

A BINARY NEUTRALIZING DETERMINANT OF GP120 DEFINED BY NUCLEOPHILIC MONOCLONAL ANTIBODIES

Yasuhiro Nishiyama¹, Stephanie Planque¹, Yukie Mitsuda¹, Hiroaki Taguchi¹, Lei Jin¹, Stephane Boivin¹, Maria Salas², Carl V. Hanson², Sudhir Paul¹

¹Chemical Immunology Research Center, Department of Pathology and Laboratory Medicine, University of Texas–Houston Medical School, Houston, Texas 77030, USA, and ²Viral and Rickettsial Disease Lab, California Department of Health Services, Richmond, California 94804, USA

INTRODUCTION

Following noncovalent substrate recognition, many enzymes employ nucleophile-electrophile interactions to form covalent reaction intermediates [1]. The presence of enzyme-like nucleophiles in antibody (Ab) combining sites is suggested by observations that haptenic electrophilic phosphonate diesters originally developed as probes for serine proteases [2] bind covalently to IgM Abs synthesized early in the ontogeny of the immune response [3]. Hypothesizing that adaptive immune processes can strengthen the nucleophilic reactivity in coordination with development of noncovalent antigen binding forces, we raised monoclonal Abs (MAbs) by immunization with HIV gp120 containing electrophilic phosphonate diesters within its antigenic epitopes (E-gp120) [4]. The MAbs displayed two types of chemical reactivities as consequences of their enhanced nucleophilic reactivity, gp120-specific proteolysis and pseudo-covalent binding [4, 5]. However, the nucleophilic strength of the MAbs did not predict their ability to neutralize primary HIV strains [5]. In this study, we studied the importance of MAb epitope specificity as a factor in viral neutralization.

MATERIALS AND METHODS

MAbs were raised by immunizations of mice with E-gp120 (30–46 phosphonates/gp120, oligomer proportions 50–80%). Preparation of E-gp120 1a was described previously [4]. 1b was prepared in a similar manner. Hybridoma culture supernatants were screened by a covalent ELISA method. Abs bound noncovalently to immobilized E-gp120 were removed by washing with 2% SDS prior to detection of immune complexes [4]. HIV neutralization was assayed using purified MAbs in PBMC cultures. The epitope reactivity was studied by competition ELISAs using 15-mer gp120 fragments and electrophoresis assays using phosphonate analogs of synthetic gp120 peptides as probes for binding activity [3–5]. E-hapten and E-VIP were prepared as described previously [6–9]. E-293-311 was obtained similarly by replacing phosphonate groups at the side chain of Lys308 and Lys310, and its structure confirmed by ESI-MS.

RESULTS

MAbs YZ18, YZ23 and SK-T03 are nucleophilic IgGs raised by immunization of mice with E-gp120 1a (Fig 1A). Denaturing electrophoresis revealed that the immunogens contain covalent oligomers (Fig 1A, monomer and oligomer proportions in the different preparations were, respectively, 20–50% and 50–80%). The oligomerization is not an indiscriminate reaction, as similar electrophilic derivatives of other proteins, e.g., the epidermal growth factor receptor, did not oligomerize detectably [8]. MAb YZ18 hydrolyzes gp120 (Fig 1B) [4], and MAbs YZ23 and SK-T03 form unusually stable gp120 complexes characterized by resistance to denaturant SDS (Fig 1C) and slow dissociation (e.g., 1% of gp120 complexes formed by SK-T03, 18.5 days; 1% for the biotin-streptavidin complex, 3.3 days) [5]. MAbs YZ18 and YZ23, but SK-T03, neutralize a clade C, R5 HIV isolate ZA009 (Fig 1D, [4, 5]).

MAbs YZ18 and YZ23 neutralized multiple CCR5-using (R5) strains drawn from clades B and C (Table 1). With a clade B strain, the potency of IgG YZ23 was comparable or superior to the reference MAb, clone b12 [10]. MAb YZ23 also neutralized the following strains with IC₅₀ values of 10–37 µg/mL: BR014 (clade B, R5), BR020 (clade B, R5), BR021 (clade B, R5), TZ013 (clade C, R5), but did not neutralize two CXCR4-using (X4) strains BZ167 (clade B) and UG046 (clade D).

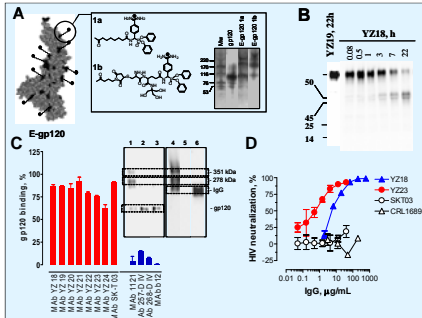


Figure 1. (A) Schematic structures of E-gp120. Two E-gp120 immunogens with the alternate linker structures (1a and 1b) were used to obtain MAbs. Electrophilic phosphonate groups were placed on Lys side chains of gp120 (30–46 phosphonates/gp120). Also shown are silver-stained SDS-gels revealing the presence of covalent oligomers in the immunogen preparations. Monomer and oligomer proportions in the different E-gp120 preparations were, respectively, 20–50% and 50–80%. (B) gp120 hydrolysis by an anti-E-gp120 MAb (IgG YZ18). Biotinylated gp120 (0.2 µM, ~1 biotin/gp120) was incubated for up to 22 h in the presence of IgG YZ18 (1 µM). Product bands at 27 kDa and 15 kDa were visible upon prolonged exposure in addition to the major 50–55 kDa bands. IgG YZ18 is a control MAb devoid of gp120 hydrolyzing activity. (C) Competition of SDS-resistant immune complexes by anti-E-gp120 MAbs. ELISA showing MAb complexes formed by incubation with gp120. MAbs (75 µg/mL) were mixed with immobilized gp120 in an ELISA plate (40 ng/well) and the wells were treated with PBS (total binding) or buffer containing 2% SDS (SDS-resistant binding). Values (means of 3 replicates) represent residual SDS-resistant binding expressed as percentage of total binding (A490 for total binding by MAbs YZ18, YZ23, YZ20, YZ21, YZ22, YZ23, YZ24, SKT03; respectively, 1.86±0.05, 0.86±0.01, 0.17±0.01, 0.32±0.03, 0.84±0.01, 1.86±0.04, 0.29±0.01, 2.08±0.04). Also shown are streptavidin-peroxidase-stained (lanes 1–3) and anti-mouse IgG-peroxidase-stained (lanes 4–6) blots of non-reducing SDS-electrophoresis gels showing SDS-resistant adducts formed by treatment of gp120 with MAb SK-T03. Lanes 1 and 4, MAb SK-T03 incubated with Bt-gp120; lane 2, isotype-matched control MAb MOPC21 incubated with Bt-gp120; lanes 3 and 5, Control Bt-gp120 alone incubated in the diluent; lane 6, MAb SK-T03 alone incubated in the diluent. Ab, 75 (lanes 1–3) or 15 µg/mL (lanes 4–6); Bt-gp120, 5 (lanes 1–3) or 27 µg/mL (lanes 4–6). Incubations for 17 h. Nominal molecular weights computed by comparison with standard proteins are indicated. (D) HIV neutralization by MAbs YZ18 and YZ23. Values are means of four replicates. HIV strain, ZA009. Phytohemagglutinin-stimulated peripheral blood mononuclear cells were added to MAb-virus mixtures and incubated for 5 days. After washing, the cells were incubated for 24 h, lysed and p24 was measured. Neutralization was computed as % decrease of p24 concentrations in MAb-containing wells compared to vehicle control. MAb CRL1689 is a control IgG without neutralizing activity.

Table 1. Neutralizing activity of MAbs YZ18 and YZ23. PHA-stimulated PBMC cells. Neutralization computed as % decrease of p24 (HIV) or p27 (SHIV) concentrations in MAb containing wells. Data are from MAb dose-response curves. NT, not tested.

Clade/Strain	MAb YZ23	MAb YZ18	MAb b12
B_SF162	4.8	2.7	14.1
B_JR-CSF	30	NT	NT
B_W61D	25	NT	NT
C_BR004	5.3	6.5	NT
C_ZA009	15	19	>20
D_UG046	>200	NT	NT
SHIV (SF162P3)	5.0	<1.9	NT

The epitope reactivity of two neutralizing MAbs (YZ18 and YZ23) and a non-neutralizing MAb (SK-T03) was tested using 15-mer peptides corresponding to gp120 residues 27–512 as competitive inhibitors. The ELISA plates contained immobilized gp120 (IgG YZ23, SK-T03) or E-gp120 (IgG YZ18; to avoid loss of gp120 by catalytic cleavage). Dose-dependent inhibition of binding of MAbs YZ18 and YZ23 by two peptide regions was observed, residues 297–315 and 417–435 (Fig 2A and B). The non-neutralizing clone SKT03 displayed a differing epitope reactivity. Only one peptide, residues 465–479, inhibited the IgG-gp120 binding competitively (Fig 2C). For both neutralizing IgGs, peptides 297–311 and 301–315 were equipotent inhibitors, suggesting the overlapping region 301–311 as a recognition element. Similarly, peptide 417–431 and 421–435 inhibited the binding with near equivalent potency, suggesting 421–431 as the second important recognition element.

The dual-reactivity was confirmed by electrophoresis and ELISA assays using electrophilic derivatives of biotinylated peptides corresponding to residues 293–311 (E-293-311) and 421–433 (E-421-433) (Fig 3A). These electrophilic analogs are sensitive probes for specific antigen-Ab binding by virtue of their ability to form covalent complexes. Both E-peptides formed covalent adducts with IgG YZ23 visible on SDS-gels at levels above the irrelevant peptide probe E-VIP (Fig 3B). Similar studies were conducted on binding of IgG YZ23 to E-293-311 and E-421-433 by ELISA (immobilized on streptavidin-coated plates). Specific binding of both peptides was evident at levels above the isotype-matched control IgG (Fig 3C). E-421-433 binding by MAb YZ23 was not inhibited by 297-311 (Fig 3D), suggesting that the MAb regions responsible for recognition of two epitope components are different.

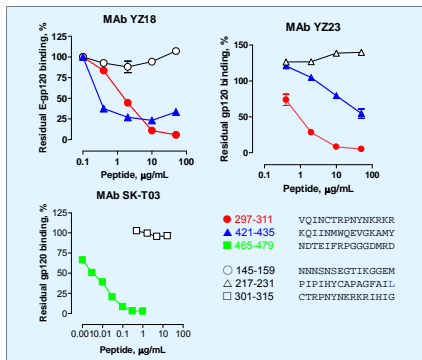


Figure 2. Inhibition of gp120-MAb binding by gp120 fragment peptides. ELISA assays were conducted using MAbs (5 µg/mL) to bind gp120 (YZ23) or E-gp120 1a (YZ18) coated plates (40 ng/well) in the presence or absence of the indicated competitor peptides. Bound MAbs were measured using anti-mouse IgG-peroxidase. Bound MAbs were measured using anti-mouse IgG-peroxidase. Peptides 301–315 and 417–431 inhibited the binding with potency equivalent to 297–311 and 421–433, respectively (not shown). The ELISA results using MAb SK-T03 (0.2 µg/mL) to bind immobilized gp120 in the presence or absence of peptide 465–479. A non-inhibitory control peptide is shown in each panel.

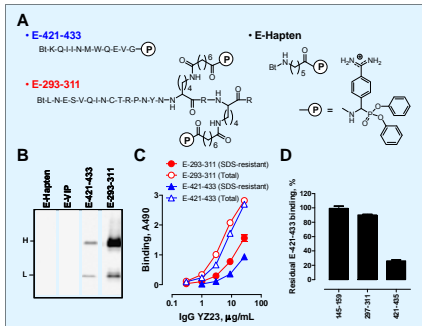


Figure 3. Identification of binary gp120 epitopes recognized by MAbs YZ18 and YZ23. (A) Structures of covalent affinity probes derived from gp120 residues 293–311 and 421–433 (E-293-311 and E-421-433). The phosphonate-derivatized peptides containing the biotin tag serve as sensitive probes for detection of Ab-peptide interactions, evident from the formation of covalent Ab-peptide adducts [3–5]. (B) Recognition of E-421-433 and E-293-311 by IgG YZ23. ELISA data using IgG YZ23 and biotinylated E-peptide probes (immobilized on streptavidin plates; 0.4 µg/well). After incubation with IgG, the wells were treated with PBS (total binding) or buffer containing 2% SDS (SDS-resistant binding). An isotype-matched irrelevant IgG (clone CRL1689) did not show detectable binding (27 ng/mL, 0.04 ± 0.01). (C) Recognition of E-421-433 and E-293-311 by IgG YZ18. ELISA data using IgG YZ18 and biotinylated E-peptide probes (immobilized on streptavidin plates; 0.4 µg/well). After incubation with IgG, the wells were treated with PBS (total binding) or buffer containing 2% SDS (SDS-resistant binding). An isotype-matched irrelevant IgG (clone CRL1689) did not show detectable binding (27 ng/mL, 0.04 ± 0.01). (D) Lack of inhibition of MAb YZ23-E-421-433 binding by 297-311. ELISA data using IgG YZ23 and immobilized E-421-433 (BSA-conjugate; ref 7) in the presence of 297-311, 421-433 or a control peptide 145–159 (50 µg/mL).

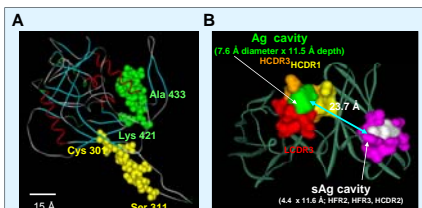


Figure 4. Dual epitope recognition model. (A) Residues 301–311 (yellow) and 421–433 (green) in the crystal structure of gp120 (PDB 2B4C, ref. 11). (B) Putative dual binding cavities in MAb YZ23. X-ray diffraction data for the YZ23 Fab crystal were collected from synchrotron radiation source and its structure was solved by molecular replacement method at 2.5 Å resolution. The putative 301–311 antigen binding cavity (Ag cavity; green) is formed by Ab CDRs. The 421–433 binding cavity (SAG cavity; white) is formed by the framework residues reported to be responsible for gp120 SAG binding.

Residues 301–311 and 421–433 are located at considerable distance in the 3-dimensional structural models of monomer human gp120 [11] (Fig 4A) and trimer simian gp120 [12] deduced from X-ray crystallography. The two peptide regions are unlikely, therefore, to form a single, contiguous epitope. Residues 301–311 and 421–433, respectively, have been suggested previously to be important in recognition of host cell CCR5 and CD4 receptors by HIV [13,14]. The region corresponding to residues 421–433 is also a component of the gp120 superantigenic (SAG) site recognized by Abs in the preimmune repertoire [15].

The V domains of IgGs YZ18 and YZ23 were sequenced using RT-PCR amplified cDNA from the hybridoma cells. Nine replacement mutations were evident in the VH and VL gene regions of each IgG, and numerous additions/deletions were observed at the V-D-J and V-J junctions (compared to the germline genes). Interestingly, the distributions of VH mutations was skewed in favor of the FRs, and all of the FR mutations were located at positions previously suggested to be important in gp120 SAG binding [16]. We must leave open the possibility, therefore, that FR mutations can improve the recognition of gp120.

The crystal structure of the Fab fragment of MAb YZ23 at 2.5 Å resolution (R factor 0.33) supported the binary recognition model (Fig 4B). Inspection of the Fab surface indicated a combining site cavity formed by the CDRs that is flanked by another, shallower cavity composed in part of certain amino acids previously suggested to contribute in binding the gp120 SAG site [16]. This supports a model of gp120 recognition in which residues 301–311 are recognized at the CDRs and residues 421–433, mainly at the FRs.

Conservation of the sequence of residues 421–433 in HIV exceeds 90% (Table 2; all strains available in the Los Alamos database; clades A, B, C, D, F, G; CR form). Except for clade D, conservation of residues 301–311 exceeds 86%. This suggests a potential basis for the neutralization resistance of the clade D strain in Table 1 (only 45% identity with consensus 301–311 sequence). The clade B non-neutralized strain BZ167 also display low identity to the consensus 301–311 sequence (64%).

A panel of MAbs raised by three separate immunizations of mice using E-gp120 immunogens with the alternate linker structures (1a and 1b in Fig 1A) were screened for dual epitope reactivity and neutralizing activity (ZA009) (n=17 MAbs, including 8 MAbs reported previously [4,5]). Ten MAbs displayed dose-dependent HIV neutralizing activity with IC₅₀ values less than 30 µg/mL. The dual epitope reactivity was studied by electrophoresis methods as in Fig 3B. Eight of the 17 MAbs displayed binding to both peptides (Fig 5A). Of the 8 dual binding Abs, 6 displayed HIV ZA009 neutralization (Fig 5B), suggesting dual recognition as a mechanism of neutralization.

Table 2. Percent conservation of residues 301–311 and 421–433. Number of HIV strains analyzed, 550; from Los Alamos Database. For each strain, the number of identities with the consensus residues in the 301–311 epitope (CTRFNNTRKRS) and the 421–433 epitope (KQKLIYVNMWVQERIGVGVGKQDR) are counted. % identities were calculated as 100 × (number of identities)/total number of residues in the peptide.

Clade*	% Identity (mean ± SD)	301–311	421–433
A (54)	88 ± 11	93 ± 6	96 ± 6
B (155)	90 ± 11	97 ± 5	97 ± 5
D (11)	62 ± 14	96 ± 11	96 ± 11
F (10)	92 ± 9	93 ± 8	93 ± 8
G (11)	91 ± 7	90 ± 4	90 ± 4
CRF (189)	86 ± 15	94 ± 7	94 ± 7

a) Numbers in parentheses are the number of strains analyzed.



Figure 5. Dual epitope reactivity of neutralizing and non-neutralizing MAbs. (A) Identification of dual binding MAbs. The anti-E-gp120 MAb panel (n=17) was assessed for dual peptide reactivity in the same manner as in Fig 3B (IgG, 0.5 µM; E-peptides, 10 µM; 3 h). MAbs positive for E-293-311 binding and E-421-433 binding were defined as those yielding band intensities >18220 and >9110 AVU (arbitrary volume unit), respectively (mean band intensity of control probe adducts of 17 MAbs, 1822 AVU). Eight of 17 MAbs were dual peptide binders. (B) Neutralizing activity of dual binding and non-dual binding MAbs. The same MAb panel was assessed for HIV neutralization using a clade C primary isolate ZA009 and PBMCs as host cells. MAbs that displayed >50% neutralization at <30 µg/mL were considered positive.

CONCLUSION

- Cross-clade neutralizing MAbs YZ18 and YZ23 recognize the binary gp120 epitope composed of residues 301–311 and 421–433.
- The dual epitope recognition property frequently results in the neutralization of HIV.
- The unique binary determinant defined by the MAbs is a promising target for induction of broadly neutralizing Abs.

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