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Coreceptor Usage of Primary Human Immunodeficiency Virus Type 1 Isolates Varies According to Biological Phenotype

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The biological phenotype of primary human immunodeficiency virus type 1 (HIV-1) isolates varies according to the severity of the HIV infection. Here we show that the two previously described groups of rapid/high, syncytium-inducing (SI) and slow/low, non-syncytium-inducing (NSI) isolates are distinguished by their ability to utilize different chemokine receptors for entry into target cells. Recent studies have identified the C-X-C chemokine receptor CXCR4 (also named fusin or Lestr) and the C-C chemokine receptor CCR5 as the principal entry cofactors for T-cell-line-tropic and non-T-cell-line-tropic HIV-1, respectively. Using U87.CD4 glioma cell lines, stably expressing the chemokine receptor CCR1, CCR2b, CCR3, CCR5, or CXCR4, we have tested chemokine receptor specificity for a panel of genetically diverse envelope glycoprotein genes cloned from primary HIV-1 isolates and have found that receptor usage was closely associated with the biological phenotype of the virus isolate but not the genetic subtype. We have also analyzed a panel of 36 well-characterized primary HIV-1 isolates for syncytium induction and replication in the same series of cell lines. Infection by slow/low viruses was restricted to cells expressing CCR5, whereas rapid/high viruses could use a variety of chemokine receptors. In addition to the regular use of CXCR4, many rapid/high viruses used CCR5 and some also used CCR3 and CCR2b. Progressive HIV-1 infection is characterized by the emergence of viruses resistant to inhibition by β-chemokines, which corresponded to changes in coreceptor usage. The broadening of the host range may even enable the use of uncharacterized coreceptors, in that two isolates from immunodeficient patients infected the parental U87.CD4 cell line lacking any engineered coreceptor. Two primary isolates with multiple coreceptor usage were shown to consist of mixed populations, one with a narrow host range using CCR5 only and the other with a broad host range using CCR3, CCR5, or CXCR4, similar to the original population. The results show that all 36 primary HIV-1 isolates induce syncytia, provided that target cells carry the particular coreceptor required by the virus.

Primary human immunodeficiency virus (HIV) isolates can be subdivided into two distinct groups according to their biological phenotype. Fast replication to high titers and syncytium formation in peripheral blood mononuclear cells (PBMC) and the capacity to infect and replicate in a broad range of T-lymphoid and monocytoid cell lines often characterize viruses isolated from immunodeficient patients (17, 37, 44). In contrast, slow replication to low titers and induction of small, if any, syncytia in PBMC are characteristic of viruses isolated from individuals with no or mild symptoms of HIV infection. These viruses lack the capacity to infect and replicate in established cell lines and thus do not induce syncytia in such cells. The two distinct groups of primary HIV isolates were classified as rapid/high or syncytium-inducing (SI) and slow/low or non-syncytium-inducing (NSI) isolates, respectively (17, 37, 45).

HIV-1 has been genetically classified into a major (M) group and a more distant outlier (O) group, and the M group has been further subdivided into nine subtypes on the basis of sequence diversity (29). Members of the same subtype differ by less than 10%, and those of different subtypes differ by 15% or more (25). Similarly to the phenotypes first described for HIV-1 of genetic subtype B, distinct biological phenotypes of subtypes A, D, E, F, and G have recently been identified (19, 21, 26, 43). All the available data suggest that the HIV-1 biological phenotype varies with the severity of HIV-1 infection across genetic subtypes (16).

Studies with HIV-1 of genetic subtype B have shown that progression of the infection from the asymptomatic phase to immunodeficiency is accompanied by a gradual increase in the replicative capacity of the viruses isolated, culminating in acquisition of the ability to induce syncytia in PBMC and replication in cell lines (5, 23, 38, 39, 45). The in vitro biological phenotype of HIV-1 has therefore been recognized as a predictive marker for progression (15, 18, 24, 35). In agreement, the early presence of rapid/high virus during seroconversion in adults or shortly after birth in infants leads to a higher rate of decline in $CD4^+$ T lymphocytes and early development of AIDS in both groups (33).

Several chemokine receptors have recently been shown to be necessary, along with CD4, for fusion of HIV-1 envelopes to the plasma membrane of target cells (reviewed in reference 12). In response to chemokines, these molecules, which belong to the family of seven transmembrane G-protein-coupled receptors, transduce signals that result in chemotaxis and, potentially, developmental processes (reviewed in reference 31).

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Since chemokines fall into two distinct groups based on the properties of their primary sequence, the corresponding receptors are named accordingly C-C or C-X-C receptors. Significantly, envelope glycoproteins from T-cell-line-adapted (T-tropic) viruses were found to utilize CXCR4 whereas envelope glycoproteins from primary non-T-cell-line adapted (also called macrophage-tropic or M-tropic) viruses utilized CCR5 and, in few cases, CCR3 (1–3, 6, 9–11, 14, 30). In line with this coreceptor usage, the β-chemokines, of the C-C type, RANTES, MIP-1α, and MIP-1β, have been shown to inhibit the infectivity of HIV-1 isolates unable to replicate in established cell lines (7, 22). These results have suggested that viruses with different biological phenotypes differ in the choice of chemokine receptor(s) (2, 6, 9, 11, 14). In the present study, we tested the receptor specificity of a set of cloned envelope glycoproteins from primary HIV-1 isolates. To further analyze the potential correlation of biological phenotype and stage of infection with coreceptor usage, a panel of 36 HIV-1 primary isolates from 30 individuals was tested for chemokine receptor specificity by infecting derivatives of the U87 glioma cell line (42) stably expressing CD4 and one of the chemokine receptors CCR1, CCR2b, CCR3, CCR5, or CXCR4. The results show that the previously established phenotypic differences between HIV-1 isolates correspond to distinct coreceptor usage.

MATERIALS AND METHODS

Patients and virus isolates. Virus isolates 92RW020, 92UG037, 92BR020, 92TH014, 92BR025, 92UG021, and 92UG024 were obtained through the WHO Network for HIV Isolation and Characterization (32, 43). Cloning and sequencing of envelopes have been described previously (19). Virus isolates A130, A136, A145, and A196 from transmitting mothers and A245 from a nontransmitting mother were characterized within the framework of studies on mother-to-child transmission (34). Isolates V2, V8, and V9 were part of a study on sexual transmission and were obtained during seroconversion (18). All other isolates were from patients with progressive HIV-1 infection (17, 23, 39, 45).

Prior to these experiments, the primary HIV-1 isolates were passaged three to five times in peripheral blood mononuclear cells (PBMC). Virus stocks were prepared by infecting 5×10^6 phytohemagglutinin P (PHA-P; Pharmacia, Uppsala, Sweden)-stimulated PBMC from two blood donors with cell culture supernatant containing 4 ng of HIV-1 p24 antigen per ml determined by an in-house HIV-1 p24 antigen enzyme-linked immunosorbent assay (36). PBMC cultures were maintained in RPMI 1640 medium (Gibco/BRL, Paisley, United Kingdom) with 10% fetal calf serum (Flow Laboratories, Costa Mesa, Calif.), 5 U of recombinant interleukin-2 (IL-2; Amersham, Little Chalfont, United Kingdom) per ml, 2 μg of Polybrene (Sigma, St. Louis, Mo.) per ml, and antibiotics (IL-2 medium). On day 7, the cultures were screened for HIV-1 p24 antigen. Subsequently, culture supernatants were harvested, clarified by centrifugation, and filtered (pore size, 0.45 μm), and aliquots were stored at −80°C until used.

Cell lines. U87.CD4 cell lines stably expressing CCR1, CCR2b, CCR3, CCR5, or CXCR4 were established as described previously (9). Briefly, cDNAs encoding the chemokine receptors were subcloned into pBABE-puro. Amphotropic virus stocks were prepared by transfecting BING packaging cells with the resulting plasmids. Supernatants were collected 48 h later and used to infect U87.CD4 cells. After another 48 h, cells were selected in medium containing 1 μg of puromycin per ml. The C-C-type chemokine receptors were expressed on the surface of the U87.CD4 cells, as assessed by mobilization of intracellular free Ca^{2+} in response to the appropriate chemokines. Expression of CXCR4 was monitored by flow cytometry with a monoclonal antibody (13).

Cell lines were maintained in Dulbecco's modified Eagle's medium (Gibco/BRL) with high glucose and with the addition of 15% fetal calf serum, 1 mM sodium pyruvate (Gibco/BRL), nonessential amino acids (Gibco/BRL), and antibiotics. The cultures were grown in 25-cm^2 tissue culture flasks (Costar) and split at a ratio of 1:3 twice a week by treatment with 5 mM EDTA (pH 8.0). For coculture experiments, the cells were seeded in 12-well plates (Costar) at a concentration of 10×10^5 cells per well in 4 ml of medium. For cell-free infection with primary isolates, the cells were seeded in 24-well plates (Costar) at 5×10^5 cells per well in 2 ml of medium. The plates were further incubated at 37°C under 5% CO_2 until cultures reached half confluence (after 1 to 3 days); they were then used for the different assays.

Infectivity assay with HIV-luciferase reporter virus. The infectivity assay with HIV-luciferase reporter virus has been described previously (8). In brief, U87.CD4 cells were seeded in 24-well tissue culture dishes (0.5×10^5 cells per well) and were infected on the following day with luciferase reporter viruses (50 ng of p24 antigen). Reporter viruses pseudotyped by a panel of genetically diverse envelopes were prepared by transfecting 293T cells with NL-luc-Env(−) and the appropriate Env expression vector (10 μg each), quantitated as previously described and stored in aliquots at −80°C. Lysates (120 μl) were prepared 2 days postinfection, and the luciferase activity in 20-μl samples was measured with commercial luciferase assay reagents (Promega) and a Wallac scintillation counter.

Cocultivation of infected PBMC with the U87.CD4 cell lines. PBMC cultures were infected with virus as described above. At 7 days postinfection, 5×10^5 to 8×10^5 infected PBMC/well were added to 12-well plates with the U87.CD4 cell lines. Noninfected PHA-P-stimulated PBMC and medium only were included as controls. At 48 h after initiation of cocultures, the plates were washed by being rinsed twice with phosphate-buffered saline (PBS), whereafter fresh medium was added. On days 2, 4 and 7, a sample of culture supernatant was withdrawn and stored at −20°C until tested for p24 antigen. Samples removed on day 2, before the washing procedure, contained input virus and were used as controls for the washing procedure. Samples from different time points within one experiment were tested for p24 antigen in the same enzyme-linked immunosorbent assay. Between days 3 and 5, wells with a confluent cell layer were treated with 5 mM EDTA and split 1:3, and fresh medium was added. Cell cultures were monitored under the microscope daily for 7 days for the presence of cytopathic effects.

Cell-free virus infection of U87.CD4 cell lines. Virus stocks were subjected to titer determination on PHA-stimulated PBMC as previously described (40). U87.CD4 cell lines were infected with 100 to 1,000 50% tissue culture infective doses ($TCID_{50}$) (corresponding to 2.5 to 30 ng of p24 antigen). Before infection, the cells were rinsed once with PBS and virus was added in a 1-ml final volume. At 24 h later, 1 ml of fresh medium was added. The cultures were rinsed twice with PBS on day 2 and observed daily for cytopathic effects, and supernatants were harvested on days 2 (before washing), 4, 6, 8, and 10 and tested for p24 antigen.

Serial passage of HIV-1 primary isolates in CCR3-, CCR5-, or CXCR4-expressing cells. Two primary isolates of the rapid/high phenotype with a broad coreceptor usage (isolates 29 and 31) were selected for this experiments. Virus stocks whose titers had been determined were used at 1,000 $TCID_{50}$ for cell-free infection of glioma cells in 24-well tissue culture plates as described above. When clear syncytium formation was observed (for CCR5- and CXCR4-expressing cells on day 4 or 5 and for CCR3-expressing cells on day 7 or later), the cell culture supernatants were harvested and centrifuged and 0.5-ml samples were used for further passage. Four passages were carried out on U87.CD4-CCR5 and U87.CD4-CXCR4 cells, and three passages were carried out on U87.CD4-CCR3 cells. Subsequently, passaged virus isolates were retested on all U87.CD4 cell lines in parallel with the original virus stocks.

Chemokine inhibition assay. A chemokine inhibition assay with sequential isolates with a phenotype switch was performed with the β-chemokines RANTES, MIP-1α, and MIP-1β as described previously (22). For the six virus isolates obtained from different genetic subtypes, chemokine inhibition was performed as follows. In brief, PHA-P-stimulated PBMC (10^5 cells/well) from one blood donor were infected with each isolate at three fivefold dilutions in a round-bottom microtiter plate (Nunc, Roskilde, Denmark). The infection was performed overnight at 37°C in the presence or absence of RANTES or MCP-1 (R & D Systems, Minneapolis, Minn.) at 250- and 62-ng/ml concentrations in duplicates. Every 48 h, the cells were washed by centrifugation and chemokine was added in fresh IL-2 medium. A $TCID_{50}$ assay of serial fivefold virus dilutions was performed in parallel. Inhibition by β-chemokines was evaluated by measuring p24 antigen production 7 to 12 days postinfection.

RESULTS

Envelopes of primary HIV-1 isolates cause segregation of chemokine receptor usage according to the biological phenotype, and not the genetic subtype of the virus. A representative panel of genetically diverse *env* genes was recently prepared from 35 primary HIV-1 isolates collected at major epicenters of the current AIDS pandemic (19). These genes were assessed for biological activity in the context of HIV-1 virions, and 15 of them were shown to encode fully functional envelope glycoproteins. We used these 15 *env* genes to generate HIV-luciferase reporter virus and tested for chemokine receptor usage on U87 cells stably expressing human CD4 and CCR1, CCR2b, CCR3, CCR5, or CXCR4 (9). The results, shown in Table 1, indicate that CCR5 and CXCR4 are the main coreceptors for HIV-1 subtypes A to E and G. The receptor usage of these isolates was closely associated with their biological phenotype but not their genotype: NSI isolates used CCR5, whereas SI isolates used CXCR4. One isolate (92HT593), previously classified as NSI, was able to use both CCR5 and CXCR4 as coreceptor. In addition, we tested the chemokine receptor usage and sensitivity to the β-chemokine RANTES of selected

TABLE 1. Coreceptor usage by envelopes of different genetic subtypes of HIV-1

Envelope[a]	Genetic subtype	Biological phenotype[b]	Luciferase activity (10^3 cps) in U87.CD4 cell lines expressing chemokine receptor[c]:				
			CCR1	CCR2b	CCR3	CCR5	CXCR4
92RW020.5	A	NA	0.1	0.1	0.1	2,896.7	0.1
92UG037.8	A	NSI	0.2	0.3	0.1	151.1	0.1
92US715.6	B	NSI	0.1	0.1	0.3	375.9	0.2
92HT593.1	B	NSI	0.1	0.1	0.3	120.8	922.6
92HT599.24	B	SI	0.2	0.5	0.4	0.1	432.3
92BR020.4	B	NSI	0.3	0.7	0.5	3,324.7	0.3
92TH014.12	B	NSI	0.1	0.1	1.9	927.2	0.3
91US005.11	B	NSI	0.6	0.1	0.1	1,787.7	0.4
92BR025.9	C	NSI	0.4	0.1	0.1	6,848.1	0.1
93MW965.26	C	NSI	0.1	0.1	0.4	128.8	0.1
92UG021.16	D	SI	0.1	0.3	0.1	0.1	221.8
92UG024.2	D	SI	0.1	0.1	0.7	0.1	38.4
93TH966.8	E	NSI	0.4	0.2	0.1	1,495.1	0.1
93TH976.17	E	NA	0.1	0.1	0.1	2,808.3	0.1
92UG975.10	G	NSI	0.4	0.1	0.1	6,761.8	0.1
HXB2[d]	B		1.7	0.3	1	0.2	952.9
ADA[e]	B		4	3.5	1.1	521.7	0.4
VSV-g[f]			691.4	540.2	432.2	165.9	170.5

[a] HIV-1 envelopes were derived from cloned virus isolates as previously described (19).
[b] The biological phenotype of all primary isolates listed was determined through syncytium induction and replication in MT-2 cells (23). NA, not assayed. In addition, isolates obtained through the WHO Network for HIV Isolation and Characterization were tested on CEM and U937 clone 2 cells and were classified as slow/low (NSI) or rapid/high (SI) (32).
[c] U87.CD4 cells expressing chemokine receptors were incubated with HIV-luc pseudotyped by the above panel of envelopes, and luciferase activity was measured as described previously (8). The values represent the mean value from duplicate experiments.
[d] HIV-1 clone derived from the primary isolate HIV-1_{IIIB}, previously described as prototype T-cell-line tropic.
[e] HIV-1 primary isolate, prototype non-T-cell-line tropic (M tropic).
[f] Envelope glycoprotein from vesicular stomatitis virus (VSV) was used as a control.

primary isolates (Table 2). With one exception, coreceptor usage of primary isolates correlated with that of the corresponding envelopes. In the exceptional case, the virus isolate (92UG024) used CCR3 in addition to CXCR4, used by its cloned envelope. Receptor usage of the primary isolates correlated with sensitivity to RANTES, in that 92UG037, 92TH014, and 92BR020, using the CCR5 receptor, were inhibited whereas 92UG024 and 92UG021, using CXCR4, were resistant. None of the isolates were inhibited by MCP-1 (data not shown).

Chemokine receptor usage of primary HIV-1 isolates from patients with HIV-1 infections of different severities. (i) Coculture of infected PBMC with the U87.CD4 cell lines. The next question was whether chemokine receptor usage of primary HIV-1 isolates would correlate with the severity of HIV-1 infection. For this purpose, a panel of 20 HIV-1 primary isolates from 19 individuals was tested for chemokine receptor usage. The 20 isolates used in these experiments showed differences in their biological phenotype according to the clinical condition of the patient at the time of virus isolation (Table 3). Patients from whom rapid/high (SI) virus was isolated had invariably low ($\leq 230 \times 10^6$ cells/liter) $CD4^+$ T-lymphocyte counts, whereas patients with slow/low (NSI) virus had $CD4^+$ counts in a higher range ($\geq 280 \times 10^6$ cells/liter). In the first series of experiments, we found that productively infected PBMC were able to infect and induce syncytia in U87.CD4 cells expressing particular chemokine receptors. The pattern of syncytium formation segregated according to the biological phenotype of the virus isolate. Beginning 24 h after coculture, syncytia were evident in CCR5-expressing cells infected with

TABLE 2. Coreceptor usage and chemokine sensitivity of primary HIV-1 isolates of different genetic subtypes[a]

Virus isolate	Genetic subtype	Biological phenotype[b]	% Inhibition by RANTES in PBMC cultures[c]:		Syncytium induction and p24 antigen production in U87.CD4 cell lines expressing chemokine receptor[d]:				
			250 ng/ml	62 ng/ml	CCR1	CCR2b	CCR3	CCR5	CXCR4
92RW020	A	NA	NA	NA	−	−	−	+++	−
92UG037	A	NSI	96	96	−	−	−	+++	−
92BR020	B	NSI	100	54	−	−	−	+++	−
92TH014	B	NSI	96	74	−	−	−	+++	−
92UG021	D	SI	0	0	−	−	−	−	+++
92UG024	D	SI	7	10	−	−	++[e]	−	+++

[a] From the WHO Network for HIV Isolation and Characterization. All patients were asymptomatic at the time of virus isolation.
[b] See Table 1, footnote *b*.
[c] Percent inhibition was evaluated at 10 to 52 $TCID_{50}$; NA, not assayed.
[d] Cells were infected with 1,000 $TCID_{50}$. Syncytium formation was evaluated on day 7 postinfection: −, no syncytia (<0.2 ng of p24 per ml); ++, large syncytia detected in every field (1 to 2 ng/ml); +++, large syncytia cover the entire well (>2 ng/ml). None of the isolates replicated or induced syncytia in the U87.CD4 parental cell line.
[e] p24 antigen values increased on days 4 to 7 postinfection; syncytium formation appeared on day 9.

TABLE 3. Cocultivation of U87.CD4 cell lines and PBMC infected with primary HIV-1 isolates obtained from patients with HIV-1 infection of different severities

Virus isolate	Patient clinical data		Virus isolate						
	Clinical status[a]	No. of $CD4^+$ cells (10^6/liter)	Replication in cell lines[b]	Biological phenotype	Syncytium induction in U87.CD4 cell lines expressing chemokine receptor[c]:				
					CCR1	CCR2b	CCR3	CCR5	CXCR4
196A	AS	183	+	Rapid/high	−	−	++	+++	+++
245A	AS	187	+	Rapid/high	−	−	−	+++	+++
31	AIDS	20	+	Rapid/high	−	−	+++[d]	+++[d]	+++
26	AIDS	230	+	Rapid/high	−	−	++[e]	−	+++
24	PGL	147	+	Rapid/high	−	−	−	−	+++
25	AIDS	100	+	Rapid/high	−	+[d]	+++	+++	+++
29	AIDS	90	+	Rapid/high	−	−	+++	+++	+++
V4	AIDS	89	+	Rapid/high	−	−	+	+++	+++
V7	AIDS	65	+	Rapid/high	−	−	−	−	+++
6A	PGL	370	−	Slow/low	−	−	±	+++	−
6B	PGL	470	−	Slow/low	−	−	−	+++	−
8	PGL	804	−	Slow/low	−	−	−	+++	−
12	PGL	330	−	Slow/low	−	−	−	+++	−
130A	AIDS	280	−	Slow/low	−	−	−	+++	−
145A	AS	600	−	Slow/low	−	−	−	+++	−
136A	AS	290	−	Slow/low	−	−	−	+++	−
V2	Seroconv.	565	−	Slow/low	−	−	−	+++	−
V6	PGL	465	−	Slow/low	−	−	−	+++	−
V8	Seroconv.	684	−	Slow/low	−	−	−	+++	−
V9	Seroconv.	382	−	Slow/low	−	−	−	+++	−
IIIB[f]					−	−	−	−	+++
BaL[g]					−	−	−	+++	−

[a] At the time of virus isolation: Seroconv., sample obtained during seroconversion; AS, asymptomatic; PGL, persistent generalized lymphadenopathy.

[b] All viruses were tested for replication in MT-2 cells: −, no replication; +, virus replication (p24 antigen-positive culture supernatant) and syncytium induction. In addition, virus isolates 130A, 136A, 145A, 196A, and 245A were tested on Jurkat and U937 clone 2 cells (34); V2, V4, V6, V7, V8, and V9 were tested on C8166 and HuT-78 cells (reference 18 and unpublished results); and 6A, 6B, 8, 12, 24, 25, 26, 29, and 31 were tested on Jurkat, CEM, H9, and U937 clone 16 cells (39, 45). Virus isolates 6A and 6B were collected from the same patient 6 months apart.

[c] Syncytium induction: −, no syncytia (<0.2 ng of p24 per ml); ±, rare small syncytia (0.2 to 0.5 ng/ml); +, small syncytia apparent in every field (0.5 to 1 ng/ml); ++, large syncytia detected in every field (1 to 2 ng/ml); +++, large syncytia covering the entire well (>2 ng/ml). Cultures were observed daily, and results obtained on day 2 are presented, except where noted. CCR3-positive isolates were tested two to five times.

[d] Results obtained on day 7.

[e] Results obtained on day 4.

[f] Laboratory strain, prototype T-cell-line adapted.

[g] Laboratory strain, prototype macrophage-tropic.

slow/low viruses and in CXCR4-expressing cells infected with rapid/high viruses. In addition, most of the rapid/high isolates were able to infect several cell lines, inducing syncytia in cells transfected with CCR3, CCR5, and, in one case, CCR2b (Table 3; Fig. 1). The cocultures were tested for p24 antigen production on days 2 (before washing), 4, and 7. Cultures with syncytia had increased levels of extracellular p24 antigen, whereas cultures negative for syncytia had decreased levels (data not shown). The results show that distinct chemokine receptor usage corresponds to the phenotype of primary HIV-1 isolates. Moreover, all primary isolates are able to induce syncytia in U87 cells, provided that the cells express the chemokine receptor used by the virus.

(ii) Cell-free infection of the U87.CD4 cell lines. Next, we analyzed the virus dose necessary to achieve productive infection and syncytium induction in the U87.CD4 cell lines. Six virus isolates were selected for these experiments, and 100 to 1,000 $TCID_{50}$ was used for infection. All viruses infected cells and induced syncytia according to the previously established pattern, provided that the virus dose was high enough, i.e., 1,000 $TCID_{50}$ (Table 4). Variable results were obtained at lower doses. In most cases, a $TCID_{50}$ of 100 was not sufficient to result in a productive infection and syncytium induction within 7 to 10 days. None of the virus isolates induced syncytia, even at the highest dose, in CCR1-expressing cells (Fig. 1A). In general, syncytia tended to be larger in CXCR4-expressing cells (Fig. 1E) than in the other cell lines, with the smallest syncytia being present in CCR2b-expressing cells (Fig. 1B).

Sequential isolates obtained from patients with progressive HIV-1 disease differ in coreceptor usage and sensitivity to β-chemokines. From a particularly interesting group of five patients with high rates of $CD4^+$ T-lymphocyte decline, pairs of virus isolates that had been obtained sequentially and had distinct phenotypes were tested for receptor usage. Viruses obtained from the same individual but differing in biological phenotype (in these cases distinguished by replication and syncytium induction in the MT-2 cell line) also differed in chemokine receptor usage. MT-2 negative (NSI) isolates used CCR5, while MT-2-tropic (SI) isolates used CXCR4 instead of or in addition to CCR5 (Table 5). Interestingly, viruses capable of using both receptors usually used CCR3 as well. Thus, the viruses appear to evolve over time to use several receptors. The results also show that β-chemokine receptor usage corresponds strictly to sensitivity to β-chemokines, expressed as the 50% inhibitory concentration in nanograms per milliliter. A mixture of RANTES, MIP-1α, and MIP-1β could block the infectivity for PBMC of viruses using CCR5 but not those using CXCR4 (Table 5) (22).

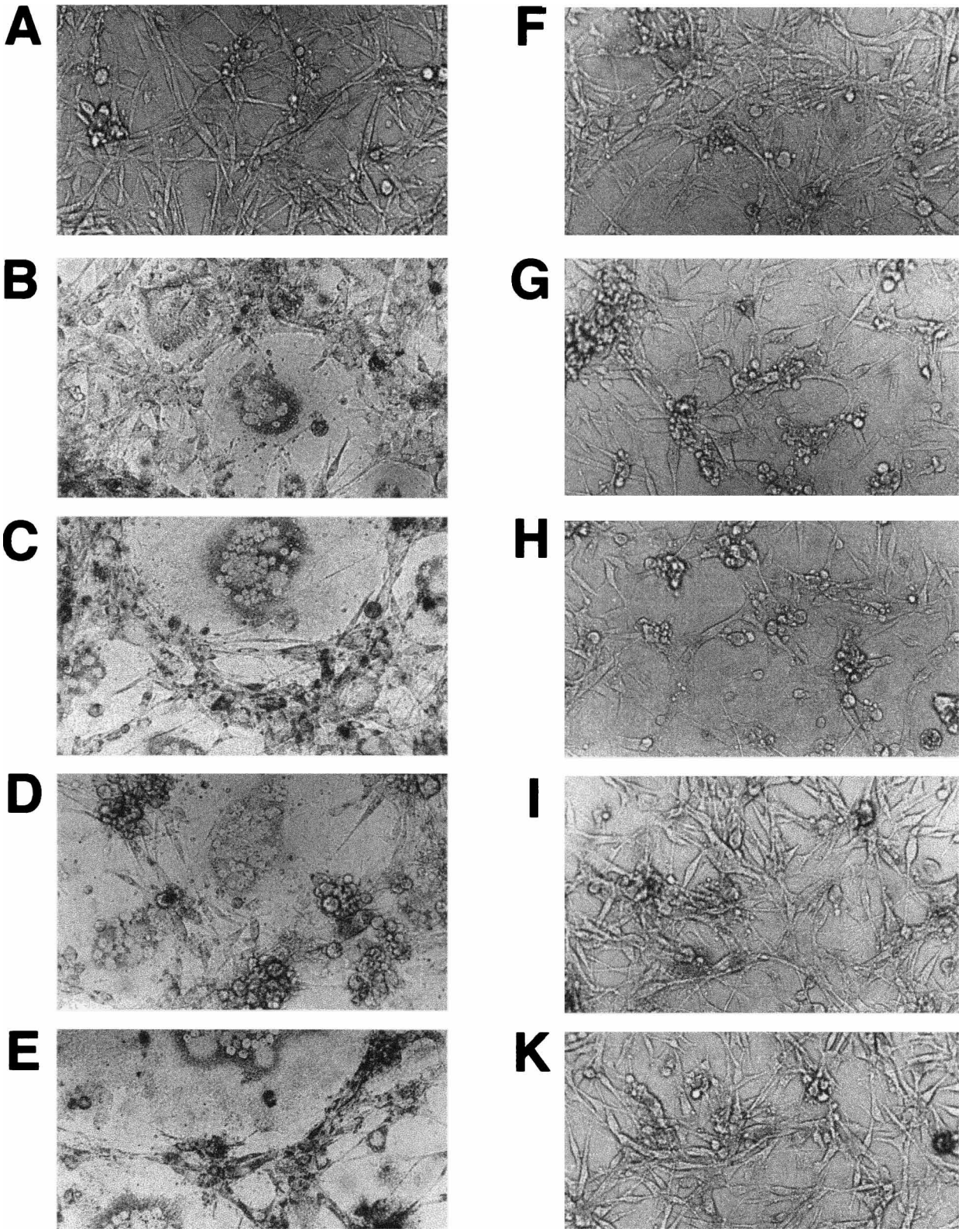

FIG. 1. Syncytium formation in U87.CD4 cell lines after direct infection with primary HIV-1 isolates. (A to E) Virus isolates 25 (A and B), 31 (C), 29 (D), and V7 (E). (F to K) Noninfected cells. The chemokine receptors used were CCR1 (A and F), CCR2b (B and G), CCR3 (C and H), CCR5 (D and I), and CXCR4 (E and K).

TABLE 4. Cell-free infection of U87.CD4 cell lines with selected primary HIV-1 isolates

Virus isolate	$TCID_{50}$[a]	Syncytium induction and p24 antigen production in U87.CD4 cell lines expressing[b]:				
		CCR1	CCR2b	CCR3	CCR5	CXCR4
31	500	NA	−	++	+++	+++
24	100	−	−	−	−	+
	500	−	−	−	−	+++
	1,000	−	−	−	−	+++
25	100	−	−	−	++[c]	+++
	500	−	±[c]	+[c]	+++	+++
	1,000	(+)[c]	±[c]	++[c]	+++	+++
29	100	−	−	±[c]	++[c]	++[c]
	500	−	−	++	+++	+++
	1,000	−	−	++	+++	+++
V7	100	−	−	−	−	++[c]
	500	−	−	−	−	+++
	1,000	−	−	−	−	+++
8	100	−	−	−	−	−
	500	−	−	−	+++	−
	1,000	−	−	−	+++	−

[a] Corresponds to 2 to 30 ng of p24 antigen per ml. Results obtained with 500 or 1,000 $TCID_{50}$ represent the mean values from two independent experiments.
[b] −, no syncytia (<0.2 ng of p24 per ml); ±, rare small syncytia detected (0.2 to 0.5 ng/ml); +, small syncytia apparent in every field detected (0.5 to 1 ng/ml); ++, large syncytia detected in every field (1 to 2 ng/ml); +++, large syncytia covering the entire well (>2 ng/ml); (+), no syncytia detected (0.5 to 1 ng/ml); NA, not assayed. Cultures were observed daily, and results obtained on day 3 are presented, except where noted. Viruses were retested two to five times with 1,000 $TCID_{50}$.
[c] Results obtained between days 7 and 10.

Serial passage in cell lines expressing individual chemokine receptors reveals heterogeneity in HIV-1 isolates. The ability of some isolates to use several different chemokine receptors was further explored. Isolates 29 and 31, which used multiple receptors, were selected for these experiments. Each of the virus isolates was passaged four times in U87.CD4-CCR5 or U87.CD4-CXCR4 cells or three times in U87.CD4-CCR3 cells and retested for receptor usage (Table 6). In both cases, passage in CCR5-expressing cells selected for virus that was able to replicate in CCR5-expressing cells but not in CXCR4- or CCR3-expressing cells. Interestingly, viruses selected for growth in cells expressing CXCR4 or CCR3 had a phenotype identical to that of the original isolate, maintaining their ability to use all three receptors. The results suggest that the primary isolates used in these experiments contained heterogeneous viral populations with regard to receptor requirements, with one using CCR5 only and the other using CCR3, CCR5, or CXCR4.

Replication kinetics of primary HIV-1 isolates using different coreceptors. The results of serial passage in cell lines expressing individual chemokine receptors also suggested that viruses using CCR5 only as the coreceptor may replicate more efficiently than multitropic viruses in the U87.CD4 cells and hence may outgrow the more promiscuous viruses in these cells. In fact, while four passages (4 to 5 days each) were carried out in the CCR5- or CXCR4-expressing cell lines, only three passages (at least 7 days each) could be carried out in the CCR3-expressing cells during the same period. Even so, one of the viruses passaged was lost (isolate 31), due to slow replication during the period allotted.

This prompted us to study the replication kinetics of three virus isolates differing in coreceptor requirements (Fig. 2). Virus levels increased rapidly in cells expressing CCR5 (Fig. 2) or CXCR4, as indicated by increasing p24 antigen values in

TABLE 5. Cell-free infection of U87.CD4 cell lines with pairs of primary HIV-1 isolates sequentially obtained from patients with progressive HIV-1 infection

Patient	Virus isolate	Clinical status[a]	$CD4^+$ T lymphocytes		Time[c] (mo)	MT-2 tropism[d]	Chemokine sensitivity (ng/ml)[e]	Syncytium induction and p24 antigen production in U87.CD4 cell lines expressing[f]:				
			Cell count (10^6/liter)	Rate of decline[b]				CCR1	CCR2b	CCR3	CCR5	CXCR4
A	J2195	AS	600			−	10	−	−	−	+++	−
	J4052	AS	260	−11, 1	20	+	>200	−	−	++[g]	+++	+++
B	J562	AS	250			−	<7	−	−	−	+++	−
	J975	AIDS	110	−5, 8	8	+	>200	−	−	++[g]	+++	+++
C	J669	AS	320			−	45	−	−	−	+++	−
	J1629	AIDS	210	−8, 5	15	+	>200	−	−	−	−	+++
D	J1874	AS	360			−	16	−	−	−	+++	−
	J2337	AS	410	−5, 5	6	+	>200	−	−	−	−	+++
E	J2090	ARC	50			−	42	−	−	−	+++	−
	J2822	ARC*	20	−6, 1	6	+	>200	(+)[g]	(+)[g]	+++[g]	+++	+++

[a] At the time of virus isolation: AS, asymptomatic; ARC, AIDS-related complex; ARC*, patient developed AIDS 4 months later.
[b] The rate of $CD4^+$ T-lymphocyte decline (10^6 cells/liter/month) was calculated by linear regression from multiple determinations during the entire observation period (40 to 100 months) (23). Patients B and C received zidovudine at the time of second isolation (virus isolates J975 and J1629, respectively); patient E received zidovudine at both samplings.
[c] Between the two samples indicated.
[d] All viruses were tested for replication (p24 antigen production) and syncytium induction in MT-2 cells: −, no syncytia/no p24; +, syncytia/p24 detected.
[e] Chemokine sensitivity as measured by the 50% inhibitory concentration of a mix of equal concentrations of chemokines MIP-1α, MIP-1β, and RANTES, starting with 200 ng of each per ml as previously described (22).
[f] For data on syncytium induction and p24 antigen production, see Table 4, footnote *b*. Cultures were observed daily, and results obtained on day 2 are presented, except where noted. CCR3-positive isolates were retested twice. Except for J2822, none of the isolates replicated or induced syncytia in the U87.CD4 parental cell line.
[g] Results obtained on day 4 or 5.

TABLE 6. Passage of primary HIV-1 isolates on chemokine receptor-expressing cells

Virus isolate	Cells used for passage	Syncytium induction and p24 antigen production in U87.CD4 cell lines expressing[a]:		
		CCR3	CCR5	CXCR4
29	U87.CD4-CCR3	+++	+++	+++
	U87.CD4-CCR5	−	+++	−
	U87.CD4-CXCR4	+++	+++	+++
31	U87.CD4-CCR3	NA	NA	NA
	U87.CD4-CCR5	−	+++	−
	U87.CD4-CXCR4	+++	+++	+++

[a] See Table 2, footnote *d*. None of the isolates replicated or induced syncytia in the parental U87.CD4 cells or cells expressing CCR1 or CCR2b.

cultures infected with viruses using these receptors (virus isolate J2822 from patient E and isolate J975 from patient B [Fig. 2A and B, respectively]). However, in CCR3-expressing cultures, an increase in p24 antigen level could not be detected before day 6. Replication of the J2822 virus in CCR2b-expressing cells showed a similar pattern. These observations may be due to relatively low-level expression of CCR2b and CCR3 or to their relatively poor function as viral receptors. Alternatively, this result may reflect the relative levels of viruses with different chemokine receptor specificities in mixed isolates.

Isolates J2822 (Fig. 2A) and 25 (data not shown) were particularly interesting because they were also able to infect the parental U87.CD4 cell line. The viruses replicated in these cells, as shown by extracellular p24 antigen production (illustrated in Fig. 2A by isolate J2822), but did not induce syncytia in these cultures or in CCR1-expressing cells. However, both isolates induced large syncytia if the cells infected expressed CCR3, CCR5, or CXCR4 in addition to CD4 (Tables 3 to 5). The two isolates differed in their capacity to induce syncytia in CCR2b-expressing cultures, in that isolate 25 induced syncytia while J2822 did not. These results suggest that in some patients HIV-1 may evolve to use coreceptors, other than those described above, that are expressed in U87 cells. Alternatively, there may be rare viruses that require only CD4 for infection, like those described by Shimizu et al. (35a).

DISCUSSION

Our results indicate that primary HIV-1 isolates with distinct biological phenotypes utilize chemokine receptors for entry into target cells according to a characteristic pattern. The ability of rapid/high (SI) viruses to infect established T-lymphoid and monocytoid cell lines is determined by their capacity to use the chemokine receptor CXCR4 as a coreceptor to CD4. This is in line with previous studies (9, 14) showing that the pattern of coreceptor usage depends on whether the envelope glycoprotein is derived from T-cell-line-adapted or nonadapted HIV-1 isolates. Furthermore, we show that among primary HIV-1 isolates, CCR5 is the most commonly used coreceptor. In general, such slow/low (NSI) viruses, unable to replicate or induce syncytia in cell lines, have a narrow host range and use CCR5 exclusively. In the present study, one slow/low virus (of 19 primary isolates tested), isolated from a patient with progressive HIV-1 infection (39), could use CCR3 in addition to CCR5, as shown by syncytium formation and p24 antigen production upon infection of cells expressing these coreceptors. In contrast, rapid/high viruses were often characterized by a broad host range and used CCR2b, CCR3, or CCR5 in addition to CXCR4. In a parallel study, Connor et al. made similar observations. A study of sequential isolates from three patients showed that dual-tropic viruses were associated with low CD4 counts in two of them (8a). In our study, this broadening of the host range may be even greater than currently suspected, since two viruses from AIDS patients were able to infect the parental U87.CD4 cells, which lack any transfected chemokine receptor. Although some viruses may evolve to infect cells through CD4 alone, it is more likely that the U87.CD4 cells express a novel coreceptor. Studies on infection of U87.CD4 with HIV-luc pseudotyped with simian immunodeficiency virus (SIV) envelope glycoproteins indicate that at least one additional coreceptor, utilized by SIV, is expressed in these cells (9a).

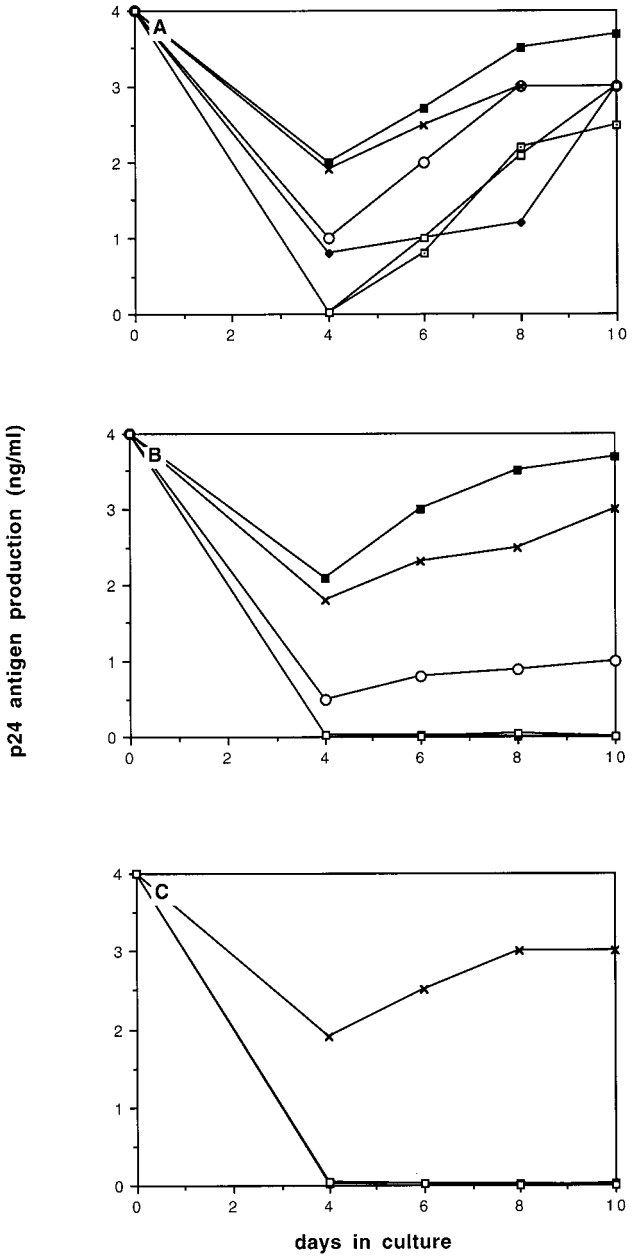

FIG. 2. Kinetics of virus replication in different U87.CD4 cell lines following infection with three selected virus isolates: patient E, isolate J2822 (A); patient B, isolate J975 (B); and patient C, isolate J669 (C). Culture supernatants were tested for p24 antigen at the times indicated. Symbols: ⊡, CCR1; ◆, CCR2b; ○, CCR3; X, CCR5; ■, CXCR4; □, CD4 only.

Since many chemokine receptors are expressed in the central nervous system, it would not be surprising to find that additional relevant receptors are expressed in this glial cell line.

Our results also show that envelopes of primary HIV-1 isolates segregate chemokine receptor usage according to the biological phenotype and not the genetic subtype of the virus. In a recent publication, Zhang et al. came to the same conclusion (44). We infected the U87.CD4 cells, in the cases where the primary virus isolate was available, and found that coreceptor usage agreed with the pattern established by using HIV-luc pseudotyped with the corresponding envelope glycoprotein. This clearly shows the role of the viral envelope glycoprotein in determining the biological phenotype of the virus. In one exceptional case (of 15 tested), the biological phenotype and chemokine receptor usage of the primary isolate and the corresponding envelope clone were discordant, indicating that a random selection of one clone from the heterogeneous population of a virus isolate may not necessarily yield viruses representative of the original virus isolate (28).

The pattern of coreceptor usage corresponded strictly to sensitivity to β-chemokines. The emerging general pattern is that viruses using the CCR5 coreceptor are sensitive to RANTES (Table 2) or to a mixture of RANTES, MIP-1α, and MIP-1β (Table 5) whereas viruses using CXCR4 are resistant. MCP-1, a chemokine not using CCR5 as the receptor, did not affect replication of the six isolates tested (data not shown), whereas isolates using the CCR5 receptor were shown to be sensitive to RANTES (Table 2). Changes in chemokine receptor usage of isolates obtained from the same patient over time paralleled changes in sensitivity to β-chemokines. Thus, progressive HIV-1 disease not only is associated with a change in the virus host range, enabling the virus to infect a different or a wider range of cells, but also seems to be linked to resistance to β-chemokines as well (22).

All primary isolates tested were able to induce syncytia provided that the cells expressed the particular coreceptor used by the virus. It is interesting that even if two virus isolates (isolates 25 and J2822) were able to infect and replicate in the parental U87.CD4 cells, syncytium induction was present only in cells coexpressing CXCR4, CCR5, CCR3, or, in one case, CCR2b. It is tempting to speculate that the presence of these chemokine receptors not only predisposes cells to infection with HIV-1 but also renders them more vulnerable to the cytopathic effect of the virus. However, it is important to recognize that the cell system used in these experiments is a model and that the U87 cell line and the in vivo target cells for HIV may show important differences. One such difference is evident when the replication kinetics of the different viruses are compared. While viruses appeared to replicate with similar kinetics in U87.CD4-CCR5 and U87.CD4-CXCR4 cells, one of the main distinctive properties of primary HIV-1 isolates was their speed of replication in primary PBMC cultures. This difference led to the previous designation of slow/low and rapid/high (45). Choe et al. (6) have pointed out the importance of the levels of cell surface expression of chemokine receptors for HIV-1 coreceptor function. Cells that stably express CD4 and the coreceptor, like the U87 series, may have relatively large amounts of these receptors, whereas lower CCR5 levels may be present in PBMC. This may explain why distinct phenotypes of primary HIV-1 isolates could be established in PBMC. If PBMC indeed have lower CCR5 concentrations than the U87.CD4-CCR5 cell line, replication by viruses using this receptor may be slow and not accompanied by syncytium induction. In line with this reasoning, PBMC and U87 cells would differ in the relative level of expression, but no intrinsic difference between the CCR5 and CXCR4 receptors would exist. In support of this model are the recent findings of Bleul et al. (4), showing that CCR5 expression is very low on unstimulated T cells (resting cells) and only weakly up-regulated by PHA-P compared to CXCR4 levels. In fact, CCR5 levels increase only after several days of IL-2 stimulation. Alternatively, a fundamental difference may exist between these receptors, and this may account for why CXCR4 usage is associated either with a more virulent phase of disease or, if such virus is transmitted, with an early and rapid decline in the number of $CD4^+$ cells. Conceivably, the CCR5 and CXCR4 receptors may set off different signalling pathways in the in vivo target cells, even if this is not evident in U87 cells.

In our study, viruses using the CCR3 receptor were able to use CCR5 or CXCR4 as well. No virus able to use CCR3 as the only coreceptor for CD4 was observed. One virus (isolate 6A) could use CCR3 or CCR5, similarly to the ADA and YU2 envelopes reported by Choe et al. (6). In our material, this phenotype was encountered once in 36 isolates and might therefore represent a rare phenotype among primary isolates. It remains to be seen if this is a true phenotype or if it reflects a mixture of viruses. Conceivably, faster replication of viruses utilizing CCR5 relative to the CCR3 coreceptor may result in selection for viruses able to use CCR5, competing out viruses using CCR3. In model experiments involving serial passage of two isolates in U87.CD4 cells expressing different coreceptors, we could show that, indeed, viruses using CCR5 only were selected from a population of viruses using CCR3, CCR5, or CXCR4. This indicates that viruses using CCR5 only as the coreceptor have a selective advantage over viruses that use several coreceptors. Recently, Simmons and colleagues compared coreceptor usage of biological clones, obtained through limiting dilution, with the parental virus isolates (35b). All the viruses were dual-tropic, indicating that the original isolates contained homogeneous populations. Different virus isolates are expected to contain different mixtures of viral populations with regard to receptor requirements; consequently, the properties of envelopes in the starting material will determine the outcome of selection, and the results may vary for different primary isolates. Again, we must remember that these results were obtained in the U87 model system, which may differ significantly from in vivo conditions. Nevertheless, we may envisage that selective mechanisms which operate at the receptor level play an important role in HIV pathogenesis.

It is intriguing that, over time in the same patient, the receptor usage and resistance to the direct inhibitory effect of β-chemokines change. Such changes may conceivably confer a replicative advantage to HIV-1 in vivo. It is also possible that the emergence of viruses with higher replicative capacity, which use the CXCR4 coreceptor, is a mere reflection of the collapse of the immune system, which finally allows uncontrolled replication. Broadening of the capacity to use several coreceptors may allow HIV-1 to infect a wide variety of cells and thereby accelerate immune deterioration. Since infection with rapid/high virus leads to a rapid loss of $CD4^+$ lymphocytes (18), it is tempting to speculate that the viral phenotype plays a major role in driving the progression of HIV-1-related disease through multiple mechanisms. Viral RNA levels in plasma have been shown to correlate with progression rates and can predict the severity of HIV-1 infection (20, 27, 41). The HIV-1 biological phenotype may be one of the determinants of viral load, of increased replicative capacity, and of broader cell tropism, which together may result in higher levels of viral RNA in plasma. Sensitivity to β-chemokines, as shown here, as well as humoral and cellular immune responses, may all contribute to the control of virus replication and thereby slow the disease process.

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