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SHORT COMMUNICATION

Breakthrough Population of HIV-1

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Certain antibody neutralization escape mutants of HIV-1 map outside of the antibody recognition epitope, thereby suggesting the presence of nonlinear conformational domains. In an effort to begin to define the interacting regions of the HIV envelope proteins, a neutralization-sensitive clone of HIV-1, HXB2/BH10Sal-Bam, was passaged in the presence of the V3-specific monoclonal antibody 0.5β. DNA sequence analysis of the V3 domain of the breakthrough viral populations revealed one population that retained the parental V3 genotype. Quantitative DNA sequence analysis of this breakthrough population revealed the presence of mutational "hotspots" in several envelope domains that are noncontiguous with V3. Mutations were seen throughout gp41 and the C1, V1/V2, C2, and C5 domains of gp120. In contrast, other regions of gp120, C3, V4, C4, and V5 remained totally unchanged. Within V1, three residues within a 14-amino acid stretch experienced five substitutions and in C5 three residues within a 7-amino acid stretch experienced four substitutions. This finding, that certain residues clustered within particular domains (V1/V2, C5, and gp41) experienced multiple substitutions under a defined environmental stressor, suggests that the degree of adaptive plasticity exhibited by the HIV envelope is limited. Based on this observation it may be possible, using a set of antibodies to various envelope epitopes, to discern a set of rules which explain the interactions of the various envelope domains with each other and with their environment. The insight gained into the physiologic constraints that the envelope proteins are subject to may be useful in developing therapeutic and vaccination strategies.

It has been previously demonstrated, both *in vivo* and *in vitro,* that under the selective pressure of V3-specific antibodies, HIV-1 neutralization escape mutants can be isolated (*1–4*). Two types of breakthrough isolates have been described, those with mutations in the V3 loop itself and those where the amino acid changes occur at one or more sites distant from the V3 loop (*5*). In the latter situation the loss of neutralization is not necessarily associated with a loss of antibody binding or even a decrease in the affinity of binding (*4*). These findings suggest that the V3 loop contains a linear epitope, but that this epitope is part of a larger three-dimensional structure that contributes to the formation of one or more conformational domains. This hypothesis would suggest that amino acids located within discrete regions throughout the envelope may contribute to the formation of various 3-D functional domains (*6*), including those for various parameters of infectivity such as tropism, viral binding, fusion and viral entry, and susceptibility to antibody-mediated neutralization.

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Previous studies have generated neutralization-resistant clones by growing HIV-1 under the selective pressure of broadly neutralizing heterotypic sera (*7, 8*). Resultant viral clones were assayed for neutralization resistance, or sensitivity, by culturing them in the presence of panels of patient sera and monoclonal antibodies (MAbs) (*9*) that were capable of neutralizing the parental virus (*10*). A single amino acid change (A582T) in the extracellular domain of gp41 was noted to confer resistance to CD4 binding domain antibodies giving credence to the hypothesis that noncontiguous regions of the viral envelope interact in the creation of functional domains.

In the current study, an HIV-1 infectious molecular clone, which is exquisitely sensitive to neutralization by the V3-specific monoclonal antibody (0.5β), was placed under selective pressure and the resultant breakthrough population was examined via quantitative DNA sequence analysis. We chose this approach, rather than cloning isolates from the breakthrough population, in an effort to better observe points of flux throughout the envelope gene. This approach allowed for the detection of the location, extent, and degree of change at a point and within a region such that multiple replacements and their ratios at a given site could be determined.

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The parental virus used in these studies was HXB2/BH10Sal-Bam, an infectious molecular clone of HIV-1 constructed by cloning the *Sal*I–*Bam*HI *env* fragment of BH10 into an HXB2 background. Virus was expressed by $CaCl_2$-mediated transfection of purified plasmid DNA into H9 cells. Cell-free viral stocks were prepared by harvesting the supernatants from the virus-producing H9 cultures at Days 7, 10, 14, and 18, and the stock with the highest titer (Day 14) was used in subsequent neutralization studies.

Neutralizing antibody assays were carried out using a modified version of the protocol described by Nara and Fischinger (*11*) with the murine monoclonal antibody 0.5β (*12*), which is specific for the V3 loop of the HIV-1 IIIB family of viruses. Briefly, 50 to 100 syncytia forming units (SFU) of the titered HXB2/BH10Sal-Bam viral stock were incubated with twofold serial dilutions of 0.5β in a 96-well, V-bottomed microtiter plate (Costar). All experiments were carried out in quadruplicate and included virus-only positive controls. Fifty microliters of the virus–antibody mixtures were used to infect DEAE-dextran-treated (20 μg/ml) MT-2 cells. Cells were plated in poly-L-lysine (Sigma)-treated 96-well flat-bottomed microtiter plates (Costar) at a concentration of 5×10^4/well in 50 μl. The virus was allowed to adsorb to the cells for 60 min at 37° in a humidified CO_2 incubator. The inoculum was then aspirated, and each well was fed with complete RPMI 1640 medium. Wells were scored for syncytia on the fifth day after infection. Neutralization resistance was determined by calculating the virus surviving fraction (V_n/V_o) at each dilution, where V_n represents the mean number of syncytia induced by the virus inoculum surviving after neutralization, and V_o represents the mean of the number of syncytia induced by virus inoculum in the absence of antibody.

The HXB2/BH10Sal-Bam virus was demonstrated to be sensitive to neutralization by MAb 0.5β. In an effort to obtain neutralization-resistant populations of this virus, the contents of wells that evidenced viral breakthrough, i.e., formation of 1–3 syncytia at the highest concentration (30 μg/ml or a 1:4 dilution) of 0.5β antibody, were centrifuged on Day 5 of the neutralization assay. The supernatants were incubated with 30 μg/ml of 0.5β and the virus–antibody mixtures were then adsorbed onto 2.3×10^5 MT-2 cells in a volume of 1 ml in 24-well microtiter plate. After 6 days, syncytia were observed and the contents of each well were transferred to a 12-well plate. Fresh MT-2 cells were added and the selective pressure was maintained for an additional 2 weeks (25 days total), at which time five neutralization-resistant breakthrough virus populations were harvested.

The V3 domains of the five breakthrough populations were screened by DNA sequence analysis. Proviral HIV-1 DNA was extracted from the infected MT-2 cells by standard procedures, to serve as the template for PCR amplification of the HIV-1 viral envelope by the primers specified in Fig. 1. Amplifications were performed under full procedural safeguards as described (*13–15*) and sample blanks were included in all amplification runs to test for the presence of carryover. The specificity of amplified products was determined by liquid hybridization and gel retardation analysis followed by autoradiography (*15–17*). Both strands of the amplified products were sequenced on an ABI 373A automated sequencing system using Taq-based cycle sequencing with dye terminator chemistry (Perkin–Elmer). Four of the five break-

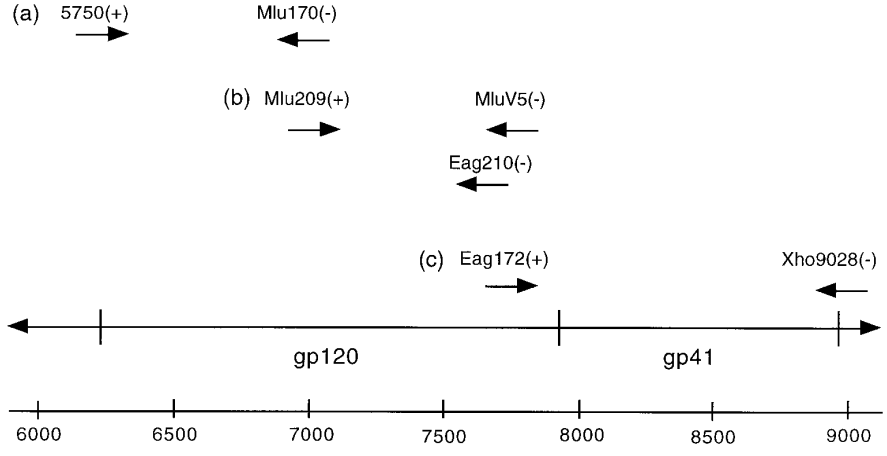

FIG. 1. Schematic representaion of PCR-based sequencing strategy for HIV env gene neutralization escape mutants. The numbered arrows indicate the location and orientation of the primers used for amplification. Primer sequences and positions () were as follows; underlined nucleotides represent changes which introduce restriction enzyme sites: 5750 TAC TTG GGC AGG AGT GGA AGC CAT (5727–5750); Mlu170 ATT GTT GTT GGG ACG CGT ACA ATT AAT TTC TA (7144–7113); Mlu209 GAA ATT AAT TGT ACG CGT CCC AAC AAC (7115–7138); MluV5 TTC TCT CTG CAC CAC GCG TCT CTT TGC CCT (7765–7736); Eag210 ATC TCC TCC TCC CGG CCG GAA GAT CTC GGA (7760–7631); Eag172 TCC GAG ATC TTC CGG CCG GGA GGA GGA GAT ATG (7631–7660); Xho9028 GGT CTT AAA GGT ACC TGA GGT GTG ACT GGA (9028–8999).

through populations were found to have mutations in the V3 loop and were eliminated from further analysis. One of the five exhibited no changes whatsoever in the V3 loop.

The neutralization phenotype of the breakthrough population that exhibited no DNA sequence alterations in the V3 domain was determined using the quantitative neutralization assay described above (Fig. 2). Assay results were compared with those for the parental virus, HXB2/BH10Sal-Bam. The parental virus was 90% neutralized at a 1:24 dilution, or 5.6 ng/ml, of antibody. In contrast, the breakthrough virus was resistant to neutralization. At a MAb 0.5β concentration of 5.6 ng/ml, the V_n/V_o value for this virus population was 0.42, indicating only 58% neutralization. Even more notable was that, at higher concentrations of antibody, there was greater divergence between the V_n/V_o values of the parental virus and the breakthrough virus. In contrast to its progenitor, HXB2/BH10Sal-Bam, which had a V_n/V_o value of only 0.018 at a 1:4 dilution of MAb 0.5β (29 μg/ml), the V_n/V_o value for the breakthrough virus was 0.20. This represents a greater than one log-fold difference in the number of surviving virus particles in the breakthrough population.

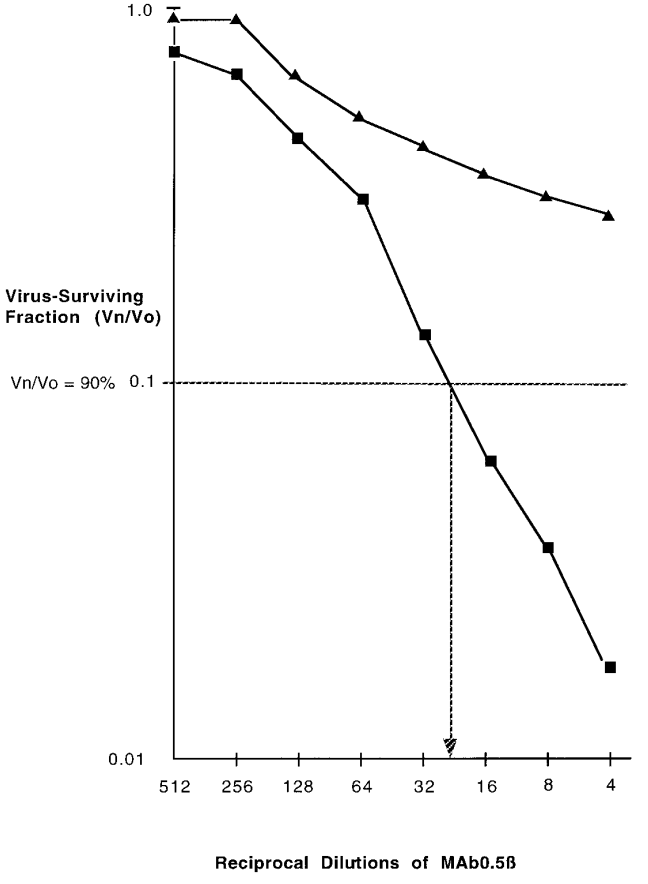

FIG. 2. The neutralization phenotype of the breakthrough virus population was determined by a quantitative neutralization assay using the selecting antibody, MAb, 0.5β. In this analysis, the breakthrough virus population (▲) was compared with its neutralization-sensitive parental virus, HXB2/BH10Sal-Bam (■). Both the parental and breakthrough viruses are identical at the antibody binding site, the tip of the V3 loop. Neutralization resistance was determined by calculating the virus surviving fraction (V_n/V_o) at each dilution, where V_n represents the mean number of syncytia induced by the virus inoculum surviving after neutralization, and V_o represents the mean number of syncytia induced by virus inoculum in the absence of antibody. A 90% neutralization titer is demonstrated at $V_n/V_o = 0.01$. The results represent the mean of two experiments, with two data points recorded per experiment at each dilution.

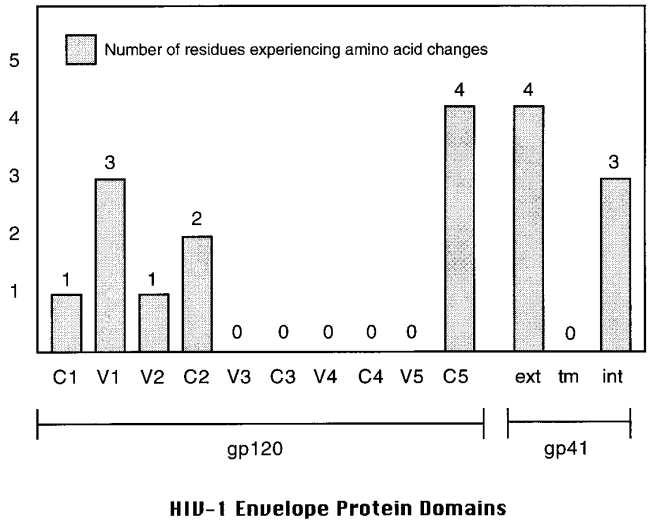

FIG. 3. Relative distribution of predicted amino acid changes in the breakthrough virus population. DNA sequence analysis showed that codons for 18 different amino acid residues throughout the envelope underwent mutation. Eleven changes were observed in the gp120, however, no changes occured within the V3 loop, the target of the selecting antibody 0.5β, or in the four contiguous domains 3′ of V3. A total of seven changes were observed in gp41, three of which occurred in the internal domain.

Both strands of the gp120 and gp41 of the breakthrough virus population were sequenced bidirectionally and in their entirety, and the sequence compared with that of the parental clone, HXB2/BH10Sal-Bam, in an effort to determine which noncontiguous regions underwent mutation and selection. DNA sequence analysis revealed that certain envelope domains had clusters of mutations, whereas, in other regions, no changes were observed. Translation of the DNA sequence analysis data predicted the accumulation of amino acid substitutions in the C1, V1/V2, C2, and C5 regions of gp120, as well as both the intracellular and the extracellular regions of gp41. The relative distribution of amino acid changes in this breakthrough population is demonstrated in Fig. 3. Of interest, was the finding that V3 and its 3′ contiguous regions C3, V4, C4, and V5 were all wild type with respect to the parental clone. However, mutations identified at 18 different codons throughout the remaining regions of the envelope gene, including gp41, would be predicted to result in a total of 22 amino acid residue changes.

TABLE 1

Amino Acid Residues in the Breakthrough Population Exhibiting Changes

Env protein	Domain	Mutation	Extent of change	Type of change
gp120	C1	T63K	Complete	Polar to basic
	V1	C131C, Y, F, S	1:1:1:1	s—s to hydrophobic
	V1	N141N, I	2:1	Loss of potential N-linked Glycosylation site
	V1	S144S, R	2:1	
	V2	E172D, Q	1:2	Acidic to uncharged
	C2	N197N, D	2:1	Loss of potential N-linked Glycosylation site; *Uncharged to acidic
	C2	A204A, G	2:1	
	C5	K487Q	Complete	Basic to uncharged
	C5	K500K, T	1:1	Basic to uncharged
	C5	A501A, T, P	3:2:1	
	C5	V506V, L	1:1	
gp41	External	G531G, A	1:1	
	External	A582S	Complete	
	External	I642T	Complete	Hydrophobic to polar
	External	E657D	Complete	
	Internal	D773D, H	1:3	Acidic to uncharged
	Internal	A839G	Complete	
	Internal	R848T	Complete	

Mutations resulting in changes within 11-amino acid residues were seen in the C1, V1/V2, C2, and C5 regions of gp120. In gp41, three of the seven amino acid residues undergoing change were localized to the intracellular domain. No changes were observed in the transmembrane domain.

Some of the mutant codons experienced complete nucleotide replacements in the breakthrough population, whereas other sites showed partial replacement with one to three changes occurring at various ratios. Table 1 demonstrates, by domain, the clustering of the amino acid residues that have undergone mutations and indicates the nature of each complete or partial amino acid replacement. Two of the gp120 amino acid residues were completely replaced by a single amino acid, and nine were partially replaced by one or more amino acids. Within the gp41 protein, five of the seven changes were complete. Two of the four gp41 mutations that arose in the extracellular region map to the putative gp120 interactive domains (*18*). It is interesting that three of the sites that underwent mutation in gp41 map to the internal domain. We have also found, through cassette mutagenesis studies, that the internal domain of gp41 contains determinants that confer neutralization resistance on HIV-1 IIIB family members (Sirko *et al.,* unpublished results 1994).

Many of the predicted amino acid changes in the breakthrough population result in the loss of interactive groups from the envelope glycoproteins (charged, polar, and glycosylated moieties), suggesting that the mechanism of viral escape may be partially mediated by reducing the number of residues which are strongly interactive with the immune system. Within the gp120 protein there was: (1) an overall decrease in the number of charged amino acid residues; (2) a net increase in hydrophobicity in the V1 region; and (3) loss of potential N-linked glycosylation sites in the V1 and C2 regions. These findings suggest that "hotspots" exist within discrete regions of the envelope, including the internal domain of gp41, that contribute to the creation of a neutralization escape phenotype from V3-specific antibodies.

In studies of immune-selected neutralization-resistant escape mutants, it has been previously noted (*4, 9, 10, 19*) that a broad spectrum of distant site compensatory mutations can occur within both gp120 and gp41. In support of a functional interaction between gp41 and V3, Back *et al.,* also reported the finding of mutations in both the external and internal domains of the gp41 gene of HIV-1 clones made from V3-specific antibody neutralization escape mutant populations (*20*). Thali *et al.,* also noted that a gp41 change conferred neutralization resistance to broadly-reactive gp120 antibodies (*21*).

The overall envelope structure within which the V3 domain is embedded appears to affect its neutralization phenotype by V3-specific antibodies and antisera. Robert-Guroff *et al.,* demonstrated that enhanced, rather than reduced, neutralization occurred by exchanging V3 loops between neutralization-sensitive isolates perhaps due to the conformation-dependent exposure of normally concealed epitopes (*22*). The structural conformation of the

entire envelope is also important in determining a number of other aspects of the HIV-1 phenotype, including compensatory changes at distant sites that affect infectivity, host range and syncytium-forming ability (*23, 24*).

The clustering of mutations at certain distant sites, in response to selective pressure against a specific epitope or functional domain in the absence of local mutations, implies that these distant-site changes permit growth and replication of the virus in the presence of the selecting agent. The finding that these distant site mutations often appear as clusters, either at a specific amino acid residue (multiple substitutions) or within a few residues of each other in a defined functional or structural domain, suggests that the HIV-1 envelope may have limited plasticity with respect to any given selecting agent. To test this hypothesis would require the application of selective pressure simultaneously against multiple envelope protein domains that have been demonstrated to interact through functional escape studies. Therefore, in future studies it may be possible to concurrently target several interacting domains by the development of peptide-derived monoclonal antibodies directed against these domains.

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