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Change in Tropism upon Immune Escape by Human Immunodeficiency Virus

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The V3 loop of human immunodeficiency virus type 1 is both a determinant of viral cell tropism and a target for neutralizing antibodies. This relationship was investigated. Selection of a dual-tropic (T cells and macrophages) virus to replicate in CD4$^+$ brain cells results in loss of macrophage tropism and of neutralization by an anti-V3 loop monoclonal antibody. Moreover, selection of the brain-selected variant to escape from V3 loop-specific neutralizing monoclonal antibodies results in the reduction or loss of brain cell tropism and the reacquisition of macrophage tropism. These data may indicate that the antigenic diversification of human immunodeficiency virus type 1 apparent after seroconversion can be selected either by immune responses or by colonization of new cell types.

Antigenic sites on gp120 of human immunodeficiency virus type 1 (HIV-1) that elicit strain-specific neutralizing antibodies (20, 38) include the variable loops V1/V2 and V3 (9, 14, 16, 19, 22, 26). These loops are also determinants of viral tropism for entry and infection of lymphocytes, T-cell lines, macrophages, and other cell types (4, 17, 18, 33, 35, 37, 39). The HIV-1 population within each infected individual has a high degree of genetic polymorphism (30) in the form of quasispecies (23). Initially, the V3 loop sequences are conserved (41, 42), but after seroconversion sequence diversity expands, yielding HIV-1 variants with distinct characteristics such as slow or rapid replication kinetics, syncytium induction, and tropism for particular cell types in addition to primary T-helper lymphocytes (2, 13, 15, 29, 31). HIV-1 variation between different tissues, such as blood, spleen, and brain, indicates that distinct quasispecies have evolved independently (3, 12, 27), although considerable variation is also seen within a single organ (10). In addition, escape from anti-V3 loop neutralizing antibodies resulting from amino acid substitutions within and outside the V3 loop has been demonstrated both in vivo and in vitro (1, 21, 24).

To define more closely the relationship between neutralization and tropism mediated through the V3 loop, we investigated the effect of a change in tropism on neutralization and the effect of escape from neutralization on cell tropism. We used viruses derived from molecular clones of the Gun-1 wild-type isolate of HIV-1 (Gun-1wt) and its brain cell-tropic variant Gun-1v with a single amino acid substitution introduced by site-directed mutagenesis (35). The two viruses differ by a proline (wt)-to-serine (variant) substitution at the V3 loop tip (GPGR→GSGR). Both viruses infect T-cell lines, but Gun-1v also infects CD4$^+$ BT cells derived from the brain (35) and a glioma cell line transfected with CD4, U87-CD4 (32). Previous studies had shown that other diverse HIV-1 strains are not able to infect U87-CD4 cells (5, 6).

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HIV-1 Gun-1wt but not Gun-1v can infect primary macrophages. Gun-1wt and Gun-1v were titrated on primary macrophages to test whether they had differential tropisms on these cells too. For comparison, the viruses were also titrated on T-cell line C8166 and on U87-CD4 cells. Primary macrophages were prepared from blood monocytes of three healthy donors. Peripheral blood mononuclear cells were separated by using standard Ficoll-Hypaque sedimentation. Monocyte-derived macrophages were further separated from lymphocytes by the adherence method, harvested, and plated on 24-well plates for infectivity studies. Infection of all cell types was measured by immunostaining as previously described (7). Tenfold serial dilutions of virus stocks were added (1 ml) to 24-well plates with appropriate cell types. After 5 days, the U87-CD4 cells were washed with phosphate-buffered saline and fixed with methanol-acetone and the C8166 cells were adhered to new 24-well trays with poly-L-lysine (Sigma) (21) and similarly fixed with methanol-acetone. Macrophage cultures were incubated for 3 weeks after infection and then washed and fixed as for U87-CD4 cells. A mouse monoclonal antibody (MAb) specific for HIV-1 anti-p24 was added to fixed cells for 1 hour, washed, and followed by an anti-mouse antibody conjugated with β-galactosidase before incubation with substrate. Foci of infection which stained blue were counted, and titers (in infectious units per milliliter) were calculated. Titers decreased linearly with dilution, and we therefore regarded each focus as arising from a single infectious unit. C8166 titrations did not yield foci of infection, and the titers are given as tissue culture infective doses, defined as the highest dilution leading to virus replication indicated by immunostained cells. Figure 1a shows that Gun-1wt infects macrophages (with varying sensitivity) as well as T-cell lines, and it therefore represents a dual-tropic HIV-1 strain. Infectivity titers of Gun-1wt on macrophages were of magnitudes comparable to those of typical macrophage-tropic strains, e.g., SF162 (data not shown). Gun-1v, however, while gaining tropism for U87-CD4 cells, has lost its tropism for primary macrophages.

A switch in tropism results in loss of susceptibility to anti-V3 loop neutralizing antibodies. MAbs were derived from rats immunized with Freund's complete adjuvant containing

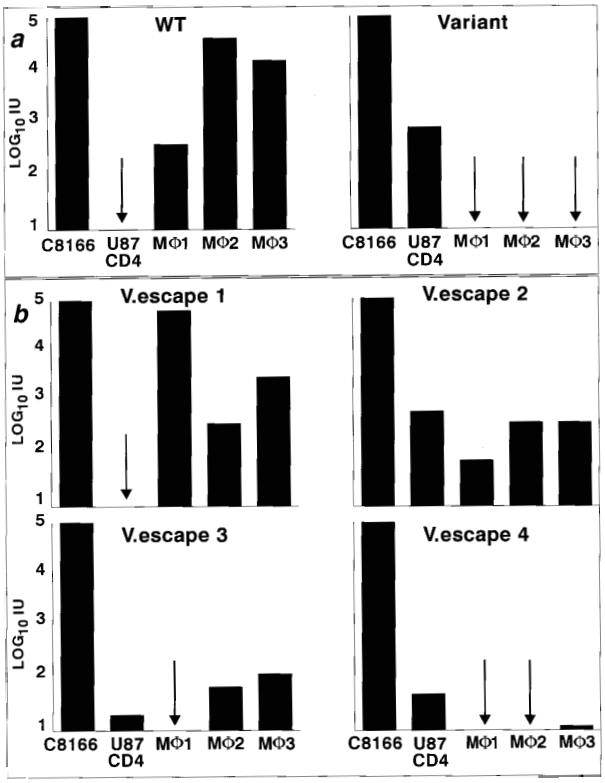

FIG. 1. Cellular tropisms of (a) Gun-1wt (WT) and Gun-1v (variant) and (b) Gun-1v anti-V3 loop escape mutants (V. escape 1, 2, 3, and 4). The viruses were titrated on the T-cell line C8166, the CD4-transfected human brain glioma cell line U87-CD4, and on three different sets of primary macrophages (Mϕ 1, 2, and 3), prepared from whole blood of three healthy HIV-negative blood donors. The virus titers are expressed as infectious units (IU) per milliliter, and arrows indicate titers of less than 10 IU/ml.

V3 loop peptides representing either Gun-1wt (KSITIG**P**GR AFHAI) or Gun-1v (KSITIG**S**GRAFHAI) conjugated to keyhole limpet hemocyanin. Aw and Bw are MAbs to wt peptide, and Dv, Fv, Gv, and Hv are MAbs to variant peptide. Gun-1wt and Gun-1v were tested for their susceptibilities to neutralization by the panel of MAbs (Aw, Bw, Dv, Fv, Gv, and Hv) and by human serum (from an individual infected with HIV-1). A sample of 1,000 tissue culture infective doses of the appropriate virus in 40 μl was incubated with 10 μl of 10-fold serial dilutions of MAb or human serum. After 1 h, 100 μl containing 2×10^4 C8166 cells was added. The virus-MAb-cell mixtures were further incubated for 3 days and examined for syncytia. Table 1 shows two weakly neutralizing MAbs raised against wt peptide (Aw and Bw) and four strongly neutralizing MAbs raised against the variant (Dv, Fv, Gv, and Hv). One of the wt MAbs (Aw) neutralized both wt and variant virus, while the other (Bw) did not cross-neutralize the variant or bind to it (fluorescence-activated cell sorter analysis; data not shown). All MAbs raised against variant peptide were specific to the variant virus. Thus, HIV-1 selected for brain cell tropism not only loses its macrophage tropism but also becomes resistant to neutralization by an anti-V3 MAb (Bw) raised against the wt virus.

TABLE 1. Neutralization of Gun-1 isolates by anti-V3 loop peptide MAbs

Gun-1 isolate	MAb titer (μg/ml)[a]						Human serum titer[b]
	Aw	Bw	Dv	Fv	Gv	Hv	
wt	10	10	>20	>20	>20	>20	1:40
Variant	10	>20	0.8	1.0	2.0	2.0	1:40
Escape mutants	>20	>20	>20	>20	>20	>20	1:80

[a] The MAb titer represents the amount of antibody which neutralized syncytia by at least 90%.

[b] The titer for human serum is the reciprocal of the highest dilution that neutralizes 90% of syncytia. All of the escape mutants yielded similar results.

Escape from V3 loop-mediated neutralization results in a switch in tropism. We next tested whether selection for escape from V3-neutralizing antibodies would result in changes of cellular tropism. We chose the four MAbs raised against Gun-1v because they exhibited much stronger neutralization than did the MAbs to Gun-1wt. The molecular clone of Gun-1v was propagated in C8166 cells in the presence of 50 μg of each of the four MAbs per ml separately and was passaged twice weekly until there was evidence of syncytia, indicative of viral replication and virus escape (about 3 weeks). Escape mutant 1 (V. escape 1) was selected with MAb Hv; V. escape 2 was selected with MAb Dv; V. escape 3 was selected with MAb Gv; and V. escape 4 was selected with MAb Fv. Each escape mutant was biologically cloned three times in the presence of the selecting MAb. The Gun-1 escape mutants were then tested for their susceptibilities to neutralization by the panel of MAbs (Aw, Bw, Dv, Fv, Gv, and Hv) and by human serum as described above. As shown in Table 1, all the escape mutants escaped neutralization from the whole panel of MAbs. Each of the escape mutants was characterized for cell tropism. The results are presented in Fig. 1b. None of the escape mutants compromised its infectivity for T-cell lines, as each maintained a high efficiency of infection for C8166 cells (and MOLT 4#8 cells; data not shown). Strikingly, three of the four escape mutants partially recovered macrophage tropism. One of these mutants (V. escape 1) reverted to the wt in entirely losing U87-CD4 tropism and regaining macrophage tropism. One mutant (V. escape 2) gained the ability to infect macrophages while maintaining its tropism for U87-CD4 cells and may thus be considered a tritropic virus, being able to infect T-cell lines, macrophages, and $CD4^+$ astroglial cells. V. escape 4 did not gain significant tropism for primary macrophages, although infectivity for U87-CD4 cells was severely impaired.

Escape from neutralization and switch in cell tropism result in amino acid changes in the V3 domain. Each escape mutant was biologically cloned three times by endpoint dilution, and its phenotype was confirmed before envelope sequences were amplified by PCR and cloned. Two clones from each virus were sequenced in both directions, and amino acid sequences were deduced. The sequences were the same for both clones of each mutant, and all amino acid substitutions in the V3 loop were found at positions 16 to 21 (see Table 2). One of these mutants (V. escape 1) reverted to the wt with an S-to-P codon change at position 16 of the V3 loop. As shown in Fig. 1, this mutant also had the same tropism as wt virus. The other three escape mutants had codon changes at position 19, 20, or 21. We also noted amino acid substitutions outside the V3 loop for each variant. These are recorded in Table 2. One change in V2, L to H at position 149 [149 L→H], which was present in each variant was also demonstrated in the virus stock used to derive the escape mutants and presumably played no part in the escape phenotype. We cannot rule out at this stage whether these other changes may also contribute to the altered phenotype. To investigate this, we are currently introducing each substitution back into cloned virus.

Our results show that selection for change in one phenotype,

TABLE 2. Summary of changes in V3 loop neutralization escape mutants[a]

Gun-1 isolate	Property				
	V3 loop MAb neutralization	Tropism			V3 loop[b] (amino acids 13–25)
		T cells	MΦ cells	U87-CD4 cells	
wt	−	+	+	−	TIGPGRAFHAIEK
Variant	+	+	−	+	TIGSGRAFHAIEK
V. escape 1	−	+	+	−	TIG**P**GRAFHAIEK
V. escape 2	−	+	+	+	TIGSGRA**IN**AIEK
V. escape 3	−	+	±	±	TIGSGR**T**F**Q**AIEK
V. escape 4	−	+	−	±	TIGSGRA**L**HAIEK

[a] Gun-1wt, Gun-1v, and the neutralizing MAb escape mutants (V. escape 1, 2, 3, and 4) were tested for their phenotypic properties of neutralization (by Dv, Fv, Gv, and Hv [Table 1]) and tropisms for T-cell lines (C8166 and MOLT 4#8), $CD4^+$ glioma cell line U87-CD4, and primary macrophages (MΦ) (Fig. 1). The amino acid sequences of gp120 of the Gun-1 isolates were deduced from the proviral DNA sequences in infected cells. Two clones from each virus were sequenced in both directions.

[b] All amino acid substitutions in the V3 loop were found at positions 16 to 21 and are shown in boldface type. Sequence changes were also noted outside the V3 loop in gp120. For the indicated isolates, these are as follows: V. escape 1, 149 L→H and 154 I→L; V. escape 2, 149 L→H; V. escape 3, 149 L→H, 241 E→K, 154 I→L, and 318 K→E; V. escape 4, 149 L→H and 154 I→L.

e.g., cell tropism, may result in concomitant alteration of another, distinct property of HIV, i.e., sensitivity to neutralizing antibodies, and vice versa. While our analysis was performed with molecularly cloned viruses and MAbs in cell cultures, there is no reason to think that selection for viral phenotype does not occur among the HIV-1 quasispecies in vivo. Although the generation of antigenic diversity is not necessarily driven by the immune system (11), subsequent selection by immune escape from neutralizing antibodies has been observed in vivo (1, 24). Thus, escape from neutralization by anti-V3 loop antibodies could be of further advantage to HIV-1 in concomitantly augmenting an array of viruses with expanded or new cell tropisms. Furthermore, since selection for one attribute alters the phenotype of the other, their independence should be borne in mind when considering viral variation in HIV infection and AIDS.

Transmission of HIV from a seropositive individual to a seronegative one often results in restriction of tropism and diversity (30, 40–42). After seroconversion, new patterns of diversification could result in part from neutralization escape. Conversely, the spread of virus to new cell types will contribute to the antigenic diversity of the HIV population in vivo. During the long, asymptomatic phase of HIV-1 infection, viral load appears to be controlled by host immune responses while viral genome diversity broadens. Eventually, viral burden increases, and often more-cytopathic, T-cell-tropic variants appear with progression to AIDS (8, 29, 31, 34) while other V3 variants appear in dementia (28). Consequently, models of viral quasispecies should take into account the interacting roles of antigenic diversity, viral replication kinetics, and cellular tropism in consideration of HIV pathogenesis (25, 36).

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