Increased Affinity and Stability of an Anti-HIV-1 Envelope Immunotoxin by Structure-based Mutagenesis*

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HIV-infected cells are selectively killed by an immunotoxin in which a truncated form of Pseudomonas exotoxin A is joined to the variable region of a broadly neutralizing antibody (3B3) that recognizes the viral envelope glycoprotein (Env). To improve the efficacy of this molecule, we used three-dimensional structural information and phage selection data to design 23 single and multiple point mutations in the antibody variable region sequences that contact Env. Substituting an aromatic residue for an aspartate in the third complementarity-determining region of VH increased the potency of the immunotoxin by ~10-fold in a cell-killing assay. Detailed analysis of one such mutant, N31H/Q100EY, revealed both a higher affinity for monomeric and cell surface Env and an increased stability against aggregation compared with the starting immunotoxin. Conversion to a disulfide-linked two-chain format further stabilized the protein. N31H/Q100EY retained the ability to bind to Env from multiple viral isolates, to inhibit Env-mediated cell fusion, and to limit spreading viral infection in peripheral blood mononuclear cells. Such site-directed mutants may increase the utility of immunotoxins for reducing or eradicating persistent HIV-1 infection in humans.

Human immunodeficiency virus type 1 (HIV-1) is a persistent virus. Although highly active antiretroviral treatment (HAART) can reduce circulating virus to below the limits of detection and partially restore immune function in many individuals, the suppression of viral replication is not permanent. When HAART is ceased, the virus almost invariably rebounds to high levels due to the existence of long-lived viral reservoirs including persistently infected macrophages, latently infected CD4+ memory T-cells, thymocytes, microglia, seminal cells, and natural killer cells (1–5). In view of the cumulative toxicity and cost of HAART, as well as the potential for the development of drug-resistant virus, it is important to develop strategies to reduce or eliminate such viral reservoirs. Immunotoxins that recognize and kill cells expressing the viral envelope glycoprotein (Env), which is displayed on the surface of many HIV-1-infected cell types, are logical candidates for this purpose (6–10).

For such immunotoxins to be clinically useful, they must bind with high affinity and specificity to a region of Env that is conserved between different viral isolates. They should also display suitable pharmacokinetic properties including stability in the circulation and minimal nonspecific toxicity. Recently Bera et al. (11) described a recombinant fusion protein that begins to meet these criteria. This molecule, termed 3B3(scFv)-PE, consists of a single-chain variable region construct (scFv) of human Fab 3B3 joined to a truncated version of Pseudomonas exotoxin A. The 3B3 Fab recognizes the highly conserved CD4-binding site of gp120, the external subunit of Env (12). 3B3 was derived by mutagenesis of b12, a Fab isolated from a combinatorial phage display library constructed from bone marrow RNA of an HIV-1 infected long-term nonprogenitor. b12 and 3B3 tightly bind to and neutralize a broad range of laboratory strains and primary isolates of HIV-1 (12–16).

The toxic portion of 3B3(scFv)-PE consists of the translocation and adenosine diphosphate-riboseylation domains of a potent toxin produced by Pseudomonas aeruginosa. The cell-binding portion of the toxin has been deleted, thereby making it independent on the antibody scFv for cell killing. Several anti-cancer immunotoxins based on this 38-kDa-truncated version of Pseudomonas exotoxin (PE38) have demonstrated efficacy in preclinical studies in animals and in human clinical trials (17–19).

The aim of the present study was to improve the biological activity of 3B3(scFv)-PE by increasing its affinity for Env while retaining its broad cross-reactivity to gp120 from multiple viral isolates. We were aided in this endeavor by the recent x-ray crystallographic structure determination of IgG1b12 and a docking model for its interaction with gp120 (20). This information, along with previous mutagenesis experiments (12, 14), allowed us to identify the antibody residues that contribute to gp120 binding. Here we describe the isolation of a 3B3(scFv)-PE variant with increased affinity for cell surface Env and biological potency. This molecule, and a disulfide-linked version derived from it, also display increased biochemical stability.

EXPERIMENTAL PROCEDURES

Mutagenesis and DNA Sequencing—Single-chain immunotoxins were derived from the previously described 3B3(scFv)-PE construct shown in Fig. 1 (11). Individual amino acid substitution mutants were constructed using the QuikChange XL site-directed mutagenesis kit (Stratagene), and multiple changes were introduced using double-stranded oligonucleotides. A disulfide-linked (dsFv) immunotoxin was expressed from two 3B3 plasmids, one carrying the triple mutation R44C/N31H/Q100EY in VH linked to PE and the second carrying the...
mutation Q100,C in V. Mutants are designated as XnnnY or XmmmY, where X is the original amino acid, Y is the new amino acid, and nnn or mmm is the position in the heavy or light chain, respectively, in the Brookhaven Protein Data Base file 1HZH (20). All constructs were verified by DNA sequencing using the dRhodamine terminator cycle sequencing kit (PE Biosystems) and an ABI 310 Genetic Analyzer.

**Expression and Purification of Immunotoxins**—The immunotoxin constructs were introduced into *Escherichia coli* BL21(DE3), and fresh transformants were grown to mid-log phase and induced with isopropyl-1-thio-β-D-galactopyranoside. Inclusion bodies were prepared and immunotoxins were extracted, reduced and denatured, refolded, and purified by ion exchange and size exclusion chromatography to greater than 95% homogeneity as determined by SDS-gel electrophoresis (11).

**Cytotoxicity Assay**—Cytotoxicity of immunotoxins was measured by the MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide) oxidation procedure using Cell Proliferation Kit I (Roche Diagnostics). ENV15 cells, a stable CHO transformant that expresses HIV-1 Env, and control CHO cells transformed with the empty expression vector, were seeded in 96-well tissue culture plates at 10⁴ cells/well. After 8 hr at 37°, various concentrations of immunotoxin were added to duplicate wells, and incubation was continued for 48 h. Then 0.01 ml of the MTT reagent was added, and 4 h later the reaction was terminated with 0.1 ml of solubilization solution. Following overnight incubation at 37° the absorbance at 595 nm was determined in a GENios Teakan microplate reader. The concentration of immunotoxin giving a 50% inhibition of cell viability (IC₅₀) was calculated by fitting the data with Prism graph software.
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**gp120 Binding**—Binding of immunotoxins to monomeric gp120 was measured by surface plasmon resonance using a BIAcore 2000 Biosensor. Purified recombinant gp120 from HIV-1 strains MN, SF2 (AIDS Research and Reference Reagent Program), or LAI deleted for the V3 loop (Progenics) was immobilized on a CM5 chip by amine coupling. Immunotoxins at concentrations of 0.1–10 μg/ml were applied to the chip for 3 min at 10 μl/min and then allowed to dissociate for 15 min. Binding kinetics were evaluated with BIAevaluation software version 3.1.

Binding of immunotoxins to trimeric gp120 on the cell surface was evaluated by FACS. HeLa cells were infected at a multiplicity of 10 plaque-forming units per cell with vaccinia virus recombinants carrying the LAV, IIIb, BaL, or JR-FL env gene or the WR vector (21) and incubated for 16 h at 31°C. The cells were harvested by centrifugation and incubated sequentially with immunotoxin (10 μg/ml), mouse anti-PE monoclonal antibody m40-1 (1,500), and FITC-conjugated goat anti-mouse IgG (1:20, Jackson Laboratories). Cell-bound FITC-labeled antibody was quantitated as the median fluorescence for 20,000 cellular events determined on a FACS Calibur (Beckton Dickinson) using CELLQuest software.

**Biochemical Stability of Immunotoxins**—Freshly purified immunotoxins were diluted to 20 μg/ml in PBS and incubated at 37°C for various intervals. Samples of 0.3 ml were analyzed by AKTA FPLC gel exclusion chromatography on a 60 cm × 7.5 mm TSK G3000SW column (TosoHaas) run at 0.5 ml/min in PBS.

**Cell-Fusion Assay and Spreading Infection in Human Peripheral Blood Mononuclear Cells (PBMC)**—Fusion between Env-expressing cells and receptor-expressing cells was assayed as described (21). To generate effector cells, HeLa cells were infected at a multiplicity of 5 plaque-forming units per cell with a mixture of vaccinia virus recombinants, one containing an HIV-1 env gene (LAV, IIIb, BaL, or JR-FL) and the other containing the T7 RNA polymerase gene. To generate target cells, HeLa cells were infected with two vaccinia virus recombinants, one containing the CD4 gene and the other containing the lacZ gene linked to the T7 promoter; when BaL or JR-FL Env was analyzed, the target cells were also infected with a vaccinia virus recombinant containing the CCR5 gene. After 16 h at 31°C, the effector cells were incubated at 37°C for 1 h with immunotoxin and then mixed with the target cells and incubated an additional 2.5 h at 37°C. The extent of cell fusion was determined by assaying β-galactosidase activity.

The ability of immunotoxins to inhibit spreading infection in PBMC was assayed by infecting phytohemagglutinin-stimulated PBMC from HIV seronegative donors with HIV-1(LAV) (120 pg of p24 per 100,000 cells) for a total of 14 days with replacement of medium and immunotoxin every 3 to 4 days (22, 23) Viral production was determined by measuring p24 production in the culture supernatant (Beckman Coulter HIV-1 p24 antigen assay kit).

**Protein Modeling**—The atomic coordinates for the docked model of IgG1b12 Fv with gp120 were kindly provided by Erica Ollmann Saphire and Ian Wilson (Scripps Research Institute). The Discover 3 package of the LAV, IIIb, BaL, or JR-FL) and env gene or WR vector (21) and incubated for 16 h at 31°C. The cells were harvested by centrifugation and incubated sequentially with immunotoxin (10 μg/ml), mouse anti-PE monoclonal antibody m40-1 (1,500), and FITC-conjugated goat anti-mouse IgG (1:20, Jackson Laboratories). Cell-bound FITC-labeled antibody was quantitated as the median fluorescence for 20,000 cellular events determined on a FACS Calibur (Beckton Dickinson) using CELLQuest software.

**RESULTS**

**CDR H3 Mutants: Effect of an Aromatic Substitution**—The crystal structure of IgG1b12, the parent antibody of 3B3, reveals an unusually long finger-like loop in CDR H3. Docking calculations suggest that this protrusion penetrates the recessed CD4 binding site of gp120 with tryptophan 100 at the apex of the loop occupying roughly the same position as the phenylalanine residue at position 43 of CD4 (20). Mutagenesis experiments have confirmed the importance of CDR H3 residues in the interaction between antibody and gp120 (12, 14).

Therefore, our initial mutagenesis experiments focused on this portion of the immunotoxin.

The biological potency of the mutant immunotoxins was determined by a cytotoxicity assay on cells that constitutively express Env from a stably transfected gene. As shown in Fig. 2, the starting 3B3 immunotoxin killed Env− cells with an IC50 of 5.7 ng/ml but had no effect on control Env− cells up to 670 ng/ml. The sequences and cytotoxicity results for all 23 of the mutants characterized in this work are summarized in Fig. 1. We first altered CDR H3 residues 100a to 100e from DDSQPQ to EMFRY to recreate the previously described phage variant H3.33, which was reported to display ∼7-fold increased binding to monomeric gp120 (14). This quintuple mutant, which alters the carboxyl-terminal portion of the CDR H3 loop, reproducibly decreased the IC50 of the immunotoxin to 1.8 ng/ml, a 3-fold increase in potency. Therefore, we tested the effect of each individual position in this region, using the same mutations present in the previously described H3.33 variant. The mutants at positions 100a to 100d all had IC50 values greater than 1 ng/ml whereas the substitution at position 100e gave a clear increase in potency to an IC50 value of 0.67 ng/ml (Fig. 1).

The Q100eY substitution replaces an amide residue with an aromatic residue. To test the effect of other aromatic substitutions at this position, mutants Q100eF and Q100eW were also constructed and tested. As shown in Fig. 2, both of these aromatic substitutions also had IC50 values less than 1 ng/ml, indicating a significant improvement over the starting molecule.

A mutation just downstream of the critical 100e residue, D100F, had no effect on the cytotoxic potency of the immunotoxin. The triple mutant CDR3-b12, which altered the three residues just upstream of the apex of the CDR3 loop from the 3B3 sequence EWF to the original IgG1b2 sequence PYS, also had no significant effect.

**CDR HH1, H2, and Multiple Mutants**—Previous mutagenesis experiments selected for either Asn or His at position 31 in CDR H1, suggesting a functional role for a side-chain amino group at this position (14). Furthermore, the crystal structure of IgG1b12 shows that this residue is located at the top of a canyon formed by CDR H1 and H3, well poised to contact gp120 at position 97 of CDR H3 was altered to Arg to potentially interact...
with the acidic gp120 residues Asp-368 or Glu-370. However, none of these mutations significantly lowered the IC<sub>50</sub> of the N31H/Q110E-Y immunotoxin.

Protein modeling suggests that the Tyr at position 100h of CDR H3 is on the same face as the new Tyr residue in the N31H/Q110E-Y mutant and forms a hydrogen bond with it as well as multiple packing interactions with gp120 (see below). As anticipated from this analysis, mutating Tyr-100h to Ala resulted in a clear decrease of cytotoxic potency from an IC<sub>50</sub> of 0.25 ng/ml to an IC<sub>50</sub> of 6.3 ng/ml (Fig. 1).

**CDR L2 and L3 Mutants**—The light chain CDR L2 has not been previously mutagenized, and the docked structure of IgG1b12 shows a paucity of contacts to Env in this region of the molecule. We mutated Ser-56L to both Met and Leu in the hope that this would fill in the “gap” in the complex, possibly by hydrophobic interaction with Leu-125 of gp120. We also mutated Ala-93L in CDR L3 to Ser in the hope that it would hydrogen bond to one of several potential acceptor sites in gp120. However, none of these mutants had improved IC<sub>50</sub> values compared with the starting N31H/Q110E-Y construct.

**Conversion of N31H/Q100E-Y to a Disulfide-linked Fv**—Many scFvs can be improved by conversion to a two-chain disulfide-linked format in which the Fv heterodimer is stabilized by a disulfide bond engineered between structurally conserved framework positions of VH and VL rather than a peptide linker (24). Accordingly, the N31H/Q100E-Y variant was further mutagenized to convert Arg-44 and Gln-100L to Cys and then refolded in a redox-shuffling buffer, the disulfide-linked form of N31H/Q100E-Y also showed a higher affinity than the initial immunotoxin for the various gp120 preparations, in this case by 1.2- to 1.7-fold.

**Binding to Monomeric gp120**—Subsequent experiments focused on N31H/Q100E-Y(scFv)-PE because it had the lowest IC<sub>50</sub> of all 23 immunotoxins analyzed in this work. Surface plasmon resonance was used to determine the affinity of this molecule, together with the starting 3B3(scFv)-PE immunotoxin and several other variants, for three different recombinant gp120 preparations differing in the HIV-strain of origin and the presence or absence of the V3 loop of the protein. As shown in Table I, the starting 3B3 immunotoxin bound about 5-fold more tightly to SF2 and LAI V3 gp120 than to MN gp120. A similar difference in binding of 3B3 Fab to gp120 from HIV-1<sub>HXB</sub> (which is nearly identical to HIV-1<sub>LAI</sub>) was noted previously (12). The affinity of the N31H/Q100E-Y mutant for gp120 was 2-fold greater than 3B3 for MN gp120 and 1.7-fold greater for SF2 and LaiΔ V3 gp120. Although these differences were small, they were highly reproducible for different immunotoxin preparations and different gp120 chips. The disulfide-linked form of N31H/Q100E-Y also showed a higher affinity than the initial immunotoxin for the various gp120 preparations, in this case by 1.2- to 1.7-fold.

Several multiple mutants were also analyzed. Compared with the original 3B3, H3.33 was previously reported to have an 8-fold increased affinity for IIb gp120 as an Fab (14); in our experiments, H3.3 bound to MN gp120 with 4-fold higher affinity, to SF2 gp120 with 30-fold higher affinity, and to LaiΔ V3 gp120 with 24-fold higher affinity. The CDR H3 variant N31H/Q100E-Y(ΔH3.33) had decreased biological activity compared with N31H/Q100E-Y, displayed reduced binding to all gp120 samples tested; its affinity was 2.2-fold lower for MN, 37-fold lower for SF2, and 36-fold lower for LaiΔ V3 gp120. By contrast, the multiple mutant CDR3-b12 bound to MN and SF2 gp120 with approximately the same affinity as the starting 3B3 immunotoxin; only with LaiΔ V3 gp120 was there significantly (5-fold) lower binding.

In summary, there was a general correspondence between the biological activity of the immunotoxins and their binding to monomeric gp120, but the relationship was not linear. In particular, N31H/Q100E-Y(scFv)-PE bound to envelope glycopro-

| Immunotoxin | gp120 | k<sub>x</sub> <i>K</i> M × 10<sup>-9</sup> | k<sub>x</sub> <i>K</i> s × 10<sup>-3</sup> | K<sub>app</sub> M × 10<sup>-9</sup> |
|------------|-------|-----------------|-----------------|-----------------|
| Wild-type 3B3 | MN | 3.8 | 1.1 | 28 |
| N31H/Q100E-Y | MN | 9.3 | 1.3 | 14 |
| N31H/Q100E-Y (dsFv) | MN | 4.9 | 1.8 | 18 |
| H3.33 | MN | 8.0 | 0.53 | 6.7 |
| N31H/Q100E-Y/Y100hA | MN | 3.2 | 2.0 | 62 |
| CDR3-b12 | MN | 4.1 | 1.2 | 29 |
| Wild-type 3B3 | SF2 | 4.0 | 0.24 | 6.0 |
| N31H/Q100E-Y | SF2 | 6.1 | 0.21 | 3.5 |
| N31H/Q100E-Y (dsFv) | SF2 | 6.1 | 0.24 | 4.6 |
| H3.33 | SF2 | 6.3 | 0.013 | 0.2 |
| N31H/Q100E-Y/Y100hA | SF2 | 4.9 | 10 | 220 |
| CDR3-b12 | SF2 | 9.6 | 42 | 4.4 |
| Wild-type 3B3 | LAI ΔV3 | 4.1 | 0.24 | 5.9 |
| N31H/Q100E-Y | LAI ΔV3 | 6.1 | 0.21 | 3.5 |
| N31H/Q100E-Y (dsFv) | LAI ΔV3 | 6.2 | 0.30 | 4.8 |
| H3.33 | LAI ΔV3 | 6.4 | 0.016 | 0.25 |
| N31H/Q100E-Y/Y100hA | LAI ΔV3 | 4.9 | 11 | 210 |
| CDR3-b12 | LAI ΔV3 | 4.4 | 1.5 | 31 |

**TABLE I**

**Affinity of immunotoxins for monomeric gp120 determined by surface plasmon resonance (BIACore)**

**FIG. 3.** FACS analysis of immunotoxin binding to the cell surface. HeLa cells were infected with vaccinia virus recombinants carrying the indicated HIV-1 env gene or with vector alone. Immunotoxin binding was determined by FACS using a mouse anti-PE monoclonal antibody and FITC-conjugated goat anti-mouse IgG. The median fluorescence for 20,000 cellular events is shown.
tein only 1.7- to 2-fold more efficiently than the starting molecule yet was at least 10-fold more potent in the cell killing assay.

Binding to Cell Surface Env—The ability of the 3B3 and N31H/Q100eY(scFv)-PE immunotoxins to bind to Env on the cell surface was evaluated by FACS experiments on cells infected with vaccinia virus recombinants expressing various env genes. After 16 h at 31°C the cells were then fused for 2.5 h with target cells infected with vaccinia virus recombinants carrying the CD4 gene and the T7 RNA polymerase gene (and the CCR5 gene for the BaL and JR-FL Env-infected effector cells) and were lysed and assayed for β-galactosidase activity. Shown are the percent β-galactosidase levels compared with control wells with no added immunotoxin.

Biochemical Stability—It was puzzling that the N31H/Q100eY(scFv)-PE immunotoxins were 15- to 23-fold more potent than the parental molecule in...
the cytotoxicity assay yet bound to gp120 only 1.5- to 2.0- fold strongly in both the BIAcore and FACS assays. Cell-killing experiments in which the duration of exposure to immunotoxin was varied suggested that this discrepancy might be due to differences in the stability of the immunotoxins (results not shown).

To directly test the effect of the N31H/Q100eY substitutions on biochemical stability, the 3B3(scFv)-PE, N31H/Q100eY(scFv)-PE, and N31H/Q100eY(dsFv)-PE immunotoxins were incubated in PBS at 37 °C for various periods of time and then analyzed by size-exclusion chromatography, which can separate the active monomeric form of the immunotoxin from inactive aggregated forms. Fig. 4 shows that there were indeed substantial differences in the stability of the three immunotoxin molecules. 3B3(scFv)-PE was quite prone to aggregate under these conditions; it was 45% aggregated within 4 h and more than 95% aggregated by 36 h. The N31H/Q100eY(scFv)-PE immunotoxin was more stable, showing less than 15% aggregation at 4 h and 50% aggregation at 60 h. Conversion of N31H/Q1200eY to a disulfide-linked format further stabilized the molecule; this immunotoxin displayed less than 15% aggregation even after 60 h of incubation. Estimated half-lives for the monomeric forms of the immunotoxins were 4.5 h for 3B3(scFv)-PE, 62 h for N31H/Q100eY(scFv)-PE, and greater than 300 h for N31H/Q100eY(dsFv)-PE.

To confirm that the species identified by size exclusion chromatography were functionally distinct, BIAcore and cytotoxicity experiments were performed on the separated monomeric and aggregated forms of N31H/Q100eY(scFv)-PE. As shown in Fig. 5, the aggregated form did not detectably bind to immobilized gp120 under the conditions employed in the BIAcore experiment and showed little cytotoxic activity on Env/IIIB cells. These results indicate that the increased biological activity of N31H/Q100eY(scFv)-PE is due to a combination of increased gp120 binding and biochemical stability.

**Inhibition of Cell Fusion and Spreading Infection**—The biological activity of N31H/Q100eY-PE was further tested by inhibition of Env-mediated cell-cell fusion and spreading infection of PBMC. In the cell fusion assay (21), effector cells expressing an env gene and a T7 promoter-β-galactosidase gene were treated with immunotoxin and then tested for fusion with effector cells expressing receptors and T7 polymerase. Fig. 6 shows that the N31H/Q100eY(scFv)-PE at 200 ng/ml was capable of inhibiting β-galactosidase activity by >80% in this assay for four different species of Env: LAV, IIIb, BaL, and JR-FL. Moreover, in each case the mutant immunotoxin was as or more effective than the initial version of the protein at the same concentration. A control experiment using 3B3 linked to an enzymatically inactive point mutant of the Pseudomonas exotoxin (3B3-PE\textsubscript{Asp553}) demonstrated that the inhibition of cell fusion was dependent on the cytotoxic activity of the immunotoxin and did not reflect direct neutralization by the antibody moiety.

For the spreading infection assays, PBMC from healthy human donors were infected with the LAV strain of HIV-1 and incubated in the absence or presence of immunotoxins for a total of 14 days. Fig. 7 shows that the N31H/Q100eY(scFv)-PE immunotoxin inhibited viral spread, as assayed by p24 production, in a dose-dependent manner. The extent of inhibition at day 7, the peak of viral production, was 38% at 3 ng/ml, 46% at

![FIG. 7. Inhibition of spreading infection of PBMC by N31H/Q100eY(scFv)-PE. PBMC from healthy human donors were infected with HIV-1 and incubated in the presence of 0, 3, 33, 325, or 3250 ng/ml of N31H/Q100eY(scFv)-PE or with 325 ng/ml of 3B3(scFv)-PE\textsubscript{Asp553} for a total of 14 days. Viral replication was assayed by ELISA of p24 in the culture fluid.](image-url)

![FIG. 8. Protein modeling. The predicted structures of the gp120 complexes with 3B3 (A) and N31H/Q100eY (B) are shown. Ribbons are colored green for gp120, yellow for the 3B3 heavy chain, and red for the 3B3 light chain. Tyr-100 (which inserts into the CD4-binding site), residue 100e (which is Gln for 3B3 and Tyr for N31H/Q100eY) and residue 31 (which is Asn for 3B3 and His for N31H/Q100eY) are rendered as CPK models. C, details of the predicted hydrogen-bonding of Tyr-100e in CDR H3 with Tyr-100h of CDR H3 and with Ser-365 and Asp-457 of gp120. All structures were minimized by 1000 iterations of steepest descent and 1000 iterations of conjugate descent.](image-url)
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...33 ng/ml, 70% at 325 ng/ml, and 82% at 3250 ng/ml. The parental immunotoxin 3b3(scFv)-PE had a similar potency (data not shown). The enzymatically inactive control immunotoxin 3B3-PE... displays negligible activity, thereby demonstrating that the inhibition resulted from the cytotoxic action of the immunotoxin rather than from simple neutralization by the 3B3 component.

Protein Modeling.—The structures of 3B3(scFv) and N31H/Q100E(scFv) complexed with Env were modeled by amino acid replacement on the published IgG1b12-gp120 docked structure (20) followed by energy minimization (1000 iterations by steepest descent followed by 1000 iterations by conjugate descent). Fig. 8, A and B show that the amino acid replacements in the mutant protein are not predicted to change the overall folding patterns of CDR H1 or H3. Interestingly, however, a detailed examination of the Env interface reveals that the hydroxyl oxygen of Tyr100e in the mutant protein has the potential to form a new hydrogen bond with Asp-457 of gp120 (Fig. 8C).

This predicted hydrogen bond has an H–O length of 1.69 Å and an O–H–O angle of 171 degrees, consistent with significant stabilization of the complexed structure. Tyr-100e also hydrogen bonds to both Tyr-100h of the VH region, which occupies the same face of the CDR H3 loop, and to Ser-365 of gp120; these contacts are also made by 3B3.

Discussion

The aim of this work was to improve the biological activity of the 3B3-PE immunotoxin by increasing its affinity for Env while preserving its cross-reactivity to various strains of HIV-1. Our main finding is that an aromatic substitution at position 100e in the extended third CDR of the heavy chain of 3B3 increases potency in a cell killing assay by 5- to 20-fold. Detailed analysis of N31H/Q100EY, in which the aromatic substitution in CDR H3 is combined with a mutation in CDR H1, showed that it retained the ability to interact with Env subtypes derived from multiple strains of HIV-1 (IIIB, LA7, MN, SF2, BaL, and JR-FL). The mutant also maintained the ability to inhibit Env-mediated cell-cell fusion and viral spread; in both cases, the activity depended on specific killing of the Env-expressing cells mediated by the PE moiety of the immunotoxin. Unexpectedly, the increased biological activities of this molecule appeared to be due to a combination of increased affinity for Env and greater biochemical stability.

N31H/Q100EY binds to both monomeric and cell-surface Env with 1.5 to 2-fold better affinity than the starting 3B3 antibody. Protein modeling suggests that this may be due to the formation of a new hydrogen bond between Tyr-100e and an Asp residue at position 457 of gp120. Close contact between this region of Env and 3B3 is supported by previous alanine-scan-mutations of gp120 (20). Moreover, an alanine substitution at position 100h of 3B3, which in the crystal structure of IgG1b12 is a close neighbor to residue 100e and in the docked structure contacts the same region of gp120, resulted in dramatically decreased binding to gp120 and biological activity. By contrast, a mutation at position 100f, which in the crystal structure is facing in the opposite direction from the CDR H3 loop and does not contact gp120, had no effect. However, it should be emphasized that the precise atomic details of the contacts between gp120 and the antibodies are speculative since neither 3B3 itself nor the complex with gp120 have been crystallized. Furthermore, the published docking model is based on the structure of gp120 bound to CD4, which may be different than either the free or antibody-bound forms of the molecule.

The N31H/Q100EY(scFv)-PE mutant had increased biochemical stability as well as affinity for gp120. This is probably due to increased hydrophobic interactions at the VH–VL heterodimer interface since other aromatic substitutions at position 100e had a similar stabilizing influence (data not shown). Furthermore, tethering the VH and VL subunits by an engineered disulfide bond completely stabilized the molecule against in vitro aggregation for up to 60 h, as has been found for many Fv molecules (24). Interestingly, the starting immunotoxin, 3B3(scFv)-PE, displayed rapid aggregation and loss of gp120-binding and biological activity when incubated at 37° in PBS, even though previous experiments showed that it retained cytotoxic activity when incubated for 24 h at 37° in 0.2% human serum albumin (11). It will be important in future animal and human studies to analyze the stability of both the starting and mutated scFvs and dsFvs in the circulation.

The current work was predicated on the idea that increases in the affinity of 3B3-PE for Env would translate into increases in biological potency, as has been observed for many cancer immunotoxins (25). Although this was generally the case, the relationship was by no means linear; for example, the multiple replacement mutant H3.33 had a lower IC50 for gp120 than did the single substitution mutant Q100eY yet had a higher IC50 in a cell-killing assay. Surprisingly, the CDR3-b12 mutant, which recreated the CDR3 sequences of IgG1b12, had the same biological potency as 3B3, even though the latter was reported to exhibit 10-fold greater affinity for monomeric gp120 when expressed as a phage Fab (12). One possible explanation for this finding is that b12 and 3B3 are more similar to one another when expressed as scFvs, an hypothesis supported by our BIAcore affinity measurements.

One concern in any mutagenesis of an anti-Env antibody, given the tremendous sequence variation of HIV-1, is that increased affinity or biological activity against one variant of the envelope glycoprotein could actually lead to decreased activity against a different variant, as has been found for some phage-selected mutants (12, 14). This does not appear to be the case for N31H/Q100EY, at least for the several different clade B Env that were examined in the present work by BIAcore, FACS, and cell-cell fusion assays. More divergent forms of Env remain to be tested.

3B3-PE is one of several different immunotoxins being considered for clinical use in HIV-1-infected persons to reduce or eliminate the viral reservoirs that remain after HAART (6–10). In view of the scarcity and long half-lives of latently infected cells, it is especially important to develop drugs that display good stability and high affinity for Env. Although 3B3-PE has been shown to be more potent than a previously described CD4-based immunotoxin (11), and to be capable of augmenting the activity of HAART in thy/liv-SCID-Hu mice (26), there are still many potential obstacles to overcome such as nonspecific toxicity, immunogenicity, instability, the high mutation rate of the virus, and the possible necessity to induce the expression of latent provirus. Structure-based mutagenesis of the sort described here will play an important role in generating the sophisticated molecular therapeutics needed to overcome these obstacles and successfully treat persistent HIV-1 disease.

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