SHORT COMMUNICATION

# Further characterization of an antigenic site of HIV-1 gp120 recognized by virus neutralizing human monoclonal antibodies

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**Objective:** The aim of this study is to characterize antigenic sites on HIV-1 gp120 which may be important for the development of active and passive immunization strategies against HIV-1 infection.

**Design:** Two HIV-1-seropositive individuals were selected from the Amsterdam cohort and Epstein–Barr virus (EBV)-transformed B cells were generated from their peripheral blood mononuclear cells, which produce HIV-1-specific human monoclonal antibodies (HuMAb).

**Methods:** HuMAb were generated and selected based on their reactivities with native gp120. Reactivity with HIV-1 strains from phylogenetically different subfamilies was determined by immunostaining and virus neutralization assays. Specificity for the CD4-binding site was tested by an inhibition enzyme-linked immunosorbent assay and amino acids (aa) involved in the binding of the HuMAb were identified with a set of gp120 molecules with single aa substitutions.

**Results:** Three HuMAb (GP13, GP44, GP68) were generated, all recognizing a conserved conformation dependent epitope within, or topographically near, the CD4-binding site of gp120. HuMAb GP13 and GP68 neutralized a broad range of HIV-1 strains from phylogenetically different subfamilies, whereas HuMAb GP44 exhibited a more restricted pattern of neutralizing activity. The patterns of gp120 aa involved in their binding were unique for each of these HuMAb.

**Conclusions:** The pattern of reactivities of these three HIV-1-neutralizing HuMAb developed in these studies is similar to, but distinct from other human and rodent MAb that recognize this antigenic site of HIV-1 gp120.

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**Keywords: HIV-1, neutralizing antibodies, human monoclonal antibody, gp120, CD4-binding site, virus neutralization, passive immunization.**

## Introduction

Several studies in which virus-neutralizing (VN) antibodies were passively transferred, indicated that antiviral antibodies play a major role in the protection from lentivirus infections [1–4]. Sera from HIV-1-infected individuals have been shown to neutralize a wide range of HIV-1 strains originating from Europe,

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the United States of America and Africa [5]. Antibodies recognizing a conformation dependent antigenic site, overlapping with the CD4-binding site on gp120 are responsible for a major part of the VN cross-reactivity in human sera [6,7]. To investigate the nature of this antigenic site in more detail, monoclonal antibodies (MAb) of this specificity are of great value. To date, a limited number of human MAb/(HuMAb) specific for this site have been derived from North American HIV-1-seropositive individuals. Furthermore, rodent MAb, which inhibit binding of gp120 to CD4, have been generated against HIV-1 IIIB gp120 [8–12]. Here, we describe the generation of VN HuMAb produced by Epstein–Barr virus (EBV) transformation of peripheral blood mononuclear cells (PBMC) from two Dutch HIV-1-seropositive individuals. The characterization of these antibodies and the corresponding epitopes are presented.

## Materials and methods

### Cell lines and virus strains

Human HeLa CD4+ cells and the human lymphoid cell lines C8166 and H9 were maintained in Dulbecco's modified Egale's medium (DMEM) or RPMI-1640 suplemented with fetal calf serum (FCS) [13]. The following virus strains, with the subtype indicated according to Meyers *et al.* [14], were used for immunostaining and virus neutralization: IIIB (LAI isolate, subtype B), RF and MN (subtype B) [15,16], SF2 (subtype B)[17], NY-5/LAV-1 (subtype B), Z34, Z84 (subtype D) and Z129 [5], GL-1 and GL-3 [9], U455 (subtype A)[5] and CBL-4 [5]. The HIV-2 strain CBL-22 [18] and ROD [19] were also used.

### Generation of human B cell clones and HuMAb

PBMC were isolated from 40 ml blood of four HIV-1-seropositive asymptomatic adult males of the Amsterdam cohort, and generation of EBV-transformed B-cell lines with these PBMC was performed as described previously [20]. For the identification and characterization of antibodies in culture supernatants, an enzyme-linked immunosorbent assay (ELISA) system described by Robinson [11] was used with recombinant HIV-1 IIIB gp120, recombinant HIV-1 IIIB gp160 and recombinant HIV-2 gp105 produced in a baculovirus expression system (American Biotechnologies Inc., Cambridge, Massachusetts, USA), feline immunodeficiency virus (FIV) glycoprotein [21] and simian immunodeficiency virus ($SIV_{mac251}$) cell lysate [22] as antigens.

### Immunostaining assay

The degree of conservation of the epitopes recognized by the HuMAb generated in these studies was tested in a previously described immunostaining assay with the above mentioned virus strains [23].

### ELISA systems

The HuMAb GP13 and GP68 generated in the course of this study were biotinylated with NHS-d-succinimidobiotin as recommended by the manufacturer (Sigma Chemie, Axel, The Netherlands). Recombinant gp120 (see above, 75 ng/ml) was coated to the ELISA plate with affinity-purified polyclonal antibody raised in sheep against the HIV-1 gp120 carboxy terminus [24,25]. gp120-coated plates were incubated for 1 h at 37°C with serial dilutions of biotinylated HuMAb and after washing incubated for 1 h at 37°C with 1 : 20 diluted StreptABComplex HRP (DAKO A/S, Glostrup, Denmark). The dilution resulting in the half maximum optical density (OD) 450 nm (mid-point titre) of the biotinylated HuMAb was determined. To determine the inhibition of the biotinylated HuMAb by the different HuMAb and soluble CD4 (sCD4), the gp120 coated plates were preincubated for 1 h with 80 µl serially diluted HuMAb or sCD4. As a negative control, a broadly reactive HuMAb (K14, immunoglobulin G1 (IgG1) specific for HIV-1 gp41 was used [20], and 20 µl of the biotinylated HuMAb (mid-point titre) was added. The ELISA was further developed as described above. These assays were performed in triplicate; the mean of the results of the three independent assays is given.

The ELISA used for the determination of the specificity of the HuMAb for a described set of mutant gp120 glycoproteins [26] was performed with a previously described assay developed by Dr J. McKeating (Chester Beatty Laboratories, London, UK) [25,27]. The reduction in binding is given as the mean percentage of the binding by the wild-type glycoproteins, and calculated from the results of two independent assays according to the formula:

$$\% = 100 \times \left( \frac{\text{OD450mutant} - \text{OD450background}}{\text{OD450WT} - \text{OD450background}} \right)$$

### VN assay

The microtiter VN assay used in these studies has been described previously [13]. Briefly, 1000 median tissue culture infective doses ($TCID_{50}$) of virus stock in 40 µl was incubated with 10 µl of serial dilutions of protein A purified HuMAb for 1 h at 37°C, then incubated with 100 µl of $2 \times 10^5$/ml C8166 or H9 cells for 1 h at 37°C. After 5 days of culture, the wells were microscopically scored for the presence of syncytia. Data presented are taken from representative experiments. Several control experiments were performed, including multiple testing of different virus stocks with different HuMAb preparations. Similar results were obtained in all of these cases.

## Results

### General properties of newly generated gp120-specific HuMAb

Screening of cloned EBV-transformed PBMC from two out of four donors (numbers 1171 and 658) yielded one B-cell line from the first donor (EBV GP13) and two from the second (EBV GP44, EBV GP68), all three produced HuMAb of the IgG1 subclass reactive in an ELISA with recombinant HIV-1 IIIB gp120 and gp160. These cell lines were subcloned until > 99.9% chance of clonality was obtained [20]. These HuMAb failed to recognize envelope glycoproteins of HIV-2, $SIV_{mac}$, and FIV in ELISA (data not shown), indicating that these three HuMAb react specifically with HIV-1 gp120. To delineate the antigenic sites recognized by these three HuMAb, we tested their capacity and that of sCD4 to inhibit the binding of biotinconjugated HuMAb GP13 (Fig. 1a) and GP68 (Fig. 1b) at their midpoint titer (18.8 and 96 ng/ml, respectivelly) to HIV-1 IIIB gp120 in the inhibition ELISA. The binding of GP13 and GP68 to gp120 was not inhibited by the gp41 specific HuMAb. GP13 and GP68 mutually inhibited each other to approximately the same extent whereas nearly 100 times more GP44 was needed for the same inhibition of the binding of both antibodies. The binding of GP13 and GP68 to gp120 could also be inhibited by pre-incubation of the wells with approximately 10 or 100 ng sCD4 per ml respectively. These data indicate that the HuMAb GP13, GP44 and GP68 recognize an antigenic site located within or topographically near the CD4-binding site of gp120.

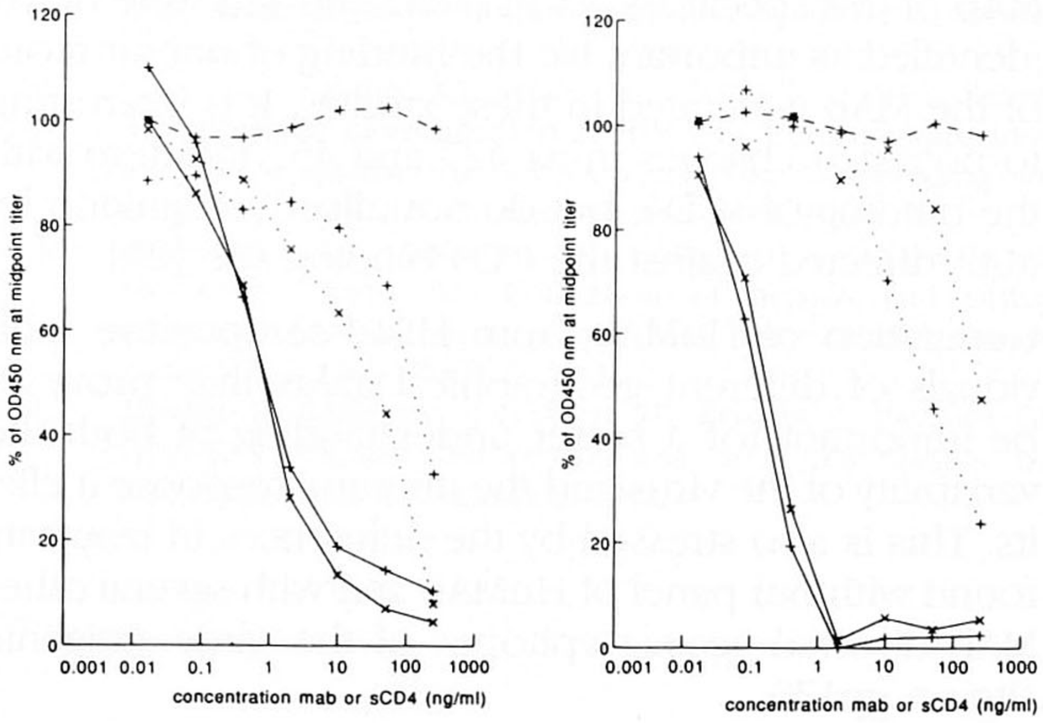


**Fig. 1.** Mean reduction percentages of OD450 nm values obtained by preincubation of gp120 coated enzyme-liked immunosorbent assay plates with non-labelled human monoclonal antibodies (HuMAb) or soluble(s) CD4, and subsequent addition of biotin labelled HuMAb GP13 (a) and GP68 (b) at midpoint titres (18.8 and 98 ng/ml respectivelly). Concentrations of non-labelled HuMAb are given in ng/ml (horizontal axis). All assays were carried out in triplicate and s.d. were less then 2% in all cases. GP13 (— + —); GP44 (···· + ····); GP68 (— × —); K14 (- - + - -); sCD4 (···· × ····).

### Specificity of HuMAb GP13, GP44 and GP68

To determine the degree of conservation of the epitope recognized by these three HuMAb, they were tested for their ability to stain cells infected with HIV isolates from three out of five phylogenetic subfamilies of HIV-1 [14] in the immunostaining assay. All HIV-1 strains tested (n = 12) were recognized by these HuMAb, whereas the two HIV-2 strains tested were not recognized in this assay (data not shown).

**Table 1.** HIV-1 neutralizing activity of human monoclonal antibodies.

| Virus | Strain | GP13 | GP44 | GP68 |
|---|---|---|---|---|
| HIV-1 | SF2 | 0.1* | 60 | 0.7 |
| HIV-1 | MN | 30 | > 240 | 11 |
| HIV-1 | IIIB | 45 | > 240 | 30 |
| HIV-1 | RF | 90 | > 240 | > 250 |
| HIV-1 | CBL4 | 12 | > 240 | 11 |
| HIV-1 | Z34 | 8 | > 240 | 11 |
| HIV-2 | ROD | > 250 | > 240 | > 250 |
| HIV-2 | CBL-22 | > 250 | > 240 | > 250 |

**In vitro* virus neurtalizing activity presented as the lowest concentration of human monoclonal activity in μg/ml, giving 90–95% reduction of the numbers of syncytia in a syncytium inhibition assay using 1000 median tissue culture infective doses ($TCID_{50}$).

The broadest HIV-1-neutralizing activity was observed for HuMAb GP13 which neutralized all the HIV-1 strains tested. The concentrations of antibody needed to neutralize 1000 $TCID_{50}$ of the different virus strains, ranged from 0.1 to 90 μg/ml for HIV-$1_{SF2}$ and for HIV-$1_{RF}$, respectively. Also, HuMAb GP68 exhibited a relatively broad VN activity. It only failed to neutralize HIV-1 RF at the antibody concentrations tested ( < 250 μg/ml). HuMAb GP44 only neutralized HIV-1 SF2 under these conditions at levels ≥ 60 μg/ml (Table 1).

### Recognition of mutant HIV-1 gp120 molecules by HuMAb GP13, GP44 and GP68

The reactivities in ELISA of these three HuMAb with a panel of mutant HIV-1 gp120, containing single amino-acid (aa) substitutions [26], are presented as percentages of their reactivities with the wild-type (HXBc2) gp120 in Fig. 2. For comparison, the reactivities of these mutant glycoproteins with sCD4 and HuMAb F105, a previously described HuMAb of similar specificity, are also shown [10,28]. Changes in threonine 257, aspartic acid 368 or glutamic acid 370 abrogated recognition by the GP13, GP44 and GP68 antibodies. Changes in recognition by these antibodies were also seen for the 256 S/Y and 262 N/T mutants, which exhibit a certain degree of conformational alteration relative to the wild-type envelop glycoproteins [26]. Recognition of the gp120 glycoprotein by the GP68 MAb was uniquely affected by changes in lysine 117 and tyrosine 435.

## Discussion

In this study we have described the generation of three EBV-transformed B-cell lines from the PBMC of

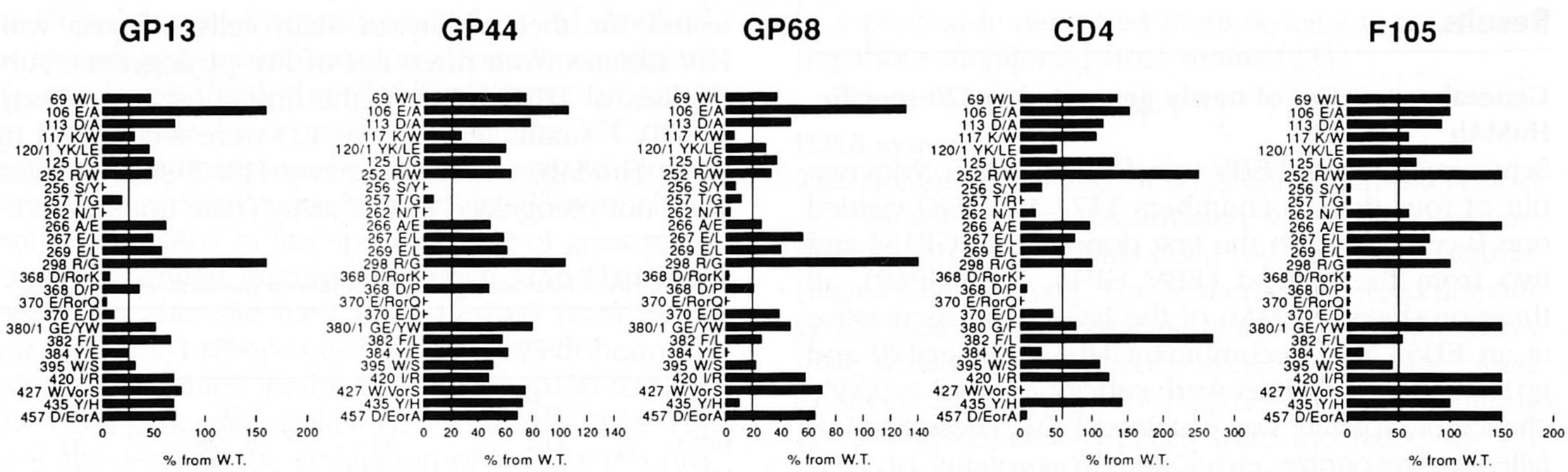


**Fig. 2.** Binding of the HuMAb and soluble(s) CD4 to mutant gp120 glycoproteins (vertical axis) expressed as percentages of binding to wild-type HIV-1 HXBc2 gp120 (horizontal axis). Other gp120 mutants tested for recognition of the newly generated HuMAb (GP13, GP44 and GP68) included 430 V/S, 314 G/W, 429 K/L, 432 K/A, 456 R/K, 457 D/G, 457D/N, 463 N/D. Recognition of these gp120 mutants was at least as efficient as that seen for the wild-type glycoproteins (not shown).

two Dutch HIV-1-seropositive individuals, which produce HIV-1-neutralizing HuMAb (GP13, GP44, GP68). These antibodies proved to be directed to a conserved conformation-dependent site that overlaps with the CD4-binding site of gp120.

These HuMAb were first shown to react specifically with HIV-1 gp120 in an ELISA using envelope glycoproteins of different primate lentiviruses and of one feline lentivirus. Their specificity for a site that overlaps with the CD4-binding site was shown in an inhibition ELISA using sCD4 as an inhibitor. The broad reactivity of these HuMAb, expected on the basis of the recognized antigenic site, was first demonstrated in an immunostaining assay with HIV-1 strains of the three phylogenetic subfamilies tested. The VN activity of HuMAb GP13, and to a lesser extent of HuMAb GP68, was also relatively broad since HIV-1 strains from different subfamilies were neutralized in a VN assay by these HuMAb. The antibody concentrations needed to neutralize the respective viruses was in the same range as described for other human and rodent MAb of similar specificity when tested in the same experiment (A. McKnight, personal observation). The VN activity of HuMAb GP44 was restricted to the HIV-1 SF2 strain, which is generally more sensitive to neutralization by sera of HIV-1-seropositive individuals, and MAb against different neutralization epitopes of HIV-1 [8] (M. Schutten, personal observation). The differences in VN activities of the three HuMAb may at least in part be explained by differences in their affinities for binding to their antigenic site on gp120. However, differences in the mechanism by which they neutralize HIV-1 may also be the cause of the differences observed. aa in this site involved in the binding of the three HuMAb were identified in an ELISA, with a previously described set of mutant gp120 molecules. In these gp120 molecules, one aa had mutated, compared with the wild-type HIV-1 HXBc2 gp120. The substituted aa were highly conserved among the gp120 molecules from different HIV-1 strains [14]. The mutant gp120 molecules had been tested previously for precursor processing, cell association and sCD4-binding activities [26]. The reactivity patterns found with the different HuMAb indicated that the three newly generated CD4-binding site-specific HuMAb have similar, yet distinct specificities. At least four aa (256, 257, 368, 370) appear to be at the core of the antigenic site, as judged from the loss of reactivity with sCD4, and with all the presently tested human and rodent MAb of this specificity. aa 117, 262 and 435 were newly identified as important for the binding of one or more of the MAb generated in these studies. It is interesting to note that changes in aa 427 and 457 interfere with the binding of sCD4, but do not affect recognition by MAb directed against the CD4-binding site [27].

Generation of HuMAb from HIV-1-seropositive individuals of different geographical areas may prove to be important for a better understanding of both the variability of the virus and the immune response it elicits. This is also stressed by the differences in reactivity found with our panel of HuMAb and with several other MAb directed against epitopes of the same antigenic site on gp120.

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