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Resistance to Neutralization by Broadly Reactive Antibodies to the Human Immunodeficiency Virus Type 1 gp120 Glycoprotein Conferred by a gp41 Amino Acid Change

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A neutralization-resistant variant of human immunodeficiency virus type 1 (HIV-1) that emerged during in vitro propagation of the virus in the presence of neutralizing serum from an infected individual has been described. A threonine-for-alanine substitution at position 582 in the gp41 transmembrane envelope glycoprotein of the variant virus was responsible for the neutralization-resistant phenotype (M.S. Reitz, Jr., C. Wilson, C. Naugle, R. C. Gallo, and M. Robert-Guroff, Cell 54:57–63, 1988). The mutant virus also exhibited reduced sensitivity to neutralization by 30% of HIV-1-positive sera that neutralized the parental virus, suggesting that a significant fraction of the neutralizing activity within these sera can be affected by the amino acid change in gp41 (C. Wilson, M. S. Reitz, Jr., K. Aldrich, P. J. Klasse, J. Blomberg, R. C. Gallo, and M. Robert-Guroff, J. Virol. 64:3240–3248, 1990). It is shown here that the change of alanine 582 to threonine specifically confers resistance to neutralization by antibodies directed against both groups of discontinuous, conserved epitopes related to the CD4 binding site on the gp120 exterior envelope glycoprotein. Only minor differences in binding of these antibodies to wild-type and mutant envelope glycoproteins were observed. Thus, the antigenic structure of gp120 can be subtly affected by an amino acid change in gp41, with important consequences for sensitivity to neutralization.

Human immunodeficiency virus type 1 (HIV-1) establishes a persistent infection in human hosts, resulting in defective cellular immunity secondary to CD4 lymphocyte depletion (10, 21). The virus can cause this gradual deterioration of the immune system despite the presence and evolution of neutralizing antibodies in the serum. The envelope glycoproteins of HIV-1, gp120 and gp41, are the major targets for these neutralizing antibodies (2, 32, 34, 44, 45). Neutralizing antibodies directed against gp120 were able to protect chimpanzees from infection following exposure to an intravenous HIV-1 challenge (3, 9). The design of effective vaccines will require an understanding of which functional regions of gp120 and gp41 are available for recognition by the humoral immune system.

Both envelope glycoproteins fulfill important tasks during the viral life cycle. While gp120 binds with high affinity to CD4, the receptor for HIV-1 on target cells, gp120 and gp41 are both involved in the fusion of viral and cellular membranes that allows virus entry (6, 13, 19, 23, 24; for a review, see reference 18). Even though the envelope glycoproteins are crucial for the infection process, a high degree of genetic heterogeneity in the envelope is found among different viral strains (5, 28). The extensive variability of gp120 provides a major hurdle for the development of a broadly protective immune response.

The maintenance of a high degree of genetic variation between different strains of HIV-1 may in part be due to continuous immune selection occurring in the host. Previous studies have shown that the in vitro passage of HIV-1 in the presence of neutralizing sera from HIV-1-infected humans can select for the growth of variants of the virus that are relatively resistant to neutralization by the selecting serum. In one of these studies, a single amino acid change (alanine to threonine) at position 582 in gp41 was responsible for the neutralization-resistant phenotype (31, 33). The site of the immune-selected amino acid change did not apparently constitute a neutralization epitope, giving rise to the speculation that this residue change might alter the epitopes of neutralizing antibodies recognizing distant sites (46). Neutralization escape was not limited to the selecting serum, since 30% of sera from individuals infected with HIV-1 that neutralized the parental virus showed decreased ability to neutralize the mutant virus.

In HIV-1-infected humans, the two major fractions of neutralizing antibodies bind either to continuous epitopes in the third variable (V3) or V2 region or to discontinuous epitopes (4, 26, 36, 37). The latter epitopes either overlap the discontinuous CD4 binding site or are better exposed upon CD4 binding (7, 14, 15, 17, 27, 29, 39, 41). The gp120 amino acids important for the formation of these epitopes are derived from the five conserved gp120 regions and have been mapped by analysis of gp120 mutants (25, 27, 39, 41, 42). In this study, we used monoclonal antibodies to test whether the change of alanine 582 to threonine (582 A/T change) allows HIV-1 to escape from antibodies directed against either variable or conserved gp120 neutralization epitopes.

MATERIALS AND METHODS

Construction of the 582 A/T mutant. The *Kpn*I-*Bam*HI fragment of plasmid pSVIIIenv was used for site-directed mutagenesis as previously described (13, 29). The presence of

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the mutation was confirmed by the generation of a novel restriction endonuclease site and by DNA sequencing. The wild-type and 582 A/T mutant glycoproteins were expressed in COS-1 cells by using either plasmid pSVIIIenv (13) or plasmid pCMVenv, in which the *rev* and *env* genes are under the control of the cytomegalovirus immediate-early promoter.

Virus neutralization assay. Complementation of a single round of replication of the *env*-deficient chloramphenicol acetyltransferase (CAT)-expressing provirus by either wild-type or mutant envelope was performed as described previously (13, 41). To inhibit viral replication, either 0, 0.1, or 10 μg of monoclonal antibody per ml was incubated with recombinant virus for 1 h at 37°C before addition of the virus to target Jurkat lymphocytes. Three days after infection, Jurkat cells were lysed and CAT activity was measured as described previously (13).

Syncytium formation assay. Three days after transfection with either wild-type or mutant envelope expressor plasmid, COS-1 cells were detached from the dishes by incubation with 50 mM EDTA. After washing, 1×10^5 to 2×10^5 cells were preincubated either in the absence of antibody or with 100 μg of antibody per ml of Dulbecco modified Eagle medium for 1 h at 37°C. Then 1×10^6 to 2×10^6 SupT1 cells were added, and syncytia were scored after 6 to 8 h of incubation at 37°C.

Binding of antibodies to envelope glycoproteins expressed on the surface of COS-1 cells. To analyze the affinity of the antibodies for the HIV-1 envelope glycoprotein complex, COS-1 cells were transfected with plasmid pCMVenv expressing either the wild-type or mutant envelope glycoproteins under the control of the cytomegalovirus immediate-early promoter. The cells were ^{35}S radiolabeled 2 days after transfection, and monoclonal antibodies were incubated with the cells for 90 min at 37°C in Dulbecco modified Eagle medium containing 10% fetal calf serum. After three washes with phosphate-buffered saline, the cells were lysed in Nonidet P-40 buffer, and bound antibody-envelope glycoprotein complexes were precipitated by protein A-Sepharose (Pharmacia) and analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Scanning densitometry was performed to determine relative antibody binding.

To assess binding of antibody in the presence of soluble CD4, COS-1 cells transfected with plasmid pSVIIIenv were incubated for 90 min with 1 ml of radiolabeled monoclonal antibody in the presence of the indicated amounts of soluble CD4. Labeling of the antibodies and detection of bound antibodies were performed as described previously (41). To minimize shedding of gp120, the incubation was performed at room temperature.

RESULTS

An amino acid change in gp41 allows HIV-1 to escape from neutralization by anti-gp120 antibodies. The 582 A/T change was introduced in the gp41 glycoprotein encoded by the HXBc2 envelope gene through the use of site-directed mutagenesis (20). Virions carrying the mutant envelope glycoprotein were tested for sensitivity to neutralization by a range of antibodies directed against conserved or variable epitopes in gp120. Recombinant viruses carrying the CAT gene and containing either wild-type or mutant (582 A/T) envelope glycoproteins were produced in COS-1 cells as described previously (13). Virus-containing supernatants were incubated with or without antibody and subsequently added to Jurkat CD4-positive lymphocytes. CAT activity in these target cells was measured to assess the degree of neutralization of this single round of infection. The data from a typical experiment are shown in Fig. 1. In contrast to viruses with the wild-type envelope glycoprotein, viruses bearing the 582 A/T change were resistant to neutralization by antibodies F105 and 48d. These two antibodies represent the two groups of broadly reactive antibodies that recognize discontinuous gp120 epitopes present only on the native glycoprotein (26, 39, 41, 42). Other antibodies of these groups, such as 21h, 15e, and 17b, also exhibited reduced ability to neutralize viruses with the 582 A/T amino acid change in gp41 relative to that seen for the wild-type glycoprotein (data not shown). In contrast, antibody 110.4, which recognizes the V3 variable region of gp120 (22), neutralized both wild-type and 582 A/T mutant viruses comparably (Fig. 1). As expected, a virus with a single amino acid change (313 P/S) in the V3 region, which disrupts the binding of antibody 110.4 (40), was not efficiently neutralized by this antibody (data not shown). Other anti-V3 loop antibodies (9284, F58/H3, and 0.5β) that were tested and monoclonal antibodies directed against the V2 loop (G3-4) or against a gp41 epitope (2F5) also neutralized the 582 A/T mutant virus (data not shown). The gp41 epitope for antibody 2F5 is located between residues 661 and 667, significantly carboxy terminal to alanine 582 (27a). Furthermore, the 582 A/T mutant was as sensitive as the wild-type virus to neutralization by soluble CD4 (data not shown). Thus, the 582 A/T change in gp41 allows virions to escape specifically from antibodies that recognize conserved regions in gp120.

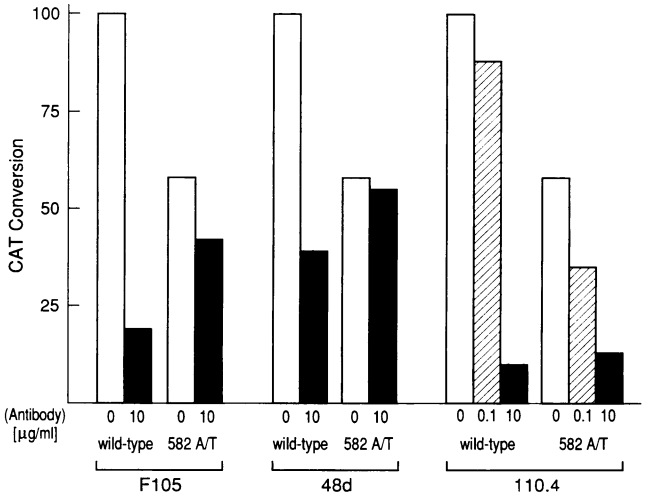

FIG. 1. Neutralization of wild-type and mutant viruses by monoclonal antibodies. Recombinant viruses carrying the CAT gene and containing either wild-type or mutant envelope glycoproteins were produced in COS-1 cells. The virions were incubated in the presence or absence of antibody F105, or 48d, or 110.4 prior to complementation with Jurkat lymphocytes. The CAT activity observed in Jurkat target cells following incubation with the indicated amounts of antibody is shown for both wild-type and 582 A/T mutant envelope glycoproteins. The CAT activity observed for the wild-type glycoproteins was arbitrarily set at 100%. The data showing inhibition by 10 μg of antibody per ml are those of a typical experiment. Also included are the results of an experiment in which 0.1 μg of antibody 110.4 per ml was used. In these experiments, the background level of CAT activity observed in the absence of envelope glycoproteins varied from 5 to 10% conversion. Values in that range therefore indicate complete neutralization, while values of 100 and 58% indicate no neutralization for viruses with the wild-type and 582 A/T envelope glycoproteins, respectively.

Wilson et al. reported that relative to that of the wild-type virus, syncytium formation by the 582 A/T mutant was less sensitive to inhibition by serum from HIV-1-positive individu-

TABLE 1. Effects of the 582 A/T amino acid change on inhibition of syncytium formation by different antibodies

Glycoprotein	% of no. of syncytia scored in the presence of[a]:		
	F105	48d	110.4
Wild type	47	50	4
582 A/T	81	85	2

[a] Antibodies were present at 100 μg/ml. In the absence of antibodies, the 582 A/T mutant envelope forms approximately one-third of the number of syncytia formed by wild-type envelope glycoproteins (100% syncytium formation in both cases).

als (46). The effect of the 582 A/T change on syncytium inhibition by monoclonal antibodies was assessed. Table 1 shows data from a typical experiment. The relative potency of antibodies F105, 48d, and 110.4 in the syncytium inhibition assay was similar to that seen in the virus neutralization assays. However, inhibition of syncytium formation required higher concentrations of the antibodies than did virus neutralization. Thus, while syncytium formation by the wild-type envelope glycoproteins was almost completely inhibited by 100 μg of anti-V3 antibody 110.4 per ml, the same concentration of antibody F105 or 48d resulted in an approximately twofold reduction in syncytia. Syncytia induced by the 582 A/T mutant glycoproteins were reduced in number by addition of antibody 110.4 comparably to that observed for the wild-type glycoprotein. This was also true at lower concentrations of antibody 110.4 that did not completely inhibit syncytium formation (data not shown). In contrast, antibodies F105 and 48d did not inhibit syncytium formation by the 582 A/T mutant glycoproteins as efficiently as they inhibited syncytium formation by the wild-type glycoproteins. Although the level of expression of the envelope glycoproteins in the transfected COS-1 cells was higher for plasmid pCMVenv than for plasmid pSVIIIenv, the results of the virus neutralization and syncytium inhibition assays did not differ (data not shown). These results suggest that the 582 A/T amino acid change confers resistance against inhibition of both virus replication and syncytium formation by antibodies directed against conserved neutralization epitopes in gp120.

Binding of anti-gp120 antibodies to the 582 A/T mutant envelope glycoproteins. The gp120 epitopes for antibodies F105 and 48d have been previously characterized (41, 42). Amino acid changes within the epitopes that render virions resistant to neutralization were shown to reduce the binding of the antibody to the envelope glycoprotein. Therefore, the effect of the 582 A/T change on F105 and 48d binding to the HIV-1 envelope glycoproteins was examined. When envelope glycoproteins from cell lysates of COS-1 cells expressing either wild-type or mutant (582 A/T) envelope glycoproteins were immunoprecipitated, no difference in the recognition of gp120 or the uncleaved precursor gp160 by antibodies F105 and 48d was observed (data not shown). To assess the binding of antibodies F105 and 48d to the multimeric envelope glycoprotein complexes on the cell surface, COS-1 cells expressing wild-type or mutant envelope glycoproteins at the surface were incubated with increasing amounts of antibody. The amount of antibody bound to cell surface envelope glycoprotein complexes at various antibody concentrations was determined to establish the binding curves shown in Fig. 2. The half-saturating concentration of antibody F105, which reflects the affinity of the antibody for the cell surface envelope glycoprotein complexes, exhibited only marginal differences between the wild-type and 582 A/T mutant glycoproteins. Relative to that

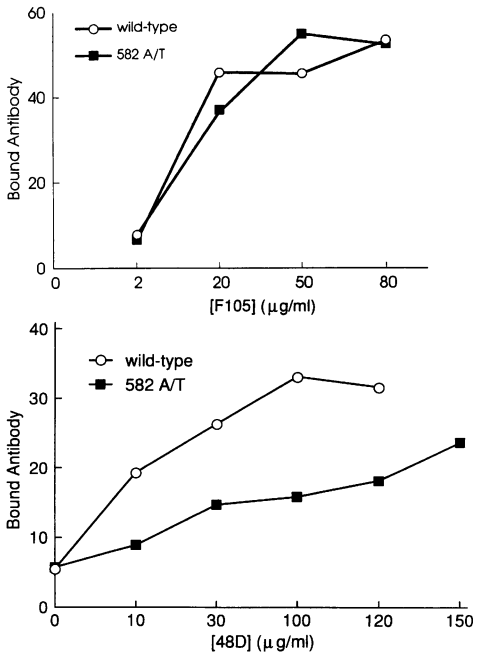

FIG. 2. F105 and 48d antibody binding to envelope glycoproteins expressed on the surface of COS-1 cells. COS-1 cells expressing either wild-type or 582 A/T mutant envelope glycoproteins were incubated with serial dilutions of antibody F105 or 48d. The amount of bound antibody at each concentration was determined as described in Materials and Methods and is expressed in arbitrary densitometric units.

of the wild-type glycoproteins, a decrease in the ratio of the 582 A/T gp120 to gp160 protein precipitated in this assay was observed for the F105 antibody, but not for the 48d or 110.4 antibodies (data not shown). This might reflect a subtle difference in the ability of the F105 antibody to recognize the fully folded gp120 form of the 582 A/T mutant and that of the wild-type glycoproteins. The differences in binding of antibody 48d were more pronounced than the differences observed for antibody F105 (Fig. 2). The observed differences for antibody 48d could be explained by a lower number of antibody binding sites, perhaps due to decreased cell surface expression of the 582 A/T mutant, as well as small decreases in antibody binding affinity.

It has been suggested that not only the affinity of an antibody for its target molecule but also the kinetics of binding may be important for successful neutralization (11). To assess the on rate of F105, this antibody was allowed to bind to wild-type or mutant envelope glycoprotein on the surface of COS-1 cells for 1, 2, 10, 30, 60, or 120 min, respectively. The amount of bound antibody increased with longer times of incubation. However, no significant difference with regard to the relative amounts of bound antibody could be observed between the wild-type and 582 A/T mutant envelope glycoproteins (Fig. 3).

Binding of antibody to envelope glycoproteins in the presence of CD4. The epitopes for both antibodies F105 and 48d are proximal to the CD4 binding site on the gp120 glycoproteins (41, 42). F105 competes with the cellular receptor for binding to the gp120 protein, whereas the binding of 48d to gp120 is enhanced in the presence of small amounts of soluble CD4 (30, 35). The binding of antibodies F105 and 48d to cell surface wild-type and 582 A/T mutant envelope glycoprotein complexes in the presence of increasing amounts of soluble CD4 was examined. In this experiment, at least part of the

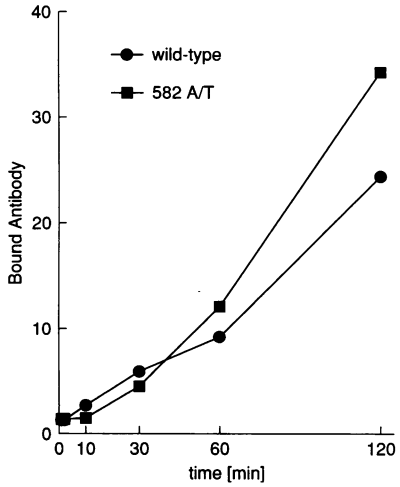

FIG. 3. Kinetics of F105 binding to the envelope glycoprotein. COS-1 cells expressing either wild-type or 582 A/T mutant envelope were incubated with radiolabeled F105 antibody for the indicated amount of time. Scanning densitometry of autoradiograms showing heavy and light chains of F105 antibody was performed to determine the relative amounts of bound antibody, which are expressed in arbitrary densitometric units.

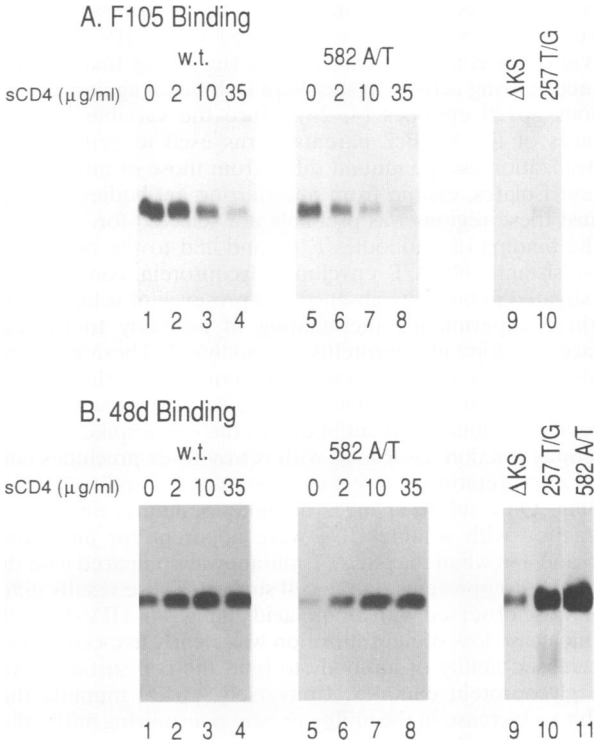

FIG. 4. Binding of antibodies F105 and 48d to envelope glycoprotein complexes in the presence of soluble CD4. COS-1 cells transfected with plasmids expressing either wild-type (w.t.) or mutant (257 T/G, 582 A/T) envelope glycoproteins or a control plasmid (pSVIIIenvΔKS) were incubated at room temperature with radiolabeled antibody (F105 or 48d) in the presence of the indicated amounts of soluble CD4 (sCD4). The data shown in lanes 9 and 10 of panel A and lanes 9 to 11 of panel B represent antibody binding in the absence of soluble CD4. Shown are the bands of the heavy chains of antibodies F105 (A) and 48d (B) as detected on SDS-polyacrylamide gels. The data shown in panel B were all derived from a single experiment. However, the data in lanes 9 to 11 were generated by a significantly longer exposure of the gels than that shown in lanes 1 to 8. The half-maximal doses of soluble CD4 for inhibition of gp120-F105 binding were 6 and 2.5 μg/ml for wild-type and 582 A/T envelope glycoproteins, respectively. For the soluble CD4-induced enhancement of gp120-48d binding, the half-maximal doses were 2 μg/ml for wild-type envelope and 4 μg/ml for the 582 A/T mutant.

small difference in binding of antibodies F105 and 48d to the mutant and wild-type envelope glycoproteins in the absence of soluble CD4 reflects the somewhat less efficient expression of the 582 A/T mutant in the transfected cells (Fig. 4A and B, lanes 1 and 5). When plasmid pSVIIIenv was used for envelope glycoprotein expression, as in the experiment shown in Fig. 4, we occasionally observed lower steady-state levels of expression for the 582 A/T mutant than for the wild-type glycoproteins. Binding of antibody F105 to the 257 T/G mutant envelope glycoprotein, which confers resistance to neutralization by that antibody, is similar to background levels of binding observed in the absence of envelope glycoproteins (Fig. 4A, lanes 9 and 10). Even though the 257 T/G mutant is more sensitive to neutralization by antibody 48d than is the 582 A/T mutant (data not shown), it is recognized less efficiently than the latter in the absence of soluble CD4 (Fig. 4B, lanes 10 and 11). In the presence of increasing amounts of soluble CD4, the binding of antibody F105 to the wild-type envelope glycoproteins was decreased. Since these experiments were conducted at room temperature, at which soluble CD4-induced shedding of the gp120 glycoprotein is less efficient, most of this decrease in F105 binding reflects direct competition of antibody binding by soluble CD4. Roughly twofold differences in the concentration of soluble CD4 required to reduce F105 binding by 50% were observed for the wild-type and 582 A/T mutant proteins. Addition of soluble CD4 resulted in an increased binding of the 48d antibody to COS-1 cells expressing the wild-type or 582 A/T envelope glycoprotein. The amount of soluble CD4 required to achieve a half-maximal increase in 48d binding was within twofold for the wild-type and 582 A/T glycoproteins. At very high soluble CD4 concentrations (35 μg/ml), the apparent decrease in 48d binding probably reflects shedding of the gp120 glycoprotein.

DISCUSSION

In vitro propagation of HIV-1 in the presence of neutralizing sera from an infected individual resulted in the generation of a variant of the virus that was resistant to 30% of sera that were able to neutralize the parental virus (33, 46). The resistant phenotype was due to a mutation in the envelope gene giving rise to a 582 A/T change gp41 (31). Here we show that the 582 A/T change in gp41 results in the relative resistance of both virus entry and syncytium formation to inhibition by antibodies that recognize gp120. The 582 A/T mutant escapes from neutralization by several monoclonal antibodies directed against conserved, discontinuous epitopes in gp120. Antibodies targeted against the CD4 binding site as well as those whose binding is enhanced by the CD4-gp120 interaction were less efficient at inhibiting the function of the 582 A/T mutant relative to that of the wild-type glycoprotein. The 582 A/T mutant was neutralized by monoclonal antibodies recognizing the V2 or V3 variable gp120 region or by antibody 2F5, which recognizes gp41. The sensitivity of the 582 A/T mutant to soluble CD4 did not differ from that of the wild-type virus. Thus, the neutralization escape by the 582 A/T mutant appears to be specific for the group of anti-gp120 antibodies recognizing relatively conserved, discontinuous epitopes. The resis-

tance of the 582 A/T mutant virus to neutralization by a substantial percentage of sera derived from HIV-1-infected individuals is consistent with reports suggesting that much of the neutralizing activity in such sera is directed against discontinuous gp120 epitopes (4, 26). Since the variable loop sequences of the HXBc2 parental virus used to generate the neutralization escape mutant differ from those of most HIV-1 patient isolates, escape from neutralizing antibodies directed against these regions was probably not selected for.

The binding of antibodies F105 and 48d to the neutralization-resistant 582 A/T envelope glycoprotein complex was investigated in both the absence and presence of soluble CD4. In these experiments, the binding of antibody to the cell surface envelope glycoproteins was analyzed. These measurements should be relevant to the inhibition of syncytium formation by the antibodies. They may also be instructive with respect to the binding of antibodies to the virion spike. The low infectious fraction associated with retroviruses precludes confident interpretation of direct measurements of antibody-virion binding. Only subtle changes in binding affinity, on rate, or interaction with soluble CD4 were apparent for antibodies F105 and 48d when the 582 A/T mutant was compared with the wild-type glycoproteins on the cell surface. These results differ from those observed with amino acid changes in HIV-1 gp120, in which case loss of neutralization was clearly associated with a decreased ability of antibody to bind the cell surface envelope glycoprotein complex. Conversely, gp120 mutants that exhibit an increase in the ability to bind neutralizing antibodies relative to the wild-type glycoproteins were also more sensitive to neutralization (39, 41, 42). For viruses other than HIV-1, loss of sensitivity to neutralization has been observed in some cases, even when the binding of the neutralizing antibody to the virus is retained (for a review, see reference 8). For such Newcastle disease virus variants, particles that remain infectious despite the presence of attached mouse monoclonal antibodies can be neutralized by the addition of rabbit anti-mouse immunoglobulin G (16). Neutralization did not result from addition of anti-human immunoglobulin G to 582 A/T virions preincubated with antibody F105 (data not shown). In addition, interference between antibodies that bind near the CD4 binding site and that fail to neutralize the 582 A/T mutant and competing antibodies that retain neutralizing ability for the mutant was not observed (38a). Thus, the available data do not allow us to conclude definitely that the mutant virions infect cells efficiently despite the presence of bound antibody.

The 582 A/T mutant specifically escapes from neutralizing antibodies directed against gp120 regions related to the CD4 binding site but not from soluble CD4 itself or from other neutralizing antibodies. The simplest physical explanation for such a specific escape is a proximity, on the intact gp120-gp41 complex, of alanine 582 and elements shared by the discontinuous, conserved gp120 neutralization epitopes. The 582 A/T change may introduce very subtle changes in the epitopes that are only marginally detectable by our assays but significantly alter neutralization.

The alanine at position 582 is highly conserved among HIV-1 isolates (28). This finding suggests that while the alteration of this residue was selected in a tissue culture setting, such a selection would not be favored in vivo. The lower replicative and syncytium-forming abilities of the 582 A/T mutant than of the wild-type glycoprotein probably account for this (46). Amino acid changes in gp120, presumably near the binding sites for the antibodies, have been previously shown to allow escape from neutralizing monoclonal antibodies directed against the CD4 binding site or the CD4-induced epitopes. In several cases, these amino acid changes exerted little effect on the efficiency of virus entry or syncytium formation (39, 41). Why did none of these changes, apparently preferred with respect to viral replicative ability, appear under the selective pressure of the HIV-1-infected patient serum? The neutralizing antibodies that compete with CD4 for gp120 binding represent a group of antibodies with different complementarity-determining region sequences and overlapping but distinct epitopes, as evidenced by different sensitivities to gp120 amino acid changes (39–42). Since the selecting serum presumably contained a mixture of different members of this antibody group, a change in gp41 probably represented the optimal alternative for simultaneous escape from this mixture. It is encouraging that this optimal alternative in tissue culture is unlikely to favor in vivo replication. This is consistent with the conservation of alanine 582 in natural HIV-1 isolates (28). Populations of HIV-1 present in infected individuals have been shown to be resistant to neutralization by the homologous serum (1, 12). In vivo selection by serum neutralizing antibodies may also be associated with a compromised viral replicative ability, through changes in amino acids other than alanine 582. An understanding of the mechanisms of natural escape from serum neutralizing ability will require further studies.

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