Selection of human anti-human immunodeficiency virus type 1 envelope single-chain antibodies from a peripheral blood cell-based phage repertoire

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Monoclonal antibodies play an important role in the development of diagnostic assays. Instead of using hybridoma technology to isolate human immunodeficiency virus type 1-specific antibodies, a phage-displayed antibody library was generated from a small number (10⁷) of peripheral blood lymphocytes from a seropositive donor. Two families of single-chain antibodies (scFvs) were selected by biopanning with the envelope precursor gp160. ELISA and competition in the BIAcore system revealed that one antibody family recognized a conformation-sensitive epitope within gp120, while the other antibody family was gp41-specific. The latter group had sequence similarity to antibodies recognizing the cluster III epitope of gp41. Binding of scFvs to gp160 could be inhibited with the donor’s serum antibodies, indicating that antibodies with a similar specificity were circulating in the donor’s blood. Competition experiments suggested that the epitope of the anti-gp41 antibodies was recognized by a broad range of patients’ sera: 21 out of 22 sera from North American and all 20 sera from African seropositive patients inhibited binding of scFvs. In contrast, three sera from this panel did not react with the epitope of the anti-gp120 antibodies. These data indicate that, because of the conserved nature of its epitope, the anti-gp41 antibody will be suitable for diagnostic applications.

Introduction

Diagnosis of human immunodeficiency virus (HIV) infection by in vitro assays is performed by detection of virus-specific antibodies or by detection of the virus itself, by means of associated antigens or by its genome and transcribed genes. Virus-directed antibodies cannot be detected with currently used assays until 6 to 8 weeks after infection; detection of antigen in immunoassays or viral genes in nucleic acid-based tests shortens this so-called ’window’ phase (Busch & Alter, 1995). Because of their high specificity, monoclonal antibodies are used for the capture and detection of antigens. These antibodies should be of high affinity and be directed against a conserved epitope. The affinity, which is directly related to the sensitivity of the assay (Lew, 1984; Devey & Steward, 1988), will determine the earliest time-point of diagnosis. Also, the epitope of the antibody must be conserved, since the heterogeneity of the virus and its high mutation rate may lead to the complete failure of assays, as was recently found with sera from individuals infected with the subtype 0 virus (Gurtler, 1996).

In addition to diagnosis of infection, immunoassays could be used for monitoring therapy. For instance, during treatment of patients with nucleoside reverse transcriptase inhibitors or protease inhibitors alone (Spector et al., 1989; Fischl et al., 1990; Volberding et al., 1990) or in combination (Harindra, 1997), replication of the virus is currently followed by CD4 counts and the measurement of virus load by amplification or...
hybridization methods. However, HIV treatment budgets are under considerable pressure. Therefore, a sensitive immunoassay (Boni et al., 1997) for detection of the capsid protein (Von Sydow et al., 1988; Graziosi et al., 1993; Baumberger et al., 1993) would be an attractive and cheap alternative to more expensive molecular diagnostics. When resistance occurs, demonstrated by a re-activation of the virus, treatment can be changed to another combination of drugs.

Immuonassays may also be used for prognosis and treatment strategy planning. The quantification of anti-gp41 and anti-p24 antibodies aids in the determination of the stage of the disease. During HIV pathogenesis, the titre of anti-gp41 antibodies remains more or less stable, while the titre of anti-p24 antibodies gradually declines. Therefore, the ratio between these titres has prognostic value and can determine the appropriate treatment (Schmidt et al., 1989; Stratthdee et al., 1995; Binley et al., 1997). To allow quantification, monoclonal antibodies directed against p24 have been labelled with a reporter enzyme and used to compete with serum antibodies for antigen binding (Janvier et al., 1991).

In this study we have evaluated phage antibody library technology (reviewed in de Haard et al., 1998a) as a substitute for the traditional hybridoma technology. Theoretically, there are several advantages in applying phage-display technology, and patient-derived human antibodies in particular, to the generation of diagnostic reagents. Firstly, the recognition of immunodominant epitopes is crucial when antibodies are used in antibody-quantification assays. The immunization of mice does not necessarily result in the isolation of monoclonal antibodies which share the immunodominant epitopes recognized by human antibodies. Moreover, the presentation of the virus antigens during immunization might be different from the situation during infection, leading to the isolation of antibodies with different recognition patterns. Secondly, the conserved nature of the epitope is especially important in antigen detection. The existence of HIV variants and the mutational drift of the virus, which occurs in response to the appearance of neutralizing antibodies during infection (Watkins et al., 1996), causes a high variability in all virus genes and their encoded proteins. The use of antibodies from different virus strains in the biopanning procedure might favour the selection of such neutralizing antibodies. Thirdly, the application of human antibodies in antigen assays may avoid false-positive reactions caused by sera with human anti-mouse reactivity (HAMA); these sera were found sporadically during the development of a p24-antigen assay. Use of human scFv fragments would even prevent problems with human anti-human responses (HAHA). Finally, the availability of the V genes permits the manipulation of antibody affinity (reviewed by Hoogenboom, 1997).

In this study we set out to generate human recombinant antibodies reactive against HIV-1, aiming mainly at their application as diagnostic reagents. As a source of antibody-producing cells, a small number of circulating lymphocytes from a seropositive individual were used for the construction of the immune library. Biopanning was performed with the envelope precursor glycoprotein gp160, which is processed into the exterior envelope protein gp120 and the transmembrane glycoprotein gp41. In addition to a family of gp120-specific antibodies, we selected a group of gp41-specific antibodies with a similar sequence to the cluster III epitope-recognizing antibodies isolated by Binley et al. (1997). The potential for both families of scFvs to be used as diagnostic reagents was examined by competition with a panel of patients’ sera that could be expected to be diverse with respect to virus subtype. Only one serum, which gave unreliable results in other HIV-1 assays, did not recognize the gp41 epitope, while three sera failed to react with the gp120 epitope. Therefore, the anti-gp41 scFv seems to be the most promising candidate for application in in vitro diagnostics.

Methods

Library construction and selections. Blood cells from a seropositive individual were separated on Ficoll Hypeque (Pharmacia) and stored in aliquots of 10^6 cells per vial in liquid nitrogen. For RNA isolation the cells from one vial were pelleted and washed with physiological salt solution. Approximately 2 μg total RNA was extracted (Chirgwin et al., 1979). One μg was reverse transcribed into random-primed cDNA. IgG-specific variable heavy (VH) chain fragments were amplified using AmpliTaq polymerase (Perkin-Elmer) for 30 cycles (94 °C for 30 s, 55 °C for 30 s and 72 °C for 2 min), with HuVHBACK and HulG4-1CHF1FOR primers (primers sequences see Marks et al., 1991). We combined the highly similar primers VH1a, VH3a and VH5a into one pool, VH4a and VH6a into a second group, and kept VH2a separate. In this way three different heavy chain amplification mixtures were obtained. The kappa light chain variable (VK) domains were amplified with the HuVKBACK and HuCKFOR primers. Primers VK1a and VK4a were combined, VK2a, VK3a and VK6a formed a second group, and VK5a was kept separate. The three heavy chain and three light chain fragments were isolated from a 15% agarose gel with the QIAex kit (QIAGen), and re-amplified with the corresponding J primers (HuJH1 and JH2 combined with JH4 and JH5, HuJH3 with JH6, HuJK1 combined with JK2, JK3 and JK4, while JK5 formed a separate group). We assembled the six heavy and six light chain fragments in PCR (Horton et al., 1989) with the corresponding linkers into 36 fusion products. These were gel-purified, digested with SfiI and NolI (Pharmacia), and cloned into the vector pVL1, which is identical to pHEN1 (Hoogenboom et al., 1991) except for the substitution of a FLAG-tag (Hopp et al., 1988) for the Myc-tag (Evan et al., 1985). Electroporation into E. coli JM101 was performed as described elsewhere (Dower et al., 1988). To establish the degree of variation of the library, a BstNI fingerprint (New England Biolabs) was performed on the PCR products of 24 individual clones (Marks et al., 1991). All gave unique patterns, suggesting that no over-represented fragments were amplified and cloned.

The rescue of phagemid particles was performed as in Marks et al. (1991) except that for panning, 100 μl 0.45 μm-filtered culture supernatant (about 3 x 10^9 transforming units) was diluted with 900 μl sample diluent (PBS, 20% normal goat serum, 1% Triton X-100). The library was selected with the envelope precursor gp160, derived from the HIV-1 subtype 111B, secreting cell line ðδ5 (Kalyanaraman et al., 1988), and purified by affinity chromatography with an anti-gp41 monoclonal antibody as described before (Kalyanaraman et al., 1990), and
with the Gag protein p24. The p24 antigen, expressed as a full-length product in E. coli under the control of the inducible lac promoter (constructed at Organon Teknika by M. Berman, unpublished results) and encoding the HIV-1 RF sequence, was affinity-purified with monoclonal antibody mAb 39B (generated at Organon Teknika, unpublished results). The proteins were coated as a mixture in 6-well tissue culture plates (Costar), at a concentration of 2.5 µg/ml for gp160 and 3 µg/ml for p24, in 50 mM Na2CO3 pH 9.6 for 16 h at 4 °C.

Screening and sequencing of clones. Soluble scFv was produced from individual clones by growth in V-shaped wells of microtitre plates, as described before (Marks et al., 1991). For ELISA, microtitre plates were coated with 3 µg/ml p24, 2.5 µg/ml gp160 or 5 µg/ml virus lysate, in 50 mM Na2CO3 pH 9.6 for 16 h at 4 °C. The plates were blocked for 2 h with 0.2% (w/v) BSA in 0.1 M Tris–HCl pH 7.4 plus 30 mM KCl. Culture supernatants and periplasmic fractions were diluted in sample diluent. After incubation at room temperature for 2 h, bound scFv was detected with anti-FLAG antibody M2 (Kodak IBI), diluted 1:4000 in sample diluent. After a 1 h incubation with 1:2000-diluted anti-mouse IgG–horseradish peroxidase conjugate (anti-mouse–HRP, DAKO), staining was performed for 30 min with tetramethylbenzidine (TMB) and urea peroxide (Organon Teknika) as substrates. The reaction was stopped by adding an equal volume of 1 M H2SO4; absorbance was measured at 450 nm.

Clones giving positive signals and with a unique BstNI fingerprint were analysed by sequencing with the T7 sequencing kit (Pharmacia) with the M13rev primer (5’ CAGGAAAAACGCTATGAC 3’), the gene III primer (5’ TGAATTTTCTGTATGAGG 3’), and two primers located in the linker (LINKFWD, 5’ GACCCACACCGCCCGAG 3’) and REV (5’ GACCCACCACGGC CCGAG 3’). Plasmid DNA was purified with the QiAgen kit (QiAgen).

Characterization of scFvs. Periplasmic fractions were prepared with borate-buffered saline (BBS) (Skerra & Pluckthun, 1991). A sample of 50 ml culture were resuspended in 1 ml BBS (200 mM borate-buffered saline (BBS) (Skerra & Pluckthun, 1991). The cells were diluted 1:4000 in sample diluent. After incubation at room temperature for 2 h, bound scFv was detected with anti-FLAG antibody M2 (Kodak IBI), diluted 1:4000 in sample diluent. After a 1 h incubation with 1:2000-diluted anti-mouse IgG–horseradish peroxidase conjugate (anti-mouse–HRP, DAKO), staining was performed for 30 min with tetramethylbenzidine (TMB) and urea peroxide (Organon Teknika) as substrates. The reaction was stopped by adding an equal volume of 1 M H2SO4; absorbance was measured at 450 nm.

Results

Library construction and panning

For the generation of a random combinatorial library, we extracted RNA from PBL of a seropositive individual. The donor, a haemophilia sufferer, was infected with HIV-1 in 1981 from a blood-derived coagulation product originating in the USA. The patient’s seroconversion was demonstrated by Western blot (Fig. 1): a response against the virus core derivatives emerged 23 months before the PBL were collected (lane 2). Seven months before harvesting, antibodies directed against the envelope derivatives gp120 and gp41 and polymerase derivatives p64, p51 and p31 were present (lane 3). A sample of 10^7 lymphocytes that had been stored in liquid nitrogen for 10 years was used for RNA isolation. The VH and Vκ chains were amplified and assembled with the linker-encoding DNA fragment into scFv gene segments. The library obtained consisted of 5 x 10^6 clones, of which 97% had a complete scFv gene (not shown).

The library was subjected to four rounds of panning with a combination coat of gp160 and p24. This selection produced a 5000-fold enrichment in the number of eluted phage particles, relative to the first round. The fraction of phage carrying a complete scFv-encoding insert in the genome was determined by colony morphology (de Haard et al., 1998b): it increased from 60% after the first round to 98% after the last round. The polyclonal phage amplification products, which were harvested before each panning round, were tested by ELISA on the combination coat of gp160 and p24 and also on gp160 alone: both assays gave increasing signals after the second round (data not shown). Twenty-four individual clones picked randomly from the third round and 24 clones from the fourth round were grown in a microtitre plate. The scFvs that leaked into the culture supernatant gave positive reactions against both the combination coat and gp160 alone, and were negative against p24 and BSA.

BstNI fingerprinting revealed the presence of two different classes of clones. One clone from each of the two classes was analysed in more detail; these were designated AB#31 and AB#32.
We repeated the panning procedure with gp160 alone, and found similar degrees of enrichment as when using the combination of antigens. BstNI fingerprints of clones were obtained for antibody fragments that gave positive signals in ELISA; the similar fingerprints suggested that the clones were closely related to those obtained in the previous panning experiment. We examined five new clones, designated #B4, #B5, #B7, #D1 and #D4, in more detail.

**Sequence analysis**

The sequences of the gp160-binding antibody fragments selected from the human library are shown in Fig. 2. Selection on gp160 yielded two different types of antibodies with heavily mutated VH genes. Comparison with the germline sequences (Tomlinson et al., 1992) shows that clone AB#31 and its derivatives #B5 and #B7 use a VH1 family-derived germline segment (DP-7). Clone AB#31 has 30 differences from the germline analogue within its nucleotide sequence, leading to 15 amino acid substitutions. In contrast, clone #32 and its derivatives #B4, #D1 and #D4 use a germline segment of the VH3 family (DP-46); for clone AB#32 there are only 17 differences in its nucleotide sequence resulting in 12 different amino acids. Alignment with the Vx germline sequences (Cox et al., 1994) showed that both types of gp160-specific antibodies use the same segment (Vx3 family; DPK22/A27), although joined to different Jx segments. The Vx segments are less mutated: only three and seven differences in nucleotide sequence, resulting in one and three amino acid substitutions, were observed for clones AB#31 and AB#32, respectively.

The use of one type of Vx for both antibodies might indicate a low complexity of the amplified Vx-fragments, but a closer examination of the sequences reveals the presence of subtype-specific residues resulting from somatic hypermutation. This can be seen with residue 77 (Fig. 2) and with the J segment-encoded residue at position 96. Ditzel et al. (1997) found a light chain nearly identical to the one used by AB#31 and AB#32, but paired with different heavy chain fragments, in their anti-gp120 antigen-binding fragment (Fab).

The heavy chain CDR3 region of the anti-gp120 antibodies is rather long (20 residues), while the one from the anti-gp41 antibody is much shorter. The existence of somatic hypermutation can be identified in the heavy chain sequences of the AB#32 family. The ancestor VH segment, which is found in clone #B4, contains three germline residues, at positions 26, 30 and 56, that are all mutated in the other members of the family (Fig. 2). A similar phenomenon is seen in the heavy chain of the AB#31 family: the germline residues 65 and 69 in clone #B7 have been mutated in clone AB#31. Finally, comparison of the sequence of the Vx region from clones #B6 and #B7 with AB#31 showed that the first two contain ten additional hypermutated residues. At present we do not know if any of these mutations lead to an increase in affinity.

In a comparison of the amino acid sequences of the VH regions using the BLAST program, a similarity was found between the scFv from AB#32 and a human monoclonal antibody, HS3D6HCV-1 (Fig. 2), directed against gp41 (Felgenhauer et al., 1990). A greater similarity existed between the Vx sequences of AB#31/AB#32 and the phage library-derived anti-gp120 Fabs p35 and p20 (Ditzel et al., 1997) (see Fig. 2). The heavy chain sequences of p20 and p35 do not resemble those of scFv from AB#31 and AB#32, although they share a long CDR3 region (17 residues for p20 and p35 compared with 20 residues for AB#32) with AB#32.

**Characterization of scFvs**

The specificity of both types of antibody was analysed with BIAcore (Malmborg & Borrebaeck, 1995). The scFv-containing periplasmic fractions were injected sequentially onto a gp160 surface (Fig. 3). When AB#32 was injected first, an additional signal was obtained from the subsequent injection of AB#31 (Fig. 3c), suggesting that the antibodies recognize distinct epitopes on gp160. The total signal obtained from both antibodies (at 1500 s the response was 450 RU) can be interpreted as the sum of the signals from both scFvs alone (the response of AB#32 at 1500 s was 130 RU (Fig. 3d) and that of
AB#31 at 800 s was 320 RU (Fig. 3a)]. Additivity can be demonstrated by overlaying the individual binding curves (Fig. 3d).

The signal obtained by injection of AB#31 followed by AB#32 (Fig. 3a) seems not to be additive, but this appearance is caused by the somewhat faster dissociation of AB#31. The curve obtained from AB#31 with a prolonged dissociation phase (Fig. 3b) shows that the total signal at 1500 s (430 RU) can also be considered as the sum of the signals of the individual antibodies: at 1500 s the signal of AB#31 was 280 RU (Fig. 3b) and at 800 s the signal of AB#32 was 150 RU (Fig. 3c).

The affinities of scFv AB#31 and AB#32, determined by kinetic analysis of association and dissociation with surface plasmon resonance (Table 1), are in the nanomolar range. AB#32 has a 12-fold higher affinity than AB#31, resulting from a 14-fold slower dissociation rate and an 8.6-fold faster association rate.

AB#31 and AB#32 were used for the detection of HIV antigens on an immunoblot (Fig. 4). Only the non-reduced forms of the envelope protein gp120 and its precursor gp160, which is actually a truncated form missing the 68 carboxy-terminal amino acids, were recognized by AB#32 (both antigens are marked in Fig. 4a). AB#31 was specific for gp160 and not for gp120, although the signal on the blot was less intense than that obtained with AB#32. Omitting boiling of the virus lysate before loading on the gel improved the sensitivity of detection: a more intense gp160 band, but also a faint gp41 band, were visible on the blot incubated with AB#31 (Fig. 4b). The bands showing up in the gp120 preparation are derived from the mouse antibody used for purification, which leaked from the affinity column; it was also detected with the anti-mouse conjugate [see left blot of Fig. 4a]. The proteins reactive with AB#32 with molecular masses greater than 200 kDa are the dimeric products of gp120 and gp160, which were also visible on a blot stained with amido black. In conclusion, AB#31 recognizes a conformation-sensitive epitope of gp160 that is probably located in the transmembrane glycoprotein gp41, and AB#32 reacts with a conformation-sensitive epitope that is present in gp120.

The specificities of the scFvs from AB#31 and AB#32 were analysed further by ELISA. Immunopurified gp120, a constrained V3 loop peptide of gp120 (Tolman et al., 1993) and a constrained peptide of gp41 (Gnann et al., 1987), all based on the HIV-1 sequence, were used to coat microtitre plates. A response was only observed for AB#32 against gp120. Therefore, the epitope of AB#31 must be located within gp41, and it differs from the ‘Gnann epitope’. The epitope of AB#32 resides in gp120, but it is not located within the immunodominant V3 loop.

We performed an inhibition ELISA in order to establish the presence of the isolated antibodies in the serum of the donor. The patient’s serum, taken before and after HIV infection, the latter with anti-gp160 reactivity, was mixed with the scFvs and incubated in wells coated with gp160. Subsequently bound scFv was detected by its FLAG-tag. As is shown in Fig. 5, binding of AB#31 to the antigen was almost completely inhibited by the serum antibodies: 94.7% inhibition was
Fig. 3. Epitope mapping with the scFv fragments from clones ABg31 and ABg32 on the BIAcore with gp160 coupled to a CM5-chip at a density of 2856 RU. (a) After 50 s the periplasmic fraction of clone ABg31 was injected, until at 640 s the Hepes-buffered saline wash started. The periplasmic fraction of clone ABg32 was injected from 800 to 1350 s. (b) Overlay of the curves from ABg31 with a prolonged dissociation phase and from ABg32 [obtained from (d)]. The curve of ABg32 was superimposed at 800 s on the level for ABg31 at 1350 s. In this way the loss of signal due to dissociation of ABg31 can be corrected, resulting in the observed end-level. (c) Sequential injection of ABg32 and ABg31 was carried out at the same time-points as in (a). Because of the lower dissociation rate of ABg32, the increased signal caused by ABg31 can be recognized more easily. The end-level at 1500 s (450 RU) is identical to that found in (a), showing that the signals are additive. (d) Overlay of the curve obtained from ABg32 with a prolonged dissociation phase, superimposed at 800 s on that obtained for ABg31 at 1350 s [obtained from (b)], confirming additivity.

Table 1. Affinities of anti-HIV-1 envelope scFvs

<table>
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<tr>
<th>Clone</th>
<th>$k_{off}$ (s$^{-1}$)</th>
<th>$k_{on}$ (M$^{-1}$s$^{-1}$)</th>
<th>$K_d$ (M)</th>
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<tbody>
<tr>
<td>ABg31</td>
<td>$(1.88 \pm 0.43) \times 10^{-3}$</td>
<td>$(5.38 \pm 0.77) \times 10^{5}$</td>
<td>$(3.49 \pm 1.29) \times 10^{-9}$</td>
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<tr>
<td>ABg32</td>
<td>$(1.33 \pm 0.10) \times 10^{-3}$</td>
<td>$(4.64 \pm 1.10) \times 10^{6}$</td>
<td>$(2.87 \pm 0.89) \times 10^{-10}$</td>
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observed with a 100-fold diluted serum and 29.0% at a 10000-fold dilution. Binding of ABg32 was also competitively inhibited, but to a lesser extent: 76.0% inhibition was observed at a 100-fold dilution. In contrast, the pre-infection serum did not affect binding of the recombinant antibodies to gp160. This experiment shows that antibodies sharing the epitopes of the library-derived scFvs were present in the serum of the donor. The antibody fragments are therefore not artificial products with unusual specificities resulting from the scrambling of VH and VL genes during library assembly.
**Fig. 4.** Analysis of the response of the recombinant antibodies against HIV-1 antigens on a non-reducing Western blot. (a) Blots were incubated with either anti-mouse–AP (α-Mo), AB#31 or AB#32, and the marker lane (M) was stained with Ponceau S. Virus lysate (about 50 µg), purified gp160 (2 µg) and gp120 (2 µg) were loaded. The positions of gp160 and gp120 are indicated. (b) Detailed analysis of reactivity of AB#31 and AB#32. Virus lysate and gp120 samples were boiled (+T) or loaded directly on the gel without heat treatment (−T). gp41 was detected by AB#31 and not by AB#32.

**Competition with heterologous sera**

To explore the possible application of the scFvs as diagnostic reagents, we analysed the epitope recognition of the selected antibody fragments in more detail. A competition assay was set up, in which a panel of anti-gp160-positive sera from 22 North American (USA and Mexico) and 20 African (Cameroon, Ivory Coast and Tanzania) patients was tested; the results obtained are shown in Fig. 6. One serum sample from a North American patient showed a low degree of inhibition.
Fig. 5. Inhibition of binding of AB\(_31\) (a) and AB\(_32\) (b) to gp160 in ELISA by serum antibodies from the donor of the PBL that were used for the construction of the library. Pre-infection serum (taken on 19 August 1980, tinted bars) and post-infection serum (taken on 17 September 1983, hatched bars) were diluted 100-fold (\(\times2\)), 1000-fold (\(\times3\)) and 10,000-fold (\(\times4\)). Results are expressed relative to those obtained with scFv without adding serum.

\(8.4\%\) of AB\(_31\) binding. The other sera competed well with AB\(_31\) for binding to gp160: mean inhibition was 95.5\% with an SD of 1.5\%. For AB\(_32\) the picture was different. The mean percentage inhibition was much lower (69.9\% with an SD of 19.9\%), and serum from three North American patients, including the one that did not compete with AB\(_31\), did not compete for binding. The serum that did not inhibit binding of either scFv to gp160 was negative on an immunoblot for binding to p18, p24, p31 and gp41, indeterminate for p51, p55, p65 and gp120 and positive for gp160. The indeterminate and the negative results against gp120 and gp41, respectively, suggest that the signal against gp160 might be caused by non-specific interactions. This serum gave a borderline signal when tested in the Clonatec assay, which contains a peptide with an immunodominant epitope of gp41. The difference between AB\(_31\) and AB\(_32\) in their sensitivity to competition can partly be explained by their affinities: binding of AB\(_32\) to gp41, with a 12-fold lower \(K_d\) than that of AB\(_31\) (Table 1), might be inhibited inefficiently.

We conclude that the epitopes of AB\(_31\) and AB\(_32\) are recognized by serum antibodies from North American and African seropositive patients. This implies that both recombinant antibodies recognize conserved epitopes, thereby making them suitable candidates for application in in vitro diagnostics.

**Discussion**

**Small numbers of PBL as a source for library construction**

Combinatorial library technology has already been applied to the generation of human Fabs directed against the HIV-1 envelope (Burton et al., 1991; Barbas et al., 1993; Binley et al.,...
Human scFv against the HIV-1 envelope

Human scFv against the HIV-1 envelope

We selected antibodies from PBL from a haemophilia sufferer (since deceased) who was infected with hepatitis C virus, and later with HIV-1, through administration of blood-derived coagulation products, and who had no acute immune response at the time of collection of the blood cells. RNA was extracted from a small number of cells \((10^7)\). In spite of the poor condition of the cells, as deduced from their low viability, we managed to isolate a small amount of RNA. After panning of the human library, two types of gp160-specific antibodies were selected, which have specificities matching serum antibodies from the donor. The two types have different sequences (VH1 and VH3 families), and recognize different epitopes of gp160. The successful selection of these antibodies from a PBL library was surprising, since it has been suggested that the preferred source of antibody-producing B cells is bone marrow (reviewed by Parren & Burton, 1997). It has been reported that the selection of anti-tetanus toxoid Fabs from PBL libraries was possible from recently boosted donors (Mullinax et al., 1990; Persson et al., 1991), but not from individuals who had not been recently immunized, in spite of the presence of high levels of circulating anti-tetanus toxoid antibodies in their blood (Persson et al., 1991). Most of the circulating lymphocytes are memory cells expressing small amounts of antibody, transcribing up to 100 copies of Ig-encoding mRNA per cell, in contrast to plasma cells, which synthesize 30,000 copies of Ig mRNA (Schibler et al., 1978). We demonstrated that serum conversion occurred in our donor approximately 30 months before the lymphocytes were collected. One year before collection, the serum contained IgG against gp120, gp41, p24 and other antigens, as detected by Western blot. These data suggest that at the time the PBL were harvested the donor did not have an acute response, in which elevated numbers of circulating plasma cells can be expected.

In spite of the presence of anti-p24 antibodies in the donor's serum, we did not retrieve such antibodies from the library during panning with the combination of gp160 and p24. This might be explained by the low complexity of the library, resulting from the small amount of RNA used for its construction, but it might also be caused by the competitive nature of panning. We believe that the outcome of selection on a mixture of antigens is the antibody with the highest affinity or antibodies with comparable affinities for one of the components, even for the protein used for blocking. This effect has been demonstrated before (Ditzel et al., 1995), during the selection of gp120-specific Fabs. The only way of selecting antibodies that were not reactive against the CD4-binding domain was to shield this epitope through binding to an antibody. Another reason for the absence of anti-p24 scFvs might be a decreasing titre of anti-p24 antibodies in the patient, as is often seen during HIV-1 pathogenesis (Forster et al., 1987; Chargelegue et al., 1993).

The anti-gp120 scFv AB#32, with a VH3 germline segment, contains a relatively long heavy chain CDR3 region (20 residues). The library-derived anti-gp120 Fabs isolated by Barbas et al. (1993) contained VH1 as well as VH3 segments, with the CDR3 length varying from 18 to 22 residues. No significant similarity was apparent to the VH sequence of our anti-gp120 scFv. However, there is a most remarkable similarity between the V\(\kappa\) sequences of two recently isolated anti-gp120 Fabs (Ditzel et al., 1997) and those of our clones. The light chains of the published Fabs were fused alternately to the JK segments, leading to a CDR3 of 9 and 10 residues in clones p35 and p20, respectively, and they paired with different heavy chains, as was also observed for AB#31 and AB#32.

Possible diagnostic applications

Our main interest is the application of monoclonal antibodies to \textit{in vitro} diagnostics. The use of antibodies of human origin in antigen assays prevents false positive reactions caused by sera with HAMA-reactivity. Moreover, the epitopes involved must be conserved and recognized by a broad range of patient's sera. Immunization of mice does not necessarily lead to the isolation of monoclonal antibodies which react with these kinds of epitopes.

The use of the BiAcore system revealed that the selected scFvs react with distinct epitopes on gp160, as confirmed by ELISA against gp120. AB#32 reacts with a conformation-sensitive epitope of gp120 different from the V3 loop. In contrast, Western blot analysis with scFv as the detection antibody suggested that AB#31 has gp41-specificity, explaining the absence of reactivity of this antibody against gp120 in ELISA and immunoblot. The epitope recognized by this scFv does not coincide with the immunodominant ‘Gnann epitope’ (Gnann et al., 1987), which is part of the cluster I epitope on gp41. According to the sequence of its VH chain, AB#31 appears to be related to the cluster III epitope-recognizing recombinant Fabs described by Binley et al. (1997).

Antibodies to this cluster use the same germline VH gene (DP-7) and share the same CDR3 length (12 residues). A faint similarity can be found between AB#31 and the cluster III clone G15 in the CDR3-encoded residues [\textit{AB}#31, PFMNQLRNLAI; G15, PRFNLI[A/P]LDL], and both use a V\(\kappa\)3 light chain gene. Comparison of clone AB#31 with G15 revealed 36 differences in the deduced amino acid sequence of VH, but 20 of the differing residues could also be found in one or more of the other cluster III clones G5, L11, and in particular L1 and A9 (data not shown). The occurrence of identical somatically mutated residues within CDR1 (residues 31 and 32) and CDR2 (residue 65) in the cluster III clones and clone AB#31 strengthens the hypothesis of a relation between these antibodies. These data suggest that AB#31, which like the cluster III Fabs of Binley et al. (1997) only reacts with native antigen on an immunoblot, recognizes a conformational epitope involving residues 619–648 and probably residues 563–583 of gp41 (for numbering of residues see Binley et al., 1997).
Inhibition ELISA with a broad range of anti-gp160-positive sera from seropositive individuals, not only from North America, but also from Africa, where variability among HIV-1 viruses is much greater than elsewhere (Nkengasong et al., 1994; Louwagie et al., 1995), showed that the epitope on gp41 is immunodominant and conserved. The presence of immunodominant and conserved epitopes on gp41 is well documented (Gnan et al., 1987; Cumming et al., 1990; Muster et al., 1993), and confirms the idea that AB#31 in particular, which is reactive against this antigen, will be useful for diagnostics.

Besides conservation of its recognized epitope, another important feature of a diagnostic antibody is affinity, since this will determine the sensitivity of the assay (Lew, 1984; Devey & Steward, 1988). Kinetic measurements with surface plasmon resonance revealed high affinities for both antibodies: AB#31 has an affinity in the nanomolar range (3-5 nM), while AB#32 has even greater affinity (0.29 nM).

One diagnostic application of these scFvs could be the quantification of anti-gp41 antibodies during HIV-1 pathogenesis. The ratio of anti-p24 to anti-gp41 antibodies has prognostic value: the titre of anti-p24 gradually declines, whereas the anti-gp41 titre remains unchanged (Schmidt et al., 1989; Strathdee et al., 1995; Binley et al., 1997). A quantitative assay for anti-gp41 antibodies could be made by construction of labelled scFv AB#31 that could be used for the competition of serum antibody-binding to immobilized antigen, as has been described before for p24 (lanvier et al., 1991). The format of the scFv can be engineered for this purpose, as has been demonstrated previously (Kerschbaumer et al., 1997).

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References


Human scFv against the HIV-1 envelope


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