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Baseline Susceptibility of Primary Human Immunodeficiency Virus Type 1 to Entry Inhibitors

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Human immunodeficiency virus type 1 plasma viruses from 29 entry inhibitor-naive patients were characterized for their susceptibilities to T-20, AMD3100, and RANTES. A strikingly wide range of susceptibilities to T-20 was observed that was influenced by coreceptor usage but not by the susceptibilities of the viruses to inhibitors that target the chemokine receptors or by polymorphisms in the gp41 N helix.

The human immunodeficiency virus type 1 (HIV-1) entry process (2, 19, 27) can be inhibited by several drugs (3, 14, 22, 23), which belong to three groups according to the step they inhibit: (i) inhibitors of the interaction between the viral surface glycoprotein (gp120) and CD4, which target the CD4-binding site on gp120; (ii) inhibitors of the interaction between gp120 and CCR5 or CXCR4 (e.g., chemokines and their derivatives or small organic molecules that antagonize chemokine receptor activity); and (iii) fusion inhibitors, which are peptides derived from the sequence of the viral transmembrane glycoprotein (gp41) that prevent the formation of a hairpin structure required for membrane fusion. One of these peptides, T-20 (enfuvirtide), is currently being evaluated in phase III clinical trials (15, 25, 26). The optimization of treatment strategies that include entry inhibitors will rely on the availability of methods capable of determining baseline viral susceptibility and acquired resistance to these drugs. In addition, the characterization of the determinants of baseline susceptibility and of acquired resistance to entry inhibitors may provide valuable information on the viral entry process and on the precise mechanism of action of these drugs.

We have developed a recombinant virus assay that permits the assessment of viral susceptibility to entry inhibitors. We modified a pNL4-3 molecular clone by deleting the region of the envelope gene encoding gp120 and the ectodomain of gp41 (positions 6480 to 8263) and replacing it with a linker that contains a unique *Mlu*I restriction site (vector 43-Δenv). Recombinant virus was produced by cotransfection of 293-T cells with the *Mlu*I-linearized 43-Δenv vector and a reverse transcription-PCR product, amplified from patient plasma samples, which encompasses the deleted region and carries short overlaps that allow homologous recombination. Virus-containing supernatants were used to infect subconfluent U373MG-CD4 cells expressing either CCR5 or CXCR4 (17), in the absence or in the presence of increasing concentrations of entry inhibitors. These target cells carry an HIV-1 long terminal repeat-*lacZ* cassette, which allows the quantification of single cycle infectivity by a colorimetric assay based on HIV-1 Tat-induced expression of β-galactosidase (24). The concentrations inhibiting 50% of virus infectivity (IC_{50}s) were calculated by using the median-effect equation (6).

The recombinant virus assay was first used to determine the baseline susceptibilities of subtype B primary viruses to the fusion inhibitor T-20 (American Peptide Company, Inc., Sunnyvale, Calif.). Plasma samples selected for the study were obtained from 29 entry inhibitor-naive patients; two patients (codes 12 and 17) were treatment naive, and the remainder had been treated with multiple reverse transcriptase and/or protease inhibitors. Samples from 23 patients harbored R5 exclusive viruses and were characterized by plasma viral loads ranging from 1,400 to 227,000 (median, 39,650) copies/ml. Patients are numbered in the order of decreasing susceptibility to T-20 (Table 1). The baseline susceptibility to T-20 for these viruses ranged from 3 to 1,002 ng/ml, with a median IC_{50} of 159 (±55) ng/ml. The variability in the range of susceptibilities to T-20 was much wider than that measured for other antiretroviral agents (13). IC_{50}s for most patients were in the range of previously reported data for T-20-naive patients, obtained by virus culture on peripheral blood mononuclear cells (8, 9).

IC_{50}s were also calculated for plasma samples from four patients with dual-tropic (R5X4) virus populations and two patients who harbored X4 exclusive viruses (Table 1). Viral load for these patients ranged from 2,000 to 75,000 copies/ml. Overall, IC_{50}s for T-20 measured on $CXCR4^+$ cells were within the range observed with $CCR5^+$ cells, varying from 32 to 408 ng/ml. It should be emphasized, however, that susceptibility could be affected by different numbers of chemokine receptor molecules expressed on target cells. Because only a small number of X4 viruses were tested, these results do not resolve the currently debated issue of the differential susceptibilities of viruses with different tropisms to T-20 (8, 9). It is interesting, however, that viruses from patients 24, 25, and 26, which displayed a mixed R5 and X4 phenotype, were 10 times more susceptible to T-20 when tested on $CCR5^+$ cells than when tested on $CXCR4^+$ cells, whereas the T-20 susceptibility of virus from patient 27 was not significantly different when measured on these cell lines. These results indicate that, for a given virus population, the usage of alternative chemokine receptors can affect the efficacy of T-20 inhibition.

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TABLE 1. Baseline susceptibilities to T-20 of primary viruses

Viral tropism	Patient code or virus	$CD4^+$ $CCR5^+$ target cells		$CD4^+$ $CXCR4^+$ target cells	
		IC_{50}[a] (ng/ml)	SD[b]	IC_{50}[a] (ng/ml)	SD[b]
R5	1	3	2.47		
	2	23	2.41		
	3	28	1.52		
	4	30	2.41		
	5	31	1.16		
	6	32	1.32		
	7	33	1.56		
	8	35	2.25		
	9	45	2.96		
	10	45	1.25		
	11	50	1.15		
	12	95	1.24		
	13	101	1.22		
	14	103	2.84		
	15	115	1.37		
	16	122	1.64		
	17	134	1.46		
	18	193	1.4		
	19	220	1.53		
	20	286	1.77		
	21	309	1.38		
	22	616	1.26		
	23	1,002	1.21		
R5X4	24	2	1.84	90	1.62
	25	3	1.31	32	1.83
	26	28	1.11	356	1.39
	27	136	1.59	61	1.57
X4	NL4-3[c]			30	1.46
	HxB2[c]			2	1.17
	28			228	1.28
	29			408	2.24

[a] IC_{50}s represent the geometric means of at least three independent experiments.
[b] Standard deviations of the geometric means were calculated as the antilogarithms of the standard deviations of the logarithms of the IC_{50}s.
[c] NL4–3 and HxB2 are reference T-cell-line-adapted viruses.

The N helix of the ectodomain of gp41 is the molecular target of T-20 and other peptides based on the sequence of the C helix of gp41 (4, 16). Accordingly, the development of resistance to T-20 both in vitro and in vivo is associated with selection of mutants carrying substitutions in the $_{36}GIV_{38}$ tripeptide sequence and in other positions of the N helix, between residues 36 and 45 (20, 25; P. Sista, T. Melby, M. L. Greenberg, D. Davison, L. Jin, S. Mosier, M. Mink, E. Nelson, L. Fang, N. Cammack, M. Salgo, and T. J. Matthews, abstract from the XI International HIV Drug Resistance Workshop 2002, Antivir. Ther. **7**[Suppl. 1]**:**S23, 2002). These mutations appear to reduce virus replication capacity and are generally not found in viruses in the absence of T-20 selective pressure (20, 25, 28; J. Lu, P. Sista, N. Cammack, and D. Kuritzkes, abstract from the XI International HIV Drug Resistance Workshop 2002, Antivir. Ther. **7**[Suppl. 1]**:**S74, 2002; Sista et al., Antivir. Ther. **7:**S23, 2002). To determine whether the wide range of susceptibilities to T-20 observed for the primary viruses analyzed here depended on polymorphism in this region, we sequenced the N-helix region from 16 plasma viruses characterized by different tropisms and by different levels of baseline susceptibility to T-20, including the most and the least sensitive viruses (Table 2). All samples carried the wild-type $_{36}GIV_{38}$ tripeptide sequence. In several samples, polymorphisms were detected at positions of the N helix that have previously been shown to be subject to natural variation in T-20-naive patients (18). None of these polymorphisms was repeatedly found in samples characterized by low or high susceptibility to T-20. Our data suggest that the determinants for the natural susceptibility to T-20 are different from currently described determinants of acquired resistance and reside outside the gp41 N helix.

We reasoned that a virus characterized by efficient interaction with chemokine receptors could be expected to display reduced unmasking of the gp41 ectodomain and/or accelerated kinetics of the entry process, thereby reducing the time that the

TABLE 2. Sequence comparison of the N helix of the gp41 ectodomain (residues 30 to 79) from primary isolates characterized by different susceptibilities to T-20

Patient code or virus	Viral tropism	N-helix sequence				
		30	40	50	60	70
HxB2[a]	X4	ARQLLSGIVQ	QQNNLLRAIE	AQQHLLQLTV	WGIKQLQARI	LAVERYLKDQ
NL4-3[a]	X4	------D---	----------	----------	----------	----------
1	R5	--L-------	------K---	----------	----------	----------
4	R5	----------	----------	----------	----------	-------Q--
6	R5	--S-------	----------	----------	----------	-------Q--
7	R5	----------	----------	----------	----------	------R--
8	R5	--S-------	----------	----------	----------	----------
9	R5	----------	--S---K---	------K---	----------	----------
13	R5	----------	----------	----------	----------	-------Q--
15	R5	----------	------K---	----------	----------	---L---Q--
19	R5	----------	----------	----M-----	----------	----------
21	R5	----------	----------	----------	----------	----------
22	R5	----------	--S---M---	----M-----	---------L	----------
23	R5	----------	------Q---	----------	----------	----------
25	R5X4	----------	----------	----------	----------	-------R--
26	R5X4	--L-------	--S-------	----------	----------	-------R--
27	R5X4	--L-------	--S-------	----------	----------	--L----R--
29	X4	--L-------	----------	----------	----------	-------R--

[a] NL4-3 and HxB2 are reference T-cell-line-adapted viruses.

TABLE 3. Baseline susceptibilities to AMD3100 of primary viruses on $CD4^+$ $CXCR4^+$ target cells

Patient code	Viral tropism	IC_{50}[a] (ng/ml)	SD[b]
26	R5X4	0.08	1.83
25	R5X4	0.28	1.37
28	X4	0.32	1.42
27	R5X4	0.48	2.01
24	R5X4	0.96	1.84
29	X4	3.57	1.49

[a] IC_{50}s represent the geometric means of at least three independent experiments.
[b] Standard deviations of the geometric means were calculated as described in footnote *b*, Table 1.

molecular target of T-20 is exposed. In the absence of a method to accurately measure the avidity of interactions between virus and chemokine receptors expressed on the cellular membrane, we used the susceptibilities to AMD3100 (a generous gift from E. De Clercq) and RANTES (R & D Systems, Minneapolis, Minn.) to estimate the efficiency of the interaction with the chemokine receptors. We measured the susceptibilities of the four R5X4 viruses and the two X4 viruses to AMD3100, which inhibits the interaction with CXCR4 (10, 21) (Table 3). Their baseline susceptibilities, expressed as IC_{50}s, ranged from 0.08 to 3.57 ng/ml. No correlation was found between susceptibility to AMD3100 and that to T-20 (R^2 = 0.2906, P = 0.2697). Similarly, we measured the susceptibilities of 11 of the 23 R5 viruses to RANTES, a natural ligand of CCR5 (1, 5, 7, 11, 12). Inhibition of HIV infection by RANTES is due both to competition for CCR5 and to down-modulation of CCR5 surface expression by endocytosis. To reduce the influence of CCR5 down-modulation, which is dependent on the concentration of RANTES, we compared the susceptibilities of different R5 viruses at a fixed concentration of RANTES. The percentage of inhibition of infection at 250 ng/ml ranged from 20 to 60.2% (Table 4). Again, there was no correlation between viral susceptibility to T-20 and that to RANTES (R^2 = 0.0154, P = 0.7164). These data suggest that the efficiency of interaction with chemokine receptors was not a major determinant of the natural susceptibility of viruses to T-20.

In conclusion, our approach permits the accurate measurement of the susceptibility of plasma virus to entry inhibitors targeting virus adsorption and membrane fusion (AMD3100, RANTES, and T-20). Our system may provide a valuable tool for the optimization of treatment strategies that include entry inhibitors and for detection of the appearance of phenotypic resistance in treated patients. We measured a wide range of baseline susceptibilities to T-20, which were not associated with polymorphism in the N helix of gp41. Thus, the domains that determine baseline susceptibility to T-20 are, at least in part, distinct from those implicated in the development of acquired resistance to this drug. We showed that, for dual-tropic viruses, the alternative usage of CCR5 or CXCR4 could affect their susceptibility to T-20. We did not find a correlation between the susceptibilities of plasma viruses to entry inhibitors that target different steps in the entry process. Sequence analysis of the entire envelope cassette from a large number of plasma viruses, selected on the basis of their different susceptibilities to fusion inhibitors, will help to identify the determinants of this characteristic and may contribute to further understanding of the fusion process.

TABLE 4. Inhibition of infection by RANTES (250 ng/ml) of primary viruses on $CD4^+$ $CCR5^+$ target cells

Patient code	Viral tropism	% Inhibition of infection	SD[a]
3	R5	20.0	1.00
18	R5	20.7	1.51
23	R5	31.4	1.08
13	R5	36.2	1.27
8	R5	37.0	1.06
7	R5	37.3	1.47
1	R5	37.9	1.44
21	R5	40.3	1.19
9	R5	46.9	1.19
4	R5	49.2	1.11
22	R5	60.2	1.29

[a] Standard deviations of the geometric means were calculated as described in footnote *b*, Table 1.

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REFERENCES

1. **Alkhatib, G., C. Combadiere, C. C. Broder, Y. Feng, P. E. Kennedy, P. M. Murphy, and E. A. Berger.** 1996. CC CKR5: a RANTES, MIP-1α, MIP-1β receptor as a fusion cofactor for macrophage-tropic HIV-1. Science **272:** 1955–1958.
2. **Berger, E. A., P. M. Murphy, and J. M. Farber.** 1999. Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease. Annu. Rev. Immunol. **17:**657–700.
3. **Brelot, A., and M. Alizon.** 2001. HIV-1 entry and how to block it. AIDS **15**(Suppl. 5)**:**S3–S11.
4. **Chan, D. C., and P. S. Kim.** 1998. HIV entry and its inhibition. Cell **93:**681–684.
5. **Choe, H., M. Farzan, Y. Sun, N. Sullivan, B. Rollins, P. D. Ponath, L. Wu, C. R. Mackay, G. LaRosa, W. Newman, N. Gerard, C. Gerard, and J. Sodroski.** 1996. The beta-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. Cell **85:**1135–1148.
6. **Chou, T.-C.** 1991. The median-effect principle and the combination index for quantitation of synergism and antagonism, p. 61–102. *In* T.-C. Chou and D. C. Rideout (ed.), Synergism and antagonism in chemotherapy. Academic Press, San Diego, Calif.
7. **Deng, H., R. Liu, W. Ellmeier, S. Choe, D. Unutmaz, M. Burkhart, P. Di Marzio, S. Marmon, R. E. Sutton, C. M. Hill, C. B. Davis, S. C. Peiper, T. J. Schall, D. R. Littman, and N. R. Landau.** 1996. Identification of a major co-receptor for primary isolates of HIV-1. Nature **381:**661–666.
8. **Derdeyn, C. A., J. M. Decker, J. N. Sfakianos, X. Wu, W. A. O'Brien, L. Ratner, J. C. Kappes, G. M. Shaw, and E. Hunter.** 2000. Sensitivity of human immunodeficiency virus type 1 to the fusion inhibitor T-20 is modulated by coreceptor specificity defined by the V3 loop of gp120. J. Virol. **74:**8358–8367.
9. **Derdeyn, C. A., J. M. Decker, J. N. Sfakianos, Z. Zhang, W. A. O'Brien, L. Ratner, G. M. Shaw, and E. Hunter.** 2001. Sensitivity of human immunodeficiency virus type 1 to fusion inhibitors targeted to the gp41 first heptad repeat involves distinct regions of gp41 and is consistently modulated by gp120 interactions with the coreceptor. J. Virol. **75:**8605–8614.
10. **Donzella, G. A., D. Schols, S. W. Lin, J. A. Este, K. A. Nagashima, P. J. Maddon, G. P. Allaway, T. P. Sakmar, G. Henson, E. De Clercq, and J. P. Moore.** 1998. AMD3100, a small molecule inhibitor of HIV-1 entry via the CXCR4 co-receptor. Nat. Med. **4:**72–77.
11. **Doranz, B. J., J. Rucker, Y. Yi, R. J. Smyth, M. Samson, S. C. Peiper, M. Parmentier, R. G. Collman, and R. W. Doms.** 1996. A dual-tropic primary HIV-1 isolate that uses fusin and the beta-chemokine receptors CKR-5, CKR-3, and CKR-2b as fusion cofactors. Cell **85:**1149–1158.
12. **Dragic, T., V. Litwin, G. P. Allaway, S. R. Martin, Y. Huang, K. A. Nagashima, C. Cayanan, P. J. Maddon, R. A. Koup, J. P. Moore, and W. A. Paxton.** 1996. HIV-1 entry into $CD4^+$ cells is mediated by the chemokine receptor CC-CKR-5. Nature **381:**667–673.

13. **Harrigan, P. R., J. S. Montaner, S. A. Wegner, W. Verbiest, V. Miller, R. Wood, and B. A. Larder.** 2001. World-wide variation in HIV-1 phenotypic susceptibility in untreated individuals: biologically relevant values for resistance testing. AIDS **15:**1671–1677.
14. **Heveker, N.** 2001. Chemokine receptors as anti-retroviral targets. Curr. Drug Targets **2:**21–39.
15. **Kilby, J. M., S. Hopkins, T. M. Venetta, B. DiMassimo, G. A. Cloud, J. Y. Lee, L. Alldredge, E. Hunter, D. Lambert, D. Bolognesi, T. Matthews, M. R. Johnson, M. A. Nowak, G. M. Shaw, and M. S. Saag.** 1998. Potent suppression of HIV-1 replication in humans by T-20, a peptide inhibitor of gp41-mediated virus entry. Nat. Med. **4:**1302–1307.
16. **Kliger, Y., and Y. Shai.** 2000. Inhibition of HIV-1 entry before gp41 folds into its fusion-active conformation. J. Mol. Biol. **295:**163–168.
17. **Labrosse, B., A. Brelot, N. Heveker, N. Sol, D. Schols, E. De Clercq, and M. Alizon.** 1998. Determinants for sensitivity of human immunodeficiency virus coreceptor CXCR4 to the bicyclam AMD3100. J. Virol. **72:**6381–6388.
18. **Myers, G., B. T. Korber, B. T. Foley, K.-T. Jeang, J. W. Mellors, and S. Wain-Hobson.** 1997. Human retroviruses and AIDS 1997: a compilation and analysis of nucleic acid and amino acid sequences. Theoretical Biological and Biophysics Group, Los Alamos National Laboratory, Los Alamos, N.Mex.
19. **Poignard, P., E. O. Saphire, P. W. Parren, and D. R. Burton.** 2001. Gp120: biologic aspects of structural features. Annu. Rev. Immunol. **19:**253–274.
20. **Rimsky, L. T., D. C. Shugars, and T. J. Matthews.** 1998. Determinants of human immunodeficiency virus type 1 resistance to gp41-derived inhibitory peptides. J. Virol. **72:**986–993.
21. **Schols, D., S. Struyf, J. Van Damme, J. A. Este, G. Henson, and E. De Clercq.** 1997. Inhibition of T-tropic HIV strains by selective antagonization of the chemokine receptor CXCR4. J. Exp. Med. **186:**1383–1388.
22. **Sodroski, J. G.** 1999. HIV-1 entry inhibitors in the side pocket. Cell **99:**243–246.
23. **Stephenson, J.** 2002. Researchers explore new anti-HIV agents. JAMA **287:**1635–1637.
24. **Trouplin, V., F. Salvatori, F. Cappello, V. Obry, A. Brelot, N. Heveker, M. Alizon, G. Scarlatti, F. Clavel, and F. Mammano.** 2001. Determination of coreceptor usage of human immunodeficiency virus type 1 from patient plasma samples by using a recombinant phenotypic assay. J. Virol. **75:**251–259.
25. **Wei, X., J. M. Decker, H. Liu, Z. Zhang, R. B. Arani, J. M. Kilby, M. S. Saag, X. Wu, G. M. Shaw, and J. C. Kappes.** 2002. Emergence of resistant human immunodeficiency virus type 1 in patients receiving fusion inhibitor (T-20) monotherapy. Antimicrob. Agents Chemother. **46:**1896–1905.
26. **Wild, C. T., D. C. Shugars, T. K. Greenwell, C. B. McDanal, and T. J. Matthews.** 1994. Peptides corresponding to a predictive alpha-helical domain of human immunodeficiency virus type 1 gp41 are potent inhibitors of virus infection. Proc. Natl. Acad. Sci. USA **91:**9770–9774.
27. **Wyatt, R., and J. Sodroski.** 1998. The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens. Science **280:**1884–1888.
28. **Zollner, B., H. H. Feucht, M. Schroter, P. Schafer, A. Plettenberg, A. Stoehr, and R. Laufs.** 2001. Primary genotypic resistance of HIV-1 to the fusion inhibitor T-20 in long-term infected patients. AIDS **15:**935–936.