CCR3, CCR5, CCR8, CXCR4, CXCR5, D6, FPRL1, and GPR1 had been confirmed in NP-2/CD4/GPCR cells [14–16,22,32–34]. We detected comparable levels of the mRNA expression among GPCRs (Fig. 1a). However, unavoidable unevenness of the expression levels among GPCRs in NP-2/CD4/GPCR cells might affect the efficiency of HIV-1 isolation. HIV-1 was isolated from 11 of 17 PBLs by coculture with NP-2/CD4/GPCR cells (Fig. 2 and Table 1). HIV-1 was efficiently isolated from NP-2/CD4/FPRL1 or NP-2/CD4/GPR1 cells cocultured with 05JAGU-#07, #11, and #15 PBLs. Interestingly, HIV-1 infection was not detected in NP-2/CD4/CCR5 and NP-2/CD4/CXCR4 cells but in NP-2/CD4/FPRL1 or NP-2/CD4/GPR1 cells cocultured with 05JAGU-#05 and #6 PBLs, although abundant viral propagation was not observed in these cocultures. In fact, HIV-1 proviral DNA was detected in 05JAGU-#05 PBL (Fig. 1c) and NP-2/CD4/GPR1 cells cocultured with the PBL (data not shown). The involvement of its longer V3 loop in coreceptor usage should be investigated further (Table 3). These results suggest that FPRL1 and GPR1 may work as significant coreceptors *in vivo* next to CCR5 and CXCR4, and HIV-1 populations that use FPRL1 or GPR1 instead of CCR5 and CXCR4 certainly exist in the blood of HIV-1-positive patients.

HIV-1 could be isolated from PBLs with less than 1000 copies/ml of HIV-1 RNA in their plasma, such as 05JAGU-#06 and #12 PBLs (Table 1). HIV-1 proviral DNA was, however, clearly detected in these PBLs (Fig. 1c), suggesting that the amounts of HIV-1 produced by them were enough to infect NP-2/CD4/GPCR cells. Conversely, HIV-1 could not be isolated from 05JAGU-#03 and #09 PBLs, although these blood samples contained higher copy numbers of HIV-1 RNA. Thus, no apparent correlation was observed between HIV-1 isolation by coculture using NP-2/CD4/GPCR cells and the copy number of the HIV-1 genome in plasma or PBLs (Table 1). This discrepancy implies that factors other than the copy numbers of HIV-1 in plasma or PBLs affect the efficiency of HIV-1 isolation by coculturing NP-2/CD4/GPCR cells with PBLs.

It is assumed that the efficiency of cell-free infection of HIV-1 is comparable with that of cell-to-cell infection [35]. Our results, however, showed that the primary HIV-1 isolates produced by NP-2/CD4/GPCR cells cocultured with HIV-1-positive PBLs have a much broader spectrum of GPCR usage than those detected in coculture (Table 2). Moreover, we found that three CKRs (i.e. CCR9B, CCR10, and XCR1) work as novel coreceptors of primary HIV-1 isolates. Markedly expanded GPCR usage was detected in primary HIV-1 isolates derived from 05JAGU-#11, #12, and #15 PBLs (Table 2); however, these isolates could, unexpectedly, use none of the novel coreceptors identified here. These novel coreceptors also contain tyrosines in their NTRs like the other HIV-1 coreceptors identified so far. These results support the hypothesis that GPCRs harboring tyrosines in their NTRs have potential as HIV-1 coreceptors.

Table 2. Coreceptor usage of primary HIV-1 isolates.

PBL	Infection[b]	Virus producer cells	HIV-1 susceptibility[a] of NP-2/CD4-expressing cells										
			CCR5	CXCR4	FPRL1	GPR1	CCR1	CCR3	CCR8	CCR9B	CCR10	XCR1	D6
5JAGU-													
#01	Coculture	PBL	+++	−	−	−	−	−	−	−	−	−	−
	Isolate	NP-2/CD4/CCR5	+++	+++	−	−	−	+	−	−	−	−	−
#11	Coculture	PBL	+++	++	−	++	−	−	−	−	−	−	−
	Isolate	NP-2/CD4/CCR5	+++	+++	+	−	++	+++	+++	−	−	−	++
	Isolate	NP-2/CD4/CXCR4	+++	+++	++	+	++	+++	+++	−	−	−	−
	Isolate	NP-2/CD4/GPR1	+++	+++	+	++	++	+++	+	−	−	−	−
#12	Coculture	PBL	+++	+++	−	−	−	−	−	−	−	−	−
	Isolate	NP-2/CD4/CCR5	+++	+++	−	−	−	+++	+++	−	++	−	+
	Isolate	NP-2/CD4/CXCR4	+++	+++	+	−	−	+++	++	+	++	+	+++
#14	Coculture	PBL	++	−	−	−	−	−	−	−	−	−	−
	Isolate	NP-2/CD4/CCR5	+++	+++	−	−	−	−	−	−	−	−	−
#15	Coculture	PBL	++	+++	++	++	−	−	−	−	−	−	−
	Isolate	NP-2/CD4/CXCR4	+++	+++	++	−	+	+++	+	−	−	+	−

FPRL1, formylpeptide receptor; GPCR, G protein-coupled receptor; PBL, peripheral blood lymphocyte.
[a]Ratios of NP-2/CD4/GPCR cells infected by HIV-1 during 4-week coculture with HIV-1-positive PBLs or on day 6 after inoculation of primary HIV-1 isolates are shown as follows: +++, ≥ 50%; ++, 10–49%; +, 1–9%; −, <1%.
[b]Coculture indicates GPCR use by HIV-1 in the coculture of NP-2/CD4/GPCR cells with HIV-1-positive PBLs. Isolate indicates GPCR use by primary HIV-1 isolates.

Table 3. Amino acid sequences of the V3 region of HIV-1 in 05JAGU-#05 and #11 peripheral blood lymphocytes.

Target cells	Clone No.[a]	Amino acid sequence of the V3 region[b]
1. Cocultured with 05JAGU-#11 PBL		1 10 20 30 40 50 60
NP-2/CD4/CCR5	1	NAKTIIVQLNRTVQINCTRPNNNTRKGIHIGPGRARFYATKIIGDIRQAYCNISRAAWND
	2	.....L.....
NP-2/CD4/CXCR4	3	.....R.....L.....
	4	.....R.....L.....
NP-2/CD4/GPR1	5	.....R.....T.....L.....
2. Inoculated with primary isolate HIV-#11/CCR5		
NP-2/CD4/CCR1	6	.....L.....
	7	.....
	8	.....K.....L.....
	9	.....K.....
NP-2/CD4/CCR3	10	.....
	11	.....L.....
NP-2/CD4/CCR5	12	.....
	13	.....L.....
NP-2/CD4/CCR8	14	.....
	15	.....L.....
NP-2/CD4/D6	16	.....
	17	.....L.....
NP-2/CD4/CXCR4	18	.....R.....L.....
NP-2/CD4/GPR1	19	.....R.....T.....L.....
3. Cocultured with 05JAGU-#05 PBL		1 10 20 30 40 50 60
	NL4-3	NAKTIIVQLNTSVEINCTRPNNNTRKSIRIQR-GPGRAFVTIGKIGNMRQAHCNISRAKWNA
NP-2/CD4/GPR1	20	.....ET.V..... GHI....V.YGTNI..DI.....T...N

PBL, peripheral blood lymphocyte.
[a]Clone numbers were attached for convenience.
[b]HIV-1 proviral DNA was amplified by PCR and cloned into the TA vector.
Nucleotide sequences of the V3 region of several clones were determined and amino acid sequences were deduced. Symbols, '.' and '–' indicate similar and blank amino acid positions, respectively, to the representative amino acid sequence shown at the top of the amino acid alignments.

Different coreceptor usage patterns were detected in primary HIV-1 isolates that originated from the same PBL, 05JAGU-#11 or #12, but produced from different NP-2/CD4/GPCR cells (Table 2), suggesting that these PBLs contain distinct HIV-1 subpopulations with different coreceptor usage. Our coculture method using NP-2/CD4/GPCR cells enabled us to separately and directly isolate these HIV-1 subpopulations from PBLs. Separation of the HIV-1 subpopulation was confirmed by amino acid sequences of the V3 region of primary HIV-1 isolates from 05JAGU-#11 PBL (Table 3). Distinct amino acid sequences were detected in NP-2/CD4/CCR5, NP-2/CD4/CXCR4, and NP-2/CD4/GPR1 cells cocultured with this PBL; however, all of these amino acid sequences detected in coculture were also observed in the primary HIV-1 isolate produced by NP-2/CD4/CCR5 cells (i.e. HIV-#11/CCR5). These results support the usefulness of NP-2/CD4/GPCR cell to directly isolate HIV-1 subpopulations with different coreceptor usage from PBLs. We have reported that amino acid substitution at the 32nd position in Table 3 is responsible for HIV-1 to use GPR1 [22,36]. Amino acid substitutions at the 26th, 39th, or 53rd position may have a role in the multiple uses of GPCRs as coreceptors by some HIV-1 isolates. A charged amino acid, arginine, at the 26th position has been shown to be critical for CXCR4 use of HIV-1 [37]. This substitution is possibly also important for HIV-1 to use GPR1. An amino acid substitution in the carboxyl-terminal region of the V3 loop may also play a role in expansion of the GPCR usage of HIV-1.

Some cell surface molecules incorporated into HIV-1 have been shown to enhance infectivity [38]. Similarly, factors of NP-2/CD4/GPCR cells might give HIV-1 the ability to efficiently use multiple GPCRs as coreceptors. Recently, Gorry *et al.* [31] reported that HIV-1 isolated from PBLs of an individual with a CCR5 delta-32 heterozygosity can use various GPCRs such as APJ, CCR2b, CCR3, CCR5, CCR8, CX3CR1, GPR1, and GPR15. In the same report, it was also shown that HIV-1 strains that can use multiple coreceptors are generated in patient PBLs in accordance with progression to AIDS [31]; however, we isolated HIV-1, which can use various GPCRs as coreceptors from PBLs of asymptomatic HIV-1 carriers (Table 1). Our results indicate that HIV-1 populations that can use various GPCRs other than CCR5 or CXCR4 because coreceptors have already been generated *in vivo* without the host condition of delta-32 heterozygosity or low immunity.

In the present study, we propose that FPRL1 and GPR1 are also significant coreceptors *in vivo* next to CCR5 and CXCR4. We have found that FPRL1 works as a coreceptor not only for primary HIV-1 isolates, but also for some laboratory-adapted strains of HIV-1, HIV type-2 (HIV-2), and simian immunodeficiency viruses (SIVs) [33]. FPRL1 expression was detected in various types of cells, such as neutrophils, phagocytes, and some organs, although their roles have not been elucidated well [39]. One of its ligands, lipoxin A4, to FPRL1 caused an activation and anti-inflammatory response in neutrophils [40]. GPR1 expression was abundant in the human brain hippocampus [31]. GPR1 mRNA was detected in T cell lines and PBLs [22]. HIV-1 infection mediated through FPRL1 and GPR1 *in vivo* should be further investigated. HIV-1 has been developing the potential to use various GPCRs other than CCR5 and CXCR4 as coreceptors by its high rate mutation. We conclude that the usage spectrum of GPCRs as coreceptors by HIV-1 must be wider than has been identified. The involvement of HIV-1 infection mediated through GPCRs other than CCR5 and CXCR4 in AIDS progression and HIV-1 reservation *in vivo* should be studied further.

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N.S. conceived and designed this study, carried out the molecular genetic and virology studies, and drafted the article. A.T. established the cell lines. A.O. and T.M. carried out biochemical studies. C.A., T.O., H.U., and Y.N. participated in PBL preparation and their characterization. H.H. played important roles in the coordination of this study and helped to draft the article.

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Virus Entry via the Alternative Coreceptors CCR3 and FPRL1 Differs by Human Immunodeficiency Virus Type 1 Subtype▽†

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Human immunodeficiency virus type 1 (HIV-1) infects target cells by binding to CD4 and a chemokine receptor, most commonly CCR5. CXCR4 is a frequent alternative coreceptor (CoR) in subtype B and D HIV-1 infection, but the importance of many other alternative CoRs remains elusive. We have analyzed HIV-1 envelope (Env) proteins from 66 individuals infected with the major subtypes of HIV-1 to determine if virus entry into highly permissive NP-2 cell lines expressing most known alternative CoRs differed by HIV-1 subtype. We also performed linear regression analysis to determine if virus entry via the major CoR CCR5 correlated with use of any alternative CoR and if this correlation differed by subtype. Virus pseudotyped with subtype B Env showed robust entry via CCR3 that was highly correlated with CCR5 entry efficiency. By contrast, viruses pseudotyped with subtype A and C Env proteins were able to use the recently described alternative CoR FPRL1 more efficiently than CCR3, and use of FPRL1 was correlated with CCR5 entry. Subtype D Env was unable to use either CCR3 or FPRL1 efficiently, a unique pattern of alternative CoR use. These results suggest that each subtype of circulating HIV-1 may be subject to somewhat different selective pressures for Env-mediated entry into target cells and suggest that CCR3 may be used as a surrogate CoR by subtype B while FPRL1 may be used as a surrogate CoR by subtypes A and C. These data may provide insight into development of resistance to CCR5-targeted entry inhibitors and alternative entry pathways for each HIV-1 subtype.

Human immunodeficiency virus type 1 (HIV-1) infects target cells by binding first to CD4 and then to a coreceptor (CoR), of which C-C chemokine receptor 5 (CCR5) is the most common (6, 53). CXCR4 is an additional CoR for up to 50% of subtype B and D HIV-1 isolates at very late stages of disease (4, 7, 28, 35). Many other seven-membrane-spanning G-protein-coupled receptors (GPCRs) have been identified as alternative CoRs when expressed on various target cell lines in vitro, including CCR1 (76, 79), CCR2b (24), CCR3 (3, 5, 17, 32, 60), CCR8 (18, 34, 38), GPR1 (27, 65), GPR15/BOB (22), CXCR5 (39), CXCR6/Bonzo/STRL33/TYMSTR (9, 22, 25, 45, 46), APJ (26), CMKLR1/ChemR23 (49, 62), FPLR1 (67, 68), RDC1 (66), and D6 (55). HIV-2 and simian immunodeficiency virus SIVmac isolates more frequently show expanded use of these alternative CoRs than HIV-1 isolates (12, 30, 51, 74), and evidence that alternative CoRs other than CXCR4 mediate infection of primary target cells by HIV-1 isolates is sparse (18, 30, 53, 81). Genetic deficiency in CCR5 expression is highly protective against HIV-1 transmission (21, 36), establishing CCR5 as the primary CoR. The importance of alternative CoRs other than CXCR4 has remained elusive despite many studies (1, 30, 70, 81). Expansion of CoR use from CCR5 to include CXCR4 is frequently associated with the ability to use additional alternative CoRs for viral entry (8, 16, 20, 63, 79) in most but not all studies (29, 33, 40, 77, 78). This finding suggests that the sequence changes in HIV-1 *env* required for use of CXCR4 as an additional or alternative CoR (14, 15, 31, 37, 41, 57) are likely to increase the potential to use other alternative CoRs.

We have used the highly permissive NP-2/CD4 human glioma cell line developed by Soda et al. (69) to classify virus entry via the alternative CoRs CCR1, CCR3, CCR8, GPR1, CXCR6, APJ, CMKLR1/ChemR23, FPRL1, and CXCR4. Full-length molecular clones of 66 *env* genes from most prevalent HIV-1 subtypes were used to generate infectious virus pseudotypes expressing a luciferase reporter construct (19, 57). Two types of analysis were performed: the level of virus entry mediated by each alternative CoR and linear regression of entry mediated by CCR5 versus all other alternative CoRs. We thus were able to identify patterns of alternative CoR use that were subtype specific and to determine if use of any alternative CoR was correlated or independent of CCR5-mediated entry. The results obtained have implications for the evolution of *env* function, and the analyses

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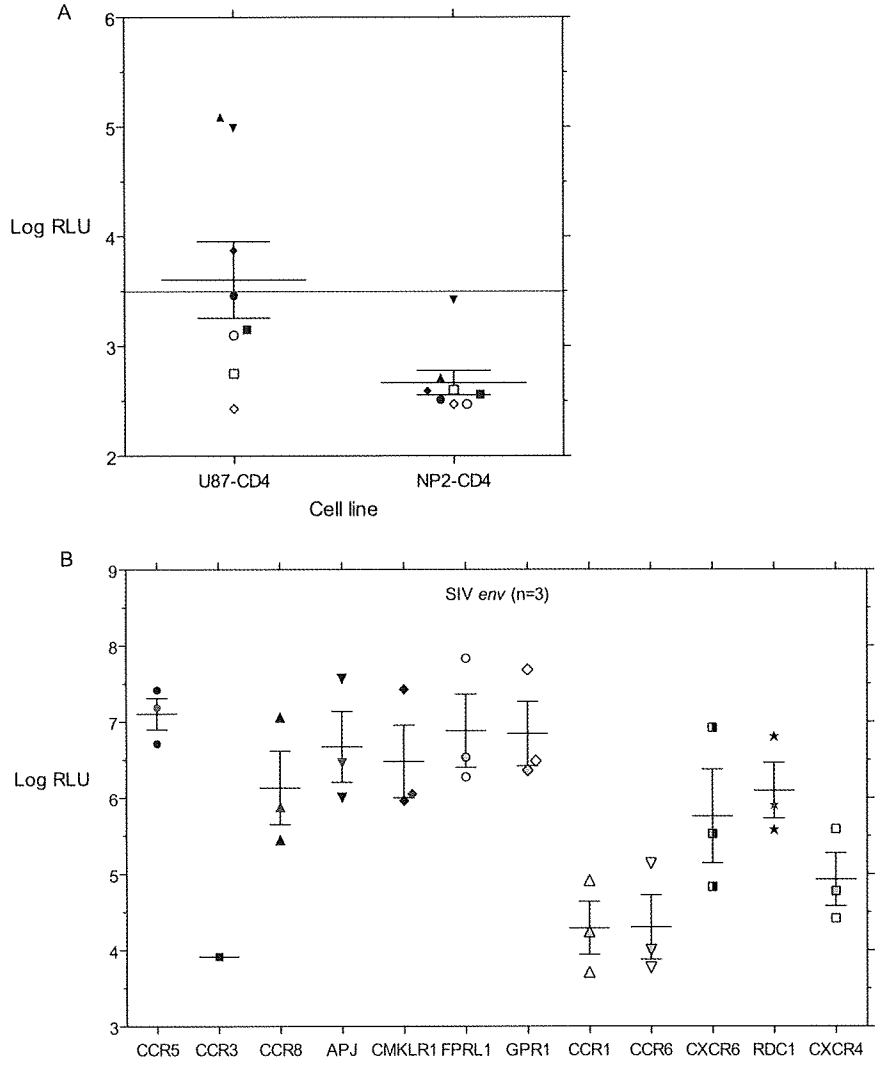

FIG. 1. (A) Entry mediated by eight, late-stage subtype C *env* clones determined on U87-CD4 or NP2/CD4 cell lines lacking any exogenous CoR. Two independent *env* clones from the same patient (4-1, triangle; 4-3, inverted triangle), and one *env* clone from a separate patient (11-1, filled diamond) were able to mediate virus entry into U87-CD4 cells well above the background of the assay, whereas no *env* clone could use endogenous coreceptors expressed on NP2-CD4 cells at levels that were scored positive (3.5 log RLU). (B) Entry mediated by two SIVmac239 *env* clones (one gp160 and a second gp140) and one SIVmac251 gp140 *env* clone on NP-2.CD4 cells expressing the indicated alternative CoR. The SIVmac239 results are shown in black or open symbols, while the SIVmac251 results are shown as gray-filled symbols.

revealed important differences between subtype B Env function and all other HIV-1 subtypes.

MATERIALS AND METHODS

Typing of alternative coreceptor use. NP-2/CD4 cells engineered to express the GPCR proteins CCR5, CCR3, CMKLR1/ChemR23, APJ, CCR1, CCR6, CCR8, CXCR6/Strl33/BONZO, GPR1, RDC1, FPRL1, or CXCR4 (49, 65–69) were used as target cells for infection by luciferase reporter viruses (19) pseudotyped with Env proteins expressed from full-length *env* clones, as described previously (56, 57). The NP-2/CD4/CoR cell lines were kindly provided by the Hoshino (all but CMKLR1/ChemR23) and Mårtensson (CMKLR1/ChemR23) laboratories. NP-2 glioma cells were chosen for target cells because the more commonly used U87/CD4 cells could be infected by virus pseudotyped by several different Env proteins in the absence of any exogenous CoR expression (see Fig. 1A), confirming a previous report of GPR1 (66) and an unidentified endogenous CoR present in this cell line (74). Each NP-2/CD4/CoR line was permissive for high-level entry ($>10^6$ relative light units [RLU]) mediated by at least two independent Env proteins, and most are documented to mediate entry by at least a subset of HIV-1 Env proteins (49, 58, 65, 74). To confirm expression of functional CoRs, we performed entry assays with viruses pseudotyped with two SIVmac239 *env* clones (one gp160 and a second gp140) and one SIVmac251 gp140 *env* clone (see Fig. 1B). NP-2/CD4 cells expressing CCR1 or CCR6 gave relatively low signals with SIV Env-mediated entry and did not support entry above background levels for any of the HIV-1 *env* clones studied here. All other alternative CoRs supported robust entry function mediated by SIV Env (except CCR3) or by many HIV-1 Env proteins. Entry was scored as positive if the RLU signal was >log 3.5, the highest background value observed on NP-2/CD4 cells in the absence of an identified CoR (Fig. 1A).

***env* molecular clones.** We collected a total of 66 full-length *env* clones, each derived from an individual patient infected with subtype A, B, C, or D HIV-1. Standard panels of 12 subtype B *env* clones from the United States, Italy, and Trinidad; 12 subtype C *env* clones from South Africa and Zambia (43, 44, 50); and 10 subtype C *env* clones from India were obtained from the NIH AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, NIH (Indian subtype C clones were from R. Paranjape, S. Kulkarni, and D. Montefiori). All of these clones were isolated directly from the blood of recently infected individuals and thus represented

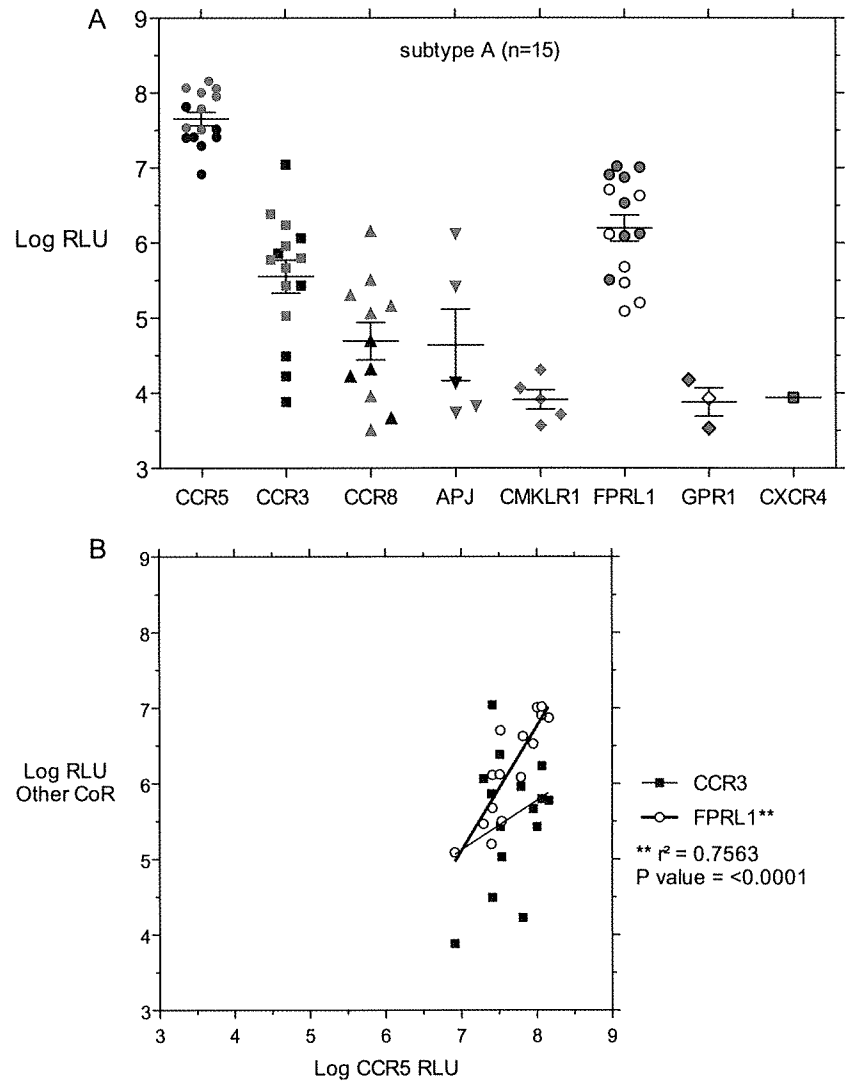

FIG. 2. (A) Entry mediated by 15 full-length *env* clones from different HIV-1 subtype A-infected patients via CCR5 or the alternative CoRs CCR3, CCR8, APJ, CMKLR1 (ChemR23), FPRL1, GPR1, or CXCR4. Data are expressed as log RLU, with the mean ± standard error shown by horizontal lines. Only data for positive use of CoR are shown, defined as RLU of $>3.2 \times 10^3$ (log 3.5), with background of <500. *env* clones from chronic infection are shown in black or open symbols while *env* clones from recent transmission are shown in gray. (B) Linear regression analysis of entry via CCR5 (*x* axis) versus entry via alternative coreceptors. The bold line indicates significant correlation between the regression lines fitted by Prism, version 5.0, for CCR5 and FPRL1. Use of CCR5 and other CoRs (including CCR3; shown here for comparison with Fig. 3) did not show statistically significant correlation.

early/transmitted strains. Several *env* clones from subtype B laboratory strains (BaL, ADA, SF162, and JR-CSF) were included in the analysis, bringing the total to 18. Subtype A *env* clones were from Kenya and included envelope variants from seven chronically infected mothers and two infants as well as six variants from recently infected women (10, 47, 59, 75), for a total of 15. Most subtype D *env* clones were from Uganda and have been previously described (13), but two were added from one transmission case (QB857 [11]) and an early infection sample from the Rakai cohort (42) that was cloned in our laboratory (GenBank accession number GQ245681), for a total of 11 clones. Two SIVmac239 *env* clones and one SIVmac251 *env* clone were also tested for comparison (see above and Fig. 1B). All *env*-pseudotyped viruses were freshly prepared for each assay since we observed that freeze-thawing cycles reduced infectivity.

Statistical analysis. Linear regression analysis of log-transformed RLU for CCR5-mediated entry versus entry mediated by individual alternative CoRs was performed using Prism 5 statistical programs (GraphPad Software, San Diego, CA). Pairwise comparison of the level of entry mediated by *env* clones from different subtypes via the same CoR was performed with the Mann-Whitney U test (two-tailed) implemented with Prism 5. Analysis of the differences between the fraction of positive and negative *env* clones for one alternative CoR was performed using two-by-two contingency tables and a Fisher's exact test, also implemented with Prism 5.

RESULTS

Use of alternative coreceptors by SIVmac Env proteins. As noted above, SIVmac239 and SIVmac251 Env proteins could mediate entry via all the alternative CoRs expressed including CCR1, CCR6, and CXCR4 (Fig. 1B) although entry via CCR3 was at a very low level. These results confirm the previously reported promiscuous use of alternative CoRs by SIV isolates (26, 27) and show a surprisingly high level of entry via CXCR4.

Use of alternative CoRs by subtype A HIV-1 Env proteins. Fifteen *env* clones from 15 different individuals infected with

TABLE 1. Summary of alternative coreceptor use by HIV-1 subtype

Receptor	CoR use by the indicated HIV-1 subtype												Efficiency of CoR use for all subtypes[a]
	A			B			C			D			
	Efficiency of CoR use[a]	Level of entry (log_{10} RLU) with CoR		Efficiency of CoR use[a]	Level of entry (log_{10} RLU) with CoR		Efficiency of CoR use[a]	Level of entry (log_{10} RLU) with CoR		Efficiency of CoR use[a]	Level of entry (log_{10} RLU) with CoR		
		Mean	95% CI		Mean	95% CI		Mean	95% CI		Mean	95% CI	
CCR5	**15/15 (100)**	**7.66**	7.46–7.85	**18/18 (100)**	**6.99**	6.63–7.35	**22/22 (100)**	**7.00**	6.71–7.29	**11/11 (100)**	**7.04**	6.52–7.56	**100**
CCR3	**15/15 (100)**	5.55	5.08–6.02	**18/18 (100)**	**6.90**	6.52–7.27	18/22 (82)	4.99	4.53–5.45	**11/11 (100)**	**5.14**	4.59–5.69	95
CCR8	11/15 (73)	4.69	4.14–5.25	14/18 (78)	4.69	4.35–5.03	17/22 (77)	5.23	4.71–5.75	6/11 (55)	4.42	3.52–5.32	71
APJ	6/15 (40)	4.43	3.30–5.56	15/18 (84)[b]	4.64	4.25–5.02	9/22 (41)	4.55	4.09–5.01	3/11 (27)	4.66	2.49–6.84	48
CMKLR1	5/15 (33)	3.91	3.55–4.27	11/18 (61)	4.12	3.68–4.56	3/22 (14)[c]	3.64	3.28–4.00	6/11 (55)	4.24	3.77–4.71	41
FPRL1	**15/15 (100)**	**6.19**	5.82–6.57	11/18 (61)[d]	4.68	4.16–5.19	**22/22 (100)**	**5.83**	5.36–6.30	8/11 (73)	5.02	4.40–5.65	84
CXCR4	1/15 (7)	3.94	NA[f]	5/18 (28)	3.91	3.69–4.12	5/22 (23)	4.18	3.94–4.42	7/11 (64)[e]	4.32	3.03–5.61	31

[a] Calculated as the number of positive *env* clones/number of clones tested (% positive). Boldface indicates 100% positive.
[b] Percentage of subtype B *env* clones using APJ is significantly higher than the percentage of subtype A ($P = 0.0120$, Fisher's exact test), subtype C ($P = 0.0092$), or subtype D ($P = 0.0045$).
[c] Percentage of subtype C *env* clones using CMKLR1 is significantly lower than the percentage of subtype B ($P = 0.0014$) but not significantly lower than the percentage of subtype A or D.
[d] Percentage of subtype B *env* clones using FPRL1 is significantly lower than the percentage of subtypes A or C ($P = 0.0008$).
[e] Percentage of subtype D *env* clones using CXCR4 is significantly higher than the percentage of subtype A ($P = 0.0005$) but not significantly higher than the percentage of subtype B or C.
[f] NA, not available.

subtype A HIV-1 were used to generate pseudotyped virus for alternative CoR determination. The results are depicted in Fig. 2A for all *env* clones that mediated entry above background (>log 3.5 RLU). Three CoRs were permissive for virus entry by all 15 subtype A *env* clones: CCR5, CCR3, and FPRL1 (Table 1). CCR5 was the best CoR, FPRL1 was the next most efficient CoR (1.47 log lower than CCR5), and CCR3 was less efficient (2.22 log lower than CCR5) (Fig. 2A and Table 1). Other alternative CoRs were used by fewer *env* clones and with less efficiency. Eleven of 15 *env* clones mediated entry via CCR8, 6/15 clones mediated entry via APJ, and 5/15 mediated entry via CMKLR1. Only three *env* clones could utilize GPR1 for entry, and only one *env* could mediate entry via CXCR4 at low levels.

Eight of the subtype A *env* clones were from acute/early infection, and seven were from chronically infected mothers who transmitted infection to their infants. The data in Fig. 2A suggest that *env* clones from acute/early infection showed trends toward a higher level of entry via CCR5, CCR8, APJ, CMKLR1, and FPRL1 than for *env* clones from chronic infection. More samples will be needed to verify these trends.

Linear regression analysis was performed to determine if entry mediated via CCR5 correlated with entry via other CoRs. The results are shown in Fig. 2B and demonstrate that entry mediated by FPRL1 was highly significantly correlated with entry via CCR5 (*P* value of <0.0005), whereas entry via CCR3 was not significantly correlated with entry via CCR5. The best alternative CoR for subtype A samples was thus FPRL1, and CCR3 was a less efficient and less predictable alternative CoR.

Alternative CoR use by subtype B HIV-1 Env proteins. A similar analysis was performed with viruses pseudotyped with 18 subtype B Env proteins expressed from the panel of subtype B *env* molecular clones (mostly from early infection) described in Materials and Methods. All 18 subtype B *env* clones could mediate entry via both CCR5 and CCR3. Although CCR5 was highly efficient for mediating virus entry (Fig. 3A and Table 1), CCR3 was statistically equivalent to CCR5 (Table 1). APJ and CCR8 could be used by a majority of *env* clones with lesser efficiency: 15/18 could use APJ, and 14/18 could use CCR8. Fewer *env* clones mediated entry via CMKLR1 or FPRL1: 11/18 could use CMKLR1, and 11/18 could use FPRL1. Only 5/18 *env* clones could use CXCR4 and all with poor efficiency (Table 1), as might be expected given the early stage of infection from which the isolates were derived.

Figure 3B shows the results of the linear regression analysis of entry mediated by CCR5 versus other CoRs. Entry via CCR5 and CCR3 was equivalent and highly significantly correlated, and entry via CCR5 and APJ or CCR8 showed a correlation that was less significant. By contrast to subtype A Env function, there was no correlation between entry via CCR5 and FPRL1.

Alternative CoR use by subtype C HIV-1 Env proteins. We proceeded to analyze virus entry mediated by 22 subtype C Env proteins incorporated in pseudotyped viruses using the 22 *env* molecular clones described in Materials and Methods. The results of the entry assays are shown in Fig. 4A and Table 1. All 22 *env* clones mediated entry via CCR5 more efficiently than any alternative CoR. The second most efficient CoR was FPRL1, which could also be used by all 22 *env* clones. CCR3 and CCR8 were able to mediate virus entry for the majority of subtype C *env* clones: 18/22 clones could use CCR3, and 17/22 clones could use CCR8. APJ could be used for entry by 9/22 clones, whereas only 5 clones could use CXCR4 and only 3 could use CMKLR1 at levels barely above background.

The regression analysis displayed in Fig. 4B shows that entry via CCR5 was highly correlated with entry via FPRL1 and also that entry via CCR5 and CCR3 or CCR8 was significantly correlated although less impressively so than for FPRL1.

Alternative CoR use by subtype D HIV-1 Env proteins. A similar analysis of CoR use was performed with 11 Env proteins from subtype D isolates from nine different patients described in Brown et al. (13) and two additional *env* clones described in Materials and Methods. One of these isolates, 93/UG/065, was known to use CXCR4 and not CCR5 for

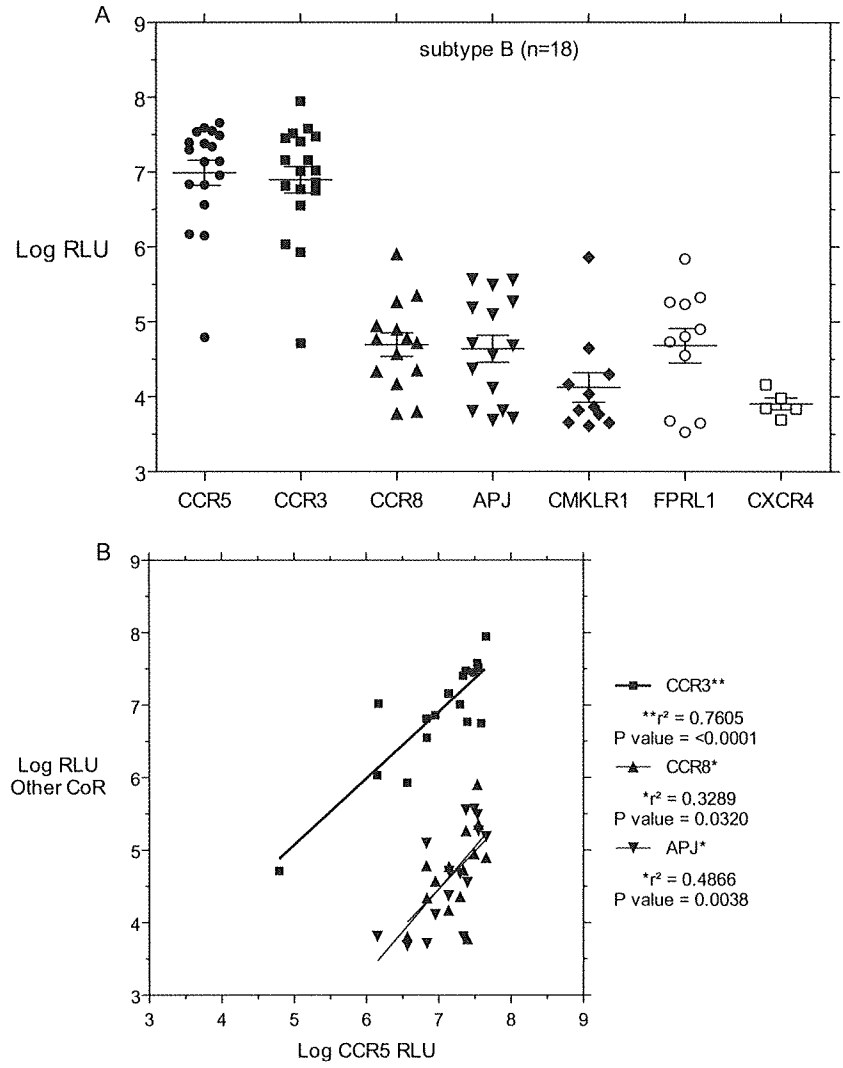

FIG. 3. (A) Entry mediated by 18 subtype B *env* clones from different patients plotted as described in the legend of Fig. 2. (B) Linear regression analysis of entry via CCR5 versus via alternative CoR, analyzed as described in the legend of Fig. 2. The bold regression lines for CCR3 and APJ indicate significant correlation with CCR5-mediated entry, but entry via FPRL1 did not show significant correlation with CCR5.

infection (13), while the remaining 10 *env* clones used CCR5 much better than CXCR4 or other alternative CoRs (Fig. 5A and Table 1). Only CCR5 and CCR3 were able to mediate entry by all 11 subtype D *env* clones. A majority of subtype D *env* clones could mediate virus entry via other alternative CoRs. FPRL1 was used by 8/11 clones, and CCR8 and CMKLR1 were used by 6/11 clones. Only 3/11 subtype D *env* clones could mediate entry via APJ, but 7/11 clones could use CXCR4, with clone 93/UG/065 capable of robust entry via CXCR4 (Fig. 5A and Table 1). The previously characterized X4 93/UG/065 *env* clone was the poorest at mediating entry via CCR5 and the best at mediating entry via CCR3 and CXCR4 (Fig. 5A) but probably should be characterized as "dual/X4" based on our results. Excluding that one high value (7.41 log RLU), the mean entry level via CXCR4 for the remaining six clones was 3.87 log RLU (95% confidence interval [CI], 3.44 to 4.18).

Linear regression analysis of virus entry via CCR5 versus any other CoR did not show any significant correlation (Fig. 5B). The smaller *env* clone sample size would make significant correlation more difficult to observe, but the plotted regression lines shown in Fig. 5B show no hint of positive correlation.

Comparison of entry via alternative CoRs between different HIV-1 subtypes. Table 1 and Fig. 6 show summary data for all experiments, and Table S1 in the supplemental material gives the *env* clone name, accession number, and entry data for each CoR assayed. The major differences between entry mediated by *env* clones from different subtypes was in the use of CCR3 and FPRL1, so only data for CCR5, CCR3, and FPRL1 are shown in Fig. 6. Use of CCR5 was not significantly different between subtypes although subtype A *env* clones showed a trend toward a higher level of entry. Entry function via CCR3 was much higher for subtype B *env* clones than for other subtypes, and this difference was very highly significant ($P < 0.0001$). Subtype A and C *env* clones

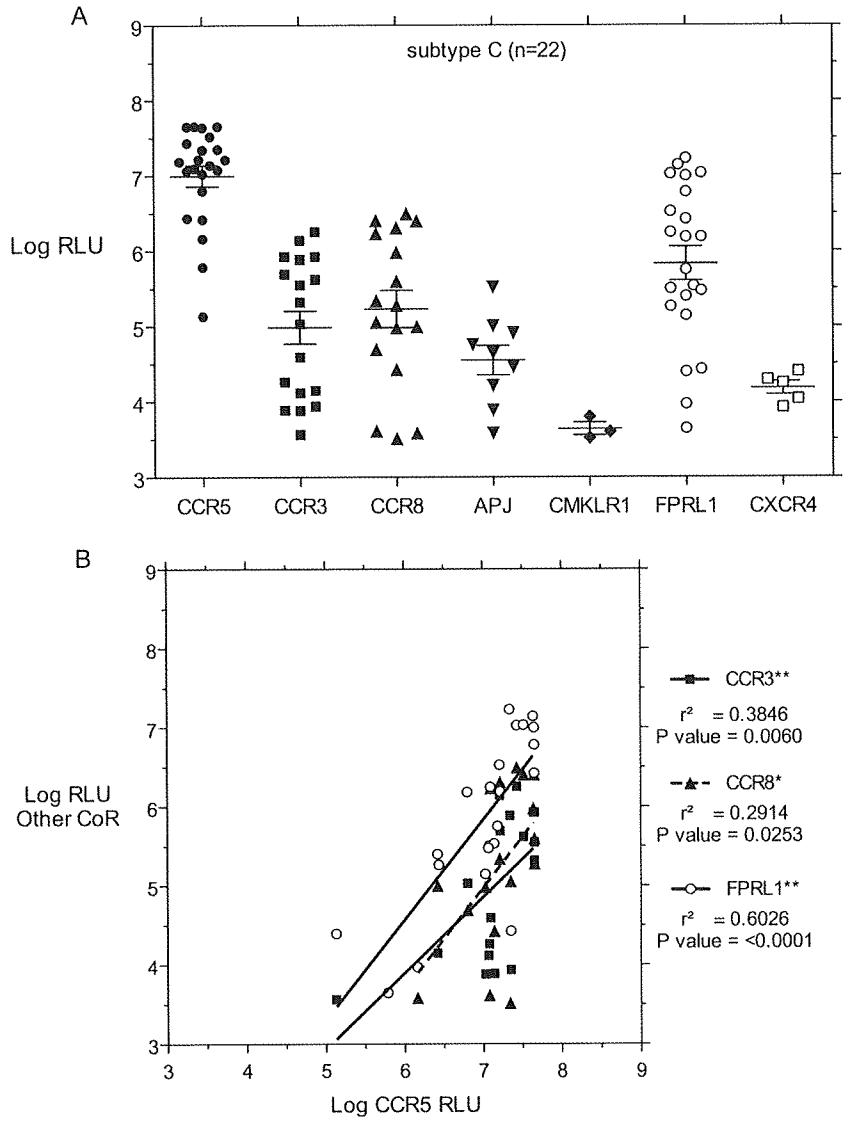

FIG. 4. (A) Entry mediated by 22 subtype C *env* clones from different patients at early stages of infection plotted as described in the legend of Fig. 2. (B) Linear regression analysis of entry via CCR5 versus three alternative CoRs showing significant correlation with CCR5: CCR3, CCR8, and FPRL1.

mediated significantly higher levels of entry via FPRL1 than subtype B or D *env* clones. Subtype D had a unique phenotype, with a low level of entry via CCR3, like subtypes A and C, and a low level of entry via FPRL1, like subtype B and unlike subtypes A and C.

Additional significant differences between HIV-1 subtypes were a larger fraction of subtype B *env* clones than all other subtypes that could use APJ for virus entry, a smaller fraction of subtype C *env* clones than subtype B that could use CMKLR1, and a larger fraction of subtype D *env* clones than subtype A that could use CXCR4 (Table 1).

DISCUSSION

The most striking finding of these studies is that we were able to define an alternative CoR entry phenotype that distinguished each HIV-1 subtype examined (Fig. 6). Robust use of the primary CCR5 CoR was common to HIV-1 Env from all subtypes, but subtypes A and C shared low levels of infection via CCR3 and high levels via FPRL1, in contrast to subtype B, which displayed high levels of infection via CCR3 and low levels via FPRL1. Subtype D Env proteins were poor at mediating infection via either CCR3 or FPRL1. All of these differences in entry phenotypes were highly significant (Fig. 6, legend). These subtype-specific patterns of CoR use go beyond differences previously observed (8, 61, 71), primarily because of the use of a highly permissive indicator cell line, stringent criteria for positive entry, and the addition of FPRL1 (67) to the list of alternative CoRs assayed. The implications of these results are that HIV-1 *env* has evolved differently in subtypes A and C compared to either subtype B or D infection, perhaps in response to subtle selection pressure influenced by the host genetics of the infected population or founder effects from

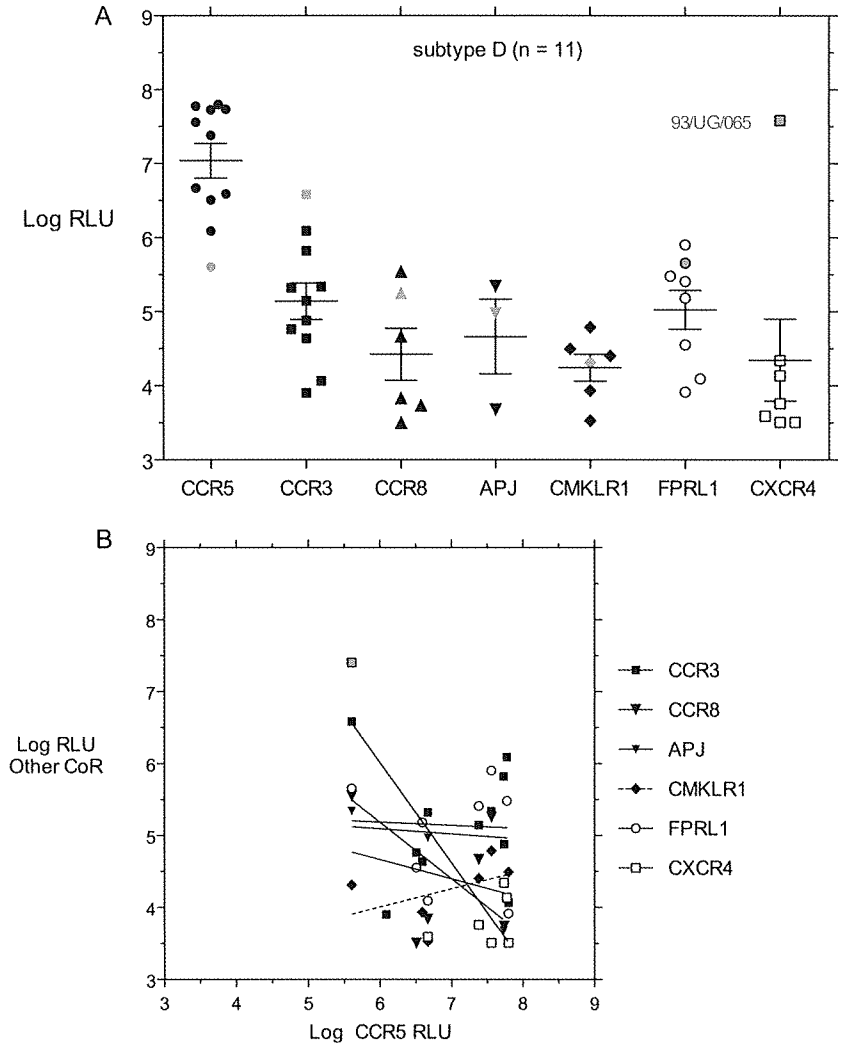

FIG. 5. (A) Entry mediated by 11 subtype D *env* clones from different patients plotted as described in the legend of Fig. 2. One subtype D *env* clone (93/UG/065) is depicted in lighter fill because it was the only one capable of robust entry via CXCR4. (B) Linear regression analysis of entry via CCR5 versus via alternative CoRs. No significant correlations were observed.

early in the epidemic. CCR3 and FPRL1, the two favored alternative CoRs, show little primary sequence homology in their extracellular domains (49), and neither has a particularly close homology to CCR5. Subtle differences in CCR5 binding by CD4-triggered Env protein may contribute to the subtype-specific patterns of alternative CoR use we have identified and may possibly relate to the development of resistance to CCR5-targeted entry inhibitors (72).

These experiments were predicated on the hypothesis that the ability of HIV-1 isolates to utilize CoRs other than CCR5 (clearly the primary CoR) might fall into two or more categories that would have implications for how Env evolution impacts CoR use. The first category would be defined by use of alternative CoRs that was correlated with entry activity mediated by CCR5, a finding that would suggest that alternative CoRs are surrogates for CCR5 and would generally be less efficient than CCR5 at mediating virus entry. The second category would be defined by independent use of alternative CoRs that is not predicted by entry efficiency via CCR5, and entry efficiency via the alternative CoRs might be greater or less than via CCR5. In the first category, the driving force for Env evolution is improved ability to use CCR5 for entry, and expanded use of alternative CoRs is a direct consequence of this evolutionary pathway. In the second category, Env evolution is more complicated, and pathways leading to CCR5 or alternative CoR use must diverge at some point prior to sampling of the HIV-1 quasispecies.

The results obtained favor the first hypothesis, namely, that the use of the preferred alternative CoR is correlated with (or predicted by) virus entry efficiency via CCR5. Linear regression analysis of the correlation between entry via CCR5 and other CoRs revealed a subtype-specific phenotype. For subtype A Env proteins, entry via CCR5 and FPRL1 was highly correlated (Fig. 2B). For subtype B, entry via CCR5 and CCR3 was highly correlated (Fig. 3B). For subtype C, entry via CCR5 was highly correlated with entry via FPRL1, CCR3, and CCR8 (Fig. 4B). For subtype D, entry via CCR5 did not correlate with entry mediated by any alternative CoR (Fig. 5B). We also

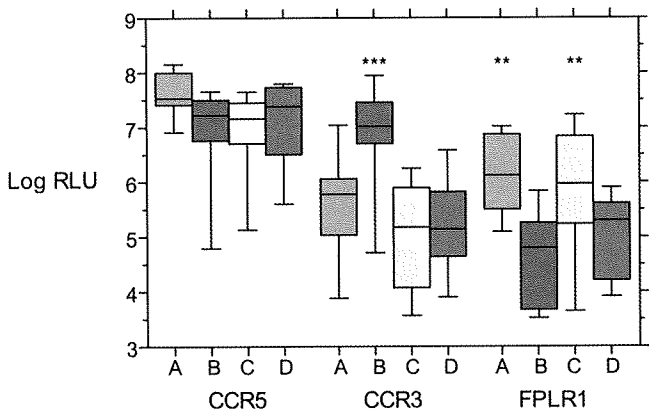

FIG. 6. Comparison of virus entry mediated by *env* clones from different subtypes (A, B, C, or D), with log RLU values depicted in box-and-whisker plots such that the vertical bars represent the range of observed values and the box represents the 25th to 75th percentile. Results for each subtype are color-coded with subtype A in blue, subtype B in magenta, subtype C in green, and subtype D in salmon. The difference in virus entry mediated by CCR3 for subtype B versus subtypes A, C, and D is highly significant (***, $P < 0.0001$; Mann-Whitney U test). The difference in virus entry mediated by FPRL1 for subtype B versus subtype A (**, $P = 0.005$) or versus subtype C (**, $P = 0.0094$) is significant, but there is no significant difference between subtype B and subtype D entry via FPRL1.

noted some subtype-specific differences in the frequency with which alternative CoRs could be used for entry (Table 1). Significant differences were a more frequent use of APJ in subtype B, a less frequent use of CMKLR1 in subtype C, a less frequent use of FPRL1 in subtype B, and a more frequent use of CXCR4 in subtype D.

Several prior reports have noted the high frequency of R5R3 viruses that show efficient entry via both CCR5 and CCR3 (1–3, 5, 17, 61), and some (77, 78) suggest possible target cell adaptation by this prevalent subset of HIV-1 phenotypes. Differences in cell lines used for typing CCR3 use could have contributed to distinct outcomes between our results and prior reports; specifically, U87/CD4/CCR3 cells obtained from the AIDS Research and Reference Reagent Program and used in the study by Morris et al. (54) express 100-fold lower levels of CCR3 than the NP-2/CD4/CCR3 cells we have employed (data not shown). Other studies employing transiently transfected target cell lines have also found a lower incidence of R5R3 viruses (see, e.g., reference 81), so it is likely that typing of CCR3 use is highly dependent upon the level of CCR3 expressed on a given target cell. The demonstration of HIV-1 entry via alterative CoRs other than CCR5 or CXCR4 is often dismissed as an artifact of using target cell lines that overexpress a given GPCR, a view that is bolstered by blocking infection of primary target cells by either CCR5 or CXCR4 inhibitors (30, 53, 80, 81). The phenotypes that we have observed are entirely based on overexpression of alternative CoRs on one cell line and do not address the important issue of whether or not these CoRs can be used for productive infection of natural target cells in HIV-1-infected individuals. However, we speculate that high entry efficiency via a CoR expressed at high levels may translate into low entry efficiency via the same CoR expressed at physiological levels.

FPRL1 emerged as a potentially important CoR in our studies. There have been prior studies suggesting that a peptide derived from either the C4-V4 region of subtype B Env (23) or from the V3 region (64) could react with FPRL1, but its use as an alternative CoR has only recently been documented (67). FPRL1 is expressed on a variety of human cell types, including monocytes, dendritic cells, and T cells (52), that potentially could serve as targets for HIV-1 infection. The importance of FPRL1 for infection of natural target cells remains to be established, but our results suggest that this may be a fruitful area for further investigation.

Use of CMKLR1/ChemR23 was less frequent in subtype C, in agreement with prior reports (49, 62). However, entry function via CMKLR1/ChemR23 did not correlate with CCR3 use despite the homology in the extracellular domains noted in a prior report (49). CMKLR1 was efficiently used for entry by SIVmac *env*-pseudotyped viruses (Fig. 1B), and we used the same cell line employed in prior studies, so these differences in results appear to reflect the selection of distinct *env* clones. We also noted a higher than expected frequency (5/22) of subtype C *env* clones that could mediate low-level entry via CXCR4. This may reflect our unique assay conditions since SIVmac *env* clones could also infect CXCR4-expressing target cells (Fig. 1B). No unique features of the five subtype C *env* clones capable of CXCR4 use were found, either in time of isolation or use of other CoRs, when they were compared to the other 17 clones incapable of mediating entry via CXCR4.

The results in Fig. 2 to 6 show only a subset of alternative CoRs. The primary reason for this is that few HIV-1 *env* clones were able to generate pseudotyped virus that could enter target cells via GPR1, CCR1, CCR6, CXCR6, or RDC1 although SIVmac *env* clones could mediate entry via these CoRs (Fig. 1B). We also did not have access to NP-2/CD4 target cells expressing GPR15 or D6, two alternative CoRs previously reported to mediate infection of other target cell lines (22, 55). It is possible that examination of these two additional alternative CoRs could add to the differences between HIV-1 subtypes that we observed. We also had access to only limited numbers of *env* clones from subtypes E, A/D, A/C, CRF01_AE, or CRF02_AG HIV-1 infections, and thus we cannot comment on the properties of these variants. Future analysis of a larger collection of natural recombinant viruses may be of use in dissecting the determinants for the subtype differences observed here.

Expansion of alternative CoR use to receptors other than CXCR4 and the relationship between CCR5-mediated entry and alternative CoR use distinguish different HIV-1 subtypes. Much of the data on alternative CoR use has been derived from the study of subtype B isolates, and larger sample sets from other subtypes need to be carefully examined for alternative CoR use. Even in subtype B-infected patients, reduction in levels of CCR5 or treatment with CCR5 inhibitors may increase the selective pressure to expand CoR use beyond CXCR4, as in the well-studied patient of Gorry et al. (30). Although there are many pathways to CCR5 inhibitor resistance (48, 73), these data suggest that we should remain alert for selection of rare variants with expanded CoR use beyond CXCR4, particularly in patients with non-subtype B HIV-1 infection.

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BIOLOGY CONTRIBUTION

IRRADIATION WITH CARBON ION BEAMS INDUCES APOPTOSIS, AUTOPHAGY, AND CELLULAR SENESCENCE IN A HUMAN GLIOMA-DERIVED CELL LINE

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Purpose: We examined biological responses of human glioma cells to irradiation with carbon ion beams (C-ions).
Methods and Materials: A human glioma-derived cell line, NP-2, was irradiated with C-ions. Apoptotic cell nuclei were stained with Hoechst 33342. Induction of autophagy was examined either by staining cells with monodansylcadaverine (MDC) or by Western blotting to detect conversion of microtuble-associated protein light chain 3 (MAP-LC3) (LC3-I) to the membrane-bound form (LC3-II). Cellular senescence markers including induction of senescence-associated β-galactosidase (SA-β-gal) were examined. The mean telomere length of irradiated cells was determined by Southern blot hybridization. Expression of tumor suppressor p53 and cyclin/cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ in the irradiated cells was analyzed by Western blotting.
Results: When NP-2 cells were irradiated with C-ions at 6 Gy, the major population of the cells died of apoptosis and autophagy. The residual fraction of attached cells (<1% of initially irradiated cells) could not form a colony: however, they showed a morphological phenotype consistent with cellular senescence, that is, enlarged and flattened appearance. The senescent nature of these attached cells was further indicated by staining for SA-β-gal. The mean telomere length was not changed after irradiation with C-ions. Phosphorylation of p53 at serine 15 as well as the expression of $p21^{WAF1/CIP1}$ was induced in NP-2 cells after irradiation. Furthermore, we found that irradiation with C-ions induced cellular senescence in a human glioma cell line lacking functional p53.
Conclusions: Irradiation with C-ions induced apoptosis, autophagy, and cellular senescence in human glioma cells. © 2010 Elsevier Inc.

Heavy ion beam, Glioma, Apoptosis, Autophagy, Cellular senescence, p53.

INTRODUCTION

Carbon, neon, and other heavy ions are charged particles with high linear energy transfer (LET), and their irradiation has shown higher relative biological effectiveness than low-LET radiation such as X-rays (1). In addition, the dose distribution of heavy ion beams exhibits a steep fall-off after the Bragg peak, and thus more precise dose localization can be achieved. Consequently, cancer radiotherapy using heavy ion beams have shown that serious damage to surrounding normal tissues can be markedly reduced. For clinical trials, carbon ion beams (C-ions) have been selected, and the efficacy of C-ions therapy has been demonstrated in various cancers (2).

It has been reported that irradiation with C-ions causes DNA damage, cell cycle arrest, apoptosis, or differentiation

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(3–6). Recently, nonapoptotic cell death, or autophagy, has been revealed as a novel response of cancer cells to ionizing radiation (7). In addition, ionizing radiation also induces cellular senescence, which shows characteristic changes in morphology, such as enlarged and flattened cell shape, and terminal growth arrest (8). However, induction of autophagy and cellular senescence in human glioma cells after irradiation with C-ions has not been well studied. In this study, to elucidate the biological responses of cultured cells to irradiation with C-ions, we examined the induction of apoptosis, autophagy, and cellular senescence in human glioma cells.

METHODS AND MATERIALS

Cells and cell culture

A human glioma cell line, NP-2 (a gift from Dr. T. Kumanishi, Niigata University, Niigata, Japan) at passage number around 70 was used. We also used human glioma-derived cell lines, U-87 MG at passage number around 40 and U-251 MG at passage number around 30. These cells were maintained in Eagle's minimum essential medium containing 10% fetal calf serum at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Irradiation

C-ions were accelerated by an azimuthally varying field (AVF) cyclotron in the Takasaki Ion Accelerator for Advance Radiation Application (TIARA) at the Japan Atomic Energy Agency (JAEA) as described previously (6,9). Accelerated C-ions passed through 30-μm-thick titanium foil, 10-cm-thick helium gas phase, 8-μm-thick Kapton polymidine film (DuPont-Toray Co., Tokyo, Japan), and 1-cm-thick air phase before reaching the cell monolayer. The penetration depth of C-ions used in this experiment was 1 mm. The LET at the cell surface was calculated according to the kinetic energy loss (E_{loss}), assuming that cells are equivalent to water. The dose rates of the C-ions were 1 Gy/s for doses greater than 5 Gy, 0.1 Gy/s for doses less than 5 Gy, and 0.01 Gy/s for doses less than 0.5 Gy.

One day before irradiation, 5 to 8 $\times$ 10^5 cells were seeded in 60-mm culture dishes, and exponentially growing cells were irradiated at room temperature with C-ions (18.3 MeV/u ^{12}C, LET = 108 keV/μm). Before irradiation, culture medium was removed and dishes were covered with 8-μm-thick Kapton polymidine films. NP-2 cells were also irradiated with X-ray machine at a dose rate of 0.83 Gy/min (Siemens-Asahi Medical Technologies, Tokyo, Japan).

Cell proliferation assay and clonogenic assay

NP-2 cells were washed with PBS, trypsinized, and counted at indicated intervals to examine the growth of cells. For clonogenic assays, irradiated cells were counted and plated in 60-mm dishes, cultured for 10 days, and stained with 0.2% methylene blue in 30% methanol. Colonies containing more than 50 cells were scored as survivors.

Apoptosis detection assay

For analysis of apoptotic cell death, Hoechst 33342 dye (Cambrex, Walkersville, MD) was added to cell culture medium at a final concentration of 1 μg/ml, incubated at 37°C for 30 min, and observed under a fluorescence microscope (DMI4000 B; Leica). Cells with chromatin condensation or nuclear fragmentation were considered as apoptotic cells. At least 300 cells in each dish from six to seven randomly selected fields were counted, and ratios of apoptotic cells were expressed as percentages of total cell counts. Apoptosis was also evaluated by detection of cleaved poly (ADP-ribose) polymerase–1 (PARP-1) fragment by Western blotting as described below.

Autophagy detection assay

It has been reported that autophagic vacuoles are specifically labeled with monodansylcadaverine (MDC) (10,11). NP-2 cells cultured on the slides were incubated with 0.05 mmol/l MDC (Sigma, St. Louis, MO) at 37°C for 60 min, washed twice with PBS, and fixed with 4% paraformaldehyde. After incubation at room temperature for 15 min, cells were washed with PBS and analyzed by a fluorescence microscope. At least 300 cells in each slide from six to seven randomly selected fields were counted, and ratios of MDC-positive punctuated cells were expressed as percentage of total number of cells, which had been attached to slides. Irradiated NP-2 cells were also treated with 5 mmol/l 3-methyladenine (3-MA) (Sigma) to inhibit early stages of the autophagic process (12). To induce autophagy of cells by amino acid starvation, cells were washed three times with PBS and incubated with Hank's balanced salt solution (HBSS) at 37°C for 2 h. The expression of microtubule-associated protein light chain 3 (MAP-LC3) (LC3) in the cells, which is a well-established marker of autophagosome (13), was also detected by Western blotting as described below.

Detection of senescence markers

Senescence-associated β-galactosidase (SA-β-gal) activity was detected as described previously (14). For detection of lipofuscin by Schmorl reaction (15), cells were fixed with 4% paraformaldehyde in PBS for 10 min, rinsed in PBS, and incubated for 5 min in a solution containing 0.75% ferric chloride and 0.1% potassium ferricyanide.

Detection of incorporation of BrdU into SA-β-gal–positive cells

Irradiated cells were labeled with 10 μmol/l of BrdU (Sigma) for 4 h at 37°C, washed, and fixed to detect SA-β-gal. After staining with SA-β-gal, cells were treated with 70% ethanol, washed, incubated with 2 N HCl at 37°C for 30 min, and neutralized with 0.1 mol/l Tris-HCl (pH 8.2). They were then probed with an anti-BrdU antibody (Merck Biosciences, Darmstadt, Germany) followed by peroxidase-labeled secondary antibody. After washing, color was developed using 3,3′-diaminobenzidine as a substrate.

Telomere restriction fragment analysis

The mean telomere lengths were determined by telomere restriction fragment (TRF) assay (16). Briefly, genomic DNA was prepared by Wizard Genomic DNA purification kit (Promega, Madison, WI), digested with *HinfI* and *RsaI*, separated through a 0.7% agarose gel, and stained with ethidium bromide. DNA was then transferred onto a nylon membrane, hybridized with 50 ng/ml of digoxigenin (DIG)–labeled telomeric oligonucleotide probe ($[TTAGGG]_4$), and the hybridized probe was incubated with a DIG-specific antibody coupled to alkaline phosphatase. Finally, telomere probe was detected by a chemiluminescent system (Roche Diagnostics, Mannheim, Germany). Hybridization signals were quantitated by using a CS analyzer (ATTO, Tokyo, Japan). The mean telomere lengths of samples were estimated by measuring migration distances of bands with the highest intensity.