Antigenic conservation and immunogenicity of the HIV coreceptor binding site

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Immunogenic, broadly reactive epitopes of the HIV-1 envelope glycoprotein could serve as important targets of the adaptive humoral immune response in natural infection and, potentially, as components of an acquired immune deficiency syndrome vaccine. However, variability in exposed epitopes and a combination of highly effective envelope-cloaking strategies have made the identification of such epitopes problematic. Here, we show that the chemokine coreceptor binding site of HIV-1 from clade A, B, C, D, F, G, and H and circulating recombinant form (CRF)01, CRF02, and CRF11, elicits high titers of CD4-induced (CD4i) antibody during natural human infection and that these antibodies bind and neutralize viruses as divergent as HIV-2 in the presence of soluble CD4 (sCD4). 178 out of 189 (94%) HIV-1–infected patients had CD4i antibodies that neutralized sCD4-pretreated HIV-2 in titers (50% inhibitory concentration) as high as 1:143,000. CD4i monoclonal antibodies elicited by HIV-1 infection also neutralized HIV-2 pretreated with sCD4, and polyclonal antibodies from HIV-1–infected humans competed specifically with such monoclonal antibodies for binding. In vivo, variants of HIV-1 with spontaneously exposed coreceptor binding surfaces were detected in human plasma; these viruses were neutralized directly by CD4i antibodies. Despite remarkable evolutionary diversity among primate lentiviruses, functional constraints on receptor binding create opportunities for broad humoral immune recognition, which in turn serves to constrain the viral quasispecies.
these epitopes in humans are generally unknown. This is in part a consequence of technical difficulty in identifying epitope-specific neutralizing antibody responses within a larger context of polyclonal neutralizing and nonneutralizing antibody reactivities (15–17).

In the present study, we sought to identify immunogenic, broadly cross-reactive epitopes on the HIV-1 envelope glycoprotein that might serve as targets of the adaptive humoral immune response in naturally infected humans. We hypothesized that conserved requirements for coreceptor binding among diverse lineages of human or simian immunodeficiency viruses might be reflected in conserved antigenicity at the corresponding envelope surface. As a strategy, we took advantage of the wide evolutionary distance that exists between HIV-1 and HIV-2 lineages to probe for conserved neutralization epitopes. The envelope glycoproteins of HIV-1 and HIV-2 are only ~40% homologous in amino acid sequences (18). As a consequence, they generally exhibit weak antigenic cross-reactivity, and sera from HIV-1–infected individuals cross-neutralize HIV-2 poorly, if at all (19–21). Nonetheless, HIV-1 and HIV-2 each require chemokine coreceptor binding for cell entry, with primary non–T cell line–adapted viruses of both types generally using CCR5 (22, 23). Binding of CD4 to HIV-1 gp120 induces conformational changes in the outer and inner envelope domains, the bridging sheet, and the positioning of variable loops V1/V2 and V3 (24–30). These changes lead to exposure of the envelope coreceptor binding site, comprised of the bridging sheet, adjacent surfaces, and possibly the tip of V3. Antibodies that bind to HIV-1 gp120 preferentially (or only) after CD4 engagement are referred to as CD4-induced (CD4i). Typically, these antibodies bind to surfaces that include or are proximal to the bridging sheet where they compete with coreceptor binding and broadly (but not potently) neutralize different HIV-1 strains (28–33). Cross-reactivity between HIV-1–induced CD4i antibodies and HIV-2 has not been reported. Here, we explore the antigenic cross-reactivity and inherent immunogenicity of the coreceptor binding surfaces of HIV-1 and HIV-2 and assess whether HIV-2, in complex with soluble CD4 (sCD4), might be useful as a specific probe for HIV-1–elicited, CD4i-neutralizing antibodies in humans infected by HIV-1 or immunized with candidate HIV-1 vaccines.

RESULTS

Plasma from HIV-1–infected patients neutralizes sCD4–induced HIV-2

Table I shows the extent and kinetics of the Nab response to autologous HIV-1 virus in a patient (133M) after subtype C HIV-1 infection. Nab titers against the earliest detectable virus reached 1:2,500 (50% inhibitory concentration [IC50]) by 11 mo of infection and then subsided. Such a response is typical of patients with newly acquired HIV-1 infection, and it generally followed rapidly by virus mutation and escape from neutralization (3, 4). To look for more broadly reactive Nabs in this subject, we applied these same plasma specimens to the HIV-2 strain 7312A, a primary CD4–dependent R5 virus (22, 23, 34). As expected, plasma from this HIV-1–infected patient (133M) exhibited no detectable neutralizing activity against HIV-2_{7312A}, a finding consistent with prior studies showing little neutralization cross-reactivity between these highly divergent viral lineages (19, 20). However, when HIV-2_{7312A} was pretreated for 1 h with 9nM sCD4 (equal to the IC50 for this virus), the virus became remarkably susceptible to neutralization by 133M plasma, with titers of Nab reaching 1:12,500 by 26 mo after infection (Table I). Similar results were obtained in six additional subjects with primary subtype C HIV-1 infection, whose Nab titers to sCD4–pretreated HIV-2_{7312A} ranged from 1:53 to 1:3,361 and peaked between 8 and 24 mo after acute infection. To determine if the CD4–dependent Nab activity that we observed in plasma from subtype C patients was limited to this virus clade, we studied additional patients chronically infected with HIV-1 subtypes A, B, C, or D. Fig. 1 A depicts the neutralization profile of plasma from four such patients against HIV-2_{7312A} in the absence or presence of sCD4. In each case, there was a dramatic sCD4–dependent shift of 100–10,000-fold in the susceptibility of HIV-2 to neutralization. IC50 titers of CD4i Nab titers in these four individuals ranged from 1:750 to 1:20,000. 15 uninfected normal donors had no detectable Nabs to HIV-2_{7312A} with or without sCD4.

HIV-1 CD4i monoclonal antibodies neutralize sCD4–induced HIV-2

If the broadly cross-reactive neutralizing antibody activity that we observed in HIV-1–infected patient plasma is due to classical CD4i antibodies, then prototypic CD4i monoclonal antibodies derived from HIV-1–infected patients, which have been extensively characterized against HIV-1 envelope glycoproteins (28–33), might be expected to cross-neutralize HIV-2 in a CD4–dependent fashion. Fig. 1 B demonstrates this to be the case. Without sCD4, the CD4i monoclonals 17b, 21c, and...
19e failed to neutralize HIV-2\textsubscript{7312A}. In the presence of sCD4, a dramatic shift in the neutralization curves was observed with all three antibodies neutralizing HIV-2\textsubscript{7312A} potently (Fig. 1 B). It is notable that for both the CD4i polyclonal (Fig. 1 A) and monoclonal (Fig. 1 B) antibodies, the extent of neutralization reached only \approx 90\%, and in the case of the clade D plasma KAMW, 80\%. This is due in part to a time- and concentration-dependent interaction between sCD4 and the gp120 envelope glycoprotein because higher sCD4 concentrations and more prolonged preincubation times (30–120 min) increased the extent of HIV-2\textsubscript{7312A} neutralization by both monoclonal and polyclonal CD4i antibodies (unpublished data). Steric accessibility or affinity of CD4i antibodies to their cognate epitopes may also influence the extent of virus neutralization because a single mutation (V434M) in the bridging sheet of HIV-2\textsubscript{7312A}, making this amino acid the same as in HIV-1 (see Site-directed mutagenesis of the HIV-2 bridging sheet alters HIV-1 CD4i antibody recognition), resulted in a marked shift of the neutralization curves of 17b and 19e and of three HIV-1 patient plasmas (shown in Fig. 1 C) to the left and downward, resulting in 100\% neutralization of infectious virus.

**Multiple primary HIV-2 strains are susceptible to HIV-1 CD4i antibody neutralization**

Neutralization of HIV-2 by HIV-1–elicited CD4i antibodies is not restricted to HIV-2\textsubscript{7312A} and derivative strains. HIV-
Table II. Neutralization titers of HIV-1 monoclonal antibodies and patient plasma against different HIV-2 viruses

| Moab    | Epitope     | 7312A | UC-1  | V434M | 7312A | 7312A | H419R | Q422L |
|---------|-------------|-------|-------|-------|-------|-------|-------|-------|
| E51     | CD4i        | −/−   | −1/3.0| −4.0  | −22.0 | −/−   | −/−   | −/−   |
| 17b     | CD4i        | −/0.16| −/9.4 | 8.0/0.002| 15.0/0.002| −/− | −/−   | −/−   |
| 48d     | CD4i        | −/−   | −/−   | −/−   | −/−   | −/−   | −/−   | −/−   |
| 31H     | CD4i        | −/3.71| −/1.58| −/0.62| −/1.42| −/−   | −/−   | −/−   |
| 23c     | CD4i        | −/−   | −/−   | −/−   | −/−   | −/−   | −/−   | −/−   |
| 21c     | CD4i        | −/0.11| −/0.005| −/0.94| −/0.014| −/−   | −/−   | −/−   |
| X5      | CD4i        | −/−   | −/−   | −/2.5 | −/−   | −/−   | −/−   | −/−   |
| 412d    | CD4i        | −/−   | −/−   | −/−   | −/−   | −/−   | −/−   | −/−   |
| 19e     | CD4i        | −/0.017| −/0.009| −/0.006| −/0.005| −/−   | −/−   | −/−   |
| ED47    | CD4i        | −/−   | −/−   | −/−   | −/−   | −/−   | −/−   | −/−   |
| ED49    | CD4i        | −/5.4 | −/12.0| −/2.4 | −/3.3 | −/−   | −/−   | −/−   |
| b12     | CD4bs       | −/−   | −/−   | ND    | ND    | ND    | ND    | ND    |
| F105    | CD4bs       | −/−   | −/−   | ND    | ND    | ND    | ND    | ND    |
| F91     | CD4bs       | −/−   | −/−   | ND    | ND    | ND    | ND    | ND    |
| 15e     | CD4bs       | −/−   | −/−   | ND    | ND    | ND    | ND    | ND    |
| 2F5     | gp12        | −/−   | −/−   | ND    | ND    | ND    | ND    | ND    |
| 447-52D | V3          | −/−   | −/−   | ND    | ND    | ND    | ND    | ND    |
| 19b     | V3          | −/−   | −/−   | ND    | ND    | ND    | ND    | ND    |
| C011    | V3          | −/−   | −/−   | ND    | ND    | ND    | ND    | ND    |
| 2580    | V3          | −/−   | −/−   | ND    | ND    | ND    | ND    | ND    |
| 2442    | V3          | −/−   | −/−   | ND    | ND    | ND    | ND    | ND    |
| 2G12    | 35Kican     | −/−   | −/−   | ND    | ND    | ND    | ND    | ND    |
| A32     | gp120       | −/−   | −/−   | ND    | ND    | ND    | ND    | ND    |
| C11     | gp120       | −/−   | −/−   | ND    | ND    | ND    | ND    | ND    |
| 2.6C    | HIV-2/gp120 | −/−   | −/−   | ND    | ND    | ND    | ND    | ND    |
| 1.7A    | HIV-2/gp120 | 0.016/0.011| 0.005/0.007| 0.017/0.009| 0.023/0.017| 0.009/0.009 |

| Patient ID | HIV-1 subtype | 7312A | UC-1  | V434M | 7312A | 7312A | H419R | Q422L |
|------------|---------------|-------|-------|-------|-------|-------|-------|-------|
| 6X4F       | A             | −/10,000| 370/76,923| 20/41,667| 4,065/96,937| ND    | ND    | ND    |
| 21X0F      | A             | −/6,667 | 500/13,699| 63/17,241| 222/47,619| ND    | ND    | ND    |
| 37X4F      | A             | −/3,846 | −/1,333 | 59/68,027| 435/65,240| ND    | ND    | ND    |
| BAMA0037   | B             | 36/4,167| 83/3,448 | 40/16,667| 48/4,167 | ND    | ND    | ND    |
| SMST1012   | B             | 67/7,692| 370/9,090| 48/13,514| 192/4,348| ND    | ND    | ND    |
| KIMA9001   | B             | 31/1,136| 36/1,563 | 37/6,250 | 21/1,612| ND    | ND    | ND    |
| 200M       | C             | −/2,941 | 91/5,000 | 31/4,348 | 77/7,692 | ND    | ND    | ND    |
| 49M        | C             | −/17,241| 385/17,241| 45/27,027| 333/65,189| ND    | ND    | ND    |
| A2F        | C             | −/5,000 | 263/6,251| −/52,632 | −/18,181| ND    | ND    | ND    |
| KAMW       | D             | −/18,868| 53/18,519| 143/33,333| 27/26,316| ND    | ND    | ND    |

| sCD4      | 9 nM          | 3 nM   | 15 nM | 28 nM | 6 nM  |
|-----------|---------------|--------|-------|-------|-------|

\(^a\text{Values preceding the slash marks denote the IC}_{50} \text{ in } \mu \text{g/mL for monoclonal antibodies and in reciprocal dilutions for patient plasma specimens, each in the absence of sCD4. Values following the slash marks denote IC}_{50} \text{ values in the presence of sCD4. sCD4 concentrations were adjusted to correspond to the IC}_{50} \text{ specific for each virus as indicated in the bottom row. Dashes denote absent neutralization defined as IC}_{50} > 25 \mu \text{g/mL for monoclonal antibodies or } < 1:20 \text{ for human plasma. Neutralization assays were performed in JCS3BL-13 cells (reference 3).}\)
man monoclonal antibodies, including those targeting the CD4 binding site (CD4bs), V3 loop, surface glycans, and gp41. HIV-2UC-1 was also compared with HIV-2 7312A in its susceptibility to neutralization by a subset of 10 HIV-1 clade A, B, C, and D patient plasmas (Table II, bottom). CD4-dependent Nab titers against HIV-2UC-1 were at least two-fold higher than for HIV-2 7312A in two patients (6X4F and 21X0F), threefold lower in one patient (37X4F), and not substantially different in seven others. For each HIV-1 antibody-positive plasma specimen tested, there was a one-to-three log CD4-dependent shift in the HIV-2UC-1 neutralization curve (Table II, bottom).

HIV-1 CD4i antibody binding to HIV-2 glycoprotein correlates with neutralization

CD4i antibodies in HIV-1 plasma that neutralize HIV-2 infection might also be expected to compete directly with HIV-1 CD4i monoclonal antibodies for binding to HIV-2 gp120–sCD4 complexes. Fig. 2 shows the results of an assay using 16 human plasma samples (11 HIV-1 positive; 5 normal uninfected controls) to compete with biotin-conjugated 19e for binding to HIV-2 7312A, HIV-2 MVP15132, or HIV-1 JR-FL gp120–sCD4 complexes. A mock-treated sample did not inhibit biotin-labeled 19e binding, which was normalized to 100%. Unlabeled 19e competed efficiently with biotin-labeled 19e binding to each of the three HIV glycoproteins. The five normal control specimens (sample nos. 1–5) showed no significant competition for biotinylated 19e binding to any of the three HIV envelope glycoproteins. The 11 HIV-1–positive patient specimens, however, competed variably with 19e for binding to both HIV-1 and HIV-2 glycoproteins. Sample nos. 13–16 showed the strongest competition against 19e for HIV-2 7312A binding, and these samples also exhibited the highest neutralization titers against HIV-2 7312A (reciprocal mean IC50 = 0.00007 ± 0.00005). Sample nos. 6–9 showed the least competition with 19e for binding HIV-2 7312A, and these had the lowest Nab titers against this virus (IC50 = 0.023 ± 0.024). Other samples were intermediate in binding and neutralization activity. There was a highly significant correlation between the titers of Nab measured against HIV-2 7312A and the efficiency with which these plasma specimens competed with 19e for HIV-2 7312A binding (R² = 0.94; r = 0.97; P < 0.0001). With the exception of sample no. 10, the HIV-1–positive patient plasma specimens competed for 19e binding to the HIV-1 JR-FL glycoprotein more efficiently than to either of the two HIV-2 glycoproteins.

To further examine the correlation between antibody binding and neutralization, we tested a large number of biotin-labeled HIV-1 CD4i antibodies for binding to HIV-2 7312A envelope glycoprotein with and without sCD4. Fig. 3 A shows that the HIV-1–elicited CD4i antibodies that were found in Table II to neutralize HIV-2 7312A most efficiently (19e, 17b, 31H, and 21c) also bound the HIV-2 7312A glycoprotein most efficiently in a CD4-dependent manner, whereas those antibodies that neutralized poorly, bound poorly. To further evaluate the breadth of HIV-1 CD4i monoclonal antibody binding, we tested three antibodies (19e, 21c, and 17b) for reactivity against additional primate lentiviruses (Fig. 3 B). The HIV-1 CD4i monoclonal anti-
bodies bound not only HIV-2\textsubscript{7312A} Env–sCD4 complexes, but also HIV-2\textsubscript{CBL20}, HIV-2\textsubscript{MVP15132}, SIVmac239, SIVmne, and as a control, HIV-1\textsubscript{BAL}. It is again noteworthy that gp120–sCD4 complexes from different HIV-2 and SIV strains were recognized variably by the three HIV-1 CD4i monoclonal antibodies, with 19e exhibiting the strongest reactivity to all viral envelopes, followed by 21c, and then 17b. These findings, together with the neutralization results, indicate that the CD4i chemokine receptor binding surfaces of HIV-2 strains 7312A, UC-1, ST/SXB1, CBL20, and MVP15132, as well as SIVmac239 and SIVmne, all share substantial antigenic cross-reactivity with each other and with HIV-1.

Site-directed mutagenesis of the HIV-2 bridging sheet alters HIV-1 CD4i antibody recognition

HIV-2 neutralization by HIV-1 CD4i monoclonal and polyclonal antibodies is best explained by antibodies binding to the conserved chemokine coreceptor binding surface, including the bridging sheet. To evaluate this hypothesis directly, we performed site-directed mutagenesis on the HIV-2 bridging sheet region (36). The primary amino acid sequence of the bridging sheet of HIV-1 and the corresponding sequence of HIV-2 is conserved but not identical (Fig. 4). Substitutions were made at three positions in the HIV-2\textsubscript{7312A} sequence at or near the binding footprints of monoclonals 17b, 21c, and 19e in the corresponding HIV-1 sequence (8, 9, 31). The effects of these mutations on the susceptibility of the respective viruses to neutralization by HIV-1 monoclonal and polyclonal antibodies were substantial (Fig. 1 C and Table II). Mutations V434M and H419R (HXB2 numbering system; Fig. 4) made the HIV-2 sequence at these positions the same as HIV-1, and thus would be expected to enhance HIV-1 CD4i–antibody binding. The V434M substitution led to an 80-fold enhancement of 17b neutralization, at least 10-fold enhancement of X5 neutralization, 6-fold increase in E51 and 31H neutralization, and 2–3-fold enhancement of ED49 and 19e neutralization. Neutralization enhancement was not global, however, because there was a concomitant 85-fold decrease in 21c susceptibility and no change in susceptibility to the HIV-2 monoclonal 1.7A, which binds a conserved epitope distant from the bridging sheet (Table II). Similarly, the H419R mutation led to a 2- to 80-fold enhancement in neutralization by 17b, 31H, 19e, ED47, and ED49, but little or no change in susceptibility to E51, 21c, or
1.7A. In addition to mutations expected to enhance HIV-1 CD4i antibody binding, we also tested a Q422L mutant, which had been shown in HIV-1 to reduce CD4i–antibody binding (e.g., 17b), while allowing the envelope to otherwise retain its normal receptor binding and entry functions (31). The Q422L mutation in 7312A resulted in complete loss of 17b neutralization (>150-fold change), complete loss in 31H neutralization (>7-fold change), and a 3-fold decrease in 21e neutralization, but had little effect on 19e-, ED49-, or 1.7A-mediated neutralization. Enhanced susceptibility of the V434M and H419R mutants to neutralization was also observed with most of the HIV-1 patient plasmas tested (Table II).

Prevalence and titers of CD4i-neutralizing antibodies in patients infected by diverse HIV-1 subtypes
Plasma samples from 189 individuals infected by HIV-1 clade A, B, C, D, F, G, or H, or by circulating recombinant form (CRF)01, CRF02, or CRF11, were tested for CD4i Nabs against HIV-2. In preliminary studies, we tested a subset of 69 of these specimens for reactivity against the wild-type HIV-2 strain 7312A and its derivative 7312A/V434M. This pilot study showed that the frequency of detection of HIV-2 cross-reactive CD4i Nabs was modestly higher for the V434M virus (94%) compared with 7312A (87%). Based on the enhanced sensitivity of HIV-2/7312A/V434M, we used this virus to test all 189 patient plasma specimens for CD4i Nabs (Table III). CD4i Nabs were detected in 174 (92%) of patients, with median IC₅₀ titers of 0.0004 (1:2,500) and mean titers of 0.004 (1:250). Titers of CD4i Nab in plasma from clade D and CRF11 patients, considered separately or as a group, were significantly greater than for patients in the remaining groups (P < 0.0001). We considered the possibility that, despite the overall similarity in neutralization patterns observed for the HIV-2 strains depicted in Table II, divergent HIV-2 strains might detect CD4i Nabs in some of the patients’ plasma that tested negative against HIV-2/7312A/V434M. Thus, we retested the 15 negative samples, first by Western immunoblot to confirm HIV-1 positivity, and then by neutralization assay against three different HIV-2 strains: UC-1, ST/SXB1, and 7312A. All 15 samples were Western immunoblot positive against HIV-1 proteins. Four samples were found to have CD4i Nabs against one or more of these viruses in titers ranging from 1:25 to 1:750. Thus, overall, out of 189 HIV-1–infected patients tested, 178 (94%) had detectable neutralizing CD4i antibodies against HIV-2.

Role of CD4i antibodies in natural HIV-1 infection
Previous studies have shown that HIV-1 CD4i antibodies are largely excluded by steric hindrance from the virus–cell interface after CD4 engagement, and as a consequence, CD4i antibodies generally neutralize HIV-1 inefficiently (12, 28). However, this steric restriction could be overcome experimentally by using CD4i antibody fragments (Fab or sFv) or by dissociating (spatially or temporally) envelope–CD4 engagement from envelope–coreceptor engagement (12, 28). Given these constraints on CD4i antibody-mediated neutralization, we sought to examine what role CD4i antibodies might play in vivo. Sodroski et al. (37) first postulated that CD4i antibodies might constrain virus to CD4 dependence by selecting against envelope mutations that lead to spontaneous exposure of the viral coreceptor binding surface (38, 39). Our results support this hypothesis by showing in naturally infected humans that CD4i antibodies are prevalent, high-titer, and so broadly cross-reactive that they neutralize even HIV-2. However, to test more directly if CD4i antibodies might be active in constraining HIV-1 to CD4 dependence in vivo, we examined sequential uncultured plasma specimens from four HIV-1–infected patients (133M, WEAU0575, SUMA0874, BORJ0637) for evidence of viruses that contain mutations in envelope that result in greater spontaneous exposure of the receptor binding surfaces. 74 full-length, functional gp160 envelope clones were derived by PCR amplification of plasma virion RNA and used to pseudotype env-deficient HIV-1 virus for entry in JC53BL-13 cells. Two clones from patient SUMA0874 (S736-68 and S736-75) were found to be uniquely sensitive to neutralization by sCD4 (IC₅₀ <0.05 μg/ml), indicating that they might exhibit greater spontaneous exposure of receptor-binding surfaces than is generally observed in primary HIV-1 strains (40). These same two clones were also distinguished from all others that we examined by an isoleucine (I) to threonine (T) substitution at position 309 (HXB2 numbering system) immediately 5′ of the GPGR crown of the V3 loop (Fig. S1, available a http://www.jem.org/cgi/content/full/jem.20042510/DC1), a position reported by Quinnan et al. (41) to confer CD4-independent infectivity and enhanced susceptibility to neutralization in an unrelated primary HIV-1 strain. We, therefore, first tested clones S736-68 and S736-75, along with other SUMA clones lacking the I309T mutation (including S736-68m/TI), for CD4-independent fusion and infectivity in C2Th-synCCR5 cells, a canine thymocyte cell line that expresses human CCR5 but lacks CD4 on its surface (42). The S736-68 and S736-75 envelopes, but not isogenic envelopes lacking the I309T mutation, supported CD4-independent virus fusion and entry, and this was abolished by treatment with 17b and other HIV-1 CD4i antibodies (unpublished data). We next tested the S736-68 envelope clone, along with a site-directed mutant that restored the more common isoleucine at position 309 (S736-68m/TI), for their susceptibility to sCD4, to an anti-CD4 monoclonal antibody, to the CD4i monoclonal 17b, and to autologous SUMA plasma in JC53BL-13 cells (Fig. 5). The S736-68 pseudotyped virus was far more sensitive compared with the isogenic S736-68m/TI mutant to neutralization by sCD4, 17b, and autologous plasma, and it was less sensitive to inhibition by anti-CD4 antibody. Similar findings were made with S736-75. These data suggest that the S736-68 and S736-75 envelopes, like those from some T cell line adapted viruses, have a spontaneously exposed chemokine coreceptor binding site
Figure 4. Envelope gp120 alignments for HIV-2 (7312A and UC1), SIV (Mac239 and Ver-Tyo1), and HIV-1 (YU2 and HXB2). Bridging sheet, variable loops, amino acid identities, and site-directed mutations (H419R, Q422L, and V434M) are indicated. The signal peptide-gp120 cleavage position for HIV-1 is shown. Variable loops (V1/V2, V3, and V4) have conventionally been defined by disulfide-linked cysteine residues at their bases as depicted. However, the actual limits of variable loops have been resolved structurally in the HXB2–CD4–17b crystal complex (reference 8), and these sequences are indicated by green bars. It is possible that structural details diverge in the more distantly related HIV/SIV sequences. The amino acids contributing to the bridging sheet are highlighted in yellow. Blue dots indicate residues contributing to chemokine coreceptor binding based on site-directed mutagenesis studies (references 29, 30). Additional amino acids within the stem of V3, including 298R, 301N, 303T, 323I, 325N, 326M, and 327R, may contribute to gp120 interaction with CCR5 (reference 76). Red dots indicate HIV-1 contact residues for CD4 based on crystal structure analyses (reference 8). Asterisks below the sequence indicate conservation of amino acid identity across all five virus strains. Overall gp120 sequence identity was calculated based on amino acid residues exclusive of the initiator methionine of the (cleaved) signal peptide and a gap-stripped alignment of the sequences shown. Except for SIVverTYO1, sequences were obtained from the HIV Sequence Compen...

**Sequence Identity**

| gp120 | 57 |
|--------|----|
| UC1    | 57 |
| MAC239 | 57 |
| VER-Tyo1 | 415 |
| YU2    | 415 |
| HXB2   | 415 |

**Figure 4.** Envelope gp120 alignments for HIV-2 (7312A and UC1), SIV (Mac239 and Ver-Tyo1), and HIV-1 (YU2 and HXB2). Bridging sheet, variable loops, amino acid identities, and site-directed mutations (H419R, Q422L, and V434M) are indicated. The signal peptide-gp120 cleavage position for HIV-1 is shown. Variable loops (V1/V2, V3, and V4) have conventionally been defined by disulfide-linked cysteine residues at their bases as depicted. However, the actual limits of variable loops have been resolved structurally in the HXB2–CD4–17b crystal complex (reference 8), and these sequences are indicated by green bars. It is possible that structural details diverge in the more distantly related HIV/SIV sequences. The amino acids contributing to the bridging sheet are highlighted in yellow. Blue dots indicate residues contributing to chemokine coreceptor binding based on site-directed mutagenesis studies (references 29, 30). Additional amino acids within the stem of V3, including 298R, 301N, 303T, 323I, 325N, 326M, and 327R, may contribute to gp120 interaction with CCR5 (reference 76). Red dots indicate HIV-1 contact residues for CD4 based on crystal structure analyses (reference 8). Asterisks below the sequence indicate conservation of amino acid identity across all five virus strains. Overall gp120 sequence identity was calculated based on amino acid residues exclusive of the initiator methionine of the (cleaved) signal peptide and a gap-striped alignment of the sequences shown. Except for SIVverTYO1, sequences were obtained from the HIV Sequence Compen...
and is less dependent on CD4 binding for entry compared with most primary viruses. Thus, exposure of the coreceptor binding surface on primary HIV-1 viral envelopes occurs spontaneously in vivo, but such viruses are exquisitely sensitive to neutralization by antibodies including those targeting CD4i epitopes.

**Breadth of antigenic cross-reactivity in the HIV and SIV coreceptor binding sites**

To examine the breadth of antigenic cross-reactivity in the coreceptor binding sites of HIV-1, HIV-2, SIVsm, and SIVagm, we preincubated strains of each virus with CD4i monoclonal antibodies or plasma from infected subjects (with and without sCD4) and assayed for virus neutralization or fusion inhibition. The results showed that natural infection by these lentiviruses elicits antibodies that neutralize the homologous virus as well as the evolutionarily divergent viruses. Fig. S2 (available at http://www.jem.org/cgi/content/full/jem.20042510/DC1) depicts potent neutralization of the homologous virus as well as the evolutionarily divergent viruses. The results extend the findings of Berger et al., who observed that HIV-1 subtypes A, B, C, D, E, and F were all susceptible to neutralization by the HIV-1 CD4i monoclonal antibody 17b (28).

**DISCUSSION**

Although much is known about the HIV-1 envelope glycoprotein (7–17, 24–33), the present study provides new insight into its immunogenicity and antigenic conservation. Previous studies suggested that the conformationally dependent coreceptor binding surface on HIV-1 was only weakly immunogenic and CD4i antibodies were relatively uncommon (31–33). This paper indicates quite the opposite to be the case. We find the vast majority (94%) of HIV-1–infected patients infected by any 1 out of 10 different clades or CRFs harbor HIV-specific CD4i Nabs with IC50 titers ranging from 1:20 to >1:100,000. The mean CD4i Nab titer against HIV-2_3312A/V434M among 189 subjects was 1:250 and the median titer was 1:2,500. 114 subjects had Nab titers ≥1:1,000, the highest reaching 1:143,000. In a related study, we found that 8 out of 10 healthy, uninfected human volunteers immunized with ALVAC vCP1452 HIV-1 gp140 alone or in combination with soluble monomeric HIV-1 gp120 (AIDS-VAX B/B) developed HIV-1 CD4i-neutralizing antibodies against HIV-2_3312A, compared with 0 out of 5 control subjects who were vaccinated with placebo (unpublished data). To explain the elicitation of CD4i Nabs by soluble HIV-1 gp120 or expressed gp140, we suspect that envelope glycoprotein is bound to cell surface–associated CD4, undergoes conformational change, and elicits a CD4i antibody response.

The observation that CD4i antibodies elicited by HIV-1 infection potently neutralized multiple strains of HIV-2 came as a surprise. Although most primary human and simian lentiviruses use CCR5 as a coreceptor for cell attachment and entry (23), functionally important amino acids in the HIV-1 envelope coreceptor binding region identified by mutagenesis experiments (8, 29, 30) are only partially conserved in HIV-2, SIVmac, and SIVagm (Fig. 4). Moreover, conserved receptor binding would not necessarily be expected to be reflected in conserved receptor antigenicity (43–45). Thus, the finding that HIV-1 CD4i monoclonal antibodies such as 19e and 21c could bind viral glycoproteins as divergent as those from HIV-1, HIV-2, SIVsm, SIVmac, and SIVagm in a CD4-dependent fashion (Fig. 3, A and B), and that monoclonal and polyclonal antibodies from HIV-1–infected humans routinely neutralized sCD4-triggered HIV-2 (Tables II and III), was quite unexpected. We even found that sCD4-treated SIVverTy1 from African green monkey is susceptible to CD4i neutralization by some HIV-1–infected patient samples in titers as high as 1:1,000 (Fig. S2). Together, these observations highlight the extraordinary degree of antigenic conservation linked to coreceptor binding exhibited by diverse HIV-1 and HIV-2 lineages, and at the same time, an ability of the human humoral immune system to exploit these constraints.

It is of interest to consider the cooperative interactions that may be occurring among sCD4, the HIV-2 envelope glycoprotein, and CD4i antibody that result in potent virus neutralization. We have ruled out the possibility that HIV-1–elicited CD4i antibodies neutralize HIV-2 by binding directly to CD4 because a scorpion toxin-based CD4 mimetic that differs substantially from sCD4.

### Table III. Prevalence and titers of CD4i-neutralizing antibodies against HIV-2_3312A/V434M in plasma of HIV-1–infected subjects

| HIV-1 plasma | n  | Positive (%) | Mean CD4i Nab titer SD  | Median CD4i Nab titer |
|-------------|----|--------------|-------------------------|-----------------------|
| Clade A     | 39 | 35 (90)      | 0.029 0.0052 0.0007     |                       |
| Clade B     | 25 | 24 (96)      | 0.0047 0.0105 0.0003    |                       |
| Clade C     | 23 | 22 (96)      | 0.0051 0.0118 0.0004    |                       |
| Clade D     | 7  | 7 (100)      | 0.00007 0.00006 0.00007 |                       |
| Clade F     | 6  | 5 (83)       | 0.0008 0.0005 0.001     |                       |
| Clade G     | 5  | 3 (60)       | 0.0061 0.0092 0.0015    |                       |
| Clade H     | 2  | 2 (100)      | 0.002 0.0028 0.002     |                       |
| CRF01       | 1  | 1 (100)      | 0.003 – 0.003           |                       |
| CRF02       | 77 | 72 (94)      | 0.0053 0.0106 0.0008    |                       |
| CRF11       | 4  | 3 (75)       | 0.00005 0.00002 0.00004 |                       |
| Total       | 189| 174 (92)     | 0.004 0.0093 0.0004     |                       |

*Reciprocal IC50 titers of CD4i-neutralizing antibodies against HIV-2_3312A/V434M pre-treated with 15 nM sCD4.*
stantially in amino acid sequence from CD4 also results in conformational change and exposure of CD4i epitopes, but in a cooperative manner because the magnitude of HIV-2 neutralization we observe is far greater than would be expected on the basis of additive stoichiometry. Of note, Berger et al. (47) have demonstrated cooperative interactions between different gp120 protomers within a trimer complex of HIV-1.

A role for CD4i antibodies in natural HIV-1 infection may become apparent. Our data, together with other results (37, 41), suggest that HIV-1 variants with exposed coreceptor binding surfaces and varying degrees of CD4 independence, are generated spontaneously in vivo where they are almost certainly neutralized by CD4i or other HIV-1–specific antibodies. In fact, four studies have now shown that single amino acid substitutions in the HIV-1 glycoprotein, either at the base of V1/V2 (3, 48) or in the V3 loop (the present text and reference 41), are sufficient to confer on the virus varying degrees of CD4 independence, spontaneous exposure of the coreceptor binding site, and enhanced susceptibility to CD4i Nabs. Principles of viral dynamics suggest that such mutations must be occurring in vivo on a virtually continuous basis, as has been documented for comparable mutations leading to antiretroviral drug resistance (49). Thus, CD4i antibodies may influence HIV-1 natural history and pathogenesis to a greater extent than is currently recognized by constraining virus to CD4 dependence. Consistent with this interpretation, Gabuzda et al. have shown that HIV-1 virus within the central nervous system (where circulating antibodies are relatively excluded) has less dependence on cell surface–bound CD4 for attachment and entry (50). CD4i antibodies could also influence the frequency of R5/X4 coreceptor switching (51) and target viruses with short or otherwise constrained envelope variable loop sequences (52).

The discovery that sCD4–triggered HIV-2 is susceptible to binding and neutralization by HIV-1–elicited CD4i antibodies has practical application in studies of HIV-1 natural history and vaccine assessment. A number of investigative groups have attempted to stabilize the HIV-1 envelope glycoprotein in a CD4-bound configuration to use it as an immunogen designed to elicit antibodies against viral receptor surfaces or other intermediate envelope structures (53–55). But methods to selectively identify and titer Nabs specific for such epitopes have been limited. Here, we show that neutralization of sCD4–treated HIV-2 represents an extremely sensitive and specific assay to detect HIV-1–elicited CD4i antibodies. Investigators have also targeted the membrane-proximal external region (MPER) of HIV-1 gp41 for vaccine development (56–66) because conserved epitopes in this region are capable of eliciting broadly reactive Nabs in natural infection (56–58, 65). But again, neutralization assays are lacking that allow for the sensitive and specific detection of MPER epitope–specific Nabs (17). We considered the possibility that HIV-2 could act as a “molecular scaffold” on which to present these and other HIV-1 epitope–specific antigens in the context of a functional envelope glycoprotein that does not otherwise cross-react with HIV-1–neutralizing antibodies. In recent studies, we have identified and modi-
fied by site-directed mutagenesis HIV-2 strains that can be used to detect and tier neutralization by the HIV-1 gp41 MPER–specific human monoclonal antibodies 4E10 and 2F5 with high sensitivity and specificity (unpublished data). Thus, the strategy described in this paper of using HIV-2 envelope glycoproteins in the context of infectious virions or as isolated proteins to detect HIV-1 epitope-specific neutralizing antibodies may find application in the assessment of candidate vaccines and in studies of HIV-1 natural history.

MATERIALS AND METHODS

Plasma specimens. Pre-existing coded plasma samples from 189 HIV-1–infected subjects and 15 uninfected normal control individuals were analyzed. Human subjects gave informed consent and protocols received (University of Alabama at Birmingham) institutional review board approvals.

Cell entry and neutralization assays. Plasma samples and monoclonal antibodies were assayed for Nab activity using a modification of recently described HIV entry and fusion assays (3, 32, 67). These assay systems employ the HeLa cell-derived JC53BL-13 cell line (National Institutes of Health AIDS Research and Reference Reagent Program catalogue no. 8129, TZM-b1), which has been genetically modified to constitutively express CD4, CCR5, and CXCR4, and the canarypox transgene cell line C2Th-synCCL5R3, which expresses human CCR5 but not CD4 (42).

Virus stocks. HIV-2 proviral clones pJK7312A (GenBank/EMBL/DDBJ accession no. L36874), pJK7312A/V343M, pJK7312A/H419R, pJK7312A/Q422L, and pSP4-27 (ST/SXBl; references 22, 68–70) were used to transfect 293T cells. HIV-2 UC-1 env (22, 35) and HIV-1 133M env, cloned in pSM and pCR3.1, respectively, were cotransfected with pSG3deltaEnv or pJK7312AdeltaEnv to create infectious pseudovirions, as described previously (3). HIV-1 env genes cloned in pcDNA3.1 were cotransfected with an HIV-1 reporter virus (pNLENG1-ES-IRES) containing an enhanced green fluorescence gene (67) for virus entry studies in C2Th-synCCL5R3 cells.

Binding and competition assays. Biotinylated monoclonal antibodies (31–33, 71–76) were tested for binding to HIV-2, SIV, or HIV-1 gp120 envelope glycoproteins (34, 35, 68–70, 77–79) captured on microtiter plates coated with mAb 2.6C or EH21, as previously described (31, 32). Before the addition of biotin-labeled antibodies, gp120 was preincubated with 1–10 μg/ml sCD4 (R&D Systems) or a mock preparation and with or without competing plasma specimens.

Monoclonal antibodies. mAbs are described in supplemental Materials and methods (available at http://www.jem.org/cgi/content/full/jem.20042510/DC1).

Molecular cloning, sequencing, and mutagenesis. Full-length gp160 envelope genes were amplified by nested PCR from plasma HIV-1 RNA, cloned, and sequenced as previously described (3, 49). Sequences are deposited in GenBank/EMBL/DDBJ (accession nos. AY223761–90, AY223720–S4, AY885850).

Statistical analyses. Linear regression, Pearson correlations, Fisher’s exact test, and Wilcoxon rank sum test were performed on primary and log transformed datasets. Calculations were performed in SAS.

Online supplemental material. Fig. S1 shows the complete amino acid sequences for 31 gp160 envelope clones derived from plasma virus from subject SUMA0874. Four additional gp160 sequences corresponding to site-directed mutants of wild-type clones S736-68 and S736-73 containing substitutions at positions 308 or 309 (HXB2 numbering system) are designated S736-68m/TI, S736-68m/PI, S736-73m/TT, and S736-73m/PI. Fig. S2 depicts sCD4-dependent neutralization of different HIV and SIV viruses by HIV-1–elicited CD4+ antibodies. Included in supplemental Materials and methods are detailed descriptions of all materials and methods. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20042510/DC1.

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