Cryptic Nature of a Conserved, CD4-Inducible V3 Loop Neutralization Epitope in the Native Envelope Glycoprotein Oligomer of CCR5-Restricted, but Not CXCR4-Using, Primary Human Immunodeficiency Virus Type 1 Strains

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The envelope subunit of the human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein (Env), gp120, contains conserved regions that mediate sequential interactions with two cellular receptor molecules, CD4 and a chemokine receptor, most commonly CCR5 or CXCR4. However, antibody accessibility to such regions is hindered by diverse protective mechanisms, including shielding by variable loops, conformational flexibility and extensive glycosylation. For the conserved neutralization epitopes hitherto described, antibody accessibility is reportedly unrelated to the viral coreceptor usage phenotype. Here, we characterize a novel, conserved gp120 neutralization epitope, recognized by a murine monoclonal antibody (MAb), D19, which is differentially accessible in the native HIV-1 Env according to its coreceptor specificity. The D19 epitope is contained within the third variable (V3) domain of gp120 and is distinct from those recognized by other V3-specific MAb.

To study the reactivity of MAb D19 with the native oligomeric Env, we generated a panel of PM1 cells persistently infected with diverse primary HIV-1 strains. The D19 epitope was conserved in the majority (23/29; 79.3%) of the subtype-B strains tested, as well as in selected strains from other genetic subtypes. Strikingly, in CCR5-restricted (R5) isolates, the D19 epitope was invariably cryptic, although it could be exposed by addition of soluble CD4 (sCD4); epitope masking was dependent on the native oligomeric structure of Env, since it was not observed with the corresponding monomeric gp120 molecules. By contrast, in CXCR4-using strains (X4 and R5X4), the epitope was constitutively accessible. In accordance with these results, R5 isolates were resistant to neutralization by MAb D19, becoming sensitive only upon addition of sCD4, whereas CXCR4-using isolates were neutralized regardless of the presence of sCD4. Other V3 epitopes examined did not display a similar divergence in accessibility based on coreceptor usage phenotype. These results provide the first evidence of a correlation between HIV-1 biological phenotype and neutralization sensitivity, raising the possibility that the in vivo evolution of HIV-1 coreceptor usage may be influenced by the selective pressure of specific host antibodies.
lar receptor structures restricts the degree of variation that the virus can tolerate at selected gp120 sites. Such conserved regions include the CD4-binding site, a discontinuous region at the interface between the inner and outer domains of gp120 (27), and the so-called “bridging sheet,” which connects the two gp120 domains and contributes the largest surface of the coreceptor-binding site (27, 44). To compensate for the obligatory antigenic conservation of such gp120 regions, the virus has nevertheless evolved an extraordinary array of protective mechanisms that provide an effective shield from neutralizing antibodies (66). One of the most remarkable mechanisms is the cryptic conformation of the coreceptor-binding site, which is poorly accessible on the native virion surface but is either unmasked, stabilized, or de novo formed after CD4 binding (27, 44), a process that induces dramatic conformational changes in gp120 (34, 46, 55, 56, 57, 63, 65); this complex region encompasses most of the so-called CD4-induced (CD4i) epitopes (56, 65, 67). Another major protection device is the physical shield provided by the variable loops V1/V2 and V3. In native virions, such structures are positioned to cover an extended surface of gp120, which includes most of the coreceptor-binding site and part of the CD4-binding site, but they are displaced away following CD4 binding (3, 27, 34, 55, 65). The variable loops represent poor targets for broadly neutralizing antibodies, due to a high degree of tolerance to antigenic variation. Additional mechanisms of gp120 evasion from antibody-mediated control include oligomerization, as illustrated by the lack of correlation between antibody binding to monomeric gp120 and neutralization (15, 37, 47); conformational flexibility (26), which reduces the immune system ability to obtain a sharply focused image of the major neutralization epitopes; and an extraordinary degree of surface glycosylation (up to 50% of the entire gp120 molecular mass), which can disguise epitopes to appear as “self” to the immune system (27, 64). All the above protective mechanisms are fully operational in primary HIV-1 strains, which generally display a neutralization-resistant phenotype, but their effectiveness is progressively reduced after multiple in vitro passages, as is the case for X4 strains adapted to growth in continuous T-cell lines, which exhibit the highest sensitivity to antibody-mediated neutralization (2, 12, 17, 33, 57, 62, 68).

Several studies with primary HIV-1 strains have suggested that neutralization by defined monoclonal antibodies (MAbs), as well as by sera from HIV-infected patients, is independent of the viral coreceptor preference (6, 28, 36, 53), leading to the conclusion that the selective pressure exerted by specific host antibodies is not a major factor driving the phenotypic switch of the viral coreceptor preference (6, 28, 36, 53). Thus, the relationship between HIV-1 coreceptor phenotype and neutralization sensitivity has yet to be definitively elucidated.

In this study, we characterized a novel conserved neutralization epitope in the V3 domain of HIV-1 gp120. By analysis of a large panel of primary and laboratory HIV-1 isolates, we documented a consistent correlation between the exposure of such epitopes in the native Env oligomer and viral coreceptor usage phenotype: the epitope was constitutively exposed in CCR4-using strains, but it was invariably cryptic in X4 strains, although it became detectable upon CD4 binding, providing the first example of a CD4i epitope within the V3 loop. These results may help to elucidate the mechanisms underlying the coreceptor evolution of HIV-1 in vivo and may be relevant to current efforts to develop a vaccine capable of eliciting broadly neutralizing antibodies against HIV-1.

**MATERIALS AND METHODS**

**Monoclonal and polyclonal antibodies.** MAbs D19 was obtained by standard technology from a hybridoma derived by fusing mouse myeloma Sp20 with splenocytes of mice immunized with recombinant oligomeric preparations of uncleaved gp140 from a molecular clone (BH8) derived from the T-cell line-adapted X4 HIV-1 isolate IIB (16). The human anti-V3 loop MAb B4e8 (5) and the anti-gp120 MN goat antiserum (PB1 MN), directed against a long V3-containing linear peptide (PB1; amino acids 295 to 474), were obtained from the National Institutes of Health (NIH) AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases (NIAID), Rockville, MD. Human anti-V3 MAbs 268-D and 447-52D (9, 19) were a kind gift of S. Zolla-Pazner, New York, N.Y. The hyperimmune serum against the V3 

**Recombinant molecules and synthetic peptides.** A recombinant, soluble, truncated molecule encompassing the two outer domains (D1 and D2) of CD4 (sCD4); recombinant HIV-1 gp120 proteins from HIV-1 isolates BaL, IIB, SF162, MN and SF2, all produced in mammalian cells; and overlapping 15-mer synthetic peptides derived from gp120 of HIV-1 MN were obtained from the NIH AIDS Research and Reference Reagent Program. All the other synthetic peptides were produced by standard solid-phase protocols using 9-fluorenylmethoxycarbonyl chemistry and purified by reverse-phase high-performance liquid chromatography to >95% purity.

**Viruses and persistently infected cell lines.** HIV-1 isolates 91US714, 93BR0120, 93SU076, 92SU077, 92U2024, 93SU145, SF162, BaL, JR-FL, JR-CSF, IIB, SF2, and MN were obtained from the NIH AIDS Research and Reference Reagent Program. Pediatric primary isolates from northern Italy (IT224-1, IT224-18, IT306-9, IT306-0, IT306-3, IT145, IT140, IT136, and IT193-21) were derived in the laboratory of one of us (G.S.) by cocultivation of patient peripheral blood mononuclear cells (PBMC) with activated PBMC from healthy blood donors, as previously described (49). All the remaining primary HIV-1 isolates, with the exception of B117, which was kindly provided by Eva M. Fenyo (Lund University, Sweden), were obtained from the NIBSC-MRC AIDS Reagent Project (London, United Kingdom) within the framework of the World Health Organization-Joint United Nations Programme on HIV/AIDS Network for the Characterization of Globally Prevalent HIV-1 Strains in Relation to Vaccine Development. All HIV-1 isolates were expanded and titrated in activated human PBMC, as previously reported (49). Primary isolates were minimally passaged in vitro. For isolates CM235, CM243, 89.6, and BK132, the cloned Env genes were expressed in HeLa cells using recombinant vaccinia virus vectors.

The PM1 cell clone and its persistent infection with biologically diverse HIV-1 strains have previously been described (38). Chronically infected PM1 cell lines were obtained for all the aforementioned HIV-1 isolates, with the exception of isolate IIB and its molecular clone, which were chronically infected SupT1 cell lines were derived. To produce the chronically infected cell lines, the cells were exposed to the viral stocks at an approximate multiplicity of infection of 0.1; cultured in complete RPMI 1640 medium (Gibco) supplemented with 10%
heat-inactivated fetal bovine serum (HyClone), 2 mM t-glutamine, and antibiotics (Gibco); and monitored daily for cytopathic effects and for extracellular p24 antigen release by enzyme-linked immunosorbent assay (ELISA) with commercial antibodies (Aalto Bio Reagents, Dublin, Ireland). At the peak of the cytopathic effects (typically, day 7 to 10 postinfection), the cells were pelleted, washed once in prewarmed complete medium, and then cultured at low cellular density (5 × 10⁶ cells/ml) with daily replacement of half of the culture medium in the presence of conditioned supernatants (20% [vol/vol]) from uninfected PM1 (or SupT1). In most cases, this treatment resulted in the appearance within 10 to 14 days of small clusters of healthy-appearing cells that rapidly colonized the cultures. These outgrowing cell lines were shown to be chronically infected by their stable cell surface expression of surface viral Env over time and by their sustained release of extracellular p24 antigen.

Sequence analysis of the V3 loop. Sequence analysis of the V3 loop of isolates for which no sequence was available in public databases was performed as previously described (48). Briefly, DNA extracted from 2 × 10⁶ PBMC infected with each isolate was amplified by nested PCR (outer primers, JA9 and JA12; nested primers, JA53 and JA10). The amplified products were directly sequenced using the Thermosequenase sequencing kit (Amersham Pharmacia) according to the manufacturer’s indications, with both forward and reverse fluorescent-labeled primers in an automated laser fluorescent sequencing apparatus (ALF Red; Pharmacia LKB).

Fluorocytometry and MAb-binding competition assays. For fluorocytometric analysis, 5 × 10⁶ cells were incubated for 30 min at 4°C with 0.2 µg of the relevant MAb in 100 µl of phosphate-buffered saline (PBS) (Gibco); in some experiments, the cells were previously incubated with recombinant sCD4 (0.5 µg/ml). Cells treated with an irrelevant, isotype-matched MAb were used as negative controls. Affinity-purified, phycoerythrin-conjugated polyclonal goat anti-mouse or anti-human antibody (Sigma, St. Louis, MO) was used as a secondary reagent. Samples were analyzed with a FACSscan cytometer (Becton-Dickinson) acquiring at least 10,000 events for each sample.

ELISA assays. Flat-bottom, 96-well ELISA plates (Immunol; PBI International, Milan, Italy) were coated with synthetic peptides (0.5 µg/well) or with recombinant HIV-1 gp120 (0.05 µg/well) in PBS (50 µl/well) for 18 h at 4°C. Bovine serum albumin (1% [wt/vol]) was used for blocking the plates. Antibodies were added in 100 µl PBS and incubated for 1 h at room temperature, after which the plate was washed and incubated for an additional hour with the secondary antibody. Peroxidase-conjugated, affinity-purified goat anti-mouse immunoglobulin G (IgG), goat anti-human IgG, goat anti-rabbit IgG, or swine anti-goat IgG (all from Sigma) were used as secondary antibodies. After the plates were washed, the reaction was revealed with an appropriate substrate.

Acute HIV-1 infection assays. PBMC were isolated by Ficoll-Hypaque (Pharmacia) gradient centrifugation from concentrated leukocytes of healthy blood donors and stimulated with 10 µg/ml phytohemagglutinin (Sigma) in complete RPMI medium. Infection was performed by exposing cells (10⁶/well) to the viral stocks (50% tissue culture infective doses/well) in duplicate 96-well round-bottom microwell plates in 200 µl of complete RPMI medium supplemented with 20% (vol/vol) of recombinant interleukin-2 in the presence or absence of inhibitors. After incubation at 37°C for 16 h, the wells were washed twice and recultured in complete medium with or without the inhibitors. After 48 h, 75% of the culture supernatant was removed from each well for HIV-1 p24 antigen measurement and replaced by an equal volume of fresh medium containing interleukin-2 and the respective inhibitors. Virus replication was assayed by p24 antigen ELISA at days 3, 5, and 7 postinfection.

HIV-1 Env-mediated cell fusion assays. To assess HIV-1 Env-mediated fusion, effector cells expressing HIV-1 Env (infected with vaccinia recombinant v7T encoding bacteriophage T7 RNA polymerase) were mixed with target cells expressing the appropriate receptors (infected with vaccinia recombinant vCB21R containing the Echerichia coli lacZ reporter gene linked to the T7 promoter) in 96-well plates (10⁵ each cell type per well) and incubated at 37°C for 2 h. The cells were lysed with a nonionic detergent, and the extracellular portion of gp120 (14). For a more detailed epitope mapping, we screened by immunoprecipitation of the gp140 protein, thus allowing for the identification of MAbs recognizing conformational as well as linear epitopes. Preliminary epitope characterization indicated that MAb D19 reacts with the V3 loop region of gp120 (14). For a more detailed epitope mapping, we tested MAb D19 reactivity with a series of V3-derived synthetic peptides by ELISA; for comparison, three previously described V3-specific, isolate-unrestricted human MAbs, B4e8, 268-D, and 447-52D, were tested in parallel. As shown in Table 1, two anti-V3 hyperimmune rabbit sera used as positive controls (PB1MN and RoV3BaL) recognized all the V3-derived peptides tested. MAb D19 reacted strongly with a IIIB-derived 23-mer linear peptide (D19B4e8 268-D 447-52), which is also recognized by MAb RoV3BaL.

RESULTS

Characterization of the D19 epitope. MAb D19 was obtained by immunizing mice with a recombinant oligomeric purified preparation of HIV-1 gp140, derived from the BH8 molecular clone of IIIB, a T-cell line-adapted X4 isolate. gp140 is an uncleaved, truncated, soluble glycoprotein containing the entire extracellular region of HIV-1 Env, i.e., gp120 and the extracellular portion of gp41 (14). Hybridomas were screened by immunoprecipitation of the gp140 protein, thus allowing for the identification of MAbs recognizing conformational as well as linear epitopes. Preliminary epitope characterization indicated that MAb D19 reacts with the V3 loop region of gp120 (14). For a more detailed epitope mapping, we tested MAb D19 reactivity with a series of V3-derived synthetic peptides by ELISA; for comparison, three previously described V3-specific, isolate-unrestricted human MAbs, B4e8, 268-D, and 447-52D, were tested in parallel. As shown in Table 1, two anti-V3 hyperimmune rabbit sera used as positive controls (PB1MN and RoV3BaL) recognized all the V3-derived peptides tested. MAb D19 reacted strongly with a IIIB-derived 23-mer linear peptide (D19 7-29), centered around the GPGR motif at the tip of the V3 loop, as well as with peptides derived from two distinct HIV-1 isolates, a colinear peptide from HIV-1 MN (MN 6-28) and a cyclic, disulfide-bonded peptide encompassing the entire V3 loop sequence of HIV-1 BaL (BaL 1-36cyc), thus demonstrating that MAb D19 reactivity is not iso specific.

### TABLE 1. Peptide mapping analysis of V3-specific monoclonal and polyclonal antibodies

<table>
<thead>
<tr>
<th>V3 peptide</th>
<th>Amino acid sequence</th>
<th>Reactivity*</th>
</tr>
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<tbody>
<tr>
<td>IIIB 7–29</td>
<td>NTRKSI1RQPGRAFVTGK-1G</td>
<td>++ ++ NT ++</td>
</tr>
<tr>
<td>BaL 1–36cyc</td>
<td>CTRPNNYKKRHI--GPGRAFYTTIIDGIRQAHKC</td>
<td>++ ++ + + + ++</td>
</tr>
<tr>
<td>MN 6–28</td>
<td>YNKKRIHI--GPGRAFYTTKN-IIG</td>
<td>++ ++ + + + ++</td>
</tr>
<tr>
<td>MN 1–15</td>
<td>CTRPNNYKKRHI--G</td>
<td>-- -- -- ++ ++</td>
</tr>
<tr>
<td>MN 5–19</td>
<td>NYNKKRIHI--GPGRA</td>
<td>-- -- ++ ++ ++</td>
</tr>
<tr>
<td>MN 9–23</td>
<td>KRRIHI--GPGRAFYTT</td>
<td>++ ++ + + + +</td>
</tr>
<tr>
<td>MN 13–27</td>
<td>HI--GPGRAFYTTKN-II</td>
<td>-- -- -- ++ ++</td>
</tr>
<tr>
<td>MN 17–31</td>
<td>GRAFYTTKN-IIGTIRQAHC</td>
<td>-- -- -- ++ ++</td>
</tr>
<tr>
<td>MN 21–35</td>
<td>YTTKN-IIGTIRQAHC</td>
<td>-- -- ++ ++ + +</td>
</tr>
<tr>
<td>MN 9–18</td>
<td>RKRRIHI--GPGRA</td>
<td>-- -- ++ ++ + +</td>
</tr>
</tbody>
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* Reactivity was scored as follows: -, below the threshold of background signal; calculated as the mean ELISA OD values from replicate negative controls (no peptide bound) plus 3 times the standard deviation of the negative controls; +, OD of < 0.5; ++, OD of 0.5 to 1.0; ++++, OD of > 1.5; ++, OD of >1.5; NT, not tested.

All the MAbs were tested at a concentration of 2.5 µg/ml, the hyperimmune antisera were used at a final dilution of 1:40.
Next, we tested a series of overlapping 15-mer peptides spanning the entire V3 loop sequence of isolate HIV-1 MN. Strikingly, MAb D19 was reactive with only one such peptide (MN 9-23), which encompasses the conserved V3 loop tip motif (GPG), as well as flanking sequences on both sides, while no reactivity was observed with the two contiguous peptides, MN 5-19 and MN 13-27, containing only two amino acids on the C- and N-terminal sides, respectively, of the tip; likewise, no signal was detected against a 10-mer peptide (MN 9-18) nested within peptide MN 5-19. Thus, the integrity of the D19 epitope requires the presence of flanking sequences on both sides of the V3 loop tip.

A reactivity pattern similar to that of MAb D19 was seen with the human MAbs B4e8 (5) even though, in accordance with previous data using IIIb-derived recombinant gp120, MAbs B4e8 failed to recognize the IIIb-derived peptide MN 7-29. A distinct peptide recognition pattern was observed with MAbs 447-52D, which recognizes a wide range of HIV-1 strains of different genetic subtypes (9, 18, 19), and with 268-D, which is more restricted in its reactivity (19). Both MAbs reacted not only with the tip-centered peptide (MN 9-23), but also with the contiguous peptide on the N-terminal side (MN 5-19), as well as with the 10-mer peptide V3MN 9-18, suggesting that the presence of sequences on the C-terminal side of the loop is not essential for their epitope configuration. These data are consistent with the previously suggested minimal core epitope requirements for the two MAbs (GPxR for 447-52D; HIGPGR for 268-D) (19), as recently confirmed for 447-52D by both nuclear magnetic resonance (51) and crystal structural studies (54). Altogether, these observations suggest that the D19 epitope is distinct from those recognized by B4e8, 268-D, or 447-52D.

Monomeric HIV-1 gp120: D19-epitope accessibility is unrelated to coreceptor usage phenotype. To evaluate whether the D19 epitope is accessible on the correctly folded gp120 glycoprotein, we tested the ELISA reactivity of MAbs D19 with recombinant gp120 (predominantly monomeric) obtained from three distinct HIV-1 isolates, two R5 (BaL and SF162) and one X4 (IIIb). As illustrated in Fig. 1, MAb D19 was able to bind monomeric gp120 from all three isolates in a dose-dependent fashion. As a proof of specificity, preadsorption of gp120 with peptide BaL 1-36cyc at 100 μg/ml virtually abrogated the reactivity. With all the gp120 preparations tested, pretreatment with sCD4 induced a very limited increase in MAbs D19-binding activity. Similar results, i.e., binding to recombinant gp120 with limited increase upon sCD4 treatment, were obtained using other MAbs directed against the V3 loop (447-52D and 268-D) or against well-characterized CD4i epitopes (17b and 48d) (data not shown). These results indicate that the D19 epitope is readily accessible on monomeric gp120 molecules derived from different HIV-1 strains, regardless of their coreceptor specificities.

Native oligomeric HIV-1 Env: D19-epitope accessibility correlates with coreceptor usage phenotype. To evaluate the reactivity of MAbs D19 within the context of the native, oligomeric Env, we derived T-cell lines persistently infected with different HIV-1 isolates; the parental cell was a unique CD4+ T-cell clone, PM1, which expresses both CXCR4 and CCR5 and shows a broad sensitivity to infection by, as well as primary, laboratory-adapted HIV-1 isolates, irrespective of their coreceptor usage phenotype and genetic subtype (30). PM1 cells were infected with four viral isolates displaying different coreceptor usage phenotypes, i.e., IIIb (X4), 92US077 (R5X4), BaL (R5), and 91US714 (R5); since efforts to obtain PM1 cells stably infected with isolate IIIb were unsuccessful, the CD4+ T-cell line SupT1 was utilized for this strain. Stable populations of chronically infected cells were obtained after several in vitro passages. All the cell lines continued to release extracellular p24 antigen for prolonged periods (>3 months) in the absence of syncytium formation or other evident cytopathic effects; moreover, they stably expressed the native Env on the cell surface, as shown by immunostaining using a panel of specific MAbs and by testing their fusogenic activities in a vaccinia-based assay (data not shown). Of note, all persistently infected cell lines exhibited no detectable surface CD4 expression, thus ruling out the possible formation of cell surface-bound complexes between residual CD4 and released virions or shed soluble gp120, which is a potential confounding factor in the analysis of the native Env on infected cells (38). Absence of syncytium formation in the analysis of the native Env on infected cells (38). Absence of syncytium formation is a consequence of the absence of coreceptor usage phenotype and genetic subtype (30). PM1 cells were infected with four viral isolates displaying different coreceptor usage phenotypes, i.e., IIIb (X4), 92US077 (R5X4), BaL (R5), and 91US714 (R5); since efforts to obtain PM1 cells stably infected with isolate IIIb were unsuccessful, the CD4+ T-cell line SupT1 was utilized for this strain. Stable populations of chronically infected cells were obtained after several in vitro passages. All the cell lines continued to release extracellular p24 antigen for prolonged periods (>3 months) in the absence of syncytium formation or other evident cytopathic effects; moreover, they stably expressed the native Env on the cell surface, as shown by immunostaining using a panel of specific MAbs and by testing their fusogenic activities in a vaccinia-based assay (data not shown). Of note, all persistently infected cell lines exhibited no detectable surface CD4 expression, thus ruling out the possible formation of cell surface-bound complexes between residual CD4 and released virions or shed soluble gp120, which is a potential confounding factor in the analysis of the native Env on infected cells (38). Absence of syncytium formation is a consequence of the absence of syncytium formation is a consequence of
The results obtained with monomeric versus oligomeric gp120 derived from the same isolate (BaL) suggest that the cryptic nature of the D19 epitope in the native, cell surface-expressed Env conformation is due to oligomerization-dependent masking.

Induction of D19-epitope accessibility in native R5 Envs by CD4 binding. To evaluate whether the antibody accessibility of the D19 epitope was affected by the gp120 conformational changes associated with CD4 binding, fluorocytometric analysis was performed after treatment of Env-expressing cells with sCD4. Strikingly, in cells infected with the R5 isolates BaL or 91US714, the D19 epitope became accessible to antibody binding upon treatment with sCD4 (Fig. 2, bottom two panels), while its accessibility was only modestly enhanced in cells infected with CXCR4-using isolates (Fig. 2, top two panels). These findings suggest that in the native Env oligomer of R5 isolates, the D19 epitope is present in a cryptic conformation but is either unmasked, stabilized, or de novo formed upon CD4 binding, thereby fulfilling the definition of CD4i epitope. Indeed, a similar pattern of sCD4-mediated epitope inducibility in PM1 cells persistently infected with BaL or 91US714 was observed with two reference anti-CD4i human MAbs, 17b and 48d, directed against the “bridging-sheet” surface (not shown).

By contrast, other V3-specific reagents (human MAbs 447-52D, 268D, and B4e8 and hyperimmune serum RoV3) were able to bind to PM1 cells infected with HIV-1 BaL or HIV-1 91US714 regardless of sCD4 treatment (not shown), indicating that the lack of basal recognition by MAb D19 does not reflect a cryptic conformation of the entire V3 domain. In accordance with previous results (34, 53), however, all these antibodies showed increased binding after treatment with sCD4.

Reactivity of MAb D19 with a panel of native HIV-1 Envs: consistent correlation between epitope accessibility and coreceptor usage phenotype. To assess the breadth of reactivity of MAb D19 and the consistency of the correlation between D19 epitope accessibility in native oligomeric Env and coreceptor usage phenotype, we studied a large panel (n = 43) of cell surface-expressed Envs from both primary and laboratory-passage HIV-1 isolates (Table 2): 22 CCR5 specific (R5), 12 CXCR4 specific (X4), and 9 capable of using both coreceptors (R5X4). Twenty-nine of the Envs tested belonged to genetic subtype B (13 R5, 9 X4, and 7 R5X4), 5 to A (both R5), 2 to F (R5X4). Most of the Envs were analyzed using chronically infected PM1 cell lines; the only exceptions were Envs 89.6, BK132, CM235, and CM243, which were used, and Envs 89.6, BK132, CM235, and CM243, which were analyzed using recombinant vaccinia vectors. Cell fusion assays indicated that the Envs of isolates grown in chronically infected PM1 or SupT1 cells retained the same coreceptor usage phenotype as the original isolate from which they were derived; moreover, their reactivity with a panel of well-characterized human MAbs (e.g., 2G12, IgGl b12, 2F5, 17b, 48d, 447-52D, and 268-D) was unmodified (data not shown).

Analysis by fluorocytometry demonstrated the presence of the D19 epitope in 25 (58.1%) of the 43 Envs tested, with a similar prevalence among CCR5-specific (12/22; 54.5%) and CXCR4-using (13/21; 61.9%) strains (Table 2). The most striking observation emerging from the analysis of the D19-reactive
Envs was the strict correlation between epitope accessibility in the native, cell surface-expressed Env and coreceptor usage phenotype. Thus, for all 12 R5 Envs, the D19 epitope was invariably cryptic, although inducible upon CD4 binding; by contrast, for all 13 CXCR4-using Envs (7 X4 and 6 R5X4), the epitope was constitutively exposed independent of CD4 binding (Table 2).

The D19 epitope was highly conserved within genetic subtype B (23/29; 79%), which was the most represented subtype in our panel. Among the non-B strains tested, the overall frequency of MAb D19 reactivity was significantly lower (2/14; 14%), and variable between different subtypes: the two reactive Envs belonged to subtypes A and F, while no reactive Envs were found within subtypes C, D, and E (Table 2). Although the number on non-B strains analyzed was small, these data nevertheless indicate that MAb D19 reactivity is not restricted to genetic subtype B. Assessment of the relationship between conservation of the D19 epitope

\*a\* The amino-acid (aa) sequences of the V3 loop of all the isolates studied have been aligned; amino acid differences from the consensus sequences are in boldface type.

\*b\* Amino acid differences from the consensus sequence of D19-reactive isolates are in boldface and underlined.
and genetic subtype awaits the analysis of a larger panel of primary isolates.

Further definition of the D19 epitope by comparative analysis of V3 loop sequences from reactive and nonreactive HIV-1 isolates. In an attempt to refine our definition of the antigenic boundaries of the D19 epitope, we compared the V3 loop amino acid sequences from all the D19-reactive and -nonreactive HIV-1 isolates included in this study (Table 2). The V3 consensus sequences of D19-reactive (n = 25) and -nonreactive (n = 18) isolates differed at four positions (H13→R, R16→Q, T22→A, and E23→D); among such differences, however, only R18→Q clearly distinguished the two groups, being specifically associated with the lack of MAb D19 reactivity, whereas H13→R discriminated the two groups only within the R5 subset. Overall, we were unable to identify any other specific pattern that permitted the unambiguous identification of either group; nonetheless, some distinctive features were more commonly associated with the absence of the D19 epitope, irrespective of the viral coreceptor usage phenotype. Besides the Env s (n = 9) displaying a Q at position 18 (GPGQ or GQGQ motifs), several D19-nonreactive Env s showed conservative amino acid changes on the C-terminal side of the tip of the loop, including replacement of R18 by Y, replacement of A, or T23 by positively charged residues, replacement of Y23, by F, or deletion of T23; also, in some D19-unreactive isolates, there were 1- or 3-aa insertions N-terminal to the GPG motif or amino acid changes in the GPG tip motif itself (G15→V, P16→Q, and P16→L). The tip motif was instead 100% conserved among D19-reactive isolates. Other residues that were remarkably conserved among D19-reactive strains included the positively charged residues at positions 10 and 18 and the isoleucine at position 12. In conclusion, despite the lack of specific “signature” motifs consistently associated with the presence or absence of the D19 epitope, this analysis reinforced the concept that the lack of recognition of certain HIV-1 isolates by MAb D19 can be ascribed to genetic heterogeneity within the V3 domain, which is subjected to a strong selective pressure by the host humoral immune system.

Neutralization of primary HIV-1 strains by MAb D19: dependence on sCD4 for neutralization of CCR5-dependent but not CXCR4-using isolates. To evaluate the biological relevance of the reactivity pattern of MAB D19 in relation to the HIV-1 coreceptor usage phenotype, we investigated the ability of this MAB to neutralize HIV-1 infection and Env-mediated cell fusion. First, we tested neutralization of acute HIV-1 infection in primary human PBMC using four viral strains of different coreceptor usage phenotype: two extensively in vitro passaged isolates, IIIB (X4) and BaL (R5), and two primary isolates, 92US077 (R5X4) and 91US714 (R5). The latter were grown exclusively in primary PBMC for a limited number of in vitro passages. As shown in Fig. 3, left, MAB D19 effectively neutralized both of the CXCR4-using isolates, with mean 50% inhibitory doses (ID_{50}) of 0.16 and 0.38 μg/ml, respectively; by contrast, it had no effect against the two R5 isolates up to a concentration of 100 μg/ml. The neutralization tests were also performed in the presence of subinhibitory concentrations of sCD4 (10% inhibitory dose [ID_{10}]), which were predetermed for each isolate by PBMC infection assays. For the two R5 isolates, such doses were nonetheless sufficient to induce D19 epitope exposure in persistently infected PM1 cells (not shown). The efficacy of MAB D19 against IIIB and 92US077 was virtually unaffected by sCD4 at ID_{10} concentrations. Strikingly, however, the concomitant presence of sCD4 enabled MAB D19 to neutralize both BaL and 91US714, with ID_{50} values of 0.65 and 15.4 μg/ml, respectively. Thus, in agreement with its binding pattern against the corresponding Env oligomers expressed in chronically infected PM1 or SupT1 cells, MAB D19 was able to constitutively neutralize the CXCR4-using isolates, while neutralization of R5 isolates could only be achieved in the presence of sCD4.

To extend the analysis of MAB D19 neutralization of primary and laboratory-adapted HIV-1 isolates, we performed cell fusion assays, employing as effectors chronically infected PM1 or SupT1 cells expressing physiological levels of surface Env and cells expressing recombinant receptors as targets. Passage in PM1 had no evident effects on several primary HIV-1 strains with respect to their profiles of neutralization by a panel of well-characterized human MAbs (2G12, IgG1b12, 2F5, 17b, 48d, 1570, 447-52D, and 268D) or by a reference pool of human immune sera obtained from infected individuals (HIV-1g) (data not shown). Table 3 shows the neutralization sensitivities of different HIV-1 isolates in two types of cell fusion assay. In accordance with its binding pattern to the respective Env oligomers, MAB D19 was able to block all the CXCR4-using strains tested (one X4 and three R5X4) in the standard fusion assay (target cells expressing CD4 and coreceptor), albeit with different efficiencies; by contrast, it had no measurable effects on any of the R5 strains tested, at doses up to 100 μg/ml. In agreement with previous observations (6, 28, 36, 58), individual R5X4 viruses showed similar neutralization profiles irrespective of the corecep-
tor expressed on the target cells (CCR5 or CXCR4), supporting the concept that the sensitivity to virus-neutralizing antibodies is an inherent property of the viral Env and not of the composition of the cellular receptor complex.

To ascertain whether CD4 binding-induced exposure of the D19 epitope could render R5 isolates sensitive to neutralization by MAb D19, we also used a sCD4-activated assay in which target cells express the relevant coreceptor but not CD4; fusion is then induced by addition of sCD4, which binds to Env, triggering the gp120 conformational changes required for coreceptor binding. In this assay (Table 3), all the CXCR4-using Envs displayed sensitivity to MAb D19, generally at slightly lower doses than in the standard test. Most dramatically, in contrast with the results obtained in the standard assay, all the R5 Envs were highly sensitive to neutralization by MAb D19 in the sCD4-activated assay. Taken together, these data demonstrate a clear correlation between MAb D19 neutralization and binding to native Env: CCR5-restricted Envs consistently display a cryptic D19 epitope that becomes accessible only upon binding to CD4, while CXCR4-using Envs constitutively expose the epitope both in the absence and in the presence of sCD4.

Neutralization of R5 HIV-1 infection by other V3-specific antibodies. To directly compare the neutralization pattern of MAb D19 with that of other well-characterized antibodies to the V3 loop, we evaluated the neutralizing activity of MAbs B4e8 and 447-52D in the acute HIV-1 BaL infection assay in primary human PBMC. The prototype CD4i MAb 17b, which recognizes a complex “bridging-sheet” epitope, was used as a control. Of note, MAbs B4e8 and 447-52D were able to bind to the native cell surface-expressed HIV-1 BaL Env oligomer in the absence of sCD4, whereas MAb 17b was not; however, treatment with sCD4 induced de novo epitope accessibility for MAb 17b and increased the binding of MAbs B4e8 and 447-52D (data not shown). Figure 4 shows that the two anti-V3 MAbs were capable of neutralizing HIV-1 BaL in the absence of sCD4, with mean ID_{50} values of 0.75 and 0.22 μg/ml, respectively; addition of sCD4 at subneutralizing concentration (ID_{10}) did not significantly enhance their inhibitory activity, suggesting that even a limited epitope accessibility is sufficient for effective neutralization by such MAbs. By contrast, as documented with MAb D19, the CD4i MAb 17b was per se ineffective against HIV-1 BaL, but it was able to effectively neutralize viral replication when used in combination with sCD4. Altogether, these results further distinguish MAb D19 from the other V3-specific MAbs tested, whereas the biological similarity to MAb 17b reinforces the concept that the D19 epitope behaves as a bona fide CD4i epitope, albeit in an R5-restricted fashion.

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<th>Assay</th>
<th>Coreceptor expressed on target cells</th>
<th>HIV-1 Envs expressed on isolate effector cells</th>
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<td>CCR5</td>
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<td>sCD4 activated</td>
<td>CXCR4</td>
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* Complete designations of primary isolates: CW, 93USCW; 077, 92US077; 714, 91US714. All the Envs were expressed on the surface of chronically infected T-cell lines with the exception of 89.6, which was expressed in HeLa cells using a recombinant vaccinia virus vector. Values are ID_{50} values (μg/ml) with respect to antibody-untreated cells used as a control. —, not applicable (no cell fusion with the indicated coreceptor); NT, not tested.

**DISCUSSION**

The antigenic evolution of the HIV-1 Env in vivo is primarily driven by the selective pressure of the host adaptive immune responses, particularly neutralizing antibodies. Based on in vitro neutralization studies, however, several authors have concluded that neutralizing antibodies do not influence the evo-
olution of the HIV-1 coreceptor usage phenotype (28, 36, 58), even though the two major gp120 structures that dictate the use of different coreceptors, i.e., the V3 and V1/V2 loops, are highly variable, as is characteristic of epitopes that are subject to selective immune pressure. In addition, several clinical observations suggest the existence of an as-yet-undefined negative pressure that hinders selectively the emergence of CXCR4-using (i.e., X4 and R5X4) HIV-1 in individuals who are still immunocompetent: (i) the exceptionally low rate of established HIV-1 infection (de rigueur with CXCR4-using strains) in people homozygous for the CCR5-Δ32 allele (1), (ii) the very infrequent expansion of CXCR4-using strains during the asymptomatic phase of HIV-1 infection (10, 23, 25, 48, 49, 60), (iii) the often-transient replication of such strains even in patients with progressive disease (21-23, 50), (iv) the preferential suppression of CXCR4-specific strains after initiation of antiviral therapy (23, 43), (v) the lack of sustained increase of CXCR4-tropic SHIV replication after treatment with a CCR5-specific inhibitor in macaques dually infected with R5 and X4 viruses (61), and (vi) the selective clearance of CXCR4-using strains from an initially mixed viral population (R5 plus X4) documented in a few cases of acute primary infection (11, 29).

In the latter cases, the purging of CXCR4-using variants was temporally associated with antibody seroconversion and in one case specifically with the appearance of neutralizing antibodies against the autologous virus (11, 29).

Altogether, the above considerations provide a rationale for reevaluating the role played by neutralizing antibodies in the evolution of coreceptor usage phenotype in HIV-infected people. In this report, we provide the first conclusive evidence, to our knowledge, of a correlation between antibody-mediated HIV-1 neutralization and coreceptor usage phenotype, thus establishing a proof of principle that antibodies may indeed exert an influence on the coreceptor specificity of HIV-1. In fact, we identified a unique V3 loop epitope (D19) that is constitutively exposed by HIV-1 isolates that can use CXCR4 but is maintained in a cryptic conformation by CCR5-specific strains, becoming accessible only after CD4 binding. It is conceivable that the adoption of a more protected V3 loop conformation confers a selective advantage to R5 strains in an immunocompetent host environment, as occurs during the asymptomatic phase of the infection; given the crucial role of the V3 loop in the HIV-1 infectivity process, however, a partially cryptic conformation may be less favorable in terms of viral fitness. By contrast, a better exposed V3 loop on the one hand implies a reduced protection from antibody-mediated neutralization, but on the other hand may facilitate infectivity, as well as utilization of CXCR4, a homoeostatic chemokine receptor with a much wider in vivo distribution than CCR5 (20).

Thus, our results corroborate the hypothesis that a lowered antibody-mediated selective pressure may be one of the factors favoring the phenotypic switch from CCR5 to CXCR4 usage during the progression of HIV-1 disease.

The epitope recognized by MAb D19 is the first example, to our knowledge, of a bona fide CD4-induced epitope mapping to the V3 loop of HIV-1, although this definition applies selectively to strains that depend on CCR5 for entry. In fact, other known CD4i epitopes of gp120 map to the highly conserved “bridging-sheet” surface and do not apparently differentiate between CCR5-restricted and CXCR4-using strains (56, 65, 67). Within the V3 loop, the D19 epitope appears to be unique, as none of the V3-specific antibodies tested in our study or reported previously (5, 9, 15, 17-19, 28, 34, 37, 39, 45, 47, 52, 58, 59) shows an identical reactivity pattern. Specifically, none of the epitopes recognized by other MAbs displays the same dichotomous behavior as MAb D19 in CCR5- versus CXCR4-using HIV-1 isolates.

The structural basis of the cryptic nature of the D19 epitope in R5 isolates remains unknown, although it may be related either to an alternative conformation of the V3 region or to steric occlusion by other gp120 structures. Our results with monomeric gp120 imply that D19 epitope shielding is directly related to the oligomeric structure of the HIV-1 Env. It is well documented that several gp120 epitopes are markedly less exposed on the Env oligomer than on the monomer (15, 37, 42, 53), fingering oligomerization as one of the most efficacious strategies enacted by HIV-1 to protect critical neutralization targets on the external Env surface. Indeed, the reactivity of antibodies with monomeric gp120 is not predictive of their capacity to bind the Env spikes or, therefore, of their neutralizing capability (15, 33, 37, 38, 42, 47, 53, 59). Oligomerization-mediated epitope protection may be achieved, at least in part, through the extensive glycosylation of the exposed surface of gp120. Accessibility of the D19 epitope can be induced upon binding of native Env to CD4, which causes dramatic conformational changes in gp120, including displacement of the prominent V1/V2-loop structure, as well as stabilization and/or de novo formation of the “bridging sheet” that is the major coreceptor-binding interface of gp120 (27, 44).

Previous work with V1/V2 loop-deleted mutants has documented the critical role of this structure in concealing the coreceptor-binding region (3, 55, 65), which includes a portion of the V3 loop (34, 44, 57, 63, 64). Thus, we can speculate that in the Env oligomeric conformation the V3 loop in each protomer may be partially shrouded by the V1/V2 loop from a neighboring protomer or, alternatively, that within each protomer the trimeric structure forces the V1/V2 loop into a constrained position, resulting in an intrinsically more protected conformation of the entire coreceptor-binding region. The concept that the V3 loop is more accessible in monomeric gp120 was indirectly confirmed by mouse immunization studies using oligomeric gp140, which yielded markedly lower numbers of V3-directed MAbs than using monomeric gp120 (7% versus 50%, respectively, of the total) (14).

Studies are under way to evaluate the impact of V1/V2-loop truncation or of targeted deglycosylation of gp120 on the antibody accessibility of the D19 epitope.

Analysis of a large panel of primary HIV-1 isolates of different genetic subtypes showed that, despite its cryptic conformation in the native R5 Env, the D19 epitope is relatively well conserved, being present in more than half of the viral isolates tested, as previously documented for other V3 epitopes (2, 5, 9, 18, 19, 39). Of note, primary HIV-1 isolates recognized by MAb D19 were found within three different genetic subtypes (A, B, and F). The conservation of selected V3 loop epitopes, which is critical for the direct interaction of this domain with the highly conserved coreceptor molecules, most likely results from both secondary and tertiary structural elements. Evidence suggesting a critical role of the tridimensional structure in the antigenic makeup of the V3 loop was obtained in studies with escape mutants (13, 35, 40), as well as with natively folded
V3 chimeric proteins (24). By comparing the amino acid sequences of MAb D19-reactive and -nonreactive HIV-1 isolates, we were unable to identify specific motifs, besides the R→Q substitution at the tip of the loop, that were consistently associated with reactivity or nonreactivity, reinforcing the concept that the structural configuration of the loop, besides its primary sequence, is critical for D19 epitope integrity. Unfortunately, no high-resolution structural information of the V3 loop within the context of the native HIV-1 Env is currently available, as gp120 crystals could only be obtained thus far after truncation of this and other variable loops (27). However, recent nuclear magnetic resonance and crystal structural data on molecular complexes between V3-derived linear peptides and a fragment of the broadly reactive human MAb 447-52D have provided new insights into the structure of the V3 loop (51, 54). According to these models, the distal portion of the V3 loop adopts a β-hairpin conformation, consisting of two antiparallel β-strands (residues K101-T14 and A192-T33) linked by either an inverse γ-turn (51) or a type II β-turn (54) and corresponding to the highly conserved GPG motif at the tip of the loop. In the crystal structure, the peptide N-terminal β-strand has extensive main-chain interactions with the antibody CDR3 H3 loop, resulting in the formation of a three-stranded mixed β-sheet (54). Interestingly, the 447-52D epitope is partly conformation dependent, as shown by its higher binding affinity for a folded V3 fusion protein than for linear V3 peptides (19). Since our peptide mapping analysis showed that D19 recognizes the presence of sequences on both sides of the GPG motif for its integrity, it is plausible that MAb D19 recognizes the conserved β-hairpin structure of the V3 loop, but it is unable to bind to any purely linear sequences. This hypothesis is corroborated by the observed loss of MAb D19 reactivity with HIV-1 isolates displaying nonconservative substitutions in the C-terminal β-strand of the putative hairpin structure, which may result in the disruption of the strand conformation.

In conclusion, our data illustrate a novel mechanism of epitope protection that is differentially implemented by the two major biological variants of HIV-1. This novel paradigm may provide a physiological key for interpreting the viral phenotypic switch that occurs in a proportion of patients during the progression of the disease, as well as for elucidating the relative role played by R5- versus CXCR4-using isolates in the natural history of HIV-1 disease. Further studies will be important to verify this hypothesis in vivo, by correlating the emergence of CXCR4-tropic viral strains with a lowered humoral surveillance against this V3 loop epitope or other Env epitopes that may differentially accessible in R5- versus CXCR4-using HIV-1 strains. Regarding the efforts aimed at developing a protective vaccine against HIV-1, our data provide further ground to the recent rehabilitation of the V3 loop as a potential vaccine target, confirming the presence of broadly conserved neutralization epitopes in this region. The fact that the majority of subtype B isolates, but none of the subtype C or E isolates, was recognized by MAb D19 suggests that different serotypes may be defined based on epitopes present in this region, all of which should be adequately represented to generate a broadly effective vaccine. However, the cryptic nature of the D19 epitope in all R5 isolates, those accounting for the vast majority of HIV-1 transmissions worldwide, will reduce the potential value of at least this specific V3 epitope for the induction of protective neutralizing antibodies.

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