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Human Immunodeficiency Virus Type 1 Fitness Is a Determining Factor in Viral Rebound and Set Point in Chronic Infection

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Human immunodeficiency virus type 1 (HIV-1) isolates from 20 chronically infected patients who participated in a structured treatment interruption (STI) trial were studied to determine whether viral fitness influences reestablishment of viremia. Viruses derived from individuals who spontaneously controlled viremia had significantly lower in vitro replication capacities than viruses derived from individuals that did not control viremia after interruption of antiretroviral therapy (ART), and replication capacities correlated with pre-ART and post-STI viral set points. Of note, no clinically relevant improvement of viral loads upon STI occurred. Virus isolates from controlling and noncontrolling patients were indistinguishable in terms of coreceptor usage, genetic subtype, and sensitivity to neutralizing antibodies. In contrast, viruses from controlling patients exhibited increased sensitivity to inhibition by chemokines. Sensitivity to inhibition by RANTES correlated strongly with slower replication kinetics of the virus isolates, suggesting a marked dependency of these virus isolates on high coreceptor densities on the target cells. In summary, our data indicate that viral fitness is a driving factor in determining the magnitude of viral rebound and viral set point in chronic HIV-1 infection, and thus fitness should be considered as a parameter influencing the outcome of therapeutic intervention in chronic infection.

The level of human immunodeficiency virus type 1 (HIV-1) viremia that patients reach and maintain after the acute infection phase predicts disease progression (26). This steady-state level of plasma viral load (VL) is referred to as viral set point and can vary more than 1,000-fold between individuals (35). Viral set points are a consequence of the interplay of viral, immunological, and host genetic factors (8, 36), including maintenance and specificity of anti-HIV CD4 and CD8 T-cell responses (2), neutralizing antibodies (33), target cell availability (13), genetic polymorphisms of the viral coreceptors, and the HLA type (38).

Likewise, biological properties of HIV-1, namely, tropism, cytopathicity, and replication rate, are relevant parameters in AIDS pathogenesis. The switch in coreceptor usage from CCR5 to CXCR4, which occurs in approximately 50% of patients, is associated with more-vigorous viral replication and rapid disease progression (5, 6, 12, 20, 40). In recent years, investigations of viral features have shifted to evaluation of overall viral fitness (36). Viral fitness reflects the aptitude of a viral isolate to replicate in a given host system and is a consequence of the capacity of the virus to efficiently enter and infect target cells and to establish and spread the infection (8, 36). The efficacy of this process is further influenced by the availability of target cells, adaptive and innate immune responses, genetic host factors, and antivirals. Estimation of viral fitness has gained particular interest in the investigation of viral strains with drug resistance mutations, since these mutations are frequently accompanied by a loss of replicative capacity (7, 14, 24, 36). The outcome of the Swiss-Spanish intermittent treatment trial (SSITT) with 133 chronically infected patients was previously reported (15, 29–31). No clinically relevant impact of structured treatment interruption (STI) on improvement of viremia control was found. A boost of cytotoxic T lymphocyte and T helper responses occurred in most patients but did not correlate with viremia control (15, 31). In total, 17% of the SSITT patients potently suppressed VLs to levels below 5,000 RNA copies/ml without treatment after completion of the trial. However, as observed in similar studies (18), these patients had significantly lower viral set points before the initial onset of antiretroviral therapy (ART). No further decrease in their VLs upon STI was found (15). This result indicates strongly that preexisting viral and immune properties determined the outcome of this STI trial. Here we investigate the impact of fitness and intrinsic biological properties of the patient viruses on the extent of viremia rebound and the manifestation of viral set point during STI in a subgroup of 20 patients participating in the SSITT.

MATERIALS AND METHODS

Patients. Twenty chronically infected patients (Table 1) participating in the SSITT (15) at the University Hospital Zurich, Zurich, Switzerland, were studied. Patients underwent four consecutive STI cycles (2 weeks off and 8 weeks on

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TABLE 1. Patient and virus characteristics

Patient	Age (yrs)	Gender[a]	Characteristics before ART			Baseline data for STI trial			Post-STI characteristics		Autologous virus characteristics		
			Amt of time (mos) HIV-1 positive	Clinical stage	No. of RNA copies/ml	ART[b]	Mos at VL of <50 copies/ml	No. of CD4 cells/mm^3	No. of RNA copies/ml	No. of CD4 cells/mm^3	Coreceptor[c]		HIV subtype
											First cycle	Fifth cycle	
Controlling group													
106	39	M	>24	A	402	AZT, 3TC	36	878	5,128	650	ND	R5	B
107	44	F	>24	A	5,216	AZT, 3TC, NFV	25	544	2,551	537	ND	R5	B
112	46	M	6–12	A	32,140	AZT, 3TC, IDV	25	347	6,866	431	ND	R5	B
117	33	F	12–24	A	29,344	AZT, 3TC, RTV	36	489	13,007	341	ND	R5	B
118	33	M	12–24	A	16,927	AZT, 3TC, IDV	30	832	3,099	785	R5	R5	B
125	35	F	>24	A	11,298	ddI, d4T, NFV	23	777	4,873	882	R5	R5	E/CRF1
130	66	M	6–12	A	537	AZT, 3TC, NFV	30	829	107	729	ND	R5	A
Noncontrolling group													
102	40	M	>24	C	561,831	AZT, 3TC, IDV	32	723	76,805	491	R5	R5	B
109	38	M	>24	B	34,752	AZT, 3TC, RTV	31	1115	148,191	806	R5	R5	B
111	38	M	>24	A	122,729	ddI, d4T, NFV	11	422	22,030	362	R5	R5	B
113	59	M	12–24	A	107,303	AZT, 3TC, RTV	20	995	34,110	792	ND	R5	B
114	32	M	>24	A	9,276	AZT, 3TC, RTV	29	907	28,478	784	ND	R5	B
116	53	M	>24	C	467,593	AZT, 3TC, IDV	32	350	31,500	202	R5×4	R5×4	B
119	35	M	12–24	A	113,052	d4T, 3TC, SQV, RTV	12	440	99,274	394	ND	R5	B
120	55	M	12–24	A	150,390	AZT, 3TC, IDV	28	766	38,252	511	R5	R5	B
121	38	M	3–6	A	164,772	d4T, 3TC, NFV	12	591	67,321	380	R5	R5	B
122	40	M	>24	A	13,317	AZT, 3TC, IDV	30	669	24,982	481	ND	R5	B
126	49	M	>24	A	63,698	AZT, 3TC, RTV	34	842	19,782	441	ND	R5	B
127	52	F	>24	A	25,417	d4T, 3TC, NFV	22	839	106,923	614	R5	R5	B
128	43	F	>24	A	9,404	AZT, ddI, NFV	25	749	20,236	949	R5	R5	B

[a] M, male; F, female.
[b] AZT, zidovudine; 3TC, lamivudine; NFV, nelfinavir; IDV, indinavir; RTV, ritonavir; ddI, dideoxyinosine; d4T, stavudine; SQV, saquinavir.
[c] ND, not determined.

treatment) followed by a fifth long treatment interruption (a minimum of 12 weeks off treatment if no adverse effects occurred). Patients had never experienced drug failure and had undetectable VLs (<50 RNA copies/ml) for ≥6 months. Detailed descriptions of the respective clinical trial and patient characteristics have been reported elsewhere (15, 30). Written informed consent was obtained from all patients according to the guidelines of the University Hospital Zurich.

Quantification of plasma VL. Plasma HIV RNA was quantified by using the Amplicor HIV-1 Monitor test, version 1.5 (Roche Diagnostics, Rotkreuz, Switzerland) with a detection limit of <50 copies/ml (30).

Genotypic analysis. Peripheral blood mononuclear cell (PBMC) DNA from participants was genotyped at CCR5 G-2455A, CCR5 delta 32, RANTES G-403A, RANTES C-28G, macrophage inflammatory protein 1α (MIP-1α), T113C, and SDF-1 3′A genes by using TaqMan allelic discrimination techniques (Applied Biosystems). The contribution of the various alleles to HIV-1 susceptibility has been described elsewhere (41). Determination of HLA genotypes was performed as described previously (9).

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

Autologous patient viruses. Autologous virus was isolated from patient PBMCs during the first interruption cycle (week 2 of the trial) and the beginning of the fifth interruption cycle (weeks 42 to 50) by coculturing patient $CD4^+$ T cells with stimulated PBMCs (49). The 50% tissue culture infectious dose and coreceptor usage of the obtained virus stocks were determined as described previously (4, 43, 44).

In vitro replicative capacity. Virus inoculum (100 50% tissue culture infectious doses in 50 μl) was added to 12 replicate wells of a 96-well culture plate containing 2×10^5 stimulated PBMCs in 150 μl of culture medium. Culture supernatant was assayed for p24 antigen on days 4, 6, 10, and 14 postinfection (p.i.) by using an in-house p24 antigen enzyme-linked immunosorbent assay as described previously (27, 46). As the virus inoculum was not washed out at any stage of the experiment, the residual input p24 concentration was measured and subtracted from all test results. Cultures were fed 100 μl of medium on days 6 and 10 p.i.

Inhibition by chemokines. Inhibition of infection by chemokines was assessed on stimulated CD8-depleted PBMCs (45). The calculated inhibitory doses refer to the final concentrations of chemokines in the cultures on day 0. Virus production in the absence of chemokines was designated as 100%, and the ratios of p24 antigen production in chemokine-containing cultures were calculated relative to this value. The chemokine concentrations (in nanograms per milliliter) causing 50, 70, and 90% reduction in p24 antigen production (50, 70, and 90% inhibitory doses) were determined by linear regression analysis. If the appropriate degree of inhibition was not achieved at the highest or lowest chemokine concentration, a value of more than or less than was recorded and these upper or lower limits were used for statistical analysis.

Neutralization assay. Neutralization activity was evaluated as described previously (4, 44). MAbs 2F5 (28) and 2G12 (47) were gifts from H. Katinger, MAb IgG_1b12 (3) was from D. Burton, and the CD4-immunoglobulin CD4-IgG_2 molecule was from P. Maddon (1). Briefly, virus inoculum was incubated with serial dilutions of antibodies for 1 h at 37°C. Then stimulated PBMCs were infected with aliquots of this preincubation mixture. Three days postinfection, cultures were washed three times and supplemented with fresh medium and fresh stimulated PBMCs. Cultures were incubated for 6 to10 days and assayed for p24 antigen. Calculation of inhibitory doses was as described above.

Data analysis. For each patient, the following viral life history parameters were calculated.

(i) Pre-ART VL. The pretreatment VL corresponds to the last plasma HIV RNA value recorded before ART or, if two measurements within 6 months before initiation of ART were available, to the geometric mean of those levels.

(ii) Post-STI VL. The post-STI VL value reflects the viral set point, i.e., the plateau of viremia post-STI, and was determined as the geometric mean of plasma HIV RNA levels measured after week 40, when a steady state was reached (usually between weeks 46 and 64). Three patients (patients 102, 109, and 116) had plateau VLs calculated from the two or three time points just prior to the restart of therapy. For two patients (patients 107 and 130), the week 46 data point was part of a peak and was therefore omitted from the estimation of the plateau. For the 17 patients who remained off therapy for extended periods, an average of 9.06 data points (range, 6 to 12) was used to calculate the plateau VLs.

(iii) Control of viremia. Patients were classified into a controlling group and a noncontrolling group according to their abilities to control viremia in the absence of ART between weeks 40 and 76. Control of viremia was defined as maintenance of a VL of <5,000 RNA copies/ml for at least 8 weeks during this time period. The cutoff of 5,000 RNA copies/ml was set in the SSITT trial as a value above which patients had to reinitiate ART (15).

(iv) Improvement of viral set point. For differences between pre-ART VLs and post-STI VLs, positive values indicate decreases (improvement) in VLs and negative values indicate increases. A decrease in VL of 0.5 logs is considered to be a significant change.

(v) Cumulative VL. The total amount of virus produced during the individual cycles was estimated by calculating the area under the curve (AUC). The AUC between successive time points was calculated using the following formula: $AUC = (VL2 - VL1) \times (T2 - T1)/\ln(VL2/VL1)$, where 1 and 2 indicate values at first and second time points and time T is measured in days after start of therapy. The total AUC is determined by adding the AUCs between successive time points. Since for some patients RNA levels at weeks 10, 20, 30, and 40 were not available, AUC values for all patients from these time points were derived from RNA levels at weeks 2, 9, 12, 19, 22, 29, 32, and 39 by extrapolation: e.g., VL at week 10 (VLwk10) = $VLwk9 \times \exp\{[\ln(VLwk9) - \ln(VLwk2)]/[(T \text{ at week } 9) - (T \text{ at week } 2)] \times 7\}$. When a given VL was undetectable, the limit of detection was used as the anchor for the extrapolation.

(vi) In vitro replication capacity (slope between values from days 0 and 6). Slopes were calculated by performing linear regression analysis using the natural logarithm of p24 antigen values obtained on days 0, 4, and 6 p.i.

Statistical analyses. Statistical analyses were performed using GraphPad Prism version 4.0 (GraphPad Software Inc., San Diego, Calif.). Patient groups were compared by using nonparametric (Mann-Whitney) tests unless stated otherwise.

RESULTS

Phenotypic and genotypic evaluation of autologous patient isolates. Twenty patients participating in the SSITT were studied to determine the impact of intrinsic patient virus characteristics and overall viral fitness on the level of viremia rebound and manifestation of viral set point upon STI. Patients were classified into controlling and noncontrolling groups according to their abilities to control viremia in the absence of drugs after STI (Table 1). The controlling and noncontrolling groups differed significantly in their pretreatment VLs ($P = 0.016$) and their post-STI VL plateaus ($P = 0.0004$), but no difference in the abilities to improve VLs upon STI was observed (see also reference 15). Thus, potent control of viremia upon STI was not a consequence of treatment interruptions but correlated strongly with pre-ART VLs.

Virus isolates from patient PBMCs were collected during the first interruption cycle (week 2 of the trial) and the fifth interruption cycle (weeks 42 to 50). Isolation of virus during the first cycle was not possible in all cases because some patients had no or extremely low viral rebound during this cycle. Altogether, 10 first-cycle and 20 fifth-cycle virus isolates were obtained (Table 1). With one exception (patient 116), all patients were infected with R5 virus isolates both during the first STI and after completion of the STI (Table 1). The isolate from patient 116 utilized CCR5 and CXCR4 before and after the STI. Thus, viremia control in these patients was not determined by a specific viral tropism nor did we find evidence that STI provoked changes in coreceptor utilization patterns. Equally, the distribution of HIV subtypes among patients does not suggest that spontaneous control of plasma viremia was influenced by the genetic subtype (Table 1).

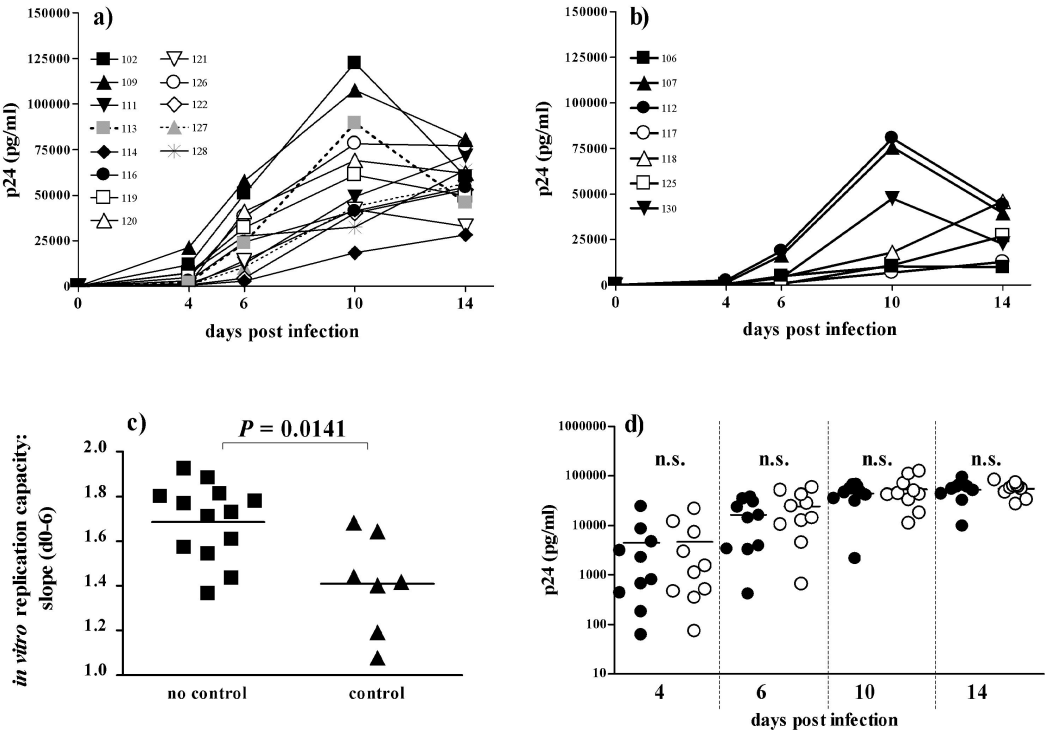

FIG. 1. In vitro replication kinetics of patient isolates. (a and b) Profile of p24 antigen production in cultures of CD8-depleted PBMCs infected with isolates derived during the fifth cycle from patients in the noncontrolling group (a) and the controlling group (b). (c) In vitro replication capacities. Slopes of viral antigen production between days 0 and 6 [slope (d0-6)] were calculated by performing linear regression analysis using the natural logarithm of p24 antigen values obtained on days 0, 4, and 6 p.i. Slopes for viruses from controlling (triangles) and noncontrolling (squares) patients were compared using the Mann-Whitney test. Data are means of results from two independent experiments. (d) The extent of viral replication of first-cycle and fifth-cycle virus pairs from eight patients of the noncontrolling group and two patients of the controlling group on CD8-depleted PBMCs is depicted as p24 antigen production on days 4, 6, 10, and 14 p.i. Antigen production levels of first-cycle (closed circles) and fifth-cycle (open circles) virus pairs were compared using the Wilcoxon signed-rank test. n.s., not significant. Bars indicate means.

In vitro replicative capacity. To compare the efficacies of patient isolates in infection of target cells, we evaluated their in vitro replication capacities on stimulated primary $CD4^+$ T cells. In order to allow interisolate comparisons, infection experiments for all isolates were performed on the same day by using the same target cells from healthy donors, which eliminates possible distortion of the results by donor cell variability. For the assessment of the viral replication capacity, the absolute amount of viral antigen generated was taken as a measure of viral productiveness. Therefore, this analysis relies on the assumption that the p24 antigens from all isolates are detected equally well by the p24 antigen detection system used. In fact, all virus isolates were readily detectable by our p24 antigen enzyme-linked immunosorbent assay, irrespective of their genetic subtypes (data not shown). A range of methods have been developed with the aim of evaluating viral fitness or the contribution of specific viral genes to the overall replicative capacities of viral variants (36). The strength but also the limitations of the assay used in our studies lie in the use of replication-competent patient isolates and primary cells as target cells. This allowed us to investigate the influences of all viral genes on replication in the natural target cells in order to get an initial estimate of the relative fitness levels of the diverse patient isolates. Although useful for the analysis described here, exact measurements of the relative contributions of specific viral genes are not possible with this method.

We observed a striking difference between the cultures infected with virus isolates from the controlling and noncontrolling groups in both the amount of p24 antigen produced and the timing of production (Fig. 1a and b). The levels of p24 antigen generated by days 4 and 6 p.i. were significantly lower in the controlling group ($P = 0.0216$ and 0.0175, respectively). In the majority of cultures in both groups, the peak of infection was reached by day 10 p.i. and virus production decreased thereafter.

To obtain a numerical measure of the in vitro replicative capacities, we calculated the slopes of viral antigen production between days 0 and 6. During this early phase before replication peaks, both groups of viruses have the same growth conditions in terms of supply of target cells and nutrition and thus a logarithmic growth phase can be safely assumed. Again, a significant difference between the controlling and noncontrolling groups was found using these slopes as a measure of the in vitro replicative capacities (Fig. 1c).

The in vitro replicative capacity does not change during short-term STI. It cannot be excluded that isolates derived during the fifth cycle evolved as a consequence of immune escape and viral evolution during STI (23). Specimens for virus

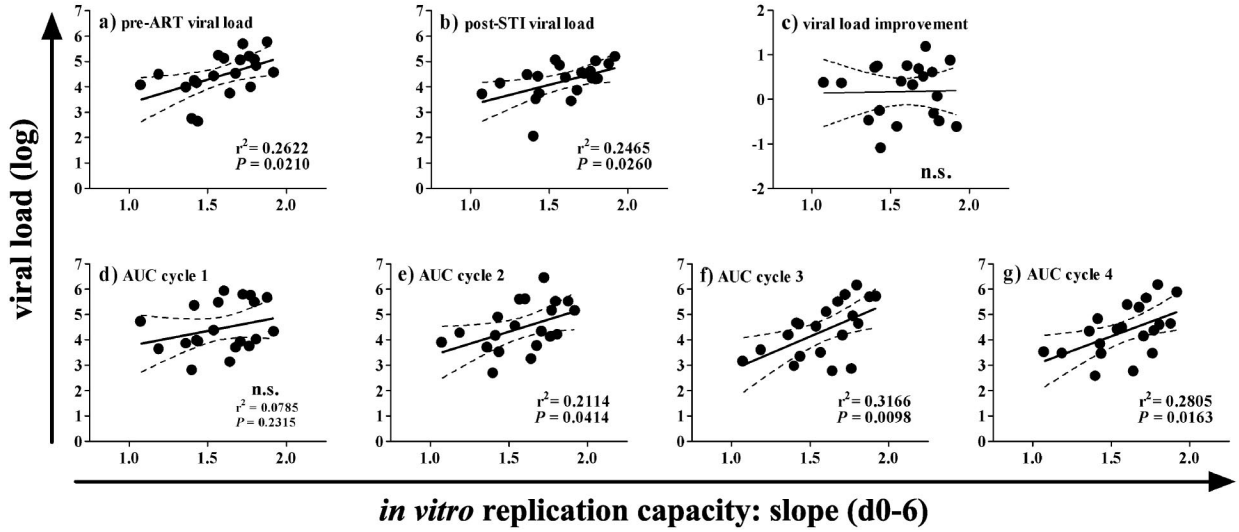

FIG. 2. In vitro replication kinetics correlate with in vivo VLs. Results of the correlation analysis of in vitro replication capacities {slopes of viral antigen production between days 0 and 6 [slope (d0-6)]} and pre-ART VLs (a), post-STI VLs (b), levels of VL improvement (c), and cumulative levels of viral replication (AUC) during cycles 1 (d), 2 (e), 3 (f), and 4 (g) are shown. n.s., not significant.

isolation at pre-ART time points were not available, but it was recently shown that virus evolving early in STI represents pre-ART quasipecies (B. Joos, M. Fischer, A. Trkola, J. Böni, H. Kuster, A. Oxenius, J. Wong, B. Hirschel, R. Weber, and H. Günthard, 9th Conf. Retrovir. Opportunistic Infect., abstr. 531-M, 2002). We therefore probed whether or not STI induced changes in the replication potencies of individual strains by comparing the growth characteristics of first-cycle and fifth-cycle virus pairs from 10 patients (8 noncontrolling and 2 controlling patients) (Table 1 and Fig. 1d). We observed no tendency towards the evolution of more rapidly replicating or more slowly replicating strains. No significant differences between total levels of viral antigen produced by early and late isolate pairs were detected (Wilcoxon signed-rank test) (Fig. 1d), nor was a difference in replication slopes detectable (data not shown). This excludes the possibility that short-term STI induced changes in virulence and indicates a preexisting infection with these slow-replicating isolates before initiation of ART.

Relationship between viral replicative capacities and viremia levels. A central point of our investigation was to determine whether viral replication capacity in vitro substantially impacts viral replication in vivo. We observed that low replication capacities of the patient isolates correlated with both low pre-ART and low post-STI plateaus but not with the patients' abilities to improve viral set points upon STI (Fig. 2a to c).

Our data provide further evidence that the in vitro replication capacity of a viral isolate influences the total amount of virus replication during the short-term treatment interruption. The in vitro replication capacities of patient virus isolates correlated with the cumulative viral production levels (AUC) during cycles 2, 3, and 4 but did not reach statistical significance for the first cycle (Fig. 2d to g). This suggests that viral rebound may be driven at least in part by viral properties.

Sensitivity to inhibition by chemokines. Viral fitness in vivo will not only be a consequence of the efficacy of virus-target cell interaction but will also be steered by the presence of and susceptibility to antiviral agents. Since the virus isolates investigated here were fully suppressed by the antiretroviral drugs prescribed, we focused our investigation on naturally occurring antiviral agents, namely chemokines and neutralizing antibodies. Differential susceptibilities to one or both of these classes of inhibitors could potentially affect viral replication in vivo.

We assessed the inhibitory effects of the chemokines RANTES/CCL5 and MIP-1α/CCL3 on viral replication in stimulated healthy donor $CD4^+$ T cells. To rule out distortion of the results by donor cell influences, all isolates were analyzed on cells from the same donors. The R5X4 isolate from patient 116 was excluded from this analysis.

We observed in both the controlling and the noncontrolling patient groups chemokine-sensitive and -insensitive isolates. In agreement with previous observations (45), we found that isolates were overall more susceptible to inhibition by RANTES than by MIP-1α (median 70% inhibitory doses were 36 and 393 ng/ml, respectively). Isolates from the controlling group showed an increased sensitivity to inhibition by chemokines compared to isolates from the noncontrolling group (Table 2; Fig. 3a and b). The median inhibitory doses of RANTES differed significantly between the two patient groups. A difference in susceptibilities to inhibition by MIP-1α was also observed but was less pronounced. This is probably due to the lower intrinsic inhibitory activity of MIP-1α. Only 6 of the 19 isolates were inhibited to 90% at the highest concentration tested (500 ng/ml).

Most strikingly, a strong correlation between low replication capacities of viral isolates in vitro and high sensitivities to RANTES was found (Fig. 3c). In addition, sensitivities to inhibition by RANTES correlated with pre-ART VLs and post-STI viral set points (Fig. 3d and e). Increased sensitivity of

TABLE 2. Inhibitory doses (ID)[a] of chemokines, neutralizing antibodies, and tetrameric CD4-IgG_2

Patient	RANTES			MIP-1α			2F5 ID_{90} (mg/ml)	2G12 ID_{90} (mg/ml)	IgG_1 b12 ID_{90} (mg/ml)	CD4-IgG_2 ID_{90} (mg/ml)
	ID_{50} (ng/ml)	ID_{70} (ng/ml)	ID_{90} (ng/ml)	ID_{50} (ng/ml)	ID_{70} (ng/ml)	ID_{90} (ng/ml)				
Control group										
106	16	46	81	218	>343	491	13.9	>25	5.4	19.2
107	<3	13	34	58	>458	>500	16.2	0.8	>10	24.3
112	9	24	101	65	341	>500	12.7	>25	7.9	14.1
117	11	17	42	62	262	>500	3.7	2.3	4.4	>25
118	<10	12	16	37	53	288	19.3	>25	>10	24.3
125	11	18	26	33	56	225	22.9	>25	>10	>25
130	4	7	20	45	81	300	17.7	>25	9.6	22.4
Noncontrol group										
102	14	>98	>150	>500	>500	>500	20.5	1.4	8.5	22.3
109	27	91	>148	>500	>500	>500	21.3	>25	>10	9.5
111	30	57	83	164	>393	>500	17.4	16.9	9.5	15.4
113	48	107	>150	>273	>500	>500	>25	4.7	>10	24.1
114	32	60	89	95	>461	>500	>25	24.8	>10	17.5
116	ND	ND	ND	ND	ND	ND	21.3	7.0	5.9	18.1
119	18	25	81	235	>412	>500	19.8	>25	>10	>25
120	27	56	99	136	247	>500	7.9	22.4	>10	13.1
121	4	20	>125	>296	>500	>500	3.6	0.6	6.9	9.4
122	5	32	71	100	197	>488	11.8	8.1	5.2	7.4
126	19	36	>126	190	>307	>498	22.1	>25	>10	23.1
127	18	47	>149	303	>450	>500	19.8	17.0	9.6	10.3
128	59	87	>140	>500	>500	>500	16.3	10.0	9.5	11.1

[a] ID_{50}, ID_{70}, and ID_{90} are 50, 70, and 90% inhibitory doses, respectively. ND, not determined.

low-replicating isolates to inhibition by CCR5-targeting chemokines could indicate that these viruses require high coreceptor densities on target cells for entry. A low availability of cells expressing high levels of CCR5 together with the increased sensitivity to inhibition by endogenous chemokines could therefore contribute to reduced fitness and consequently lower replication of these viruses in vivo. Of note, no influence of genetic polymorphisms in the genes for CCR5, RANTES, and MIP-1α on viremia control was observed in our subset of patients (Table 3).

Sensitivity to inhibition by neutralizing antibodies. Given that high sensitivity to inhibition by chemokines was strongly associated with low in vitro replication capacity of a virus isolate, one could reason that the observed effect of chemokines may be simply a consequence of the decreased infectivity in vitro. This may potentially be the case, since efficacy of inhibition was assessed over multiple rounds of infection and, therefore, over time inhibitory agents will have to counter vastly different amounts of virus. In order to probe whether viruses with low replication capacities are in general more

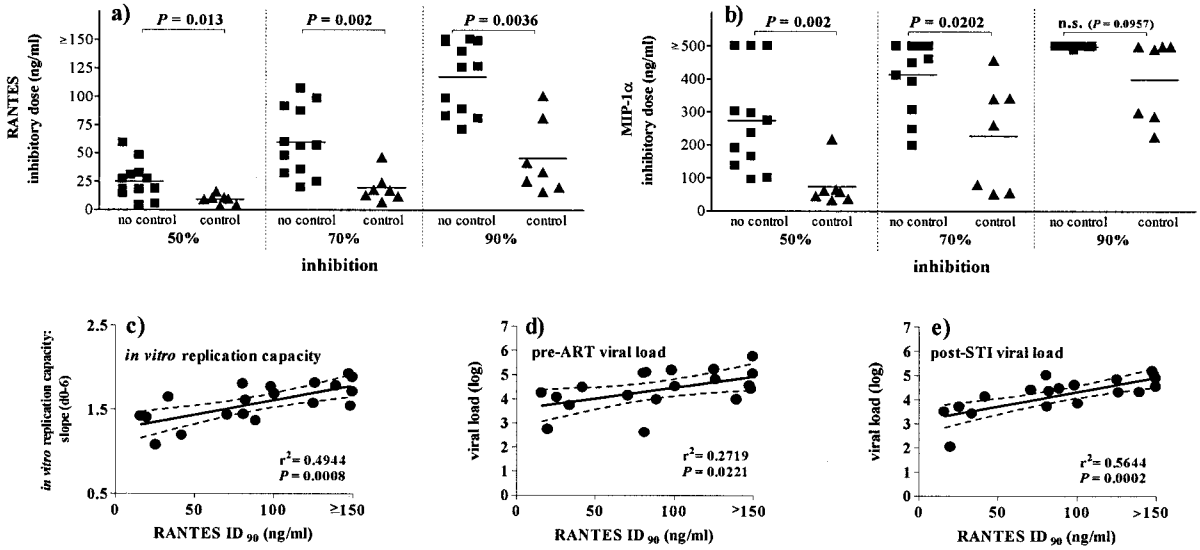

FIG. 3. Sensitivity to inhibition by chemokines. The 50, 70, and 90% inhibitory doses of RANTES (a) and MIP-1α (b) in CD8-depleted PBMC cultures are shown. Inhibitory doses for viruses from controlling (triangles) and noncontrolling (squares) patients were compared using the Mann-Whitney test. Data are means of results from two independent experiments. Results of the correlation analysis of the 90% inhibitory doses (ID_{90}) of RANTES with in vitro replication capacities {slopes of viral antigen production between days 0 and 6 [slope (d0-6)]} (c), pre-ART VLs (d), and post-STI VLs (e) are displayed. n.s., not significant. Bars indicate means.

TABLE 3. Genotypic analysis[a]

Patient	Characterization of genes for:						
	CCR5 G-2455A	CCR5 delta 32	CCR2 V64I	RANTES G-403A	RANTES C-28G	MIP-1α T113C	SDF-1 3′A
Controlling group							
106	1	1	1	1	1	2	1
107	3	2	1	1	1	1	1
112	3	2	1	1	1	2	1
117	1	1	1	2	1	2	1
118	3	1	2	1	1	1	1
125	2	1	1	2	1	2	3
130	2	1	1	1	1	2	2
Noncontrolling group							
102	2	1	1	1	1	2	1
109	2	2	1	1	1	2	1
111	2	1	1	1	1	1	1
113	3	1	2	1	1	2	1
114	2	1	1	1	1	2	1
116	1	1	1	1	1	1	1
119	2	2	1	1	1	1	3
120	2	2	1	1	1	2	3
121	2	2	1	1	1	2	1
122	2	1	1	1	1	2	1
126	2	1	1	1	1	1	2
127	2	1	1	1	1	2	1
128	3	1	2	1	1	1	2

[a] 1 indicates wild-type genes, and 2 and 3 indicate heterozygosity and homozygosity, respectively, for the mutant gene.

susceptible to antivirals, we investigated their sensitivities to inhibition by the potent neutralizing anti-gp41 MAb 2F5 and the anti-gp120 MAbs 2G12 and IgG_1b12 as well as the tetrameric CD4 molecule, CD4-IgG_2. In contrast to results for inhibition by chemokines, we found no significant differences in sensitivities to these antibodies. Of note, virus isolates from the controlling group proved to be slightly more resistant to CD4-IgG_2 (Table 2; Fig. 4). Therefore, the increased sensitivities of virus isolates with slow replication capacities to inhibition by chemokines is a distinct feature of these virus strains and does not reflect a general sensitivity to entry inhibitors.

HLA genotypes and viremia control. Our analysis of virus isolates from chronically HIV-1-infected individuals thus far leaves open the question of whether virus isolates with low replication capacities were transmitted or emerged over time as a result of an active immune defense. In order to resolve the issue of whether low viral fitness is more a consequence of selection during transmission or a reflection of the impact of immune pressure, an extensive, longitudinal monitoring of patients from acute to chronic disease stages will be necessary. While this type of analysis could not be performed since natural history samples from the acute infection stage were not available for our patient cohort, we further investigated the potential influence of host genetic factors on viral replication and control. This is of particular interest since both transmission and emergence of viral isolates with low fitness will be influenced by genetic host factors, either directly as in the case of the chemokine and chemokine receptor polymorphisms described above or indirectly through control of immune responses. Several HLA genotypes been shown to influence disease progression and viremia control and are considered to drive immune defense and thus viral replication (10, 22, 42). We therefore determined HLA genotypes in our cohort (Table 4) and explored whether the distribution of HLA genotypes which are described to be associated with susceptibility (HLA-B*08, HLA-B*3501, HLA-B*45, HLA-B*53, and HLA-Cw*04) or protection (HLA-A2/*6802, HLA-A*11, HLA-B*27, HLA-B*51, HLA-B*57, HLA-B*58, HLA-Cw*08, and HLA DRB*01) may indicate why viral variants with low or high replication capacities were selected in our patient cohort. We found that neither genes considered to confer protection nor genes associated with increased viral replication and disease progression were present at higher frequencies in either the controlling or the noncontrolling group. However, it has to be taken into consideration that our cohort of patients is small, and given the vast polymorphism of HLA genes, it is not too surprising that no such associations were found. Although it does not appear that HLA genotypes were a driving force in the establishment of viral fitness in our patients, it nevertheless cannot be ruled out that these genes in concert with other immune and genetic parameters contributed to the selection of viral variants.

DISCUSSION

In this study, we investigated the generic fitness of virus isolates derived from patients participating in the SSITT (15,

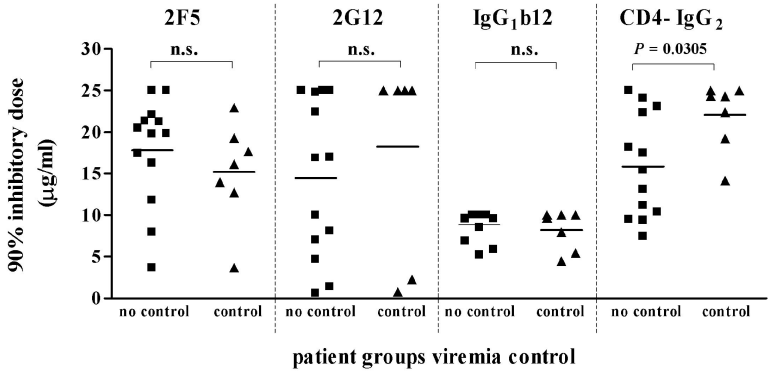

FIG. 4. Sensitivity to inhibition by neutralizing antibodies and CD4-IgG_2. The 90% inhibitory doses for the neutralizing anti-gp41 MAb 2F5, the anti-gp120 MAbs 2G12 and IgG_1b12, and the tetrameric CD4 molecule, CD4-IgG_2, are shown. Inhibitory doses for viruses from controlling (triangles) and noncontrolling (squares) patients were compared using the Mann-Whitney test. Data are means of results from two to four independent experiments. n.s., not significant. Bars indicate means.

TABLE 4. HLA genotypes[a]

Patient	Genotype of HLA-I			Genotype of HLA-II			
	A	B	Cw	DRB1	DRB3	DRB4	DRB5
Controlling group							
106	*31, *2601/02/08	*14, *40	*0304/06/08/05, *0802/04	*04, *1101/04	*02	*01	
107	*02, *−	*44, *5201	*0303, *0501	*1301/02, *−	*0101, *0301		
112	*02, *03	*07, *51	*0702, *1203	*0101/03/04, *1301/02	*0301		
117	*02, *68	*4901, *57	*0602/03, *07	*1101/04, *07	*02	*01	
118	*0101-05N	*08, *57	*0602, *07	*1501/03, *03011/05	*0101		*0101
125	*11, *33	*5801, *15	*1203, *0302/05	*12, *1301/02	*0301, *0301		
130	*02, *03	*15, *35	*0304/06/08/09, *0401/04/05	*0101/04, *04		*01	
Noncontrolling group							
102	*2301, *32	*35, *38	*0401/04/05, *1203	*1101/04, *1303	*0101		
109	*11, *25	*18, *−	*02022/024, *0701/05/06	*1101/04, *0701	*02	*01	
111	*02, *03	*07, *−	*07, *−	*1501*03, *−			*0101
113	*03, *−	*15, *57	*0304/06/08/09, *0602/03	*04, *07		*01, *01	
114	*02, *31012/3102	*27, *51	*0102/03, *1502/05/06	*0101/04, *1101/04	*02		
116	*02, *03	*44, *4901	*02, *07	*1101/04, *04	*02	*01	
119	*24, *68	*15, *44	*0303, *0704	*04, *1101/04	*02	*01	
120	*02, *24	*27, *44	*0102/03, *1601	*0103, *07		*01	
121	*01, *−	*07, *27	*0702, *02	*1501/03, *0901		*01	*0101
122	*03, *24	*57, *15	*0303, *0602/03	*07, *1301/02	*02	*01	
126	*03, *33	*14, *18	*0701/05/06, *0802/04	*11, *13	*02, *0101		
127	*01, *11	*08, *15	*0701/05/06, *0102/03	*04, *1102/03	*02	*01	
128	*03, *24	*35, *57	*0602/03, *0401/02/04/05	*0101/04, *07		*01	

[a] Dashes indicate homozygosity.

32). A fraction of patients (17%) participating in this trial potently suppressed viremia upon final cessation of ART. However, this control of viremia was not induced by STI but reflected pre-ART viral set points (15, 32). Thus far no parameter could be identified that provided insight into how and why these patients maintained low VLs in the absence of treatment (15, 29–32).

Observations made in long-term nonprogressing patients and analysis of drug-resistant strains of HIV-1 strongly suggest that low viral replication capacity in vitro may be directly associated with a decreased viral burden in vivo and predicts slower disease progression (5, 14, 16, 17, 19, 20, 36, 37, 40, 48). In agreement with the results of previous studies, we found a significant correlation between viral replication capacities in vitro and viral set points in our group of 20 chronically infected patients.

Previous reports have by and large focused on cross-sectional analyses of patients. The advantage of the present study lies in the nature of the STI, in which a coherent group of patients, fully sensitive to antiretroviral drugs, underwent several controlled viremic episodes. The close longitudinal monitoring of these patients provided the empirical data to probe whether viral fitness steers the magnitude of the rebounding viremia. We were able to demonstrate that the difference in replication capacities as measured in vitro remains proportional to the replication rate in vivo over several short-term intervals of viral replication. Collectively, although the investigated group of patients is small, these data support the hypothesis that viral rebound in chronic HIV-1 infection is in part driven by viral fitness.

Of note is that the determination of the replicative capacity in vitro excludes several potential factors affecting viral replication in vivo. Thus, in vivo viral fitness will not be driven only by the virus's access to target cells and its aptitude to efficiently enter these cells and establish productive infection but also by its sensitivity to inhibitory agents such as drugs, neutralizing antibodies, and antiviral chemokines.

With one exception, all virus isolates tested utilized solely R5 for entry and should thus in principle be able to infect the same pool of target cells. HIV-1 isolates are known to differ greatly in their susceptibilities to inhibition by chemokines (45). Whether or not the antiviral potency of chemokines substantially contributes to viremia control in vivo has not yet been conclusively determined (11, 21, 25, 34). The efficacy of chemokine inhibition will be governed by the sensitivity of the individual virus strain to inhibition, by the magnitude of chemokine secretion, and also by the genetically and cell type-dependent density of chemokine receptor expression.

A key finding of the present study was the observation that viruses from patients who control viremia at low levels are more susceptible to inhibition by CCR5-targeting chemokines. This correlation argues for a low affinity of the respective viral envelope proteins in the interaction with the receptor and/or a particular need of these viruses for high levels of CCR5 expression on their target cells. The restriction in replication could be to some extent due to the lack of appropriate target cells expressing the required high density of receptors. Taken together, our data suggest that the low replication capacities of these viruses are at least in part due to a limitation in viral entry. We are currently cloning the envelopes of these viruses to investigate whether the ensuing low replication capacity is mainly the result of a less efficient entry process or whether additional viral genes participate in manifesting the low-replication phenotype.

Due to their reduced virulence, viruses with low replication capacities will cause less destruction to the immune system and

thus might be more successful in inducing and maintaining immune responses (5, 17, 39). Likewise, at a reduced replication rate, mechanisms of escape from immune responses and antiretroviral drugs will appear less rapidly (8, 36). Whether or not low viral fitness was a cause or an effect of an improved anti-HIV immune response could not be determined conclusively in this cohort of patients. Despite significant increases in HIV-specific cytotoxic T-cell responses and T helper responses, no correlation between these cellular immune responses and control of viremia was found (32). However, autologous neutralization responses pre-STI were found to be higher among patients who suppressed viremia (A. Trkola and H. F. Günthard, unpublished data). Determining when and why these viruses with low replicating capacities evolved will require further investigations. At present it remains unclear whether viruses with decreased replication capacities were already present at the stage of transmission or were selected over time as a consequence of an active immune defense and/or genetic host factors. To fully unravel the dependencies of virus and immune responses, a longitudinal analysis of a larger set of patients from acute to chronic disease stages will be required.

Our data provide substantial evidence that viral fitness was a determining factor of low viral set points detected in a fraction of patients participating in the SSITT. Furthermore, our observations strongly suggest that low-replicating viruses were present before ART was initiated and therefore are not a consequence of STI. Thus, to fully understand the prospects of therapeutic interventions in chronic infection, it is pivotal to consider viral fitness and its consequences for activation and maintenance of immune responses or emergence of drug resistance.

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