Identification of Envelope V3 Loop as the Major Determinant of CD4 Neutralization Sensitivity of HIV-1

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Laboratory isolates of human immunodeficiency virus type–1 (HIV-1) such as HTLV-IIIB are generally T cell line–tropic and highly sensitive to neutralization by soluble CD4 (sCD4), a potential antiviral agent that is undergoing clinical trial. However, many primary HIV-1 isolates are macrophage-tropic and sCD4-resistant. Envelope V3 loop sequences derived from primary HIV-1 isolates were sufficient to confer on HTLV-IIIB not only the tissue tropism but also the degree of sCD4 neutralization resistance characteristic of their HIV-1 strains of origin. Single amino acid changes in the V3 loop enhanced sCD4 resistance by up to tenfold. These observations suggest that the tissue tropism and sCD4 neutralization sensitivity of HIV-1 isolates are regulated by similar mechanisms.

The human membrane glycoprotein CD4, found on the surface of helper T cells and cells of the monocyte-macrophage lineage, is the primary receptor for HIV-1 (*1*). Several groups have reported that soluble, recombinant forms of CD4 can block the replication of diverse strains of HIV-1 in culture, presumably by competing with cell-surface CD4 for binding to the gp120 component of envelope (*2*). The sCD4 can also protect chimpanzees against experimental infection with HIV-1 (*3*). These observations have encouraged the initiation of clinical trials of sCD4 in HIV-1–infected individuals (*4*).

It has been reported that low concentrations of sCD4 (<1 μg/ml) that can efficiently neutralize laboratory isolates of HIV-1 are unable to significantly inhibit the replication of many primary HIV-1 isolates (*5*). The majority of HIV-1 primary isolates are tropic for macrophages, whereas laboratory isolates have been selected for efficient replication on transformed T cell lines (*6*). We wished to examine whether this apparent correlation was indeed valid and whether these two properties were functionally linked. We therefore measured the sensitivity of two distinct T cell line (T)–tropic isolates of HIV-1 (HTLV-IIIB and SF2) and three diverse macrophage (MT)-tropic isolates (BaL, JR-FL, and SF162) (*7–9*) to neutralization by a range of concentrations of recombinant sCD4 (Table 1). All neutralization assays were performed with viral stocks that had been grown (*10*) and titered (*11*) on activated human peripheral blood lymphocytes (PBLs) (*10*) and used a similar challenge dose. All assays were performed on human PBLs as these are fully permissive for both T-tropic and MT-tropic HIV-1 isolates (*6–9*).

As we predicted, the T-tropic HIV-1 laboratory isolates HTLV-IIIB and SF2 were highly sensitive to neutralization by sCD4 (Table 1). In contrast, the MT-tropic isolates displayed an approximately 10-fold (BaL, SF162) to ~100-fold (JR-FL) higher resistance to sCD4 neutralization (Table 1). We therefore conclude, on the basis of this limited sample, that MT-tropic HIV-1 strains appear to be significantly more resistant to sCD4 neutralization than are T-tropic HIV-1 isolates. In addition, we conclude that MT-tropic isolates can display either moderate (BaL, SF162) or high (JR-FL) resistance to sCD4 neutralizations. These differences are revealed in Fig. 1A, which compares the sCD4 neutralization profiles of the HTLV-IIIB, BaL, and JR-FL strains.

The V3 loop of the gp120 envelope protein is a disulfide-bonded protein domain of ~35 amino acids that serves as the primary neutralization determinant of HIV-1 (*12*) and as the major determinant of HIV-1 cell tropism (*7–9, 13*). Mutation of the V3 loop or blockade by monoclonal antibodies specific to V3 has no detectable effect on binding to CD4 but efficiently blocks the fusion of HIV-1 with susceptible $CD4^+$ cells (*14*).

We have described a T-tropic HIV-1 proviral clone, pIIIB, that contains a modified form of the replication-competent HXB-3 provirus derived from the HTLV-IIIB isolate (*7*). We have also described two MT-tropic derivatives of pIIIB that contain either the entire envelope gene of

Table 1. Relative sCD4 neutralization sensitivity of HIV-1 isolates and proviral clones. We performed assays of the in vitro sensitivity of HIV-1 to neutralization by sCD4 essentially as described (*5, 26*). Virus stocks (*10*) derived from the indicated HIV-1 proviral clones or isolates were first titrated (*11*) and then diluted to 1250 $TCID_{50}$ per milliliter of medium. Total concentrations of HIV-1 virions per milliliter were assessed by measurement of p24 Gag concentrations. We prepared a threefold dilution series of recombinant sCD4 extending from 1000 μg/ml to 0.46 μg/ml and then diluted each concentration 1 to 10 into 450 μl of each viral stock. After the virus and sCD4 stocks were mixed, the mixtures were incubated at 37°C for 30 min before addition to 2×10^6 phytohemagglutinin-stimulated PBLs. Forty-eight hours after infection, the PBL cultures were centrifuged, washed, and supplemented with sufficient activated PBLs from the same donor to give 2×10^6 cells per milliliter in 10 ml of fresh medium. At approximately 5 days after infection, supernatant media were sampled and p24 Gag concentrations determined by a quantitative enzyme-linked immunosorbent assay (DuPont Biotechnology). For each virus stock, we calculated the percentage reduction in p24 Gag expression relative to untreated controls observed for each concentration of sCD4. Representative sCD4 neutralization profiles are given in Fig. 1. The sCD4 dose that inhibited p24 Gag expression by 50% (ID_{50}) is indicated for each of two separate experiments (Exp) in which different PBL donors were used. Additional experiments yielded comparable results (*16*). The tissue tropism of each HIV-1 proviral clone or isolate has been reported (*7–9*) with the exception of pIIIB/V3–JR-FL, which was determined as described (*7*).

	Input virus		sCD4 ID_{50} (μg/ml)		Tropism
	TCID (per milliliter)	p24 (ng/ml)	Exp 1	Exp 2	
	HIV-1 molecular clones				
pIIIB	1250	10.8	0.4	0.12	T
pBaL	1250	6.9	3.0	1.5	MT
pIIIB/V3-BaL	1250	4.0	4.0	2.8	MT
pIIIB/V3–JR-FL	1250	7.0	21	36	MT
	HIV-1 isolates				
HTLV-IIIB	1250	8.3	0.5	0.23	T
BaL	1250	5.6	5.0	1.9	MT
JR-FL	1250	11.2	22	21	MT
SF2	1250	15.1	0.4	0.1	T
SF162	1250	8.3	8.0	4.0	MT

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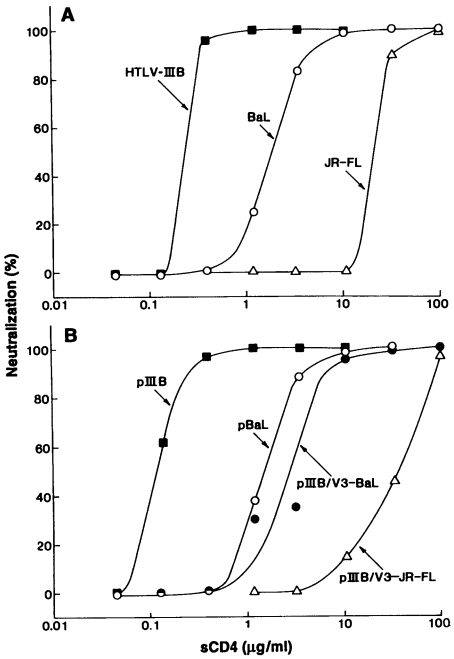

Fig. 1. Concentration-dependent neutralization of distinct HIV-1 isolates (**A**) or proviral clones (**B**) of HIV-1 by sCD4. The percentage of inhibition of HIV-1 replication induced by the indicated concentrations of recombinant sCD4 was determined as described in Table 1.

BaL (pBaL) or only the V3 loop of BaL (pIIIB/V3-BaL) substituted into the IIIB context (*7*). A virus stock derived from the pIIIB provirus displayed an sCD4 neutralization profile comparable to that observed with the HTLV-IIIB isolate (Table 1 and Fig. 1). Because CD4 is believed to interact exclusively with the viral envelope protein (*1*), we predicted that substitution of the BaL envelope gene, in pBaL, would result in the approximately tenfold higher resistance to sCD4 characteristic of this isolate (Table 1 and Fig. 1B). Substitution of only the V3 loop of BaL in pIIIB/V3-BaL also conferred an sCD4 neutralization profile similar to that seen with either the BaL isolate or the pBaL proviral clone. Therefore, the V3 loop of BaL is sufficient to confer not only the MT tropism (*7*) but also the CD4 resistance of the BaL isolate on this HTLV-IIIB–derived proviral cone.

As shown in Table 1 and Fig. 1A, the MT-tropic JR-FL isolate of HIV-1 is approximately tenfold more resistant to sCD4 than is the BaL isolate. However, the 35–amino acid V3 loop of JR-FL differs at only one amino acid residue from that observed in BaL (the BaL sequence is GPGRALYT; the JR-FL sequence is GPGRAFYT) (*7, 8, 15*). Modification of this amino acid in the pIIIB/V3-BaL context to give pIIIB/V3–JR-FL led to an sCD4 neutralization profile (Fig. 1B) and median inhibitory dose (ID_{50}) (Table 1) that were indistinguishable from those observed with the JR-FL isolate but distinct from those observed for the pBaL and pIIIB/V3-BaL proviral clones. As we expected, the pIIIB/V3–JR-FL provirus continued to display an MT-tropic phenotype in culture (*16*). Experiments with pIIIB derivatives that contained the V3 loop of the sCD4-resistant isolate SF162 (pIIIB/V3-SF162) or the sCD4-sensitive isolate SF2 (pIIIB/V3-SF2) indicate that these observations may be a pattern. In particular, we observed the pIIIB/V3-SF162 provirus to be resistant to sCD4 (ID_{50} = 10 to 20 μg/ml) and MT-tropic. In contrast, the pIIIB/V3-SF2 provirus displayed a high degree of sensitivity to sCD4 (ID_{50} ≥ 0.1 μg/ml) and a preferential tropism for transformed T cells (*16*).

It could be hypothesized that MT-tropic isolates of HIV-1 are resistant to sCD4 neutralization because they are able to infect cells by means of an alternate receptor. However, it has been demonstrated that treatment of target cells with a monoclonal antibody to CD4 (Leu3A) effectively blocks infection by both T-tropic and MT-tropic HIV-1 isolates (*14*), and we have confirmed this result (*17*). Differences in sCD4 sensitivity also do not result from strain-specific differences in the ratio of infectious to total HIV-1 particles; this latter value, as measured by p24 Gag concentration, varied only slightly between the different viral stocks used in these assays (Table 1). Finally, we note that all viral stocks were prepared by passage in human PBLs and these differences cannot therefore result from differences in the cellular origin of virions.

The direct interaction of CD4 with the HIV-1 envelope protein induces one or more conformational changes that increase the accessibility of the V3 loop to antibodies and proteases and that also expose epitopes on the gp41 component of the envelope, which may include the hydrophobic NH_2-terminal domain (*18*). This ordered conformational change has been proposed (*18*) to form a critically important step in the fusion of the HIV-1 virion with the target cell membrane, a process that remains poorly understood. It is becoming clear that the envelope V3 loop is not merely a passive participant in this process but instead forms a key regulatory component. Thus, inactivation of the V3 loop can preclude viral fusion but does not inhibit binding to either soluble or cell-surface CD4 (*14*). The sequence of the V3 loop has been shown to determine the pattern of HIV-1 tissue tropism, and this regulation is also believed to be entirely at the level of viral entry (*7–9, 13, 19*). Here, we demonstrate that the resistance of HIV-1 virions to sCD4 neutralization is also largely determined by the sequence of the V3 loop. Although our data do not suffice to prove a mechanistic connection between HIV-1 cell tropism and sCD4 sensitivity, they do demonstrate that these attributes are tightly linked.

The relative resistance of HIV-1 isolates to sCD4 neutralization does not correlate with differences in the intrinsic affinity of the viral envelope proteins for sCD4 (*20*). It is hypothesized that the conformational change undergone by gp120 subsequent to sCD4 binding can result in the removal of gp120 from the virions of sensitive isolates (*21*). V3 loop sequences might therefore modulate resistance to sCD4 by altering the nature of these conformational changes or by affecting the stability of the gp120-gp41 interaction. Similarly, differences in viral tropism might also result from V3 loop–regulated differences in the conformational change of envelope subsequent to binding to cell-surface CD4. These distinct conformational states could affect fusion by regulating the access of different domains of envelope to secondary cell-surface receptors or proteases (*7–9, 22*). However, the molecular basis for the determination of HIV-1 tropism and sCD4 resistance and the connection between these two properties remain unclear.

The V3 loop sequence of the JR-FL isolate is identical to the statistically prevalent or consensus V3 loop sequence derived from analysis of 245 distinct HIV-1 isolates (*7, 8, 23*). This correlation suggests that the tissue tropism and relative sCD4 resistance that are characteristic of JR-FL will also be prevalent among primary HIV-1 isolates. Analyses of HIV-1 isolates obtained from patients during the long asymptomatic period confirm the predominance of MT tropism (*6, 24*). However, more pathogenic T-tropic isolates of HIV-1 increase in prevalence during the later, symptomatic phases of this disease (*24*), and T-tropic isolates may also play a critical role during the initial, acute infection with HIV-1 (*25*). The data presented here suggest the treatment with sCD4 would be likely to selectively suppress the replication of these T-tropic variants. If the appearance of such T-tropic HIV-1 strains does contribute to disease progression in vivo, as has been suggested (*24*), then sCD4 might prove clinically useful despite its relative ineffectiveness against the more prevalent MT-tropic HIV-1 isolates.

Note added in proof: O'Brien and coworkers (*27*) have reported that a 160–amino acid JR-FL envelope sequence that includes the V3 loop was sufficient to confer sCD4 resistance on the otherwise highly sensitive NL4-3 HIV-1 provirus.

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10. Human PBLs were isolated, activated, and maintained as described (*7*). Activated PBL preparations were routinely found to consist of >50% $CD4^+$ T cells. Virus stocks were prepared from proviral clones after transfection into COS cell cultures (35-mm plates) as described (*7*). At ~72 hours after transfection, the COS cultures were fed again with 2 ml of fresh medium containing 2 × 10^6 activated PBLs. Virus stocks specific for the various HIV-1 isolates (*7–9*) were prepared by addition of infectious virus to 4 × 10^6 activated PBLs in a total volume of 2 ml. At 48 hours after infection, all PBL cultures were harvested, centrifuged, washed, and resuspended in 10 ml of fresh media. The cultures were then adjusted to 2 × 10^6 cells per milliliter by supplementation with uninfected cells derived from the same donor. Supernatant media were harvested 48 to 72 hours later and passed through a 0.45-μM filter. The virus stocks were then divided into 1-ml aliquots and frozen at −70°C.
11. After all the viral stocks had been prepared, an aliquot of each was thawed and titrated by the end-point dilution method (*5*). All stocks were tritrated in parallel on PBLs derived from a single donor. The median number of tissue culture infectious doses ($TCID_{50}$) of HIV-1 per milliliter was taken as the inverse of the highest dilution that on average resulted in infection of the PBL cultures. To confirm reproducibility, we titrated the virus stocks a second time with a different PBL donor; comparable results were observed.
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15. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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28. The following reagents were obtained through the Acquired Immunodeficiency Syndrome (AIDS) Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health: HIV-1 isolate JR-FL from I. Chen and isolates SF2 and SF162 from J. Levy. We also thank Biogen Corporation for their gift of sCD4 and S. Goodwin for secretarial assistance. Supported by the Howard Hughes Medical Institute, by PHS grants AI28233 and AI28662 from the National Institute of Allergy and Infectious Diseases, and by funds from the Duke Department of Surgery.

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Stress-Induced Facilitation of Classical Conditioning

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Stress has been shown to impair subsequent learning. To determine whether stress would impair classical conditioning, rats were exposed to inescapable, low-intensity tail shock and subsequently classically conditioned under freely moving conditions with a brief periorbital shock unconditioned stimulus and a white noise conditioned stimulus. Unexpectedly stressed rats exhibited significantly more conditioned eyeblink responses and the magnitude of their individual responses was also enhanced. These results stand in contrast to the learning deficits typically observed and suggest that stress can enhance the acquisition of discrete conditioned responses.

Excessive environmental stimulation is capable of imposing far-ranging consequences on behavior, specifically on the ability to process and synthesize new information. In animals, exposure to inescapable shock typically impairs learning and is referred to as "learned helplessness" (*1*). This phenomenon affects a wide range of learning paradigms but is especially prevalent during instrumental learning (*2*). In contrast, we report here an increase in the acquisition of associative classical conditioning after exposure to inescapable shock.

Male Fischer 344 rats (n = 24) were implanted with two wires around the upper eyelid to record electromyographic (EMG) activity from obicularis oculi and two wires around the lower lid to deliver a periorbital shock. After at least 4 days of recovery, rats were adapted to a conditioning chamber for 1 hour. Half of the rats were then placed in a restraining tube in a different chamber in a different room and exposed to 90 1-s shocks (1 mA, 60 Hz) to the tail, one per min for 90 min, and the other half were returned to their home cages. Twenty-four hours later, stress was reinstated with five 1-s shocks (1 mA), one per minute. After 30 min, the animals were transferred to the conditioning chamber for training. This procedure was repeated each day for 4 days. Unstressed control rats (n = 8) were taken directly from their home cage for training. Four rats were trained per day, two stressed and two unstressed.

On the first day of training, rats were observed for orienting or startle responses to the white noise stimulus, and shock thresholds needed to elicit a blink were obtained. Training consisted of pairing a 350-ms white noise conditioning stimulus (CS) (85 dB, 5 ms rise and fall time) with a coterminating 100-ms shock to the periorbital muscles (2 mA, 60 Hz, ac). Each daily training session consisted of ten blocks of ten trials. Each block consisted of a noise-alone trial, four paired trials, a shock-alone trial, and four additional paired trials. Intertrial intervals were randomized between 20 and 40 s (mean, 30 s). Two groups (stressed, n = 4; unstressed, n = 4) were exposed to the same number of stimuli as in paired training, except that stimuli were explicitly unpaired and presented between 10 and 20 s (mean, 15 s) (*3*).

Immediately after the last training session, rats were killed and trunk blood was collected for radioimmunoassay of serum corticosterone. Trunk blood was also obtained from a group of rats (n = 4) exposed to the same amount of restraint, tail shock, and time in the conditioning chamber (without stimuli) and from a group of naïve controls (n = 4) taken directly from their home cage.

Compared to unstressed controls, rats

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