Human Monoclonal Antibody 2G12 Defines a Distinctive Neutralization Epitope on the gp120 Glycoprotein of Human Immunodeficiency Virus Type 1

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We have isolated and characterized human monoclonal antibody 2G12 to the gp120 surface glycoprotein of human immunodeficiency virus type 1 (HIV-1). This antibody potently and broadly neutralizes primary and T-cell line-adapted clade B strains of HIV-1 in a peripheral blood mononuclear cell-based assay and inhibits syncytium formation in the AA-2 cell line. Furthermore, 2G12 possesses neutralizing activity against strains from clade A but not from clade E. Complement- and antibody-dependent cellular cytotoxicity-activating functions of 2G12 were also defined. The gp120 epitope recognized by 2G12 was found to be distinctive; binding of 2G12 to LAI recombinant gp120 was abolished by amino acid substitutions removing N-linked carbohydrates in the C2, C3, V4, and C4 regions of gp120. This gp120 mutant recognition pattern has not previously been observed, indicating that the 2G12 epitope is unusual. Consistent with this, antibodies able to block 2G12 binding to recombinant gp120 were not detected in significant quantities in 16 HIV-positive human serum samples.

Characterization of the immune response to human immunodeficiency virus type 1 (HIV-1) infection may yield information useful in the development of effective passive or active antiviral immunization strategies. Neutralizing antibodies have been considered an important component of a protective immune response, as indicated by studies involving long-term nonprogressors (6), although proof of antibody efficacy *in vivo* is lacking (33). Identification of the antigenic sites of HIV-1 able to elicit antibodies that possess biological activity against primary strains will assist in defining the potential of the humoral immune response to control HIV-1 replication.

To date, many continuous and discontinuous epitopes within the HIV-1 envelope glycoproteins gp120 and gp41 have been shown to elicit neutralizing antibodies. Human and murine monoclonal antibodies (MAbs) to the V3 loop can neutralize HIV-1 strains, although usually only in a type-restricted manner (12, 19, 23, 36, 45, 49, 53). Antibodies to epitopes overlapping the CD4-binding site on gp120 are highly prevalent in patient sera and have been shown to neutralize divergent HIV-1 isolates with moderate (4, 17, 32, 41, 59, 61, 63, 64) or strong potency (5). Antibodies directed against sites in the V2 region of gp120 (10, 13, 16, 29, 66) and a group of antibodies that display increased binding to gp120 after CD4 binding (62) have also been found to possess some neutralizing activity. A further strong neutralizing domain has been identified in gp41 (8, 38, 44, 65). There are, however, only a few human MAbs

that are able to neutralize primary isolates of HIV-1 efficiently (5, 38, 44).

Neutralization of cell-free virus is not, however, the only mechanism by which antibodies can counter HIV-1. Via their Fc regions, MAbs can mediate antibody-dependent cellular cytotoxicity (ADCC) against cells infected with HIV-1 and also activate the complement system (22, 42). However, these effector function-mediated activities have not been well characterized for most human MAbs.

Here we show that human MAb 2G12 can activate both ADCC- and complement-mediated activities and that it possesses broad and potent neutralizing activity against primary and T-cell line-adapted strains of HIV-1. Furthermore, we show that the gp120 epitope recognized by 2G12 is a heretofore undefined structure dependent on N-linked glycans in the C2, C3, V4, and C4 domain, and that antibodies to the 2G12 epitope are rare in sera from HIV-1-infected humans.

MATERIALS AND METHODS

Viruses, cells, and MAb 2G12. The production of human MAbs 2G12, 1B1, and 3D6 has been described previously (3). The cell line H9/HTLV_{III} was obtained from the American Type Culture Collection (Rockville, Md.). The following cell lines were obtained through the AIDS Research and Reference Program, Division of AIDS, National Institute of Allergy and Infectious Diseases: AA-2 (7), H9 (40), CEM.NKR (18), Molt-4 clone 8 (9), HUT78/HIV-1_{SF2} (25, 51), H9/HTLV_{III}_{MN} NIH-1984 (11, 54), and H9/HTLV_{III}_{RF} NIH-1983 (40, 58).

All primary isolates were grown in mitogen-stimulated peripheral blood mononuclear cells (PBMC). Primary isolates designated by a code in the format exemplified by 92RW009 were provided by the World Health Organization (21). Primary isolates WYG, WRF, WHM, and WRB were isolated from Viennese patients with various disease statuses, as described previously (44). WYG, WRF, and WRB are syncytium-inducing isolates that productively infect and induce syncytia in AA-2 and MT-2 cells, while WHM is a non-syncytium-inducing isolate unable to infect these cell lines (data not shown). Cultivation of noninfected and

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infected cell lines, preparation of PBMC, and production of virus stocks were carried out as described previously (44).

Syncytium inhibition assay. Syncytium inhibition was assessed with AA-2 cells as the indicator cell line, with syncytium production quantitated by light microscopy as described previously (44). The presence of one syncytium per culture well was considered to be an indication of HIV-1 infection. The 50% effective concentration (EC_{50}) and the 50% tissue culture infective dose were calculated by the method of Reed and Muench (46). The EC_{50} is defined as the antibody concentration resulting in 100% inhibition of syncytium formation in 50% of the wells. In some experiments, p24 antigen production was measured at the same time as the observation of syncytium formation with a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Coulter Corp., Chicago, Ill.), used according to the manufacturer's instructions, or with an in-house ELISA as described previously (44). The ratios of p24 antigen production in MAb-containing cultures to p24 antigen production in the absence of MAb were estimated, and the MAb concentrations (in micrograms per milliliter) causing 50, 90, and 99% inhibition (the ID_{50} , ID_{90} , and ID_{99}) were determined by linear regression analysis.

Neutralization assay. Virus neutralization was assessed with phytohemagglutinin-stimulated PBMC as indicator cells, with determination of p24 antigen production as the end point. PBMC were stimulated with phytohemagglutinin for 48 h before removal of the mitogen by washing. Assays were performed as described previously (44). The calculated neutralization titers refer to the MAb concentrations present during the preincubation step of MABs with virus. The MAB-virus mixture was incubated for 7 days with stimulated PBMC. The cultures were then collected and treated with 1% Nonidet P-40 detergent. The contents of replicate wells were pooled before determination of their p24 antigen concentration by the in-house ELISA. The ID_{50} , ID_{90} , and ID_{99} were calculated as described above.

Deposition of complement factor C3 on infected cells. Indirect immunofluorescence staining was performed prior to the complement-binding assay to examine the MAB reactivity with oligomeric gp120 on cells infected with the HIV-1 strains IIIB, MN, and RF and to monitor the percentage of infected cells in the cultures used for the complement experiments and ADCC assays. Assays were performed as described previously (44).

For the complement factor C3 deposition experiments, slides with HIV-1-infected and -uninfected CEM.NKR cells were prepared as described previously (44). All samples and reagents were diluted in phosphate-buffered saline (PBS) and applied to the reaction fields in a volume of 20 μ l. Cells were treated with PBS containing 1% bovine serum albumin (BSA), and subsequently, human MABs 2G12, 3D6, 2F5 (38), 1B1 (3), and 257-D (57; a gift from S. Zolla-Pazner) at 25 μ g/ml or human immunoglobulin G (IgG) preparation from a serum pool of HIV-1-infected donors (HIV⁺ hIgG; provided by P. N. Barrett, Immuno AG, Vienna, Austria), diluted 1:40, was added to the cells for 1 h at room temperature (RT). After this, and between all following incubation steps, the slides were washed twice in PBS. In the following steps, the samples were incubated with PBS containing 1% BSA and then with human complement serum (Sigma, St. Louis, Mo.) diluted 1:12. Negative controls included the use of human serum heated (30 min at 56°C) to inactivate complement and of PBS-BSA buffer. After the complement incubation, the slides were washed and then blocked with PBS-BSA. The cells were next incubated for 30 min on ice with goat anti-C3 antiserum (Sigma) at a 1:60 dilution and then blocked with heat-inactivated mouse serum. Finally, fluorescein isothiocyanate-conjugated mouse anti-goat Ig (dilution, 1:100; Sigma) was applied to the samples. After three washing steps, the slides were mounted in 50% glycerol-PBS and viewed with a confocal microscope with an argon laser (Bio-Rad MRC 600).

Syncytium inhibition in presence of active human complement. The assay was performed as described above except that 50 μ l of active human complement serum (diluted 1:4 in assay medium; Sigma) was added to each well during the preincubation step. To control for nonspecific inhibitory effects, antibodies were also incubated with heat-inactivated human complement at the same concentration. As an additional control, samples were tested in medium lacking human serum. These controls were performed with eight replicates per MAB dilution step, whereas samples with the active and inactivated complement sera were performed with four replicates. The input virus inoculum was titrated in assay medium as well as in medium containing active or inactivated human complement serum to ensure that an appropriate degree of virus infection had occurred. A 50- μ l aliquot of AA-2 cells (8×10^5 /ml) was added after the preincubation period. The cultures were incubated for 5 days, and syncytium production was determined as described above.

ADCC assay. The ADCC activities of 2G12 and control sera were determined in a standard 5-h, 51 Cr release assay (28). CEM.NKR cells infected with the HIV-1 strains IIIB, MN, or RF were used as target cells. Cells were only used after at least 90% of them had become infected, as monitored by indirect immunofluorescence staining with the anti-gp41 human MAB 3D6. In addition to infected cells, uninfected CEM.NKR cells with adsorbed recombinant gp120 (gp120/IIIB baculovirus expressed; American Biotechnologies Inc., Cambridge, Mass.) were used as target cells (28). Target cells were labelled with 100 μ Ci of 51 Cr ($Na_2^{51}CrO_4$; Amersham, Bucks, United Kingdom) for 1 h at 37°C. PBMC were isolated from healthy HIV-1-negative donors and incubated overnight. Subsequently, nonadherent cells were collected and used as effector cells. Target cells, effector cells (effector/target ratio, 20:1), and serial dilutions of the samples

were incubated for 5 h at 37°C, and supernatants were harvested. As a negative control, uninfected CEM.NKR cells were included as targets in all assays. Concentrations presented in Fig. 2 correspond to the MAB dilution in the total 150- μ l reaction mixture volume. Spontaneous release controls were determined by incubating the target cell suspension with assay medium. The maximum release value was obtained by lysis of the target cell suspension with PBS containing 1% Triton X-100. All samples were tested in duplicate, except that control samples were tested in quadruplicate. Experimental data were used for analysis only if the spontaneous release values were less than 15% of the maximum release value. The percentage of cell lysis was calculated by the following formula: (experimental release - spontaneous release)/(maximum release - spontaneous release) $\times$ 100. None of the reagents tested lysed uninfected cells.

Effect of sCD4 on 2G12 binding to monomeric gp120. A competition assay was performed to examine whether the binding of 2G12 to its epitope interfered with the binding of sCD4 to gp120. Recombinant vaccinia virus-derived gp160/IIIB (a gift from Immuno AG) was coated onto microtiter plates at a concentration of 5 μ g/ml. Twofold serial dilutions, starting at 10 μ g/ml, of human MABs 2G12 and 1B1 (3) in PBS containing 1% BSA were transferred to the plates. After 1 h of incubation at 37°C, 100 μ l of sCD4 (2 μ g/ml; American Biotechnologies) was added to each reaction mixture and the plates were incubated for 1 h at RT. After washing and blocking for 1 h at RT with PBS containing 1% BSA, the plates were incubated with the murine anti-CD4 MAB OKT4 (Ortho Diagnostics, Raritan, N.J.) and diluted 1:50 in PBS for 1 h at RT. Finally, bound OKT4 was detected by subjecting the plates to treatment with horseradish peroxidase-conjugated goat anti-mouse IgG (diluted 1:1,000; Zymed, San Francisco, Calif.) and then incubation with 1,2-*o*-phenylenediamine dihydrochloride solution containing 0.03% hydrogen peroxide. The optical density was read at 492 nm (reference wavelength, 620 nm).

Binding of 2G12 to denatured, reduced, and deglycosylated gp120 and gp160. To further determine the nature of the 2G12 epitope, we performed binding assays with recombinant native, denatured, and denatured and reduced recombinant gp120/IIIB. These proteins were either CHO cell expressed (kindly provided by H. Stoiber) or baculovirus expressed (American Biotechnologies). The CHO and baculovirus proteins were coated onto microtiter plates at concentrations of 5 μ g/ml and 200 ng/ml, respectively. Denaturation of gp120 was performed by boiling for 10 min in PBS containing 6 M urea. Reduction of gp120 was performed by boiling the samples in PBS containing 6 M urea and 2% β -mercaptoethanol. Free sulfhydryl groups were acetylated by treatment with iodoacetamide (30 mg/ml in 1 M Tris, pH 8.5) for 15 min at 37°C. Serial twofold dilutions of MAB 2G12 in PBS containing 1% BSA were incubated with the various gp120 samples for 1 h at RT. Bound antibody was detected as described above.

For deglycosylation experiments, 500 ng of gp160/MN (vaccinia virus expressed; Immuno AG) was boiled for 10 min in denaturation buffer (0.5% SDS-1% β -mercaptoethanol). Then, 1/10 volume of 10 $\times$ peptide-N-glycosidase F (PNGaseF) enzyme buffer (Boehringer Mannheim), 10% Nonidet P-40, and 2,000 U of PNGaseF were added and the samples were incubated for 12 h at 37°C. Free sulfhydryl groups were not acetylated in order to allow oxidation and refolding during the 12-h incubation. Untreated gp160, gp160 denatured without PNGaseF treatment, and gp160 both denatured and PNGaseF treated were separated by polyacrylamide gel electrophoresis on 10 to 20% Tris-glycine gels. Proteins were blotted onto a nitrocellulose membrane and incubated with MAB 2G12 (5 μ g/ml) or control MAB 2F5 (5 μ g/ml) (3, 38). Bound antibody was detected by incubation with alkaline phosphatase-conjugated anti-human IgG (Sigma). Antibody-antigen complexes were visualized with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (NBT-BCIP; Sigma).

Seroprevalence of 2G12-like antibodies. To determine whether 2G12-like antibodies are prevalent in sera from HIV-1-infected people, we performed competition experiments with biotin-labelled 2G12 and patient sera (1:100 dilution) to detect serum antibody inhibition of 2G12 binding to recombinant gp120. Assays were performed essentially as described previously (31), except that detergents were omitted from the assay buffers. Recombinant gp120 molecules from the LAI (BH10; Celltech Ltd., Slough, United Kingdom), JR-FL (Progenics Inc., Tarrytown, N.Y.), and SF-2 (Chiron Inc., Emeryville, Calif.) strains were used.

Epitope mapping by ELISA with HxBc2 gp120 mutants. ELISA binding studies with HxBc2 gp120 mutants were performed as described previously (35, 37), with the addition of the following mutants to the test panel: the mutant with a K-to-A substitution at position 59 (59K/A), 64E/A, 67N/A, 97K/V, 106E/K, 110S/Q, 113D/G, 114Q/T, 130K/N, 177Y/F, 178K/E, 179L/V, 181I/M, 254V/I, 258Q/H, 286V/A, 295N/S, 327R/H, 334S/N, 342L/G, 363Q/R, 372V/T, 375T/M, 375S/A, 423I/F, 425N/A, 426M/L, 426M/R, 427W/F, 427W/R, 428Q/A, 482E/S, 491I/L, Δ 128-195, Δ 136-152, and Δ 303-323.

Briefly, soluble gp120 molecules were captured onto a solid phase via adsorbed antibody D7324 (Aalto BioReagents, Dublin, Ireland) to the carboxy-terminal 15 amino acids. MAB 2G12 was bound onto gp120 in TMSS buffer (2% nonfat milk powder-20% sheep serum in Tris-buffered saline). Bound human MAB was detected with alkaline phosphatase-conjugated goat anti-human IgG (Accurate Chemicals, Westbury, N.Y.) followed by the AMPAK amplification system (Dako Diagnostics, Carpinteria, Calif.).

Escape from neutralization by 2G12. To examine whether the amino acid changes that were shown to affect 2G12 recognition in the ELISA binding studies

TABLE 1. Syncytium inhibition titers of human MAb 2G12^a

Strain	EC ₅₀ (μg/ml)
IIIB	4.6
MN	>60
RF	0.5
SF2	55
WYG	1.0
WRF	4.2

^a EC₅₀s of MAb 2G12 are from syncytium inhibition assays, against primary isolates and T-cell line-adapted strains, with cell line AA-2. The absence of neutralization is indicated as an EC₅₀ of >60 μg/ml. Values shown are the means from two to three independent experiments.

reduced the ability of a virus to be neutralized by this MAb, an *env* complementation assay was used as described previously (15, 60, 62). Briefly, complementation of a single round of replication of the *env*-deficient chloramphenicol acetyltransferase-expressing provirus by the various envelope glycoproteins was performed. The recombinant virions were incubated in the presence or absence of 2G12 prior to addition to Molt-4 clone 8 target cells. The degree of neutralization was assessed by comparing the level of chloramphenicol acetyltransferase activity in the target cells following exposure to viruses incubated with antibody with that in target cells following exposure to viruses incubated without antibody.

RESULTS

Syncytium inhibition. The virus-inhibiting activity of human MAb 2G12 was assessed with AA-2 cells as the indicator cell line. Syncytium inhibition was tested with the T-cell line-adapted strains IIIB, MN, RF, and SF2, as well as with two PBMC-grown, syncytium-inducing primary isolates, WYG and WRF. The EC₅₀s for syncytium inhibition by 2G12 against these isolates are listed in Table 1, and the ID₉₉, ID₉₀, and ID₅₀ titers calculated from the p24 endpoint readings are shown in Table 2. The microscopical evaluation of syncytium formation is very sensitive, as cultures with one single syncytium are scored as HIV-1 infected. The recorded EC₅₀s therefore represent an antibody dilution at which 100% neutralization in 50% of the cultures was achieved. These values are similar to the ID₉₀s estimated from the p24 readout.

MAb 2G12 inhibited syncytium formation by IIIB, RF, WYG, and WRF strains very potently (EC₅₀, 0.5 to 4.6 μg/ml), while the SF2 strain was only weakly sensitive (EC₅₀, 55 μg/ml). 2G12 was unable to inhibit syncytium formation by HIV-1_{MN} (Table 1). A low concentration of 2G12 neutralized cell-free HIV-1_{MN} by 50%, but 90% neutralization of this virus by 2G12 was not achieved. This contrasts with the potent neutralization of IIIB and RF by 2G12 (Table 2). We noted that 2G12 was completely unable to bind to recombinant MN gp120 (Genentech Inc., South San Francisco, Calif.) of a fixed clonal composition (data not shown), yet 2G12 could bind to MN-infected cells (see C3 deposition experiments and ADCC assay above). One explanation for these paradoxical observations could be clonal variation in uncloned stocks of HIV-1_{MN}; both

TABLE 3. Neutralization titers of human MAb 2G12 against primary HIV-1 isolates^a

Isolate	Clade	ID ₉₉ (μg/ml)	ID ₉₀ (μg/ml)	ID ₅₀ (μg/ml)
92RW009	A	2.35	0.13	0.01
92RW021	A	22.0	1.04	0.15
92UG037	A	>50.0	35.7	0.07
92TH014	B	0.14	0.10	<0.01
92BR021	B	>50.0	>50.0	>50.0
92BR030	B	1.34	0.27	<0.01
92TH021	E	>50.0	>50.0	>50.0
92TH024	E	>50.0	>50.0	>50.0
WYG	ND ^b	<0.20	<0.20	<0.20
WRF	ND	>50.0	0.13	<0.01
WHM	ND	>50.0	43.4	<0.01
WRB	ND	1.94	0.61	<0.15

^a Neutralization titers of MAb 2G12 against primary viruses in a PBMC-based assay are shown. The absence of neutralization is indicated as an ID of >50 μg/ml. Values shown are the means from two to three independent experiments.

^b ND, not determined.

neutralization-sensitive and -insensitive fractions may be present, corresponding to gp120 molecules containing or lacking the 2G12 epitope.

Primary virus neutralization. To examine whether 2G12 could reduce the infectivity of primary isolates, we performed neutralization assays with phytohemagglutinin-stimulated PBMC as target cells. MAb 2G12 was tested against three isolates from clade A, three from clade B, and two from clade E, as well as against four primary isolates of undetermined clades isolated from Viennese patients. Neutralization titers for 2G12 against these 12 isolates are listed in Table 3. Seven of the isolates were very sensitive to neutralization by 2G12 (ID₉₀, ≤2 μg/ml), and six of these isolates could be also neutralized by 99%. Nine isolates were neutralized with ID₉₀s of ≤50 μg/ml. However, 2G12 completely lacked activity against both of the clade E isolates and one Brazilian clade B isolate. These data are consistent with the results of a more extensive analysis (65). The above-mentioned observations indicate that 2G12 is one of the broader and more potent human MAbs described to date.

Additional neutralization assays were performed with envelope glycoproteins of primary viruses YU2, ADA, and 89.6 in the single-round complementation assay. While less sensitive to 2G12 than the T-cell line-adapted strain HxBc2 (82% inhibition at 20 μg of 2G12 per ml), two of the viruses containing primary strain envelopes were neutralized by 20 μg of 2G12 per ml. Thus, the infectivities of ADA and 89.6 were reduced to 52 and 57% of control, respectively, whereas YU2 infectivity could only be reduced to 23% of control at this concentration of 2G12. The same rank order of sensitivity to neutralizing MAbs directed against the CD4-binding site was previously observed for this group of viruses (60).

Deposition of complement factor C3 on infected cells. We determined whether the binding of 2G12 to infected cells allowed activation of the complement system. Complement activation was determined by measuring complement factor C3 deposition onto infected cells by immunofluorescence staining. Prior to the C3 deposition experiments, we confirmed by indirect immunofluorescence staining that MAbs 2G12 and 3D6, as well as HIV⁺ hlgG, bound strongly to CEM.NKR cells infected with the HIV-1 strains IIIB, MN, and RF (data not shown). However, none of the reagents bound nonspecifically to uninfected CEM.NKR cells. Photographs depicting complement deposition onto MN-infected cells are shown in Fig. 1;

TABLE 2. Neutralization titers of human MAb 2G12^a

Strain	ID ₉₉ (μg/ml)	ID ₉₀ (μg/ml)	ID ₅₀ (μg/ml)
IIIB	4.29	0.33	0.02
MN	>50.0	>50.0	1.56
RF	3.83	0.66	0.13

^a Inhibition of virus production was assessed by measuring p24 antigen levels in assays with AA-2 cells. The absence of neutralization is indicated as an ID of >50 μg/ml. Values shown are the means from two to three independent experiments.

FIG. 1. Deposition of complement factor C3 on CEM.NKR cells infected with HIV-1_{MN}. (A) Control staining with active complement in the absence of antibody; (B) MAb 2G12 with active complement; (C) MAb 3D6 with active complement (negative control); (D) HIV⁺ hIgG with active complement.

similar results (not shown) were obtained with IIIB- and RF-infected cells. For controls, we performed complement deposition experiments with several other antibodies, including MAbs 2F5 (3, 38), 1B1 (3), and 257-DIV (57); none of these antibodies caused complement deposition to an extent comparable with the effect of 2G12 (data not shown).

Syncytium inhibition in the presence of active human complement. To examine whether 2G12-mediated activation of the complement system could influence the neutralizing ability of this MAb, we performed syncytium inhibition assays with HIV-1_{MN} and HIV-1_{RF} in the presence and absence of active human complement. MAb 2G12 could efficiently inhibit syncytium formation by the MN strain only in the presence of active complement (17 μ g/ml). When HIV-1_{RF}, an isolate that is very sensitive to neutralization by 2G12, was used, we found a two- to threefold increase in syncytium inhibition in the presence of active complement compared with that of control cultures lacking complement (EC_{50} in the presence of complement, 0.33 μ g/ml; EC_{50} without complement, 0.78 μ g/ml). The mechanism by which the neutralizing activity of 2G12 is increased in the presence of complement has yet to be determined. No evidence for antibody- and/or complement-directed lysis of MN-infected CEM.NKR cells by 2G12 and active complement was found (data not shown).

ADCC. MAb 2G12 was tested for its ability to mediate ADCC against HIV-1-infected cells. A representative experi-

ment with uninfected targets with surface-bound recombinant gp120/IIIB and cells infected with HIV-1 strains IIIB, MN, and RF is shown in Fig. 2. As a positive control, a serum sample from an HIV-1-positive patient was included in all experiments. A 1:270 dilution of this serum caused maximal 51 Cr releases from the gp120/IIIB, IIIB, and MN targets of 28, 30, and 25%, respectively. Maximum 51 Cr release for this patient serum was 30% at a 1:90 dilution when RF-infected targets were used. A negative control serum from an HIV-1-negative donor caused less than 8% release in all experiments.

MAb 2G12 is a potent mediator of ADCC as it induces specific lysis of three of the tested targets (gp120/IIIB, IIIB, and MN) at concentrations of <1 μ g/ml. Lysis of RF-infected targets by 2G12 was weaker, but the lysis of these target cells mediated by the positive-control HIV-1⁺ serum was also weaker (data not shown). This suggests that there may be a lower level of expression of the envelope glycoproteins on the surface of the RF-infected cells rather than a reduced activity of 2G12 against HIV-1_{RF}.

Characterization of the 2G12 epitope. In a first step to clarify the nature of the 2G12 epitope, we examined whether 2G12 could interfere with the binding of sCD4 to vaccinia virus-derived recombinant gp120 (Fig. 3). While human MAb 1B1 directed to an epitope overlapping the CD4-binding site (2) strongly inhibited sCD4 binding, 2G12 had very little effect.

To obtain initial information on the 2G12 epitope, we per-

FIG. 2. ADCC mediated by MAb 2G12. ADCC activity of MAb 2G12 was measured against CEM.NKR cells coated with recombinant gp120/IIIB (■) or infected with HIV-1 strains IIIB (●), MN (▲), and RF (◆). Lysis values exceeding 10% (dashed line) are considered significant, as uninfected cells yielded values of <10% lysis in all experiments.

formed binding studies with native gp120, denatured gp120, and denatured and reduced gp120 molecules, expressed in both mammalian and insect cells. We found that 2G12 bound to both native and urea-denatured forms of both mammalian and insect cell-derived gp120. However, 2G12 binding was completely abolished when the mammalian protein was reduced with β -mercaptoethanol (data not shown).

We also examined whether deglycosylation of gp120 affected binding of 2G12 to sodium dodecyl sulfate (SDS)-denatured mammalian gp160. Although 2G12 bound to denatured mammalian gp160, it was completely unable to react with deglycosylated denatured gp160. As a control, MAb 2F5 to a linear epitope in the ectodomain of gp41 (3, 38) was shown to bind

FIG. 3. Effect of sCD4 on 2G12 and 1B1 binding to gp120. MAbs 2G12 (●) and 1B1 (▲) were bound to gp160 coated onto ELISA plates, and then incubation with sCD4 and detection of bound sCD4 by the murine anti-CD4 MAb OKT4 were performed. The mean binding value (mean of four replicate wells) obtained with sCD4 in the absence of MAb is represented by the dashed line. OD492, optical density at 492 nm.

TABLE 4. Epitope mapping with HxBc2 mutants

HxBc2 mutant	Binding ratio ^a
Down mutants	
295N/S.....	0.00
392N/E+397N/E.....	0.00
334S/N.....	0.10
386N/Q.....	0.21
450T/N.....	0.36
Δ303-323.....	0.45
Up mutants	
256S/Y.....	2.69
262N/T.....	2.20
103Q/F.....	1.72
88N/P.....	1.50
Selected neutral mutants	
281A/V.....	0.74
286V/A.....	1.15
298R/G.....	0.85
Δ298-327.....	0.81
342L/G.....	1.24
349L/A.....	1.45
356N/I.....	0.70
363Q/R.....	0.98
384Y/E.....	1.16
395W/S.....	0.62
406N/G.....	0.74
463N/D.....	0.57

^a Binding ratios of ≤ 0.5 and ≥ 1.5 are considered indicative of down- and up-mutants, respectively (see text).

equally well to the native, denatured, and deglycosylated forms of gp160 (data not shown).

To further characterize the 2G12 epitope, we determined how its binding to recombinant gp120 from the HxBc2 clone of HIV-1 LAI was affected by amino acid substitutions throughout the gp120 molecule. The binding of 2G12 to HxBc2 gp120 was abolished or significantly impaired by amino acid substitutions affecting glycosylation sites in the C2 and C3 domains near the base of the V3 loop, in the amino-terminal flank of the V4 loop, and in the C4 region (Table 4; Fig. 4). These amino acid substitutions do not globally disrupt the conformation of gp120, for they do not affect the binding of CD4 or of other MAbs to discontinuous gp120 epitopes (35, 61–63). Data in support of the mutant binding analysis comes from escape mutant studies; among residues in HIV-1 LAI that are changed in 2G12 escape mutants are those at positions 297 and 341, which destroy the canonical N-linked glycosylation sites at residues 295 and 339, respectively (Fig. 4) (43).

Note that although deletion of a major segment (residues 303 to 323) of the V3 loop very weakly impaired 2G12 binding, another V3 deletion mutant lacking residues 298 to 327 bound 2G12 with wild-type efficiency, and several point substitutions in the V3 loop had no effect on 2G12 binding (Table 4 and data not shown). Neither does 2G12 recognize V3 loop peptides (data not shown), so it is not a V3-directed antibody.

A few gp120 mutants bound 2G12 significantly better than did wild-type HxBc2 gp120 (Table 4). These up-substitutions include four changes in putatively interacting alpha-helical segments of the C1 and C2 regions (37); we presume that alteration in the conserved core structure of gp120 causes the 2G12 epitope to be better exposed on monomeric gp120.

To gain further insights into the nature of the 2G12 epitope, we performed cross-competition experiments with 45 human

FIG. 4. HxBc2 mutant binding analysis of MAb 2G12. The linear, disulfide linkage map of the gp120/IIIB sequence (24) is shown. Amino acids shown as solid ovals represent changes that resulted in reduced binding of 2G12, boxed amino acids represent changes that increased binding of 2G12, and arrows indicate selected neutral changes. Amino acid changes at positions 297 and 341 have been identified in 2G12 escape mutant studies (43). Whether substitutions at these positions affect 2G12 binding has not been tested directly.

and rodent MAbs to continuous or discontinuous gp120 epitopes. The competing MAbs included those to the V2 and V3 loops, to the C1, C4, and C5 regions, to the discontinuous epitopes overlapping the CD4-binding site, to CD4-induced epitopes, and to other discontinuous gp120 epitopes. Not one of these test MAbs significantly cross-blocked 2G12 binding or vice versa (data not shown). A few MAbs to V3, C4, and CD4-binding site-related epitopes modestly increased 2G12 binding to gp120, presumably by altering the conformation of monomeric gp120 to better expose the 2G12 epitope. Further details of these cross-competition experiments may be found

elsewhere (34). We conclude that 2G12 binds to a distinctive epitope that is recognized by no other anti-gp120 MAb of which we are aware.

Taken together, our data suggest that the 2G12 epitope is discontinuous, in that reduction of gp120 destroys it, but is not particularly sensitive to gp120 conformation, in that it survives urea treatment. The studies with gp120 mutants imply that the 2G12 epitope is probably centered around the C3/V4 domain of gp120, and it is clearly sensitive to the presence of N-linked glycosylation sites in that area of the molecule. The results of the deglycosylation experiments cannot be interpreted unam-

FIG. 5. Neutralization of HxBc2 glycosylation site mutants. The wild-type HxBc2 envelope and mutant envelope glycoproteins (334S/N, 386N/Q, 450N/T, and 392N/E+397N/E) were compared in a single-round complementation assay for their sensitivities to neutralization by MAb 2G12 at a fixed concentration of 10 μ g/ml. CAT, chloramphenicol acetyltransferase.

biguously, for removal of sugars from SDS-treated gp120 might have unpredictable effects on a purely peptidic epitope. Thus, it remains possible either that the 2G12 epitope is peptidic but influenced by the presence of N-linked glycans or that it involves glycans directly. If the latter is true, the precise nature of the glycan residues seems unimportant, as 2G12 bound to both mammalian and insect cell-expressed gp120 molecules, whose glycans differ in form.

Sensitivity of selected HxBc2 glycosylation site mutants to neutralization by 2G12. To study the biological relevance of the amino acid substitutions in HxBc2 identified above as diminishing 2G12 binding to gp120, we tested the HxBc2 wild-type clone of HIV-1_{LAI} and the mutants 334S/N, 386N/Q, 450N/T, and 392N/E+397N/E (a double mutant) in a single-round complementation assay. The ability of mutants to escape neutralization by 2G12 clearly corresponded to the pattern of 2G12 binding to the envelope glycoproteins. Thus, the mutants 334S/N and 392N/E+397N/E, which showed the most dramatic decrease in 2G12 binding (Table 4), were also the least sensitive to neutralization by 2G12 (Fig. 5). Furthermore, other mutants such as 386N/Q and 450N/T to which 2G12 binding was impaired, but not abolished, were partially sensitive to neutralization by 2G12.

Seroprevalence of 2G12-like antibodies. To determine how common antibodies to the 2G12 epitope were in sera from HIV-1-infected people, we performed competition experiments designed to detect serum antibody inhibition of biotin-labelled 2G12 binding to recombinant gp120 from the LAI (BH10), JR-FL, and SF-2 strains. A panel comprising seven sera from long-term survivors of HIV-1 infection, nine sera from individuals progressing to AIDS (6), and four HIV-negative sera was assembled. Each serum was tested at a 1:100 dilution against each of the three gp120s in two similar experiments, with the biotin-labelled 2G12 concentration differing by threefold between the two experiments. Unlabelled 2G12 (10 μ g/ml) blocked biotin-2G12 binding almost completely (residual binding, 7% of control), but none of the sera strongly inhibited biotin-2G12 binding to any of the three gp120 molecules (data not shown). A single progressor serum reduced biotin-2G12 binding to SF-2 gp120 to levels of $77\% \pm 2\%$ and $66\% \pm 2\%$ of control in the two experiments (means $\pm$ standard errors of the means) but was without effect on biotin-2G12 binding to the other two gp120s. No other serum caused even this modest level of inhibition, and no serum caused

significant enhancement of biotin-2G12 binding to any of the three gp120 molecules.

We conclude that antibodies to the 2G12 epitope are unlikely to be common in HIV-1⁺ human sera. This probably explains why human MAbs to this epitope have not previously been isolated. It remains to be determined whether the 2G12 epitope is an inherently poor antigen or whether immunological parameters, for example, self-tolerance to glycan-dependent epitopes, dominate the poor response to this gp120 site.

DISCUSSION

In this paper we have described a newly discovered human MAb, 2G12, characterized its virus-neutralizing and effector-mediated functions, and partially defined its distinctive epitope on HIV-1 gp120. The identification of this new epitope contributes to our understanding of the exposure of immunogenic determinants and functional domains on gp120.

Much emphasis has rightly been placed on characterizing the virus-neutralizing capacity of antibodies to HIV-1 in human serum. Here we show that human MAb 2G12 broadly and potentially neutralized the majority of clade B primary and T-cell line-adapted strains against which it was tested and also that it has neutralizing activity against viruses of clade A (Table 3) and other subtypes (65). However, HIV-1 antibodies have also been shown to mediate lysis of cell-free virus through activation of the complement system (14, 56, 57), although the relevance of this mechanism to virus neutralization in vivo is uncertain (50). In vitro, the neutralizing activity of HIV-1⁺ sera can be increased significantly in the presence of active complement (55); we show in this work that the virus-inhibiting capacity of 2G12 was also increased by active complement. In contrast, complement- and antibody-dependent enhancement of infection has also been observed with HIV-1⁺ sera under certain experimental conditions (30, 47); we noted no such enhancement mediated by 2G12. Although caution is required when extrapolating these in vitro results to determine what might happen in vivo, we have clearly shown that 2G12 can interact effectively with the human complement system. MAb 2G12 also has ADCC activity against virus-infected cells. High titers of antibodies able to mediate ADCC against cells infected with HIV-1 can be found in HIV-1⁺ sera (20, 26, 39, 48). The presence of high ADCC titers in sera has been suggested to correlate with a good prognosis in adults and children (26, 27, 52).

Having the above-mentioned effector-mediated properties as well as virus-neutralizing activities, 2G12 is clearly a good candidate for inclusion in passive immunotherapeutic regimens designed to treat HIV-1 infection or to prevent HIV-1 transmission from an infected mother to her infant. It is generally considered that passive immunotherapy would be most likely to succeed if MAbs were used in combination; we note that the unusual characteristics of the 2G12 epitope on gp120 render this MAb suitable for combining with several others. Thus, 2G12 neither cross-blocked nor was cross-blocked by 45 other anti-gp120 MAbs, including MAbs targeted at other neutralization epitopes in the V2 or V3 loops or around the CD4-binding site (34). It is therefore quite feasible that combining 2G12 with other MAbs able to neutralize primary strains, such as 2F5 to a gp41 epitope (38, 44) or IgG1b12 or CD4-IgG2 to CD4-binding site-related structures (1, 5), would strengthen and/or broaden the neutralizing effect of 2G12. In vitro experiments designed to explore the effects of combining 2G12 with other reagents are in progress.

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