Plasma specimens. Preexisting coded plasma samples from 189 HIV-1–infected subjects and 15 uninfected normal control individuals were analyzed. Six of the HIV-1 clade C–infected subjects included subject 133M in Table I were acutely infected partners in a heterosexual discordant couple previously described (32). Blood was generally collected in acid citrate dextrose, planar-free plasma prepared by sequential 10-min centrifugations at 2000 g and 1,000 × g, and 1-ml aliquots stored at −20 °C or −70 °C. Before use, plasma was thawed, heat inactivated at 56 °C for 30 min, and clarified by centrifugation at 3,000 g for 5 min. Human subjects gave informed consent and protocols received institutional review board approval.

Cell entry and neutralization assays. Plasma samples and monoclonal antibodies were assayed for NAb activity using a modification of a recently described HIV entry assay (3) that uses the surface adherent HEK cell–derived JCSBL-13 cell line (National Institutes of Health AIDS Research and Reference Reagent Program, T20-84), which has been genetically modified and selected so as to constitutively express CD4, CXCR3, and CCR5. The cells contain integrated lacZase and β-galactosidase (β-gal) genes under tight regulatory control of an HIV-1 LTR. Viruses were stocks were obtained by transfection of 293T cells and were titrated by β-gal expression on JCSBL-13 cells, as described previously (3). 7 × 10^5 JCSBL-13 cells were plated in 96-well tissue culture plates (Falcon) and cultured overnight in DMEM supplemented with 10% FCS. 3,000 infectious units of virus were combined in a total volume of 60 μl with or without a 2X concentration of cDNA in DMEM with 6% FCS and 80 μg/ml DEAE-dextran. After 1 h at 37 °C, an equal volume of test or control plasma (50% vol/vol in DMEM plus 6% FCS or ficolled aliquots thereof) or monoclonal antibody was added. This brought the total concentration of DEAE dextran to 40 μg/ml and that of human plasma to 5%. It is important to note that sufficient normal human plasma (NHP) was added to each well so as to maintain a constant final human plasma concentration of 5% in each virus + cDNA + test plasma mixture. Concentrations of NHP (or test plasma) that exceed 5% commonly result in nonspecific inhibition of virus entry (3), and thus samples are not tested for neutralizing activity at dilutions <1:20. The concentration of cDNA was chosen so that the final 1X concentration after the addition of test plasma corresponds to the IC50 of cDNA specific for each virus. The virus + cDNA + test plasma (or monoclonal antibody) mixture was incubated for 1 h at 37 °C. Media was removed entirely from the adherent JCSBL-13 monolayer just before the addition of the virus + cDNA + test plasma (or monoclonal antibody) to the cells. Cells were incubated at 37 °C for 24 h and analyzed for luciferase expression, as described previously (3). Controls included cells exposed to no virus and to virus premixed with NHP or control monoclonal antibodies only. Relative infectivity was calculated by dividing the number of luciferase units at each dilution of test plasma or monoclonal antibodies by values in wells containing NHP but no test plasma or monoclonal antibodies. Neutralization was assayed by 50% inhibitory concentration (IC50) determined by linear regression using a four-parameter method. All samples were tested in duplicate and all experiments repeated at least three times to ensure reproducibility.

A 293T–CD4–CCR5 cell assay was used to test viruses for CD4-independent cell fusion and entry. Envelope glycoproteins from plasma derived virus RNA–DNA were expressed in 293T cells and used to pseudotype an ex-vivo–defective HIV-1 reporter virus [pNL4-3.G-B-IRES] containing an enhanced GFP gene (67). Infectious titers of pseudotyped virus were determined first in JCSBL-13 cells so that virus inocula could be standardized. CD4+–CCR5+ cells (42), which express human CCR5 but not CD4, were plated in 24-well tissue culture plates at a density of 4 × 10^4 cells/well and cultured overnight in standard medium (90% DMEM, 10% FBS, 1.5 mg/ml G418, 3.0 mg/ml streptomycin, 100 U/ml penicillin, and 100 μg/ml streptomycin) at 37 °C and 5% CO2. Virus, with or without preincubation with cDNA, and with or without preincubation with monoclonal antibodies or test plasma, was added in a total volume of 0.25 ml of standard culture medium and incubated for 5 h at 37 °C. Neutralization assays were performed with human plasma, attention was again given to ensure that 5% vol/vol total concentration of plasma was maintained in all wells, as aforementioned in the JCSBL-13 assay. An additional 0.25 ml of medium was added and the cultures were maintained for 48 h at 37 °C. Thereafter, cells were washed in PBS and visualized directly for GFP expression or incubated 30 min with 1:20 dilution of Cy2–anti-GFP antibody (Jackson Immuno Research, West Grove, PA) and a 1:500 dilution of Cy2–conjugated avidin (Jackson Immuno Research) and visualized using a modified confocal microscope. The concentration of virus was varied in the range of 0.001 to 100 infectious units per well, plasma was varied from 0% to 10%, and the monoclonal antibodies were varied from 0.01 to 100 μg/ml. Relative infectivity was calculated by dividing the number of GFP-positive cells by the number of cells plated. The IC50 was determined by linear regression using a four-parameter method. All samples were tested in duplicate and all experiments repeated at least three times to ensure reproducibility.

Virus stocks. For neutralization experiments in JCSBL-13 cells, HIV-2 proviral clones pK7312A (Gbenchka/EMBL/DDBJ accession no. L0674; references 14, 43, 44) and pK7312B (SVK251, each cloned in pBluescript SK II at NcoI–NheI sites, and pK7312/YU2/3) (references 24, 35) were used to transfect 293T cells. HIV-2 UC-1.2, HIV-2 133M, HIV-1 MN, or HIV-1 JR-FL, all cloned in pSM and pCR3.1, respectively, were cotransfected with pBluescript SK II or pK7312A/B to create infectious pseudovirions, as described previously (5). For cell entry experiments using p24Gag–lacZase+ virus, the following plasmids were cotransfected: pNL4-3.G-B-IRES, pCMV-gfp (67) and pCMV-Gal (67) using the FuGENE 6 transfection kit (Roche Diagnostics). For antibody binding studies, HIV and SIV envelope glycoproteins captured in wells of microtiter plates coated with mAb 2.6C or EH21, as previously described (31, 32). Before the addition of biotin–labeled mAbs, plates were coated with immobilized gp120–sCD4 complexes and determining binding of biotin–labeled mAbs at subsaturating concentrations, as described previously (31, 32).

Antibody binding and competition assays. Biotinylated monoclonal antibodies were tested for binding to HIV-2, SIV, or HIV-1 gp120 envelope glycoprotein captured in wells of microtiter plates coated with mAb 2.6C or EH21, as previously described (31, 32). Before the addition of biotin–labeled antibodies, cDNA was precipitated with 1-10 μg/ml 50% cDNA (R&D Systems) or a mock preparation. Binding was quantified by the reaction of polystyrene conjugated streptavidin and subsequent color development with submaximal TMB-H2O2. Competition assays were performed by preincubating plasma samples with immobilized gp120–cDNA complexes and determining binding of biotin–labeled mAbs at saturating concentrations, as described previously (31, 32).

Monoclonal antibodies. The proteotypic CD4 mAbs 17b and 46d, and several recent recombinant CD4 mAbs, 23e, 21c, 4L1p, 41L2, E51, and CM15 have been described previously (32, 33, 71–73). Additional CD4 mAbs used in this study were isolated from HIV–1–infected subjects sampled on HAART during acute infection. These include 17b, 21c, 41L2, 2D7, 4E10, 2G12, 3B11, 58H, and 2B. All of the CD4 mAbs bind to the HIV-1 gp120 glycoprotein: the common binding surface that is created (or exposed) after cCD4 binding or deletion or reinsertion of V1-V2 variable loop sequences. But these of the mAbs, 17b,
ED47, and ED49, are unusual in that they bind poorly, or not at all, to V1/V2 deleted HIV-1 gp120. Hence, their binding is CD4-dependent. Further characteristics of these mAbs will be presented in a separate publication. The other mAbs specific for the HIV-1 CD4 binding site, variable loops, surface glycans, and other gp120 and gp41 epitopes have been described previously (73). Human mAbs 1.7 and 2.6C have specificity for HIV-2 gp120 and were isolated from an HIV-2 infected West African patient, as previously described (74, 75). The anti-CD4 mAb from clone RPA-T4 was obtained from BD Biosciences.

Molecular cloning, sequencing, and mutagenesis. Full-length gp160 envelope genes were amplified by nested PCR from plasma HIV-1 RNA. Virion-associated plasma RNA was prepared using the QiPrep Viral RNA Mini Kit (QIAGEN) as previously described (3, 49). From each time point, replicate plasma virus RNA preparations (4,000–8,000 RNA molecules per reaction) were subjected to cDNA synthesis using SuperScript II (Invitrogen). Replicate viral cDNA samples (1, 10, 100, or 1,000 molecules each) were subjected to nested PCR amplification as described, using the following primers: outer sense primer (5′-TAGAGGCCTGTGGAAGCATCCAGGAAG-3′, nt 5852–5876), outer antisense primer (5′-TTGCTACTTGTGATTGCTCCATGT-3′, nt 8912–8935), inner sense primer (5′-GATCAAGCTTGTGGAAGCATCCAGGAAG-3′, nt 5852–5876), outer antisense primer (5′-TTGCTACTTGTGATTGCTCCATGT-3′, nt 8912–8935), outer sense primer (5′-GATCAAGCTTGTGGAAGCATCCAGGAAG-3′, nt 5852–5876), and inner antisense primer (5′-AGCCTGTGGCTGGAAGCATCCAGGAAG-3′, nt 5852–5876).inner primers contain additional 5′ sequences and restriction sites to facilitate cloning. The PCR products of the full-length env genes were cloned into pcDNA3.1 (Invitrogen) for expression. All clones, including those modified by site-directed mutagenesis, were sequenced using an ABI 3100 Genetic Analyzer and dideoxy methodology. Sequences have been deposited in GenBank/EMBL/DDBJ (accession nos. AY223761-90; AY223720-54; AY858550). To ensure that molecular clones of HIV-1 envelope amplified from plasma viral RNA were representative of plasma virus, replicate PCR reactions were performed on primary samples at varying endpoint titrations of viral cDNA and on separate days. Site-directed mutagenesis was done using the QuikChange site-directed mutagenesis kit (Stratagene Inc.). 125 ng of complementary primers with mutant sequences and 20 ng of template plasmid DNA 1:1 were used for each PCR amplification. PCR conditions were as follows: 95°C for 50 s, 60°C for 50 s, and 68°C for 10 min. After 16 cycles, the PCR product was digested with 10 U of PstI to cleave template DNA at 37°C for 1 h. Mutants were identified and confirmed by nucleotide sequencing.