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Human Immunodeficiency Virus Type 1 Mutants That Escape Neutralization by Human Monoclonal Antibody IgG1b12

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IgG1b12, a human monoclonal antibody (MAb) to an epitope overlapping the CD4-binding site on gp120, has broad and potent neutralizing activity against most primary human immunodeficiency virus type 1 (HIV-1) isolates. To assess whether and how escape mutants resistant to IgG1b12 can be generated, we cultured primary HIV-1 strain JRCSF in its presence. An escape mutant emerged which was approximately 100-fold more resistant to neutralization by IgG1b12. Both virion-associated and solubilized gp120 from this variant had a reduced affinity for IgG1b12, and sequencing of its *env* gene showed that amino acid substitutions had occurred at three positions within gp120. Two (D164N and D182N) were located in V2, and one (P365L) was in C3. By site-directed mutagenesis, we demonstrated that the D182N and P365L mutations, but not D164N, contribute to the IgG1b12-resistant phenotype. However, the former two substitutions, individually or in combination, hinder the replication of the neutralization-resistant virus. Introduction of the D164N substitution into the P365L variant results in a nonviable virus (D164N/P365L). In contrast, addition of D164N to the D182N or D182N/P365L mutant partially restored replicative function to near wild-type levels. Furthermore, we found that all of the IgG1b12-resistant mutant viruses remained sensitive to other human MAbs, such as 2G12 and 2F5, and to the CD4-IgG molecule, except that the P365L-containing mutant was slightly resistant to CD4-IgG. These results suggest that escape from IgG1b12 neutralization is due to a local rather than a global modification of the gp120 structure. Our findings have implications for the therapeutic and prophylactic applications of antibodies for HIV-1 infection.

Human immunodeficiency virus type 1 (HIV-1) primary strains are relatively resistant to neutralization by antibodies and sCD4 compared to variants selected for growth in permanent cell lines (3, 11, 18, 24, 25, 29). However, a few human monoclonal antibodies (MAbs) are known which possess broad and potent neutralizing activity against primary isolates (4, 5, 10, 27, 36, 37). These antibodies, perhaps, represent the upper bounds of the power of the human immune system to counter HIV-1 infection, at least with respect to the virus neutralization response. Information on how these antibodies interact with HIV-1 is therefore useful for vaccine design strategies based on the induction of humoral immunity and for passive immunotherapeutic approaches aimed at treating established HIV-1 infection.

One major problem facing the humoral immune system in terms of countering HIV-1 is the enormous mutability of this pathogen. As a consequence of the rapid rate of HIV-1 replication in vivo and the error-prone nature of the reverse transcriptase enzyme (9, 15, 38), myriad variants of HIV-1 are generated daily. It is inevitable that some of these variants will be able to evade the immune response and therefore gain a selective advantage (1, 2, 12, 16, 17, 34). To understand how virus mutation impacts on the activity of a broadly neutralizing antibody, we subjected the molecularly cloned primary HIV-1 strain JRCSF (HIV-1_{JRCSF}) to the selection pressure of human MAb IgG1b12 in vitro. This antibody was generated by recombinant DNA technology (5, 6) and is one of the three human anti-HIV-1 MAbs yet described that have truly broad and potent neutralizing activity against primary strains (4, 5, 8, 10, 12a, 36). MAb IgG1b12 recognizes a conformation-sensitive epitope that overlaps, but is not precisely contiguous with, the CD4-binding site on the HIV-1 surface glycoprotein gp120 (27). Uniquely among MAbs that recognize this epitope cluster, IgG1b12 binds equivalently or better to the oligomeric form of the envelope glycoprotein; this probably accounts for its exceptional potency (13, 27).

Here we show that HIV-1_{JRCSF} variants that are resistant to IgG1b12 can arise in culture, and we demonstrate that only a limited number of amino acid substitutions are required to confer the resistant phenotype. However, the escape mutant strain has a lower rate of replication and remains as sensitive as the wild-type virus to two other broadly neutralizing MAbs, 2F5 and 2G12. This encourages the notion that a combination of potent antibodies might have significant antiviral activity in vivo.

MATERIALS AND METHODS

MAbs. IgG1b12 is a human recombinant antibody initially isolated as a Fab fragment by screening against gp120 from the LAI strain (5, 6). Dimeric CD4-IgG was obtained from Genentech Inc. (South San Francisco, Calif.) (8). Human MAb 21h recognizes a discontinuous epitope overlapping the CD4-binding site that has been described previously (14). Human MAb 2G12 recognizes a conformationally sensitive gp120 epitope unrelated to the V1, V2, or V3 loop or to the CD4-binding site (4, 37). 2F5 is an anti-gp41 human MAb that has been mapped to the sequence ELDKWA (10).

Generation of neutralization-resistant virus. IgG1b12 (0.001 μg/ml) was incubated with supernatant from 293 cells transfected with pYK-JRCSF plasmid DNA from the molecular clone JRCSF for 1 h at 37°C, and 200 μl of the mixture was added to 2×10^6 mitogen-stimulated primary peripheral blood mononuclear cells (PBMCs). The inoculum was removed the next day, and the cells were cultured in 1 ml of RPMI 1640 medium (with 10% fetal calf serum and interleukin 2) in the presence of IgG1b12 (0.001 μg/ml). After 7 days, the cell-free supernatant was harvested and used to infect fresh PBMCs (second passage). In subsequent passages, the concentration of IgG1b12 was gradually increased.

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After 10 rounds of passage, the virus grew well in the presence of 10 μg of IgG1b12 per ml. This virus was designated P10.

HIV neutralization assay. Neutralization assays were performed as described previously (7). Briefly, 500 μl of virus (1,000 50% tissue culture-infective doses/ml) was incubated with 500-μl volumes of serial dilutions of each MAb at 37°C for 1 h. Aliquots (200 μl) of the virus-antibody mixture were added to 4 wells of a 24-well plate which contained 2×10^6 phytohemagglutinin-stimulated PBMCs. To provide a calibration curve, the same viral inoculum (100 tissue culture-infective doses/well) and 1:3 and 1:9 dilutions of the inoculum were added to 2×10^6 PBMCs. Excess virus and antibody were removed by extensive washing 24 h later, and the cultures were maintained for 7 days. Virus production (p24 antigen) was measured by using a commercial kit (Abbott Laboratories, Abbott Park, Ill.). All data points represent the means of quadruplicate determinations. Percent neutralization was calculated by determining the reduction in supernatant p24 antigen production in the presence of the MAb compared to that in control cultures lacking the MAb.

Amplification of proviral DNA and nucleotide sequencing. Proviral HIV-1 DNA was extracted from the P10-infected PBMCs by standard procedures. Nested PCR was performed to amplify the gp160 coding region as described previously (39). The primers used for the first round were REC-07, which hybridizes to the minus strand at positions 5569 to 5988 of the NL4-3 sequence, and PX2, hybridizing to the plus strand at positions 8998 to 9024; primers REC-27, hybridizing to the minus strand at positions 6202 to 6226, and PX1, hybridizing to the plus strand at positions 8900 to 8923, were used for the second round. The products of the nested PCR were inserted in the TA Vector (Invitrogen). Double-stranded DNAs from recombinant clones were sequenced by the dideoxynucleotide chain termination method.

Monomeric gp120 binding assay. MAb binding to monomeric gp120 was assessed by enzyme-linked immunosorbent assay as described previously (19, 20, 23, 39). Briefly, virus-containing culture supernatant was inactivated with 1% Nonidet P-40 nonionic detergent to provide a source of monomeric gp120. A sheep anti-gp120 antibody, D7324, which recognizes a 15-amino-acid peptide from the C terminus of gp120, was used to capture gp120 on Immulon-2 plates (18, 20). After washing out of the unbound gp120, MAbs were added in Tris-buffered saline containing 2% nonfat milk powder, 20% sheep serum, and 0.5% Tween 20. After 1 h, unbound antibodies were removed by washing, bound antibodies were detected by incubation for 1 h with the appropriate alkaline phosphatase-conjugated anti-species antibody, and the signal was amplified with the AMPAK system (Dako Diagnostics). Binding curves based on readings of optical density at 490 nm against the MAb concentrations were plotted.

Antibody-virus binding. The assay was performed as described previously (30). Briefly, sucrose-purified infectious virions (free of soluble gp120 molecules) were incubated with increasing concentrations of MAbs (0.1 to 50 μg/ml) for 3 h at 37°C in Tris-buffered saline containing 4% nonfat dry milk and 10% fetal calf serum. The virion-MAb complexes were separated from free MAbs by centrifugation (1 h, 4°C, 12,500 × *g* in a Sorvall RC-5C centrifuge with an SH-MT rotor). The viral pellet was lysed with Nonidet P-40 detergent (1% vol/vol), and the monomeric gp120-MAb complexes were captured on 96-well plates coated with D7324 as described above. To monitor for MAb-mediated gp120 dissociation from virions, supernatant from the virion-MAb pellet was collected and added to enzyme-linked immunosorbent assay wells coated with D7324. The sum of the optical densities at 490 nm of the viral pellets and the supernatant represents the total amount of MAb bound to virion gp120.

Site-directed mutagenesis. A 2.689-kb *Sal*I fragment of pYK-JRCSF was cloned into pALTER (Promega), and purified single-stranded DNA was used as the template for mutagenesis. The site-directed mutagenesis reaction was carried out by using the Altered Site in vitro Mutagenesis System (Promega, Inc.) in accordance with the manufacturer's instructions. Following confirmation of the desired mutation by direct sequencing, a 2.689-kb *Sal*I fragment containing the gp120 coding region was purified and ligated into the JRCSF molecular clone plasmid. After transformation of JM109 cells, plasmid DNA was purified and sequenced for verification of the desired mutation. The mutagenized JRCSF was subsequently transfected into 293 cells by the calcium phosphate precipitation method. On day 2 posttransfection, the culture medium was changed; at 72 h posttransfection, the medium was collected and filtered to provide a source of infectious HIV-1 virions.

RESULTS

Selection of IgG1b12-resistant variant. To select for IgG1b12 escape mutants, we cultured the molecularly cloned primary strain HIV-1_{JRCSF} in the presence of the MAbs in mitogen-stimulated human PBMCs. After 10 rounds of passage, a viral variant (designated P10) arose that grew in the presence of 10 μg of IgG1b12 per ml (the 50% inhibitory concentration for neutralizing wild-type HIV-1_{JRCSF} is 0.1 μg/ml). Thus, an approximately 100-fold higher concentration of IgG1b12 was required to neutralize P10 than to neutralize HIV-1_{JRCSF} to an equivalent extent (Fig. 1).

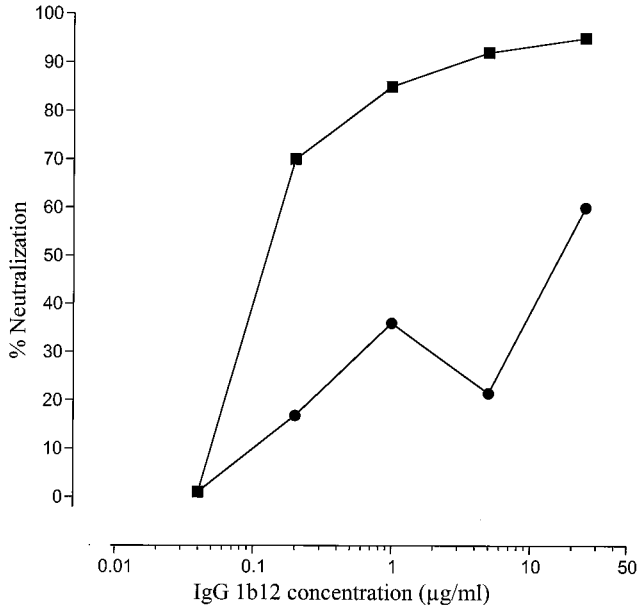

FIG. 1. IgG1b12 neutralization of HIV-1_{JRCSF} (■) and the escape mutant P10 (●).

Reactivity of MAbs with oligomeric and monomeric gp120 from the escape variant. To address the mechanism of resistance of P10, we performed binding assays by using virion-associated gp120 and monomeric gp120 from the wild-type and variant viruses. As shown in Fig. 2a, the virion-associated gp120 of P10 was completely unable to bind to IgG1b12 at concentrations of up to 10 μg/ml while wild-type HIV-1_{JRCSF} bound IgG1b12 half maximally at 0.2 μg/ml.

To see whether this loss of IgG1b12 reactivity was specific to oligomeric gp120-gp41 complexes on the P10 virus or was due to loss of the IgG1b12 epitope from monomeric gp120, we performed gp120-binding assays by using nonionic-detergent-treated culture supernatants. The gp120 derived from the variant P10 failed to bind to IgG1b12 completely at concentrations of up to 5 μg/ml (Fig. 2b). There was also a modest (three- to fivefold) reduction in the binding of P10 gp120 to the CD4-IgG molecule (Fig. 2c). However, both gp120s bound a pool of HIV-1-positive human serum equally well (Fig. 2d) and had indistinguishable affinities for MAb 21h (Fig. 2e). MAbs 21h and IgG1b12 recognize very similar, overlapping epitopes near the CD4-binding site on gp120 (4, 14, 21, 27, 32, 36). The failure of P10 gp120 to bind MAb IgG1b12 while retaining its ability to bind 21h implies that the loss of IgG1b12 binding is a specific selection process due to the MAb pressure (Fig. 2).

gp160 sequence of escape mutants. To determine the genetic basis of the resistance of variant P10, its *env* gene was amplified and sequenced. Three amino acid substitutions from the parental sequence were noted consistently in clones (five of six) from the P10 variant, while two other changes (E143K and N287K) occurred infrequently (Fig. 3). One of six clones from P10 had the wild-type HIV-1_{JRCSF} sequence. Of the three consistent changes, two were in the V2 region of gp120 (D164N and D182N) and one was in the C3 region (P365L). These were of clear significance, based on our prior studies on the epitope of MAb IgG1b12 (27). Unusually among MAbs to CD4-binding site-associated epitopes, IgG1b12 is sensitive to amino acid changes in the V2 loop (in the background of HIV-1_{HxBc2}) (27). Roben et al. (27) also showed that the par-

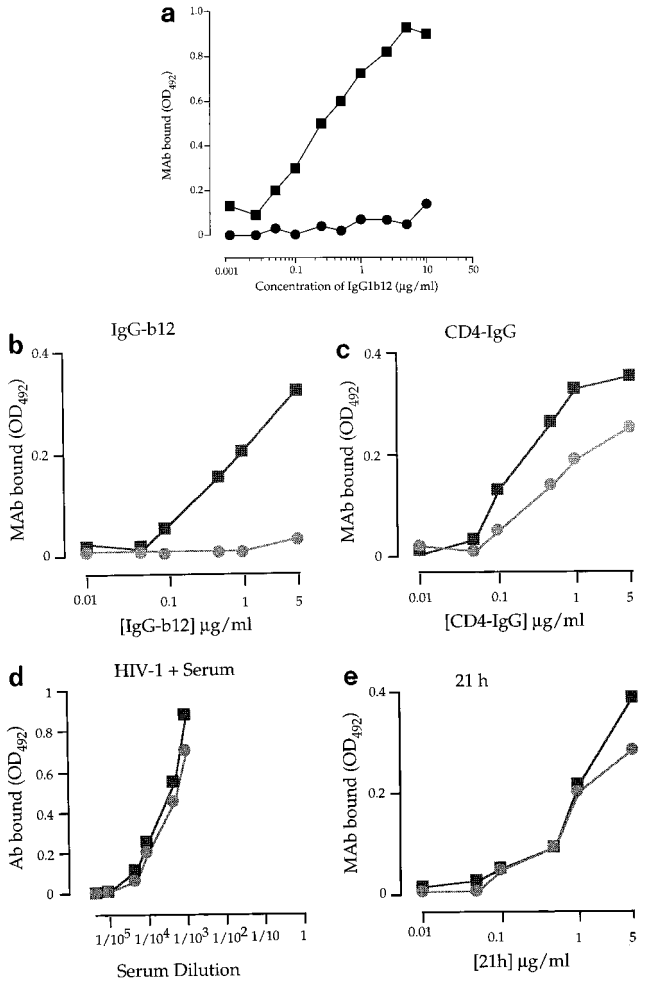

FIG. 2. Binding of virion-associated gp120 to IgG1b12 (a) and monomeric gp120 to IgG1b12 and other antibodies (b, c, d, and e). Symbols: ■, represents HIV-1_{JRCSF}; ●, P10. OD_{492}, optical density at 490 and 492 nm.

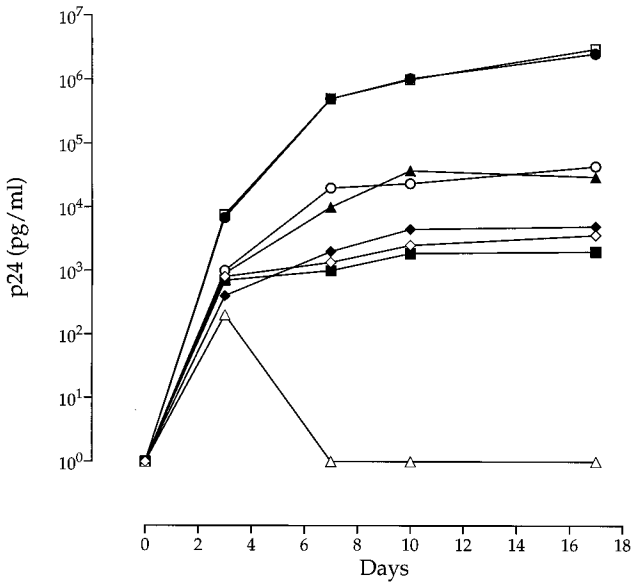

FIG. 4. Replication kinetics of HIV-1_{JRCSF} and created mutants in PBMCs. Symbols: □, HIV-1_{JRCSF}; ▲, D164N; ◆, D182N; ■, P365L; ○, D164N/D182N; △, D164N/P365L; ◇, D182N/P365L; ●, TM5.

ticular V2 changes in HIV-1_{HxBc2} residues 183 and 184, which correspond to HIV-1_{JRCSF} gp120 positions 180 and 181, reduced the binding affinity of monomeric gp120 for IgG1b12; substitutions at the HIV-1_{HxBc2} residues corresponding to amino acids 164 and 182 of HIV-1_{JRCSF} gp120 were not made in this study. The third change (P365L) was also of note: amino acid 365 in gp120 corresponds to residue 369 of HIV-1_{HxBc2} gp120, and a substitution at either adjacent position (i.e., D368R or E370R) destroys both the CD4-binding site (33) and the epitopes for IgG1b12 (27, 36) and almost all known MAbs to CD4-binding site-related structures (14, 20, 28, 32, 33). Thus, the IgG1b12 escape variant has an amino acid substitution of a highly conserved amino acid (proline 365) that is in close proximity to residues implicated in forming the CD4-binding site, as well as two changes in a region of the V2 loop that is known to influence the formation of the IgG1b12 epitope (27).

Replication kinetics of mutants. To confirm which, if any, of the observed sequence changes in the P10 variant created the neutralization-resistant phenotype, we introduced the changes, individually and in combination, into the parental HIV-1_{JRCSF} clone (Fig. 4). The D182N and P365L single mutants and the D182N/P365L double mutant replicated poorly, and another double mutant (D164N/P365L) was unable to sustain significant replication in culture. However, the D164N and D164N/D182N mutants grew relatively well and the triple mutant TM-5 (D164N/D182N/P365L) replicated as efficiently as wild-type HIV-1_{JRCSF}. Of note is the fact that certain of the mutants (e.g., D164N/P365L) replicate initially but fail to establish a productive infection. The reasons for this are not known. Overall, the substitutions at residues 182 and 365 create a poorly viable (presumably IgG1b12-resistant) virus and the introduction of an additional change at residue 164 (D164N) to D182N-containing mutants restores replication competence.

Neutralization of mutagenized HIV-1_{JRCSF} variants. The sensitivity of the replication-competent clones to neutralization by IgG1b12 was assessed next (Fig. 5). All mutants with the D182N and/or P365L substitution (the D182N and P365L single mutants, the D164N/D182N and D182N/P365L double

	V1			V2						
	141 150	160	170	180	190	280	290	360	370	
	* *	*	*	*	*		*		*	
WT	SSEGMMERGE	IKNCSFNITK	SIRDKVQKEY	ALFYKLDVVP	IDNKNNTKYR	----------	TIIVQLNESV	----------	SGGDPEIVMH	FREQUENCY
P10	..K.....	....	...N.....	....	.N.....	....	....	....	....L.....	1/6
	....	....	...N.....	....	.N.....	....	......K...	....	....L....	1/6
	....	....	...N.....	....	.N.....	....	....	....	....L.....	3/6
	....	....	....	....	....	....	....	....	....	1/6

FIG. 3. Deduced amino acid sequences of the V1, V2, and C3 regions of gp120 from wild-type (WT) HIV-1_{JRCSF} and mutant P10.

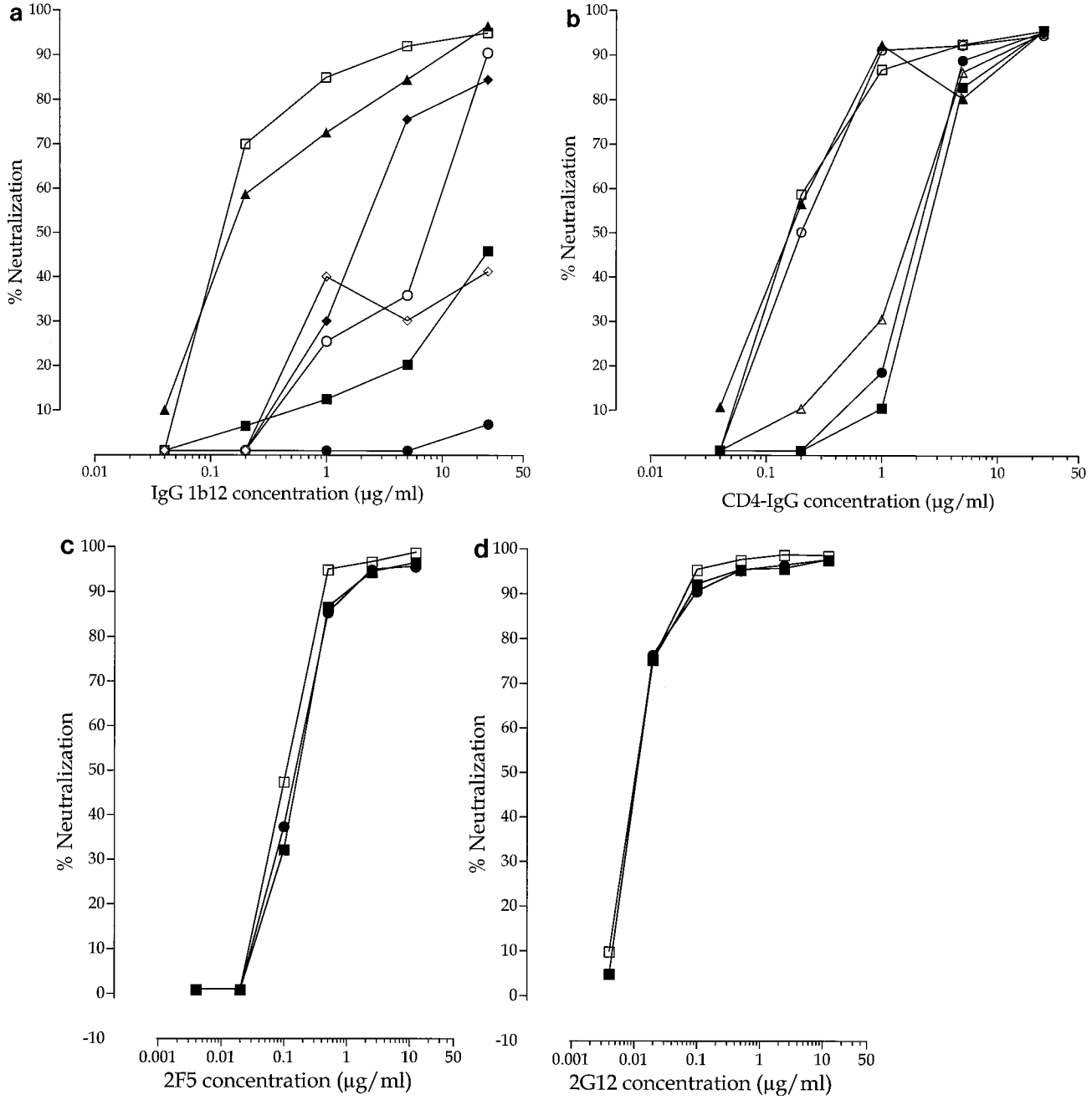

FIG. 5. Neutralization of HIV-1_{JRCSF} (□) and mutants D164N (▲), D182N (◆), P365L (■), D164N/D182N (○), D182N/P365L (◇), and TM5 (●) by IgG1b12 (a); HIV-1_{JRCSF}, D164N, P365L, D164N/D182N, TM5, and P10 (△) by CD4-IgG (b); and JRCSF, P365L, and TM5 by 2F5 (c) and 2G12 (d).

mutants, and the TM-5 triple mutant) were significantly resistant to IgG1b12, whereas a mutant with a change only at residue 164 remained sensitive to IgG1b12 (Fig. 5a). The P365L-containing mutants, but not the others, were also slightly resistant to neutralization by CD4-IgG, although much less so than they were to IgG1b12 (Fig. 5b).

We then determined whether the IgG1b12 escape variant viruses remained sensitive to two other broadly neutralizing antibodies, 2F5 and 2G12 (Fig. 5c and d). Both the P365L and TM-5 viruses had unchanged sensitivities to MAbs 2F5 (Fig. 5c) and 2G12 (Fig. 5d), which recognize conserved epitopes in gp41 (10) and gp120 (4, 37), respectively. Thus, the neutralization escape mechanism is specific to the IgG1b12 selection MAb and the escape viruses can be neutralized by other antibodies. There was, however, slight resistance of P365L variants to neutralization by CD4-IgG (Fig. 5b).

IgG1b12-binding affinity of gp120 from escape mutants. To assess whether the pattern of IgG1b12 neutralization resistance shown by the escape mutants had a correlate, we performed the oligomeric and monomeric gp120-binding assays. IgG1b12 bound to the oligomeric gp120 of wild-type HIV-1_{JRCSF} but failed to bind significantly to the oligomeric gp120 from TM-5 (Fig. 6a). This result is consistent with the loss of binding to IgG1b12 by the P10 variant (Fig. 2a). IgG1b12

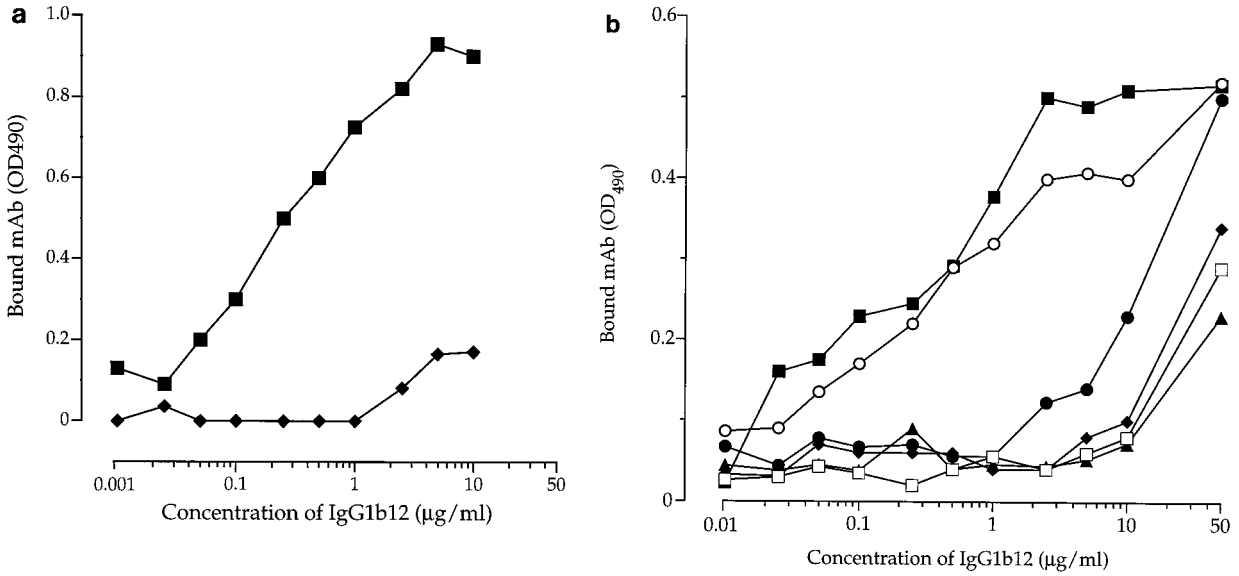

FIG. 6. IgG1b12 binding to oligomeric gp120 of HIV-1_{JRCSF} (■) and TM5 (◆) (a) and monomeric gp120 of HIV-1_{JRCSF} (■), D164N (○), D182N (◆), P365L (●), D164N/D182N (▲), and D182N/P365L (□) (b). OD_{490}, optical density at 490 nm.

binding to virion-associated gp120 from other mutant viruses could not be assessed accurately because insufficient gp120 was associated with them (data not shown). This suggests, perhaps, that the slow growth kinetics of these mutant viruses are partially due to poor incorporation or retention of gp120 molecules on the virion surface; substitutions in V2 can have this effect (31). The binding of IgG1b12 to monomeric gp120 from all of the mutants is shown in Fig. 6b. All gp120s which contained either D182N or P365L (the D182N and P365L single mutants, the D164N/D182N and D182N/P365L double mutants, and the TM-5 triple mutant) did not bind IgG1b12 except at a high IgG1b12 concentration (25 μg/ml) (Fig. 6b). However, binding of IgG1b12 to D164N gp120 was similar to its binding to wild-type gp120.

DISCUSSION

We have described the selection and characterization of a passaged escape mutant (P10) and genetically engineered mutants of the HIV-1 primary virus HIV-1_{JRCSF} that resist neutralization by the broadly and potently neutralizing human MAb IgG1b12 (5). The critical amino acid substitutions for the resistant phenotype are at residues proline 365 (P365L) and aspartic acid 182 (D182N), which are equivalent to residues 369 and 185 of HIV-1_{HxBc2} gp120, respectively. Highly conserved residue 365 is immediately adjacent in the primary sequence to amino acids implicated in CD4 binding (22, 26) and also in the formation of the IgG1b12 epitope (27). The V2 change D182N is proximal to positions in the HIV-1_{HxBc2} sequence that also have a major influence on the structure of the IgG1b12 epitope on monomeric gp120 (27). Both of the engineered, neutralization-resistant single mutants (D182N and P365L), although replication competent, do have a significantly reduced rate of replication. The other mutation (D164N) found in P10 does not, by itself, confer significant resistance to IgG1b12, even though the possibility that it makes a minor contribution to resistance in the context of other changes cannot be excluded (Fig. 5); however, it does appear to increase the replication competence of the otherwise partially defective D182N/P365L variant. The triple mutant (TM5), like P10, was fully replication competent. To create a replication-competent, IgG1b12-resistant virus, it therefore seems that three amino acid substitutions have to occur together.

The P365L-containing mutant viruses also slightly resist neutralization by the CD4-IgG molecule. It is, however, significant that the P10 (TM5) escape mutant remains completely sensitive to other human MAbs, 2G12 and 2F5. These antibodies recognize epitopes that do not overlap the binding sites for IgG1b12 and CD4-IgG2 (10, 21, 37), and we have found that combination of these MAbs with CD4-IgG2 or IgG1b12 cause extremely potent suppression of primary virus replication in vitro (35, 37). As we have shown that escape from a single MAb (IgG1b12) requires several genetic changes and the escape variants remain sensitive to other MAbs, this gives encouragement to the concept that combinations of broad and potent MAbs might be hard for a virus to escape from. To do so might require multiple, independent mutations which, in combination, could have a significant deleterious impact on virus replication. Thus, passive immunotherapy with cocktails of active MAbs such as IgG1b12, 2G12, and 2F5 (or the CD4-IgG2 molecule) to prevent or treat HIV-1 infection remains a viable concept.

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