Adaptation to Persistent Growth in the H9 Cell Line Renders a Primary Isolate of Human Immunodeficiency Virus Type 1 Sensitive to Neutralization by Vaccine Sera

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Seven diverse primary isolates of human immunodeficiency virus type 1 (HIV-1) were examined and found to be refractory to neutralization by antisera to recombinant gp120 (rgp120) protein from HIV-1 MN. This stands in marked contrast to the sensitivity exhibited by certain laboratory-adapted viruses. To understand the difference between primary and laboratory-adapted viruses, we adapted the primary virus ACH 168.10 to growth in the FDA/H9 cell line. ACH 168.10 was chosen because the V3 region of gp120 closely matches that of MN. After 4 weeks, infection became evident. The virus (168A) replicated in FDA/H9 cells with extensive cytopathic effect but was unchanged in sensitivity to antibody-mediated neutralization. Thus, growth in cell lines is not sufficient to render primary virus sensitive to neutralization. The 168A virus was, however, partially sensitive to CD4 immunoadhesin (CD4-Ig). Adaptation was continued to produce a persistently infected FDA/H9 culture that displayed minimal cytopathic effect. The virus (168C) was now sensitive to neutralization by MN rgp120 vaccine sera and by MN-specific monoclonal antibodies and showed increased sensitivity to HIVIG and CD4-Ig. 168C encoded three amino acid changes in gp120, including one within the V3 loop (I-166→R, I-282→N, G-318→R). MN-specific monoclonal antibodies bound equally to the surface of cells infected with either neutralization-resistant or -sensitive virus. The coincidence of changes in neutralization sensitivity with changes in cell tropism and cytopathic effect suggests a common underlying mechanism(s) acting through the whole of the envelope protein complex.

Primary isolates of human immunodeficiency virus type 1 (HIV-1) are obtained by limited cultivation of patient peripheral blood mononuclear cells (PBMCs) or plasma with uninfected PBMCs. These viruses differ in a number of ways from the commonly used laboratory-adapted viruses such as IIIb/LAI and MN, which have been passaged over time in human T-lymphoid cell lines (3, 18, 42). First, most primary isolates do not readily grow in cell lines. Among primary isolates that induce syncytium formation in PBMC culture (SI isolates), most will replicate in MT2 cells, but few will replicate in less permissive cell lines such as CEM or H9 (23). Non-syncytium-inducing (NSI) primary isolates replicate only in primary T cells.

Another key difference between primary and laboratory-adapted viruses relates to sensitivity to in vitro neutralization by soluble forms of the viral receptor protein CD4 (sCD4) (12). The relative resistance of primary viruses to sCD4-mediated neutralization may explain the failure of sCD4 therapy to exert an antiviral effect in HIV-1-infected persons (21, 27, 53). Although the molecular basis for the resistance of primary virus isolates to sCD4 remains uncertain (22), it is useful to point out that primary viruses can be rendered sensitive to sCD4-mediated neutralization by adaptation to growth in T-cell lines (12, 31, 63).

Recently, another difference between primary and laboratory-adapted viruses has been noted with regard to antibody-mediated virus neutralization. Candidate vaccines, comprising recombinant DNA-derived forms of the viral surface protein gp120 (rgp120), have been shown to elicit antibodies in immunized persons that are capable of neutralizing the infectivity of homologous and some heterologous laboratory-adapted viruses (1, 4). These antisera fail, however, to neutralize primary virus isolates in PBMC culture (11). Similarly, primary virus isolates appear less sensitive to neutralization by antisera from HIV-1-infected persons (28).

In this report, we confirm the observation that primary isolates of HIV-1 are resistant to neutralization by vaccine sera and we explore the basis for this resistance. We report that adaptation of a primary virus to persistent growth in a T-cell line renders the virus sensitive to neutralization by MN rgp120 vaccine sera, by monoclonal antibodies (MAbs), and by HIVIG.

MATERIALS AND METHODS

Cells and viruses. HIV-1 MN (18) was obtained as a persistently infected H9 cell culture from R. C. Gallo (National Cancer Institute). ACH 168.10 was isolated from an individual in the Amsterdam cohort after progression to AIDS (13, 59). 301593 was isolated from an asymptomatic woman in Haiti (N. Halsey and R. Boulos, Johns Hopkins University School of Medicine) and was provided by J. Bradac (National Institute of Allergy and Infectious Diseases). Viruses VS and RA were obtained during primary HIV infection (V and R, respectively, in reference 67) and were provided by D. Ho (Aaron Diamond AIDS Research Center). RVL04 and RVL05 were isolated from PBMCs obtained from two subjects participating in a phase I therapeutic study of MN rgp120 vaccine in persons with CD4 cell counts of >500/mm³ (1). Viruses were isolated by coculture according to the AIDS Clinical Trials Group consensus protocol (19). JR-CSF virus was derived by Lipofectin (Gibco BRL)-mediated transfection of PBMCs with a molecularly cloned provirus provided by I. S. Y. Chen (University of California at Los Angeles). The molecular clone was from virus isolated from the cerebrospinal fluid of a person with AIDS (24).

PBMCs were obtained from buffy coats of HIV-1-seronegative donors (Irwin Memorial Blood Bank) and were isolated by centrifugation in Ficoll-Paque

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(Pharmacia). PBMCs were cultured according to the AIDS Clinical Trials Group consensus protocol (19).

In brief, PBMCs were activated with phytohemagglutinin-P (3 μg/ml [Sigma]) in growth medium containing interleukin 2 (RPMI 1640 medium with glutamine, 20% heat-inactivated fetal bovine serum [Hyclone], gentamicin (50 μg/ml [Sigma]), and 4% interleukin 2 [Boehringer Mannheim]). After 24 h, cells were washed free of phytohemagglutinin-P and maintained at 2×10^6 cells per ml in growth medium containing interleukin 2. Cultured PBMCs were used in virus neutralization assays within 1 week of preparation.

MT4 (30) and FDA/H9 human T-lymphoid cells were grown in RPMI 1640 medium with glutamine and 10% heat-inactivated fetal bovine serum (Hyclone). FDA/H9 cells are an early clone of H9 (42) obtained by the Bureau of Biologics, Food and Drug Administration, and were provided by B. Potts (Repligen Corporation).

Virus neutralization. We developed an assay to assess antibody-mediated neutralization of primary isolates in PBMC culture. The assay is contained entirely in flat-bottom, 96-well microculture dishes (Costar Corporation, Cambridge, Mass.) and requires 5 days. Initially, primary virus was titrated within the assay format to determine the amount that reproducibly yields significant but submaximal p24 antigen production. In neutralization assays, this virus input was incubated, in quadruplicate, with serial dilutions of test antiserum (37°C for 1 h) and PBMCs were then added (2.5×10^5 cells per 250 μl per well). The culture was continued for 3 days, at which time cells were washed to remove virus and antiserum. Cultures were washed three times by centrifugation in microculture plates (300 × g for 7 min) and resuspended in growth medium containing interleukin 2. Extensive washing is critical to ensure that antibody directed against HIV-1 p24 will not interfere with the ultimate detection of p24 antigen. The washed cultures were continued for an additional day. Accumulated p24 antigen was subsequently detected by enzyme-linked immunosorbent assay (ELISA [Coulter Corporation, Hialeah, Fla.]). Maximal p24 antigen accumulation was determined from cultures incubated in the absence of antiserum (virus control), and test cultures were subsequently diluted appropriately for analysis of neutralization.

Recently, we found that the assay can also be performed in MultiScreen microculture plates (0.65-μm-pore-size Durapore filter [Millipore Corporation, Bedford, Mass.]). Cultures can be washed by filtration, avoiding centrifugation.

Virus neutralization was also determined with MT4 cells, a human T-cell leukemia virus type 1-transformed cell line that is highly susceptible to HIV-1 infection and cytopathic effect. In some assays, MT4 cells were used in the PBMC assay format (2.5×10^4 cells per well). In other virus neutralization assays, including those using laboratory-adapted viruses, the cytopathic effect of HIV-1 infection was quantitated after 7 days by MTT dye conversion (49) and 50% inhibitory concentrations of antibody were determined spectrophotometrically at 570 nm.

Antibodies. Murine MAbs 1034 (2.8 mg/ml) and 1024 (3.6 mg/ml) were obtained from P. Berman (Genentech) (37). These MAbs were raised against MN rgp120 (5) and recognize epitopes within the V3 and C4 regions, respectively. Murine MAb 50.1 (1 mg/ml) was raised against a synthetic peptide derived from the V3 loop of HIV-1 MN and was purchased from Repligen Corporation (Cambridge, Mass.) (66). The epitope recognized by this MAb has been defined, by peptide binding assays (46) and crystallographic structure determination (48), as KRIxIGP. The human MAb 2F5 (0.5 mg/ml) recognizes a conserved epitope on gp41 (ELDKWA) (36) and was purchased from Viral Testing Systems Corporation (Houston, Tex.). HIVIG (50 mg/ml), a purified immunoglobulin (Ig) preparation from pooled plasmas of infected individuals (44, 45), was kindly provided by A. Prince (New York Blood Center). The recombinant immunoadhesin molecule CD4-Ig (4.5 mg/ml) was provided by S. Chamow (Genentech) (57). Pooled sera from guinea pigs immunized with MN rgp120 and QS-21 adjuvant (39) were provided by P. Berman and M. Powell (Genentech). Sera from uninfected volunteers participating in phase I clinical studies of MN and IIIb rgp120 vaccines (4, 55) were kindly provided by M. Chernow (Genentech). All antisera were heat inactivated (56°C for 30 min) prior to use in virus neutralization assays.

Antibody binding studies. Indirect immunofluorescence was used to measure the spread of HIV-1 infection in the FDA/H9 cell culture. Cells were fixed with a 50:50 mixture of methanol-acetone (−20°C) and were subsequently stained with serum from an HIV-1-infected individual and fluoresceinated secondary antibody (goat anti-human IgG [Organon Teknika]).

gp120 protein was quantitated by ELISA (32). Assay reagents and MN rgp120 quantitation standard were kindly provided by A. Gray and P. Berman (Genentech). In brief, cell culture supernatant containing virus was made to 0.5% Nonidet P-40 and gp120 was immobilized in microtiter wells with sheep polyclonal antibody specific for the C terminus of gp120 (D7324 [International Enzymes, Fallbrook, Calif.]). Bound gp120 was detected with a rabbit serum raised against MN rgp120 (5), horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit IgG [Organon Teknika]), and o-phenylenediamine chromogen. This ELISA was also used to titrate MAb binding to gp120. In this case, microtiter wells containing a constant amount of captured gp120 were incubated with serial dilutions of the test antibody and developed with horseradish peroxidase-conjugated secondary antibody (goat anti-mouse IgG [Organon Teknika]).

For flow cytometric analysis, PBMCs were infected with a 1:10 dilution of 168 virus stock. After 4 days of infection, a fivefold excess of uninfected PBMCs was added to allow synchronous infection and virus expression. These cultures were stained 2 days later.

Antibody staining for flow cytometry was performed in microculture plates containing 1.5×10^6 cells per well in physiologic saline containing 1% fetal bovine serum and 0.01% NaN$_3$. Primary antibodies were MAbs 1034, 50.1, and 1024 (each used at 1:50), HIVIG (at 1:50), and normal mouse gamma globulin (at 70 μg/ml [Jackson Immuno Research]). Incubations were for 1 h at 37°C. Cells were washed twice by centrifugation, and the appropriate secondary (R-phycoerythrin [PE]-conjugated) antibody was applied [1:500 dilution of goat anti-mouse IgG (H&L) F(ab′)$_2$ fragment, or goat anti-human IgG F$_c$ F(ab′)$_2$ fragment; Jackson Immuno Research]. After incubation, cells were washed and stained for CD4 antigen with fluorescein isothiocyanate (FITC)-conjugated OKT4 MAb (Ortho Diagnostics). Cells were then washed in physiologic saline containing 0.01% NaN$_3$, and each well was resuspended in 0.5 ml of ice-cold physiologic saline containing 1% formalin. After overnight fixation at 4°C, the cells were used for flow cytometry.

Flow cytometric analyses were performed with an EPICS Elite flow cytometer (Coulter Corporation) and two lasers: FITC was excited with the 488-nm line of an argon ion laser tuned to 488 nm, and PE was excited with a mixed-gas argon-krypton laser tuned to 531 nm. Dual-parameter immunofluorescence and 90°C light-scatter analyses were performed with three photomultipliers and 488-nm dichroic and bandpass filters for 90° light scatter, 550-nm longpass and 530-nm shortpass filters for FITC, and 550-nm longpass and 575-nm bandpass filters for PE. Antibody staining of the CD4$^-$ lymphocyte population served as an internal background control for specific staining of HIV-1-infected CD4$^+$ lymphocytes.

Nucleic acid techniques. Provirus DNA sequences were obtained by PCR amplification of total DNA from infected PBMCs. Cellular DNA was obtained by using the QIAamp DNA purification kit (QIAGEN, Inc., Chatsworth, Calif.). The oligonucleotide primers used in PCR are listed as follows, with the nucleotide position of the 3′ terminus of the primer, relative to the genome of HIV-1 MN (18), indicated: JM11a (5′CTCGAGCTCCTGAAGACAGTCAGACTCATCAAG3′; 6043) and JM12 (5′CTCCTAAGAACCCAAGGAACAGAGCTCC3′; 7787) (25); 750isR (5′GGTCTAGAAGCTTTACAATTTCTGGRTCYCCTCCT3′; 7342) and 750isF (5′GGGAATTCGGATCCGGATCAAAGYCTAAARCCATGTGT3′; 6594 [suggested by A. Gray, Genentech]); and JM12 rev (5′GGAGCTCTGTTCCTTGGGTTCTTAGGAG3′; 7814), nef84-3′ (5′GTCTCGAGATACTGCTCCCACCC3′; 8902), and C4rev5′ (5′AGGGCTRCTATTAACAAGAGAT3′; 7609).

The following primer pairs were used to amplify overlapping portions of the envelope gene: JM11a-750isR (1.3 kb), 750isF-JM12 (1.2 kb), JM12R-nef84-3′ (1.1 kb), and C4rev5′-nef84-3′ (1.3 kb). Amplification utilized Taq polymerase (Perkin-Elmer) and 30 cycles of PCR (94°C for 0.5 min, 60°C for 0.5 min, and 72°C for 2 min) (51). The PCR product was purified by size-exclusion chromatography (Quick Spin Linker 6; Boehringer Mannheim), and DNA sequences were obtained with the AmpliTaq Cycle Sequencing kit (Perkin-Elmer). Approximately 22 oligonucleotide primers were used, as appropriate, to obtain unambiguous DNA sequence from the entire envelope gene. Primers were labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. Cycle sequencing was performed as described in the kit, except that 20 cycles (95°C for 1 min, 65°C for 3 min, 75°C for 3 min) were used. Sequencing products were resolved with 6% polyacrylamide–urea gels (Gibco BRL), and sequences were compiled and analyzed with MacDNASIS software (Hitachi, San Bruno, Calif.).

Nucleotide sequence accession number. The DNA sequences of the envelope genes of 168A, 168C, and 168P have been submitted to GenBank (accession numbers U15030 to U15032, respectively).

RESULTS

Primary virus neutralization. Several primary isolates of HIV-1 were tested for sensitivity to neutralization by antisera elicited by immunization with rgp120 derived from the prototypic laboratory-adapted strain HIV-1 MN. Table 1 summarizes the complete absence of detectable neutralization with antisera from either MN rgp120-immunized guinea pigs or human volunteers. The primary viruses tested include SI viruses and NSI viruses, newly isolated viruses and those that had been passed minimally in PBMCs beyond the initial isolation, viruses isolated during acute HIV infection and through progression to AIDS, and virus derived from a homogeneous molecularly cloned provirus.

These negative neutralization results with diverse primary viruses are in contrast to the potent neutralization obtained with the laboratory-adapted virus MN and the MT4 human

TABLE 1. Neutralization of primary isolates of HIV-1

Virus	Virus neutralization (IC$_{50}$)[a]		Comments[b]	Source
	HIVIG	MN rgp120		
JR-CSF	500	<10[c] <10[d]	NSI; AIDS; molecular clone	I. S. Y. Chen (24)
RVL 04	100	<20 <20	NSI; CD4 count, >500	Genentech Research Virology Laboratory
RVL 05	1,000	<20 <20	NSI; CD4 count, >500	Genentech Research Virology Laboratory
VS	50	<10 <10	NSI; primary infection	D. D. Ho (67)
RA	50	<10 <10	NSI; primary infection	D. D. Ho (67)
301593	100	<10 ND[e]	SI; asymptomatic; Haiti	J. Bradac and N. Halsey
ACH 168.10	100	<10 <10	SI; AIDS	H. Schuitemaker (13, 59)
MN (in MT4 cell assay)	1,000	600 200	AIDS; laboratory adapted	R. C. Gallo (18)

[a] IC$_{50}$s (50% inhibitory concentrations) are interpolated and expressed as reciprocal dilutions.
[b] Comments include SI or NSI phenotype, patient status at the time of virus isolation, and other relevant features of the virus.
[c] Guinea pig antisera to MN rgp120.
[d] Human antiserum to MN rgp120.
[e] ND, not determined.

T-lymphoid cell line (30, 49) (Table 1). Similarly, primary viruses were resistant to neutralization by MN-specific, V3-directed MAbs that exhibit potent neutralization against MN. The epitope recognized by MAb 50.1 has been well characterized (KRIxIGP [48, 66]) and is predicted by amino acid sequence to be present on the ACH 168.10 primary virus gp120 (13). Nonetheless, neutralization by MAb 50.1 is not observed (see Fig. 3). Another MN-specific, V3-directed MAb, 1034 (37), also fails to neutralize this primary virus (Fig. 3).

In contrast, these primary viruses are sensitive to neutralization by HIVIG, a concentrated Ig preparation from pooled plasma of infected persons (44, 45) (Table 1). The sensitivity of the laboratory-adapted virus MN to neutralization by HIVIG is similar to that by the MN rgp120 guinea pig serum, which failed to neutralize these primary viruses.

The apparent failure of MN rgp120 sera and MAbs to neutralize primary viruses in PBMC culture, while neutralizing laboratory-adapted viruses in T-cell lines, could be a property of the virus or of the cell substrate used in the assay. To explore the role of the cell substrate, we examined the neutralization sensitivity of the primary SI virus ACH 168.10 by using either PBMCs or MT4 cells (Fig. 1A). The two MN rgp120 sera again failed to neutralize, regardless of the cell type. Fifty percent inhibitory concentrations for HIVIG were comparable in the two assays. Thus, the use of MT4 cells as the cell substrate in the assay did not render the primary virus sensitive to neutralization by vaccine sera.

We also wanted to determine whether the laboratory-adapted virus MN, a virus sensitive to neutralization by MN rgp120 sera and MAbs in the MT4 cell assay, would remain sensitive when assayed with PBMCs. The virus was passaged once in PBMCs, and this stock was used in the standard PBMC neutralization assay. Figure 1B demonstrates that neutralization by HIVIG, by guinea pig MN rgp120 serum, and by MAb 1034 was potent and unchanged when assayed with PBMCs.

These observations suggested to us that the inability of MN rgp120 vaccine sera to neutralize primary viruses when assayed in PBMC culture resides not with the PBMC assay per se but rather with the primary virus.

Adaptation to growth in the FDA/H9 cell line. Adaptation of

sCD4-resistant primary virus to growth in T-cell lines renders the resulting virus sensitive to sCD4-mediated neutralization (12, 31, 63). Thus, we were interested to explore further the relationship between adaptation to growth in cell lines and sensitivity to antibody-mediated virus neutralization. We chose the primary isolate ACH 168.10 for these studies because the V3 region of this virus matches closely that of the prototypic laboratory-adapted virus MN (13) and thus that of the rgp120 vaccine immunogen.

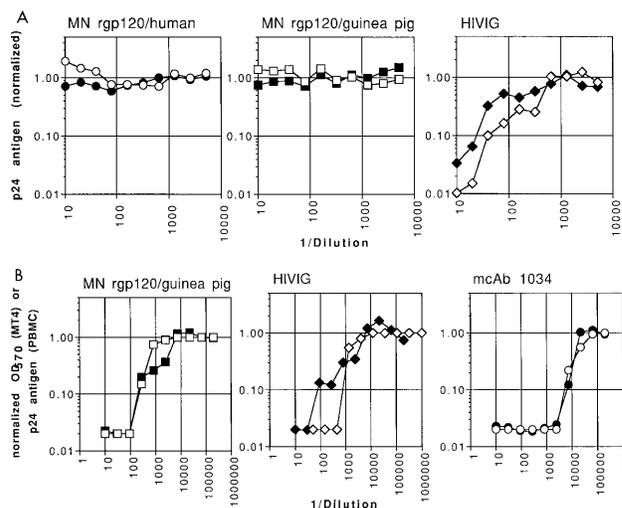FDA/H9 cells, a clonal isolate of the human T-lymphoid H9

FIG. 1. Virus neutralization with PBMCs or MT4 cells. (A) The primary virus ACH 168.10 was tested for sensitivity to human or guinea pig MN rgp120 antiserum and to HIVIG, as indicated. The standard PBMC assay used either PBMCs (solid symbols) or MT4 cells (open symbols). p24 antigen production was normalized to that of cells infected in the absence of specific antiserum (virus control). (B) The laboratory-adapted virus MN was expanded in PBMCs and tested for sensitivity to guinea pig MN rgp120 antiserum, to HIVIG, and to the V3-directed MAb (mcAb) 1034, as indicated. The standard PBMC assay (solid symbols) was compared with the standard MT4 cell line assay (open symbols). p24 antigen was normalized to that of the virus control; MTT dye conversion was normalized within the range of optical density at 570 nm (OD$_{570}$) values defined by uninfected cells and the virus control.

TABLE 2. Properties of 168 virus stocks

Virus	CPE in FDA/H9 culture[a]	Titer in PBMC culture[b]	Amt (ng/ml) of antigen in virus stock	
			p24[c]	gp120[d]
168P	NA	30	340	5.7
168A	+++	200	490	14.8
168C	±	20	54	2.2

[a] CPE (cytopathic effect) was assessed microscopically. +++ represents extensive syncytium formation and cell killing. NA (not applicable) indicates that the virus did not replicate appreciably in FDA/H9 cells.

[b] Titers represent reciprocal virus dilutions that generate 50% of maximal p24 yield in the standard PBMC assay format.

[c] The amount of p24 antigen in virus stock was determined by ELISA (Coulter).

[d] The amount of gp120 antigen in virus stock was determined by immune capture ELISA (33) with polyclonal rabbit anti-MN rgp120 serum for detection and MN rgp120 protein for quantitation.

cell line (42), were inoculated with a large multiplicity of PBMC-derived ACH 168.10 (168P), and the culture was passaged. Cytopathic effect and HIV-1 p24 production were first evident at week 3. By week 4, the culture demonstrated extensive syncytium formation. Culture supernatant was harvested at this time, to produce an acute infection virus stock (168A), and the culture was continued for an additional 7 weeks. During this period, there was a reduction in cytopathic effect and an increase in the percentage of infected FDA/H9 cells. At this time, approximately 35% of the cells could be stained by indirect immunofluorescence with HIV-positive serum. Culture supernatant was harvested to produce a chronic infection virus stock (168C). A summary of the properties of the three 168 virus stocks is presented in Table 2.

The persistently infected 168C culture demonstrated very little cytopathic effect. Because this culture grew out after extensive cell killing during the acute infection, we wanted to confirm that the 168C culture represented a cell line-adapted virus and not a virus-adapted cell line. Aliquots of 168P, 168A, and 168C virus containing approximately equal amounts of infectious virus (as measured in PBMC culture) were used to infect naive FDA/H9 cells. Virus growth was observed microscopically and by indirect immunofluorescence. In all cases, the phenotype of the virus was maintained (Fig. 2). 168C rapidly infected naive FDA/H9 cell cultures (100% infection at 7 days) with minimal cytopathic effect. 168A was less efficient in infecting FDA/H9 cultures (5% infection at 7 days) but induced extensive cytopathic effect in infected cells. We could discern no evidence of infection by 168P at 7 days. Thus, the biological properties of the acute and chronic cultures reflected those of the adapted virus populations.

Neutralization of adapted viruses. The sensitivities to neutralization of the primary virus 168P and the cell line-adapted virus 168C were compared in the PBMC culture (Fig. 3). 168C demonstrated increased sensitivity to several MN-specific reagents, including MN rgp120 sera and V3-directed MAbs 50.1 and 1034, and to the gp41-directed human MAb 2F5 (36). Epitopes recognized by these MAbs have been mapped, and neutralization is consistent with the deduced amino acid sequence of the virus envelope protein (36, 37, 48, 66) (see Fig. 5). 168C also demonstrated increased sensitivity to HIVIG. 168C was not sensitive to neutralization by human serum to IIIb rgp120 vaccine or to several other MAbs, such as the C4 MAb 1024, whose epitope is absent (37). The simultaneous increase in sensitivity observed with diverse immunologic reagents suggests a mechanism that is not local or epitope

specific but rather one that operates in the larger context of the envelope protein complex. As anticipated, 168C also showed increased sensitivity to CD4-Ig immunoadhesin-mediated neutralization.

Adaptation to growth in FDA/H9 cells is, however, not sufficient to render virus sensitive to antibody-mediated neutralization; the acute virus 168A remained resistant to MN rgp120 sera and to the MN-specific MAbs 50.1 and 1034 and was unchanged in sensitivity to HIVIG (Fig. 4). Thus, growth of a primary virus in a T-cell line does not cause the virus to be sensitive to antibody-mediated neutralization; an additional change must take place before the cell line-adapted virus becomes sensitive to antibody. Interestingly, the antibody-resistant 168A virus was partially sensitive to neutralization by CD4-Ig immunoadhesin (Fig. 4).

DNA sequence analysis of envelope genes. The DNA sequences of the envelope genes of the three 168 virus populations were obtained by direct DNA sequencing of PCR-amplified products. The deduced amino acid sequences of the envelope proteins are shown in Fig. 5. The neutralization-sensitive virus 168C encodes three amino acid changes relative to the parental primary virus 168P. These changes lie within the V2 region of gp120 (I-166→R), the C2 region (I-282→N), and the V3 region (G-318→R). No amino acid changes were detected within gp41 of 168C. Neither were amino acid changes detected within the entire envelope protein of the cell line-adapted, CD4-Ig-sensitive virus 168A.

The specific amino acid changes in 168C are not expected to directly affect the binding of the MAbs used in these studies. The amino acid change within the V3 region (G-318→R) falls outside of the epitope of MAb 50.1 (46, 48, 66). Likewise, changes in gp120 are not expected to directly affect the binding of MAb 2F5 to its linear epitope in gp41 (36).

MAb binding to envelope protein. To investigate further the relationship between virus neutralization and antibody binding, we measured the binding of MAb 50.1 to gp120 protein in viral lysates. Viral envelope protein was immobilized in microtiter wells by using a sheep polyclonal antibody specific for the C terminus of gp120 (32), and binding of MAb 50.1 was detected by ELISA. MAb 50.1 bound equally to lysates of the neutralization-resistant 168P virus and the neutralization-sensitive 168C virus (Fig. 6). Binding was comparable to that seen with purified MN rgp120. The differential sensitivity of 168P and 168C to neutralization apparently does not reflect differential binding of MAb 50.1.

Binding studies done with solubilized envelope protein carry some artifice. To approach more realistically the question of whether these MAbs bind native, oligomeric envelope protein, we used flow cytometry to study binding to the surface of infected PBMCs. Intact cells were stained at 37°C with the V3-specific MAbs 50.1 and 1034 or a polyclonal HIV antiserum (HIVIG). PE-labeled secondary antibodies were used for analysis. Cells were additionally stained with FITC-labeled OKT4, a MAb that recognizes CD4 but does not compete with gp120 for binding. Cells were subsequently fixed with 1% formalin prior to dual-parameter flow cytometric analysis (PE, 532 nm; FITC, 488 nm). PE staining of the CD4+ lymphocyte population was compared with PE staining of the CD4− population (presumed background). The results of this analysis are presented in Fig. 7. Specific binding by both V3-directed MAbs is evident in the CD4+ lymphocyte population infected with the neutralization-resistant viruses 168P and 168A, as well as that infected with the neutralization-sensitive virus 168C. On the basis of staining, it appears that a majority of CD4+ lymphocytes in each culture are infected. The C4-directed MAb 1024, whose epitope is absent on all 168 viruses, does not
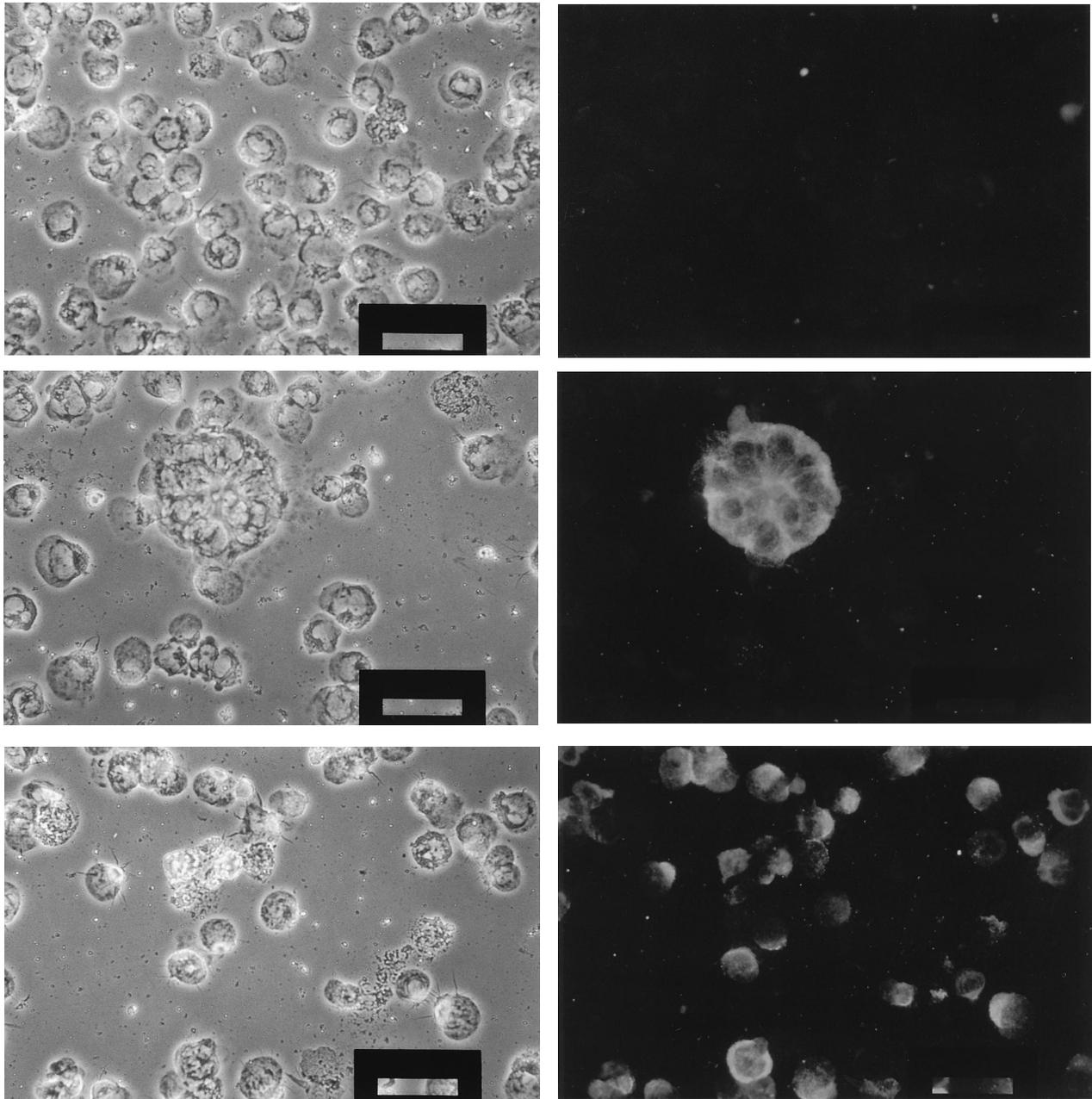
FIG. 2. Indirect immunofluorescence of FDA/H9 cells infected with the 168 viruses. FDA/H9 cells were infected with approximately equal PBMC-infectious titers of 168P (top), 168A (middle), or 168C (bottom) viruses. After 7 days of culture, cells were fixed and stained for indirect immunofluorescence with serum from an infected person. Infected cells were visualized by epifluorescence (right panels); the corresponding visible-light fields are shown to the left. The scale mask (left panels) denotes 50 μm.

stain, nor does normal mouse IgG. Only background PE staining is observed in uninfected PBMC cultures.

The V3-directed MAbs increased PE fluorescence of all three 168-infected CD4$^+$ populations approximately 1 decade over that of the uninfected CD4$^-$ population. Because fluorescence intensity reflects both binding and gp120 density, we attempted to normalize gp120 density by using the broadly reactive polyclonal antibody HIVIG. Although HIVIG discriminates among the 168 viruses by neutralization, we expect less discrimination based solely on binding. Despite a generalized increase in background fluorescence with HIVIG, comparable increases in staining were observed in the three 168

virus-infected populations, indicating comparable levels of gp120 expression. A similar conclusion is derived from a comparison of virus accumulation in supernatants from these cultures as well as from an analysis of viral gp120 content (Table 2). Thus, V3-directed MAbs bind comparably to the envelope protein complex of PBMCs infected with neutralization-sensitive or neutralization-resistant 168 viruses.

DISCUSSION

Central to our study is the need to understand the basis for the failure of MN rgp120 vaccine sera and MN-neutralizing
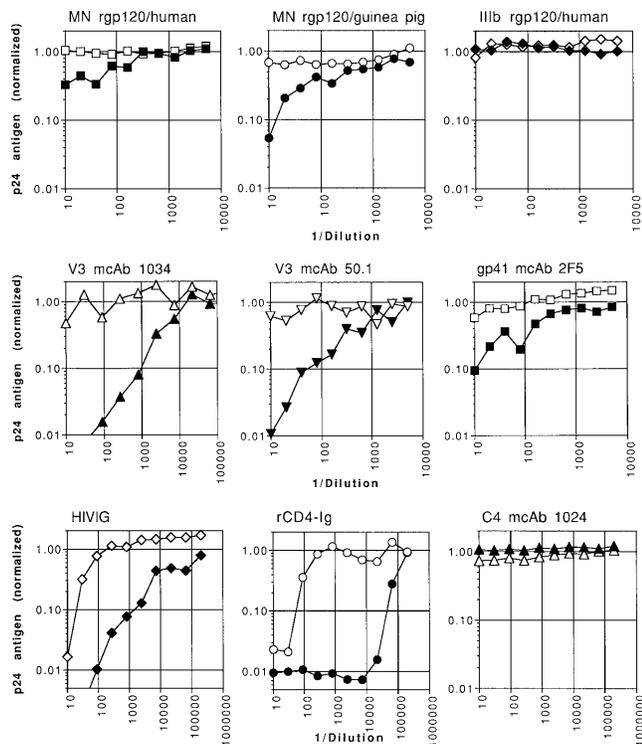
FIG. 3. Virus neutralization of 168P and 168C viruses. Neutralization sensitivities of the primary 168P virus (open symbols) and the chronic, cell-adapted 168C virus (solid symbols) were compared in the standard PBMC assay. The antibodies used are indicated. p24 antigen production was normalized to that of the virus control. mcAb, MAb.
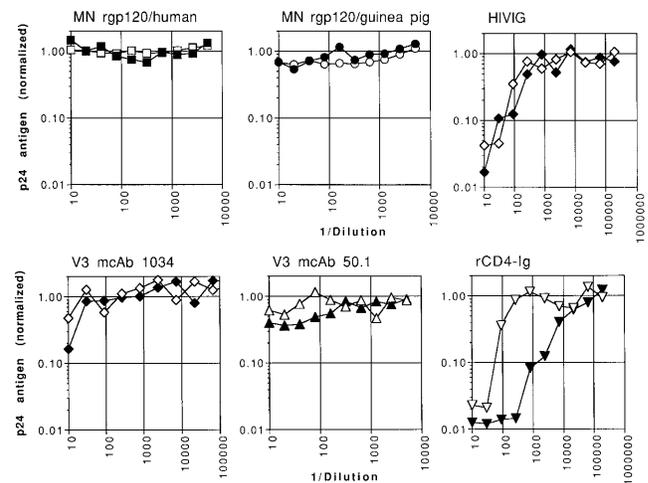

FIG. 4. Virus neutralization of 168P and 168A viruses. Neutralization sensitivity of the acute 168A virus (solid symbols) was measured in the standard PBMC assay. The antibodies used are indicated. p24 antigen production was normalized to that of the virus control. In all experiments except that with HIVIG, primary 168P virus data (open symbols) are from Fig. 3 and are included for comparison. mcAb, MAb.

MAbs to neutralize primary isolates of HIV-1 in PBMC culture. In particular, we sought to understand better the differences between primary isolates in PBMC culture and the more commonly used cell line-adapted laboratory strains of the virus. Our initial studies compared primary virus neutralization with either PBMCs or the MT4 cell line as the cell substrate in the assay. We also compared neutralization of the laboratory-adapted virus MN in PBMC or MT4 cell assays. These studies suggested that neutralization sensitivity or resistance resided not with the cell substrate but rather with the virus. To explore further the basis for the failure of these MN-specific antibodies to neutralize primary viruses, we passaged the primary SI virus ACH 168.10 in the FDA/H9 cell line in order to establish a cell line-adapted virus population. ACH 168.10 was chosen for these studies because the principal neutralizing determinant within the V3 region of this virus closely matches that of MN. We established a chronic, persistently infected culture (168C) and compared the neutralization sensitivity of this virus with that of the parental primary virus (168P). 168C was now sensitive to neutralization by MN rgp120 vaccine serum and by MN V3-directed MAbs. Relative to 168P, 168C was also substantially more sensitive to neutralization by HIVIG. The coincidental increase in sensitivity observed with diverse immunologic reagents suggests a mechanism that operates not locally but rather on a larger scale, comprising perhaps the entire envelope complex.

The failure of V3-directed MAbs to neutralize 168P is not attributable to the absence of the respective epitopes on the viral gp120. The well-characterized linear epitope of MAb 50.1 is present within the V3 amino acid sequence of 168-type viruses, and this MAb binds equally to lysates of 168P and

168C virus. More significantly, flow cytometric analysis demonstrates that MAbs 50.1 and 1034 bind equally to 168P and 168C envelope proteins expressed on the surface of infected lymphocytes. Additional detailed studies of MAb binding affinity and kinetics are necessary to assess further possible biochemical differences in binding. If extrapolated to virion particles, our results suggest that the critical differences in antibody-mediated neutralization of primary and cell adapted viruses result not from differential binding but from a differential effect of binding. We have used MAbs to probe specific neutralizing epitopes; we presume that critical, although undefined, neutralizing antibodies present in polyclonal vaccine sera also bind, but fail to neutralize, primary viruses.

Sensitivity to antibody-mediated neutralization arises coincidentally with adaptation to persistent growth in cell lines. The mechanistic relationship between these properties is not clear. It has been proposed that rapid growth in T-cell lines may select for rapid virus binding and entry, at the expense of protective barriers to neutralization (31). Alternatively, the selection for rapid growth in T-cell lines might enhance the use of ancillary aids to binding and entry, such as the proposed role of the highly positively charged V3 loop of laboratory-adapted viruses (13) in scanning negatively charged cell surface molecules to enhance the opportunity for binding to CD4 (8). This process would render cell line-adapted viruses more sensitive to neutralization by V3-directed antibodies. Conversely, primary viruses might take advantage of alternate pathways of binding and entry available on PBMCs but not on cell lines, perhaps via ancillary coreceptors (7, 58) or via virion-associated lymphocyte proteins that bind ligands on PBMCs (2). Although growth in cell lines per se does not abrogate neutralization resistance (168A), adaptation to persistent growth in cell lines may select against retention of the ability to utilize these alternate pathways and thus may render the virus more sensitive to neutralization.

We have identified three amino acid changes in the envelope glycoprotein of the cell line-adapted and neutralization-sensitive virus 168C. None of the changes coincides precisely with those previously identified in primary viruses that have been newly adapted to growth in cell lines (6, 31, 52). Whether this

```
                          ↓ gp120
168P    1  MRVKGIRKNYLWRWGMMLLGMLICSATEKLWVTVYYGVPVWKEATTTLFCASDAKAYDTEVHNVWATHACVPTD
168A       ..........................................................................
168C       ..........................................................................

168P   76  PNPQEVVLGNVTENFNMWKNNMVEQMHEDIISLWDQSLKP CVKLTPLCVTLDCTDVNVTDTNSTTNATIGSWEKM
168A       ..........................................................................
168C       .....V1V2.................................................................

168P  151  EKGEIKNCSFNITTS I RDKGQKEYALFYRHDVVPINTTKYRLISCNTSVITQAC PKVSFEPIPIHYCAPAGFAIL
168A       ...............-..........................................................
168C       ...............R..........................................................

168P  226  KCNNKTFNGKGPCTNVSTVQCTHGIRPVVSTQLLLNGSLAEEEIVIRSENFTNNAK I IIVQLNVSVDIN CTRPNN
168A       .......................................................-..................
168C       .......................................................N..................

                              V3
168P  301  NIRKRIHIGPGRAFYT T GQIIGNIRQAHC NISKTRWNNTLNQIAKKLREKFGNKTIVFNQSSGGDPEIVMHSFNC
168A       ...............-..........................................................
168C       ...............R.....................V4...................................

168P  376  GGEFFY CNTTGLFNSTWNGTEEIVTERSNRTGENDTLILQC KIRQIVNLWQQVGKAMYAPPIQGQISCSSNITGL
168A       ..........................................................................
168C       ..........................................................................
                                                                  ↓ gp41
168P  451  LLTRDGGNNNSSNNNTEIFRPGGGDMRDNWRSELYKYKVVKIEPLGLAPTKAKRRVVQREKRAVGIGVLFLGFLG
168A       ..........................................................................
168C       ..........................................................................

168P  526  AAGSTMGAAAVTLTVQARQLLSGIVQQQNNLLRAIEAQQHLLQLTVWGIKQLQARILAVERYLKDQQLLGIWGCS
168A       ..........................................................................
168C       ..........................................................................

168P  601  GKLICTTTVPWNASWSNKSLDMIWNNMTWMEWEREIDNYTGLIYNLIEQSQNQQEKNEQELLELDKWANLWSWFS
168A       ..........................................................................
168C       ..........................................................................

168P  676  ITNWLWYIRLFIMIVGGLIGLRIVFAVLAIVKRVRQGYSPLSFQTHLPAQRGPDRPEGTEEEGGERDRDRSTRLV
168A       ..........................................................................
168C       ..........................................................................

168P  751  DGFLALIWDDLRSLFLFSYHRLRDLLLIVTRIVELLGRRGWEILKYWWNLLQYWSQELKNSAVSLLNATAVAVAE
168A       ..........................................................................
168C       ..........................................................................

168P  826  GTDRVVEVLQRAGRAVLHIPRRIRQGLERALL
168A       ................................
168C       ................................
```
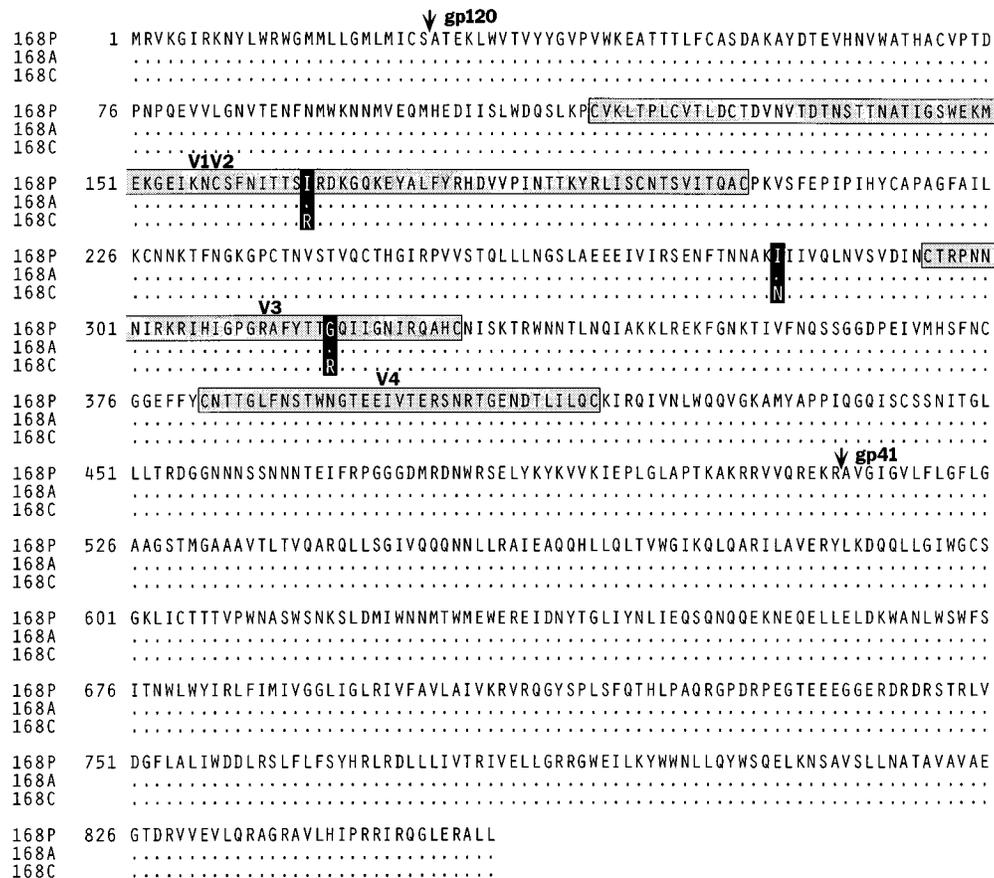
FIG. 5. Deduced amino acid sequences of the envelope proteins of the 168 viruses. Amino acid changes relative to the primary 168P virus are indicated in black boxes. A silent nucleotide change was also observed at codon Q-342 in 168C. For comparison, the V3 loop of HIV-1 MN contains CTRPNYNKRKRIHIGPGRAFYT TKNIIGTIRQAHC (18, 47).

reflects different virus backgrounds or cell lines or different degrees of progression toward stable adaptation or whether the different amino acid changes are functionally equivalent is not known.

In our studies, it is particularly interesting that one of the amino acid changes identified resides within the V3 loop of gp120. This region not only serves as a major target for virus neutralization (14, 50, 66) but is also a major determinant of cell tropism. Changes in this region affect the ability of virus to grow in primary monocyte-derived macrophages as well as in established cell lines (20, 41, 56, 65). The V3 region is also a major determinant of syncytium formation among primary isolates of HIV-1 (13, 15). In turn, most SI primary viruses will grow in the MT2 cell line (23) and most NSI viruses exhibit monocytotropism (54). Sensitivity to neutralization by sCD4 has also been linked to cell tropism (40, 63) and to the V3 region (40).

DNA sequence analysis has revealed a pattern of increased positive charge within the V3 region of SI viruses, including frequent changes at V3 positions 11 and 28 (15). The primary virus 168P induces syncytia in PBMC culture and encodes positively charged amino acids at V3 positions 11 and 28. The 168C virus encodes an additional positively charged amino acid at V3 position 27 (G-318→R). This change increases the positive charge of the V3 region to +7, similar to that observed in the prototypic H9 cell line-adapted viruses RF, MN, and HXB2 (+7 to +9) (13). Positively charged amino acids at V3 position 27 are occasionally found among primary SI viruses (15).

The central role of the V3 region in cell tropism, in syncytium formation, and in antibody-mediated neutralization suggests that these phenotypes reflect aspects of a fundamental continuum among HIVs. These variations within the basic and essential viral process of binding and fusion may be mediated in part by the positive charge displayed by the V3 region of gp120. The V3 loop may interact with negatively charged molecules on the cell surface prior to CD4 binding (8) or may participate in other inter- or intramolecular interactions during CD4 binding and membrane fusion.
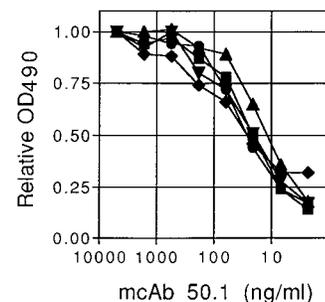

FIG. 6. Binding of MAb (mcAb) 50.1 to lysates of the 168 viruses. Viral lysates of 168P (●), 168A (■), 168C (◆), and MN (▼) were used, as was MN rgp120 (▲). gp120 was captured onto microtiter wells by using a sheep polyclonal antibody specific for the C terminus of gp120 (32). Serial dilutions of MAb 50.1 were tested for binding by ELISA. Because of differences in the amount of bound gp120, optical density at 490 nm (OD_{490}) values were normalized to saturating levels of antibody (5 μg/ml) for comparison.
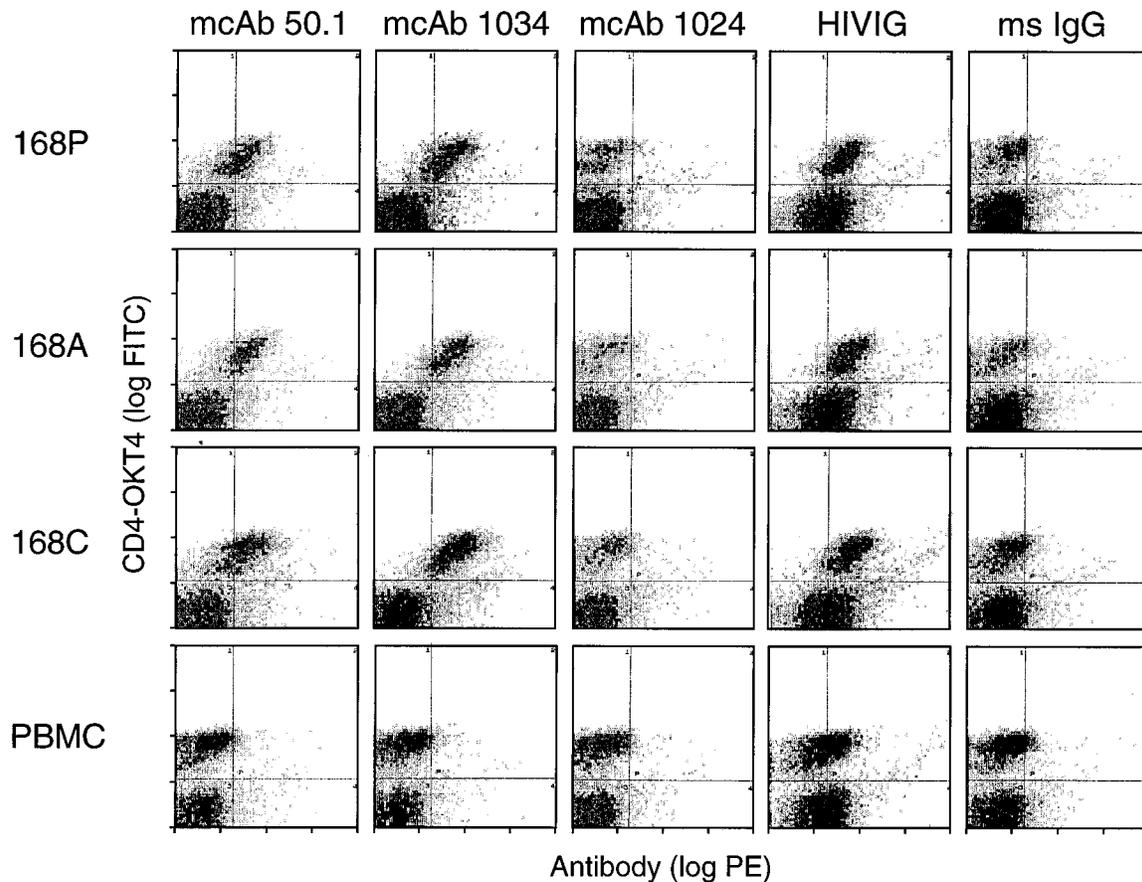
FIG. 7. Flow cytometric analysis of binding to PBMCs infected with the 168 viruses. PBMCs were infected with approximately equal infectious titers of 168P, 168A, or 168C virus. Uninfected PBMCs were used as controls. Cells were stained with the indicated HIV-specific reagents (and controls) and PE-labeled secondary antibody. Cells were also stained for CD4 with FITC-OKT4. Dual-parameter immunofluorescence was analyzed with a Coulter EPICS Elite flow cytometer. The dual-parameter analysis allows for comparison of PE fluorescence in $CD4^+$ and $CD4^-$ lymphocytes. FITC fluorescence is displayed along the y axis of the scatterplot, and PE fluorescence is displayed along the x axis. mcAb, MAb; ms, mouse.

The individual amino acid changes identified within the V2 and C2 regions of the gp120 of 168C are less easily understood than the change within the V3 region. The V1/V2 region can also serve as a target for neutralizing MAbs (17, 29). As has been shown for the V3 region (9, 35, 43, 60, 62), events at the CD4-binding site of gp120 can also affect the binding of V1/V2-directed MAbs (16, 17, 31, 34, 61). An amino acid change in the C2 region of HXB2 (A-281→V, analogous to A-280 in 168-type viruses) has been reported to confer resistance to neutralization by some sera from HIV-1-infected persons (64); this change also affects neutralization by V3-directed antibodies. The complexity of potential intramolecular interactions, in the folding of the protein or during the conformational changes that transduce CD4 binding to allow fusion, renders it difficult to ascribe specific phenotypic consequences to the individual amino acid changes identified within the gp120 protein of 168C. Until individual recombinant viruses are studied the significance of any of the amino acid changes identified remains speculative.

One surprising finding from our adaptation studies is that the acute infection virus 168A is unchanged in antibody-mediated neutralization sensitivity relative to the primary virus 168P. Thus, neither adaptation to growth nor growth per se in cell lines is sufficient to render virus sensitive to neutralization. Hanson and colleagues were first to describe the phenomenon wherein passage of a primary virus in H9 cell culture renders the virus sensitive to antibody-mediated neutralization (52).

Although the authors hypothesize that selection for growth in H9 cells could drive genetic changes in the virus, the focus of their speculation is more directly on the host cell and on potential epigenetic differences arising as a result of simple growth in a different cellular milieu. On the basis of our findings, we favor the former hypothesis. The remarkable ability of populations of HIV-1 quasispecies to respond to changing growth conditions, such as those imposed by antiviral drugs (10, 26, 38), is well known. We suggest that it is genetic changes associated with adaptation to persistent growth in cell lines that give rise to the so-called "host cell effect" (52).

Also informative is the observation that the acute infection virus 168A encodes an envelope protein that is identical in deduced amino acid sequence to that of the primary virus 168P. Thus, the initial adaptation to growth in FDA/H9 cells may involve viral elements other than envelope.

The emergence of partial sensitivity to CD4-Ig in 168A, a virus that remains resistant to antibody-mediated neutralization, suggests that some mechanism(s) involved in CD4-Ig sensitivity can be distinct from those that render a cell line-adapted virus sensitive to antibody. Whether these early changes in 168A are necessary preconditions for the subsequent emergence of antibody sensitivity is unknown.

Immunization with MN rgp120 elicits antibodies that neutralize cell line-adapted viruses, such as MN and 168C, but fail to neutralize primary isolates of HIV-1. Our studies focus on the differences between the neutralization-resistant primary

virus and the neutralization-sensitive cell line-adapted virus. Our studies also point to differences between vaccine sera and sera from infected persons. All primary isolates of HIV-1 tested are sensitive to neutralization by HIVIG. Primary viruses are also sensitive to neutralization by individual serum samples from infected persons, albeit with variable potency and partial range (28, 66a). At present it is not known whether the failure of vaccine sera to neutralize primary viruses is due to qualitative differences in the type of antibody or to quantitative shortfalls in antibody titer.

The relationship between the in vitro neutralization of primary viruses by sera elicited by MN rgp120 vaccine and the in vivo efficacy of vaccination is, of course, unknown. A better understanding of the factors that influence the sensitivity of HIV-1 to in vitro neutralization may provide guidance for the development of future HIV-1 vaccine immunogens.

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