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Quaternary Epitope Specificities of Anti-HIV-1 Neutralizing Antibodies Generated in Rhesus Macaques Infected by the Simian/Human Immunodeficiency Virus $SHIV_{SF162P4}$ ▽

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Monoclonal antibodies (MAbs) that neutralize human immunodeficiency virus type 1 (HIV-1) have been isolated from HIV-1-infected individuals or animals immunized with recombinant HIV-1 envelope (Env) glycoprotein constructs. The epitopes of these neutralizing antibodies (NAbs) were shown to be located on either the variable or conserved regions of the HIV-1 Env and to be linear or conformational. However, one neutralizing MAb, 2909, which was isolated from an HIV-1-infected subject, recognizes a more complex, quaternary epitope that is present on the virion-associated functional trimeric Env spike of the SF162 HIV-1 isolate. Here, we discuss the isolation of 11 anti-HIV NAbs that were isolated from three rhesus macaques infected with the simian/human immunodeficiency virus $SHIV_{SF162P4}$ and that also recognize quaternary epitopes. A detailed epitope mapping analysis of three of these rhesus antibodies revealed that their epitopes overlap that of the human MAb 2909. Despite this overall similarity in binding, however, differences in specific amino acid and glycosylation pattern requirements for MAb 2909 and the rhesus MAbs were identified. These results highlight similarities in the B-cell responses of humans and macaques to structurally complex neutralization epitopes on related viruses, HIV-1 and SHIV.

HIV-1 infection typically elicits high levels of antibodies directed against the viral surface envelope (Env) glycoprotein, gp160. The initial anti-Env antibody response is nonneutralizing (28), but within 1 or 2 months after infection, neutralizing antibodies (NAbs) emerge which tend to be highly strain specific for the autologous virus and exhibit little or no neutralizing activity against heterologous HIV-1 strains (10, 22). However, several recent reports have indicated that approximately 25% of HIV-1-infected, antiretroviral-naïve patients develop broad cross-neutralizing antibody responses (5, 23, 26). In some cases, these broad neutralizing antibody responses can be mapped to the CD4-binding site of Env while in most cases a single epitope specificity cannot be identified to recapitulate the neutralizing breadth of the corresponding plasma (1, 4, 14, 15, 23, 25). Detailed analyses of the epitope specificities of broad plasma neutralizing antibody responses performed by several groups revealed the presence in HIV-positive (HIV^+) plasmas of NAbs with as yet undefined epitope specificities (1, 15, 18, 23). It is possible that these undefined specificities include quaternary neutralizing epitopes (QNEs) and/or sugar molecules which coat the HIV Env spike expressed on the surface of viral particles.

The human monoclonal antibody (MAb) 2909 recognizes a QNE present on the oligomeric Env spike present on the surface of HIV-1 SF162 virions (8). MAb 2909 can bind and neutralize SF162 virions but does not bind to the corresponding soluble SF162 Env. The binding of MAb 2909 to its QNE depends on the presence of the second and third variable regions of gp120 (the V2 and V3 loops, respectively). One particular amino acid at the amino terminal side of the V2 loop (K at position 158, based on the SF162 numbering, or position 160, based on the strain HxB2 numbering) appears to be critical for its binding (11). MAb 2909 was isolated from a person who was not infected with SF162, but a virus isolated from the donor of MAb 2909 bears a V2 loop with similarities to that of SF162 and, in particular, possesses the same K158 residue (M. K. Gorny, unpublished data). More recently, two additional human MAbs, PG9 and PG16, were isolated from a subject infected with clade A HIV-1 and were shown to bind to a QNE that also includes the V2 and V3 loops (30). In contrast, however, to the narrow neutralizing potential of MAb 2909, MAbs PG9 and PG16 display far broader neutralizing abilities.

Similar to the infection of humans by HIV-1, chronic infection of rhesus macaques by simian/human immunodeficiency viruses (SHIVs) or chimpanzees by HIV-1 also results in the elicitation of potent NAbs against the autologous virus and, to a much lesser extent, against heterologous SHIV isolates or

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HIV-1 viruses (3, 6, 12, 17). Here, we describe a panel of MAbs from $SHIV_{SF162P4}$-infected rhesus macaques that demonstrates extremely potent neutralization against the homologous virus (that expresses the same Env as HIV-1 SF162) and that recognizes QNEs present on the surface of intact virions. Similar to the human MAbs 2909, PG9, and PG16, these rhesus macaque monoclonal antibodies (RhMAbs) recognize QNEs that include the V2 and V3 loops. Also, similar to MAb 2909, the RhMAbs neutralize only viruses expressing the SF162 Env. Consequently, we compared the fine epitope specificities of these RhMAbs to the epitope specificity of the human MAb 2909. Our detailed epitope mapping analysis reveals that although the human MAb 2909 and the RhMAbs recognize that same overall Env complex region, their specific requirements for binding differ. Thus, these studies of human and rhesus MAbs indicate that infection of humans and rhesus macaques with viruses expressing distinct Envs can result in the elicitation of antibodies that bind to overlapping conserved quaternary epitopes.

MATERIALS AND METHODS

Cells and plasmids. Cryopreserved mononuclear cells were obtained from rhesus macaques infected with the $SHIV_{SF162P4}$ virus. Animals C640 and A141 were infected by intravenous viral administration (12) while animal A5005 was infected by intrarectal viral administration. TZM-bl and 293T cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Plasmids expressing the HIV-1 SF162 Env and the deletion mutants of SF162 (ΔV1, ΔV2, and ΔV3) were previously described (24). Plasmid pSG3Δenv contained the HIV-1 full-length sequence in which the envelope gene has been deleted.

Transfection for antigen and pseudovirus generation. Transfection of 293T cells was performed using Fugene HD reagent following the manufacturer's protocol. For Env production, 5×10^6 293T cells were plated in T75 flasks and grown overnight prior to transfection with 12 μg of plasmids expressing the HIV-1 Env gp160 of SF162. Cell supernatants were harvested 72 h after transfection and inactivated with Triton-X. For pseudovirus production, 5×10^6 293T cells were plated in T75 flasks and grown overnight prior to transfection with 4 μg of *env* expression plasmids and 8 μg of pSG3Δenv. Medium containing pseudoviruses was harvested 72 h after transfection, filtered using a 45-μm-pore-size filter, and stored at −80°C until used in neutralization or virion capture assays.

Transformation of rhesus B cells. The method used to produce RhMAbs has been previously described (33). The B cells were inoculated with a rhesus lymphocryptovirus, herein referred to as RhEBV (where EBV is Epstein-Barr virus), capable of transforming rhesus B cells into continuous B-cell lines. RhEBV-inoculated cells were plated at low cell density in multiple 96-well tissue culture plates containing irradiated, mature human macrophage feeder cells prepared from HIV-seronegative subjects. Wells containing oligoclonal B-cell lines were screened using both the enzyme-linked immunosorbent assay (ELISA) and neutralization techniques discussed below. As reported by others, this dual screening leads to the identification of antibodies that can neutralize HIV but do not bind to the recombinant Env (8, 30). Eventually, wells of interest were cloned by limiting dilution, as previously described (33).

Antibodies and other ligands. MAbs 447-52D and 39F are human anti-V3 antibodies (9, 20). MAb 860-55 is a human anti-parvovirus B19 MAb (7). The human MAb 830A binds to the V2 loop of gp120 (21). MAb 2909 is specific for a QNE of the SF162 Env and was isolated from a chronically infected HIV-1 subject (8). CD4-IgG2 (PRO542) and soluble CD4 (sCD4) were obtained from Progenics. MAb IgG1b12 was obtained from Denis Burton (The Scripps Research Institute, La Jolla, CA).

Screening ELISA. For the initial antibody screening, we used a reverse capture immunoassay, as previously described (33). Briefly, supernatants containing antibodies from the EBV-transformed rhesus B cells were incubated in plates coated with mouse anti-monkey IgG antibodies. Detergent (Triton X-100)-treated supernatants from cells transfected with a DNA plasmid that expresses the full-length SF162 gp160 with an intact gp120-gp41 cleavage site were added, and the bound HIV-1 Env was detected using a mixture of biotin-labeled human MAbs specific for several nonoverlapping conserved sites in gp120 and gp41. The biotin mixture contained three anti-gp120 MAbs and two anti-gp41 MAbs. The anti-gp120 MAbs were the following: A32, a nonneutralizing human MAb recognizing a conserved epitope involving the C1 and C4 regions of HIV-1 Env and having an unusual property of enhancing the binding of MAb 17b and other CD4-induced (CD4i) antibodies (32); 17B, which binds to a CD4-induced epitope that participates in the formation of the coreceptor binding site (33); and EH21, which binds to a highly conserved linear epitope near the N terminus of gp120 (29). The anti-gp41 MAbs were 7B2 (cluster I) and 22B (cluster II), both nonneutralizing (2). Signal was developed using peroxidase-streptavidin and 3,3′,5,5′-tetramethylbenzidine (TMB)-H_2O_2 as a substrate. The color reaction was stopped with 1 M phosphoric acid, and color was read as the optical density (OD)/absorbance at 450 nm. An excess concentration (>100 μg/ml) of human IgG was added to the dilution buffers to prevent the capture of the biotin-labeled human MAbs by the mouse anti-monkey IgG, which would otherwise cause high background. Positive reactions give OD readings of >1.500 (usually, >2.400), and background OD is usually <0.200.

Neutralization assays. The neutralizing activities of antibodies were evaluated in three laboratories using a single-round competent-virion neutralization assay as described previously (31).

(i) Robinson lab. To identify the B cells that secrete anti-Env antibodies with neutralizing activities, we determined the neutralizing activities of the supernatants from EBV-transformed rhesus B cells. Briefly, supernatants from EBV-transformed rhesus B cells were incubated with HIV-1 SF162 pseudovirus (previously titrated to identify a dilution that produced an optimal relative light unit [RLU] signal, typically between 50,000 to 100,000) in black 96-well culture plates. TZM-bl cells were added to a final concentration of 5×10^3 cells/well with 37.5 μg/ml DEAE-dextran, and the plates were incubated for 48 h. Neutralization was assessed by analyzing the amount of luciferase produced from the pseudovirus-infected cells. Luciferase was quantified using a commercially available kit (Promega BriteGlo). To define the neutralizing activity of the isolated and purified MAbs, serial dilutions of the MAbs were incubated with the pseudovirus. The assay was then performed exactly as discussed above, and the percent inhibition of viral replication at each antibody dilution was determined.

(ii) Stamatatos lab. The neutralizing activity of MAbs against mutagenized SF162 virus was evaluated using the TZM-bl–pseudovirus neutralization assay, as previously described (23). Briefly, a predetermined amount of virus was mixed, or not, with serially diluted MAbs, and the mixture was added to wells of flat-bottom 96-well tissue culture plates containing 3×10^3 Polybrene-treated TZM-bl cells for 3 days at 37°C. The cell supernatants were then removed by aspiration, and 100 μl of Steady Glo (Promega) was added to each well for 15 min at room temperature. The number of relative light units associated with 75 μl of cell lysate was determined on a Fluoroskan Ascent FL (Thermo Labsystems). Percent neutralizations were calculated as previously described (24).

(iii) Montefiori lab. The neutralizing activities of purified MAbs against HIV-1 isolates other than SF162 were evaluated as described previously (13).

Virion capture assay. This assay was previously described (8). Briefly, antibodies to be tested were added to plates coated with anti-human IgG antibodies at a concentration of 5 μg/ml and incubated for 1 h. The plates were washed thoroughly, and virus was added to the antibodies and incubated for 1 h. The plates were washed again and incubated with Triton-X (1% in phosphate-buffered saline [PBS]) to release p24 from the virus bound to the test antibody, and the amount of released p24 was determined using a noncommercial p24 ELISA (19).

The above virion capture assay was adapted as a competition assay in which 100 μl of SF162 virions containing 100 ng of p24/ml was preincubated with 100 μl of various human MAbs or sCD4 at a concentration of 20 μg/ml. The MAbs included 2909, 447-52D (anti-V3), 830A (anti-V2), and an irrelevant MAb 860-55 (anti-parvovirus B19) (7). The SF162-MAb complexes were then added to ELISA plates coated with 10 μg/ml of one of three RhMAbs (2.3E, 2.2G, or 2.5B), 2909, 447, and control MAb 860-55. After the wells were washed extensively, the amount of bound virus was determined by disrupting the viral membrane with Triton X-100 and releasing the p24 antigen, whose amount was subsequently determined as discussed above.

Mutagenesis of SF162 gp160 *env*. Point mutations were introduced into SF162 gp160 *env* in the pEMC* vector to generate single amino acid substitutions. Position 158 based on the SF162 numbering (160 based on the HxB2 numbering) was changed from lysine to either asparagine (K158N; K158N forward primer, AAAAATTGCTCTTTCAACGTCACCACAAGCATAAGA; K158N reverse primer, TCTTATGCTTGTGGTGACGTTGAAAGAGCAATTTTT) or alanine (K158A; K158A forward primer, AAAAATTGCTCTTTCGCGGTCACC ACAAGCATAAGA; K158A reverse primer, TCTTATGCTTGTGGTGACCG CGAAAGAGCAATTTTT), and position 159 was changed from valine to either isoleucine (V159I; V159I forward primer, AAAAATTGCTCTTTCAAGATCA

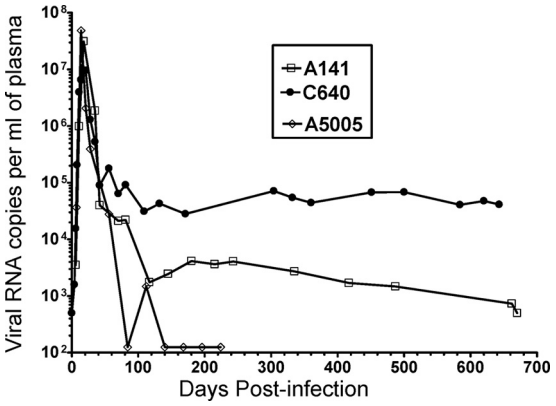


FIG. 1. Plasma viremia in $SHIV_{SF162P4}$-infected macaques. Summary of plasma viral loads in three $SHIV_{SF162P4}$-infected rhesus macaques from which we isolated the RhMAbs discussed in this study.

CCACAAGCATAAGA; and V159I reverse primer, TCTTATGCTTGTGGTG ATCTTGAAAGAGCAATTTTT) or alanine (V159A; V159A forward primer, AAAAATTGCTCTTTCAAGGCCACCACAAGCATAAGA; and V159A reverse primer, TCTTATGCTTGTGGTGGCCTTGAAAGAGCAATTTTT). The mutagenesis reaction mixtures consisted of 25 ng of template DNA, a 0.4 μM concentration of each mutagenesis primer, and PFX SuperMix (12344-040; Invitrogen Corp, CA). The reaction conditions were 95°C for 5 min followed by 30 cycles of 95°C for 30 s, 56°C for 1 min, and 68°C for 7 min, with a final extension of 68°C for 10 min. All mutations were confirmed by sequencing. The generation of the N-linked glycosylation SF162 Env mutants was previously discussed (16).

RESULTS

Isolation of HIV-1 envelope-specific MAbs from $SHIV_{SF162P4}$-infected macaques. One of the objectives of this study was to survey the rhesus macaque B-cell anti-HIV-1 neutralizing antibody response to chronic R5-$SHIV_{SF162P4}$ infection in three animals (C640, A141, and A5005). The infection process in animals C640 and A141 was previously reported (12). A141 and C640 were viremic throughout the course of infection (Fig. 1). In contrast, plasma viremia in animal A5005 decreased to undetectable levels following the acute phase of infection. All three monkeys developed high titers of serum NAbs against the human virus strain homologous to $SHIV_{SF162P4}$, HIV-1 SF162, but animals A141 and C640 also developed heterologous NAbs against diverse HIV-1 viruses (12).

B cells from cryopreserved peripheral blood mononuclear cells (PBMCs) from these animals were immortalized by a rhesus lymphocryptovirus as previously described (33). PBMCs collected 220 days postinfection from animal A05005 were tested. PBMCs collected 180 and 487 days postinfection from animal A141 were tested. PBMCs collected at 106, 171, 407, 451, 519, 584, and 643 days postinfection from animal C640 were tested. Spleen cells as well as axillary, mesenteric, inguinal, and iliac lymph nodes collected 643 days postinfection from animal C640 and A141 (except iliac lymph nodes) were also tested. Using the above-mentioned Robinson neutralization assay (see Materials and Methods for details), we screened immortalized B-cell cultures for antibodies that neutralized the homologous virus, SF162.

A total of 14 rhesus MAbs were derived in these experiments (Table 1). Despite the fact that we attempted to generate viable B-cell hybridomas from multiple time points during infection, due to poor cell viability, we were successful in generating viable hybridomas from only a few time points. This limited our ability to examine longitudinally the anti-HIV Env rhesus B-cell responses within each animal. Five of these 11 MAbs (2.2G, 2.3E, 2.5B, 2.10A, and 1.8E) were derived from macaque A5005; seven MAbs (LV23, LV24, LW26, LW10E, 1.6F, 1.10D, and 2.8F) were derived from macaque A141; and two MAbs (3.9A and MB25) were derived from animal C640. On the basis of sequence analysis of their light and heavy chains, two of the RhMAbs, 2.2 G and 2.3E, were found to be clonal, differing in one amino acid position in each of the variable regions of the heavy (V_H) and light (V_L) chains (M. K. Gorny, unpublished data).

Binding and neutralization properties of RhMAbs. Almost all Env-specific human MAbs previously produced had been detected by assays that measure antibody binding to viral Env glycoproteins, missing the predominant antibodies identified by the neutralization screen. More recently, however, MAbs that do not react in conventional binding assays but neutralize HIV-1 were isolated and characterized from HIV-1-infected

TABLE 1. Neutralization and binding properties of rhesus MAbs

Monkey	MAb	B-cell source	No. of days postinfection	Screening result		Site
				Neutralization assay	ELISA	
A5005	2.2G	PBMCs	220	+	−	QNE
A5005	2.3E	PBMCs	220	+	−	QNE
A5005	2.5B	PBMCs	220	+	−	QNE
A5005	1.8E	PBMCs	220	+	−	QNE
A5005	2.10A	PBMCs	220	+	+	V3
A141	LV23	PBMCs	180	+	−	QNE
A141	LV24	PBMCs	180	+	+	V3
A141	LW26	PBMCs	180	+	−	QNE
A141	LW10E	PBMCs	180	+	−	QNE
A141	1.6F	Lymph node	643	+	−	QNE
A141	1.10D	Lymph node	643	+	−	QNE
A141	2.8F	Spleen	643	+	−	QNE
C640	3.9A	PBMCs	584	+	−	QNE
C640	MB25	PBMCs	451	+	+	V3

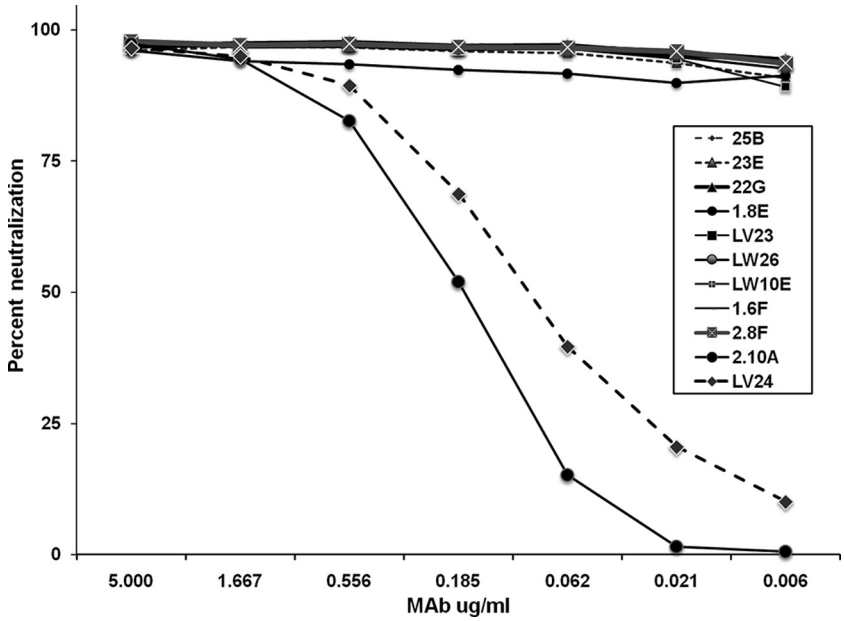


FIG. 2. HIV-1 SF162 neutralization susceptibility by RhMAbs. The neutralizing activity of the RhMAbs against HIV-1 SF162 was determined using the TZM-bl pseudovirus neutralization assay. MAbs 2.10A and LV24 are specific to V3 while the remaining RhMAbs are directed to quaternary epitopes; MAbs LV24, 1.10D, and 3.9A were not tested. HIV-1 SF162 and $SHIV_{SF162P4}$ express the same Env proteins (12).

subjects (8, 30). These MAbs were shown to recognize complex epitopes formed by quaternary structures present on virion surfaces. One of these MAbs, MAb 2909, manifests unusually potent neutralizing activity that is highly restricted for HIV-1 SF162 even though the subject from which MAb 2909 was isolated was not infected by SF162. The other two MAbs, PG9 and PG16, display broad anti-HIV-1 neutralizing activities.

To determine if any of the above-mentioned rhesus antibodies displayed similar properties as MAbs 2909, PG9, and PG16, the supernatants from the transformed rhesus B cells were screened for reactivity to the SF162 Env by ELISA (Table 1). This initial screen indicated that 11 out of 14 hybridomas failed to recognize the SF162 Env although, as discussed above, they displayed anti-SF162 neutralizing activity. Four of these 11 antibodies (2.2G, 2.3E, 2.5B, and 1.8E) were derived from macaque A5005; six (LV23, LW26, LW10E, 1.6F, 1.10D, and 2.8F) were derived from macaque A141; and one (3.9A) was derived from animal C640. The other three antibodies (2.10A, LV24, and MB25, one from each monkey) were both neutralization and ELISA positive and were subsequently shown to recognize linear epitopes of SF162 V3 based on peptide reactivity and competition binding assays with other V3 MAbs (data not shown).

All of the RhMAbs were determined to be IgGs and were readily purified by protein A affinity chromatography. Known concentrations of purified RhMAbs derived from each B-cell line were tested for reactivity with soluble SF162 Env glycoproteins by ELISA. The results with the purified RhMAbs confirmed the above-mentioned initial results with the corresponding hybridoma supernatants. Possibly the "nonreactivity" by ELISA could have been due to masking of the epitopes of the detecting antibodies by the RhMAbs already bound on the SF162 Env. However, we subsequently examined if three of these MAbs (2.2G, 2.3E, and 2.5B, isolated from A05005) could directly immunoprecipitate recombinant soluble SF162 gp140 trimeric proteins (with a mutagenized gp120-gp41 cleavage site [27]). No reactivity with such protein constructs was evident (L. Stamatatos, unpublished data). Since these results were similar to those reported with MAb 2909 (8), they suggested that the RhMAbs, like human MAb 2909, might recognize quaternary structures on intact virions which are not present on soluble SF162 Env proteins or recombinant SF162 gp140 constructs.

We next determined the neutralization potency of the purified RhMAbs against the homologous isolate, SF162 (Fig. 2). Uniformly, the group of ELISA-negative RhMAbs showed unusually potent neutralizing activities against the homologous virus, achieving >90% neutralization at MAb concentrations at or below 0.006 μg/ml without reaching a definitive endpoint (Robinson neutralization assay; see Materials and Methods for details). In contrast, the V3-specific RhMAbs 2.10A and LV24 showed neutralizing potency against SF162, with 50% neutralizing levels of <0.2 μg/ml. None of the ELISA-negative but neutralization-positive RhMAbs was able to neutralize a panel of heterologous isolates when tested at concentrations as high as 20 μg/ml (Table 2) (Montefiori neutralization assay; see Materials and Methods for details). Collectively, these results demonstrate that the $SHIV_{SF162P4}$-infected monkeys developed potent neutralizing antibodies that were highly specific for the autologous strain and suggested that these antibodies recognized one or more, complex QNE(s) present on Env spikes on the surface of infectious virions.

Epitope specificities of RhMAbs. Since it is known that the QNE recognized by MAb 2909 involves the V2 and V3 loops of SF162 gp120, we next determined the dependency for RhMAb-binding of the variable Env regions V1, V2, and V3 using a virion capture assay, as previously described (8). Since SF162 was the only virus susceptible to neutralization by the

TABLE 2. Neutralization is restricted to autologous isolate

Virus	MAb ID_{50} in TZM-bl cells (μg/ml)[a]										
	2.2G	2.3E	2.5B	1.8E	LV23	LW26	LW10E	1.6F	1.10D	2.8F	3.9A
SF162	<0.01[1]	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
TRO	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20
BAL	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20
AC10.0.29	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20
AC13	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20
6535.3	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20
QH0692.42	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20

[a] Values indicate the concentration at which the numbers of RLU were reduced by >50% compared to the number of RLU in virus control wells (no test sample).

RhMAbs, during these virion capture experiments we used the SF162 virus as well as several mutant versions of SF162 that contain deletions in the V1, V2, or V3 loop (24) (Fig. 3). We included MAb 2909, the anti-CD4-binding site (BS) MAb IgG1b12, and the V3 MAb 3.9F. RhMAbs 2.3E, 2.2G, 2.5B, LV23, LW10E, LW26, 1.6F, and 3.9A showed the same pattern of reactivity as MAb 2909; that is, they captured the SF162 and ΔV1 virions but not the ΔV2 or ΔV3 virions. In contrast, MAb IgGb12 bound to all virions, and MAb 3.9F bound to SF162 and ΔV1 and ΔV2 but not to ΔV3 variants. These results and those presented in Fig. 2 indicated that the V2 and V3 loops, but not the V1 loop, are involved in forming the QNE(s) recognized by eight RhMAbs and human MAb 2909 (8). The requirement of the V2 loop, but not that of the V1 loop, for the binding of the RhMAbs was confirmed by neutralization experiments during which it was observed that the RhMAbs identified as recognizing QNEs neutralized the SF162 and ΔV1 viruses but not the ΔV2 viruses (Table 3). MAb 2.10A which binds to the V3 loop (Table 1) neutralized the SF162 virus and both the ΔV1 and ΔV2 mutants (Table 3). Thus, the neutralization data indicate that the V2 loop of SF162 contributes to the QNE recognized by the virion-specific RhMAbs, as has been shown previously with MAb 2909. As noted above, the RhMAbs do not bind to SF162 ΔV3, indicating that V3 is an essential component of the binding site; but since the SF162ΔV3 Env does not support virus-cell entry (24), we were unable to directly evaluate the role of the V3 loop on the neutralizing activities of the RhMAbs.

Antibody competition of virion binding. We adapted the virion capture assay discussed above (Fig. 3) to evaluate the ability of different ligands of known specificity to compete for the binding of the RhMAbs to virion Env spikes (Fig. 4). In these experiments we tested three of the RhMAbs identified to recognize QNEs, MAbs 2.2G, 2.3E, and 2.5B. SF162 pseudovirions were first incubated with anti-V3 MAb 447, anti-V2 MAb 830A, sCD4, MAb 2909, or anti-parvovirus MAb 860-55. The virion-MAb mixtures were then added to MAbs immobilized in the wells, and the amount of captured virions was determined. Preincubation of virions with MAb 447 blocked the capture of virions by the three RhMAbs, MAb 2909, and by MAb 447 itself. These results support the idea that the V3 loop belongs to the QNEs recognized by these

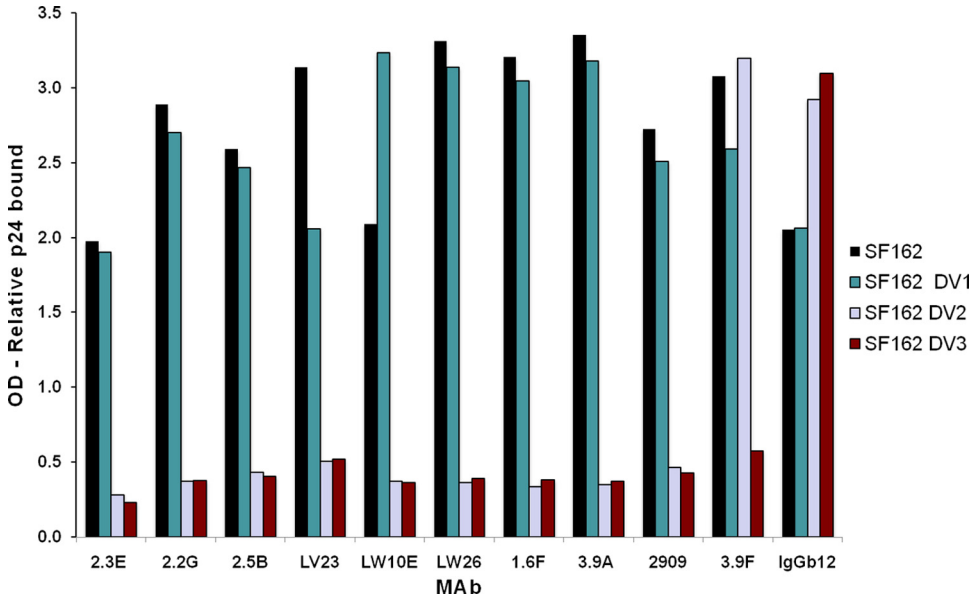


FIG. 3. Effect of variable loop deletion on virion capture by human and rhesus MAbs. SF162-derived virions without or with deletions in the V1, V2, or V3 loop (DV1, DV2, or DV3, respectively) were added to ELISA wells coated with the indicated MAbs. Detection of virion capturing was determined as discussed in the Materials and Methods section. MAbs 2909, 3.9F, and IgGb12 are human MAbs directed to quaternary epitope, V3, and CD4-binding domain, respectively.

TABLE 3. Effect of V1 and V2 loop deletions on neutralization by RhMAbs

Virus[a]	RhMAb ID_{90} (μg/ml)[b]										
	2.2G	2.3E	2.5B	1.8E	LV23	LW26	LW10E	1.6F	1.10D	2.8F	2.10A
SF162	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	3.3
SF162 DV1	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	<0.003	3.3
SF162 DV2	>20	>20	>20	>20	>20	>20	>20	>20	>20	>20	0.041

[a] DV1 and DV2, deletions of V1 and V2 regions, respectively.
[b] Values are the concentrations at which the numbers of RLU were reduced by >90% compared to the number of RLU in virus control wells (no test sample).

RhMAbs. MAb 2909 blocked the capture of virions by itself and by all three of the RhMAbs and partially blocked the capture by MAb 447. The anti-V2 MAb 830A partially blocked the capture of virions by the three RhMAbs and MAb 2909 (between 50% and 70%). Similarly, sCD4 partially blocked the capture of virions by the three RhMAbs and MAb 2909 (between 50% and 90%); sCD4 did not block the capture of virions by MAbs 447 or 860-55. As expected from the negative-control MAb, the anti-parvovirus MAb 860-55 had no effect on the capture of virions by any of the anti-Env MAbs.

Effect of amino acid substitutions in V2 on MAb neutralizing activity. The KV dipeptide within the SF162 V2 at positions 158 to 159 (160 to 161 based on the HxB2 numbering) has been previously shown to be critically important for the binding and neutralizing activity of MAb 2909 (11). When this dipeptide is reverted to the consensus B *env* sequence (NI instead of KV), MAb 2909 is no longer able to bind to and neutralize SF162. To examine whether the neutralizing activities of the RhMAbs were also dependent on the presence of the KV dipeptide, we generated the following individual mutations on the SF162 Env background: K158N, K158A, V159A, and V159I. We then compared the effects of these mutations on the neutralizing activities of MAb 2909 and three RhMAbs, 2.3E, 2.2G, and 2.5B (Fig. 5) (Stamatatos neutralization assay; see Materials and Methods for details).

The virus with the K158N mutation, which at residue 158 bears a glycosylation site that is absent from SF162, was completely resistant to all the MAbs (Fig. 5A). To determine whether the amino acid lysine at position 158 is critical for the neutralizing activities of the three RhMAbs, we generated the K158A mutation (Fig. 5C). In that case, only the neutralizing activity of MAb 2909 was abrogated. In contrast, approximately 2-fold higher concentrations of RhMAbs 2.2G and 2.3E were required for 50% neutralization while approximately a 1 order of magnitude higher concentration of RhMAb 2.5B was required for 50% neutralization. Thus, the presence of lysine at position 158 (or potentially another positively charged amino acid) is required for neutralization by MAb 2909, and it

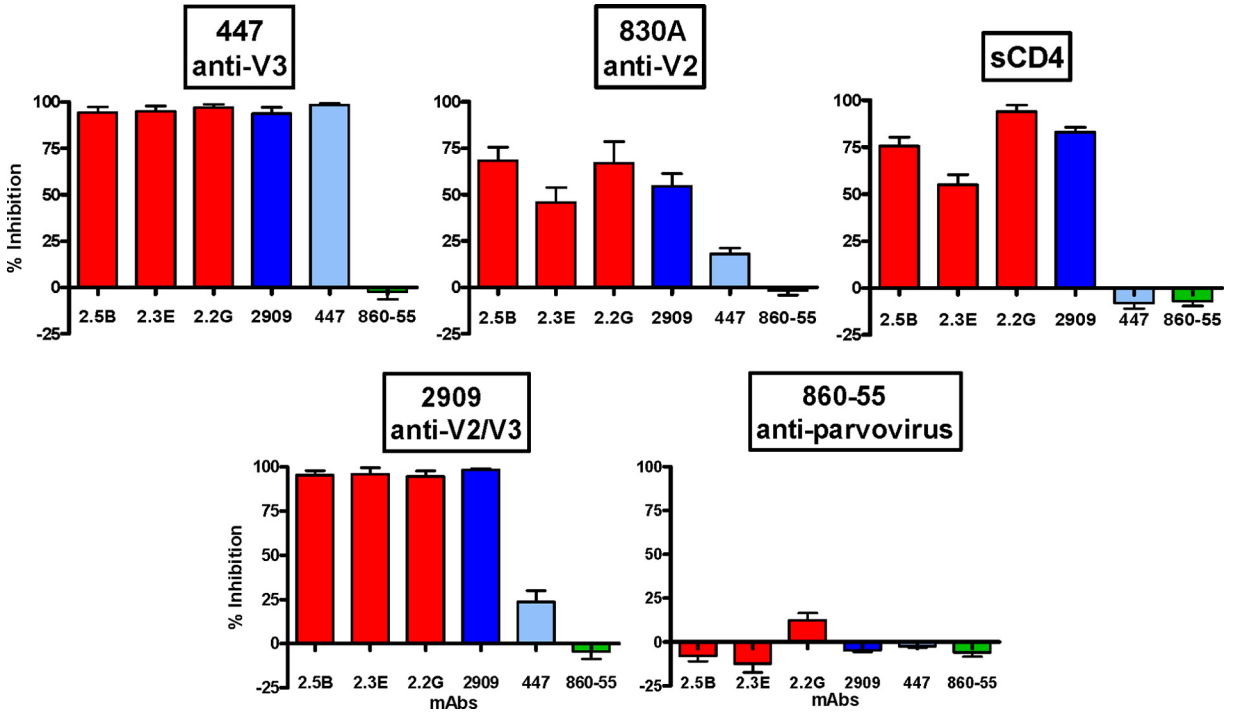
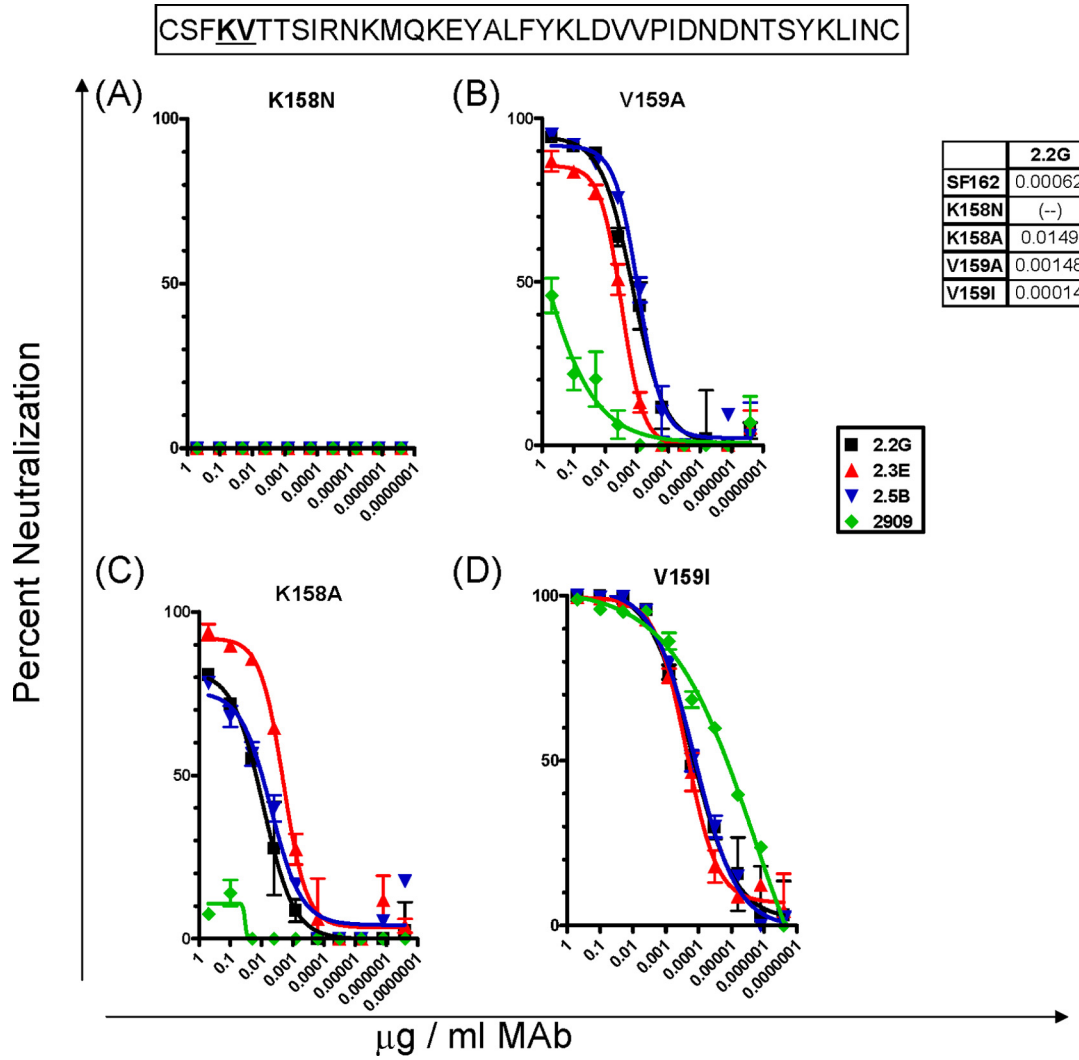


FIG. 4. Competing the capturing of virions by RhMAbs with human MAbs and sCD4. SF162 virions were first incubated with the human anti-HIV-1 Env MAbs 447 (anti-V3), 830A (anti-V2), 2909 (anti-quaternary epitope), 860-55 (anti-parvovirus, used as a negative internal control), or soluble CD4 and then added to wells coated with the indicated MAbs (*x* axis). Inhibition of capturing is represented as the percent reduction in virion capturing (*y* axis) compared to the capturing of SF162 virions that were not preincubated with human MAbs or sCD4.



	2.2G	2.3E	2.5B	2909
SF162	0.00062	0.00112	0.0008	0.00064
K158N	(--)	(--)	(--)	(--)
K158A	0.0149	0.00218	0.00964	(--)
V159A	0.00148	0.00397	0.001	(--)
V159I	0.00014	0.0002	0.00013	0.00001

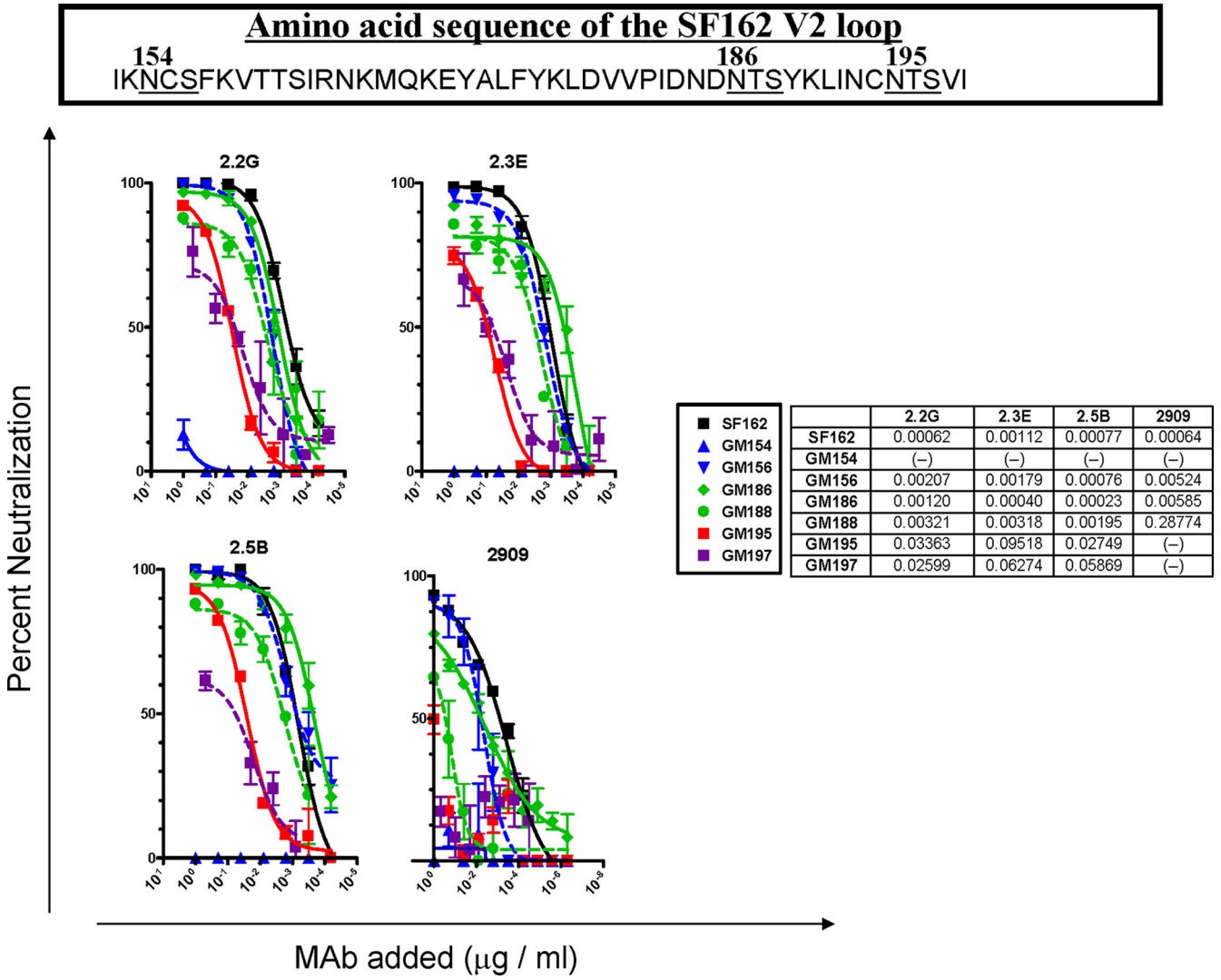
FIG. 5. Role of the KV dipeptide in V2 on MAb neutralizing activities. The lysine at position 158 (position 160, based on the HxB2 numbering) in the V2 loop of the SF162 Env was altered to either an asparagine (K158N) (A) or alanine (K158A) (C), and the valine at position 159 was altered to either alanine (V159A) (B) or isoleucine (V159I) (D). The neutralizing susceptibilities of the corresponding mutant viruses to the RhMAbs 2.2G, 2.3E, 2.5B, and the human MAb 2909 were determined. In the inset, the neutralizing titers (50% inhibitory concentrations) of each MAb against SF162 and the KV mutants are presented. The amino acid sequence (from cysteine to cysteine) of the SF162 V2 loop is shown, and the KV dipeptide is underlined.

improves the neutralizing activities of the three RhMAbs. The results also suggest that the presence of a carbohydrate moiety at position 158 in most clade B Envs prevents neutralization by the three RhMAbs to their overlapping epitopes.

The virus with the V159A mutation (Fig. 5B) was significantly resistant to MAb 2909 but still susceptible to the three RhMAbs (although a 2- to 3-fold reduction in neutralizing activity versus SF162 was recorded). Interestingly, the neutralizing activities of the RhMAbs and MAb 2909 were unaffected when the valine at position 159 was mutated into an isoleucine (V159I) (Fig. 5D). In fact MAbs 2.2G, 2.3E, and especially 2909 neutralized the V159I virus more efficiently than SF162.

In summary, the neutralizing activities of MAb 2909 and of the three RhMAbs are differentially affected by the amino acids present at position 158/159 of the V2 loop. Specifically, the amino acid K at 158 is critical for MAb 2909 binding, as previously reported (11), but has a lesser effect on the binding of the three RhMAbs, and the presence of sugars at position 158 appears to block the binding of the three RhMAbs to Envs that contain the NX(T/S) motif (which is absent from the SF162 V2 loop).

Effect of V2 loop glycosylation on MAb neutralizing activity. The SF162 V2 loop has three N-linked glycosylation sites ([NLGSs] positions 154, 186, and 195, based on the SF162 sequence numbering) (Fig. 6). All three sites are utilized, and they participate in defining the neutralization susceptibility of that virus (16). We investigated whether the neutralizing activities of the three RhMAbs and of MAb 2909 depended on these three glycosylation sites. To that end, the glycosylation sites were eliminated individually by mutagenesis: by either mutating the asparagines to glutamines or by mutating the serines into alanines (in the N-X-S glycosylation motif).



	2.2G	2.3E	2.5B	2909
SF162	0.00062	0.00112	0.00077	0.00064
GM154	(–)	(–)	(–)	(–)
GM156	0.00207	0.00179	0.00076	0.00524
GM186	0.00120	0.00040	0.00023	0.00585
GM188	0.00321	0.00318	0.00195	0.28774
GM195	0.03363	0.09518	0.02749	(–)
GM197	0.02599	0.06274	0.05869	(–)

FIG. 6. Effect of V2 loop NLGSs on the neutralizing activity of RhMAbs and MAb 2909. The V2 glycosylation sites (154, 186, and 195) were eliminated from the SF162 Env by mutating either the asparagines to glutamines or the serines to alanines. The amino acid sequence of the SF162 V2 loop is shown (numbering is based on SF162). The N-linked glycosylation sites are underlined. In the inset, the neutralizing titers (50% inhibitory concentrations) of each MAb against each deglycosylated variant of SF162 are presented.

When the asparagine at position 154 was changed to glutamine (glycosylation mutant 154 [GM154]) the neutralizing activities of all three RhMAbs and MAb 2909 were abrogated. However, when the serine at position 156 was changed into an alanine (GM156), the neutralizing activities of RhMAbs 2.3E and 2.5B were unaffected, that of RhMAb 2.2G was reduced by 3-fold, and that of MAb 2909 was reduced by approximately 1 order of magnitude. It appears therefore that the amino acid asparagine at position 154 but not the sugar molecules at that position are necessary for the binding and neutralization of these RhMAbs and MAb 2909. The serine at position 156 appears to be important for the neutralizing activity of MAb 2909.

The neutralizing activities of these three RhMAbs were minimally affected by the elimination of the glycosylation site at position 186 (GM186 or GM188). Therefore, the neutralizing activities of the three RhMAbs are independent of the presence of sugar molecules at that site and of the amino acids asparagine at position 186 and serine at position 188. In contrast, although the neutralizing activity of MAb 2909 was unaffected by the GM186 mutation (N186Q), the GM188 (S188A) mutation significantly reduced (by more than 3 orders of magnitude) the neutralizing activity of this MAb. In contrast, therefore, to the observations made with the RhMAbs, the neutralizing activity of MAb 2909 is affected by the mutation of the serine residue at position 188.

The elimination of the glycosylation site at position 195 due to either the N195Q substitution (GM195) or the S197A substitution (GM197) significantly reduced (by 1.5 to 2.0 orders of magnitude) the neutralizing activities of the RhMAbs and abrogated the neutralizing activity of MAb 2909. It is possible, therefore, that the sugars at that position are part of the 2909 epitope.

Overall, these results indicate that the three RhMAbs and MAb 2909 recognize overlapping epitopes (that may include

carbohydrate moieties) but that their binding is differentially affected by the amino acid composition and glycosylation pattern of the V2 loop of SF162.

DISCUSSION

In this study we report on the characterization of several new MAbs that recognize QNEs on the trimeric HIV-1 Env spike of the SF162 isolate. These MAbs were not isolated from HIV-1-infected subjects but from $SHIV_{SF162P4}$-infected rhesus macaques. We screened rhesus macaque EBV-transformed B-cell hybridoma supernatants for both anti-SF162 neutralizing activity and for reactivity with SF162-derived soluble Env proteins by ELISA. A total of 14 new MAbs were isolated based on the neutralization screening assay, but only three of those RhMAbs were also detected by ELISA.

Using the SF162-based neutralization screening assay, Gorny et al. previously reported on the isolation of an anti-HIV neutralizing antibody (MAb 2909) from an HIV-1-infected subject (8) that binds to a QNE on the HIV Env spike. We therefore examined whether the 11 RhMAbs that neutralize SF162 but do not bind efficiently to its soluble Env recognize the same or different QNEs as those recognized by the human MAb 2909. Our data indicate that although the 11 RhMAbs and the human MAb 2909 bind to the same overall QNE, they possess distinct amino acid and Env glycosylation pattern requirements.

Like MAb 2909, the binding and neutralizing potentials of the RhMAbs depend on the presence of regions of the V2 and V3 loops but not the V1 loop. Our data extend previous observations that the narrow neutralizing specificity of MAb 2909 is highly dependent on the presence of a lysine at position 158 (160 based on the HxB2 numbering) within the V2 loop (11). Most HIV-1 isolates have an asparagine at that position, which creates an N-linked glycosylation site (NIT in the consensus B Env sequence). It was thought that the presence of sugar molecules at that particular position in V2 abrogated the binding and the neutralizing activity of MAb 2909. Our data, however, indicate that MAb 2909 actually may recognize the amino acid K158 itself. In contrast, the binding and neutralizing activities of the three RhMAbs that we examined in fine detail (2.2G, 2.3E and 2.5B) do not depend on the presence of the amino acid K158 per se since they bind and neutralize viruses expressing an alanine at position 158; however, the K158A mutation decreases their activity. The reason that these RhMAbs do not neutralize isolates other than SF162 may be the contributions to the epitope by both the lysine residue and the carbohydrate moiety (Fig. 5).

The nature of the amino acid at position 159 is also important for the neutralizing activity of MAb 2909 and crucial for that of the RhMAbs. The neutralizing activity of MAb 2909 was abrogated when the valine at position 159 was altered to alanine, but the neutralizing activities of the RhMAbs were reduced only by approximately 2-fold. Interestingly, the neutralizing activities of two of the three RhMAbs and that of MAb 2909 improved when the valine at position 159 was altered to isoleucine (V159I). We hypothesize that the length and/or structure of the alanine side chain reduces the binding of the RhMAbs and abrogates the binding of MAb 2909. In contrast, the presence of the amino acid isoleucine perhaps alters the local conformation of that region and facilitates the binding of both MAb 2909 and the RhMAbs.

There is one NLGS within the V2 loop of SF162 and two NLGSs immediately adjacent to the two cysteines at the amino and carboxy termini of that region (Fig. 6). All three sites are utilized during *in vitro* SF162 replication in PBMCs (16). Our results indicate that the sugar molecules at these three positions and the underlying amino acids differentially affect the neutralizing activities of the RhMAbs and MAb 2909. In both the case of the RhMAbs and MAb 2909, it appears that the asparagines at position 154 but not the sugar molecules at that position are crucial for their neutralizing activities. Similarly, the sugar molecules on asparagine at position 186 are not part of the epitopes of MAb 2909 and the RhMAbs, but the amino acid at position 188 is part of the MAb 2909 epitope and potentially of the RhMAbs epitope. Interestingly, our data indicate that the sugars present at the carboxy terminus of the V2 loop (position 195, using the SF612 numbering) are important for the neutralizing activity of the three RhMAbs and critical for the neutralizing activity of MAb 2909. Therefore, the epitope of 2909 may partially be made out of sugar molecules.

Overall, although MAb 2909 and the RhMAbs 2.2G, 2.3E, and 2.5B recognize the same quaternary region of the SF162 Env, there are significant differences in their recognition patterns. This is not unexpected since MAb 2909 was isolated from an asymptomatic individual infected with a virus other than SF162. Hence, despite the remarkable potency of MAb 2909 against SF162, the epitope presented by this virus is probably not the best fit for this MAb. In contrast, because the RhMAbs arose in monkeys infected with $SHIV_{SF162P4}$, which expresses the same Env as HIV-1 SF162 (12), their activities more truly reflect the natural B-cell recognition of autologous the virus. We do want to emphasize, however, that the epitope-mapping studies presented here were conducted with three RhMAbs derived from the same animal (A0005, which developed only autologous plasma neutralizing antibody responses). Although antibodies that recognize a QNE on SF162 Env were isolated from other animals (Table 1), this does not necessarily mean that they recognize the same epitope, and a detailed analysis of the epitopes of the RhMAbs isolated from the other animals is important.

It is as yet uncertain whether the RhMAbs and MAb 2909 actually make contact with the CD4-BS. During the virion binding competition assays, we observed that sCD4 binding to the viral Env spikes partially reduces the binding of these MAbs to their epitopes. RhMAbs 2.2G and 2.5B and MAb 2909 were especially affected by the binding of sCD4 to the virion Env (~75 to 85% inhibition of binding to the virion surface) while MAb 2.3E was less affected (~50% inhibition of binding to the virion surface). These observations are not, however, proof that the CD4-BS is part of the QNEs of these MAbs. The preincubation of virions with sCD4 induces a conformational change in Env which involves a spatial rearrangement of the V2 and V3 loops. Such structural rearrangements could disrupt the QNE recognized by MAb 2909 and the RhMAbs. We speculate that if the QNE of these RhMAbs and MAb 2909 depends on the close association of V2 and V3 loops, either within or between the gp160 protomers forming one Env spike, then these MAbs may block the engagement of

cellular CD4 by cross-linking V2 and V3 loops. This could partially account for the potency of these MAbs.

A topic not addressed here is how the amino acid and glycosylation patterns of the V3 loop may affect the neutralizing activities of these RhMAbs and MAb 2909. Unfortunately, partial or complete deletion of the V3 loop from SF162 Env renders it nonfusogenic (24), so we could not perform neutralization assays. Previous studies with MAb 2909 showed that while specific features of the SF162 V3 sequence were required for the optimal recognition of viruses, this MAb retained sufficient affinity for many SF162 Env chimeras expressing variant V3 sequences corresponding to consensus sequences of multiple viral subtypes to mediate relatively potent neutralization (11). It is imperative, however, that the specific role of the V3 loop in the binding of the RhMAbs be examined in detail in the future.

It would also be important eventually to determine the *in vivo* antiviral activities of antibodies that recognize complex QNEs. The dependency on the K160 residue for the neutralizing activities of the RhMAbs examined would predict that the $SHIV_{SF162P4}$ replicating virus would be able to easily evade their action. It is thus interesting that for the virus in animals C640 and A141, one of the first mutations (within the first 70 days of infection) that we recorded in the infected animals showed a change from K to N at that specific position (12). The resulting virus is completely resistant to these RhMAbs (data not shown). The dependency of the three RhMAbs for the K at position 158 of the V2 loop may (in part) explain why these antibodies are SF162 specific. The human MAbs PG9 and PG16 also recognize quaternary epitopes that include elements of the V2 loop, but they do not neutralize SF162 unless the K is mutated into an N (30). It would therefore be very informative for future Env-based immunogen design efforts if the quaternary epitopes of PG9, PG16, 2909, and the three RhMAbs discussed here are compared.

Our results and those mentioned above suggest, therefore, that a quaternary epitope that encompasses elements of both the V2 and V3 loops is immunogenic within the trimeric HIV-1 Env spike. This epitope is the target of NAbs with narrow or broad cross-neutralizing activities. Rhesus B cells "see" this epitope in a similar way as human B cells. In fact, a previous study indicated that the neutralization epitopes in macaques infected with the X4-tropic SHIV-KB9 virus were located in both the V2 and V3 loops of that Env (6). Therefore, rhesus macaques can be used to test HIV Env-based vaccine candidates that aim at the elicitation of antibodies to complex, quaternary epitopes of the HIV-1 Env spike. Whether smaller animals, such as rabbits or guinea pigs, which are routinely used for the comparative evaluation of HIV Env-based immunogens, respond similarly to such complex QNEs should be determined.

As reported earlier, animal C640 (and, to a lesser extent, animal A141) developed cross-reactive plasma neutralizing antibody responses (12). One of reasons to conduct this study was to isolate novel cross-neutralizing antibodies from SHIV-infected macaques. However, the use of SF162 as the initial screening virus resulted in the isolation of MAbs that were highly specific for this isolate. Screening with a heterologous isolate might have resulted in the isolation of MAbs with cross-neutralizing activities. The low viability of frozen PBMCs from these animals renders such assays very difficult. We are, however, pursuing our efforts to isolate cross-neutralizing MAbs from C640 and A141 using heterologous HIV-1 isolates for screening with the few samples remaining.

Finally, it is noteworthy that the RhMAbs discussed here and the human MAbs 2909, PG9, and PG16 (8, 30) recognize QNEs composed (partially or entirely) of regions in V2 and V3 loops. In contrast to the RhMAbs discussed here and MAb 2909, MAbs PG9 and PG16 are broadly neutralizing. For immunogen design purposes it would be important to better define similarities as well as differences in the epitopes recognized by these MAbs. It would also be important to better define the frequency of NAbs that bind QNEs in HIV-1^+ plasmas and understand the implications of their generation on viral fitness.

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