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Recognition Properties of a Panel of Human Recombinant Fab Fragments to the CD4 Binding Site of gp120 That Show Differing Abilities To Neutralize Human Immunodeficiency Virus Type 1

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Six recombinant human Fab fragments that were derived from the same human immunodeficiency virus type 1 (HIV-1)-infected individual and are directed against the CD4 binding site (CD4bs) of the gp120 envelope glycoprotein were studied. A range of neutralizing activity against the HIV-1 (HXBc2) isolate was observed, with Fab b12 exhibiting the greatest potency among the Fabs tested. The neutralizing potency of Fab b12 was better than that of monoclonal whole antibodies directed against the third variable (V3) region of gp120. To explore the basis for the efficient neutralizing activity of b12, the recognition of a panel of HIV-1 gp120 mutants by the six Fabs was studied. The patterns of sensitivity to particular gp120 amino acid changes were similar for all six Fabs to those seen for anti-CD4bs monoclonal antibodies derived from HIV-1-infected individuals by conventional means. In addition, recognition by Fab b12 demonstrated an atypical sensitivity to changes in the V1 and V2 variable regions. Next, the binding of the Fabs to monomeric gp120 and to the envelope glycoprotein complex was examined. Neither the binding properties of the b12 Fab to monomeric gp120 nor the ability of the Fab to compete with soluble CD4 for monomeric gp120 binding appeared to account for the greater neutralizing potency. However, both quantitative and qualitative differences between the binding of b12 and that of less potent Fabs to the cell surface envelope glycoprotein complex were observed. Relative to less potently neutralizing Fabs, Fab b12 exhibited a higher affinity for a subpopulation of cell surface envelope glycoproteins, the conformation of which was best approximated by the mature gp120 glycoprotein. Apparently, subtle differences in the gp120 epitope recognized allow some members of the group of anti-CD4bs antibodies to bind to the functionally relevant envelope glycoprotein complex and to neutralize virus more efficiently.

Antibody is capable of neutralizing human immunodeficiency virus type 1 (HIV-1) in vitro and in affording protection against viral challenge in vivo in chimpanzees (6, 15, 16, 18, 20). All of the neutralizing activity in human sera has been associated with reactivity to the envelope glycoproteins gp120 and gp41 and in particular to the third hypervariable domain (V3 loop) and CD4 binding site (CD4bs) of gp120 (for reviews, see references 26, 30, 31, 35, 36, and 38). Recently the potential importance of antibodies to the V2 loop of gp120 in the neutralizing response has also been reported (29, 32). To study HIV-1 neutralization at the molecular level and to generate reagents of potential prophylactic and therapeutic value requires rapid access to human monoclonal antibodies to HIV-1. However, "present technology is tedious and inefficient, making it imperative that quicker and easier methods be devised for obtaining human monoclonal antibodies" (26).

Combinatorial library technology, i.e., the use of antigen to select from antibody libraries generated on the surface of bacteriophage, offers a new approach to the generation of monoclonal antibodies (4, 9, 13, 24). Indeed, we have shown that a panel of 36 Fabs reacting with gp120 can be derived from a library prepared from the bone marrow of an asymptomatic long-term HIV-1-seropositive individual. Most of these Fabs have a high apparent affinity for recombinant gp120 from the strains LAI (IIIB), SF2, and MN, as assessed by inhibition enzyme-linked immunosorbent assay (ELISA) (1, 2, 10). Most react with a conformational epitope(s) overlapping the CD4 bs of gp120, as judged by the ability of soluble CD4 to inhibit Fab binding to recombinant LAI gp120 in an ELISA. This is consistent with the observation that antibodies to this epitope are highly prevalent in HIV-1-positive human sera (30). A number of the Fabs have been shown to neutralize laboratory strains of HIV-1 (LAI and MN) in different assays (1). Typical 50% neutralization titers of the most effective Fabs are of the order of 1 μg/ml. However, many of the Fabs are weak or nonneutralizing under comparable conditions despite being competitive with CD4 for binding to gp120, leading us to speculate that neutralization by these antibodies may not involve a simple receptor blocking mechanism.

Sequence analysis of 33 Fabs, all inhibited from binding to gp120 by CD4, revealed that the heavy chains could be organized into seven groups (2). Each group contained members with identical V-D and D-J joining regions, implying a common clonal origin, with various numbers of differences elsewhere in the VH domain (2). When the corresponding light chains were examined, more diversity was observed. In some groups, many of the light chains were closely related, leading to identification of a predominant heavy-light chain

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combination. In other groups there was great variation in the light-chain sequences, i.e., identical or related heavy chains were found paired with very different light chains. These combinations, many of which may not occur in vivo, arise because of the random recombination of heavy and light chains in the construction of the phage libraries. In a sense, therefore, the library-derived Fabs are artificial antibodies (19). However, we have argued that the heavy chains associated with antigen binding in vitro are likely to be also involved with binding in vivo (2, 7). Further, since chain-shuffling experiments (14) point to the preeminence of heavy chains in dictating specificity in the cases examined, the library-derived antibodies may be of value in reflecting features of the in vivo response (2, 7).

In this paper, the neutralization, binding, and specificity properties of six anti-gp120 recombinant Fabs are examined. The six were chosen as representatives of each of the groups referenced above (the seventh, a single-member group, was not studied). The principal aim of the study is to investigate whether any feature of Fab binding to recombinant or cell surface gp120 can be correlated with neutralizing ability. Another aim is to compare the specificity properties of the recombinant Fabs with those of human antibodies derived by conventional means. Neutralization ability is assessed in a quantitative envelope complementation assay with a molecular clone of HIV-1. Binding affinity to gp120 is determined by inhibition ELISA and surface plasmon resonance. Competition with CD4 for gp120 is determined by ELISA. Initial epitope characterization is carried out using ELISA by the competition of the set of Fabs with a whole immunoglobulin G (IgG) molecule derived from one member of the set and using surface plasmon resonance by competition of the Fabs with one another. More precise mapping is carried out by monitoring binding of Fabs to an array of mutant gp120s in ELISA. Finally, the binding of Fabs to gp120 and gp160 expressed on the surface of COS-1 cells is assessed by an immunoprecipitation assay.

MATERIALS AND METHODS

Generation of antigen-binding clones. The clones numbered b3, b6, b11, b12, b13, and b14 were obtained from a combinatorial library as described previously (1, 2, 8, 10). Briefly, RNA was prepared from 5 ml of bone marrow from an asymptomatic HIV-1-seropositive individual and used as the starting material to generate an IgG1κ library of 10^7 members on the surface of filamentous phage, using the pComb3 system. The library was panned four times against baculovirus-derived gp120 from LAV-1 BRU (now designated HIV-1 LAI [12, 44]) (American Biotechnologies, Inc., Cambridge, Mass.) to yield a library highly enriched for specific phage Fabs. This library was then converted to a library of phagemids secreting soluble Fabs, and positive clones were identified by ELISA of bacterial supernatants, with gp120 as antigen.

Preparation of purified Fab fragments. Bacterial cultures of the above clones were each grown in 1 liter of superbroth (for 1 liter: 10 g of 3-(*N*-morpholino)propanesulfonic acid, 30 g of tryptone, and 20 g of yeast extract [pH 7.0]) containing 0.5% glucose, tetracycline (10 μg/ml), and carbenicillin (100 μg/ml). The flasks were incubated, with shaking, at 37°C for 8 h, after which time 1 mM isopropyl-β-D-thiogalactopyranoside was added to each culture. These cultures were then incubated for a further 12 h at 25°C. The cells were collected by centrifugation (4,000 rpm in a Sorvall RC-5 rotor for 10 min at 4°C), and the pellets were resuspended in 10 ml of phosphate-buffered saline (PBS) containing 34 μg of phenylmethylsulfonyl fluoride per ml and 1.5% streptomycin sulfate. The suspension was subjected to three freeze-thaw cycles and then centrifuged (17,000 rpm in a Sorvall RC-5 rotor for 30 min at 4°C). The supernatants were collected and cleared by filtration through 0.2-μm-pore-size filters. Soluble Fab was purified by a single pass of the supernatants over an affinity column as follows. The column, consisting of goat anti-human $F(ab')_2$ antibody (Pierce) linked to Gamma Bind G Sepharose (Pharmacia), was equilibrated in 3 column volumes of 87.2% phosphate buffer (0.1 M sodium phosphate [dibasic], 0.5 M sodium chloride)–12.8% citrate buffer (0.05 M citric acid, 0.5 M sodium chloride). The supernatants were loaded in the same buffer, and the column was washed until the optical density at 280 nm of the pass-through returned to a baseline level. The Fab was then eluted in 10.8% phosphate buffer–89.2% citrate buffer, and the collected fractions were neutralized with 1 M Tris-HCl (pH 9.0) and concentrated to a final volume of approximately 1 ml (typical concentration, 100 to 800 μg/ml).

Neutralization of HIV-1 in an envelope complementation assay. The ability of recombinant Fabs to neutralize the HXBc2 molecular clone of the HTLV-IIIB (LAI) isolate was assessed in an envelope complementation assay (22). Briefly, COS-1 cells were cotransfected with a plasmid expressing envelope glycoproteins and a plasmid containing an *env*-defective HIV-1 virus encoding the bacterial chloramphenicol acetyltransferase (CAT) gene. Equal fractions of the cell supernatants containing recombinant virions were incubated at 37°C for 1 h with various concentrations of Fab prior to incubation with Jurkat cells. Three days postinfection, Jurkat cells were lysed and CAT activity was measured.

Inhibition ELISAs. Microtiter wells were coated with 0.1 μg of HIV-1 LAI gp120, and relative affinities were determined by gp120 inhibition ELISA as reported elsewhere (2). CD4 (American Biotechnologies) inhibition ELISAs were performed by the same method.

Competition ELISAs between individual Fabs and b13 whole IgG1 antibody were also performed. The whole antibody was obtained by splicing constant domain genes to the b13 Fab and expressing the protein in Chinese hamster ovary cells (5, 25). b13 IgG was used at a constant dilution of 1:10,000 in the ELISA. The Fab fragments were serially diluted from 1:100 to 1:32,000 and incubated with the IgG in the ELISA plate for 2 h. After a washing, the amount of whole antibody remaining bound to the plate was detected with a peroxidase-labeled antibody specific for the Fc portion of IgG.

Determination of binding affinities by surface plasmon resonance. A Pharmacia BIAcore machine was used (27, 28, 43) for determination of binding affinities. Optimization for the Fab fragments involved a number of steps. Two separate channels on a biosensor chip were coated with HIV-1 LAI gp120 (Repligen, Cambridge, Mass.) such that one channel could be used for the determination of on-rate constants (k_{on}) and the other could be used for the determination of off-rate constants (k_{off}).

For immobilization of antigen on the sensor surfaces, a flow rate of 5 μl of PBS (pH 7.4) per min over the biosensor chip was first established. The chip was activated by injecting 30 μl of activation solution [Pharmacia Biosensor: 50% 0.2 M *N*-ethyl-*N'*-(3-diethylaminopropyl)-carbodiimide, 50% *N*-hydroxysuccinimide]. The flow rate was then adjusted to 10 μl/min, and the antigen was injected in 10 mM sodium acetate buffer (pH 4.5). When association rates were to be determined, 25 μl of gp120 at 10 μg/ml was injected (a final level of 4,000 response units [RU] [27, 28]), whereas 20 μl of gp120 at 2 μg/ml was injected for the determination of dissociation constants (a final level of 800 RU). In both cases, a flow rate of 5 μl/min was reestablished following the gp120 injection, and the chip was

blocked from any further immobilization by injecting 30 μl of 1 M ethanolamine (pH 8.5; Pharmacia Biosensor).

For determination of on-rate constants (k_{on}), a series of dilutions was made for each Fab to give final concentrations in the range of 1 to 20 μg/ml. A total of 30 μl of each dilution was injected, in turn, over the immobilized antigen at a flow rate of 5 μl/min. The change in response per unit time (dR/dt) was plotted against time (t) for each concentration. The slopes of each of these graphs were then plotted against their corresponding concentrations to give a final graph from which the on-rate constant could be read.

For determination of off-rate constants (k_{off}), 30 μl of each Fab solution at 150 μl/ml was injected over the immobilized antigen at a flow rate of 5 μl/min. Once the reaction had reached equilibrium, the Fab was removed from the antigen at a constant flow rate of 50 μl/min. A plot was then made of $\ln(R_i/R_0)$ against $t_i - t_0$ for the dissociation phase (R_i is the response at time t_i; R_0 is the initial response at time t_0), and the slope of this graph was taken to be the off-rate constant. Affinities were then calculated as k_{on}/k_{off}.

Epitope mapping by surface plasmon resonance. A flow rate of 5 μl of PBS (pH 7.4) per min was established, and the biosensor chip was activated as described above. A total of 40 μl of goat anti-human F(ab')$_2$ (Pierce) at 40 μg/ml was injected in 10 mM sodium acetate buffer (pH 4.5) to give a final level of immobilization of 10,000 RU. The chip was then blocked as described above. The flow rate was adjusted to 1 μl/min, and 4 μl of the first Fab at 100 μg/ml was injected and immediately followed by 4 μl of an anti-cytomegalovirus Fab at 150 μg/ml to block any remaining sites on the surface. Next, 4 μl of gp120 at 10 μg/ml was injected over this, followed by 4 μl of the second Fab, also at 100 μg/ml. The entire surface was regenerated with 25 μl of 60 mM HCl so that the next cycle could be run. Each Fab pair was examined with one of the Fabs immobilized to give a mosaic of binding patterns. All values were obtained as the averages of two measurements.

Epitope mapping with gp120 mutants. HIV-1 envelope glycoproteins were obtained by using culture supernatants from COS-1 cells transfected with plasmids expressing either wild-type or mutant gp120 from the HXBc2 clone of the HIV-1 LAI isolate. These molecules were then captured onto the surface of an ELISA plate by using antibody D7324 (Aalto BioReagents, Dublin, Ireland), which binds to the conserved 15-amino-acid sequence at the carboxy terminus of gp120. The binding of a reference HIV-1-positive human serum pool (1/3,000 dilution) to each mutant was assayed by incubating the serum pool with the immobilized gp120 in the presence of 0.5% Tween 20, and bound antibody was detected by means of a second, enzyme-conjugated antibody. The resultant reading ($n = 4$) was taken as the reference value for each mutant. Recombinant Fabs were then assessed for binding to the mutant panel in the same way, and the binding ratio of the test antibody to reference serum was determined for each gp120 mutant. The average ratio for the entire panel was calculated, and any individual ratio deviating from the mean by less than 0.5 times was considered to indicate a gp120 amino acid change that decreased Fab recognition, while those deviating by more than 1.5 times indicated an enhancing amino acid change. In this way, a map of mutations affecting the binding of the Fab to gp120 was obtained for each clone, essentially as described previously (22, 32, 37).

Binding of Fabs to envelope glycoproteins expressed on the surface of COS-1 cells. COS-1 cells were transfected with pCMVenv plasmids expressing HXBc2 envelope glycoproteins under the control of the cytomegalovirus immediate-early promoter. The cells were [^{35}S]cysteine radiolabeled 2 days after transfection and Fabs 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 PBS, the cells were lysed in Nonidet P40 buffer and Fab-envelope glycoprotein complexes were precipitated with goat anti-human F(ab')$_2$ (Pierce) and protein G-Sepharose (Pharmacia) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Scanning densitometry was performed to quantitate relative Fab binding.

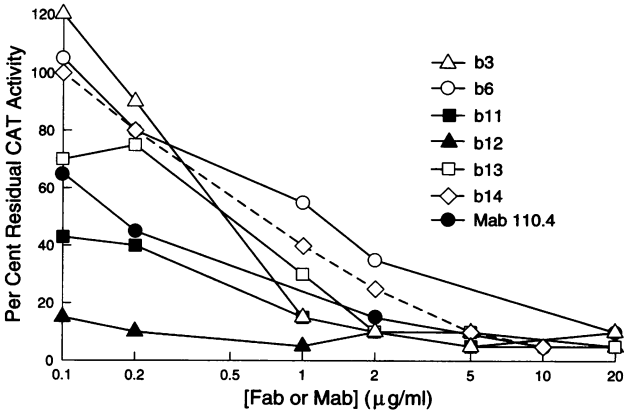

FIG. 1. Neutralization of HIV-1 HXBc2 clone by the panel of recombinant Fabs, using an envelope glycoprotein complementation assay. Neutralization, expressed as a decrease in the percentage of residual CAT activity, is shown as a function of the concentration of Fab with which cells were incubated. Details of the assay are described in the text.

RESULTS

Neutralization of HIV-1 by recombinant Fabs. Figure 1 shows the concentration dependence of Fab neutralization of the HXBc2 clone as measured in the envelope complementation assay. All of the Fabs neutralize effectively at the highest concentration measured, i.e., 20 μg/ml. Irrelevant Fabs, e.g., those directed to surface glycoproteins on other viruses such as respiratory syncytial virus, do not neutralize in this assay. Examination of the data obtained at the lower Fab concentrations clearly reveals that Fab b12 is the most effective neutralizer. The neutralizing potency of Fab b12 was greater than that of the 110.4 whole monoclonal antibody tested in parallel. The 110.4 antibody is one of the most potent antibodies directed against the V3 loop of the HXBc2 HIV-1 strain (41). Generally Fab b12 is found to show exceptional neutralizing ability towards laboratory (1, 3) and field isolates (3, 23) of HIV-1.

Affinities of recombinant Fabs for LAI gp120. Previously the apparent affinities of the panel of recombinant Fabs isolated from the donor studied here have been reported as about 10^8 M^{-1} (2, 10). These values were obtained by inhibition ELISA, in which soluble and immobilized gp120 competed for Fab in bacterial supernatants. Figure 2 shows inhibition data for the representative clones investigated in this study by using purified Fab preparations and again indicates apparent affinities of the order of 10^8 M^{-1}. One set of data is presented, but similar results were obtained in repeat experiments. Such a methodology is appropriate only to give an approximate measure of affinity. We therefore chose to measure the affinities of a number of these clones by using real-time biospecific interaction analysis (surface plasmon resonance) in order to obtain more rigorous values (27, 28). The affinities for a number of

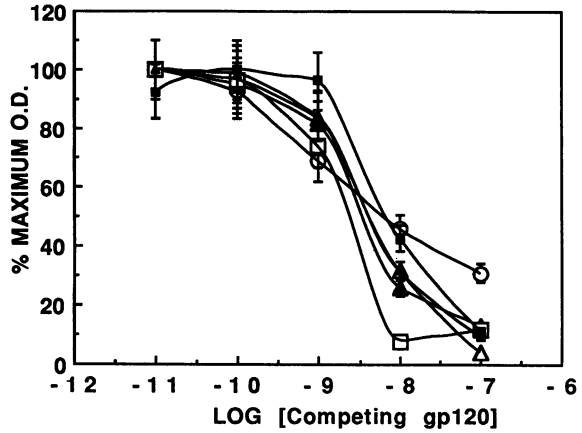

FIG. 2. Relative affinities of the panel of recombinant Fabs for LAI gp120 as indicated by inhibition ELISA. Increasing concentrations of soluble gp120 compete with immobilized gp120 for binding to a fixed concentration of Fab. After a washing, Fab bound to immobilized gp120 is detected in a colorimetric assay. Details are described in the text. Symbols: ○, b12; △, b13; ▲, b11; □, b6; ■, b14; ▲, b3. OD, optical density.

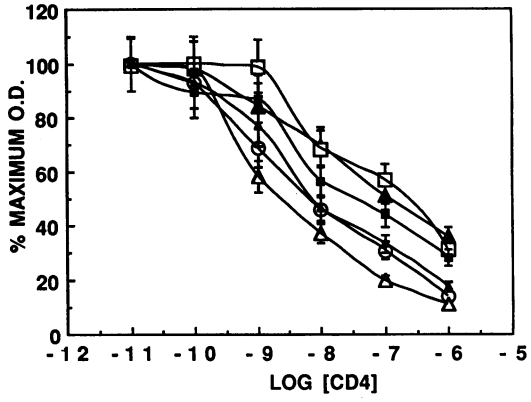

FIG. 3. Competition of soluble CD4 and the panel of recombinant Fabs for binding to LAI gp120 assessed by ELISA. The amount of Fab bound to immobilized gp120 in the presence of an increasing concentration of soluble CD4 is monitored colorimetrically. Details are described in the text. See legend to Fig. 2 for symbol definitions. OD, optical density.

clones used in this study, as measured by surface plasmon resonance, are shown in Table 1. These results are reproducible, with a standard deviation from the mean of approximately 5%, as determined by calculating a number of these constants in triplicate. All Fabs examined have affinities in the range of 5×10^7 to 1×10^8 M^{-1}. These values are in broad agreement with those derived from inhibition ELISA and imply no correlation between affinity for recombinant LAI gp120 and ability to neutralize the HXBc2 clone of HIV-1 derived from the LAI isolate as assessed here (compare, e.g., Fabs b6, b12, and b14).

Competition of soluble CD4 and recombinant Fabs for binding to gp120. The ability of soluble CD4 to compete for binding to gp120 with most of the recombinant Fabs from this donor has been described previously (1, 2, 10). Those studies used bacterial supernatants containing Fabs. Competition data for purified preparations of the representative panel of Fabs under study here are presented in Fig. 3. There is clearly variation in the ability of soluble CD4 to compete with the different Fabs, but it is notable that Fab b12, the most potent neutralizer, is more readily inhibited by sCD4 than by all but one (b13) of the other Fabs. Again, one set of data is shown, although the overall pattern was reproduced in several experiments.

Binding of Fabs to HIV-1 envelope proteins expressed on the surface of COS-1 cells. Given the lack of correlation of Fab neutralization with binding parameters assessed by using recombinant gp120, we compared the binding of Fabs b3, b6, and b12 to COS-1 cells expressing the HXBc2 envelope glycoproteins. We used Fab b3, the poorest neutralizer; Fab b6, also relatively poor; and Fab b12, the most effective Fab at neutralization (Fig. 1). In this assay, different concentrations of Fab were incubated with radiolabeled, envelope glycoprotein-expressing COS-1 cells, after which the cells were washed and lysed. After addition of goat anti-F(ab')$_2$ antibody, the labeled gp120 and gp160 envelope glycoproteins were precipitated and analyzed. Since the amount of HIV-1 envelope glycoprotein expressed on the surface of transfected COS-1 cells is small compared with the amount present intracellularly after cell lysis, the bound Fab is presented with a large excess of both mature gp120 and gp160 precursor forms. The total amount of envelope glycoproteins precipitated thus provides an indication of the amount of Fab bound to the cell surface. The immunoprecipitations are shown in Fig. 4A, and the quantitative binding curves for the total envelope glycoproteins and for the gp120 glycoprotein are given in Fig. 4B. Although the lack of saturation for Fabs b6 and b3 precludes a precise estimate of affinity, it is clear that Fab b3 exhibits a lower affinity for the envelope glycoprotein complex than either Fab b6 or Fab b12. When the binding of Fab b12 and b6 is compared, several differences are apparent. Assuming that Fab 6 achieves saturation at concentrations slightly higher than 150 μg/ml, the estimated affinities of Fab b12 and b6 for the total population of envelope glycoproteins recognized differ only marginally. The most striking difference in the binding of Fab b12 and b6 to the multimeric envelope glycoprotein complex is the preferential detection of gp120 relative to gp160 by the bound Fab b12 (compare the relative intensities of gp120 and gp160 bands for b6 and b12 in Fig. 4A). The estimated affinities, based on the Fab concentrations at which half-maximal binding to gp120 is observed, are 3×10^7 and $<6 \times 10^6$ M^{-1} for Fabs b12 and b6, respectively (Fig. 4B). In addition to these differences in gp120 recognition, the number of binding sites for Fab b6, and probably Fab b3 as well, appears to be greater than that of Fab b12.

Epitope mapping by competition with IgG1 b13. We sought to determine if the epitopes recognized by the Fabs on recombinant gp120 can be distinguished. For one of the clones, b13, the Fab had been spliced to the Fc to generate a whole

TABLE 1. Kinetic constants and calculated affinity constants for the binding of selected Fabs to LAI gp120, measured by surface plasmon resonance

Fab	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_a (M^{-1})
b3	9.6×10^3	1.8×10^{-4}	5.1×10^7
b6	1.6×10^4	1.6×10^{-4}	9.7×10^7
b11	5.6×10^4	4.3×10^{-4}	1.3×10^8
b12	4.5×10^4	4.3×10^{-4}	1.1×10^8
b13	1.1×10^4	1.4×10^{-4}	7.9×10^7
b14	6.0×10^4	6.5×10^{-4}	9.2×10^7

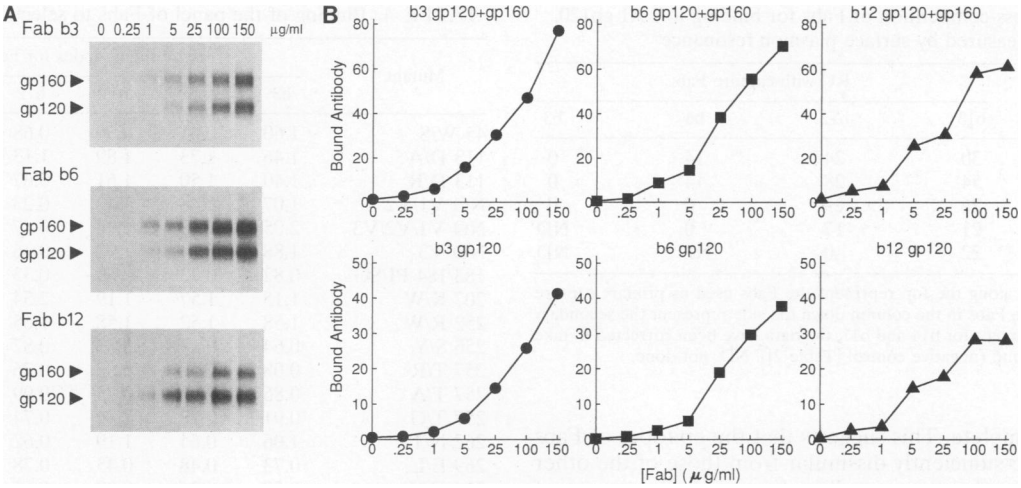

FIG. 4. Fab binding to envelope glycoproteins expressed on the surface of COS-1 cells. (A) Autoradiographs of immunoprecipitated complexes of Fabs b3, b6, and b12 with gp120 and gp160 following incubation of cells with various concentrations of Fabs. See text for details. (B) Scanning densitometry profiles derived from the autoradiographs shown in panel A expressed in arbitrary densitometric units. The lower curves show the profiles for Fab binding to mature gp120, and the upper curves show the profiles for Fab binding to the total envelope glycoprotein (gp120 plus gp160).

IgG1 molecule. Therefore, it was convenient to perform a competition ELISA in which the Fabs competed with IgG1 b13 for binding to immobilized gp120, since the IgG could be readily monitored with a labeled anti-Fc reagent. Figure 5 shows that all of the Fabs are competitive with IgG1 b13, indicating they are probably binding to proximal, if not identical, sites. A control anti-tetanus toxoid Fab did not compete with IgG1 b13.

Epitope mapping by surface plasmon resonance. An alternative and more precise method for determining similarity of epitopes, using surface plasmon resonance, is possible. The procedure adopted here was to immobilize a polyclonal anti-human $F(ab')_2$ on the sensor chip and use this to capture a recombinant Fab. This in turn was used to capture LAI gp120. Finally, a second recombinant Fab was injected. A response at this stage suggests that the epitopes recognized by the two Fabs are distinct from one another and that binding of the second Fab to gp120 is possible in the presence of the first Fab.

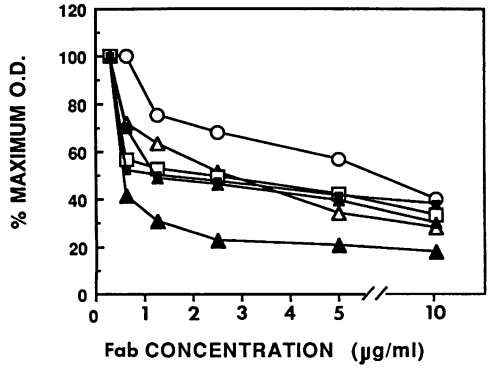

FIG. 5. Competition of IgG1 b13 and the panel of Fabs for binding to LAI gp120 assessed by ELISA. The amount of IgG1 b13 bound to gp120 in the presence of an increasing concentration of Fab is monitored colorimetrically. Details are described in the text. ○, b3; △, b6; ▲, b11; □, b12; ■, b13; ▲, b14. OD, optical density.

Conversely, a lack of binding by the second antibody indicates that the two Fabs share the same or similar epitopes. The results of this experiment are shown in Tables 2 and 3.

Table 2 shows the positive and negative controls for the clones used. The positive controls are the RU levels obtained when the first Fab used is the clone as indicated and the second Fab is an anti-gp120 V3 loop Fab, which neither competes with soluble CD4 nor competes with anti-CD4 site Fabs (data not shown). As can be seen from the table, all positive controls result in significant values of 125 or more, indicating the validity of the technique in the case of nonidentical epitopes. The negative controls are the values obtained when both the first and the second Fabs are the same in each case, as indicated. This gives background values, and these values were subtracted from measured values in subsequent experiments.

An epitope map (Table 3) was then constructed. It can be seen from this map that Fabs b3, b6, b11, and b14 form a set of Fabs which compete with one another highly effectively. For the most part, a member of the set competes as well with another member as it does with itself (RU = 0 [Table 3]). On the other hand, b12 and b13 appear somewhat different in that they do compete with members of the above set but not as effectively as within the set. Further competition between b12

TABLE 2. Control values for cross-competition of Fabs for binding to LAI gp120[a]

Capture Fab	Control RU	
	Positive	Negative
b3	129	24
b6	128	38
b11	131	ND
b12	125	17
b13	135	15
b14	134	ND

[a] Values represent the RU detected with the addition of secondary Fab. Positive control values result from the Fab together with an anti-V3 loop Fab (loop2/35 [2]), and negative control values result from the same Fab used as the primary and secondary antibody. ND, not done.

TABLE 3. Cross-competition of Fabs for binding to LAI gp120, as measured by surface plasmon resonance[a]

Secondary Fab	RU with capture Fab:			
	b13	b12	b6	b3
b14	30	24	14	0
b11	54	28	14	0
b3	26	29	0	0
b6	21	17	0	ND
b12	22	0	ND	ND

[a] The Fabs listed along the top represent the Fabs used as primary capture antibodies, while the Fabs in the column down the side represent the secondary added antibodies. Except for b14 and b11, the data have been corrected to take account of background (negative control [Table 2]). ND, not done.

and b13 is incomplete. This suggests that the epitopes of Fabs b12 and b13 are sufficiently dissimilar from those of the other four and from each other, to allow for a certain amount of binding when they are used in combination with any of the other clones. It may therefore be concluded that clones b3, b6, b11, and b14 bind similar epitopes, with Fabs b12 and b13 showing some distinct features not found in the others.

Epitope mapping with gp120 mutants. To examine specificity differences at a greater resolution, the reactivity of the panel of Fabs with a panel of HXBc2 gp120 mutants altered in conserved residues was investigated. These mutants have been previously characterized with respect to gp160 precursor processing, gp120-gp41 association, and CD4 binding ability (37). Wild-type and mutant gp120s were tested for their abilities to bind a saturating concentration of each Fab, the results being expressed as the ratio of Fab bound to mutant gp120 or wild-type gp120 (binding index [Table 4]) (34).

Sensitivity to certain mutations in residues, particularly towards the C terminus of gp120, has previously been associated with CD4bs antibodies (39, 40). These mutations include residue 257 mutated from threonine to arginine (257 T/R), 368 D/R, 370 E/R, 457 D/A, and 477 D/V. As can be seen from Table 4, most of these mutations abrogate Fab binding or reduce it to low levels, consistent with our assignment of the recombinant Fabs as reacting with the CD4 site.

Some of the other major features of this study are also shown in Table 4. In one particular mutant of gp120, the V1 and V2 loops (residues 119 to 205) are completely removed. This change enhances the binding of Fabs b6, b11, and b14 but significantly decreases the binding of Fab b12. Deletion of the V3 loop produces a more modest decrease in Fab b12 binding while generally enhancing the binding of the other Fabs. The 314 G/W change in the V3 loop produces a decrease in binding of all the Fabs. This effect has been observed for other CD4bs antibodies (33). In detail, each Fab has a unique mutant binding profile. For instance Fab b14 binding is eliminated by the 113D/A change, whereas the binding of the other Fabs is unchanged or enhanced; Fab b3 and b11 binding is reduced by the 475 M/S mutation, but binding by the other Fabs is unchanged, and the 370 E/Q change reduces binding of all the Fabs except for b6 and possibly b11.

TABLE 4. Binding of the panel of Fabs to selected gp120 mutants

Mutant	Binding index for Fab[a]:					
	b3	b6	b11	b12	b13	b14
45 W/S	1.60	0.61	0.50	0.68	1.20	0.28
113 D/A	1.46	1.73	1.89	1.13	0.99	0.00
113 D/R	1.40	1.50	1.61	0.67	0.71	0.00
NO V1/V2	1.07	1.48	1.42	0.23	0.86	1.68
NO V1/V2/V3	2.05	1.48	1.94	0.47	0.95	1.60
NO V3	1.88	1.64	1.92	0.46	1.08	1.72
183/184 PI/SG	0.82	0.73	0.69	0.33	0.92	0.32
207 K/W	1.15	1.57	1.19	2.54	1.30	1.36
252 R/W	1.58	1.52	1.58	1.65	1.39	2.04
256 S/Y	0.64	0.14	0.33	0.82	1.15	0.00
257 T/R	0.08	0.59	0.00	0.76	0.22	0.00
257 T/A	0.86	0.93	0.75	0.99	0.68	0.40
257 T/G	0.91	0.70	1.14	0.74	0.75	0.00
262 N/T	1.06	0.64	1.19	0.62	0.72	0.24
269 E/L	0.73	0.48	0.45	0.78	0.83	0.20
314 G/W	0.59	0.36	0.39	0.65	0.71	0.28
356 N/I	0.67	0.66	0.39	0.92	0.80	0.52
368 D/R	0.19	0.18	0.00	0.04	0.00	0.00
368 D/T	0.28	0.20	0.00	0.03	0.02	0.00
370 E/R	0.01	0.25	0.17	0.07	0.00	0.00
370 E/Q	0.25	0.89	0.58	0.46	0.14	0.00
384 Y/E	1.21	1.02	1.11	0.25	0.02	0.88
386 N/Q	0.88	0.59	0.31	1.05	0.01	0.36
395 W/S	0.92	0.59	0.47	1.00	1.05	0.12
427 W/S	1.57	1.11	1.53	0.63	0.98	0.00
435 Y/S	1.93	1.16	1.58	1.41	1.24	2.04
450 T/N	0.62	0.48	0.58	0.75	0.75	0.60
457 D/A	0.62	0.39	0.44	0.28	0.62	0.20
457 D/R	0.84	0.55	0.92	0.32	0.58	0.56
470 P/L	0.80	0.64	0.72	0.72	0.18	0.24
475 M/S	0.06	1.02	0.33	1.50	1.39	0.92
477 D/V	0.50	0.09	0.00	0.07	0.52	0.00

[a] Binding indices for each Fab to each mutant were obtained as described in the text. The following mutants were also included in the test panel but did not produce a binding index of <0.5 or of >2.0 for any of the Fabs tested: 36 V/L, 40 Y/D, 69 W/L, 76 P/Y, 80 N/R, 88 N/P, 102 E/L, 103 Q/F, 106 E/A, 117 K/W, 120/121 VK/LE, 125 L/G, 152/153 GE/SM, 168 K/L, 176/177 FY/AT, 179/180 LD/DL, 191/193 YSL/GSS, 266 A/E, 267 E/L, 281 A/V, 298 R/G, MT 33+1, 313 P/S, 380 G/F, 382 F/L, 381 E/P, 392 N/E, 397 N/E, 406 N/G, 420 I/R, 421 K/L, 427 W/V, 429 K/L, 430 V/S, 432 K/A, 433 A/L, 435 Y/H, 438 P/R, 456 R/K, 463 N/D, 470 P/G, 485 K/V, 491 L/F, 493 P/K, and 495 G/K.

DISCUSSION

A previous study (1) has shown that Fab fragments reacting with gp120 can neutralize HIV-1 in vitro. As Fabs are monomeric, the study implies that neither cross-linking of virus particles nor cross-linking of envelope glycoproteins on the viral surface is required for virus neutralization. Further, the study indicates that Fab fragments could be useful tools in exploring the mechanism(s) of virus neutralization. The initial experiments gave no indication that neutralization by Fabs could be correlated with either affinity or ability to compete with CD4. Here we have carried out a more systematic and quantitative study of the relationship between binding parameters and neutralization and looked more closely at the epitopes involved in Fab recognition. It should be stressed that this controlled study has employed a molecular clone of HIV-1, and its extension to primary isolates will be the subject of further investigations.

A set of six recombinant Fabs reacting with the CD4 site of gp120 and isolated from a single donor library has been examined in a quantitative neutralization assay. The Fabs show a spectrum of neutralizing ability for a molecular clone derived from the LAI isolate of HIV-1. Fab b12 exhibits the greatest potency of neutralization and is even more effective in this assay than a whole antibody directed to the V3 loop of gp120. Neutralizing ability is not correlated with either the apparent affinity of the Fab for recombinant LAI gp120 estimated by inhibition ELISA or the affinity for LAI gp120 determined by surface plasmon resonance. For instance, Fabs b6, b12, and b14 have very similar affinities by surface plasmon resonance

(Table 1) but different neutralization abilities (Fig. 1). Similarly, neutralization is not correlated with the ability of the Fab to compete with soluble CD4 in a competition ELISA (Fig. 3).

The binding of the Fabs to the multimeric envelope glycoprotein complex on the transfected COS-1 cell surface provides some insights into the observed differences in neutralization potency. The binding of the most potent neutralizing Fab, b12, achieves saturation at roughly 100 μg/ml, whereas neither of the less potent neutralizing Fabs achieves saturation even at 150 μg/ml. Fab b3 clearly exhibits a lower affinity for the cell surface envelope glycoprotein complex than do the other two Fabs tested, b12 and b6. The most striking difference in the binding of b12 and b6 to the multimeric envelope glycoprotein complex is the preferential precipitation of gp120 relative to gp160 by the bound Fab b12 (Fig. 4A). In addition to these differences in gp120 recognition, it appears that the overall number of cell surface envelope glycoproteins capable of being recognized by the less neutralizing Fabs is greater than that seen for Fab b12. These differences suggest that Fab b12 recognizes a more limited subset of envelope glycoprotein conformations and that these conformations are better approximated by the mature gp120 glycoprotein in the cell lysates. It is known that the gp160 precursor assumes a greater variety of conformations during the maturation process than does the fully folded gp120 product (17, 42). The enhanced neutralization ability of Fab b12 could reflect a higher affinity for a restricted gp120 conformation present in the functionally relevant subset of envelope glycoprotein spikes. Such a functionally relevant group of envelope glycoprotein moieties probably represents a small subset of the total population, consistent with the low infectious fraction associated with HIV-1 and other retroviral virus preparations.

One caveat to the above is that the glycosylation of gp120 expressed as a recombinant protein in baculovirus or on the surface of CHO cells is likely to differ and this could affect Fab b12 binding. However, no difference in affinity for CD4bs antibodies between the two forms of gp120 has been observed previously, using a range of antibodies (33).

It should also be noted that the observation of a relative preference of Fab b12 for mature gp120 made by radioimmunoprecipitation could likely not be made with flow cytometry or immunofluorescence, which would simply reflect the total envelope glycoprotein population (equivalent to the upper curves of Fig. 4B).

To probe differences between the Fabs in their recognition of gp120, a number of cross-competition and mutant gp120 binding studies were carried out. Competition monitored in an ELISA format showed that all of the Fabs compete with one of the panel as a whole IgG. More compellingly, all of the Fabs are cross-competitive as measured by surface plasmon resonance. However, there is also an indication that Fabs b12 and b13 are distinct in that they are somewhat less effective in cross-competition than the other members of the panel.

The effects on Fab binding of a series of point mutations in gp120 afford the opportunity to look more closely at recognition differences. The general patterns observed are broadly reminiscent of many CD4bs antibodies and of soluble CD4 itself. Fab b12 is distinguished by its decreased binding to a mutant in which the V1 and V2 loops are deleted. This may or may not be related to the enhanced neutralizing ability of Fab b12. It will be necessary to study a number of variants of Fab b12, which could be produced by chain shuffling or mutation, to answer this question. However, it is clear that the V1 and V2 loops and the V3 loop can affect antibody binding to the CD4bs either by direct contact or by transmitted conformational effects.

As has been commented on above, Fabs derived from libraries may be viewed as artificial. However, as shown here, the recognition properties of a set of antibodies directed to the CD4 site of gp120 show many features in common with those derived by conventional means (39). They also show many features in common with one another, suggesting that, with the caveats inherent in the library approach (2, 7), one individual produces several clearly distinct antibodies directed to a common structural feature, i.e., the CD4bs. This is in agreement with observations made on anti-CD4bs antibodies by using anti-idiotype antibodies (11, 21). One advantage of producing several antibodies is that escape (at least in binding terms) is made more difficult. The only mutations shown in Table 4 which essentially eliminate the binding of all the antibodies also reduce CD4 binding ability.

The observations presented here have significance for vaccine development. The most effective vaccine may need to induce antibodies to the CD4bs with properties similar to those of Fab b12. Given the data above, recombinant gp120 offers no special qualities in this regard. Further, the Fab b12 type of antibody formed only about 10% (4 of 33 Fabs) of the cloned response of the library donor (2) and has not been described amongst the human antibodies derived by other means, suggesting it is a minor component of typical responses. It is clearly of some interest for vaccine design to define more precisely the structure recognized by Fab b12.

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