The Neutralization Properties of a HIV-Specific Antibody Are Markedly Altered by Glycosylation Events Outside the Antigen-Binding Domain[1]

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Neutralizing Abs constitute a pivotal mechanism of the adaptive immune response against HIV-1 infection. Yet, most of the Abs that appear in the circulation during HIV infection are nonneutralizing. In this study, we report a dramatic change of the neutralizing properties of a human Ab reactive with the nonneutralizing epitope termed cluster I on the HIV-1 transmembrane protein gp41 when the Ab was produced in Chinese hamster ovary (CHO)-K1 cells. Our laboratory has previously reported that the Ab F240, when produced in a hybridoma, is nonneutralizing as assessed by standard neutralization assays. The F240 IgG1 Ab expressed in CHO cells acquired a strong neutralization activity against a broad range of HIV isolates without a change in immunoreactivity. Sequencing of the F240 mRNAs produced in the parental hybridoma and CHO cells revealed identical sequences, suggesting that acquired neutralization resulted from cell-specific posttranslational modifications. We found that the Ab produced by CHO cells is glycosylated to a greater extent than the parental Ab produced by the hybridoma. Moreover, treatment with peptide *N*-glycosidase F abrogated F240 neutralization, in an isolate-specific manner, but not Ab b12 neutralization. Interestingly, the F240 isotype-switched variants IgG3 and IgG4, also expressed in CHO cells, exhibited identical immunoreactivity to IgG1 isotypes but had clear differences in viral neutralization. These results suggest that structural features of the Ig molecule other than the primary sequence of the variable regions play a more prominent role in HIV neutralization than anticipated. ***The Journal of Immunology,*** **2007, 178: 7132–7138.**

Human immunodeficiency virus type 1-specific neutralizing Abs, i.e., Abs that block virus entry into the host cell, have an important role in controlling HIV spread in vivo as suggested by the rapid and constant selection of neutralizing Ab-escape variants during the course of infection (1, 2). This class of Abs is directed against the viral envelope glycoprotein, a heterotrimer composed of the transmembrane gp41 subunit and the heavily glycosylated CD4-binding subunit gp120 (3–5). HIV-1 possesses multiple mechanisms that allow escape from immune surveillance. For example, evasion from Ab neutralization is facilitated by the fact that the accessible regions of the viral envelope are highly variable while important neutralizing epitopes remain concealed within the heterotrimeric complex (6–8). Similarly, some epitopes that are deeply buried in the envelope may only be transiently exposed during viral entry, limiting the time window for effective Ab binding (9, 10). Furthermore, glycosylation on gp120 blocks access to the conserved envelope core, and the evolving asparagine-linked (N-linked) glycosylation pattern facilitates viral escape from the host's Ab repertoire (2, 11).

Only a limited number of highly and broadly HIV-neutralizing human mAbs have been isolated and characterized. They include the gp41 Abs 2F5 and 4E10 and the gp120 Abs 2G12 and b12 (12–17). Data derived from the crystal structure of envelope-Ab complexes suggest that the neutralizing activity of these Abs (except for 2G12) is mediated by an unusually long CDR H3 loop that penetrates deeply into the Ag cleft, which is obscured in the heterotrimeric envelope (12, 15, 18). Unfortunately, broad HIV neutralizing Abs occur only rarely in patients, while experimental vaccines have failed to induce a significant Ab response to autologous primary isolates (19–25) let alone nonautologous strains (26, 27).

The work of our laboratory and that of others (28–30), has demonstrated that when the isotype (e.g., IgG to IgA) or subclass (e.g., IgG1 to IgG3) of an anti-HIV Ab is switched, the binding properties of the parental Ab are retained. Typically, in vitro Ab isotype switching involves the insertion of the Ag binding variable regions into a plasmid containing the Ab effector constant domain and subsequently the plasmid is expressed in host cells such as the Chinese hamster ovary (CHO)[3] cell line, which is one of the first mammalian cell lines successfully developed for use in the production of therapeutically valuable proteins. In our study we found that a nonneutralizing but broadly reactive anti-gp41 Ab, F240 (26, 31), originally produced in a hybridoma, acquired the capacity to neutralize HIV-1 upon expression in CHO cells. The F240 human mAb was originally isolated in our laboratory. This Ab recognizes the immunodominant epitope of gp41, which spans residues 592–

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[3] Abbreviations used in this paper: CHO, Chinese hamster ovary; AAL, *Aleuria aurantia* lectin; IRES, internal ribosome entry site; N-linked, asparagine-linked; OPD, orthophenylenediamine; PNGase F, peptide *N*-glycosidase F; RCAI, *Ricinus communis* agglutinin I; SNA, *Sambucus nigra* agglutinin.

604, and is commonly found among HIV-1 primary isolates (26, 31). Sequencing analysis of the variable regions of the Ab in the parental hybridoma as well as in CHO cells showed identical primary sequences, suggesting that this change in function is likely the result of distinct posttranslational modifications conferred by production in different cell types. Indeed, our experiments revealed different extents of glycosylation between the Abs expressed in these two cell lines, and the specific sugar moieties added to F240 are critical for the change in neutralizing activity. Moreover, no glycosylation sites are predicted within the variable domains of the Ab and, thus, acquired neutralization seems to be dependent on the Fc region. This proposal is further reinforced by the fact that isotype variants of F240 produced in CHO cells distinctly neutralize virus isolates. The precise mechanisms by which Ab glycosylation mediates neutralization of HIV-1 are under investigation.

Materials and Methods

Cell lines and reagents

CHO cells (CHO-K1) and 293T cells were obtained from the American Type Culture Collection (ATCC number CCL-61). TZM-bl cells were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program (ARRRP). CHO-K1 cells were grown in RPMI 1640 medium (Mediatech) and 293T and TZM-bl cells were grown in DMEM (Invitrogen Life Technologies). All media contained 10% FBS (HyClone), 10 μg/ml gentamicin (Invitrogen Life Technologies), and 2 mM HEPES (Mediatech). Luria-Bertani broth and agar and ampicillin were obtained from Fisher Scientific. Puromycin was purchased from Acros Organics. G418 was obtained from Mediatech. Restriction enzymes were obtained through New England Biolabs. Other chemicals were obtained from Sigma-Aldrich.

Oligonucleotide primers

Oligo$(dT)_{20}$ was obtained through Invitrogen Life Technologies. The Ig oligonucleotides used for PCR were synthesized by Operon. They include the F240 κ-chain sense (5′-ATATATGCTAGCCGCCACCATGGTGTTG CAGACCCAG-3′) and antisense (5′-ATATATTGCGGCCGCTCGTCTG ATTTCCACCCT-3′ primers) and the H chain sense (5′-ATATATGCTA GCCGCCACCATGGAGTTTGGGCTGAGC-3′) and antisense (5′-ATAT ATGAAGCTTGCTGAGGAGACGGTGACC-3′) primers. To generate RT-PCR products for sequencing analysis of the variable regions of the F240 Ab expressed in the parental hybridoma and CHO cells, the primers used were the κ constant region (5′-ACACTCTCCCCTGTTGAA-3′) and IgG1 constant region (5′-CAACTTTCTTGTCCACCTTGG-3′) antisense primers. These primers were used in the RT-PCR together with the F240 κ and H chain variable region primers, respectively.

cDNA synthesis

RNA was isolated from 10^7 to 10^8 cells by using the FastTrack mRNA isolation kit (Invitrogen Life Technologies) according to the manufacturer's instructions. cDNAs were synthesized using oligo$(dT)_{20}$, Ig-specific primers, and high fidelity *Taq* DNA polymerase (Invitrogen Life Technologies).

Abs and viruses

The IgG1κ F240 hybridoma was created from B cells isolated from an HIV-1 seropositive individual (31). The broadly neutralizing mAb IgG1b12 was obtained from the National Institutes of Health ARRRP. The HIV-1 clade B primary isolates BaL (macrophage-tropic), JR-FL (R5-tropic), SF162 (R5-tropic), 67970 (X4-tropic), and 89.6 (R5X4-tropic) and the clade C isolate 301906 (R5-tropic) were obtained through the National Institutes of Health ARRRP. The primary viruses were propagated in PHA-stimulated PBMCs. Peripheral blood was collected from normal healthy donors following informed consent and the study was approved by the Beth Israel Deaconess Medical Center Institutional Review Board (Boston, MA). HIV-1 envelope-pseudotyped viruses were generated by the transfection of 293T cells as previously described (32).

Plasmids

The vectors pLC-huCk (κ L chain), pHC-huCγ1 (IgG1), pHC-huCγ3 (IgG3), and pHC-huCγ4 (IgG4) encoding the Ig constant regions were obtained from Dr. G. McLean (University of Texas Health Sciences Center, Houston, TX) (33). The bicistronic mammalian expression vectors pIRESneo3 and pIRESpuro3 were purchased from BD Biosciences-Clontech. Plasmids expressing the tier 2 HIV-1 clade B envelopes PVO.4, QHO692.42, and RHPA4259.7, the clade C envelopes Du156.12 and Du422.01, and the clade A envelopes Q23 and Q769 were obtained from the National Institutes of Health ARRRP. Plasmids expressing the tier 1 clade B envelopes SF162.LS and BaL.26 were the generous gifts of D. Montefiori (Duke University Medical Center, Durham, NC) and J. Mascola (National Institutes of Health Vaccine Research Center, Bethesda, MD), respectively. Tier 1 viruses are well-characterized neutralization-sensitive strains of HIV-1, whereas tier 2 viruses are primary isolates that are not overly sensitive or resistant to Ab-mediated neutralization.

Molecular cloning of Abs

In vitro generation of IgG F240 Abs was achieved by using a multistep process. The variable regions of the L (V_L) and H chains (V_H) were isolated by RT-PCR-mediated amplification with specific oligonucleotide primers to each polypeptide chain from RNA isolated from the F240 hybridoma. The primers contained unique restriction enzyme cut sites at the ends of the V_L (*Nhe*I-*Not*I) and V_H (*Nhe*I-*Hin*dIII) cDNAs. To generate the H and L chains, the VL and V_H PCR products were appropriately digested and cloned in-frame into the corresponding sites of the vectors pLC-huCk and pHC-huCγ1/pHC-huCγ3/pHC-huCγ4, respectively. Full-length sequencing of the constructs was performed to exclude adventitious mutations that may have been introduced during the procedure. The F240 L and H chain cassettes were subsequently cloned into internal ribosome entry site (IRES)-based bicistronic expression vectors. To this end, the L chain was digested with appropriate enzymes and cloned into the pIRESneo3 vector, whereas the F240 H chain cassettes were cloned into the pIRESpuro3 vector.

Generation of recombinant Abs

Bicistronic plasmids carrying either the L or H chains were purified with the Maxiprep kit (Qiagen) and the L chain vector was transfected into CHO-K1 cells using Lipofectamine 2000 (Invitrogen Life Technologies). After ~2 wk of selection in RPMI 1640 containing 800 μg/ml G418, colonies were isolated and L chain expression was monitored by Western blotting of cell lysates. Cell clones expressing the F240 L chain were propagated and subsequently subjected to a second round of transfection with the pIRESpuro3-F240 H chain plasmids to generate the mature Ig. After 2 wk of clonal selection in medium containing 10 μg/ml puromycin and 800 μg/ml G418, individual culture supernatants were screened for IgG production by subclass specific ELISA. To this end, ELISA plates were sensitized with goat anti-human IgG (Southern Biotechnology Associates) and blocked with 1% BSA. Ab-containing medium was added to the wells and bound Ab was detected using murine Abs to IgG subclasses (HP6001 (IgG1), HP6050 (IgG3), and HP6025 (IgG4)) followed by HRP-conjugated goat anti-mouse IgG Ab and detection using an orthophenylenediamine (OPD) substrate. The murine Abs were purified from hybridomas obtained from the ATCC. Known standards of IgG subclasses (EMD Biosciences-Calbiochem) were used as controls.

Western blots

For Western blot analysis, CHO cells were washed with PBS and the pellet was treated with 100 μl lysis buffer (1% TX-100, 0.4% deoxycholic acid, 100 mM PMSF, and 1× Halt protease inhibitor mixture (Pierce) in PBS). The lysates were clarified by centrifugation and SDS loading buffer was added to 100 μg of the clarified supernatant. Proteins were subjected to SDS-PAGE (8–20%) and transferred (16 h) onto Immobilon membranes (Millipore) that were incubated with the primary Ab followed by a HRP-conjugated secondary Ab and revealed by using the SuperSignal West Pico chemiluminescence system (Pierce).

Purification and quantitation of recombinant Abs

For Ab production, cell clones were grown in medium without selection antibiotics for 4–7 days and the supernatant was collected. Ab was purified from the supernatant by protein G affinity chromatography. Purified Ab was quantitated by ELISA using known concentrations of isotype control proteins (EMD Biosciences-Calbiochem) to generate a standard curve. All preparations were prepared under endotoxin-free conditions and purity was assessed by SDS-PAGE.

Immunoreactivity of recombinant Abs

ELISA plates were sensitized overnight with recombinant gp41 (VTI-310) from Vybion at a concentration of 2 μg/ml in PBS. Plates were blocked with 1% blocker BSA (Pierce) for 2 h at room temperature before use. Serial, 2-fold dilutions of Ab were added in triplicate to blocked plates and

incubated for 30 min at room temperature. Unbound Ab was removed by washing and bound Ab was detected using a HRP-conjugated goat anti-human κ-chain Ab (Southern Biotechnology Associates) which reacts identically to the L chains shared by the recombinant Abs. After 30 min at room temperature, the plates were washed and developed using OPD substrate for 15 min. OD was determined using an automated plate reader.

Neutralization assays

Neutralizing Ab responses against primary isolate and envelope-pseudotyped HIV-1 viruses were measured using a luciferase-based assay in TZM-bl cells as previously described (32). This assay measures the reduction in luciferase reporter gene expression in TZM-bl cells following a single round of virus infection. The IC_{50} was calculated as the concentration that caused a 50% reduction in relative luminescence units compared with the virus control wells after the subtraction of cell control relative luminescence units. Alternatively, for the isotype switch mutants the reporter readout was based on β-galactosidase production, which was measured by ELISA (Pierce). A PBMC-based assay (26, 34) was also used to confirm HIV-1 neutralization in a more biologically relevant model. Briefly, serial 2-fold dilutions of a mAb were incubated with virus stock diluted to 200 50% tissue culture infective dose $TCID_{50}$ for 1 h at 37°C before the addition of PHA-stimulated PBMC target cells (1×10^5 cells/well). The plates were incubated for 4 h at 37°C with 5% CO_2, after which they were washed and the medium replaced with fresh growth medium. Seven days after the infection was initiated, the supernatant was removed and tested for p24 by ELISA using murine anti-p24 Ab to capture p24, which is detected by sandwich ELISA using IgG purified from pooled HIV sera followed by goat anti-human IgG biotin and HRP-conjugated streptavidin with OPD as the substrate. Concentrations of p24 were derived from a standard curve using known concentrations of p24. IC_{50} was calculated as the concentration of the Ab that reduced p24 levels by 50% from that observed with a non-HIV IgG control Ab.

Lectin binding ELISA

ELISA using biotinylated lectins was used to determine the glycosylation of Abs produced in hybridoma and CHO cells. ELISA plates were sensitized overnight with purified Ab at 2 μg/ml in PBS. Plates were blocked as described above and the biotin-labeled lectins Con A, *Sambucus nigra* agglutinin (SNA), *Aleuria aurantia* lectin (AAL), and *Ricinus communis* agglutinin I (RCAI) (Vector Laboratories) added at 1/10,000 to 1/20,000 dilution for 30 min at room temperature. After washing, HRP-conjugated streptavidin was added for 30 min, the plates washed, and the OPD substrate added. The OD was determined at 490 nm. Biotinylated goat anti-human κ-chain or IgG Fc Abs were added to control wells to ensure that the Abs of interest bound to the plate equivalently.

Peptide N-glycosidase F (PNGase F) treatment

This procedure was performed following the manufacturer's directions. Briefly, b12 or F240 IgG1 Abs at 340 μg/ml were incubated overnight at 37°C under nondenaturing conditions with 5 U of PNGase F (New England Biolabs) in a 200-μl reaction. PNGase F-treated Abs were sensitized to ELISA plates to determine the extent of deglycosylation and used in a standard neutralization assay as indicated above.

Results

In vitro generation of recombinant F240

F240 is an IgG1κ human mAb originally isolated in our laboratory. It recognizes an extracellular conserved epitope (residues 592–604) on gp41 within the epitope termed class I. F240 reacts with primary isolates from all clades of HIV-1 (31) and, while nonneutralizing, enhances neutralization by other Abs (26).

The generation of IgG F240 isotypes in CHO-K1 cells was achieved by using a multistep strategy. Essentially, the Ab variable regions were isolated by RT-PCR from the original hybridoma and subsequently cloned into plasmid DNA vectors that encode either the constant region of the κ L chain (pLC-huCk) or the constant region of the IgG subclasses (pHC-huCγ1, 3, or 4). Although these vectors are capable of driving Ig expression in mammalian cells (33), to further facilitate the selection of cells stably expressing the Ab of interest we subcloned the full-length F240 L and H chain cassettes into IRES-based bicistronic expression vectors. The plasmids were then purified and the L chain vector was transfected into

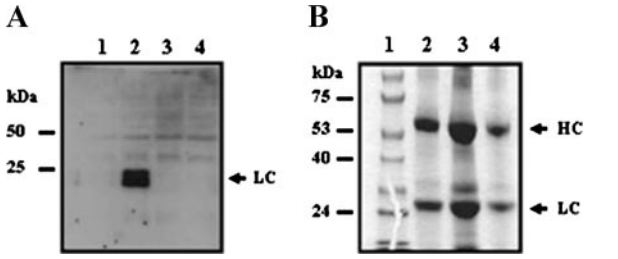

FIGURE 1. Generation of F240 IgG Abs in CHO cells. *A*, Western blot of lysates from CHO colonies resistant to G418. F240 L chain expression was monitored by using a goat anti-human κ-chain Ab conjugated to HRP. *Lane 1*, Cells stably transfected with vector alone; *lanes 2–4*, three cell clones stably transfected with the F240 L chain vector. All cell clones were grown under identical conditions. *B*, SDS-PAGE of purified Abs. *Lane 1*, m.w. marker; *lane 2*, IgG1 F240 purified from the hybridoma; *lane 3*, IgG1 F240; *lane 4*, IgG4 F240. Abs were produced, purified, and stored under identical conditions. HC, H chain; LC, L chain.

CHO-K1 cells. After ~2 wk of selection in RPMI 1640 containing G418, colonies were isolated and L chain expression was confirmed by Western blot analysis of lysed cells. Fig. 1*A* shows that the κ L chain of the F240 Ab is retained intracellularly in the CHO cell clones. Although notably rare, intracellular retention of L chains has been observed and could be the result of somatic hypermutations of L chain residues (our unpublished observations).

To generate mature immunoglobulins, cells expressing the F240 L chain were transfected with the pIRESpuro3-F240 γ1, 3, and 4 plasmids. After two weeks of clonal selection in medium containing puromycin and G418, individual culture supernatants were screened for IgG production by ELISA. Stable cell clones produced moderate levels of Ab (0.01–0.05 μg/ml; 10^6 cells; 24 h). Finally, cells expressing the highest levels of F240 IgG isotypes were propagated in culture triple flasks and the Ab was purified from the culture medium using protein G (IgG) affinity chromatography, quantified, and subjected to SDS-PAGE analysis to verify the presence of the L and H chains and assess purity (Fig. 1*B*). The results show that by using this method we have produced in vitro the Abs F240-IgG1, F240-IgG3, and F240-IgG4. The class of the purified Abs was verified by class-specific ELISA.

Immunoreactivity of isotype switched F240

F240 Abs produced in CHO cells were tested for immunoreactivity with recombinant gp41 by ELISA to assess whether class switch led to alterations of their binding properties. F240 bound to gp41 was detected using a goat anti-κ-chain Ab for comparative purposes. As shown in Fig. 2, the reactivity of recombinant Abs generated in CHO cells, including IgG1, IgG3, and IgG4, paralleled that observed for the parental F240 produced by hybridoma cells. F240 constructs were also tested for reactivity with primary isolate virions from clades B and C, some of which are considered either neutralization resistant or sensitive (34), and identical results were obtained (data not shown). Thus, CHO-derived F240 isotypes retained broad reactivity with gp41.

Neutralization of HIV by F240 generated in CHO cells

F240 isotype-switched Abs were tested for neutralization of HIV using a TZM-bl based reporter assay. Primary isolate virus was grown in PBMCs and an envelope-pseudotyped virus was isolated from transfected 293T cells. In contrast to the parental F240 IgG1 Ab produced in hybridoma cells (F240-H), F240-IgG1 purified from CHO cells neutralized a range of HIV isolates with an IC_{50} in the range of that observed with the broadly neutralizing anti-

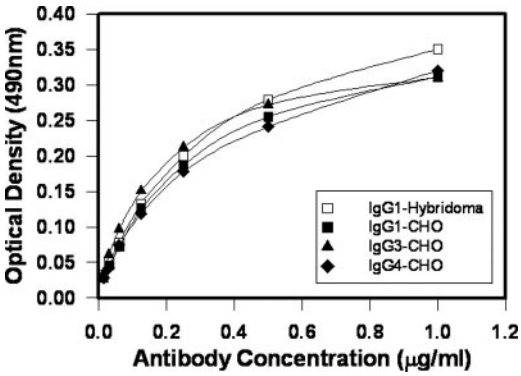

FIGURE 2. Immunoreactivity of F240 generated in CHO cells with gp41. F240 IgG1 (■), IgG3 (▲), and IgG4 (◆) along with F240 from the parental hybridoma (□) were added to ELISA plates sensitized with gp41. The bound F240 Ab was detected using HRP-conjugated goat anti-human L chain Ab and OD was determined at 490 nm after the addition of the OPD substrate. Results are the mean of triplicate wells and are representative of at least three independent experiments.

CD4 binding site Ab, b12 (Table I). F240-IgG1 generated in CHO cells neutralized the majority of clade B isolates, including those designated tier 1 and tier 2 (35), similar to b12. Tier 1 viruses are well-characterized neutralization-sensitive strains of HIV-1, whereas tier 2 viruses are primary isolates that are not overly sensitive or resistant to Ab-mediated neutralization. Although tier 2 clade A isolates were resistant to neutralization by either F240-IgG1 or b12, two tier 2 clade C isolates were neutralized by both Abs. When the IgG3 and IgG4 variants of F240 were compared with the F240-IgG1, despite similar immunoreactivity with HIV, there were subclass-specific differences in neutralization (Table II). The IgG1 and IgG3 subclasses were much more effective at neutralizing the virus than the IgG4 subclass, and the IgG3 appeared to be most potent. In addition to neutralizing the primary isolate virus when measured using TZM-bl indicator cells, F240 IgG1 expressed in CHO cells also neutralized the primary isolate virus when tested using a PBMC-based assay (Table I). The neutralization of SF162 in the PBMC-based assay requires significantly more F240-G1 produced in CHO cells than that required for neutralization in the TZM-bl assay. This difference likely reflects differences in the source of the virus (PBMC vs pseudovirus), the target cell, and the rounds of infection (multiple for PBMC and single for TZM-bl).

Table I. *Neutralization of HIV primary isolates by F240-IgG1*

	IC_{50} (μg/ml)[a,b]		
TZM-bl Assay	F240-H[c]	F240-G1[d]	b12[e]
Clade B, tier 1			
SF162.LS	>25	0.09	0.25
BaL.26	>25	0.4	0.28
Clade B, tier 2			
PVO.4	>25	>25	>25
QH0692.42	>25	7.12	4.44
RHPA4259	>25	2.22	1.03
Clade A, tier 2			
Q23	>25	>25	>25
Q769	>25	>25	>25
Clade C, tier 2			
Du156.12	>25	7.67	3.84
Du422.01	>25	5.89	2.76
	IC_{50}(μg/ml)[b,f]		
PBMC Assay	F240-H	F240-G1	b12
67960	>25	0.15	0.4
SF162	>25	4.6	0.3

[a] Concentration of Ab (μg/ml) required for 50% inhibition of virus.
[b] IC_{50} values are the mean of triplicate wells and are representative of at least two independent experiments.
[c] F240 IgG1 parental hybridoma.
[d] F240 IgG1 CHO-K1 cells.
[e] b12 IgG1 CHO-K1 cells.
[f] Concentration of antibody (μg/ml) required for 50% inhibition of virus as measured by a reduction of p24 produced after 7 days.

Table II. *Neutralization of HIV-1 primary isolates by F240 IgG antibodies generated in CHO cells*

	IC_{50}[a,b]		
Isolate	F240-IgG1	F240-IgG3	F240-IgG4
67970	0.3	0.4	9.7
SF162	0.2	0.4	2.6
BaL	22.3	1.2	>40
89.6	3.0	1.6	>40
JR-FL	12.6	1.6	>40
93MW960	22.7	>40	>40

[a] Concentration of Ab (μg/ml) required for 50% inhibition of virus.
[b] IC_{50} values are the mean of triplicate wells and representative of at least three independent experiments.

Sequence comparison of F240 Abs generated in two different cell types

To exclude any artifacts that may have been produced during integration of the F240 L and H chains plasmids into the genome of CHO cells, we performed RT-PCR on mRNA isolated from the F240 parental hybridoma as well as CHO-K1 cells expressing the Ab, using specific primers derived from the signal sequence of the L and H chains and primers from the corresponding constant regions. The sequencing analysis demonstrated that there are no differences in the nucleotide sequences of the L and H chain variable regions of the F240 Abs produced in both cell types (data not shown).

Glycosylation of recombinant F240

Glycoproteins are differentially glycosylated depending upon cell type and growth conditions, and differences in glycosylation are known to affect Ab function (36–43) There is an N-linked glycosylation site at N297 of the CH2 domain of IgG with an α-linked mannose core. There is heterogeneity in the terminal glycans resulting from differences in fucose, bisecting *N*-acetylglucosamine, galactose, and sialic residues. The terminal glycans on the F240 Abs produced in CHO cells were compared with the parental hybridoma Ab by binding to lectins (lectin binding ELISA) specific for defined glycans. As expected, there was no difference in the binding of Con A, which reacts with α-linked mannose, to either CHO-derived or hybridoma Ab (Fig. 3). However, there was marked elevation in sialic acid, fucose, and *N*-acetylglucosamine as evidenced by increased binding of the lectins SNA, AAL, and RCAI to the Abs produced in CHO cells as compared with Ab purified from the hybridoma. Therefore, there was an increase in the glycans associated with the Ab generated in CHO cells, which is neutralizing, as compared with the Ab purified from the parental hybridoma, which fails to neutralize. By sequence analysis, a potential *O*-linked glycosylation site was found in the variable region of the L chain; however, when tested by Western blotting there was no difference in lectin binding to the L chain but there were differences in binding to the H chain (data not shown).

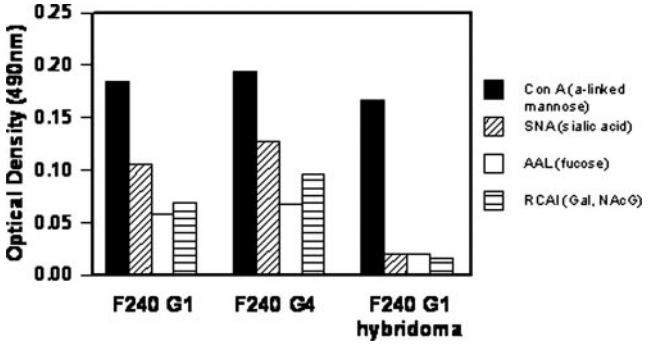

FIGURE 3. Determination of recombinant F240 glycosylation by lectin ELISA. Biotinylated lectins with defined specificity (ConA, α-linked mannose (filled bars); SNA, sialic acid (diagonal bars); AAL, fucose (open bars); and RCAI, *N*-acetylglucosamine and galactose (horizontal bars)) were reacted with ELISA plates sensitized with F240 generated in CHO cells or the hybridoma. Bound lectin was detected using HRP-conjugated streptavidin and the OPD substrate and OD was determined at 490 nm. Results are the mean of triplicate wells and representative of at least three independent experiments.

To further test the role of glycosylation on F240 neutralization activity, Abs purified from CHO cells were treated with *N*-glycosidase F (PNGase F), which removes all types of N-linked glycosylation. Overnight treatment of recombinant F240 with PNGase F significantly reduced, but did not eliminate, the glycans associated with the recombinant Ab as assessed by the lectin binding ELISA. PNGase F treatment reduced AAL binding by 70%, RCAI by 36%, SNA by 45%, and ConA by 15%. Similarly, PNGase F treatment of the control anti-gp120 human mAb b12 IgG1 (also purified from CHO cells) reduced AAL binding completely, RCAI by 69%, SNA completely, and Con A by 68%. Moreover, PNGase F treatment did not affect the binding properties of the recombinant F240 Ab produced in CHO cells (Fig. 4), nor did it affect b12 binding to recombinant gp120. More importantly, PNGase F treatment significantly abolished the neutralization activity of recombinant F240 with isolate 89.6 ($p < 0.001$). Further, more PNGase F-treated recombinant F240 IgG1 Ab was required to neutralize the 67970 isolate ($p < 0.05$). This effect appears to be isolate specific inasmuch as PNGase F treatment had no effect on the SF162 and 93MW960 isolates, which may be a function of the neutralization sensitivity/resistance of these isolates. Importantly, treatment did not affect the activity of the control b12 IgG1 Ab (Table III). These data support the proposal that glycans are critical for the neutralization of HIV-1 isolates by CHO-produced recombinant F240.

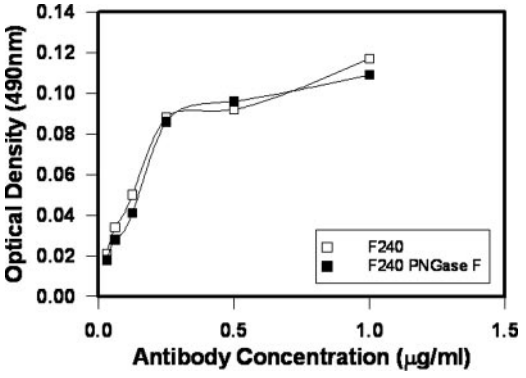

FIGURE 4. Deglycosylation by PNGase F of the F240 Ab produced in CHO cells does not alter binding to Ag. IgG1 untreated (□), and PNGase F-treated (■) F240 Abs were added to ELISA plates sensitized with gp41. Bound F240 Ab was detected using a HRP-conjugated goat anti-human L chain Ab and OD was determined at 490 nm after the addition of the OPD substrate. Results are the mean of triplicate wells and representative of at least three independent experiments.

Table III. *Neutralization of HIV-1 primary isolates by F240 IgG1 produced in CHO cells after PNGase F treatment*

	IC_{50}[a,b]			
Isolate	F240-IgG1	F240-IgG1 PNGase F	b12-IgG1	b12-IgG1 PNGase F
67970	0.3	3.6	ND	ND
93MW960	22	34.2	ND	ND
SF162	0.5	1.1	0.001	0.003
89.6	5.7	>40	8.1	11.1
JR-FL	ND	ND	0.2	0.3

[a] Concentration of Ab (μg/ml) required for 50% inhibition of virus.
[b] IC_{50} values are the mean of triplicate wells and are representative of at least two independent experiments.

Discussion

In recent years there has been a dramatic increase in the number of mAbs either approved or under clinical development to treat a range of diseases (reviewed by Laffly and Sodoyer in Ref. 44). The majority of these Abs, as well as isotype-switched Abs, have been produced by expressing transfected genes in CHO cells with the retention of Ab immunoreactivity and/or function. In this report we describe the acquired capacity of the human F240 mAb to the immunodominant region of gp41 to neutralize infection in a traditional neutralization assay when it is generated as a recombinant Ab and expressed in CHO cells. It is worth noting that although the F240 Ab has been previously characterized as nonneutralizing, a recent report has shown that this Ab (and other nonneutralizing Abs) is capable of inhibiting viral replication by mechanisms distinct from those mediated on the virion surface by the Ab Ag-binding regions (45).

Isotype-switched F240 (IgG1, IgG3 and IgG4) was purified from CHO cells in a manner identical with that of an Ab purified from the parental hybridoma and found to be equivalently immunoreactive with recombinant gp41 and primary isolate viruses. However, in contrast to the Ab produced by the hybridoma, isotype-switched Abs purified from CHO cells neutralized HIV infection as measured in a TZM-bl assay, and also in PBMCs. In some cases, F240 as an IgG3 was more potent than the IgG1 isotype (e.g., BaL and JR-FL), whereas there was no difference for the other isolates. In all cases, F240 as an IgG4 molecule was less able to neutralize infection. These isotypic differences are likely a function of the flexibility of the IgG subclasses. It is known that IgG3 molecules tend to be more flexible in binding than IgG1 molecules because of the extended hinge region, whereas IgG4 molecules tend to be relatively rigid in structure (46). We have shown previously that when the CD4bs Ab F105 is changed from an IgG1 Ab to IgG3, both forms of the Ab had equivalent binding to HIV (28). However, F105 IgG3 is able to neutralize an isolate that the IgG1 failed to neutralize. Thus, an increase in Ab flexibility resulted in altered Ab/Ag interactions such that an isolate resistant to the IgG1 subclass was neutralized by the IgG3 subclass. Polyclonal serum IgG3 was found to have more neutralizing activity than IgG1 or IgG2 (47). More flexible IgG3 Abs might be capable of increased access to epitopes and efficacy than the more prevalent IgG1, and the ability of IgG3 Abs to form higher order complexes might also enhance functional effects (48–50). Thus, crosslinking of specific epitopes of HIV by flexible IgG3 Abs may result in changes in the oligomer that reduce viral infection.

Because there are no differences in the sequences of the F240 Abs produced by CHO cells and the parental hybridoma, the acquired neutralization activity of this Ab is likely the result of distinct posttranslational modifications conferred by these two cell lines. Among the posttranslational modifications that Abs undergo during their intracellular maturation process, tyrosine sulfation and N-linked glycosylation are the best characterized. For HIV-1 Abs, Ab tyrosine sulfation has been shown to be important for the ability of the Ab to neutralize the virus. Yet, this type of modification has been shown to be relevant for Abs that recognize the CCR5-binding region of gp120 only (51, 52) and, hence, it is unlikely to play a significant role in the acquired neutralization of the gp41 Ab F240 upon expression in CHO cells. Importantly, it is widely acknowledged that an Ab expressed in CHO cells is differentially glycosylated as compared with an Ab produced in other mammalian cells (36, 37). There is an N-linked glycosylation site located at the N297 of the CH2 domain. The core of this complex biantennary type of sugar is a heptasaccharide consisting of *N*-acetylglucosamine and mannose. The variation in glycosylation is due to differences in terminal sialic acid, galactose, *N*-acetylglucosamine, and fucosylation of the core. It was found that the F240 Ab produced in CHO cells had significantly more of these terminal glycans and core fucose when compared with the F240 Ab from the hybridoma, despite similar levels of core mannose. Ab glycosylation stabilizes IgG molecules from degradation (53, 54) and affects IgG binding to Fc receptors, possibly by influencing the structural conformation of the IgG molecule (55, 56).

It is also worth noting that the extent of neutralization observed within the same subclass appears to be isolate specific. This observation is unlikely to be the result of subtle differences within the sequence of the immunodominant domain of gp41 that F240 recognizes because this Ab reacts identically with virus isolates considered either neutralization sensitive or resistant. Rather, it is widely believed in the field that divergent glycosylation patterns of the viral envelope are responsible for the different sensitivities of HIV isolates to broadly neutralizing Abs. This could account for the dissimilar results with the isolates herein studied. Of particular relevance are our observations involving PNGase F treatment of CHO-derived Abs. In fact, in the present study Abs treated with the glycosidase produced the largest neutralizing effect with the HIV-1 isolate 89.6, whereas no significant differences in neutralization were observed with the isolates SF162 and 93MW960 (see *Results* and Table III). We think that this effect is due to the intrinsic resistance of these viruses to neutralizing Abs. Although SF162 is known to be extremely sensitive to Ab-mediated neutralization, the opposite is true for the 93MW960 isolate, which is not neutralized by a number of neutralizing Abs. Therefore, a significant difference in the extent of neutralization of Abs treated with PNGase F is not expected with the latter two isolates. Apart from these observations, differences in glycosylation are also known to profoundly affect Ab function with Ab-dependent cellular cytotoxicity activity, presumably due to differences in Ab interaction with Fc receptors (38–43).

Finally, in the absence of structural data we speculate that the F240 Ab produced in CHO cells may more effectively impact on viral infection as a result of the interaction of its terminal glycans with HIV after F240 binding to its epitope on gp41, hence interfering with the conformational changes and energy relationships among the trimers or between the trimers and the receptors on the cell surface. Indeed, from the point of view of the Ag/Ab interaction, it has been shown that sugar moieties that apparently lie apart from the antibody's binding site can severely influence the access of the Ab to neutralizing epitopes (57). Thus, by analogy glycans present on the Fc region of F240 can dramatically affect the virus/Ab interaction. An alternative mechanism would involve alterations in the structure of the Fab domain by the oligosaccharides present in the Fc region. However, the fact that the flexible hinge region structurally isolates both Ab domains makes this hypothesis less plausible. It has been shown by Kaneko et al. that the degree of sialylation of the Fc domain of IgG regulates its interaction with FcγRs (58). Although it can be speculated that the higher content of the terminal saccharide sialic acid in the F240 Ab derived from CHO cells facilitates the engagement of immune complexes with Fc receptors on neutrophils or NK cells resulting in virus inactivation, this is unlikely to occur in the 7-day PBMC-based assay or in the TZM-bl assay. Studies are underway to determine the contribution of sialic acid in our observations and also to find out whether F240 blocks binding, engagement with the chemokine coreceptor, or fusion and whether glycosylation alters binding on/off rates.

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Disclosures

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