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Epitope mapping and characterization of a novel CD4-induced human monoclonal antibody capable of neutralizing primary HIV-1 strains

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Abstract

Human immunodeficiency virus (HIV-1) enters target cells by binding its gp120 exterior envelope glycoprotein to CD4 and one of the chemokine receptors, CCR5 or CXCR4. CD4-induced (CD4i) antibodies bind gp120 more efficiently after CD4 binding and block the interaction with the chemokine receptor. Examples of CD4i antibodies are limited, and the prototypes of the CD4i antibodies exhibit only weak neutralizing activity against primary, clinical HIV-1 isolates. Here we report the identification of a novel antibody, E51, that exhibits CD4-induced binding to gp120 and neutralizes primary HIV-1 more efficiently than the prototypic CD4i antibodies. The E51 antibody blocks the interaction of gp120–CD4 complexes with CCR5 and binds to a highly conserved, basic gp120 element composed of the β19-strand and surrounding structures. Thus, on primary HIV-1 isolates, this gp120 region, which has been previously implicated in chemokine receptor binding, is accessible to a subset of CD4i antibodies.

Keywords: Human immunodeficiency virus (HIV-1); Primary HIV-1 isolates; CD4-induced (CD4i) antibodies; Neutralizing antibodies; Epitope mapping; Human monoclonal antibody E51; Structured treatment interruption (STI); Soluble CD4 (sCD4); Envelope glycoprotein (gp120)

Introduction

The production of neutralizing antibodies is a major defense against viral infections (Burton, 2002; Iwasaki and Nozima, 1977; Lehner, 1989; Morris, 2002; Zinkernagel et al., 2001). Human immunodeficiency virus (HIV-1) establishes persistent infections and has evolved to minimize the elicitation and impact of neutralizing antibodies (Kwong et al., 1998, 2002; Parren et al., 1999; Wei et al., 2003; Wyatt and Sodroski, 1998). In most HIV-1-infected individuals, the majority of the antibodies elicited are nonneutralizing, even though they are directed against the viral envelope glycoproteins, the targets for antibodies with neutralizing capacity. Early-arising neutralizing antibodies typically exhibit strain specificity and thus HIV-1 can escape from these antibodies by altering elements in the envelope glycoproteins (Arendrup et al., 1992; Wei et al., 2003). Most HIV-1-infected individuals, after a period of months or years, elicit cross-reactive neutralizing antibodies. Particularly in subjects with preservation of immune system function, such as long-term nonprogressors, considerable titers of broadly neutralizing antibodies can be produced. Although primary, clinical isolates of HIV-1 are resistant to neutralization compared with tissue culture-passaged viruses (Burton et al., 1994; Moore et al., 1995; Parren et al., 1998a, 1998b, Sullivan et al., 1995, 1998; Zwick et al., 2001), the more potent subset of broadly neutralizing antibodies can inhibit infection of tissue-cultured cells by primary isolates.

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The in vivo role of antibodies in modulating the level of HIV-1 infection or disease progression is uncertain, but several reports suggest that passive administration of HIV-1-neutralizing antibodies can protect against infection or mitigate the pathologic outcome of infection (Ferrantelli and Ruprecht, 2002; Gauduin et al., 1997; Hinkula et al., 1994; Jackson et al., 1988; Jacobson, 1998; Jacobson et al., 1993; Katinger, 1994; Laal et al., 1994; Mascola et al., 1997; Morand-Joubert et al., 1997). A major goal in the quest for an HIV-1 vaccine is the identification of approaches that efficiently elicit antibodies with broad and potent neutralizing capacity.

Neutralizing antibodies recognize the HIV-1 envelope glycoproteins, which consist of the gp120 exterior glycoprotein and the gp41 transmembrane glycoprotein. Most neutralizing antibodies recognize the gp120 glycoprotein, which has surface-exposed variable loops (Javaherian et al., 1990; Spear et al., 1994) and carbohydrates that are thought to facilitate resistance to the binding of inhibitory antibodies (Reitter et al., 1998; Wei et al., 2003). The gp120 glycoprotein binds the target cell receptors, CD4, and the chemokine receptors, CCR5 or CXCR4 (Alkhatib et al., 1996; Choe et al., 1996; Deng et al., 1996; Dragic et al., 1996; Kwong et al., 1998; Oberlin et al., 1996; Rizzuto et al., 1998; Sattentau et al., 1986; Trkola et al., 1996; Wu et al., 1996). The receptor-binding regions of gp120 are potentially vulnerable to neutralization, as they must be conserved among HIV-1 strains and must be exposed at some point during the process of virus entry. Two groups of neutralizing antibodies directed against conserved gp120 elements are the CD4-binding site (CD4BS) antibodies and the CD4-induced (CD4i) antibodies (Burton, 2002; Burton and Montefiori, 1997; Moore and Burton, 1999; Wyatt et al., 1998). CD4BS antibodies can block gp120 binding to CD4 and to the chemokine receptors (Raja et al., 2003) and vary in neutralizing potency from weak to very strong (Ho et al., 1991; McInerney et al., 1997; Posner et al., 1991, 1992, 1993; Burton et al., 1994; Saphire et al., 2001; Xiang et al., 2002b). CD4i antibodies block CCR5 or CXCR4 binding by gp120–CD4 complexes (Babcock et al., 2001; Trkola et al., 1996; Wu et al., 1996). Only a handful of CD4i antibodies, all of which are derived from HIV-1-infected humans, have been reported (Sullivan et al., 1998; Thali et al., 1993; Xiang et al., 2002a). The prototypic CD4i antibodies, of which 17b and 48d represent two examples, exhibit weak or modest neutralizing potency against primary HIV-1 isolates (Sullivan et al., 1998; Xiang et al., 2002a). The variable loops and the conformational flexibility of gp120 contribute to the resistance of primary HIV-1 isolates to these CD4i antibodies (Sullivan et al., 1998; Cao et al., 1997; Kwong et al., 2002). Moreover, CD4i antibodies encounter steric restrictions to binding gp120 after cell-surface CD4 has been engaged by the virus (A.F. Labrijn et al., submitted for publication). Thus, single-chain sFv or Fab fragments of CD4i antibodies exhibit greater neutralizing potency than complete immunoglobulin molecules (A.F. Labrijn et al., submitted for publication). Thus, although the gp120 epitope for CD4i antibodies is critical for CCR5 and CXCR4 binding and is highly conserved (Xiang et al., 2002a), the ability of antibodies to target this region efficiently is still uncertain.

The low number of CD4i antibodies identified to date suggested that their elicitation may not be efficient during most cases of natural HIV-1 infection. This has prompted a search for these antibodies in individuals that have been exposed to HIV-1 but who retain relatively intact immune systems. One such group consists of individuals whose HIV-1 infection was recognized very early and who received highly active antiretroviral therapy (HAART). This early intervention has been shown to preserve HIV-1-specific helper T cells and may predispose such individuals to control virus infection (Allen et al., 2002; Altfeld et al., 2002; Rosenberg et al., 1999, 2000). Indeed, upon structured treatment interruptions (STI) (Allen et al., 2002), some of these individuals appeared to dampen the resurgence of viremia in a progressive fashion, suggesting the development of more effective immunological control following intermittent exposure to viral antigens (Rosenberg et al., 2000). One individual who was treated early after infection and experienced several STIs was intensively studied with respect to the generation of antibodies directed against the HIV-1 envelope glycoproteins (Montefiori et al., 2003). This individual was found to have a high frequency of CD4i antibodies among the Env-reactive antibodies (J. Robinson and E.S. Rosenberg, unpublished observations). Several of these CD4i antibodies are novel with respect to their heavy-chain CDR3 primary sequences, which resemble that of the CCR5 N-terminus and are unusually long (Choe et al., 2003). Moreover, it has been recently shown that the heavy-chain CDR3 sequences of some of these antibodies are posttranslationally modified by tyrosine sulfation (Choe et al., 2003), as is the CCR5 N-terminus (Farzan et al., 1999). Thus, antibodies that mimic the CCR5 chemokine receptor are elicited in some HIV-1-infected individuals. Here we study the envelope glycoprotein binding and virus neutralization properties of one of these novel, sulfated CD4i antibodies, E51.

Results

E51 is a CD4-induced antibody

The properties of the E51 antibody were compared with those of the 17b antibody, which exhibits binding and neutralization activity typical of those of currently characterized CD4i antibodies (Xiang et al., 2002a). To test whether binding of the E51 antibody to HIV-1 gp120 is influenced by CD4 binding, wild-type soluble gp120 derived from the YU2 strain was captured on an ELISA plate by the D7324 antibody directed against the gp120 carboxyl-terminus (Moore et al., 1994). The binding of the prototypic CD4i

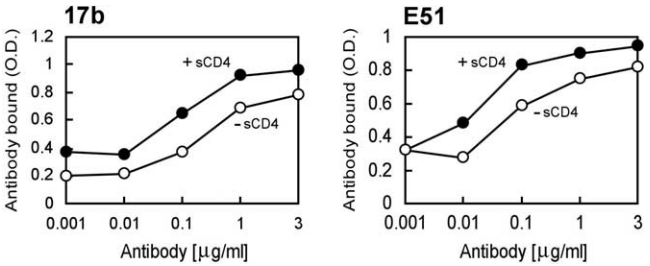

Fig. 1. CD4 induction of the binding of the E51 antibody to HIV-1 gp120. The YU2 HIV-1 gp120 glycoprotein was captured on an ELISA plate using the D7324 antibody, which is directed against the gp120 carboxyl-terminus. The binding of the 17b and E51 antibodies to the captured gp120 glycoprotein was studied in the absence (open circles) or presence (filled circles) of sCD4 (8 μg/ml).

antibody 17b and the E51 antibody was studied in the absence or presence of sCD4. The binding of both antibodies was increased in the presence of sCD4 (Fig. 1). Thus, E51 is a CD4i antibody.

E51 blocks gp120 binding to CCR5

CD4i antibodies are able to block the binding of gp120–sCD4 complexes to the CCR5 or CXCR4 chemokine receptors (Babcock et al., 2001; Trkola et al., 1996; Wu et al., 1996). Radiolabeled gp120 from the R5 YU2 HIV-1 isolate was incubated in the presence or absence of sCD4 with CCR5-expressing cells (Cf2Th-CCR5 cells). After washing, the bound gp120 was precipitated by a mixture of sera from HIV-1-infected individuals and analyzed by SDS–PAGE (Fig. 2). Inclusion of the E51 or 17b antibodies in the assay resulted in complete inhibition of gp120–sCD4 binding to CCR5-expressing cells. By contrast, the C11 antibody, which recognizes a gp120 epitope distant from the chemokine receptor-binding site (Moore et al., 1994), did not exhibit inhibitory activity in this assay. Thus, E51, similar to other CD4i antibodies, can block gp120 interaction with the CCR5 chemokine receptor.

Mapping the E51 epitope on gp120

To gain insight into the gp120 epitope recognized by the E51 antibody, the ability of the antibody to precipitate a panel of mutant gp120 derivatives was examined. The mutants consist of variants of the wtΔ protein derived from the YU2 HIV-1 strain (Rizzuto and Sodroski, 2000; Rizzuto et al., 1998). The wtΔ protein corresponds to a gp120 glycoprotein missing the N-terminus and V1/V2 variable loops. The use of this protein minimizes the potential effects of V1/V2 loop movement on epitope exposure and allows the results to be compared with a large preexisting body of data on mutant binding to CD4, CCR5, and monoclonal antibodies. Table 1 shows the binding of the E51 antibody to the panel of mutant envelope glycoproteins, relative to that observed for the wtΔ glycoprotein. The results are compared with previously generated data on the relative binding of the mutants to sCD4, CCR5, a CD4BS antibody (F105), and a CD4i antibody (17b) (Rizzuto and Sodroski, 2000; Rizzuto et al., 1998). The binding of the antibodies to the mutant glycoproteins was initially assessed in the absence of sCD4. Under these conditions, changes in several gp120 regions resulted in decreases in E51 binding. These regions include the bridging sheet and underlying substructures as well as the adjacent V3 variable loop. Most of the mutants that were not recognized efficiently by the E51 antibody are not globally misfolded proteins, as they were recognized by at least one conformation-dependent ligand. One exception is the 383 F/S mutant, which is poorly recognized by a number of diverse conformation-dependent antibodies and CD4. The location of the gp120 residues in which changes resulted in decreases in E51 binding in the absence of sCD4 is shown in Fig. 3, right panel (green and red residues). The gp120 surface implicated by these data overlaps that involved in CCR5 binding (Fig. 3, left panel).

When the relative binding of the E51 antibody to a mutant glycoprotein was decreased in the absence of sCD4, the antibody recognition of the mutant in the presence of sCD4 was assessed (Fig. 4). E51 recognition was restored in many cases by sCD4 binding to the mutant wtΔ glycoprotein. This indicates that the involved gp120 residue is not absolutely required for E51 binding. Changes, including complete deletion, of the gp120 V3 loop fall into this category (see ΔV3 in Fig. 4). On the other hand, sCD4 binding did not restore E51 recognition of the mutants that involve β19-strand residues lysine 421 and glutamine 422. As these mutants retain the ability to bind sCD4 (Table 1), these results suggest the critical importance of these residues in E51 recognition. Even in the presence of sCD4, E51 precipitation of the 420 I/R and 423 I/S mutants, which contain alterations in the β19 strand, was lower than that of the wtΔ protein. These results suggest that the E51 antibody mainly recognizes highly conserved β19-strand residues 420–423, which have been previously implicated in CCR5 binding (Rizzuto and Sodroski, 2000; Rizzuto et al., 1998).

Mutual induction of E51 and CD4 binding

Previous studies have suggested that CD4, the chemokine receptors, and CD4i antibodies all recognize a similar

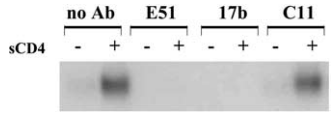

Fig. 2. Inhibition of gp120 binding to CCR5 by the E51 antibody. Radiolabeled YU2 HIV-1 gp120 glycoprotein was incubated, in either the absence (−) or the presence (+) of sCD4 (8 μg/ml), with Cf2Th-CCR5 cells. The incubation was performed in the absence (no Ab) or presence of the E51, 17b, or C11 antibodies. The bound gp120 glycoprotein was detected by lysis of the Cf2Th-CCR5 cells and precipitation with sera that was pooled from HIV-1-infected individuals.

Table 1
Recognition of HIV-1 gp120 Mutants by Ligands[a]

gp120 region	Envelope protein	sCD4	F105	CCR5	17b	E51
	YU2 wtΔ	1.00	1.00	1.00	1.00	1.00
C1	107 D/R	1.02	1.14	1.02	0.97	1.24
	114 Q/L	0.79	0.75	1.22	0.73	1.22
	117 K/D	0.74	0.83	0.15	0.64	0.07
	121 K/D	0.73	0.99	0.07	0.00	0.03
	122 L/S	0.84	1.11	0.98	1.07	1.03
	123 T/D	0.99	1.25	0.08	1.06	0.97
C2	197 N/D	1.34	1.11	1.33	0.80	1.52
	199 S/L	1.32	1.04	1.50	0.94	1.15
	200 V/S	0.91	1.06	0.84	1.05	0.97
	201 I/A	0.90	0.81	0.46	0.67	1.77
	203 Q/L	0.85	0.93	0.68	0.88	0.07
	207 K/D	0.85	0.98	0.00	0.10	0.08
	209 S/L	1.11	1.00	1.00	0.85	1.13
	210 F/S	0.81	0.74	0.65	0.81	1.06
	211 E/K	1.13	1.24	0.73	1.03	1.26
	257 T/D	0.00	0.00	0.05	0.80	0.12
V3	295 N/E	0.75	0.79	0.86	0.73	1.05
	298 R/G	1.03	1.14	0.10	0.15	0.02
	308 N/D	1.10	1.03	0.31	0.89	1.11
	311 L/S	1.12	1.03	0.08	1.05	1.48
	330 H/A	0.75	0.64	0.22	0.55	1.42
	Δ298–329 (ΔV3)	0.80	0.93	0.00	0.00	0.00
C3	370 E/Q	0.00	0.00	0.17	1.04	1.29
	372 V/S	1.03	0.44	0.85	1.08	1.35
	373 T/D	1.12	1.10	0.48	1.10	0.76
	375 S/W	1.74	0.00	0.59	0.90	0.55
	377 N/E	0.71	0.60	0.22	0.52	0.85
	381 E/R	0.81	0.96	0.07	0.20	0.01
	383 F/S	0.00	0.00	0.04	0.02	0.04
	386 N/D	1.14	0.97	1.22	0.97	0.63
C4	419 R/D	0.86	0.82	0.19	0.00	0.03
	420 I/R	0.59	0.72	0.06	0.00	0.03
	421 K/D	0.86	0.00	0.07	0.00	0.03
	422 Q/L	0.53	0.55	0.07	0.00	0.05
	423 I/S	0.97	1.03	0.61	0.00	0.07
	424 I/S	0.25	0.81	0.37	0.48	0.52
	426 M/A	0.69	1.11	0.75	0.69	0.83
	429 E/R	1.17	0.82	1.54	1.00	0.88
	432 K/A	1.00	1.45	0.06	0.92	0.24
	434 M/A	0.90	1.04	1.22	0.65	0.12
	435 Y/S	0.33	1.00	0.21	0.00	0.09
	436 A/S	1.05	1.23	0.98	0.91	0.36
	437 P/A	0.80	0.82	1.79	0.68	0.52
	438 P/A	1.18	1.18	0.06	1.00	0.23
	439 I/A	0.68	0.84	0.45	0.76	0.69
	440 R/D	1.03	1.13	0.09	1.05	0.59
	441 G/V	0.67	0.78	0.00	0.70	0.47
	442 Q/L	1.11	0.83	2.00	0.74	0.63
	444 R/D	0.79	0.74	0.25	0.67	0.68
C5	474D/R	0.59	0.00	1.03	0.81	0.49

[a] The residue number of the mutants is based on the sequence of the prototype strain HXBc2 (Korber et al., 1998). The value for ligand binding was determined by immunoprecipitation of radiolabeled protein with a mixture of sera from HIV-1-infected individuals and with specific antibodies/ligands, and quantified by a PhosphorImager. The values were normalized by the following formula: $(\text{mutant protein/wt protein})_{\text{ligand}} \times (\text{wt protein/mutant protein})_{\text{serum mixture}}$. Values for sCD4, F105, CCR5, and 17b binding are from Rizzuto et al. (1998). E51 binding was increased by incubation with sCD4 for all of the mutants except those contained in the box.

conformation of the structurally flexible gp120 glycoprotein (Kwong et al., 1998; Xiang et al., 2002a, 2002b). In one of these studies (Xiang et al., 2002b), some mutant wtΔ glycoproteins bound poorly to either CD4 or a CD4i antibody, but were recognized more efficiently in the presence of both CD4 and CD4i antibody. To determine whether this applied

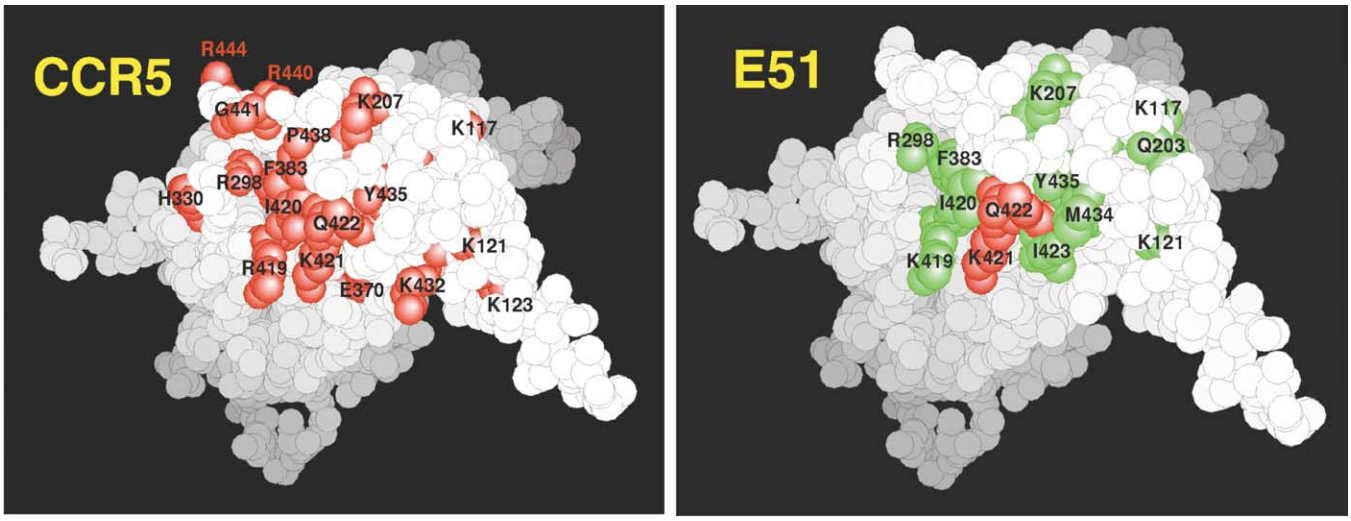

Fig. 3. Mapping the E51 epitope on the gp120 surface. The HIV-1 gp120 glycoprotein core in the CD4-bound state (Kwong et al:, 1998) is shown from the perspective of the target cell. In the left panel, the residues colored red are those in which changes resulted in greater than 75% reduction in CCR5 binding in the presence of sCD4 (Rizzuto and Sodroski, 2000; Rizzuto et al., 1998). In the right panel, residues in which changes result in greater than 80% reduction in E51 precipitation in the absence of sCD4 are colored. E51 precipitation was completely or partially restored in the presence of sCD4 for those wtΔ mutants in which the altered residue is colored green. E51 precipitation was undetectable, even in the presence of sCD4, for those wtΔ mutants in which the altered residue is colored red.

to the E51 antibody, three mutant wtΔ glycoproteins previously shown to exhibit mutual induction of CD4 and CD4i antibody binding (Xiang et al., 2002a) were incubated with a CD4-immunoglobulin fusion (CD4-lg) protein, the E51 antibody, or both sCD4 and E51. As a control, the wtΔ and mutant glycoproteins were precipitated by a mixture of sera from HIV-1-infected individuals. Fig. 5 shows that the 257 T/D mutant, which alters the hydrophobic internal cavity (the "Phe 43 cavity") in the CD4-bound conformation of gp120 (Kwong et al., 1998), was not recognized well by either CD4-lg or E51. However, when both ligands were present, precipitation of the 257 T/D mutant was efficient. A similar but less dramatic effect was observed with the 383 F/S and 435 Y/S mutants. Because of the minimal surface

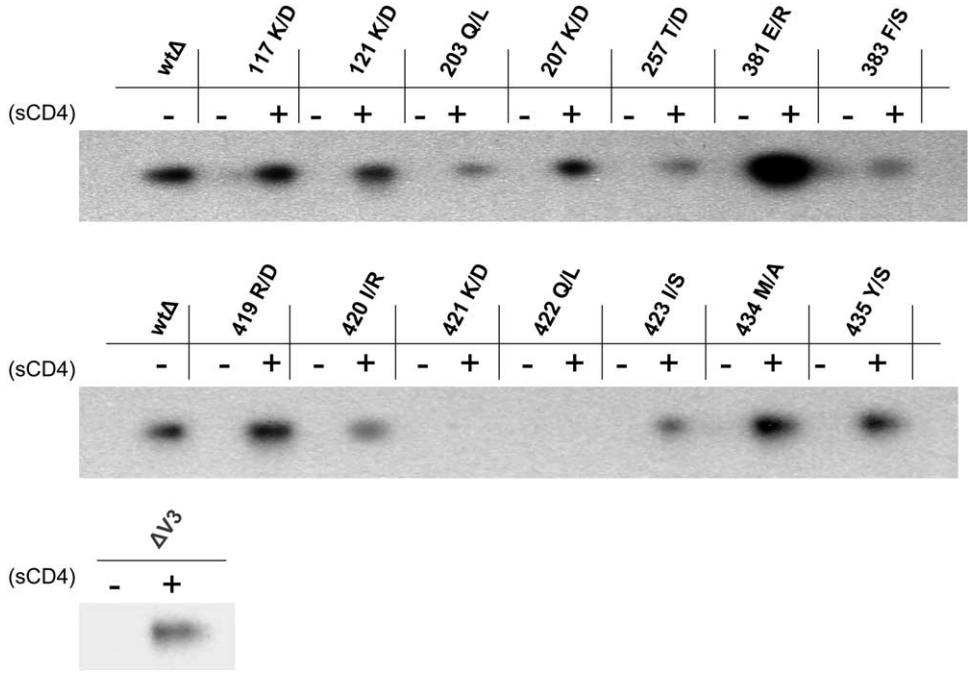

Fig. 4. Precipitation of HIV-1 gp120 mutants by the E51 antibody. Radiolabeled wtΔ or mutant derivatives were incubated with the E51 antibody in the absence (−) or presence (+) of sCD4 (8 μg/ml). The precipitated proteins are shown.

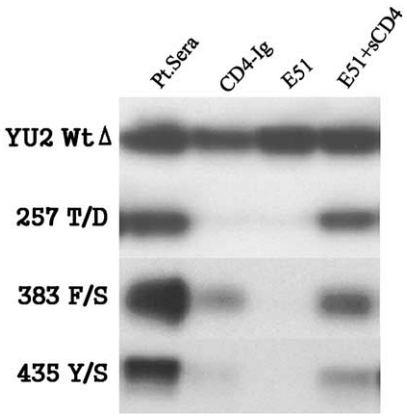

Fig. 5. Mutual induction of gp120 binding by sCD4 and the E51 antibody. Precipitation of the YU2 HIV-1 wtΔ glycoprotein and mutant derivatives by soluble forms of CD4 and the E51 antibody, alone or in combination, is shown. The wtΔ and mutant glycoproteins were also precipitated by a mixture of sera from HIV-1-infected individuals (Pt. Sera).

exposure of threonine 257, phenylalanine 383, and tyrosine 435 in the CD4-bound conformation of gp120 (Kwong et al., 1998), the effects of the introduced changes in these residues on the binding of CD4 and E51 probably occur indirectly, through alterations in gp120 conformation. Thus, achieving a conformation mutually recognized by both E51 and soluble forms of CD4 is optimal when both ligands are present. These results support a model in which CD4 and the E51 antibody recognize a similar gp120 conformation.

The neutralizing activity of the E51 antibody

The neutralizing activity of the E51 antibody was compared with that of the prototypic CD4i antibody 17b. The neutralizing activity of the 17b antibody is typical of that of previously described CD4i antibodies (Xiang et al., 2002a). The neutralization assays were performed according to two protocols. In the first protocol, viruses and antibodies were preincubated for 1 h at 37°C before addition of the target cells (Fig. 6A). The 17b and E51 antibodies both neutralized the viruses with the envelope glycoproteins of the clade B laboratory-adapted HXBc2 strain of HIV-1 (Fig. 6A). Both antibodies also demonstrated some inhibition of infection by viruses with the envelope glycoproteins of the primary ADA isolate, also from clade B. Although infection by the ADA virus could be inhibited by 50% at modest concentrations of the antibodies, 90% neutralization was not achieved even at 50 μg/ml of antibody. Viruses with the envelope glycoproteins of two other primary clade B HIV-1 isolates (89.6 and JR-FL) were neutralized by the E51 antibody but not by the 17b antibody. We also examined the ability of the 17b and E51 antibodies to neutralize viruses with envelope glycoproteins derived from the globally prevalent phylogenetic clade C (MCGP1.3 and SA32 in Fig. 6A). Both 17b and E51 inhibited infection by these viruses, with E51 exhibiting greater potency against the SA32 virus. These results indicate that, for numerous HIV-1 isolates, E51 exhibits neutralizing activity that is as good as or better than that of 17b.

It has been previously demonstrated that HIV-1 neutralization by CD4i antibodies is less efficient after the virus has attached to the cell surface, due to steric limitations on antibody binding to an envelope glycoprotein–CD4 complex (A.F. Labrijn et al., submitted for publication). To examine whether the E51 antibody could neutralize HIV-1 under such circumstances, recombinant HIV-1 with the HXBc2, 89.6, or ADA envelope glycoproteins was incubated with the appropriate target cells at 4°C to allow virus attachment but not entry. The virus–cell mixtures were then washed and incubated in the absence or presence of either 17b or E51 antibody at 37°C. Under these conditions, the E51 antibody was as effective as the 17b antibody in neutralizing viruses with the HXBc2 and ADA envelope glycoproteins (Fig. 6B). The E51 antibody was slightly more effective than the 17b antibody in neutralizing viruses with the 89.6 envelope glycoproteins under these assay conditions. Neutralization of the viruses with the HXBc2 and 89.6 envelope glycoproteins by the E51 antibody was less efficient in this assay format, compared with the standard neutralization assay (compare Figs. 6A and B). Thus, the neutralizing efficiency of the E51 antibody was influenced by the prior attachment of the virus to the target cell.

Steric restrictions on neutralization by the CD4i antibodies can be at least partially bypassed by single-chain sFv or Fab fragments of these antibodies (A.F. Labrijn et al., submitted for publication). We examined the ability of the 17b and E51 antibodies, or Fab fragments of these antibodies, to inhibit infection of viruses with envelope glycoproteins derived from the very neutralization-resistant YU2 HIV-1 isolate. The 17b and E51 antibodies demonstrated little neutralization of the YU2 virus, at concentrations up to 50 μg/ml (Fig. 6C). The 17b and E51 Fab fragments exhibited greater neutralization of the YU2 virus, compared with the complete antibodies. This suggests that the E51 antibody is also subject to steric restrictions that decrease its potential neutralizing activity against primary HIV-1 isolates.

Discussion

Heretofore, the CD4i antibodies have been identified only rarely in HIV-1-infected individuals, suggesting that they may not be efficiently elicited by the viral envelope glycoproteins (Thali et al., 1993). Moreover, the neutralizing activity of the prototypic CD4i antibodies against primary HIV-1 isolates is weak or modest (Xiang et al., 2002a). Several factors, including the conformational flexibility and variable loop masking of gp120, and steric constraints on antibody binding, are thought to contribute to the poor efficacy and inefficient elicitation of CD4i antibodies. Thus, despite the conservation of this gp120 epitope, which reflects its involvement in chemokine receptor binding,

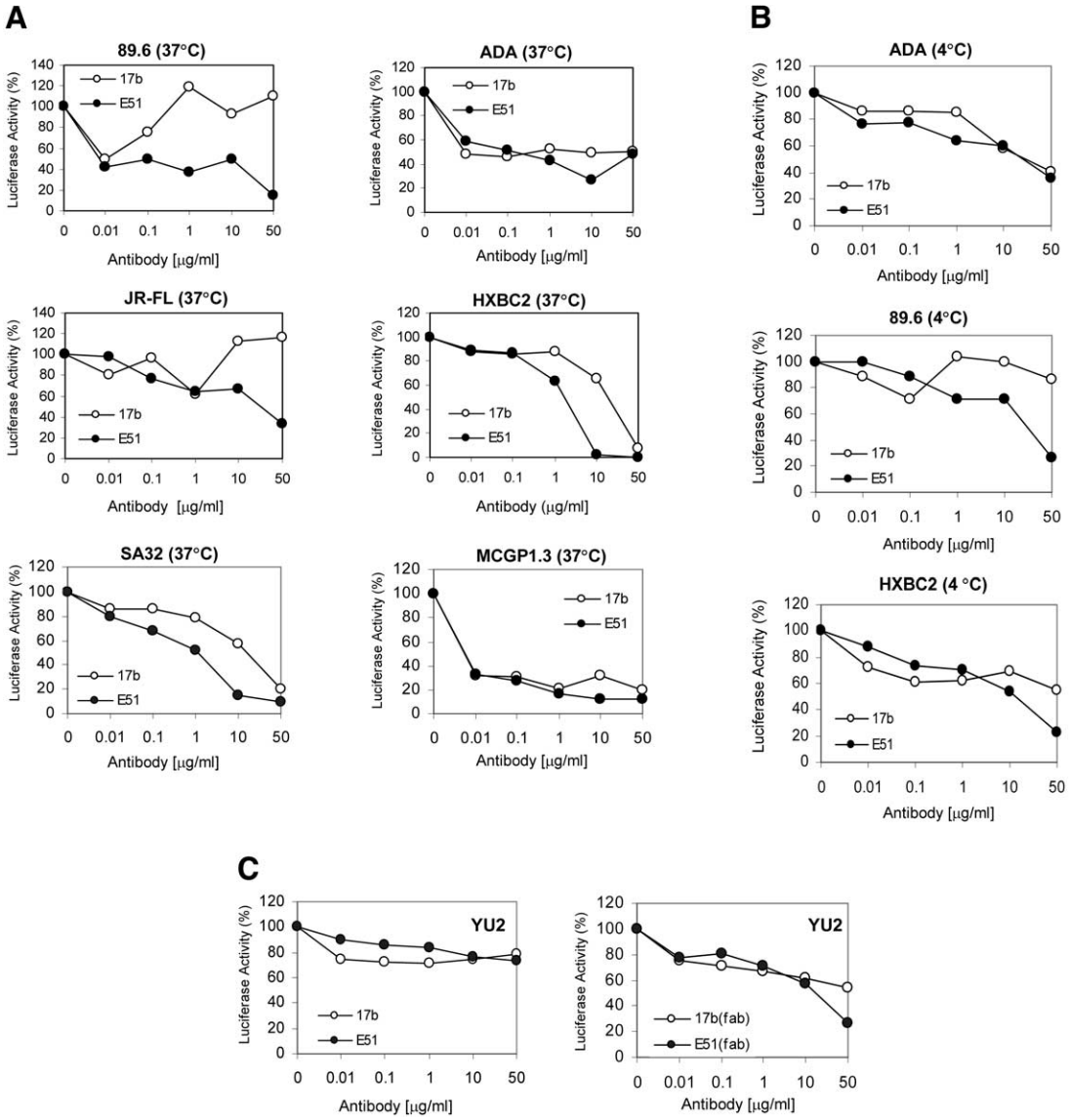

Fig. 6. Neutralization of HIV-1 by CD4i antibodies. Recombinant HIV-1 viruses expressing luciferase and containing *env* deletions were pseudotyped with HIV-1 envelope glycoproteins from the HXBc2, ADA, JR-FL, 89.6, MCGP1.3, SA32, or YU2 isolates. Neutralization by the 17b antibody (○) or E51 antibody (●) was examined. All assays were conducted in triplicate, and the results shown are typical of those obtained in three independent experiments. (A) The viruses were incubated with the indicated concentration of antibody for 1 h at 37°C prior to exposure to the appropriate target cells. Luciferase activity in the target cells was measured as described under Materials and methods and is reported as the percentage of activity relative to that observed in the absence of antibody. (B) The recombinant viruses were incubated with the target cells at 4°C for 2 h, after which antibodies were added at the indicated concentrations and the temperature was raised to 37°C. After 48 h, luciferase activity in the target cells was measured. (C) Recombinant viruses with YU2 HIV-1 envelope glycoproteins were incubated with the indicated concentrations of 17b (open circles) or E51 (filled circles) antibodies (left) or Fab fragments (right) for 1 h at 37°C. The viruses were then added to target cells and luciferase activity was measured as described under Materials and methods.

there has been considerable uncertainty about the feasibility of targeting this region with antibodies. Here, we show that primary HIV-1 isolates can be neutralized by a CD4i antibody, E51, derived from an HIV-1-infected individual undergoing STI (Rosenberg et al., 2000). This setting might be ideal for the generation of antibodies optimized for binding the HIV-1 envelope glycoproteins, as antiviral helper T cell responses are preserved by early intervention and exposure to viral antigens is considerable. Indeed, a high percentage of HIV-1 envelope glycoprotein-specific antibodies generated in some STI patients are CD4i antibodies (J. Robinson and E.S. Rosenberg, unpublished observations). Many of these resemble E51 with respect to the possession of long heavy-chain CDR3 loops that contain acidic and sulfated tyrosine residues (Choe et al., 2003). The resemblance of these CDR3 sequences to that of the CCR5 amino-terminus suggests that these antibodies act as soluble mimics of the chemokine receptor.

Several of the features of the E51 antibody observed in this study support its resemblance to the chemokine recep-

tor. First, E51 binds gp120–CD4 complexes and competes for CCR5 binding. Second, both E51 and the chemokine receptors preferentially bind the CD4-bound conformation of gp120. Third, a β19-strand structure on the gp120 glycoprotein is important for binding E51, CCR5, and CXCR4. The binding of these ligands is also influenced by the structure of the adjacent V3 loop. Requirements for binding the conserved gp120 β19 structure, which is recessed due to the adjacent V2 and V3 variable loops, may include long projections, such as those of the E51 heavy-chain CDR3 or the surface-exposed N-termini of the CCR5 and CXCR4 chemokine receptors. The sulfation of tyrosine residues in the gp120-binding regions of these ligands increases the opportunities for interactions involving longer side chains and electrostatic bonds (Choe et al., 2003, Farzan et al., 1999). These adaptations may allow antibodies such as E51 to achieve efficient binding to HIV-1 before virus attachment to the target cell CD4, when steric constraints on antibody binding minimize the effectiveness of the CD4i antibodies (A.F. Labrijn et al., submitted for publication). Our results indicate that the E51 antibody is subject to some extent to these steric constraints.

The demonstration that the chemokine receptor-binding region of the viral envelope glycoproteins can serve as a neutralization target on primary HIV-1 isolates should encourage efforts to generate antibodies directed against this region. An understanding of the mechanisms used by HIV-1 to protect this conserved region from neutralizing antibodies should assist these efforts.

Materials and methods

Cell lines

293T cells were used for expressing HIV-1 gp120 variants and for producing recombinant viruses for the study of virus entry and neutralization. Cf2Th cells expressing CD4 and CCR5 were used to study the entry of viruses with R5 envelope glycoproteins, and Cf2Th cells expressing CD4 and CXCR4 were used to study the entry of viruses with X4 or R5X4 envelope glycoproteins (Mirzabekov et al., 1999).

HIV-1 gp120 mutants

The mutants containing single amino acid substitutions in the YU2 gp120 wtΔ molecule were made by site-directed mutagenesis using the Quick-Change method. The gp120 wtΔ protein has deletions of gp120 residues 31–81 in the N-terminus and 128–194, removing the V1 and V2 variable loops but retaining the conserved V1/V2 stem. (Numbering of gp120 amino acid residues is based on the sequence of the prototypic HXBc2 strain of HIV-1, according to current convention (Korber et al., 1998; Rizzuto et al., 1998).)

Soluble CD4

Soluble CD4 (sCD4) was produced in *Drosophila* cells stably transfected with the pMT-sCD4 vector, which expresses four-domain, human sCD4 with a carboxy-terminal His_6 tag. Cells were resuspended in fresh medium and induced with 750 μM $CuSO_4$. After 7 days, the supernatants were concentrated 20-fold by using the Millipore Pellicon Ultrafiltration System and applied to an anion-exchange chromatography column (SP Sepharose Fast Flow, Amersham Pharmacia Biotech). The sCD4 was then purified by nickel-affinity chromatography (Ni-NTA Superflow, Qiagen), according to the manufacturer's instructions.

E51 antibody and Fab fragments

The human monoclonal antibody E51 was isolated from an Epstein–Barr virus-transformed B cell line, which was established from an HIV-1-infected individual (AC-01) undergoing early STI (Rosenberg et al., 2000). Epstein–Barr virus transformation of B cells was performed as previously described (Robinson et al., 1990). The antibody was purified on Protein A-Sepharose and eluted with 3 M $MgCl_2$ at room temperature.

Fab fragments of the 17b and E51 antibodies were produced by papain digestion, as described (Kwong et al., 1999).

Expression of HIV-1 gp120 variants

293T cells grown to 70% confluency in 100-mm dishes were transfected with 4 μg of the pSVIIIenv plasmid, which expresses the wtΔ and mutant glycoproteins from the YU2 HIV-1 strain, and 1 μg of a plasmid expressing the HIV-1 Tat protein, using the Effectene Transfection Reagent (Qiagen). Two days after transfection, the medium was removed; the cells were washed once with 10 ml phosphate-buffered saline (PBS), and labeling medium (4.5 ml DMEM, 0.5 ml heat-inactivated, dialyzed FBS, 50 μl penicillin-streptomycin solution and 20 μl (~230 μCi) [^{35}S]cysteine) was added. The cells were incubated at 37°C for 18 h and the medium was collected and stored at 4°C after precipitating the cell debris by centrifugation at 1000 rpm for 10 min.

Immunoprecipitation of gp120 glycoproteins

For precipitation of radiolabeled HIV-1 envelope glycoproteins, 100 μl medium containing the labeled gp120 glycoproteins was mixed with 50 μl of 10% Protein A-Sepharose (Pharmacia), 50 μl 4% bovine serum albumin, and either 1 μg E51 antibody or 2 μl pooled sera from HIV-1-infected patients. PBS was added to bring the total volume to 1 ml. The samples were rocked at 4°C overnight or at room temperature for 1–2 h. The Sepharose beads were then washed once with 1 ml 0.5 M NaCl in PBS and once with

1 ml PBS. The beads were mixed with 2× gel loading buffer and boiled for 3 min. After centrifugation to precipitate the beads, the supernatants were loaded on a 10% SDS–polyacrylamide gel. The gel was enhanced with Autofluor (National Diagnostic) for 20 min before drying at 80°C for 1.5 h and exposure to X-ray film. The gel was also used for PhosphorImager (Molecular Dynamics) analysis.

ELISA

The wild-type soluble gp120 glycoprotein of YU2 produced transiently from 293T cells was used to examine induction of antibody binding by sCD4. The ELISA plate was coated with sheep antibody D7324 (Aalto BioReagents, Dublin, Ireland), which recognizes the gp120 carboxyl-terminus. The gp120 from the 293T cell supernatants was captured on the plates. The human monoclonal antibody E51 or 17b (0.03–3.0 μg/ml) was incubated for 1.5 h at 37°C with the ELISA plates in the presence of blocking buffer (2% w/v non-fat dried milk in PBS and 5% FBS). The plates were washed with 0.2% Tween in PBS. Bound HMAb was detected with horseradish peroxidase conjugated goat anti-human IgG (Fc-specific) (Sigma). The assay was developed with TMB peroxidase EIA substrate kit (Bio-Rad). The reaction was stopped by adding 100 μl 180 mM HCl and the plate was read at 450 nm.

HIV-1 neutralization assay

The HIV-1-neutralizing ability of the human monoclonal antibody E51 was tested using a single-round virus entry assay (Yang et al., 2001). Recombinant HIV-1 expressing firefly luciferase was produced by transfection of 293T cells with the pCMV Gag-Pol packaging construct, the pSVII-Ienv plasmid expressing the envelope glycoproteins of different HIV-1 strains, and the pHIV-Luc vector. Two days after transfection, the cell supernatant containing recombinant HIV-1 was harvested and its reverse transcriptase (RT) activity was determined by measuring incorporation of ^{3}H-TTP. The recombinant viral stocks were aliquoted and frozen.

The target cells (either Cf2Th cells expressing CD4 and CCR5 or Cf2Th cells expressing CD4 and CXCR4) were seeded at a density of 6000 cells/well in a 96-well plate and cultured overnight at 37°C. About 20,000 reverse transcriptase units of virus were incubated with serial dilutions of antibody for 1 h at 37°C in a 50 μl volume. The target cells were washed once with PBS and the virus–antibody mixture was added to the cells for 18 h. The cells were then washed, and fresh medium was added. The cells were cultured for 2 days, washed once with PBS, and lysed with 30 μl luciferase lysis buffer. Luciferase assays were performed using the EG & G Berthold Microplate Luminometer LB 96V (Promega).

For some neutralization assays, recombinant viruses were added to target cells at 4°C for 2 h. Cells were then washed and incubated with the antibody for 18 h at 37°C in medium. The cells were again washed, and fresh medium was added. The cells were cultured for 48 more hours before the luciferase assays were performed, as described above.

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