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Virus Isolates during Acute and Chronic Human Immunodeficiency Virus Type 1 Infection Show Distinct Patterns of Sensitivity to Entry Inhibitors

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We studied the effect of entry inhibitors on 58 virus isolates derived during acute and chronic infection to validate these inhibitors in vitro and to probe whether viruses at early and chronic disease stages exhibit general differences in the interaction with entry receptors. We included members of all types of inhibitors currently identified: (i) agents that block gp120 binding to CD4 (CD4-IgG$_2$ and monoclonal antibody [MAb] IgG$_1$b12), (ii) compounds that block the interaction with CCR5 (the chemokine RANTES/CCL5, the small-molecule inhibitor AD101, and the anti-CCR5 antibody PRO 140), (iii) the fusion inhibitor enfuvirtide (T-20), and (iv) neutralizing antibodies directed against gp120 (MAb 2G12) and gp41 (MAbs 2F5 and 4E10). No differences between viruses from acute and chronic infections in the susceptibility to inhibitors targeting the CD4 binding site, CCR5, or fusion or to MAb 2G12 were apparent, rendering treatment with entry inhibitors feasible across disease stages. The notable exceptions were antibodies 2F5 and 4E10, which were more potent in inhibiting viruses from acute infection ($P = 0.0088$ and 0.0005, respectively), although epitopes of these MAbs were equally well preserved in both groups. Activities of these MAbs correlated significantly with each other, suggesting that common features of the viral envelope modulate their potencies.

Therapy of human immunodeficiency virus type 1 (HIV-1) infection with a combination of antiretrovirals inhibiting the viral enzymes reverse transcriptase and protease can significantly decrease HIV-related morbidity and mortality (49, 62). However, due to the toxicity of these drugs and the emergence of resistant viral variants, alternative treatment strategies are urgently needed (31, 33, 36). Entry of HIV-1 into target cells requires expression of the receptor CD4 and a fusion coreceptor, most commonly the chemokine receptors CCR5 and CXCR4 (19, 66). The entry process proceeds via a cascade of events that provide multiple opportunities for therapeutic intervention, and several agents targeting this process have been developed over recent years. Considerable effort has been put into investigating the interaction of the virus with its entry receptors and the identification of potential antiretrovirals (66). Neutralizing antibodies were among the first agents identified which block viral entry. Direct antiviral activity is attributed to antibodies directed against specific epitopes on the envelope glycoproteins gp120 and gp41, which inhibit viral entry by blocking virion attachment to its receptors or membrane fusion (65). During natural infection the effect of the autologous neutralization response appears to be limited, since the virus rapidly escapes the immune pressure in most individuals (14, 15, 54, 55, 67, 76, 101). Nevertheless, rare potent monoclonal antibodies (MAbs) with broad activity have been isolated from infected individuals. These antibodies define four neutralization-sensitive epitopes within gp120 and gp41; they are characterized by the MAbs IgG$_1$b12 (5, 13, 78), 2G12 (80, 81, 97, 98), 2F5 (59, 71, 72), and 4E10 (84, 107) and have been shown to protect against HIV-1 infection in vitro and in animal models in vivo (4, 32, 52, 53, 64, 82).

Several types of entry inhibitors have been developed that block either the interaction of the virus with CD4, the coreceptor, or the fusion reaction (66). Among the first were soluble forms of the viral receptor CD4 which impede attachment of the virus to the cell-borne receptor. While the initial versions of this inhibitor were only weakly active in vivo (3, 23, 57), the consecutively arisen multivalent CD4 molecules have shown considerable inhibitory activity in clinical application (1, 35, 38, 39, 87).

The natural ligands of the coreceptors, the CCR5 ligands CCL5 (RANTES), CCL3 (MIP-1α), and CCL4 (MIP-1β) and the CXCR4 ligand CXCL12 (SDF-1), prevent entry of HIV-1 through downregulation of these receptors and potentially also through direct competition with the viral envelope for binding to the coreceptor (2, 21, 88, 96). In addition, several types of coreceptor antagonists, small molecules, peptides, chemokine derivatives, and MAbs specific for the chemokine receptors CXCR4 and CCR5 have been developed, some of which are candidates for clinical use (66). Of these, small-molecule inhibitors are the most promising in terms of efficacy and clinical application (66). However, all of these coreceptor inhibitors, including the natural chemokines, show differential potency in

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inhibiting diverse virus strains, which is probably a consequence of the high variability of the viral envelope genes (18, 41, 46, 85, 91, 92, 96).

The fusion inhibitor T-20 (enfuvirtide) is the first of the group of entry inhibitors approved for HIV-1 therapy (34, 40, 47, 48, 66). T-20 is a synthetic peptide composed of a 36-amino-acid sequence that mimics heptad repeat region 2 (HR2) of gp41, and by binding to HR1 it blocks the formation of the heterodimeric α-helical bundle of the gp41 trimer and thereby impedes fusion (40, 66).

With T-20 as the first entry inhibitor licensed for clinical use and several others that have already entered clinical evaluation, treatment strategies that include entry inhibitors will likely shape HIV therapies in coming years. Here we studied the effect of entry inhibitors on viruses isolated during acute and chronic infection. We included members of all types of inhibitors currently identified: compounds interfering with viral binding to CD4, the coreceptor, and the fusion process as well as neutralizing antibodies targeting the viral envelope. Our aim was to validate these inhibitors in vitro and to probe whether viruses at early and chronic disease stages exhibit general differences in the interaction with entry receptors. To best optimize the use of entry inhibitors and to evaluate their potency but also to estimate potential risks and failure, it is crucial to examine their effect on viruses derived both during early and later disease stages. A clear definition of the mode of action and the interdependencies of these compounds will not only provide valuable information for their clinical use but also improve our understanding of the viral entry process.

MATERIALS AND METHODS

Patients. Patient demographics are listed in Table 1. Twenty-seven acutely infected and 31 chronically infected patients were studied. Individuals with acute infection were enrolled in our clinic in a prospective study on antiretroviral treatment of early HIV infection. Blood was sampled from acutely infected individuals before initiation of therapy. Patients with acute infection were selected as follows: (i) acute retroviral syndrome (ARS) and negative or indeterminate Western blot result and (ii) ARS, risk behavior, negative HIV screening test 1 month before ARS, and/or low avidity anti-gp120 response. Chronically infected patients were recruited among individuals that previously had received antiretroviral treatment but at the time of blood sampling had undergone treatment interruption in clinical trials (29, 30). Written informed consent was obtained from all patients according to the guidelines of the University Hospital Zurich.

Reagents. AD101 (SCH-350581) was a kind gift from B. Barhoudy (Schering Plough) (92). The anti-CCR5 antibody PRO 140 and the CD4-IgG_2 molecule (PRO 542) were described previously (1, 61). MAb IgG_1b12 was a kind gift from D. Burton (5, 13, 78). MAbs 2F5 (59), 4E10 (84, 107), and 2G12 (98) have been described previously. T-20 was provided by Roche Pharmaceuticals (40).

Stimulated primary CD8-depleted PBMC. Buffy coats obtained from three healthy blood donors were depleted of $CD8^+$ T cells by using Rosette Sep cocktail (StemCell Technologies Inc.), and peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque centrifugation. Cells were adjusted to 4×10^6 per ml in culture medium (RPMI 1640, 10% fetal calf serum, 100 U/ml interleukin-2, glutamine, and antibiotics), divided into three portions, and stimulated with either 5 μg/ml phytohemagglutinin, 0.5 μg/ml phytohemagglutinin, or anti-CD3 MAb OKT3 as described previously (94). After 72 h, cells from all three stimulations were combined and used as a source of stimulated $CD4^+$ T cells for infection and virus isolation experiments.

Autologous patient viruses. Autologous virus was isolated from patient PBMC by coculturing patient $CD4^+$ T cells with stimulated CD8-depleted PBMC (103). Only early-passage virus (passages 1 to 3) was used for these studies. The 50% tissue culture infectious dose ($TCID_{50}$) and coreceptor usage of the obtained virus stocks were determined as described previously (11, 89, 90).

Sequence analysis. Virus was pelleted from culture supernatant and viral RNA extracted using the QIAGEN RNeasy kit (QIAGEN, Basel, Switzerland). PCR was performed in a single-tube system (QIAGEN one-step reverse transcription-PCR kit; QIAGEN, Hilden, Germany), and an additional "hot start" utilizing Ampliwax (Applied Biosystems, Rotkreuz, Switzerland) to separate cDNA synthesis and PCR was as described previously (79). Aliquots of a lower-phase mix were prepared, containing 10 μl of 1×X reaction buffer (including 2.5 mM $MgCl_2$), 4.7 μM of amplification primers, 2.35 mM $MgCl_2$, and 47 ng/μl poly(A) carrier RNA. Ampliwax was added to each reaction, and lower phases were sealed by incubation at 90°C for 5 min and cooling to room temperature. Upper-phase mix containing 30 μl 1.23×X reaction buffer (including 3 mM $MgCl_2$), 0.25 μM lower-strand primer, 0.63 mM deoxynucleoside triphosphates, and 6.3% (vol/vol) enzyme mix and template RNA (7 μl) were added. cDNA synthesis and subsequent amplification were performed for one cycle (50°C for 30 min and 95°C for 15 min) and 50 cycles (95°C for 10 s, 55°C for 15 s, and 60°C 105 s). The primer pair Mf161 (5′-AGAGAAATTGACAATTACACAAGCTTAATA TA-3′) and Mf156 (5′-AATCCTCGTTACAATCAAGAGTAAGT-3′) was mainly used; tests on PCR-negative samples were repeated with the alternative set Mf159 (5′-CTGGATGAGATTTGGGATAACATGACCT-3′) and Mf83 (5′-G GATCTGTCTCTGTCTCTCTCTCCACC-3′). PCR amplicons were purified with the QIAquick PCR purification kit (QIAGEN) and sequenced in both directions (1 μl each) using the PCR primers, ABI BigDye Terminator cycle sequencing (Applied Biosystems, Rotkreuz, Switzerland), and an automated capillary sequencer (ABI 3100). The sequences were edited with Lasergene software version 5.08 (DNASTAR Inc., Madison, WI), aligned with CLUSTAL W, and analyzed with MEGA version 2.1 (43). Sequence heterogeneity was detected in some instances as a consequence of direct sequencing of PCR products derived from samples containing mixed virus populations. Only the major variants, i.e., those exhibiting the strongest signal in the chromatograms, were used in the present analysis.

Plasma antibody titers to p24 and gp120 antigen. Plasma IgG titers to recombinant gp120 from the JR-FL strain (Progenics) and recombinant p24 (Aalto BioReagents) were determined by enzyme-linked immunosorbent assay as described previously (10, 93). Bound antibody was detected using alkaline phosphatase-conjugated anti-human immunoglobulin G (Sigma) and the luminescence generating CPD-Star system (Applied Biosystems). Midpoint titers were defined by linear regression analysis as the antibody dilutions giving half-maximal binding after background subtraction. Maximal binding was defined using the antibodies 2G12 and 37G12 as references for anti-gp120 and anti-p24 detection, respectively.

To determine the avidity of the anti-gp120 response, quadruplicates of each serum sample at a fixed concentration were allowed to bind to gp120-coated plates for 1 h at room temperature. Serum dilutions were chosen depending on the total gp120 titer of the sample: sera with anti-gp120 titers of <1:200 were used at a 1:40 dilution, and sera with titers of >1:200 were analyzed at a dilution of 1:200. Plates were washed twice with Tris-buffered saline (TBS), and then duplicates of serum samples were treated with either TBS or 6 M urea in TBS for 30 min at room temperature. Bound antibody was detected as described above. Avidity indices were determined by calculating the ratio of bound antibody in the presence of urea to antibody bound in the absence of urea.

Estimation of anti-CD4BS antibody titers. CD4 binding site (CD4BS)-specific antibodies were defined by their capacity to compete off CD4 binding to gp120 in a competition enzyme-linked immunosorbent assay. CD4-IgG_2 was biotinylated using the EZ-Link sulfo-NHS-biotinylation kit (Pierce). Binding of biotinylated CD4-IgG_2 to JR-FL gp120-coated plates (see above) was monitored using alkaline phosphatase-labeled streptavidin (Pierce) and the luminescence-generating CDP-Star system (Applied Biosystems). An appropriate dilution of the biotin-labeled probe that gives 65% of the maximal binding signal was determined and biotinylated CD4-IgG_2 used at this concentration in competition experiments. For this, gp120-coated plates were incubated for 1 h with serial dilutions of the patient sera (100 μl, starting at 1:10 dilution). Unbound plasma was washed away and biotinylated CD4-IgG_2 (100 μl) added and incubated for 1 h. Unbound reagents were washed away, and bound biotin-labeled CD4-IgG_2 was detected as described above. Each test plate contained 10 control wells for 100% biotin–CD4-IgG_2 binding (no competitor sera) and 8 wells with serial dilutions of the CD4BS antibody IgG_1b12 as a reference. Maximum inhibition of biotin–CD4-IgG_2 binding by CD4BS antibodies was determined as the concentration of IgG_1b12 at which saturation of the competition is achieved. The titer of CD4BS antibodies in patient sera was then estimated as the reciprocal dilution of the serum that gives a 50% reduction of the biotin–CD4-IgG_2 binding compared to the maximum inhibition achieved by IgG_1b12.

Inhibition by chemokines and coreceptor antagonists. Inhibition of infection by CCR5-targeting substances was assessed on stimulated CD8-depleted PBMC as described previously (96). Briefly, cells were incubated with serial dilutions of inhibitory substances in 96-well culture plates for 1 h at 37°C. Virus inoculum

TABLE 1. Patient and virus characteristics

Group and patient	Age (yr)	Gender[a]	Transmission[b]	Time HIV-1$^+$ (mo)	Clinical stage	RNA (copies/ml)[c]	CD4 (cells/μl)[d]	Coreceptor	Genotype
Acute									
AK103	45	m	2	<3	A	21,100	418	R5	B
AK104	20	m	2	<3	A	54,200	264	R5	CRF0_AE
AK105	32	m	1	<3	A	340,500	470	R5	B
AK112	31	m	2	<3	A	29,700	389	R5	G
AK114	49	m	1	<3	A	4,610	474	R5	B
AK115	41	m	1	<3	C	6,040	467	R5	B
AK116	36	m	1	<3	A	105,000	327	R5	B
AK119	39	m	1	<3	A	191,500	431	R5	B
AK120	30	m	1	<3	A	27,800	429	R5	B
AK121	34	m	1	<3	A	1,550	516	R5	B
AK122	30	m	1	<3	A	2,610,000	315	R5	B
AK125	55	f	2	<3	A	124,000	483	R5	A
002	56	m	2	<3	A	261,415	832	R5	CRF01 AE
003	40	m	4	<3	A	11,053	349	R5	B
007	19	f	2	<3	A	47,681	531	R5	C
009	26	m	1	<3	A	1,490,000	250	R5	B
015	49	f	2	<3	A	37,700	445	R5	CRF01 AE
016	30	m	1	<3	A	71,700	855	R5	CRF01 AE
017	24	f	2	<3	A	1,275,000	359	R5	G
018	30	m	1	<3	A	3,925,000	159	R5	B
019	24	m	1	<3	A	1,470,000	275	R5	F1
020	45	m	1	<3	A	113,500	345	R5	B
021	33	m	2	<3	A	3,900,000	302	R5	B
022	70	m	2	<3	A	17,877,000	169	R5	B
023	34	m	1	<3	A	11,000	492	R5	B
025	31	m	1	<3	A	36,600	329	R5	B
026	29	f	2	<3	A	172,500	402	R5	B
Chronic									
102	40	m	3	>24	C	4,607	594*	R5	B
105	51	f	2	>24	A	170,265	1,228*	R5	B
106	42	m	2,3	>24	A	3,136	528	R5	B
107	46	f	2	>24	A	1,468	431	R5	B
109	38	m	2,3	>24	B	946	946*	R5	B
111	37	m	1	>24	A	<6	385*	R5	B
113	61	m	1	>24	A	99,999	977	R5	B
114	34	m	1	>24	A	17,603	811	R5	B
115	26	m	1	>24	A	59,081	463	R5	B
116	52	m	2,3	>24	C	43,594	227*	R5X4	B
117	35	f	3	>24	A	13,705	474	R5	B
118	34	m	1	>24	B	10,080	806	R5	B
119	37	m	2	>24	A	112,550	329*	R5	B
120	55	m	1	>24	A	291,771	488	R5	B
121	39	m	2	>24	A	283,140	397	R5	B
122	42	m	1	>24	A	22,510	506*	R5	B
123	42	f	3	>24	A	1,613	741	R5	B
125	34	f	2	>24	A	17,551	783*	R5	CRF01 AE
126	51	m	1	>24	A	10,000	354	R5	B
127	51	f	2	>24	A	2,692	699*	R5	B
128	43	f	2,3	>24	A	25,714	524	R5	B
130	67	m	2	>24	A	1,304	689*	R5	A
S2201	57	m	1,2	>24	A	32,773	682	R5	B
S2202	48	m	1	>24	A	146,632	315	R5	B
S2203	44	m	1	>24	A	194,754	728	R5	B
S2204	34	m	2	>24	A	8,255	902	R5	B
S2206	55	m	1	>24	A	129,088	609	R5X4	B
S2208	34	m	1	>24	A	267,336	465	R5	B
AK111[e]	41	f	2	>6	A	2,910,000	263*	R5	B
AK117[e]	39	m	1,2	>6	A	46,000	326	R5	B
027[d]	40	m	2	>6	A	112,000	132	R5	B

[a] m, male; f, female.
[b] 1, homosexual; 2, heterosexual; 3, intravenous drug use; 4, needle stick.
[c] Viral load at the day of virus isolation or the geometric mean of the two closest time points before and after.
[d] CD4 count at the day of virus isolation or the mean (*) of the two closest time points before and after.
[e] Patient recruited as acutely infected but reclassified as chronically infected according to Western blot results.

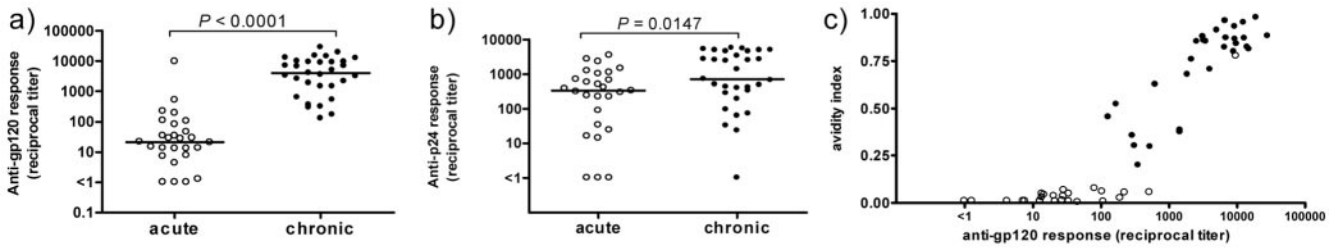

FIG. 1. Antibody titers to Env and Gag. (a and b) Binding antibody titers to (a) Env (gp120) and (b) Gag (p24) in the acute and chronic patient groups. Means from two independent experiments are shown. A value of <1 indicates undetectable reactivity to HIV-1 antigen. Groups were compared using the Mann-Whitney test. Horizontal lines indicate median values. (c) Analysis of avidity of the anti-gp120 response in acutely and chronically infected individuals. Means from two independent experiments are shown. Open and closed circles indicate acutely infected and chronically infected individuals, respectively. Significance was evaluated after Bonferroni correction.

($100\ TCID_{50}$) was then added and plates cultured for 4 to 14 days. The total infection volume was 200 μl. Culture supernatants were harvested at multiple time points between days 4 and 14, depending on the viral growth kinetics. Data for the analysis presented here are derived from the day when virus replication in the assay peaked. The calculated inhibitory doses refer to the final concentration of drugs in the culture on day 0. Virus production in the absence of drugs was designated 100%, and the ratios of p24 antigen production in drug-containing cultures were calculated relative to this. The drug concentrations (ng/ml) causing 50%, 70%, and 90% reduction in p24 antigen production were determined by linear regression analysis. If the appropriate degree of inhibition was not achieved at the highest or lowest drug concentration, a value of > or < was recorded and these upper or lower limits were used for statistical analysis. In order to control for assay variation due to differential CD4 and coreceptor expression and allow analysis of the impact of different inhibitors, assays with RANTES, PRO140, AD101, T-20, CD4-IgG_2, and IgG_1b12 were conducted for all 58 viruses using the same target cells on the same day.

Neutralization assay. The neutralization activity of agents targeting virus (2G12, 2F5, 4E10, T-20, CD4-IgG_2, and IgG_1b12) was evaluated on CD8-depleted PBMC as described previously (95). Briefly, the virus inoculum ($100\ TCID_{50}$) was incubated with serial dilutions of antibodies for 1 h at 37°C. Stimulated PBMC were then infected with aliquots of this preincubation mixture. The total infection volume was 200 μl. Cultures were incubated in 96-well culture plates and assayed for p24 antigen at multiple time points between days 4 and 14, depending on the viral growth kinetics. Data for the analysis presented here are derived from the day when virus replication in the assay peaked. Calculation of inhibitory doses was as described above. The neutralization activities of T-20, CD4-IgG_2, and IgG_1b12 were tested in parallel with the CCR5 inhibitors (see section above). The neutralization activities of 2G12, 2F5, and 4E10 were always tested in parallel, albeit for logistical reasons on different target cells.

Data analysis. Statistical analysis was performed using GraphPad Prism version 4.0, GraphPad Software Inc. Patient groups were compared using the Mann-Whitney test. Ninety percent inhibitory doses were used in this analysis in order to reflect the potency of the individual inhibitors.

To allow comparison of inhibitors with various potencies, we used the 70% inhibitory doses of the substances in the correlation analysis. For several virus-inhibitor pairs, 90% inhibition was not achieved. By using 70% inhibitory levels instead, we had more actual values for the analysis available. Using 70% inhibitory doses rather than 90% inhibitory doses has also the advantage that in most cases inhibition profiles follow a sigmoid curve where 70% inhibitory levels are more likely to be close to the point of inflection, whereas 90% is often not achieved or lies in the area of the curve where saturation is reached and calculation is more error prone. Isolate S2203, which was exorbitantly sensitive to all inhibitors tested, was excluded from regression analysis to avoid bias of the analysis.

Since in several cases multiple testing was performed, we analyzed significance on the individual and also after significance level adjustments using the Bonferroni adjustment. For group comparisons a P value for significance according to Bonferroni was 0.05 divided by the number of groups analyzed (for two groups, $P = 0.025$; for three groups, $P = 0.017$). In the correlation analysis, altogether 45 combinations were analyzed. The P value for significance according to Bonferroni was therefore $0.05/45 = 0.0011$.

RESULTS

Phenotypic and genotypic evaluation of autologous patient isolates during acute and chronic disease stages. The objective of the present study was to investigate whether viral isolates during early and later stages of the infection differ in their interaction with the viral receptors and hence display different sensitivities to inhibitors targeting the entry process.

We isolated virus from 27 patients during primary infection and from 31 chronically HIV-1-infected individuals (Table 1). Virus isolates from acutely infected individuals were from patient PBMC collected during the first 3 months of the infection. Viral strains from chronically infected individuals were isolated after a minimum of 6 months following primary infection. X4 usage is rare among virus strains derived during acute infection, and viruses isolated during earlier chronic disease stages also predominantly utilize CCR5 (19). Accordingly, the majority of the isolates in our cohort utilized solely CCR5 as a coreceptor. Only two R5X4 isolates were found in the chronic infection group (Table 1). The majority of the viral isolates in both the acutely and chronically infected patient groups were from subtype B. Nine isolates in the acute group and two isolates in the chronic group were from an alternate subtype (Table 1).

Autologous antibody titers to p24 and gp120 in acute and chronic infection. We characterized the HIV-1-specific antibody profiles in the acute and the chronic patient cohorts at the time of virus isolation to ensure that the patients classified with an acute infection did indeed show characteristics of the HIV-1-specific antibody response at this stage (that is, a low-titer, low-avidity response to selected antigens). To this end, we determined antibody titers against HIV-1 envelope (Env) and Gag proteins in the corresponding plasma samples. Antibody titers to gp120 were significantly lower in the acute patient group compared to the chronic infection group (Fig. 1a). While median anti-gp120 titers were only 1:21 in the acute group, median levels in the chronic group rose to 1:4016 ($P < 0.0001$). Anti-p24 responses were also lower in the acute group, although the differences were moderate, with median titers of 1:331 and 1:717 for acute and chronic patients, respectively ($P = 0.015$) (Fig. 1b). The anti-gp120 response in acutely infected individuals was almost exclusively a low-avidity response, while chronically infected patients had a medium- to high-avidity response to gp120 (Fig. 1c). One notable exception was patient AK120, who, despite being diagnosed with an acute infection within 3 months postexposure, had an uncharacteristically high-titer response to gp120 which also showed considerable avidity (Fig. 1c).

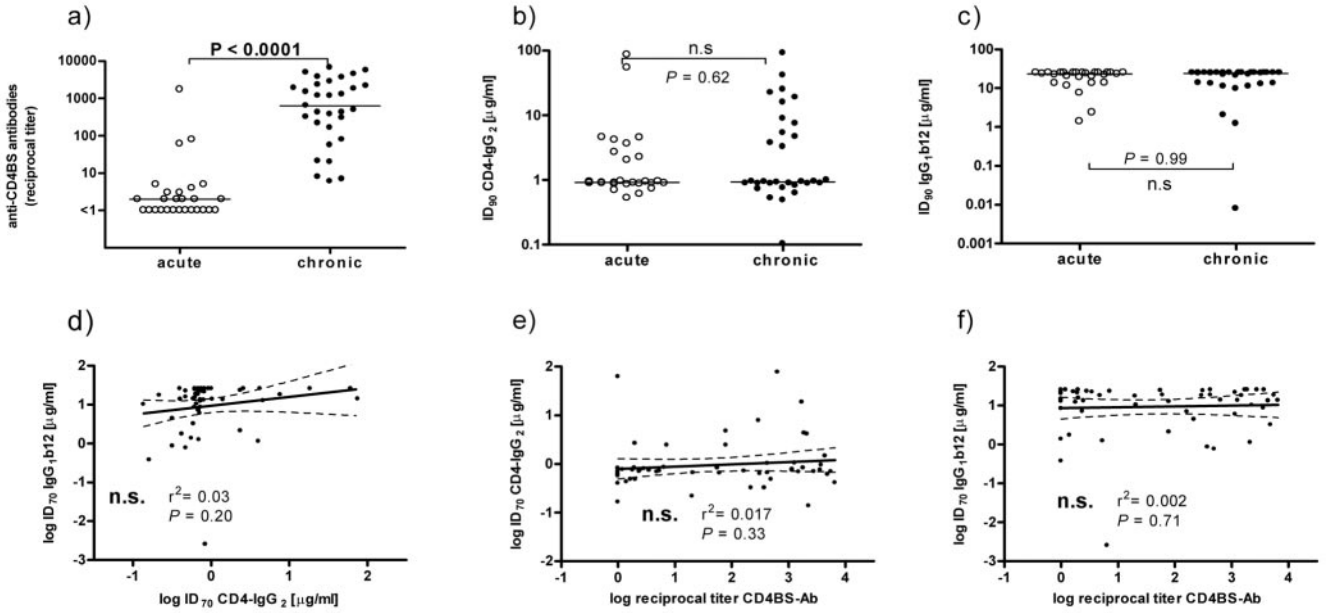

FIG. 2. CD4 binding site inhibitors. (a) Antibody titers in acute and chronic patient groups directed against the CD4BS. Means from two independent experiments are shown. A value of <1 indicates undetectable levels of CD4BS antibody titers. (b and c) Ninety percent inhibitory doses of CD4-IgG$_2$ and IgG$_1$b12 in acutely and chronically infected individuals. Means from two independent experiments are shown. Groups were compared using the Mann-Whitney test. Open and closed circles indicate acutely infected and chronically infected individuals, respectively. Horizontal lines indicate median values. *P* values for subtype B viruses only: CD4-IgG$_2$, $P = 0.71$; IgG$_1$b12, $P = 0.60$. (d) Correlation analysis of sensitivities to CD4-IgG$_2$ and IgG$_1$b12. (e and f) Correlation analysis of anti-CD4BS responses in autologous sera and sensitivities to CD4-IgG$_2$ and IgG$_1$b12, respectively. Significance was evaluated after Bonferroni correction. The solid lines are the regression lines, and the dashed lines indicate 95% confidence intervals.

Interaction with CD4. Primary HIV-1 isolates have proven to be relatively resistant to soluble CD4, probably due to the fact that these viruses have adapted to replicate in the presence of neutralizing antibodies to CD4BS, which are highly prevalent in patient sera (3, 12, 23, 37, 57, 58, 60, 63, 65, 86). HIV-1 cultured in the absence of neutralizing antibodies in vitro rapidly adapts, and more-neutralization-sensitive variants emerge (6, 56, 57, 70, 100). This change in phenotype has been attributed to an alternate interaction with CD4, potentially generating a greater affinity of the envelope for CD4 while simultaneously exposing neutralization epitopes, particularly those associated with the CD4 binding site (70). Coincidentally with the increased sensitivity to CD4BS antibodies of in vitro-cultured viral isolates, a heightened sensitivity to soluble CD4 is observed (56, 57, 70, 104). Since antibody responses are not fully matured during primary infection, it is possible that virus isolates derived early in the course of infection have not yet experienced selective pressure from CD4BS antibodies. If this is the case, then interaction of acute viruses with CD4 might reflect the situation found in vitro when viruses are cultured in the absence of antibody pressure. To explore this, we first determined the titers of CD4BS antibodies in patient sera. CD4BS antibody titers were low or undetectable in acutely infected individuals (with the exception of patient AK120) but were present in most chronically infected individuals at fairly high titers (Fig. 2a). To probe whether virus isolates in acute and chronic disease stages substantially differ in the interaction with CD4, we evaluated the sensitivity of the virus isolates to inhibition by the tetrameric CD4 molecule CD4-IgG$_2$ (PRO 542) (1, 38, 39) and the broadly active CD4BS antibody IgG$_1$b12 (13, 78). All virus isolates tested were sensitive to CD4-IgG$_2$, and 90% inhibitory doses ranged from 0.1 μg/ml to 92.0 μg/ml (median, 0.94 μg/ml) (Table 2). IgG$_1$b12 was less potent: 24 isolates were not inhibited at the highest concentration tested (25 μg/ml), and 90% inhibitory doses ranged from 8 ng/ml to >25 μg/ml. No differences between the acute and chronic patient groups in the sensitivity for CD4-IgG$_2$ or IgG$_1$b12 were found (Fig. 2b and c). Of note, although IgG$_1$b12 and CD4-IgG$_2$ target similar albeit not identical binding sites on gp120, sensitivities to these inhibitors did not correlate (Fig. 2d). Furthermore, the magnitude of autologous CD4BS antibodies did not predict sensitivity to CD4-IgG$_2$ and IgG$_1$b12 in chronically infected individuals (Fig. 2e and f).

Dependency on the interaction with the coreceptor CCR5. To evaluate whether viral isolates at early and late disease stages differ in respect to the interaction with CCR5, we assessed the sensitivity of the R5 virus isolates in our panel to inhibition by CCR5 targeting inhibitors. We assessed the inhibitory effects of the chemokine RANTES/CCL5, the small-molecular inhibitor AD101 (SCH-350581), and the anti-CCR5 antibody PRO 140 on viral replication in stimulated healthy donor CD4$^+$ T cells. Since coreceptor expression levels are known to vary substantially between individuals, all isolates were analyzed on cells from the same donors to rule out distortion of the results by target cell influences.

The three inhibitors act by different modes in perturbing HIV-1 binding to the receptor and viral entry. RANTES/CCL5, like all natural CC chemokine ligands for CCR5, blocks HIV-1 infection by potently downregulating the receptor and potentially also by direct competition with the viral envelope

TABLE 2. Ninety percent inhibitory doses of entry inhibitors

Group and patient	90% inhibitory dose								
	CD4-IgG_2 (μg/ml)	IgG_1b12 (μg/ml)	RANTES (ng/ml)	PRO 140 (μg/ml)	AD101 (nM)	T-20 (μg/ml)	2G12 (μg/ml)	2F5 (μg/ml)	4E10 (μg/ml)
Acute									
AK103	0.90	23.1	402	4.9	4.5	3.3	9.8	13.0	8.4
AK104	0.90	24.8	397	1.1	1.3	4.2	>25	8.2	1.0
AK105	0.70	13.7	313	0.24	1.9	0.46	>25	20.5	13.1
AK112	0.86	13.5	48	1.0	1.9	0.92	1.7	3.0	1.5
AK114	0.73	1.4	48	0.19	1.6	0.52	>25	6.5	1.3
AK115	0.87	21.9	438	1.3	6.2	4.0	8.1	3.8	2.9
AK116	87.0	>25	>500	>25	>25	9.5	23.7	11.5	13.9
AK119	0.89	>25	195	1.3	1.7	2.2	7.5	3.5	1.7
AK120	54.5	>25	420	2.3	10.6	4.8	1.5	4.6	2.5
AK121	2.7	>25	359	1.0	0.89	5.4	0.9	4.2	1.5
AK122	0.94	23.7	410	1.2	1.3	4.3	4.5	8.3	1.3
AK125	0.87	20.1	225	1.1	2.1	4.8	>25	4.6	4.5
002	2.3	24.0	47	2.2	1.0	0.87	>25	6.4	1.2
003	0.91	>25	126	1.0	2.0	2.1	<0.2	3.8	1.7
007	2.0	19.3	155	0.81	2.0	1.5	>25	5.7	2.1
009	0.89	>25	393	9.4	2.0	4.8	20.0	15.0	9.1
015	0.90	7.6	294	0.22	2.0	0.50	>25	3.5	7.2
016	0.95	13.5	390	1.5	2.2	0.45	>25	2.9	1.7
017	0.53	22.6	38	0.52	1.3	0.39	2.8	1.5	2.8
018	0.94	20.7	340	0.57	2.0	4.3	>25	4.8	4.1
019	1.0	>25	233	1.7	1.9	7.8	6.8	14.8	3.2
020	4.5	>25	421	21.0	19.7	4.3	10.2	8.5	4.0
021	3.7	>25	391	4.6	2.2	2.9	1.4	13.1	9.3
022	0.90	11.6	205	1.1	1.4	0.89	>25	14.0	3.0
023	4.2	>25	378	10.4	11.5	5.0	>25	>25	12.4
025	4.5	2.4	48	1.7	1.8	2.8	<0.2	3.0	3.0
026	0.61	>25	413	1.2	2.1	4.4	>25	18.8	6.3
Chronic									
102	5.4	>25	144	3.3	2.2	4.5	0.2	22.2	19.3
105	1.0	0.008	46	0.11	2.2	0.58	0.4	8.4	7.6
106	8.9	2.0	442	15.5	15.4	4.4	>25	14.0	6.6
107	0.82	13.4	257	2.1	2.2	0.86	1.1	4.4	4.3
109	0.95	22.4	345	8.1	9.9	0.87	>25	19.7	20.2
111	0.76	13.6	257	0.34	1.1	4.0	18.4	13.5	4.5
113	19.0	>25	465	8.6	7.5	2.6	18.7	23.7	>25
114	0.49	9.9	422	1.3	10.6	4.7	18.0	>25	11.4
115	3.8	>25	258	5.7	1.4	0.49	>25	>25	4.1
116	92.0	>25	>500	24.4	>25	2.9	15.8	17.1	16.3
117	0.93	>25	443	7.6	13.2	4.4	2.4	9.8	13.4
118	0.89	>25	180	1.1	1.1	9.0	14.9	8.7	3.0
119	7.5	>25	464	19.9	24.2	5.1	>25	15.5	9.9
120	25.1	>25	368	6.0	2.2	0.56	13.4	15.2	14.9
121	0.88	21.5	226	0.51	1.3	0.74	>25	23.0	23.0
122	0.53	24.4	305	0.72	1.3	4.1	0.5	2.6	1.9
123	0.88	24.2	222	0.89	10.9	2.2	>25	4.4	1.6
125	0.90	11.3	234	0.22	1.3	0.38	>25	4.8	2.8
126	15.9	>25	447	1.2	22.3	3.8	24.8	15.0	10.5
127	0.90	>25	424	2.1	2.2	0.85	>25	22.0	16.0
128	1.0	>25	215	0.25	1.6	9.2	>25	20.1	8.6
130	0.73	23.0	241	0.22	2.0	2.7	>25	7.4	19.4
S2201	4.7	>25	326	1.8	1.7	0.82	>25	>25	4.2
S2202	22.3	13.2	236	11.6	2.3	9.2	14.2	4.4	4.2
S2203	0.10	1.2	0.28	0.12	0.014	0.29	0.4	2.4	3.4
S2204	0.92	11.3	405	1.5	2.0	4.9	<0.2	3.1	2.8
S2206	41.8	24.4	>500	>25	>25	3.2	1.4	12.1	13.5
S2208	0.89	12.7	250	0.24	1.8	4.6	1.2	>25	3.1
AK111	0.63	24.9	110	0.93	0.13	0.73	>25	4.6	4.0
AK117	0.94	>25	376	10.5	1.2	7.4	>25	14.1	8.0
027	3.3	>25	447	1.7	11.0	4.1	>25	23.7	14.2

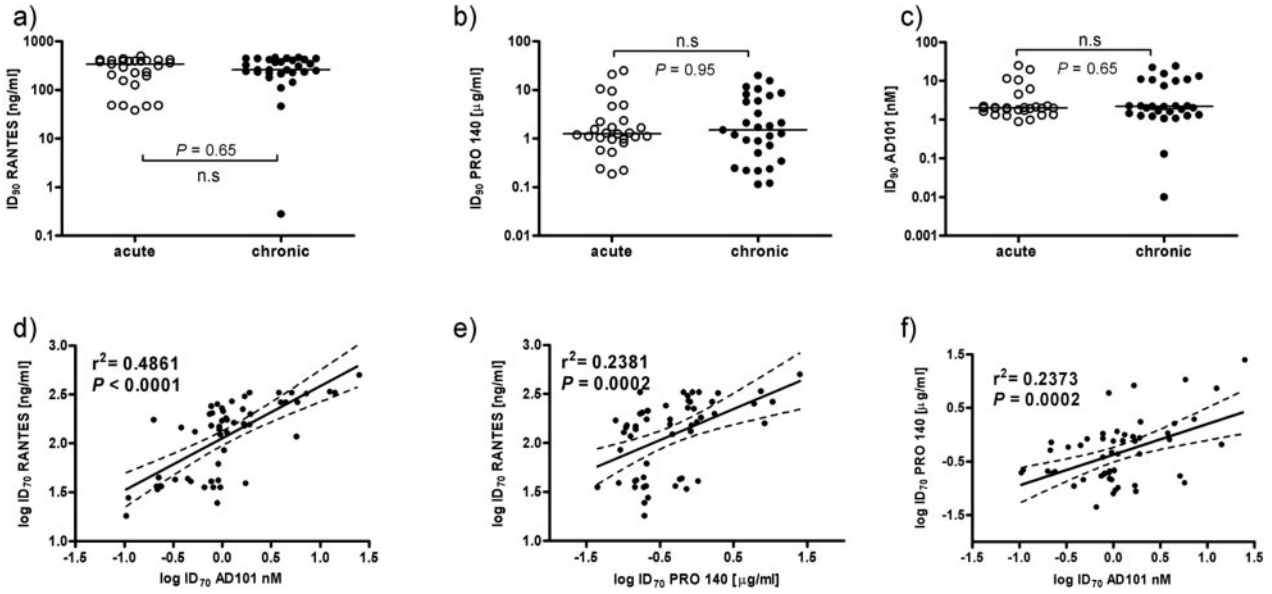

FIG. 3. CCR5 inhibitors. (a to c) Ninety percent inhibitory doses of RANTES, PRO 140, and AD101 in acutely and chronically infected individuals. Means from two independent experiments are shown. Groups were compared using the Mann-Whitney test. Open and closed circles indicate acutely infected and chronically infected individuals, respectively. Horizontal lines indicate median values. *P* values for subtype B viruses only: RANTES, $P = 0.74$; PRO 140, $P = 0.88$; AD101, $P = 0.99$. (d to f) Correlation analysis of sensitivities to RANTES, PRO 140, and AD101. Significance was evaluated after Bonferroni correction. The solid lines indicate the regression lines, and the dashed lines indicate 95% confidence intervals. R5X4 viruses were excluded from the analysis of CCR5 inhibitors.

for binding to the receptor (2, 21, 88, 96). AD101 binds to a putative ligand binding cavity formed by the transmembrane helices 1, 2, 3, and 7 of CCR5, which probably leads to conformational changes of the extracellular domains of the receptor that interfere with envelope binding and entry (27, 99). The MAb PRO 140 recognizes an epitope that includes residues in both the N terminus and the second extracellular loop domain of CCR5 and is thought to interfere with viral binding to the receptor (61, 91). Although antagonistic at higher concentration, PRO 140 also inhibits viral entry at lower concentrations that do not interfere with receptor signaling and do not induce downregulation of the receptor (61).

The median inhibitory doses of all three CCR5-targeting inhibitors tested did not differ between the acute and the chronic patient groups (Fig. 3a to c). We observed in both groups isolates that were sensitive and relatively insensitive to the three inhibitors. In agreement with previous observations (85, 91, 92, 96), we found that R5 isolates were overall susceptible to inhibition by AD101 (median 90% inhibitory concentration, 2.0 nM; range 0.014 to >25 nM), PRO 140 (median 90% inhibitory concentration, 1.3 μg/ml; range, 0.11 to >25 μg/ml), and RANTES (median 90% inhibitory concentration, 309 ng/ml; range, 0.28 to >500 ng/ml). All CCR5 inhibitors blocked 55 of 56 R5 isolates. In addition, PRO 140 inhibited one of two R5X4 viruses by >90%, whereas AD101 and RANTES were not active against these viruses. Of note, despite the different modes of action of AD101 and RANTES, we observed a strong correlation between their potencies ($r^2 = 0.4861$; $P < 0.0001$), whereas more modest correlations in activity were observed between PRO 140 and either AD101 or RANTES ($r^2 < 0.24$; $P = 0.0002$) (Fig. 3d to f). Since inhibition assays with CD4-IgG_2 and IgG_1b12 were performed in parallel to assays with the CCR5 inhibitors on the same target cells, we were able to investigate how much the sensitivities to CD4BS and CCR5 inhibitors were interdependent. We found a high degree of correlation between the activities of CD4-IgG_2 and PRO 140, and to a lesser extent also with AD101 (Fig. 4a to c). The correlation between CD4-IgG_2 and PRO 140 was greater than that between PRO 140 and the other CCR5 inhibitors. In contrast, sensitivities to CD4-IgG_2 and RANTES showed no significant interdependency. A possible explanation for this discrepancy might lie in the different mechanisms of action of the inhibitors: the major effect of RANTES is on receptor downregulation, while the other two substances interfere with gp120 binding to the receptor, which is more likely to be influenced by modifications within gp120 that also steer affinity for CD4. Sensitivity to CD4 or CCR5 inhibitors likely implies that the given virus strain has a comparatively low affinity for the respective receptor and may require higher receptor levels to be able to enter target cells. Equally, a low affinity for one receptor automatically will increase the dependency of the entry process on the other receptor. Our observations are in agreement with previous in vitro studies demonstrating that low CD4 expression on target cells can be compensated for by high coreceptor expression and vice versa (42, 68). Of note, IgG_1b12 and CCR5 inhibitors showed no correlation (Fig. 4d to f) indicating again that substantial differences in the epitopes of IgG_1b12 and CD4 exist. In summary, our data suggest that the interaction with CD4 but not the interaction with the CD4BS antibody IgG_1b12 is affected by functionally linked properties within gp120 that steer coreceptor interaction.

Sensitivity to the fusion inhibitor T-20. The fusion inhibitor T-20 (enfuvirtide) represents a novel class of entry inhibitors

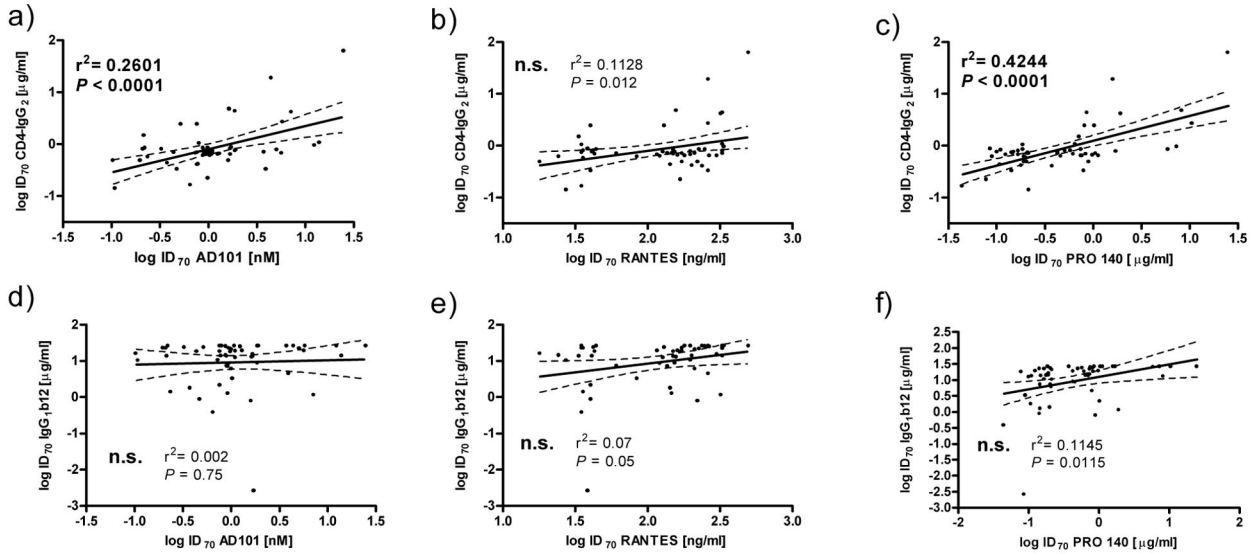

FIG. 4. Interdependencies between CD4BS and CCR5 inhibitors. (a to c) Correlation analysis of 70% inhibitory doses of CD4-IgG$_2$ and 70% inhibitory doses of RANTES, PRO 140, and AD101, respectively. (d to f) Correlation analysis of 70% inhibitory doses of IgG$_1$b12 and 70% inhibitory doses of RANTES, PRO 140, and AD101, respectively. Significance was evaluated after Bonferroni correction. The solid lines indicate the regression lines, and the dashed lines indicate 95% confidence intervals. R5X4 viruses were excluded from the analysis of CCR5 inhibitors.

(40, 47, 48, 66). Susceptibility to T-20 varies among viral strains (74). This appears not only to be a consequence of the variability in the binding efficiency to HR1 but also to be influenced by variations within gp120 (25, 26, 77). Viral strains with high affinity for CCR5 have been described to be less sensitive to inhibition by T-20 (74). The more efficient interaction with CCR5 of these strains leads to a faster fusion kinetic, reducing the window of action for T-20. Here we probed the efficacy of T-20 in inhibiting viral strains from our acute and chronic patient cohorts. All viruses tested were sensitive to T-20 (Table 2; Fig. 5). The epitope for T-20 appeared to be preserved early and late in infection: there was no difference in the sensitivities

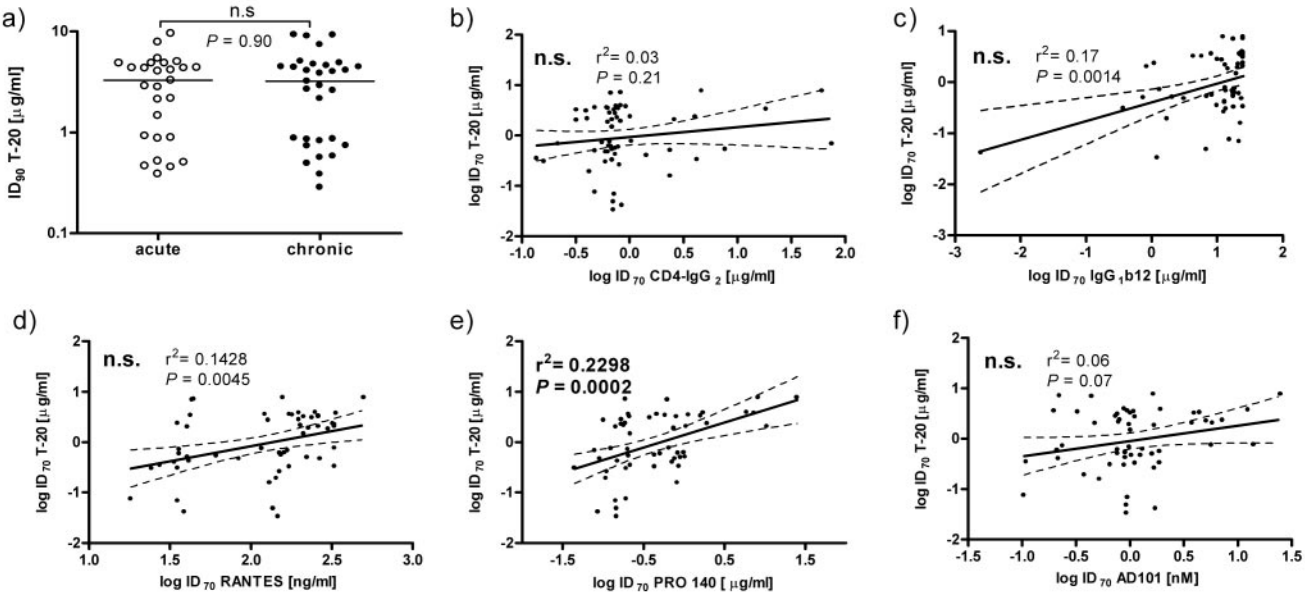

FIG. 5. Sensitivity to the fusion inhibitor T-20. (a) Ninety percent inhibitory doses of T-20 in acutely and chronically infected individuals. Means from two independent experiments are shown. Groups were compared using the Mann-Whitney test. Open and closed circles indicate acutely infected and chronically infected individuals, respectively. Horizontal lines indicate median values. For subtype B viruses only, $P = 0.62$. (b and c) Correlation analysis of 70% inhibitory doses of T-20 and sensitivities to CD4-IgG$_2$ and IgG$_1$b12, respectively. (d to f) Correlation analysis of 70% inhibitory doses of T-20 and sensitivities to RANTES, PRO 140, and AD101, respectively. Significance was evaluated after Bonferroni correction. R5X4 viruses were excluded from the analysis of CCR5 inhibitors. The solid lines indicate the regression lines, and the dashed lines indicate 95% confidence intervals.

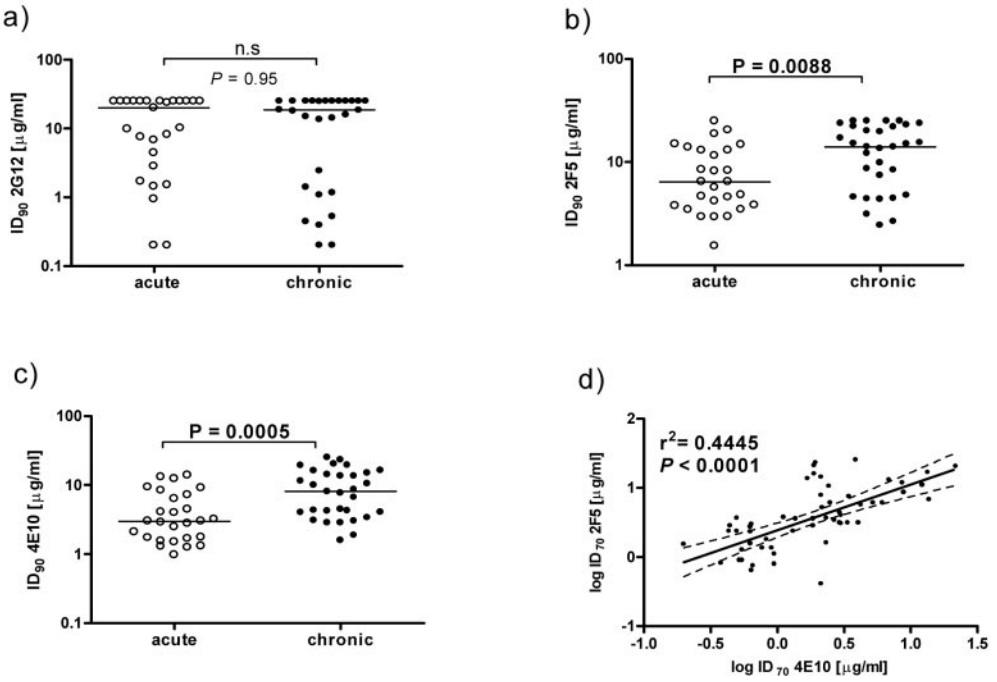

FIG. 6. Sensitivity to the neutralizing antibodies 2G12, 2F5, and 4E10. (a to c) Ninety percent inhibitory doses of 2G12, 2F5, and 4E10 in acutely and chronically infected individuals, respectively. Means from two to five independent experiments are shown. Groups were compared using the Mann-Whitney test. Open and closed circles indicate acutely infected and chronically infected individuals, respectively. Horizontal lines indicate median values. *P* values for subtype B viruses only: 2G12, $P = 0.61$; 2F5, $P = 0.057$; 4E10, $P = 0.012$. (d) Correlation analysis of 70% inhibitory doses of 2F5 and 4E10. Significance was evaluated after Bonferroni correction. The solid line indicates the regression line, and the dashed lines indicate 95% confidence intervals.

of acute and chronic isolates (Fig. 5a). To explore whether affinity for CCR5 is a major determinant in T-20 sensitivity, we probed whether sensitivity to CCR5 inhibitors (as an inverse measure for CCR5 affinity) predicts sensitivity to T-20. If such an association exists, it appears to be of negligible consequence for the entry process in primary $CD4^+$ T cells. There was only a minor level of correlation between inhibitory doses of T-20 and PRO 140 ($r^2 = 0.23$; $P = 0.0002$) (Fig. 5e). No significant correlation between inhibitory doses of T-20 and AD101 or RANTES existed (Fig. 5d to f). Equally, susceptibilities to the CD4BS inhibitors CD4-IgG_2 and IgG_1b12 did not correlate significantly with the sensitivities to T-20 (5b and c). Collectively our data suggest that on primary $CD4^+$ T cells, inhibition of entry by the fusion inhibitor T-20 appears to be largely independent of envelope determinants that are involved in the interaction with the primary receptor CD4 and the coreceptor CCR5.

Sensitivity to inhibition by the monoclonal antibody 2G12. The gp120 envelope protein is highly glycosylated. N-linked glycans alone make up nearly 50% of the gp120 mass (50, 106). The high degree of glycosylation of the gp120 molecule leads to the formation of a glycan shield which occludes potential binding sites for neutralizing antibodies and limits the immunogenicity of the envelope protein (73, 75, 101, 105). The monoclonal antibody 2G12 is unique among neutralizing antibodies as it recognizes a mannose-dependent epitope in gp120 centered on the high-mannose and/or hybrid glycans of residues 295, 332, and 392. Thus, unlike other antibodies whose epitopes are concealed by the glycan shield, this antibody depends on the glycosylation of gp120. Recent reports suggested that early in infection the gp120 envelope protein is less glycosylated and that increases in N-linked glycosylation are a result of escape from antibody-mediated neutralization (24, 101). We probed this indirectly using the monoclonal antibody 2G12. By analyzing the neutralization activity of this antibody against viral isolates from acute and chronic disease stages, we sought to determine whether isolates early in infection are less glycosylated and therefore might not harbor the mannose residues composing the epitope of 2G12. We found no evidence for a difference in the sensitivities of viruses from early and later disease stages to inhibition by this antibody (Fig. 6a; Table 2). While this finding does not exclude the possibility that differences in glycosylation at different disease stages occur, the glycosylation sites which manifest the epitope of 2G12 were considerably well conserved throughout the course of infection in our patient cohort.

Sensitivity to inhibition by monoclonal antibodies targeting the membrane-proximal external region in gp41. It has been recently proposed that viruses from acutely infected individuals are more sensitive to antibody-mediated neutralization than viruses derived during chronic disease stages (24). However, in our study, the two potent neutralizing anti-gp120 antibodies IgG_1b12 and 2G12 were equally effective against viruses from acutely and chronically infected individuals. In a next step, we therefore evaluated the sensitivity of these isolates to inhibition by the anti-gp41 antibodies 2F5 and 4E10, which bind to adjacent linear epitopes in the membrane-proximal external region of gp41 (59, 71, 72, 84, 107). Viruses from

acutely infected individuals were significantly more sensitive to inhibition by the anti-gp41 MAbs than viruses from chronically infected patients (Fig. 6b and c). As described previously, 2F5 and 4E10 were broadly reactive, neutralizing 91% and 98% of the isolates at a 90% inhibitory dose of below 25 μg/ml, respectively (Table 2). Most notably, sensitivities to these two antibodies were highly correlated (Fig. 6d) ($r^2 = 0.4445$; $P < 0.0001$). When we sequenced the epitopes for 2F5 and 4E10, we found that the binding sites of these antibodies were highly preserved in the entire cohort (Fig. 7). For 2F5, sequence changes in the core region of the epitope, DKW, were more frequent among resistant isolates. However, one isolate (isolate 019) which has a K-to-E mutation sustained its sensitivity to 2F5. Antibody 4E10 appears to allow a certain degree of promiscuity in the core epitope NWFDIT. At position 1 of this epitope, a change of N to S or T was tolerated without apparent loss of sensitivity. Likewise, substitutions of D by N or S are tolerated at position 4. Position 6 was predominantly T, but substitution to S was also tolerated. The most conserved were positions for W, F, and I within the epitope sequence; no changes in these amino acids were found in our cohort.

Virus pairs with identical epitope sequences but strikingly different sensitivities to both MAbs were detected. For instance, isolate S2204 was sensitive to both MAbs, and isolate 121 was comparatively insensitive to both; other such pairs were isolates 107 (sensitive) and 102 (insensitive) or AK112 (sensitive) and 109 (insensitive). Given the close proximity of the two epitopes, these data suggest that sensitivity to 2F5 and 4E10 is not only dependent on the presence of the actual epitope but appears to be steered also by changes in other regions of the envelope proteins that may affect epitope conformation or accessibility. Unlike the epitopes of the two anti-gp41 MAbs, the binding site for T-20 on gp41 is not accessible on the virion before receptor binding has occurred. Inhibition by the anti-gp41 MAbs and T-20 is therefore not mechanistically related and likely does not share common determinants that shape sensitivity. In support of this, we did not observe dependencies between the sensitivities of these two types of agents. Likewise, no correlation between the sensitivities to the two anti-gp41 antibodies and CCR5 inhibitors, anti-CD4BS reagents, or the two tested neutralizing anti-gp120 antibodies existed.

DISCUSSION

In the present study we investigated the potencies of entry inhibitors in suppressing the infectivities of 58 viral isolates from acute and chronic disease stages. Inhibitors included (i) agents that block the interaction of gp120 with CD4 (the tetrameric CD4 molecule CD4-IgG_2 and the CD4BS antibody IgG_1b12), (ii) compounds that block the interaction with CCR5 (the chemokine RANTES, the small-molecule inhibitor AD101, and the anti-CCR5 antibody PRO 140), (iii) the fusion inhibitor enfuvirtide (T-20), and (iv) neutralizing antibodies directed against gp120 (MAb 2G12) and gp41 (MAbs 2F5 and 4E10).

CD4 binding site-directed inhibition. The interaction of the viral envelope protein gp120 with CD4 is crucial for the virus. How the viral envelope engages CD4 and which epitopes are exposed upon CD4 binding shapes the subsequent interaction with the coreceptor but also influences the sensitivity to neutralization. In the absence of neutralizing antibodies in vitro, the virus readily adapts to optimize usage of the receptor (6, 56, 57, 70, 100). In vivo, selective pressure of antibodies directed to the CD4BS is thought to prevent this conformation of gp120. The virus still continues to utilize CD4, although in an alternate way that leads to a distinct conformation of gp120 where binding sites for neutralizing antibodies (not only those directed to the CD4BS) maybe more secluded. The epitope for CD4 on gp120 thereby appears to have a relatively high degree of plasticity. While affinity of gp120 for CD4 may be increased in the neutralization-resistant phenotype, this does not appear to dramatically affect fitness or tropism of the viral isolates both in vitro and in vivo. In our study, antibodies directed to the CD4BS were not detectable in the majority of acutely infected individuals, while they were ubiquitously produced during chronic infection. It was thus feasible to investigate to what extent escape from CD4BS antibodies directs the interaction between gp120 and CD4. Although there was a striking difference in the titers of anti-gp120 and specifically anti-CD4BS antibodies between acutely and chronically infected individuals, we noted no difference between the two groups in the sensitivity to inhibition by the two antiviral agents that target the CD4BS, i.e., the tetrameric CD4 molecule CD4-IgG_2 and the antibody IgG_1b12. Thus, we did not observe a general pattern indicating that the absence of CD4BS antibodies during the acute-infection stage influences the interaction between gp120 and CD4. Of note, also during chronic disease stages we were unable to detect a direct relationship between the magnitude of the CD4BS response and the degree of resistance to agents targeting the CD4-gp120 interaction. Taken together our data suggest that in vivo conversion to optimized CD4 usage and a neutralization-sensitive phenotype that is linked to this conformation of gp120 either does not occur as rapidly as in vitro or is not as stable. Nevertheless, we cannot rule out that sensitivity to CD4BS antibodies does indeed to some extent shape the mode of the gp120-CD4 association in vivo. Features of the cell populations that drive virus spread and persistence in vivo may differ substantially from those in in vitro studies and may not equally well support the emergence of the neutralization-sensitive phenotype. It is also possible that the neutralization-resistant phenotype may be so well adapted and bear so little fitness loss to the virus that a reversion is only rarely, and then slowly, achieved. The period that the virus replicated in absence of CD4BS antibodies during acute infection in our cohort was less than 3 months, which may be too short to allow reversion of the CD4BS-resistant phenotype (which the virus probably obtained in the previous host) to a CD4BS-sensitive stage. In comparison, emergence of CD4-sensitive quasispecies in vitro has been reported after 19 weeks of continuous culture (70) but may also occur more rapidly, depending on the virus isolate and/or target cells (6, 7, 104). It is also possible that in vivo even in the absence of neutralizing antibodies to CD4BS or additional sites, other selection pressures exist that prevent the conversion of gp120 to the neutralization-sensitive phenotype. An alternative explanation for the absence of the neutralization-sensitive phenotype in our study could be that reversion to the neutralization-sensitive phenotype occurs very rapidly but is only transiently stable in vivo. A comparatively low-level CD4BS

																																2F5	4E10
											2F5										4E10											IC_{90}[a]	IC_{90}[a]
LAI		N	E	Q	E	L	L	E	L	D	K	W	A	S	L	W	N	W	F	N	I	T	N	W	L	W	Y	I				
017	acute	.	.	.	D	.	.	A	.	.	.	.	.	.	.	.	.	.	.	D	.	S	R	.	.	.	.	.	.			1.5	2.8
S2203	chronic	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.	.	K	.	.	.	.	.	.			2.4	3.4
122	chronic	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.	.	K	.	.	.	.	.	.			2.6	1.9
016	acute	.	.	K	D	.	.	.	.	.	.	.	T	.	.	.	.	.	.	S	.	S	.	.	.	.	.	.	.			2.9	1.7
AK112	acute	.	.	.	D	.	.	A	.	.	.	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.			3.0	1.5
025	acute	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.			3.0	3.0
S2204	chronic	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	D	.	.	K	.	.	.	.	.	.			3.1	2.8
015	acute	.	.	K	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.			3.5	7.2
AK119	acute	.	.	K	.	.	.	.	.	.	.	.	.	D	.	.	.	.	.	D	.	.	K	.	.	.	.	.	.			3.5	1.7
AK115	acute	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.	S	.	.	.	.	.	.	.			3.8	2.9
007	acute	.	.	K	D	.	.	A	.	.	.	.	.	.	.	.	T	.	.	D	.	S	K	.	.	.	.	.	.			3.8	1.7
AK121	acute	.	.	.	D	.	.	.	.	.	.	.	.	N	.	.	.	.	.	D	.	S	.	.	.	.	.	.	.			4.2	1.5
S2202	chronic	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	D	.	.	Q	.	.	.	.	.	.			4.4	4.2
123	chronic	.	.	.	.	.	.	.	.	.	.	.	T	N	.	.	.	.	.	D	.	.	S	.	.	.	.	.	.			4.4	1.6
107	chronic	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.			4.4	4.3
AK125	acute	.	.	.	D	.	.	A	.	.	.	.	.	.	.	.	.	.	.	D	.	S	.	.	.	.	.	.	.			4.6	4.5
AK111	chronic	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	D	.	.	K	.	.	.	.	.	.			4.6	4.0
AK120	acute	.	.	.	D	.	.	.	.	.	.	.	.	N	.	.	.	.	.	D	.	S	.	.	.	.	.	.	.			4.6	2.5
125	chronic	.	.	.	D	.	.	A	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.			4.8	2.8
018	acute	.	.	K	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.			4.8	4.1
003	acute	.	.	K	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.	.	Q	.	.	.	.	.	.			5.7	2.1
002	acute	.	.	K	K	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.			6.4	1.2
AK114	acute	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.	S	.	.	.	.	.	.	.			6.5	1.3
130	chronic	.	.	K	D	.	.	.	.	.	.	.	.	.	.	.	T	.	.	D	.	S	K	.	.	.	.	.	.			7.4	19.4
AK104	acute	.	.	K	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.	.	S	.	.	.	.	.	.			8.2	1.0
AK122	acute	.	.	.	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	S	.	.	K	.	.	.	.	.	.			8.3	1.3
105	chronic	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	D	.	.	.	.	.	.	.	.	.			8.4	7.6
020	acute	.	.	L	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.			8.5	4.0
118	chronic	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.			8.7	3.0
117	chronic	.	.	.	D	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.			9.8	13.4
AK116	acute	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	D	.	.	.	.	.	.	.	.	.			11.5	13.9
S2206	chronic	.	.	.	.	.	.	Q	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.			12.1	13.5
AK103	acute	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.			13.0	8.4
021	acute	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	D	.	.	H	.	.	.	.	.	.			13.1	9.3
111	chronic	.	.	K	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.			13.5	4.5
022	acute	.	.	.	.	.	.	Q	.	.	.	.	.	.	.	.	.	.	.	D	.	.	H	.	.	.	.	.	.			14.0	3.0
106	chronic	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.	.	K	.	.	.	.	.	.			14.0	6.6
AK117	chronic	.	.	.	.	.	.	.	.	.	.	.	.	N	.	S	.	.	.	D	.	.	.	.	.	.	.	.	.			14.1	8.0
019	acute	.	.	.	.	.	.	A	.	.	E	.	.	N	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.			14.8	3.2
126	chronic	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	D	.	.	K	.	.	.	.	.	.			15.0	10.5
009	acute	.	.	.	.	.	.	A	.	.	.	.	.	N	.	.	.	.	.	.	.	.	Q	.	.	.	.	.	.			15.0	9.1
120	chronic	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.			15.2	14.9
119	chronic	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.	.	S	.	.	.	.	.	.			15.5	9.9
116	chronic	.	.	.	.	.	.	.	.	.	.	.	.	N	.	.	.	.	.	D	.	S	.	.	.	.	.	.	.			17.1	16.3
026	acute	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	S	.	S	.	.	.	.	.	.	.			18.8	6.3
109	chronic	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.			19.7	20.2
128	chronic	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.	S	.	.	.	.	.	.	.			20.1	8.6
AK105	acute	.	.	.	.	.	.	A	.	.	.	.	.	T	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.			20.5	13.1
127	chronic	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.			22.0	16.0
102	chronic	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.			22.2	19.3
121	chronic	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	D	.	.	K	.	.	.	.	.	.			23.0	23.0
027	chronic	.	.	.	D	.	.	A	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.			23.7	14.2
113	chronic	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	K	.	.	.	.	.	.			23.7	>25
114	chronic	.	.	.	.	.	.	A	.	.	N	.	.	.	.	.	.	.	.	D	.	.	K	.	.	.	.	.	.			>25	11.4
115	chronic	.	.	L	.	.	.	K	.	.	E	.	.	.	.	.	.	.	.	D	.	S	.	.	.	.	.	.	.			>25	4.1
S2201	chronic	.	.	.	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.			>25	4.2
S2208	chronic	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	S	.	.	S	.	.	.	.	.	.	.	.	.			>25	3.1
023	acute	.	.	L	D	.	.	.	.	.	.	.	K	N	.	.	T	.	.	D	.	.	.	.	.	.	.	.	.			>25	12.4

FIG. 7. Sequence analysis of 2F5 and 4E10 epitopes. The gp41 sequences of patient isolates are presented in comparison to that of the reference strain LAI. Core positions of the 2F5 and 4E10 epitopes are shaded. IC_{90}, 90% inhibitory dose in μg/ml.

response (below the detection level of our assay) may already suffice to induce the rearrangement of gp120, which would explain why we did not observe a direct relationship between the magnitude of the CD4BS response and resistance to CD4 inhibitors. The latter scenarios would be in agreement with previous studies showing that transfer of neutralization-sensitive virus to HIV-1 antibody-naive hosts led to a rapid conversion to a neutralization-resistant phenotype (8, 17, 28, 83).

Inhibition of the interaction with CCR5. Two regions within the highly variable envelope protein gp120, i.e., the four-stranded bridging sheet which is exposed upon binding to the receptor CD4 and the V3 loop, have been implicated in directly or indirectly participating in binding to the coreceptors (44, 45, 88). The exact epitope on gp120 that interacts with CCR5 has yet not been defined but likely varies to some degree depending on the viral strain. Virus isolates appear to differ in the coreceptor density needed for entry, and differential affinity of the viral envelopes for CCR5 was shown to influence fusion rates (68, 69, 74). In support of this, virus strains have been found to vary substantially in their susceptibility to inhibitors targeting the interaction with CCR5, which likely is a consequence of the differential binding affinities and fusion kinetics (18, 41, 46, 74, 91, 96). A recent report suggested that during the course of the infection R5 viruses may adapt to more efficiently use CCR5, a process that is associated with both higher replicative capacity and decreased sensitivity to CCR5 inhibitors (41). This observation could have substantial clinical implications: if viruses in general undergo such an evolution, then inhibitors targeting the entry process, and foremost coreceptor inhibitors, will prove to be less efficient at later disease stages. To probe this, we evaluated the sensitivities of viral isolates from acutely and chronically infected individuals to inhibition by CCR5-targeting agents. We chose substances representing the three types of CCR5 inhibition currently known: the chemokine RANTES/CCL5, the small-molecular inhibitor AD101 (SCH-350581), and the anti-CCR5 antibody PRO 140. These three inhibitors interfere with viral entry by different mechanisms: (i) as an agonist that downregulates CCR5 (RANTES), (ii) as an antagonist that disturbs CCR5 conformation (AD101), and (iii) by blocking viral attachment without receptor antagonism or agonism (PRO 140). Viruses from acutely and chronically infected individuals showed no differences in their susceptibilities to CCR5 inhibitors. In agreement with previous smaller studies, viruses in our panel were broadly inhibited by the three CCR5 inhibitors with a range of susceptibilities (18, 41, 46, 74, 91, 96). However, the purpose of the present study was not to determine the absolute breadths of potency of the various inhibitors, as this comparison would require a more detailed analysis of the contributions of interassay and intervirus variations in activity. Despite the fact that these inhibitors have substantially different modes of action, strong to moderate correlations were observed in their antiviral activities, indicating that they reflected a genuine dependency of the viruses on the expression of CCR5 levels.

Cooperation between CD4 and CCR5. Analysis of the CD4 interaction alongside the virus-CCR5 interaction in our cohort revealed that a high degree of correlation between the activity of CD4-IgG_2 and the CCR5 inhibitors PRO 140 and AD101 existed. It can be speculated that changes within the viral envelope that affect CD4 binding also influence the interaction with the coreceptor to a considerable degree. Adaptations within the CD4BS that steer binding to CD4 could either lead to conformational changes outside the epitope that have an impact on coreceptor binding or coemerge with mutations in this region. Of note, although IgG_1b12 and CD4-IgG_2 target similar albeit not identical binding sites on gp120, sensitivities to IgG_1b12 and CCR5 inhibitors showed no correlation, indicating that even though the epitopes are overlapping, only the binding sites of CD4 involve areas that are codependent with the coreceptor binding site. In general, the binding site for CD4 on the viral envelope appears to have a high degree of flexibility. Despite the extraordinary variability of the gp120 protein, the interaction with CD4 remains preserved throughout the disease course and across divergent strains. The same is not true for CD4BS antibodies; rapid escape from these antibodies appears to occur in vivo (3, 23, 70). Even the fairly cross-reactive antibody IgG_1b12 is less potent outside subtype B (97). Accordingly, several isolates in our virus panel were not inhibited by IgG_1b12 at the highest concentration tested. Although the interdependency between sensitivities to CD4-IgG_2 and CCR5 inhibitors could potentially imply that changes within the CD4BS epitope affect the binding sites for CCR5 and vice versa, any conclusions about the cooperation of the epitopes for CD4 and CCR5 on gp120 must be tentative. It is possible that this interdependency does not indicate a direct interaction between these two epitopes but reflects their documented functional cooperation. For example, a low affinity for CD4 (and with that a heightened sensitivity to CD4 inhibitors) will automatically increase the dependency of the entry process on the interaction with CCR5 and make this interaction more susceptible to inhibition. Our observations are in agreement with previous studies showing that the efficacy of the viral entry process is steered by the levels of CD4 and coreceptor expressed on the target cells (42, 68) and the affinity of the viral envelope for the receptors (74, 77). Collectively our data underscore the importance of the cooperation between the receptor binding sites on gp120 and suggest that the interaction with CD4 is affected by functionally linked properties within gp120 that steer coreceptor interaction.

Fusion inhibition. In agreement with our results on CD4 and CCR5 inhibitors, we found no difference in the susceptibility to inhibition by the fusion inhibitor enfuvirtide (T-20) between virus strains from acutely and chronically infected individuals: T-20 had considerable albeit variable potency against isolates from both groups. Since T-20 has only a short window of action (it binds to the HR1 region within gp41 that is exposed only after CD4 and coreceptor binding has occurred), its activity is strongly influenced by factors that modulate fusion efficiency. High affinity for CCR5 presumably leads to faster fusion kinetics and has been reported to diminish the effect of T-20 (74). However, when we probed for interdependencies between affinity for CCR5 and susceptibility to T-20 on primary $CD4^+$ T cells, we found no such association. In contrast to in vitro studies that utilized transformed cell lines as target cells, our studies on primary $CD4^+$ T cells showed that susceptibility to T-20 was largely independent of determinants that steer virus susceptibility to CCR5 and CD4 inhibitors on these cells.

Inhibition by MAb 2G12. HIV-1 gp120 contains both N- and O-linked glycans. While the serine or threonine residues that

carry O-linked glycans have not yet been identified, the N-linked (asparagine-linked) glycosylation sites have been studied in detail (9, 16). N-linked glycans contribute essentially to the correct folding of the viral envelope protein (51), make up nearly 50% of the gp120 mass, and are composed of high-mannose, hybrid and complex glycans (50, 106). Although several glycosylation sites appear to be conserved among divergent strains, the number of sites and exact composition of the glycosylation pattern differ among viral isolates (16, 102, 106). These sugar side chains form a "glycan shield" which protects the virus from neutralizing antibodies and also reduces the immunogenicity of the envelope protein (73, 75, 101, 105). While most antibody binding sites on gp120 are occluded by the glycan shield, the potent neutralizing antibody 2G12 binds to a mannose-dependent epitope on the viral envelope. Glycosylation patterns differ among viral isolates, and the degree of glycosylation has been proposed to be comparatively low in acute infection and to increase gradually over time, creating more-neutralization-resistant virus strains (24, 101). In our cross-sectional analysis we found that the mannose-dependent epitope of the neutralizing MAb 2G12 was equally well conserved both early in infection and at later disease stages. Thus, although glycosylation patterns may well increase over time within a patient, these differences in glycosylation do not specifically affect the epitope of 2G12, rendering therapeutic approaches with 2G12-like antibodies or other agents that target gp120 in the context of conserved glycosylation sites feasible at both early and chronic disease stages.

Inhibition by anti-gp41 MAbs. The majority of neutralizing antibodies recognize epitopes within gp120. The notable exceptions are the antibodies 2F5 and 4E10, which are directed to adjacent epitopes within the ectodomain of gp41 (59, 71, 72, 84, 107). Most striking, among all the entry inhibitors tested, only these two anti-gp41 antibodies displayed differential activity against viruses from early and late disease stages. Both antibodies inhibited more than 90% of both chronic and acute viruses tested. Viruses from acutely infected individuals, however, were significantly more sensitive to the antibodies than viruses from chronically infected patients. The sensitivities to these antibodies were highly correlated, suggesting that common factors influence the susceptibility to these antibodies. In support of this, we found that the epitopes of both antibodies were highly conserved among the 58 isolates tested, suggesting that the higher susceptibility of acute viruses to inhibition by these MAbs may be the result of a better epitope accessibility. Mutations outside the epitopes that alter the three-dimensional structure of gp41 or changes within gp120 that reshape the conformation of the envelope protein and its association with gp41 may lead to a differential exposure of the epitopes of 2F5 and 4E10 and will thus have a great influence on the activity of both of these antibodies. Considerable effort has been made over recent years towards the development of immunogens that elicit 2F5-like and 4E10-like responses. Our data here show that therapeutic vaccines based on these reactivities may bear a potential at all disease stages but could be particularly effective during early disease stages.

Summary. During the course of the infection, HIV-1 is exposed to multiple factors that counteract viral spread. Cellular and humoral immune responses, innate immunity, or antiretroviral drugs exert selective pressure. Viruses are forced to adapt to their environment and to develop strategies to escape these antiviral mechanisms. These evasion and escape pathways are major obstacles for the immune system in its fight with HIV-1, and they also present serious problems for antiviral drugs (20, 22). By comparing virus isolates from chronically and acutely infected patients, we sought to explore the efficacy of entry inhibitors in suppressing virus strains at different disease stages but also aimed to gain information on whether in vivo selection pressures exist that affect the interaction of the virus with its entry receptors and, if so, at what disease stage adaptation to the host and escape occur. Surprisingly, we found that selection pressures that shape sensitivity to CD4BS and coreceptor inhibitors must act very early in infection or reversion of the transmitted strain in the absence of selective pressures during acute infection does not occur. The decreased sensitivity to gp41 MAbs during chronic disease stages nevertheless suggests that differential selective pressure on this site may exist. Whether or not the mode of transmission or the genotype has an influence on the phenotype of the viruses circulating during acute infection and their sensitivity to entry inhibitors could not be addressed in our study. Fifty-five percent of acutely infected patients were men who had sex with men; 41% contracted HIV by heterosexual transmission. The remaining patients were infected by needle stick or could not be classified. Viruses from the majority of patients were subtype B, whereas other subtypes were only randomly detected. Of note, when we analyzed subtype B isolates alone, we found the same pattern of reactivities as for the entire group of viruses. The other subgroups were too small to evaluate whether different modes of transmission and/or genotypes may give rise to distinct virus populations with different entry inhibitor sensitivities. In summary, our comprehensive analysis of the efficacy of entry inhibitors during acute and chronic disease stages showed that all currently known types of entry inhibitors (i.e., agents targeting the interaction with CD4 or the coreceptor CCR5, agents targeting the fusion process, and neutralizing antibodies directed to the viral envelope proteins) have considerable potency against divergent viral strains. With the exception of anti-gp41 antibodies 2F5 and 4E10, which were more potent against viruses from acute infection, we found no differences in the susceptibility to entry inhibitors between viruses from acute and chronic disease stages. Accordingly, viruses from chronic disease stages are not generally more refractory to inhibition than viruses at earlier stages, and thus treatment with entry inhibitors appears to be feasible across disease stages.

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