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A Novel Antibody-Dependent Cellular Cytotoxicity Epitope in gp120 Is Identified by Two Monoclonal Antibodies Isolated from a Long-Term Survivor of Human Immunodeficiency Virus Type 1 Infection

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Two monoclonal antibodies (MAbs), 42F and 43F, were isolated some 14 months apart from a single long-term survivor of human immunodeficiency virus type 1 (HIV-1) infection. These MAbs were found to be indistinguishable in terms of their isotypes, specificities, affinities, and biological activities. Both 42F and 43F directed substantial antibody-dependent cellular cytotoxicity (ADCC) against cells infected with four divergent lab-adapted strains of HIV-1, but no neutralizing activity against these strains was detectable. The ability of MAbs 42F and 43F, as well as that of MAbs against two other gp120 epitopes, to direct ADCC against uninfected $CD4^+$ cells to which recombinant $gp120_{SF2}$ had been adsorbed (i.e., "innocent bystanders") was demonstrated to be less efficient by at least an order of magnitude than their ability to direct ADCC against HIV-1-infected cells. Flow cytometry analyses showed that 42F and 43F also bind to native primary isolate Envs from clades B and E expressed on cell surfaces. By direct binding and competition assays, it was demonstrated that the 42F/43F epitope lies in a domain of gp120 outside the previously described CD4-binding site and V3 loop ADCC epitope clusters. Immunoblot analysis revealed that the 42F/43F epitope is not dependent on disulfide bonds or N-linked glycans in gp120. Epitope mapping of 42F and 43F by binding to linear peptides demonstrated specificity of these MAbs for a sequence of 10 amino acids in the C5 domain comprising residues 491 to 500 (Los Alamos National Laboratory numbering for the HXB2 strain). Thus, 42F and 43F define a new ADCC epitope in gp120. Because of the relative conservation of this epitope and the fact that it appears to have been significantly immunogenic in the individual from which these MAbs were derived, it may prove to be a useful component of HIV vaccines. Furthermore, these MAbs may be used as tools to probe the potential importance of ADCC as an antiviral activity in HIV-1 infection.

The correlates of immunity to human immunodeficiency virus type 1 (HIV-1) infection and the role of the immune response in delaying progression to disease in HIV-1-infected individuals remain unclear despite much effort and progress in these areas. Both neutralizing antibodies (Abs) and HIV-1-suppressive $CD8^+$ cells, including cytotoxic T lymphocytes (CTL), appear to be important and desirable immune responses against HIV-1. CTL seem to be primarily responsible for clearing the viremia associated with primary HIV-1 infection (15); in addition, a high proportion of multiply exposed, HIV-seronegative individuals remain virus free but exhibit strong HIV-specific T-cell responses, including CTL (4). Long-term survivors of HIV-1 infection, compared to progressors, have higher titers of neutralizing Abs against primary isolates as well as HIV-1-suppressive $CD8^+$ cells (3). The relevance of these neutralizing Abs in long-term survivors has been questioned recently, since they often do not neutralize the autologous primary isolate(s) of HIV-1 (25). Nevertheless, a number of older studies show that neutralizing Abs, in the absence of cellular immune responses, can protect from challenge with homologous HIV-1 when they are elicited by vaccination or administered passively (reviewed in reference 28).

In contrast, the role of Ab-dependent cellular cytotoxicity (ADCC)-directing Abs in HIV-1 infection has been controversial. In this immune mechanism, anti-Env Abs direct effector cells to kill target cells bearing HIV-1 envelope on their surfaces; this is accomplished via specific binding of the Abs' antigen-binding sites to Env and their Fc regions to Fc receptors on the effector cells. Earlier in the HIV-1 epidemic, concerns were raised that shed soluble gp120 in HIV-1-infected individuals might bind to $CD4^+$ cells, including uninfected ones, and could target these cells for "innocent bystander" killing by ADCC (19). However, effector cells armed with serum Abs able to direct ADCC in vitro against either innocent bystanders or HIV-1-infected cells were found at highest frequency in asymptomatic, seropositive individuals; patients with AIDS-related complex and AIDS showed progressively diminished reactivity (43).

Other studies have caused concerns about potential pathogenic roles of anti-Env Abs in general; these studies demonstrated that anti-Env antisera or monoclonal Abs (MAbs) could enhance the infection of Fc receptor- and/or complement receptor-bearing primary cells or cell lines by HIV-1 (10, 14, 25, 32, 35, 37). Conversely, several recent studies have shown that targeting of anti-Env Ab-virus complexes to specific Fc receptors on monocytes can cause abrogation rather than

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enhancement of infection (5, 11, 20, 21). Furthermore, no consistent link for in vitro-detected Ab-dependent enhancement of HIV-1 infection with disease progression in infected individuals has been demonstrated (22, 25).

The existing in vivo data support the efficacy, rather than pathogenicity, of ADCC-directing Abs against HIV-1. An HIV-1-infected chimpanzee that developed an ADCC response similar to that seen in HIV-infected humans showed no decline in $CD4^+$ cells after over 5 years of infection (7). In another study, an immunized chimpanzee that presented both strong ADCC and high neutralizing Ab responses against HIV-1 at the time of challenge with virus was protected through at least 12 months of follow-up, while another immunized chimpanzee with neutralizing Ab but no ADCC response was protected only partially, as evidenced by delayed viremia occurring 7 months later (1a). Sawyer et al. (33) observed that in HIV-1-infected individuals, early ADCC responses correlated with a delay in the decline of $CD4^+$ cell numbers. Finally, the presence of Abs mediating ADCC and neutralization correlated with a better clinical stage in children born to HIV-1-infected mothers (18).

Because of the potential importance of ADCC-directing Abs against HIV-1 and the fact that they and neutralizing Abs are distinct, though overlapping, subsets of anti-Env Abs (reviewed in reference 40), the isolation and characterization of such MAbs from HIV-1-infected individuals is of interest. Here, we describe the isolation and primary characterization of two such MAbs that were isolated from a long-term survivor of HIV-1 infection; these MAbs define a new, conserved ADCC epitope in HIV-1 gp120.

MATERIALS AND METHODS

Human subject. Peripheral blood samples were obtained from an HIV-1-seropositive hemophiliac having normal leukocyte counts and no history of opportunistic infections. This individual continues to be followed at the Pediatric Hematology clinic at New York Hospital-Cornell Medical Center; he remains asymptomatic and has been seropositive for over 12 years. Two described anti-V3 MAbs, 4117C (39) and 41148D (29), were previously isolated from this same individual.

Isolation of human MAbs. Fresh peripheral blood mononuclear cells (PBMC) were obtained for MAb isolation upon two separate occasions that were approximately 14 months apart; MAb 42F was isolated from the first blood sample, while MAb 43F was isolated from the second blood sample. Both MAbs were isolated by the same protocol as follows. The PBMC were isolated, incubated overnight with Epstein-Barr virus (EBV), and washed and resuspended in complete medium the following day essentially as described previously (38). A proportion of the cells was plated in 96-well plates as described previously (38), while the remainder (in a few milliliters of complete medium) was transferred to a 25-cm^2 tissue culture flask in the absence of feeder cells. Approximately 4 weeks later, supernatants from the 96-well plate cultures and the single flask culture were assayed for reactivity with recombinant $gp160_{LAI}$ ($rgp160_{LAI}$; formerly referred to as $rgp160_{BRU}$) by enzyme-linked immunosorbent assay (ELISA) as described previously (38). A proportion of the plate cultures initially tested positive; these either were frozen for future analysis or were expanded and later tested negative. The flask culture was initially positive and continued to test positive over the following weeks of culture. After several weeks, the flask culture was sublined at limiting dilution, and a few hundred clonal and oligoclonal cultures resulting from this sublining were reassayed by ELISA. All of the cultures were positive, suggesting that the original culture was monoclonal. In the case of 42F, clonality was also demonstrated by Southern blot analysis as described previously (38) (data not shown).

Purification and quantitation of MAbs. The MAbs were purified, concentrated, and quantitated as described previously (28, 38).

Determination of MAb isotypes. The heavy- and light-chain isotypes were determined as described previously (38).

HIV-1 strains, HIV-1-infected cells, and recombinant HIV-1 envelope-expressing cells. HIV-1 strains IIIB, MN, SF-2, and RF have been described (reviewed in reference 28); these viruses were passaged in H9 cells.

For flow cytometric analyses and ADCC assays, CEM.NKR cells (12) and CEM.NKR cells chronically infected with IIIB were obtained from Richard Koup, Aaron Diamond AIDS Research Center, New York, N.Y. In addition, new chronically infected cell lines were obtained by infecting CEM.NKR cells independently with MN, SF-2, and RF viruses followed by passage and expansion of viable cells. The envelope expression in these chronically infected lines was assessed by flow cytometric analysis following incubation of the cells with pooled seropositive serum and then fluorescein isothiocyanate (FITC)-goat anti-human immunoglobulin G (IgG) (see below for details). This expression became stable after several cell passages and has remained so for approximately 2 years of continuous culture. The pattern of reactivity of our panel of anti-Env MAbs with these chronically infected cell lines mirrored that previously seen against H9 cells acutely infected with the corresponding HIV-1 strains (1). The level of envelope expression was consistently lower in the MN-infected CEM.NKR cells than in the other three (IIIB-, SF-2-, RF-infected) CEM.NKR lines, and so clones of these cells were isolated by limiting dilution and characterized for Env expression in attempts to identify high-level expressers. One of these MN-infected clones, clone 7, was selected for further experiments based on its higher level of Env expression than the original, uncloned MN-infected line as assessed by flow cytometric analysis as discussed above.

Molecular clones of primary isolates (clades B and E) from Thailand were isolated as described previously (24), and full-length gp160-encoding regions from clones BK132, CM235, and CM243 (Genbank accession numbers LO3697, LO3698, and LO3703, respectively) were used to create Env-expressing recombinant vaccinia viruses vCB51, vCB52, and vCB53, respectively, by Christopher Broder and Patricia Earl, National Institutes of Health, using techniques similar to those described previously (6). These recombinant viruses and vPE16 (6), which expresses the BH8 envelope, were used to infect A2.01 cells, a $CD4^-$ human T-cell line derived from a hypoxanthine-aminopterin-thymidine-sensitive derivative of CEM, A3.01 (8). These virus infections were done at a multiplicity of infection of approximately 100, and 20 h later, the cells were harvested for immunofluorescent slide assays and/or flow cytometric analyses.

Flow cytometric analyses. Approximately 5×10^5 cells determined to be $\geq$95% viable were incubated with or without Abs or MAbs for 30 min at 4°C in phosphate-buffered saline (PBS) containing 1% fetal calf serum (FCS) and 10 mM NaN_3. The cells were washed in cold PBS containing 10 mM NaN_3 and then incubated as described above in a 1/50 dilution of FITC-conjugated goat anti-human IgG (Zymed) in PBS containing 1% FCS and 10 mM NaN_3. After another wash, the cells were fixed in 100 μl of 3.7% formaldehyde in PBS containing 0.5 mM Na_2EDTA and 3 mM NaN_3 and stored in the dark at 4°C. Before flow cytometry, the cells were diluted 10-fold into PBS containing 1% FCS and 10 mM NaN_3. The fluorescence intensity was measured in an EPICS flow cytometer (Coulter Corp.).

Neutralization assay. The fluorescent focus neutralization assay was used. This technique has been described in detail elsewhere (34, 38).

Anti-HIV-1 ADCC assay. The assay was carried out as described previously (42), with minor modifications. Briefly, a buffy coat from a seronegative donor was obtained from the N.Y. Blood Center the day prior to the ADCC assay. The PBMC were isolated by Ficoll-Hypaque centrifugation, washed in RPMI 1640 containing 2 mM L-glutamine, and resuspended in RPMI 1640 supplemented with heat-inactivated 10% FBS, 2 mM L-glutamine, penicillin (50 U/ml), and streptomycin (50 μg/ml) at a density of approximately 4×10^6 cells/ml. These cells were then incubated overnight at 37°C in an 80-cm^2 tissue culture flask prior to use as effectors in the ADCC assay. The following day, target cells were labeled with $Na_2{}^{51}CrO_4$ as described by Weinhold (42) for approximately 3 h in assay medium (RPMI 1640 supplemented as described above and containing 10 mM HEPES) prior to use in the assay. These target cells were either uninfected CEM.NKR cells, CEM.NKR cells to which $rgp120_{SF2}$ (Chiron) had been adsorbed as described previously (19), or CEM.NKR cells chronically infected with one of several strains of HIV-1. Duplicate or triplicate wells for each experimental point in the assay were prepared as follows. In each well, 10^4 washed, ^{51}Cr-labeled target cells were preincubated with various dilutions of sera or concentrations of purified MAbs in a total volume of 150 μl of assay medium for 30 min at 37°C. Then 10^6 PBMC prepared as described above were added as the source of effector cells in 50 μl of assay medium, bringing the total volume to 200 μl, and the plates were spun for 5 min at 1,000 rpm in an IEC Centra 8R centrifuge to pellet the cells prior to a 4-h incubation at 37°C. At the end of this time, the cells were spun again as described above, 100-μl aliquots of cell supernatants were collected, and ^{51}Cr release was assessed by gamma scintillography in an LKB 1275 Minigamma counter. Percent specific lysis was calculated as described by Weinhold (42) as $[cpm(T + E + Ab) - cpm(T + E)]/[cpm(total\ T) - cpm(T + E)] \times 100$. The total counts in the number of target (T) cells measured from experimental wells (total T) was determined by lysis of 5,000 target cells in 7× detergent (ICN, Costa Mesa, Calif.) and counting as described above. Each assay also included targets and Ab alone; the counts per minute released in this control was always less than that for targets and effectors alone (T + E), and so the latter was routinely used as the nonspecific background in calculating the specific lysis (see above).

Competition assays. These assays were performed by ELISA on $rgp160_{LAI}$, using biotinylated 42F and various competing unlabeled MAbs as described previously (28, 38, 41).

Immunoblot analysis. $rgp160_{LAI}$ was treated with dithiothreitol or with dithiothreitol and *N*-glycosidase F (Boehringer Mannheim, Indianapolis, Ind.), and immunoblot strips of untreated and treated $rgp160_{LAI}$ were prepared essentially as described previously (41). The strips were reacted with anti-Env MAbs followed by development with recombinant protein G conjugated to alkaline phosphatase (1/250 dilution; Zymed, South San Francisco, Calif.).

TABLE 1. Epitope mapping of 42F and 43F to the C5 region of HIV-1 gp120 by peptide ELISA

Viral strain and peptide	Amino acid numbering or sequence[a]	Reactivity (optical density at 450 nm)	
		42F	43F
HIV-1 HXB2	471 491 500 520		
E48	GGGDMRDNWRSELYKYKVVK	0.02	0.16
E49	SELYKYKVVKIEPLGVAPTK	1.39	1.51
E50	IEPLGVAPTKAKRRVVQREK	0.56	0.49
E51	AKRRVVQREKRAVGIGALFL	0.06	0.19

[a] Los Alamos amino acid numbering for the HXB2 strain is shown. The HXB2 sequence is identical to the LAI and clade B consensus sequences in this region.

Epitope mapping. Synthetic peptides were used to coat polyvinyl chloride ELISA plates at 50 ng per well in Na_2CO_3-$NaHCO_3$ buffer (pH 9.8) overnight at 4°C. Following a blocking step as described previously (38), purified MAbs at 20 μg/ml were then incubated with each peptide. Diluted sera from seropositive individuals and an HIV-1-infected chimpanzee were also separately incubated with the peptides on plates and served as positive controls to ensure that each peptide had attached to the plate in reactive form. A standard ELISA protocol was used to detect bound human and chimpanzee IgG Abs (38).

Measurement of apparent affinity constants. The MAb concentration required for half-maximal binding to either $rgp160_{LAI}$ or peptide E49 (see Table 1 for sequence) in ELISA was measured, and *K* was determined as described previously (28, 38).

RESULTS

Two MAbs, 42F and 43F, were isolated approximately 14 months apart from fresh blood samples of an asymptomatic, seropositive individual. The 42F and 43F monoclonal lines were isolated by the same procedure, i.e., as sublines derived from a polyclonal culture of EBV-transformed cells. The latter was unusual, since in our hands, such polyclonal cultures were typically negative for anti-Env MAb production; EBV-transformed cells usually had to be plated at near-limiting dilution from the outset in order to detect anti-Env-producing cultures, presumably because cells producing significant levels of MAb are often at a growth disadvantage. The 42F and 43F clones have been in continuous culture for over 2 years and are stable with respect to MAb production.

Initial characterizations of MAbs 42F and 43F included determination of their heavy- and light-chain isotypes, characterization of their specificity for gp120 versus gp41, and preliminary assessment of HIV-1 strain specificity. Both 42F and 43F were found to be of the γ1 and λ heavy- and light-chain isotypes, respectively. Furthermore, both MAbs reacted with $rgp120_{SF\text{-}2}$ (Chiron) under ELISA conditions similar to those previously used to detect their reactivity with $rgp160_{LAI}$ (data not shown), indicating their specificity for gp120 rather than gp41 sequences. By immunofluorescent slide assay, both MAbs were found to react well with HXB2-, MN-, and RF-infected H9 cells that had been attached to slides and fixed prior to incubation with MAbs (data not shown). These results suggested that the epitope(s) of 42F and 43F were relatively conserved.

MAb 42F was tested for neutralizing activity against the IIIB, MN, SF-2, and RF strains of HIV-1 in a fluorescent focus neutralization assay as detailed previously (38). No neutralizing activity was detected against any of these strains at ≤20 μg of MAb per ml.

In contrast, both the 42F and 43F MAbs stained live cells infected with diverse strains of HIV-1 and directed significant ADCC against those infected cells which they recognized. Figure 1 shows representative flow cytometry analyses of 42F staining of CEM.NKR cells chronically infected with the IIIB,

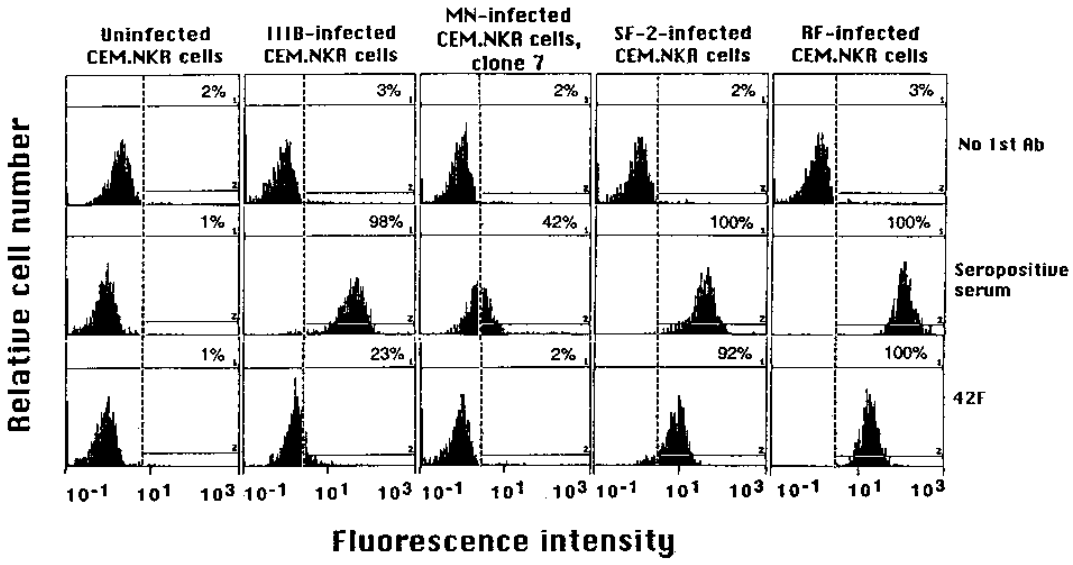

FIG. 1. Results of flow cytometry analyses on uninfected or HIV-1-infected CEM.NKR cells. The 42F MAb was used at 100 μg/ml, and the pooled seropositive serum was used at 1/250 dilution. In this experiment, gate 2 was set to exclude ≥95% of the background fluorescence observed with no primary Ab added (only FITC conjugate added) for each type of infected or uninfected cells. The percent of cells staining within gate 2 (above background) is indicated in each panel.

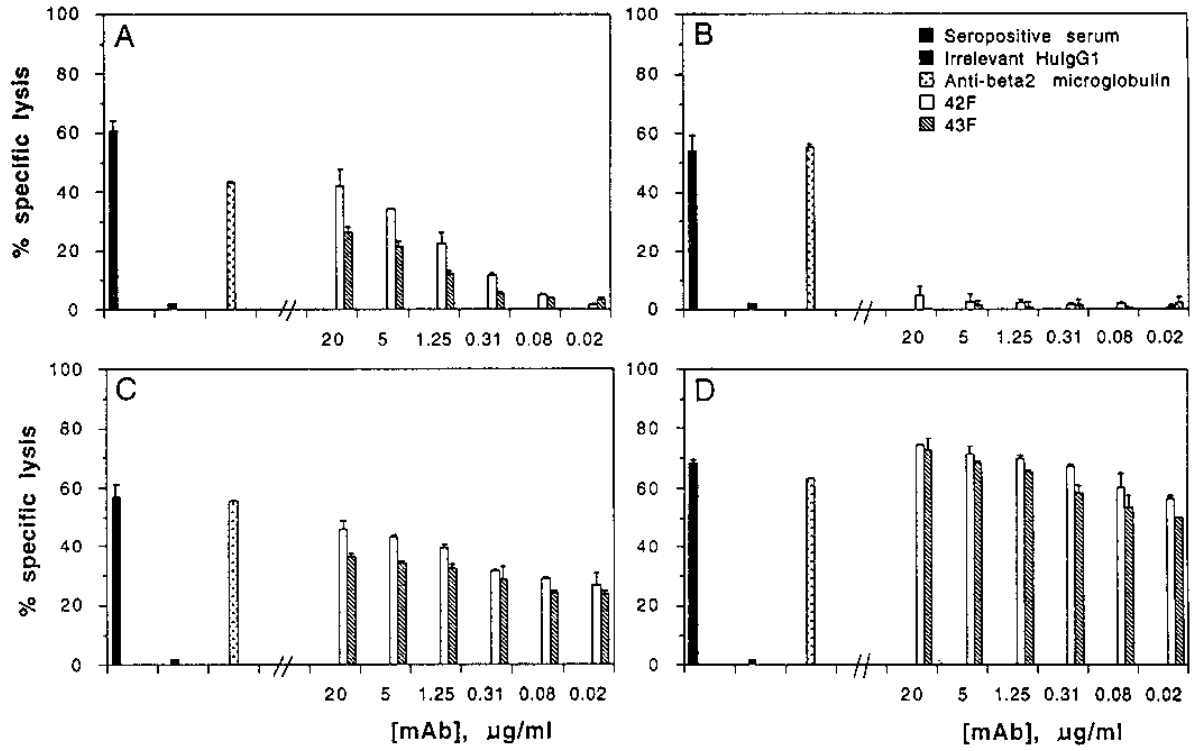

FIG. 2. Representative ADCC assay results. CEM.NKR cells were chronically infected with HIV-1 strain IIIB (A), clone 7 of MN (B), SF-2 (C), or RF (D). The left side of each panel shows results with the following controls: pooled seropositive serum at 10^{-3} dilution, irrelevant human IgG1 (HuIgG1) myeloma protein (ICN Immunobiological, Costa Mesa, Calif.) at 20 μg/ml, and rabbit antiserum against β2-microglobulin (Accurate Chemical, Westbury, N.Y.), a positive control against both uninfected and infected cells, at 33 μg/ml. The results with the latter against uninfected cells was comparable to that seen against the four infected cell lines shown, while no specific lysis above background was seen against uninfected cells for any of the other Abs or MAbs in this experiment (data not shown). The right side of each panel shows results with various concentrations of MAbs 42F and 43F. The error bars represent standard deviations of duplicate points.

MN (clone 7), SF-2, and RF strains; similar results were obtained with 43F. While 42F and 43F stained an uncloned population of MN-infected CEM.NKR cells and directed specific lysis (approximately 20% at 20 μg of MAb per ml) against them in the ADCC assay (data not shown), they did not stain a clone of such cells, designated clone 7 (Fig. 1), which was selected for these and other experiments based on its superior staining by pooled seropositive serum. Both 42F and 43F significantly stained the IIIB-, SF-2, and RF-infected cells (Fig. 1 and data not shown).

Figure 2 shows representative results of ADCC assays using 42F and 43F to direct lysis against these chronically infected cell lines. Results obtained against a given chronically infected cell line with the two MAbs were essentially the same. Furthermore, the specific lysis directed by these MAbs against each chronically infected cell line correlated in magnitude with the levels of staining of each of these cell lines by flow cytometry (compare results with 42F in Fig. 1 and 2). It is especially noteworthy that each of these MAbs directs significant lysis against the SF-2- and RF-infected targets (approximately 20 and 50% specific lysis, respectively) at as little as 20 ng of MAb per ml. We are unaware of other reports of substantial ADCC activity directed against HIV-infected cells by such low concentrations of MAb.

Because of the concern that ADCC-directing Abs against Env may contribute to pathogenesis during HIV-1 infection via innocent bystander killing (19), we measured the ability of 42F and other anti-Env Abs and MAbs to direct ADCC in vitro against uninfected $CD4^+$ cells to which saturating amounts of $rgp120_{SF2}$ had been adsorbed in comparison to their ability to direct ADCC against HIV-1-infected cells. Figure 3A to C show results obtained with the highest serum and Ab or MAb concentrations used in our standard ADCC assays (Fig. 2), while Fig. 3D to F show results obtained with 10-fold or higher concentrations of serum and Abs or MAbs. To demonstrate that the CEM.NKR cells with gp120 adsorbed were valid targets for ADCC, Fig. 3E shows that these cells can be killed when high concentrations of serum or Abs or MAbs are used in this assay. It should also be noted that nonspecific ADCC directed against untreated CEM.NKR cells by serum and HIV immune globulin (HIVIG) as well as that directed against both types of Env-bearing targets by irrelevant human IgG1 are elevated at the higher Ab or MAb concentrations used for Fig. 3D to F. Figures 3B and C show that at 20 μg of MAb/ml, 42F as well as an anti-CD4-binding-site MAb, 1125H (38), and an anti-V3 MAb, 4117C (39), mediate substantial ADCC against HIV-1-infected cells but mediate very little ADCC against cells with gp120 adsorbed. Lower MAb concentrations failed to direct any ADCC against the innocent bystanders (data not shown). In contrast, as shown in Fig. 2C and D, 42F directs substantial ADCC against cells infected with the SF-2 and RF strains down to 1,000-fold-lower concentrations (i.e., 20 ng/ml) than those used for Fig. 3A to C. Seropositive serum (at 10^{-3} dilution) also directed substantially higher ADCC against HIV-1-infected cells than against uninfected cells with gp120 adsorbed, while HIVIG at 20 μg/ml exhibited similar activities through directing a higher level of innocent bystander killing (Fig. 3B, C). In conclusion, anti-Env Abs or MAbs direct ADCC against HIV-1-infected cells much more efficiently than against $CD4^+$ cells coated with gp120. Because of the require-

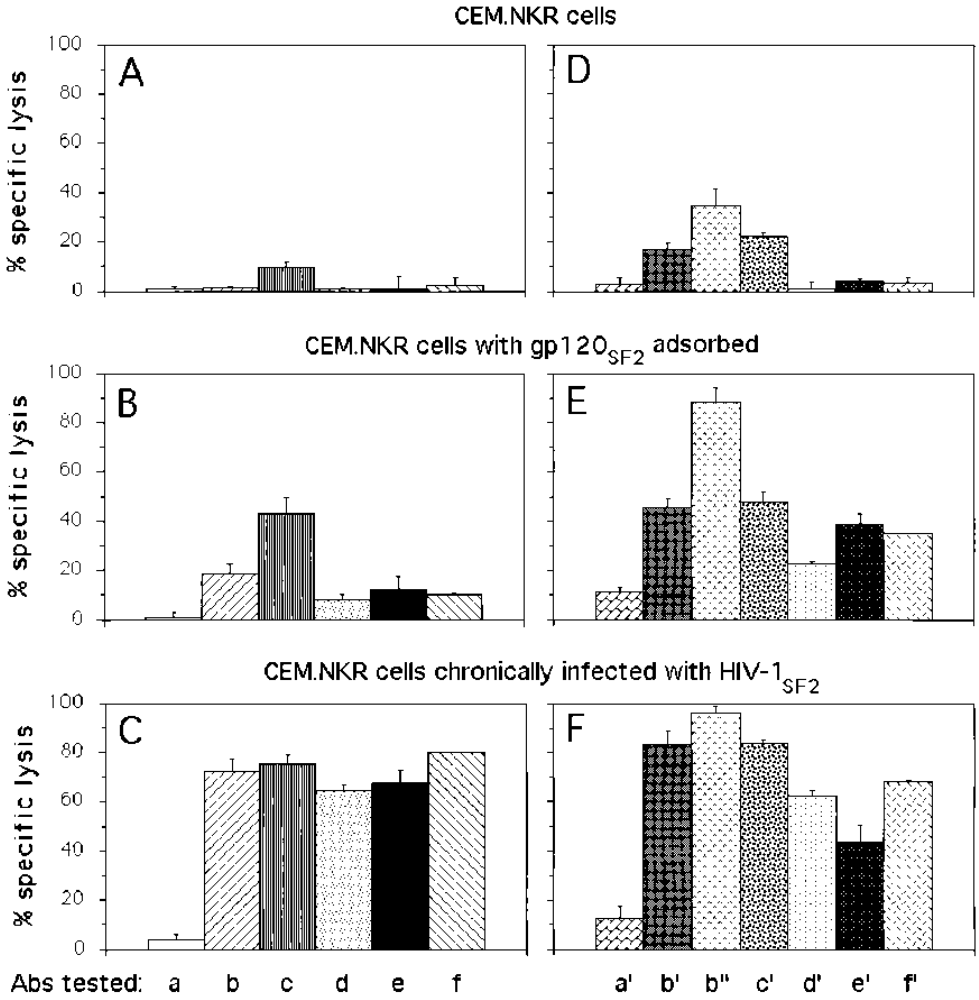

FIG. 3. ADCC assays against uninfected cells (A and D), innocent bystanders, i.e., uninfected cells with gp120 adsorbed (B and E), and HIV-1-infected cells (C and F) as targets. Abs tested in panels A to C: a, irrelevant human IgG1, 20 μg/ml; b, pooled seropositive serum, 10^{-3}; c, HIVIG, 20 μg/ml; d, 1125H, 20 μg/ml; e, 4117C, 20 μg/ml; f, 42F, 20 μg/ml. Abs tested in panels D to F: a′, irrelevant human IgG1, 200 μg/ml; b′, pooled seropositive serum, 10^{-2}; b″, pooled seropositive serum, 1/30, c′, HIVIG, 200 μg/ml; d′, 1125H, 200 μg/ml; e′, 4117C, 200 μg/ml; f′, 42F, 200 μg/ml. The error bars represent standard deviations of duplicate points.

ment for multivalent presentation of the Fc regions of antigen-bound Ab molecules to trigger killing by natural killer effectors, and the fact that amounts of free gp120 are unlikely to be sufficient to saturate cell surface CD4 molecules in vivo, this difference in efficiency of ADCC directed against bone fide HIV-1-infected cells versus innocent bystanders is likely to be even greater in vivo than in vitro.

Figure 4 shows binding of 43F to $CD4^-$ T cells infected with recombinant vaccinia viruses expressing lab-adapted and primary isolate Envs from clades B and E. The $CD4^-$ A2.01 cells were chosen for these initial experiments because we reasoned that such cells might not complex Env molecules intracellularly with CD4, thus preventing their optimal surface expression. Despite this, it should be noted that the primary isolate Envs from vCB51, vCB52, and vCB53 are apparently not expressed as well as the BH8 (IIIB-like lab-adapted strain) Env from vPE16, as assessed by lower binding (albeit above that to wild-type vaccinia virus-infected cells) of pooled seropositive serum, broadly reactive anti-CD4-binding-site MAb IgG1b12 (2), and 43F to cells expressing these Envs. Furthermore, the substantial binding of anti-vaccinia virus serum Abs to cells expressing each of these four recombinant Envs (79 to 83% of cells fluorescent above background levels) indicates that comparable levels of vaccinia virus infection were achieved with each recombinant virus. The lower expression of Envs from vCB51-53 is not surprising, since Earl et al. (6) optimized the vPE16 virus for Env expression by removing cryptic poxvirus termination signals, while this was not done for these primary isolate Env-expressing viruses. Nevertheless, 43F was demonstrated to bind specifically at levels comparable to or greater than those of pooled seropositive serum or IgG1b12 to cells expressing these primary isolate Envs (Fig. 4). This binding is somewhat greater against the two clade E Envs than against the single primary isolate clade B Env tested here; whether this represents a true preference of 42F and 43F for clade E rather than clade B or simply a difference in levels of Env expression among these recombinant vaccinia viruses remains to be determined. The reactivity of 42F and 43F with these primary isolate Envs was confirmed by immunofluorescent slide assay against fixed whole cells expressing these recombinant vaccinia viruses.

To our knowledge, the epitope clusters previously described to be targets for ADCC in HIV-1 gp120 are those overlapping the CD4-binding site and V3 loop (reviewed in reference 40).

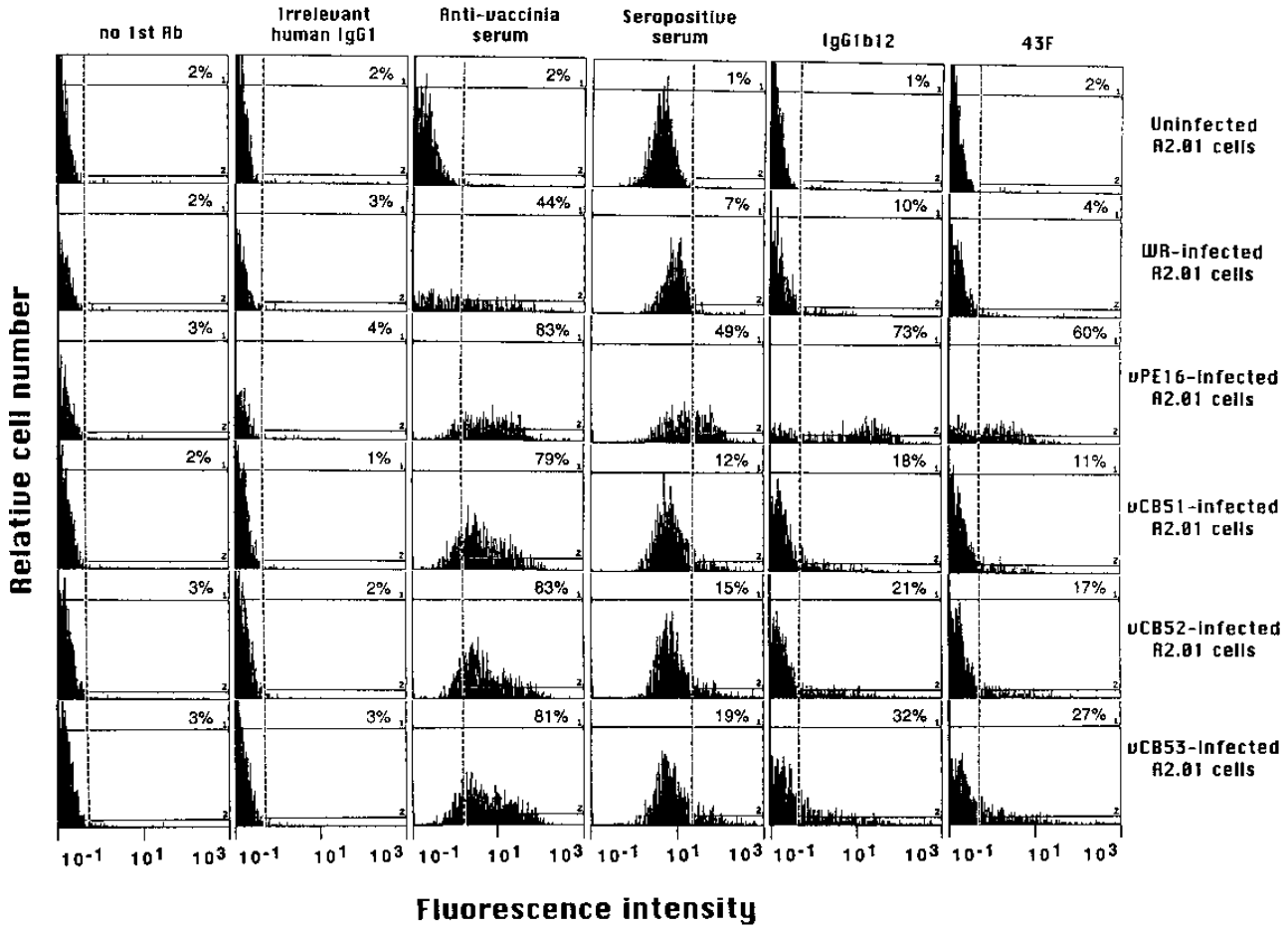

FIG. 4. Results of flow cytometry analyses on A2.01 cells that were uninfected or infected with wild-type vaccinia virus (WR) or recombinant vaccinia viruses expressing HIV-1 Envs. Abs used were irrelevant human IgG1 (myeloma protein; ICN Immunobiological) at 50 μg/ml, serum from a normal individual recently immunized against vaccinia virus (anti-vaccinia virus serum) at 10^{-2} dilution, pooled seropositive serum at 1/200 dilution, and MAbs IgG1b12 (2) and 43F, each at 50 μg/ml. The recombinant vaccinia viruses used were vPE16, expressing Env of a lab-adapted strain, BH8; vCB51, expressing a clade B primary isolate Env; and vCB52 and vCB53, expressing two different clade E primary isolate Envs (see Materials and Methods for details). In this experiment, gate 2 was set to exclude ≥98% of the background fluorescence observed against uninfected A2.01 cells with each Ab or MAb tested. The percent of cells staining within gate 2 (above background) is shown in each panel.

Using competition assays and direct epitope mapping of 42F and 43F, we have shown that these MAbs are not directed against either of these regions. Figure 5A shows representative results of a competition assay demonstrating that biotinylated 42F was not inhibited in its binding to $rgp160_{LAI}$ by MAbs against epitopes overlapping the CD4-binding site, V3 loop, or V2 domain. As a positive control, unlabeled 42F inhibited the binding of biotinylated 42F in a concentration-dependent manner in this assay. The inhibition curves of biotinylated 42F by unlabeled MAbs 42F and 43F were indistinguishable (Fig. 5B), indicating that their epitopes are identical or very similar.

The 42F/43F epitope was further characterized by determining the reactivity of 42F and 43F with reduced or reduced and deglycosylated gp160 in immunoblot analysis. Figure 6 shows that binding of 42F was retained after reduction of disulfide bonds and after reduction and deglycosylation of $rgp160_{LAI}$ with *N*-glycosidase F. The successful reduction of gp160 disulfide bonds in this experiment was documented by loss of 1125H reactivity, since the epitope of 1125H is dependent on disulfide bonds (38). The deglycosylation of gp160 was demonstrated by a marked decrease in its molecular weight as well as loss of C108G reactivity; the epitope of C108G is glycan dependent (41). Identical immunoblot results were obtained with 43F (data not shown).

The apparent lack of dependence of the 42F/43F epitope on disulfide bonds and N-linked glycans suggested that it could be mapped by using linear peptides. Since this epitope lies within gp120 and is conserved, one of the constant domains of gp120 was a good candidate for its site. Because of the lack of competition between a MAb overlapping the CD4-binding site (Fig. 5A) or soluble CD4 (data not shown) and 42F/43F, peptides covering the C2, C3, and C4 domains of gp120 were not included in the initial mapping experiments. Epitope mapping was performed by ELISA on 42F and 43F, using 20-mer synthetic peptides with 10-residue overlaps that covered the entire C1 and C5 domains of $gp120_{HXB2}$. Representative results of these experiments are shown in Table 1. Both 42F and 43F reacted specifically with peptides E49 and E50 that overlap in residues 491 to 500 from the C5 domain of $gp120_{HXB2}$, while no significant reactivity was seen with other peptides from the C5 and C1 domains (Table 1 and data not shown). Thus, the epitopes of 42F and 43F are indistinguishable and map to the 10-amino-acid overlap between peptides E49 and E50, i.e., residues 491 to 500 (underlined in Table 1).

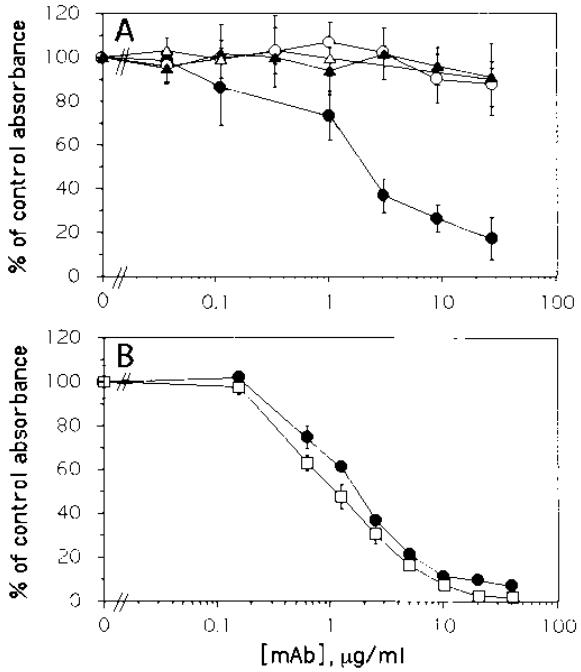

FIG. 5. Results of competition ELISAs assessing the effects of MAbs directed against various gp120 epitopes on binding of biotinylated 42F to $rgp160_{LAI}$. Error bars represent the standard deviations of duplicate points. Different preparations of biotinylated 42F were used in the two experiments shown in panels A and B; both were used at approximately the concentration required for half-maximal binding to $rgp160_{LAI}$. Symbols: ○, anti-CD4-binding-site mAb, 1125H (38); ▲, anti-V2 MAb, C108G (41); △, anti-V3 MAb, 0.5β (23); ●, 42F; □, 43F.

Measurement of the apparent affinity of 42F and 43F for $rgp160_{LAI}$ versus peptide E49 by a previously described method (28, 38) showed that both MAbs had approximately 10-fold-higher affinity (i.e., 10^9 liters/mol) for the recombinant protein than for the 20-mer peptide (i.e., 10^8 liters/mol). Similar results were found by Gorny et al. in their studies of a panel of anti-V3 human MAbs (9). Since the HXB2 amino acid sequence in residues 481 to 500 of peptide E49 is identical to that of the LAI strain in this region, the difference in affinity for $rgp160_{LAI}$ versus peptide E49 must be due to conformational elements conferred by the folding of the whole Env molecule as opposed to any differences in the epitope sequence itself.

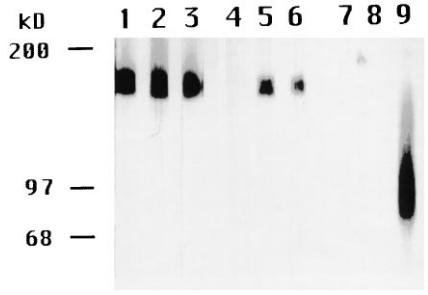

FIG. 6. Immunoblot analysis of $rgp160_{LAI}$ after either no treatment (lanes 1 to 3), reduction of disulfide bonds with dithiothreitol (lanes 4 to 6), or reduction of disulfide bonds with dithiothreitol and treatment with *N*-glycosidase F (lanes 7 to 9) as described in Materials and Methods. The blot was reacted with MAb 1125H (lanes 1, 4, and 7), C108G (lanes 2, 5, and 8), or 42F (lanes 3, 6, and 9). The molecular weight markers were prestained standards from Bethesda Research Laboratories.

DISCUSSION

Two MAbs, 42F and 43F, isolated some 14 months apart from a single long-term survivor of HIV-1 infection, were found to be indistinguishable in terms of their isotypes, specificities, affinities, and biological activities. In studies to be presented elsewhere, the variable regions of both light and heavy chains of 42F and 43F were also found to be identical; nevertheless, the cell lines producing these MAbs exhibited significantly different and stable characteristics such as rates of cellular growth and MAb production (37a). These observations, coupled with the fact that both of these monoclonal lines emerged from polyclonal cultures of EBV-transformed cells, suggest that the clonotype, i.e., antigen-binding specificity, represented by 42F and 43F is a predominant and persistent one expressed in the circulating B-cell pool in the individual from which these MAbs were derived. This hypothesis is consistent with preliminary evidence indicating a significantly higher concentration of serum antibodies in this individual that are capable of inhibiting binding of biotinylated 42F to its epitope than in a pool of serum or pooled purified antibodies from two separate groups of seropositive individuals (37a). Further studies are under way to elucidate the basis and significance of these findings.

Both 42F and 43F exhibited substantial ADCC activity against cells infected with four divergent lab-adapted strains of HIV-1 (data against uncloned MN-infected cells were not shown). The ability of MAbs 42F and 43F, as well as that of MAbs against two other gp120 epitopes, to direct ADCC against uninfected $CD4^+$ cells to which $rgp120_{SF2}$ had been adsorbed (i.e., innocent bystanders) was demonstrated to be less efficient by at least an order of magnitude than their ability to direct ADCC against HIV-1-infected cells. We would expect innocent bystander killing via ADCC to be substantially less efficient in vivo than we have found it to be in vitro, since free gp120 in vivo is unlikely to reach concentrations able to saturate CD4 molecules on the surface of $CD4^+$ cells, and because ADCC is highly dependent on multivalent presentation of Ab Fc regions bound to multivalently presented epitopes on target cells. Preliminary evidence indicates that serum concentrations of 42F/43F-like Abs in the individual from which 42F and 43F were isolated are in the range capable of directing high levels of ADCC, but not innocent bystander killing, as estimated in our in vitro assays (37a).

Specific binding of 42F and 43F to primary isolate Envs of clades B and E expressed on cell surfaces was also demonstrated (this report), and preliminary studies show that this binding correlates with ADCC activity of 42F and 43F against such cells (1). In agreement with findings of other investigators (26, 30), we have shown that the ADCC activity observed in our short-term assay using PBMC as effectors is essentially entirely attributable to natural killer cells (1). Because of these findings and the asymptomatic status of the long-term survivor from which 42F and 43F were isolated, it is tempting to speculate that this clonotype may be contributing to the immune defense and health of this individual. However, further experiments will be required to confirm or negate this hypothesis.

Prior to the current study, other investigators have characterized human polyclonal or monoclonal Abs against the C5 domain of gp120. By analysis of serum Abs, Palker et al. (27) identified a region at the C terminus of C5 (residues 497 to 511; Los Alamos National Laboratory numbering for the HXB2 strain), which overlaps the last four amino acids of the 42F/43F epitope mapped in this report, as containing an immunodominant epitope in seropositive individuals. Abs against this (these) epitope(s) had no neutralizing activity, and ADCC

activity of these Abs was not reported. Human MAbs 450-D (13) and N70-2.3a (17, 31) have been mapped to C5 regions (residues 482 to 504 and 488 to 502, respectively; numbering as above) that encompass and extend both N and C terminal to the 42F/43F epitope. While the N70-2.3a MAb was found to enhance HIV-1 infection of U937 cells (36), no neutralizing or ADCC activity of either N70-2.3a or 450-D has been reported. Despite its lack of intrinsic neutralizing activity, the 450-D MAb was reported to synergistically neutralize HIV-1 when combined with anti-CD4-binding-site MAbs (16). Further studies of 42F and 43F are under way to determine whether they exhibit any enhancing or synergistically neutralizing activity against primary isolates. At this point, characterization of MAbs 42F and 43F has resulted in identification of a new, conserved ADCC epitope in the C5 domain of HIV-1.

Because of the relative conservation of this new ADCC epitope defined by 42F and 43F, and the fact that it appears to have been significantly immunogenic in the individual from which these MAbs were derived, it may prove to be a useful component of HIV vaccines. Furthermore, these MAbs may be used as tools to probe the potential importance of ADCC as an antiviral activity in HIV-1 infection. These MAbs and/or their epitope may be used to functionally and/or physically fractionate serum Abs from seropositive individuals to determine the frequency of Abs against this epitope and their relative contribution to the ADCC response of those individuals. In addition, these MAbs could be used alone or in combination with neutralizing MAbs or their fragments in passive immunization-viral challenge experiments in an animal model(s) to sort out the antiviral effects of ADCC versus neutralization. This is important to do, because most, if not all, neutralizing MAbs characterized to date have ADCC activity as well, and so using them alone in passive immunotherapy experiments does not allow discrimination between the contributions of neutralization and ADCC to any protection observed. Since MAbs 42F and 43F apparently possess no neutralizing activity, the contribution of the latter activity to any protection observed with these MAbs might be eliminated. Theoretically, 42F and 43F may have complement-fixing and virolysis activity as well; this could be distinguished from their ADCC activity by the requirement of the latter for functional effector cells.

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