The broadly neutralizing antibody immunoglobulin G1 (IgG1) b12 neutralizes a broad range of human immunodeficiency virus-type 1 (HIV-1) isolates in vitro and is able to protect against viral challenge in animal models. Neutralization of free virus, which is an antiviral activity of antibody that generally does not require the antibody Fc fragment, likely plays an important role in the protection observed. The role of Fc-mediated effector functions, which may reduce infection by inducing phagocytosis and lysis of virions and infected cells, however, is less clear. To investigate this role, we constructed a panel of IgG1 b12 mutants with point mutations in the second domain of the antibody heavy chain constant region (CH2). These mutations, as expected, did not affect gp120 binding or HIV-1 neutralization. IgG1 b12 mediated strong antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) of HIV-1-infected cells, but these activities were reduced or abrogated for the antibody mutants. Two mutants were of particular interest. K322A showed a twofold reduction in FcγR binding affinity and ADCC, while C1q binding and CDC were abolished. A double mutant (L234A, L235A) did not bind either FcγR or C1q, and both ADCC and CDC functions were abolished. In this study, we confirmed that K322 forms part of the C1q binding site in human IgG1 and plays an important role in the molecular interactions leading to complement activation. Less expectedly, we demonstrate that the lower hinge region in human IgG1 has a strong modulating effect on C1q binding and CDC. The b12 mutants K322A and L234A, L235A are useful tools for dissecting the in vivo roles of ADCC and CDC in the anti-HIV-1 activity of neutralizing antibodies.

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Fc’s presented on the surface of an infected cell may also activate the classical pathway of complement activation ultimately leading to cell lysis (complement-dependent cytotoxicity [CDC]).

Multiple sites on IgG have been proposed to interact with FcγR. Mutagenesis studies have shown that the lower hinge region (234-LLGGPS-239) of IgG plays an important role in the binding of IgG Fc receptors (14, 19, 28, 33, 34, 38, 47, 58, 59). High-affinity binding to FcγRI is most notably affected by mutation of L235. Mouse (m)IgG2b, which does not bind FcγRI, has a glutamic acid at this position, and substitution of this residue by leucine was shown to restore the binding affinity of mIgG2b to be comparable to that of hIgG1 (19). The binding affinity of IgG for FcγRII, in contrast, seems more sensitive to mutations of L234 than L235, indicating that the FcγR interaction sites are overlapping but not identical (33). Residues in the lower part of the hinge region itself and the lower CH2 domain in addition may have a modulating effect on FcγR affinity (15, 33).

Complement activation via the classical pathway is activated through binding of C1q to the Fc domain of IgG or IgM, complexed with antigens (12, 25). Duncan and Winter (18) showed alanine substitutions in mIgG2b at positions E318, K320, K322, and N297, the last leading to the removal of carbohydrate, resulting in mutants in which binding to human C1q was strongly reduced compared to the wild type and the ability to mediate CDC was abrogated. Since E318, K320, and K322 are conserved residues in human IgG and IgG of several other species, they were designated the binding site for C1q (18). However, this binding motif has been conserved in all four human IgG subclasses which, in apparent conflict, exhibit large differences in their C1q-binding abilities. Several studies have indeed implicated additional residues in C1q binding and have suggested that the binding sites for C1q on mIgG2b and hIgG are not completely identical (8, 27, 38, 51, 59). In a recent study on rituxan, a chimeric MAb with hIgG1 constant domains used in the therapy of non-Hodgkin’s B-cell lymphomas, Idusogie and colleagues demonstrated that alanine substitution at positions D270, K322, P329, and P331 but not at positions E318 and K320 significantly reduced the ability of the chimeric MAb to bind C1q and activate complement, suggesting that E318 and K320 are only of minor importance for complement activation by hIgG1 (27). Some studies have furthermore suggested that C1q binding and complement activation may be modulated by residues in the lower hinge region (38). Thus, there are species differences in C1q binding. Finally, it has been shown that C1q binding alone is not sufficient for complement activation and complement-mediated cell lysis (51). Intrinsic factors, such as segmental flexibility of the hinge region (10, 50) but also extrinsic factors such as antigen specificity and density (5, 6), may play an important modulating role.

In the present study, we introduced point mutations in the heavy chain constant domain of IgG1 b12. Amino acid mutations were chosen on the basis of the studies discussed above. However, because of subtle species differences in C1q and FcγR binding and the possible modulation of ADCC and CDC by antigen specificity and density, the impact of these mutations on the biological activity of IgG against HIV-1-infected cells was not immediately clear. The mutants were compared for their ability to bind FcγR and C1q and to mediate ADCC and CDC. These mutants can now be used to examine the role of Fc-mediated effector function in protection against HIV-1 infection in vivo.

MATERIALS AND METHODS

Cell lines, viruses, and Mabs. Uninfected CEM-NKr and CEM-NKr cells chronically infected with HIV-1-AMN were obtained from Shermaine Tilley (Public Health Research Institute, New York, N.Y.) (1). To obtain clones of the infected CEM-NKr cells with an increased level of envelope expression, we performed a limiting dilution and characterized for Env expression by flow cytometry. A clone with a higher level of Env expression (75% of cells expressing Env) was selected for our experiments. Adult human elutriated monocytes were obtained from Advanced Biotechnologies, Columbia, Md. HIV-1 primary isolates HIV-1_F1, HIV-1_R, (contributed by Irvin Chen) (31), and HIV-1_M (contributed by Ronald Collman) (16) were obtained from the National Institutes of Health (NIH) AIDS Research and Reagent Reference Program (ARRRP). IgG1-CLB was a purified paraprotein obtained from the CLB, Amsterdam, The Netherlands. Anti-FcγRI MAb 10.1 was provided by Nancy Hogg (Leuкоocyte Adhesion Laboratory, London, United Kingdom). Fab 10.1 was prepared by papain digestion. Fab fragments of anti-FcγRII MAb IV.3 and Fab(ab’)_2 fragments of anti-FcγRIII MAb 3G8 were provided by Medarex (Anandale, N.J.). Humanized OKT3 (IgG1 and IgG4) antibody was provided by Robert A. Zvin (R.W. Johnson Pharmaceutical Research Institute, Raritan, N.J.).

Mutagenesis of heavy-chain constant domain. Mutagenesis was performed on the heavy-chain constant region derived from the IgG1 b12-expression plasmid pDR12 (see below) (13). A ScaI/SalI endonuclease restriction fragment from pDR12, containing the CH2 fragment, was subcloned into M13 mp18. Site-directed mutagenesis (32) was performed using the Mut-gene M13 in vitro mutagenesis kit (Bio-Rad, Hercules, Calif.). Five clones encoding the desired changes (K322A, L234A, L235E, G237A, and L234A, L235A) were identified by automated DNA sequencing. The mutated ScaI/SalI fragments were then cloned back into the pDR12 expression vector.

Expression and purification of antibodies. Recombinant antibody was expressed in the vector pDR12 (provided by Raju Koduri and Dean Sauer). It contains a b12 light chain and heavy-chain expression cassette in which transcription is driven from a human cytomegalovirus promoter. The heavy-chain expression cassette contains the genomic human IgG1 gene. Selection and amplification of the plasmid was done on the basis of expression of the gene for glutamine synthetase (4).

IgG1 b12 mutant DNAs, prepared as described above, were cut with SalI and transfected into Chinese hamster ovary cells (CHO-K1 cells; American Type Culture Collection, Manassas, Va.) using lipofectin reagent per the manufacturer’s recommendations (Life Technologies, Grand Island, N.Y.). Cells were distributed in six-well tissue culture plates, and clones were selected with 1-methionine sulfoximine ranging in concentration from 40 to 100 μM (Sigma, St Louis, Mo.). Wells containing discrete colonies were assayed by enzyme-linked immunosorbent assay (ELISA) for antibody production. The highest producers were cloned by limiting dilution, expanded, and grown in 3-liter spinner flasks.

Recombinant IgG1 was expressed in CHO-K1 cells in glutamine-free Glasgow minimum essential medium (GMEM supplemented with 10% dialyzed fetal bovine serum [FBS]) (Tissue Culture Biologicals, Tulare, Calif.). MEM nonessential amino acids (Gibco-BRL, Grand Island, N.Y.), 1 mM MEM sodium pyruvate (Gibco-BRL), 500 μM l-glutamic acid, 500 μM L-asparagine, 30 μM adenosine, 30 μM guanosine, 30 μM cytidine, 30 μM uridine, 10 μM thymidine (Sigma), 100 μM of penicillin/ml, 100 μg of streptomycin/ml, and 50 μM 1-methionine sulfoximine (Sigma) in a 3-liter spinner flask. The supernatants were sterile filtered and purified over protein A-Sepharose Fast Flow (Pharmacia, Arlington Heights, Ill.). The antibody was eluted in 0.1 M citric acid, pH 3.0. The pH of the antibody solution was immediately brought to neutrality by the addition of 1 M Tris (pH 9.0), and the antibody was dialyzed against phosphate-buffered saline (PBS). Antibody concentrations were determined by the absorbance at 280 nm and confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Antibody yields using this method ranged from 5 to 25 mg/liter.

Recombinant gp120 ELISA. IgG1 b12 and the Fc mutants were tested for binding to gp120 in ELISA essentially as described previously (17). Briefly, recombinant monomeric gp120 (m) (provided by Paul Maddon and Bill Olson, Progenics, Tarrytown, N.Y.) was coated to the wells of a microtiter plate by incubating overnight at 4°C. The plates were washed four times with PBS containing 0.05% (vol/vol) Tween 20 and blocked with 3% bovine serum albumin (BSA). The blocking solution was removed, and serial dilutions of the antibody set were added in duplicate (diluted in PBS–1% BSA–0.05% Tween 20) and
incubated for 1 h at 37°C. The wells were washed and incubated for 1 h at 37°C with alkaline phosphatase-labeled goat anti-human IgG F(ab')2 fragments (Pierce, Rockford, Ill.) (1:500 dilution in PBS-1% BSA-0.05% Tween 20). The plates were washed and developed with nitrophenol substrate (Sigma), and the absorbance was read at 405 nm.

**HIV-1 neutralization.** Neutralization of HIV-1 primary isolates was assessed using a phytOHemagglutinin (PHA)-activated peripheral blood mononuclear cell (PBMC)-based assay as described previously (60). PBMCs (from three CCR5 wild-type donors) were isolated and stimulated with PHA (5 µg/ml) (Sigma) for 48 h, followed by PHA and interleukin-2 (40 U/ml) (obtained from the AARRP, contributed by Hofman-La Roche) for 72 h in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 100 µg of streptomycin/ml, 100 µg of penicillin/ml, and 2 mM l-glutamine. The antibodies were diluted, and 50 µl per well was pipetted into round-bottom microtiter plates, after which an equal volume containing 100% tissue culture infective doses of HIV-1 stock was added. The antibody-virus mixture was incubated for 1 h at 37°C. Next, 100 µl of PHA-activated PBMCs (5 × 10^5/ml) was added to each well. After an overnight incubation, the cells were washed two times with tissue culture medium. On day 7, the cultures were collected and treated with 1% (vol/vol) Empigen (Calbiochem, La Jolla, Calif.). Triplicate samples were then tested for p24 content using an ELISA, as originally described by Moore et al. (37). In brief, sheep anti-p24 Ab D7320 (Aalo Bioreagents) was coated overnight on 96-well polystyrene microtiter plates. Rabbit serum (Calbiochem) was used as a source of complement, and plates were incubated for 1 h. The plates were washed and developed with nitrophenol substrate (Sigma), and the absorbance was read at 405 nm.

**Neutralization of HIV-1 primary isolates was assessed using an ELISA, as originally described by Moore et al. (37).**

**RESULTS**

**Antigen recognition and neutralization.** Five IgG1 b12 mutants were constructed by introducing point mutations in the lower hinge (L234A, L235A, G237A, and double mutant L234A, L235A and N-terminal end of the CH2 domain). Although it was unlikely that these mutations would influence the affinity of the antibody for gp120, all five antibodies were tested for binding to recombinant HIV-1_RFL gp120 in ELISA. All mutants bound similarly to gp120 as expected (Fig. 1a). In addition, we tested all mutants in a PHA-activated PBMC-based neutralization assays with HIV-1_RFL (Fig. 1b), HIV-1_HC_CSF, and HIV-1_H9 (Table 1). All mutants neutralized these primary isolates similarly (Fig. 1b; Table 1).

**Binding of wild-type and mutant IgG1 b12 to FcRy, FcRyIIa, and FcRyIIia.** The antibodies of interest were tested using ADCC assays dependent on the relative affinity of the antibody for FcRy, FcRy-II, and FcRy-III. We first measured the affinity of IgG1 b12 and the mutant antibodies described above to bind to FcRy by flow cytometry using both monomeric and dimeric IgG1 (cross-linked with an anti-light chain Fab) (26). Binding of IgG by the high-affinity receptor FcRy is usually studied using the Fab fragments of mouse anti-human IgG. After washing, cell-bound antibody was detected using flow cytometry. The assays were performed using IA1.6 cells transfected with FcRyIa and γ-chain, or FcRyIIa (H131), and Jurkat cells transfected with FcRyIa (24, 45, 54, 55).

**FcRγ binding assays.** FcRγ binding assays were performed essentially as described by Parren et al. (45). Binding of antibody to FcRγ was assessed using antibody monomers or dimers as indicated. Antibody dimers were prepared by incubating overnight with F(ab')2 fragments of mouse anti-human κ-chain MAb K35 in a molar ratio of 1:1, which resulted in stable tetrameric complexes as detailed by Huizinga et al. (26). FcRγ-transfected cells (3 × 10^5/ml in PB of 1% BSA) were incubated with 25 µl of serum dilutions of antibody monomers or dimers for 45 min at 4°C, washed, and then incubated with fluorescein isothiocyanate-labeled F(ab')2 fragments of goat anti-human IgG. After washing, cell-bound antibody was detected using flow cytometry. The results were expressed as means of triplicates.

**ADCC with TCLA HIV-1.** ADCC was assessed in standard chromium release assays (56). Effector cells were either PBMCs or adult human elutriated monocytes, as noted in the text. The PBMCs were isolated by centrifugation over Histopaque-1077 (Sigma). Cells were washed in PBS and resuspended in RPMI 1640 containing 10% FCS, 2 mM l-glutamine, penicillin (50 U/ml), and streptomycin (50 µg/ml) at a density of approximately 4 × 10^6 cells/ml and incubated overnight at 37°C to use as effector cells in ADCC. The following day, 10^6 target cells were labeled with Na 2 111 In (Amersham, Arlington Heights, Ill.) for approximately 2 h in 50 µl of fetal calf serum (FCS) containing 10 mM HEPES. These target cells were either uninfected CEM-NKr cells or CEM-NKr cells chronically infected with HIV-1_H9. After labeling, target cells were washed four times with RPMI 1640-10 mM HEPES. In each well of a microtiter plate, 10^6 washed 111 In-labeled target cells were mixed with antibody for 30 min at 37°C in a total volume of 150 µl. Then, 10^6 PBMCs or 6 × 10^6 human elutriated monocytes (monocytes were thawed rapidly at 37°C and washed once with RPMI 1640 before addition to target cells) were added as a source of effector cells in 50 µl of assay medium, bringing the total volume to 200 µl. The plates were spun at 1,000 rpm for 5 min in a Beckman GS-6R centrifuge to pellet the cells prior to a 4-h incubation at 37°C. At the end of this incubation period, plates were spun another time as described above, 100 µl of supernatant was collected, and 111 In release was measured in a gamma counter (Packard, Meriden, Conn.). The percent specific lysis was calculated as follows: (experimental release – spontaneous release)/total release × 100%. Total 111 In release was determined by substituting 50 µl of antibody for Empigen detergent (Calbiochem). All data are expressed as the means of triplicate determinations.

**CD4 assay.** Target cells (CEM-NKr cells; uninfected or infected with HIV-1) were labeled with Na 2 111 In or Na 2 114 C and as described above. After three washes, the labeled cells were sensitized by adding wild-type or mutant IgG1 b12 to the cells at a final concentration of 10 µg/ml and were incubated at 4°C for 1 h. After three washes, 2 × 10^5 sensitized target cells were dispensed into 96-well U-bottom microtiter plates. Rabbit serum (Calbiochem) was used as a source of complement and was serially diluted with RPMI 1640. The latter was added to sensitized target cells at 100 µl/well, and the plates were incubated at 37°C for 1 h. At the end of this time, plates were spun and 100 µl of supernatant was used to measure the 111 In release as described above.
Therefore, the importance of L235 for FcγRI binding was confirmed, but binding could only be completely abrogated by introducing a double mutation at L234 and L235. In contrast to previous studies on hIgG3 (34), mutagenesis of L234 as well as L235 in hIgG1 abolished both FcγRII and FcγRIII binding.

ADCC. Antibody (IgG1 b12) bound to envelope expressed on the surface of HIV-1-infected cells may recruit effector cells, such as NK cells or monocytes, by interacting with specific Fc receptors and induce ADCC leading to lysis of the infected cells. In order to measure the ability of IgG1 b12 and the mutants described above to mediate ADCC of HIV-1-infected cells, we incubated serial dilutions of these antibodies with 51Cr-labeled HIVMN-infected CEM-Nkr cells in the presence of PBMCs and purified monocytes as effector cells. As shown in Fig. 2a and b, IgG1 b12 mediated specific cell lysis of HIVMN-infected CEM-Nkr cells in the presence of both human PBMCs and purified monocytes, whereas ADCC by mutant K322A was reduced. The ability of IgG1 b12 mutants L235E, L234A, G237A, and double mutant L234A, L235A to mediate ADCC of HIV-1MN-infected CEM-Nkr cells was strongly reduced with PBMCs and abolished with monocytes as effector cells (Fig. 2a and b). The positive control serum, FDA-2, is a potently neutralizing serum with a 90% HIV-1MN neutralization titer of 1:4,000 (44); ADCC of IgG1 b12 on noninfected CEM-Nkr cells was used as a negative control.

To verify the studies with the IgG1 mutant antibodies, we examined the IgG1 b12-mediated ADCC of HIVMN-infected CEM-Nkr with PBMCs and monocytes in the presence and absence of anti-FcγR antibodies. F(ab′)2 fragments of an anti-FcγRII antibody, 3G8, efficiently inhibited the PBMC-mediated ADCC of HIVMN-infected CEM-Nkr (Fig. 3a). No inhibition of ADCC was observed with Fab fragments of anti-FcγRII 10.1 or anti-FcγRII IV.3. Thus, ADCC of HIV-infected cells by PBMCs is mediated through FcγRIIa. This is in agreement with the strong reduction of ADCC in mutants in which binding to FcγRIIa was abolished (Fig. 2a). In addition, the ability of mutant K322E to mediate ADCC was reduced about twofold compared to IgG1 b12, which corresponds with its reduction of binding to FcγRIIa (Fig. 2a; Table 2).

C1q binding and complement activation. The ability of b12 and the mutant antibodies to bind C1q and CDC of HIV-1-infected cells was determined by ELISA. Table 2 lists the binding of IgG1 b12 and IgG1 b12 Fc mutants to FcγR.

![Figure 1](https://example.com/fig1.png)

**FIG. 1.** Binding of wild-type and mutant IgG1 b12 to recombinant HIV-1JR-FL gp120 in ELISA (a) and neutralization of HIV-1JR-FL (b).

| TABLE 1. Neutralization of HIV-1 primary isolates by wild-type and mutant IgG1 b12 |
|----------------------------------|------------------|------------------|------------------|
| Antibody | HIV-1JR-FL | HIV-1JR-CSF | HIV-189.6 |
|----------|------------|------------|------------|
| IgG1 b12 | 2 | 50 | 6.3 |
| K322A    | 2 | 50 | 6.3 |
| L234A    | 2 | 50 | 6.3 |
| G237A    | 2 | 25 | 12.5 |
| L235E    | 2 | 50 | 3.1 |
| L234A, L235A | 2 | 50 | 6.3 |

| Antibodies | FcγRI (monomer binding) | FcγRI (dimer binding) | FcγRIIa | FcγRIIIa |
|------------|------------------------|-----------------------|--------|---------|
| b12        | 0.16                   | 0.3                   | 4.8    | 7       |
| K322A      | 0.17                   | 0.4                   | 8.3    | 13      |
| L234A      | 1.1                    | 1.68                  | >50    | >50     |
| G237A      | 6                      | 7.6                   | >50    | >50     |
| L235E      | 7                      | 12                    | >50    | >50     |
| L234A, L235A | >50              | >50                   | >50    | >50     |
| IgG1-CLB   | 0.12                   | 0.2                   | 1.8    | 10      |

*Data are expressed as the antibody concentration at which half maximal binding was achieved, K_{so} of >50 μg/ml indicate that this was not achieved.*
infected cells was investigated. As shown in Fig. 4a and b, wild-type IgG1 b12 bound well to C1q and mediated a potent CDC of HIV-1MN-infected CEM-Nkr cells in the presence of rabbit serum as a source for complement. The ability of the K322A mutant to bind C1q and mediate CDC was strongly reduced. All b12 variants with mutations in the lower hinge region (L235E, L234A, G237A and L234A, L235A) furthermore were reduced in their ability to mediate CDC of HIV-1-infected cells as well (Fig. 4a and b).

These results strongly suggest that for hlgG1, the residue at position 322 at the N-terminal end of the CH2 domain as well as residues in the lower hinge region are involved in C1q binding and consequent complement activation.

**DISCUSSION**

The role of effector function in the anti-HIV-1 activity of antibody is poorly understood. It has been shown in a number of passive antibody transfer studies that MAbs or polyclonal antisera capable of neutralizing the challenge virus can protect against HIV-1 infection (2, 3, 20–22, 35, 36, 40, 41, 46). Significantly, passive immunization studies using broadly neutralizing antibodies, including b12, have recently been used to protect rhesus macaques from intravenous and mucosal challenge with pathogenic primary HIV-1 isolate-derived SHIVs (35, 36, 43).

Neutralizing antibody may limit the dissemination of an enveloped virus, such as HIV-1, by at least two separate mechanisms. First, the antibody may interact with free virus and neutralize it by interfering with the attachment or fusion of its target cells, thereby protecting the cells from infection. Second, the neutralizing antibody may bind to envelope expressed on the surface of infected cells and induce cell lysis by the recruitment of effector functions, including ADCC and CDC (reviewed by Parren and Burton [40]). The relative roles of neutralization and Fc-mediated effects in HIV-1 infection have not yet been directly determined. In a recent study, Binley and colleagues (7) suggested that a transient effect on viral load by the infusion of anti-SIV antibodies was likely due to killing of SIV-infected cells, although this conclusion could only be drawn by inference.

We set out to directly study the role of Fc-mediated effector function in HIV-1 infection by the preparation of a panel of Fc
mutants of the broadly HIV-1-neutralizing MAb b12. The MAb OKT3 (IgG1 and IgG4) were used as controls.

**FIG. 4.** Binding of wild-type and mutant IgG1 b12 to C1q (a) and complement-mediated lysis of HIV-1MN-infected CEM-Nkr-MN cells by IgG1 b12 and IgG1 b12 mutants (b). Isotype variants of anti-CD3

Our results are in agreement with previous studies showing the importance of the lower hinge region of IgG1 FcRI and FcRIIIa. As shown in our results, double mutation of amino acids 234 and 235 completely abolished binding to all FcRs.

We examined the nature of the FcγRs involved in the ADCC of HIV-infected cells by using anti-FcγR antibodies. Our data indicate that ADCC by PBMCs is inhibited only in the presence of anti-FcγRII antibody, while ADCC by monocytes is abolished in the presence of anti-FcγRI antibody. Alsmadi and Tilley (1) proposed previously that ADCC of HIV-infected cells is exclusively mediated through FcγRIII expressed on the surface of CD56 + NK cells. In their study, they measured ADCC activity using cultured PBMCs as effector cells which are likely reduced in monocyte content due to the adherence of these cells to plastic. Similarly, we found that PBMC-dependent ADCC was primarily mediated through FcγRIII, even though monocytes represented up to 8% of total cells in our PBMC preparations, as measured by fluorescence-activated cell sorting (FACS) using a CD14 marker (data not shown). The effective ADCC by purified monocytes in vitro in our study, however, indicates that it is likely that monocytes or macrophages expressing FcγRI contribute to the ADCC of HIV-1-infected cells in vivo. Therefore, we suggest that ADCC of HIV-infected cells in vivo may be mediated by both FcγRI and FcγRIIIa.

Human IgG1 has the ability to bind C1q and lysy cells by activating complement through the classical pathway. The binding site for C1q on murine IgG2b was mapped to residues 318, 320, and 322 (18). We measured the ability of b12 and the mutants described to bind to C1q and to activate complement-mediated cell lysis. We used heterologous rather than homologous complement, as this resulted in more efficient lysis of HIV-1-infected (human) cells (data not shown). Changing lysine 322 to alanine completely abolished binding to C1q and complement activation, which confirms a previous report (27). Less expected was the reduction of C1q binding and complement activation by mutations in the lower hinge. In an earlier study, Morgan and colleagues had reported on the reduction of C1q binding and complement activation by changes in residues 235 and 237 for a chimeric mouse/human antibody (38). We show that in a fully human IgG1, L234, G237, and, in addition, L234 in the lower hinge region appear to play a significant direct or indirect role in C1q binding.

A concern may be that the null phenotype of the L234A, L235A double mutation for FcγR as well as C1q binding is a result of more-dramatic rearrangements of the IgG Fc structure of this mutant. However, in a recent study, we have shown that the binding of the L234A, L235A double mutant to FcγRn was only slightly reduced (<25%) compared to wild-type b12 (57). Protein A and G binding furthermore were unaffected (not shown). Proteins A, G, and FcγRn all bind to the CH2-CH3 interface. The retention of FcγRn binding, in particular, is significant as this receptor has two important functions, namely the cross-placental transport of maternal IgG to the fetus and the protection of IgG from normal serum protein catabolism (23, 29, 49). The serum half-life of the L234A, L235A mutant should therefore not be significantly affected.

In summary, we demonstrated that manipulation of residues 234, 235, and 237 in the lower hinge region of hIgG1 modulates binding to FcγRs. Our data also indicated that, in hIgG1, both the lower hinge and N-terminal end of the CH2 domain are involved in C1q binding and complement lysis. Furthermore, using both PBMCs and purified monocytes as effector cells, we found that ADCC of HIV-infected cells is mediated through both FcγRI and FcγRIIIa. As shown in our results, mutant K322A was only slightly, up to twofold, reduced in its ability to mediate ADCC and did not mediate CDC. On the other hand, the double mutant L234A, L235A mediated neither CDC nor ADCC. Both mutants were unchanged in their ability to neutralize primary HIV-1 isolates. Therefore, these mutants display specific valuable features that can be used in future in vivo studies. Our goal will be to use these mutants in vivo passive antibody transfer-SHIV challenge studies in rhesus macaques to elucidate the relative roles of neutralization and of ADCC and complement activation in protection against HIV-1 infection and pathogenesis.

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**REFERENCES**

1. Alsmadi, O., and S. A. Tilley. 1998. Antibody-dependent cellular cytotoxicity directed against cells expressing human immunodeficiency virus type 1 envelope and chimpanzee monoclonal antibodies of different epitope specificities. J. Virol. 72:286–293.
2. Anand, S. A., and J. J. Mele. 1998. Passive immunization with human immunodefi-
ciency virus type 1-neutralizing monoclonal antibody in hu-PBL-SCID mice: 1.
A role of a neutralization escape variant. J. Infect. Dis. 177:889–897.
3. Baba, T., T. Visk, A. Hofmann-Lehmann, J. Vlasak, H. Wu, S. Auyeung, L. A. Cava
cini, M. R. Posner, H. Katinger, G. Stiegler, B. J. Bernacky, T. A. Rivi
t, R. Schmidt, L. R. Hill, M. E. Keating, L. Vu, J. E. Wright, T. C. Chou, and R. M. Rup
trecht. 2000. Human neutralizing monoclonal antibodies of the IgG1 subtype protect against mucosal simian-human immunodeficiency virus infection. Nat. Med. 6:200–206.
4. Bebbington, R. A., and J. Sodroski. 1997. High-level expression of a recombinant antibody from mycelia cells using a glutamine synthetase gene as an amplifiable selectable marker. Bio/Technology 15:169–175.
5. Bindon, C. I., G. Hale, and H. Waldmann. 1988. Importance of antigen specificity for complement-mediated lysis by monoclonal antibodies. Eur. J. Immunol. 18:1507–1514.
6. Bindon, C. I., G. Hale, and H. Waldmann. 1990. Complement activation by immunoglobulin does not depend solely on C1q binding. Eur. J. Immunol. 20:277–281.
7. Binley, J. M., B. Clas, A. Gettie, M. Vesavnen, D. C. Montefiori, L. Sawyer, J. Booth, M. Lewis, P. A. Marx, S. Bonhoeffer, and J. P. Moore. 2000. Passive infusion of immune serum into simian immunodeficiency virus-infected rhesus macaques undergoing a rapid disease course has minimal effect on plasma viremia. Virology 270:237–249.
8. Breke, O. H., T. E. Michaelsen, A. Aase, R. H. Sandin, and I. Sandlie. 1994. Human IgG isotype-specific amino acid residues affecting complement-mediated lysis of virus-infected cells. Eur. J. Immunol. 24:2542–2547.
9. Buchacher, A., R. Predd, K. Strutzenberger, W. Steffineller, A. Trkola, M. Tur
certs, G. Gruber, C. Tauer, F. Steindl, A. Jungbunzlauer, and H. Katinger. 1994. Generation of human monoclonal antibodies against HIV-1 proteins; electrofusion and Epstein-Barr virus transformation for peripheral blood lymphocyte immortalization. AIDS Res. Hum. Retrovir. 10:359–369.
10. Burton, D. R. 1985. Immunoglobulin G: functional sites. Mol. Immunol. 22:199–206.
11. Burton, D. R., C. F. Barbas, I. L. M., M. A. Persson, S. Koening, R. M. Chanon, and R. A. Lerner. 1991. A large array of human monoclonal antibodies to type 1 human immunodeficiency virus from combinatorial libraries of asymptomatic seropositive individuals. Proc. Natl. Acad. Sci. USA 88:13414–13417.
12. Burton, D. R., J. Boyd, A. D. Brampton, S. B. Easterbrook-Smith, E. J. Emanuel, J. Novotny, T. W. Rademacher, M. R. van Schravendijk, M. J. E. Sternberg, and R. A. Dwek. 1980. The C1q receptor site on immunoglobulin G. Nature 288:338–344.
13. Burton, D. R., J. Ptati, R. Koduri, S. J. Sharp, G. B. Thornton, P. W. H. Parren, L. S. Sawyer, R. M. Hendry, N. Dunlop, P. L. Mara, M. Lamac
cchia, E. Garaatty, E. R. Sielen, T. J. Bryson, Y. Cao, J. P. Moore, D. B. Ho, and C. F. Barbas. 1994. Efficient neutralization of primary isolates of HIV-1 by a recombinant human monoclonal antibody. Science 266:1024–1027.
14. Burton, D. R., and J. M. Wood. 1992. Human antibody effector function. Adv. Immunol. 50:81–104.
15. Candiel, S. M., and S. L. Morrison. 1991. The binding affinity of human IgG for its high affinity Fe receptor is determined by multiple amino acids in the CH2 domain and is modulated by the hinge region. J. Exp. Med. 173:1483–1493.
16. Collman, R., J. W. Ballest, S. A. Gregory, H. Friedman, D. L. Kolson, N. Nathanson, and A. Sririvasan. 1992. An infectious molecular clone of an unusual macrophage-tropic and highly cytopathic strain of human immuno
deficiency virus type-1, J. Virol. 66:7517–7523.
17. Ditzel, H., J. P. W. H. Parren, J. M. Binley, J. Sodroski, J. P. Moore, C. F. Barbas, and D. R. Burton. 1997. Mapping the protein surface of human immunodeficiency virus type 1 gp120 using human monoclonal antibodies from phage display libraries. J. Mol. Biol. 267:684–695.
18. Duncan, A. R., and G. Winter. 1988. The binding site for C1q on IgG. Nature 332:738–740.
19. Duncan, A. R., J. M. Wood, L. J. Partridge, D. R. Burton, and G. Winter. 1990. Localization of the binding site for the human high-affinity Fe receptor on IgG, Nature 342:563–564.
20. Emin, E., S. T. Schleif, J. H. Nemberg, A. J. Conley, Y. Eda, S. Tokiyoshi, D. S. Putney, S. Matsushita, K. C. Cobb, C. M. Jett, J. W. Eichberg, and K. K. Murphy. 1992. Prevention of HIV-1 infection in chimpanzees by gp120 V3 domain-specific monoclonal antibody. Nature 355:728–730.
21. Gauduin, M. C., P. W. H. I. Parren, R. Weir, C. F. Barbas III, D. R. Burton, and R. A. Koup. 1997. Passive immunization with a human monoclonal antibody protects hu-PBL-SCID mice against challenge by primary isolates of HIV-1. Nat. Med. 3:1389–1393.
22. Gauduin, M. C., J. T. Safrit, R. Weir, M. S. Fung, and R. A. Koup. 1995. Pre-
and post-exposure protection against human immunodeficiency virus type 1 infection with a human monoclonal antibody. Infect. Dis. 171:1223–1230.
23. Ghonem, V., J. G. Hubbell, J. K. Kim, W. F. Tsen, V. Lee, and E. S. Ward. 1966. Abnormally short serum half-lives of IgG in (μ2-microglobulin-deficient mice. Eur. J. Immunol. 2:690–696.
