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A Human Recombinant Fab Identifies a Human Immunodeficiency Virus Type 1-Induced Conformational Change in Cell Surface-Expressed CD4

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To explore the role of the CD4 molecule in human immunodeficiency virus (HIV) infection following initial virus-CD4 binding, we have characterized CD4-specific antibodies raised by immunizing an HIV-1-infected human with human recombinant soluble CD4 (rsCD4). Fabs were selected from a human recombinant Fab library constructed from the bone marrow of this immunized individual. Here, we describe a human rsCD4-specific recombinant Fab clone selected by panning the library over complexes of human rsCD4 and recombinant HIV-1 envelope protein. While this Fab does not bind to CD4-positive T-cell lines or to human T lymphocytes, it recognizes cell surface-expressed CD4 following the incubation of these cells with a recombinant form of HIV-1 gp120 or with HIV-1 virions. The Fab is not HIV-1 envelope specific, since it does not bind to recombinant gp120 or to native cell surface-expressed HIV-1 envelope proteins. As confirmation of its CD4 specificity, we show that this Fab immunoprecipitates a 55-kDa protein, corresponding to the molecular mass of cellular CD4, from an H9 cell lysate. The specificity of this human Fab provides evidence for a virus-induced conformational change in cell surface-expressed CD4. The characterization of this altered CD4 conformation and its effects on the host cell will be important in defining postbinding events in HIV infection.

In serving as the human immunodeficiency virus (HIV) receptor, CD4 plays an essential role in HIV infection of lymphocytes and macrophages. The CD4 molecule is a member of the immunoglobulin (Ig) superfamily, consisting of four extracellular domains, a membrane-spanning region, and a cytoplasmic tail (17). The first of the extracellular domains consists of three segments, resembling the complementarity determining regions (CDRs) of an Ig variable domain. Mapping studies have identified the second of these Ig CDR-like domains (CDR2) in domain 1 as the HIV-binding site (1). It has been suggested that other regions of the CD4 molecule may also be important in HIV infection. While HIV is able to infect cells expressing hybrid CD4 molecules composed of CD4 domains 1 and 2 fused to CD8 hinge, transmembrane, and cytoplasmic sequences, HIV infection of these cells is significantly delayed (9). Cells expressing a CD4 molecule with a truncated cytoplasmic domain similarly demonstrate a delay in the production of HIV virions (4).

Monoclonal antibodies have proven critical in clarifying the role of CD4 in HIV infection. Murine monoclonal anti-CD4 antibodies were used to demonstrate that CD4 acts as the HIV receptor and that the virus binds to domain 1 of CD4 (7, 14). Moreover, CD4-specific antibodies generated by immunizing mice with a recombinant soluble form of the human CD4 molecule (rsCD4) have been shown to inhibit HIV infection in vitro without blocking virus binding to CD4 (3, 10, 11, 19). The epitope specificities of these antibodies have been mapped to domains 2, 3, and 4 of the CD4 molecule. The existence of such antibodies provides further evidence that CD4 may be involved in critical postbinding steps of HIV infection.

The CD4-specific antibodies used in these studies were generated by immunizing mice with human CD4. It has been assumed that the immunogenic determinants of human CD4 in these mice were defined by sequence differences between human and murine CD4. However, we have previously demonstrated that CD4-specific antibodies which inhibit simian immunodeficiency virus infection of lymphocytes and macrophages in vitro can be elicited by human rsCD4 immunization of rhesus monkeys (25). The elicitation of these antibodies was somewhat surprising, since the human and rhesus CD4 molecules share 92% sequence homology (25). We have further demonstrated that simian immunodeficiency virus-neutralizing, CD4-specific antibodies can be generated by immunizing rhesus monkeys with rhesus rsCD4 (24). The elicitation of these antibodies by immunization with a self protein demonstrates that epitopes to which these monkeys were not tolerant were presented to the immune system by rhesus monkey rsCD4.

We were interested in determining whether CD4-specific antibodies could be raised by immunizing humans with human rsCD4. Here, we describe the characterization of a human rsCD4-specific Fab clone generated from a human rsCD4-immunized, HIV-1-infected human. This clone was selected from a recombinant Fab library constructed from the bone marrow of this immunized individual. The binding characteristics of this recombinant Fab provide definitive evidence for an HIV-1-induced conformational change in cell surface-expressed CD4.

MATERIALS AND METHODS

Vector. The pCOMB3 vector (Fig. 1) was provided by the Scripps Research Institute, La Jolla, Calif.

Recombinant HIV-1 envelope protein. HIV-1_{SF2} recombinant gp120 (rgp 120) was provided by Chiron Corp., Emeryville, Calif.

Monoclonal antibodies. The monoclonal antibodies used in these studies included 5A8 and humanized 5A8 (Hu5A8), which recognize domain 2 of CD4 (Biogen, Inc., Cambridge, Mass.); OKT4, specific for CD4 domain 3 (American Type Culture Collection, Rockville, Md.); 5D4 (10), specific for an epitope in the carboxy-terminal half (domain 3/domain 4) of the CD4 molecule; M-T807, a humanized antibody specific for CD8 (Centocor, Inc., Malvern, Pa.); and L736523, a human monoclonal antibody specific for the V3 loop of HIV-1_{MN} gp120 (Merck Research Laboratories, West Point, Pa.).

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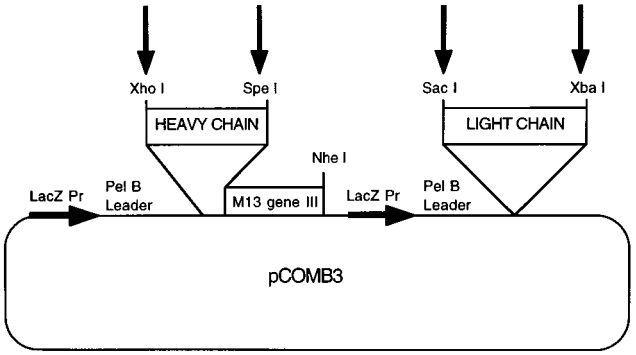

FIG. 1. Restriction site map of the phagemid pCOMB3 used for constructing a combinatorial Ig library from a human rsCD4-immunized, HIV-1-infected individual. Arrows indicate the restriction sites used for cloning. Fab expression is induced through the *lacZ* promoter with IPTG. The *pelB* leader sequence directs heavy and light chains to the periplasmic space of induced bacterial cells, where Fab assembly occurs. The heavy chain is expressed as a fusion protein with the M13 coat protein encoded by gene III, which allows for the incorporation of recombinant Fab molecules on the virion surface.

Cells. H9 and COS cells were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program, Rockville, Md. Human peripheral blood lymphocytes (PBL) were isolated from heparinized human blood by Ficoll-diatrizoate density gradient centrifugation. Chronically infected H9 cells were produced by infecting 5×10^6 H9 cells with cell-free supernatants containing 32 infectious doses of HIV_{IIIB} (1 infectious dose is defined as the minimum dilution of this stock virus-containing supernatant that infects H9 cells under these culture conditions). Staining experiments were performed after these cells had been maintained in culture for 3 weeks, at which time no surface CD4 could be detected by flow cytometry.

Viruses. The production of recombinant HIV-1 virions capable of expressing chloramphenicol acetyltransferase has been described previously (13). Briefly, COS cells were cotransfected with plasmid pHXBΔenvCAT, which encodes an HIV-1 genome with deletions in the envelope protein and an inserted chloramphenicol acetyltransferase gene, and with plasmid $pSVIIIenv_{MN}$, which encodes a full-length $HIV\text{-}1_{MN}$ envelope protein. After 3 days, recombinant virions were collected in the supernatant of these transfected cells. These virus preparations were used for the virus-binding studies (see Fig. 5).

Human rsCD4 immunization of an HIV-1-infected individual. An HIV-1-infected human with an absolute CD4 count greater than 500/μl was immunized and given intramuscular booster doses five times with 1 mg of human rsCD4 (Biogen, Inc.) formulated in an emulsion of 85% mineral oil (Drakeol-6VR; Penreco, Butler, Pa.) and 15% Arlacel A (Montamide-80; Seppic, S.A., Fairfield, N.J.).

Determination of rsCD4-specific antibody titers in the plasma of a human rsCD4-immunized, HIV-1-infected individual. Human rsCD4 was adsorbed overnight at 4°C onto Nunc Maxisorp Immunoplates at a final concentration of 0.3 μg/ml. The wells were washed three times with phosphate-buffered saline (PBS) and blocked for 2 h at room temperature with 0.5% nonfat dry milk–PBS. The wells were then washed three times with 0.5% nonfat dry milk–0.05% Tween 20–PBS. A 1-ml sample of patient plasma was heat inactivated at 56°C for 30 min and diluted 1:60, 1:180, 1:540, and 1:1,620 with 0.5% nonfat dry milk–0.05% Tween 20–PBS. A 50-μl sample of diluted plasma was then added to the appropriate wells and incubated for 2 h at room temperature. The wells were washed three times with 0.5% nonfat dry milk–0.05% Tween 20–PBS, and 50 μl of 1:50,000-diluted horseradish peroxidase-conjugated $F(ab')_2$ goat anti-human IgG was added. After 1 h at room temperature, the wells were washed three times with 0.5% nonfat dry milk–0.05% Tween 20–PBS. Color was developed by adding 100 μl of TMB one-component substrate solution (KPL, Gaithersburg, Md.). Color development was stopped by adding 100 μl of 1 N H_2SO_4. Plates were read on a Dynatech plate reader at an optical density of 450 nm (OD_{450}).

Harvesting and storage of bone marrow samples. Heparinized bone marrow cells (20 ml) were harvested from a human rsCD4-immunized, HIV-1-infected individual 200 days after the initial immunization, at the time that the peak rsCD4-specific antibody titer was measured in the serum of this patient. Lymphocytes were isolated from these samples by Ficoll-diatrizoate density gradient centrifugation, immediately frozen on dry ice, and stored at −70°C prior to RNA extraction.

Amplification of Ig DNA. mRNA was isolated from 2×10^7 bone marrow lymphocytes with the Quickprep Micro-mRNA Purification Kit (Pharmacia). Ig heavy- and light-chain cDNA was then synthesized with primers specific for the first constant domain of human Ig heavy chains, kappa light chains, and lambda light chains (Fig. 2). Specifically, 400 ng of the appropriate constant region primer was added to 30 μl of isolated mRNA. This mixture was heated to 65°C for 5 min and cooled slowly to room temperature in a water bath. Reverse transcription was initiated by adding reverse transcriptase buffer (Gibco-BRL), 80 U of rRNasin (Promega), 0.8 mM deoxynucleoside triphosphates (dNTPs; Promega), 200 U of Moloney murine leukemia virus reverse transcriptase (Gibco-BRL), and 16.7 mM dithiothreitol. After this reaction had proceeded for 2 h at 37°C, the reverse transcriptase was inactivated by heating the reaction mixture to 65°C for 20 min. The resulting cDNA was stored at −20°C prior to PCR amplification.

Two rounds of PCR amplification were used to obtain sufficient quantities of Ig heavy-chain material for cloning. The first-round PCR mixture contained 20 μl of heavy-chain-specific cDNA, 2.5 U of *Pfu* polymerase (Stratagene), *Pfu* buffer II, 0.2 mM dNTPs (Promega), 10 ng of heavy-chain constant-region primer (Operon), 10 ng of one of six variable-region primers corresponding to six heavy-chain families (VHA through VHF) (Operon) (Fig. 2), and RNase-free water to make a final volume of 100 μl. Twenty-five amplification cycles, followed by a 10-min final extension at 72°C, were performed by the hot-start technique under the following conditions: 94°C for 1.5 min, 52°C for 2.5 min, and 72°C for 3 min. A 1-μl sample of the first-round PCR product was then exposed to 25 additional cycles of amplification under the same conditions.

Ig light-chain DNA was amplified under identical conditions, with primers corresponding to the first constant domain of kappa or lambda light chains, as well as one of four variable-region primers specific for different kappa (VK1 and VK2) and lambda (VL1 and VL2) light-chain families (Fig. 2).

Cloning of Ig heavy- and light-chain DNAs into the M13 phagemid vector, pCOMB3. PCR-amplified heavy- and light-chain DNAs were gel purified. Amplified DNA from each light-chain family was then pooled, digested with the enzymes *Xba*I and *Sac*I (Boehringer Mannheim), and ligated into the pCOMB3 vector.

Electrocompetent XL1-blue cells (Stratagene) were transformed with these recombinant plasmids by electroporation. Specifically, phenol-chloroform-extracted, ethanol-precipitated ligation products were added to 300 μl of electrocompetent XL1-blue cells in prechilled 0.2-cm gene pulser cuvettes (Bio-Rad). These cells were pulsed in a Bio-Rad gene pulser apparatus set at 25 μF, 2.5 kV, and 200 Ω. SOC (0.5% yeast extract, 2% tryptone, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) (3 ml) was then added, and the cells were grown at 37°C for 1 h in a shaking incubator. Transformed cells were selected for 1 h at 37°C in 10 ml of superbroth containing 20 μg of carbenicillin per ml and 10 μg of tetracycline per ml. Finally, transformed cells were amplified overnight in a 37°C shaker in 100 ml of superbroth containing 50 μg of carbenicillin per ml and 10 μg of tetracycline per ml.

Recombinant plasmid DNA was then digested with the restriction enzymes *Xho*I and *Spe*I (Boehringer Mannheim), gel purified, and ligated with pooled *Xho*I-*Spe*I-digested, gel-purified heavy-chain PCR products. The combinatorial ligation products were then electroporated into XL1-blue cells, as described above. A restriction analysis of the resulting clones was performed to ensure that this library consisted of clones with both light- and heavy-chain inserts.

Conversion of the combinatorial library to an Fab-expressing M13 bacteriophage format. The initial combinatorial library was amplified at 37°C in a shaking incubator for 1 h in 100 ml of superbroth containing 50 μg of carbenicillin per ml and 10 μg of tetracycline per ml. The M13 helper phage VCSM13 (Stratagene) (10^{12} PFU) was then added to direct the assembly of M13 combinatorial bacteriophage. After 2 h of growth at 37°C, 70 μg of kanamycin per ml was added, and helper phage-infected cells were grown overnight at 37°C.

Precipitation of Fab-expressing M13 bacteriophage. Recombinant M13 bacteriophage in the supernatant of helper phage infected cultures were precipitated on ice for 1 h in the presence of 4% polyethylene glycol 8000 and 3% NaCl. Precipitated phage were pelleted at 9,000 rpm for 20 min at 4°C in a JA10 rotor. Finally, the phage pellet was resuspended in 2 ml of PBS and stored at −20°C.

Determination of the titer of M13 combinatorial bacteriophage. Various dilutions of bacteriophage were incubated for 15 min at room temperature with 50 μl of an XL1-blue culture grown to an OD_{600}/ml of 1.0. The infected cells were then plated onto Luria broth–50 μg of carbenicillin per ml agar plates.

Panning. Four wells of a Maxisorp 96 well plate (Nunc) were coated overnight at 4°C with 2 μg of the CD4-specific monoclonal antibody 5D4 in 100 μl of PBS per well. Unbound antibody was removed by washing three times with 350 μl of Tris-buffered saline (TBS). The wells were then blocked with 350 μl of 2% nonfat dry milk–TBS for 30 min at room temperature. After shaking out the block solution, the wells were incubated with preformed rsCD4-gp120 complexes for 2 h at room temperature. The complexes were formed by incubating rgp120 and human rsCD4 in a 1.4:1 molar ratio at 37°C for 1.5 h. Unbound proteins were removed by performing three washes with 350 μl of sterile double-distilled water. The wells were again blocked at 37°C for 1 h with 350 μl of 3% bovine serum albumin (BSA) PBS. After shaking out the block solution, 100 μl of M13 combinatorial phage (10^{12} PFU) was added to each well and the mixtures were incubated for 2 h at 37°C. Nonadherent phage were removed, and each well was washed once with sterile double-distilled water. Each well was then washed 10 times over a period of 1 h at room temperature with 350 μl of TBS–0.5% Tween 20. Detergent was removed by washing once with sterile double-distilled water, and phage were eluted by adding 100 μl of phage elution buffer (0.1 M HCl, 1 mg of BSA [pH 2.2] per ml; adjusted to pH 2.2 with glycine). The elution was allowed to proceed for 10 min at room temperature. The solution was then pipetted up and down several times, transferred to a sterile tube, and neutralized with 6 μl

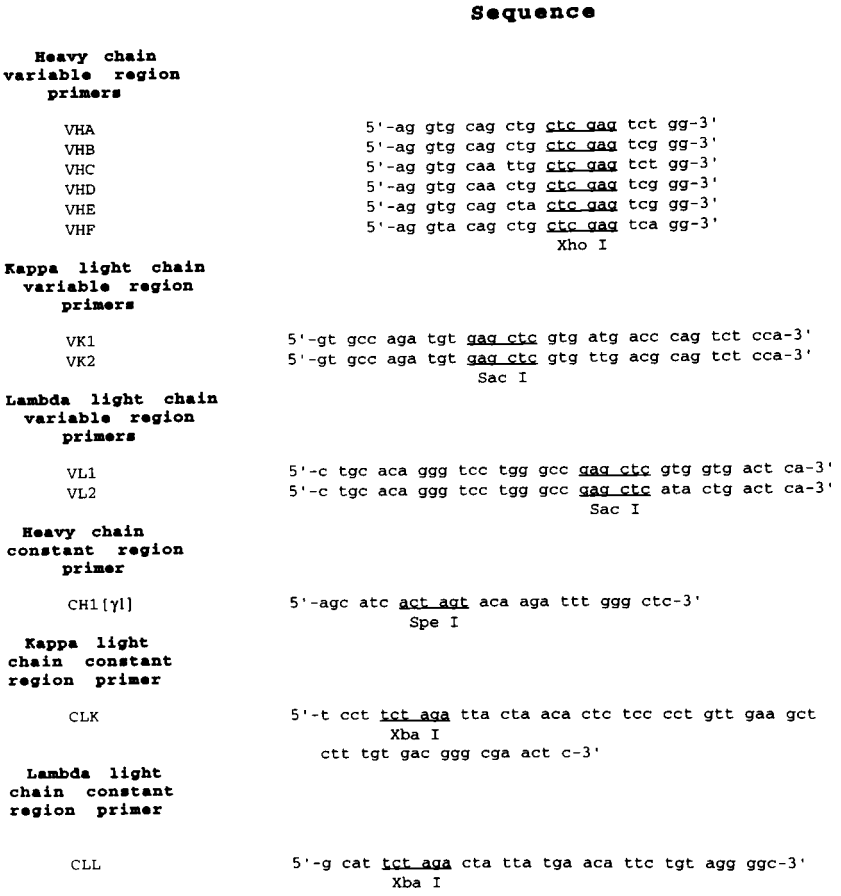

FIG. 2. Sequences of the primers used for amplification of immunoglobulin heavy- and light-chain DNA. Restriction sites incorporated into primers for cloning purposes are underlined. These primers map to the following regions of the indicated human Ig chains: VHA to VHF, heavy-chain amino acids 1 to 8; VK1 and VK2, kappa light-chain signal sequence −4 to amino acid 8; VL1 and VL2, lambda light-chain signal sequence −7 to amino acid 8; CH1, heavy-chain amino acids 226 to 232; CLK, kappa light-chain amino acids 201 to 214; CLL, lambda light-chain amino acids 210 to 215.

of 2 M Tris base per 100 μl of eluted phage. The titer of the panned phage was determined, and the phage were stored at −20°C.

Amplification of panned bacteriophage. Eluted phage were incubated with 2 ml of an XL1-Blue culture (OD_{600}/ml, 1.0) for 15 min at room temperature. The cells were then grown in a 37°C shaking incubator for 1 h in 10 ml of superbroth containing 20 μg of carbenicillin per ml and 10 μg of tetracycline per ml. These cells were then grown for an additional 1 h in 100 ml of superbroth plus 50 μg of carbenicillin per ml and 10 μg of tetracycline per ml. At this time, the cells were infected with 10^{12} PFU of the M13 helper phage VCSM13 (Stratagene) to direct the assembly of Fab-expressing M13 bacteriophage. Finally, cells which had been infected with the helper phage were selected by growing the culture overnight in the presence of 70 μg of kanamycin per ml. These amplification and panning procedures were repeated until enrichment for antigen-specific clones was achieved, as determined by the percent yield of phage (the number of phage eluted divided by the number of phage applied, multiplied by 100) after each amplification and panning cycle.

Conversion of clones to a soluble Fab expression format. To obtain soluble Fab, it was necessary to remove M13 gene III, the gene encoding the coat protein responsible for anchoring Fab molecules on the phage surface, from the combinatorial plasmids. Plasmid DNA was isolated from panned clones, digested with *Spe*I and *Nhe*I to remove gene III, and gel purified. Since the *Spe*I and *Nhe*I restriction sites are compatible, recircularized plasmid was made by religating this digested vector. The ligation product was transformed into XL1-Blue cells in preparation for the induction of Fab expression.

Induction of soluble Fab expression. Combinatorial clones were inoculated into 500 ml of superbroth containing 50 μg of carbenicillin per ml and 20 mM $MgCl_2$ and grown at 37°C in a shaking incubator to an OD_{600}/ml of 1.0. Soluble Fab expression was then induced by growing these cultures at 30°C overnight in the presence of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Stratagene) and 4 nM dibutyryl cyclic AMP (Sigma).

Isolation of soluble Fab from induced bacterial cultures. Since the *pelB* leader sequence of the pCOMB3 vector directs Fab molecules to the periplasm of induced bacterial cells, an osmotic shock procedure was performed to obtain a periplasmic extract from these cells. Specifically, induced bacterial cells were pelleted at 4000 rpm in an HS-4 rotor for 30 min at 4°C. The supernatant was discarded, and the induced bacterial cells were resuspended on ice in 20 ml of osmotic shock solution A (100 mM Tris-HCl [pH 8.6], 500 mM sucrose, 0.5 mM EDTA). The cell wall was lysed by adding 1 ml of 4-mg/ml lysozyme immediately followed by 80 ml of osmotic shock solution B (50 mM Tris-HCl [pH 8.6], 250 mM sucrose, 0.25 mM EDTA, 2.5 mM $MgCl_2$). After a 10-min incubation on ice, bacterial debris was pelleted by centrifuging the lysate in Oakridge 35-ml tubes at 12,500 rpm in an SS-34 rotor for 5 min at 4°C. The supernatant was transferred to a new Oakridge tube, and the protease inhibitor aminoethylbenzene-sulfonylfluoride (AEBSF; Calbiochem) was added to a final concentration of 1 mM. Remaining bacterial debris was removed by centrifuging the extract again at 12,500 rpm for 15 min at 4°C. Finally, the supernatant was filtered through a 0.2-μm-pore-size bottle filter system (Costar).

Purification of soluble Fab. To obtain a pure source of Fab for characterization, the periplasmic extracts of induced cells were applied to goat anti-human $F(ab')_2$ affinity columns. The affinity columns were prepared by incubating 4 mg of goat anti-human $F(ab')_2$ (Jackson Immunoresearch) with 2 ml of Gammabind G Sepharose beads (Pharmacia) for 1 h at room temperature on a rocking platform. After the conjugated beads were washed four times with 10 ml of 0.2 M sodium borate (pH 9.0), they were incubated on a rocker for 1.5 h with the coupling reagent dimethyl pimelimidate · 2HCl (DMP; Pierce) at a concentration of 20 mM in 0.2 M sodium borate (pH 9.0). The coupling reaction was terminated by washing once with 0.2 M ethanolamine (pH 8.0) and incubating the beads in ethanolamine for 2 h at room temperature on a rocker. The beads were washed three times in 0.2 M sodium borate (pH 9.0), resuspended in 10 ml of PBS–0.05% sodium azide, and stored at 4°C.

The coupled beads were loaded onto Econo-Pac columns (Bio-Rad), and sodium azide was removed by washing the column several times with PBS. The column was then equilibrated with 10 column volumes of elution buffer (3.5 M sodium thiocyanate) to remove uncoupled IgG. The columns were washed several times with PBS, and periplasmic extract isolated from a 500-ml induced culture was applied to the column at 4°C. Nonspecifically bound proteins were

washed off with 200 ml of PBS–1 mM AEBSF. Finally, bound Fab was eluted with 8 column volumes of 3.5 M NaSCN and desalted with Centriprep 30 ultrafiltration devices (Amicon).

Determination of soluble Fab concentration. Wells of a 96-well Maxisorp plate were coated overnight at 4°C with 1 μg of $F(ab')_2$ goat anti-human $F(ab')_2$ (Jackson Immunoresearch). After eight washes with TBS, the wells were blocked as described above. Various dilutions of each Fab preparation, as well as known concentrations of a purified human Fab standard (Biodesign International), were then loaded. Bound Fab was detected as described above.

Screening soluble Fabs for antigen specificity. Wells of a 96-well Maxisorp plate were coated overnight at 4°C with 1 μg of the antigen of interest in a 150-μl volume. After being washed eight times with TBS, the wells were blocked for 1 h at 37°C with 0.5% nonfat dry milk–0.5% Tween 20–TBS. Blocking solution was then removed, and the Fabs were incubated in the appropriate wells for 1 h at 37°C. Unbound Fab was removed by eight washes with TBS. Bound Fab was then detected by incubating the wells for 1 h at 37°C with a 1:50,000 dilution of horseradish peroxidase-conjugated $F(ab')_2$ goat anti-human $F(ab')_2$ (Jackson Immunoresearch). After eight TBS washes, TMB one-component substrate solution (KPL) was added. The reactions were terminated after 20 min by the addition of 2/3 N H_2SO_4, and the A_{450} was read on a Dynatech MR5000 enzyme-linked immunosorbent assay (ELISA) reader.

Cell staining. For the HIV-1 rgp120 pulsing experiments, cells were preincubated at 37°C for 1 h with either PBS or 100 μg of recombinant gp120 (rgp120; $HIV\text{-}1_{SF2}$ strain) per ml. After being washed with PBS, these cells were incubated for 20 min on ice with the indicated Fabs or monoclonal antibodies at a concentration of 2 μg/ml and washed again with PBS. The cells were then incubated at 4°C for 20 min with a 1:100 dilution of either fluorescein isothiocyanate (FITC)-conjugated $F(ab')_2$ goat anti-human $F(ab')_2$ (Jackson Immunoresearch) or FITC-conjugated $F(ab')_2$ goat anti-mouse $F(ab')_2$ (Jackson Immunoresearch). Nonspecifically bound antibodies were removed by washing the cells with PBS.

In the virus-binding experiments, H9 cells were incubated overnight at 37°C with infectious recombinant $HIV\text{-}1_{MN}$ (20 ng of p24 per 1.5×10^6 cells) in the presence of the indicated Fab preparations or monoclonal antibodies at a concentration of 2 μg/ml. After these cells were washed with PBS, bound antibodies or Fabs were detected by incubating the cells with FITC-labelled secondary antibodies, as described above.

The cross-competition studies were performed by incubating freshly isolated human PBL with medium alone or with $HIV\text{-}1_{SF2}$ rgp120, as described above. These cells were then washed with PBS and incubated with PBS or Fab clone 3-47 at 10 μg/ml for 20 min on ice. The cells were washed with PBS, and the samples were incubated with the indicated monoclonal antibodies at 10 μg/ml on ice for 20 min. These cells were then washed with PBS. Bound antibodies were detected as described above.

For each of these experiments, stained cells were fixed in 1% formalin–PBS and analyzed on an EPICS XL-MCL flow cytometer (Coulter Corp.). As a negative control, an indirect stain substituting PBS for the primary antibody was performed.

Biotin labelling of H9 surface proteins. H9 cells (3×10^7) were washed three times with PBS-Plus (PBS, 0.1 mM $CaCl_2$, 0.1 mM $MgCl_2$) and resuspended in 3 ml of 1-mg/ml Sulfo-NHS-Biotin (Pierce)/PBS-Plus. After being incubated for 1 h at 4°C with gentle agitation, the cells were washed once with RPMI 1640 and then three times with PBS-Plus.

Immunoprecipitation of biotinylated H9 proteins. The biotinylated H9 cell pellet was lysed in Triton X-100 lysis buffer (300 mM NaCl, 50 mM Tris-HCl [pH 7.6], 0.5% Triton X-100, 10 μg of leupeptin per ml, 10 μg of aprotinin per ml, 1 mM phenylmethylsulfonyl fluoride, 1.8 mg of iodoacetamide per ml) on ice for 45 min with occasional mixing. After cell debris was pelleted in a microcentrifuge for 15 min at 4°C, the supernatant was transferred to a 1.5-ml Eppendorf tube. This lysate was then incubated for 1 h at 4°C with 300 μl of a 50% suspension of Gammabind G beads (Pharmacia)–PBS with gentle agitation. These samples were centrifuged in a microcentrifuge for 1 min. The precleared lysate was then incubated overnight with the indicated antibody or Fab preparation at 15 μg/ml. These samples were incubated with 200 μl of a 75% suspension of goat anti-human IgG, $F(ab')_2$-conjugated Gammabind G beads–PBS for 1 h at 4°C with shaking. The beads were then pelleted as described above, washed three times with high-salt wash buffer (0.5 M NaCl, 20 mM Tris-HCl, 1 mM EDTA, 1% sodium deoxycholate, 0.5% Nonidet P-40, 30% sucrose), and then washed twice with low-salt wash buffer (10 mM NaCl, 10 mM Tris-HCl [pH 7.6]).

Immunoprecipitated proteins were eluted from the beads in nonreducing sample buffer (60 mM Tris-HCl [pH 6.8], 25% glycerol, 2% sodium dodecyl sulfate [SDS], 0.1% bromophenol blue) at 95°C for 10 min and loaded onto an SDS–10% polyacrylamide gel. After electrophoresis these proteins were transferred overnight to nitrocellulose. The membrane was blocked with 5% BSA–TBST (100 mM Tris-HCl [pH 7.5], 0.9% NaCl, 0.1% Tween 20) and probed with 2 μg of horseradish peroxidase-conjugated avidin (Pierce)–0.3% BSA–TBST per ml to detect biotinylated proteins. After extensive washing with TBST, biotinylated proteins were visualized by the enhanced chemiluminescence detection system (Amersham).

TABLE 1. ELISA reactivity of selected Fab clones with recombinant antigens[a]

Antigen	Reactivity (OD_{450}) of Fab clone:		
	2-6	2-36	3-47
Human rsCD4	2.411	2.433	2.368
$HIV\text{-}1_{SF2}$ rgp120	0.284	0.254	0.253

[a] Human rsCD4 or $HIV\text{-}1_{SF2}$ rgp120 (1 μg) was adsorbed onto ELISA wells. Affinity-purified Fab preparations were incubated in these wells and detected with a horseradish peroxidase-labelled secondary antibody.

RESULTS

Generation of a human recombinant Fab library from the bone marrow of a human rsCD4-immunized, HIV-1-infected individual. To characterize the antibodies elicited by human rsCD4 immunization of an HIV-1-infected human, we produced a human combinatorial Fab library. Ig heavy- and light-chain fragments, encompassing the entire variable domain and first constant region of each chain, were amplified by PCR from the bone marrow of this immunized individual and cloned into the phagemid pCOMB3 (Fig. 1). This vector directs the expression of Ig heavy- and light-chain fragments in the periplasmic space of a bacterial cell, where these chains associate to form recombinant Fabs. Since the heavy chain is expressed as a fusion protein with the M13 bacteriophage coat protein encoded by gene III, infection of the transformed bacterial cells with an M13 helper phage results in the assembly of bacteriophage particles that express recombinant Fab molecules on their surface. Antigen-specific Fab clones can be selected from this library by panning the recombinant Fab-expressing bacteriophage over the antigen of interest. The combinatorial library constructed from the bone marrow of this human rsCD4-immunized individual consisted of 3×10^6 members.

Selection of human rsCD4-specific recombinant Fab clones from the combinatorial Fab library. To select for novel human rsCD4-specific Fab clones, the library was panned against complexes of human rsCD4 and $HIV\text{-}1_{SF2}$ rgp120. After three rounds of panning, we achieved a twofold enrichment for human rsCD4-specific Fab clones as determined by the percent yield of phage. Thirty-five recombinant Fab clones from this enriched library were selected for further analysis. Soluble Fab-expressing clones were generated by removing the M13 bacteriophage gene III, which encodes the coat protein responsible for retention of the recombinant Fab on the phage surface. Soluble Fab expression was then induced from transformed bacterial cells by the addition of IPTG. Soluble Fab was isolated from the induced bacterial cells by osmotic shock and purified on goat anti-human IgG, $F(ab')_2$ affinity columns. The purified Fabs were then tested for ELISA reactivity with both human rsCD4 and $HIV\text{-}1_{SF2}$ rgp120. Of the 35 panned clones studied, 7 which bound to human rsCD4 but not to $HIV\text{-}1_{SF2}$ gp120 (selected data are given in Table 1) were identified. Three of these human rsCD4-specific Fabs were selected for further analysis.

Fab clone 3-47 binds to HIV-1 rgp120-preincubated cells but not to untreated CD4-positive cells. We next sought to determine whether we could identify human rsCD4-specific Fab clones which recognized cell surface-expressed CD4. Interestingly, none of the three human rsCD4-specific Fab clones tested bound to native CD4 expressed on the surface of H9 cells (Fig. 3). In an attempt to identify a biologically relevant form of CD4 which these Fab clones might recognize, we next assessed whether any of these Fabs, selected by panning the

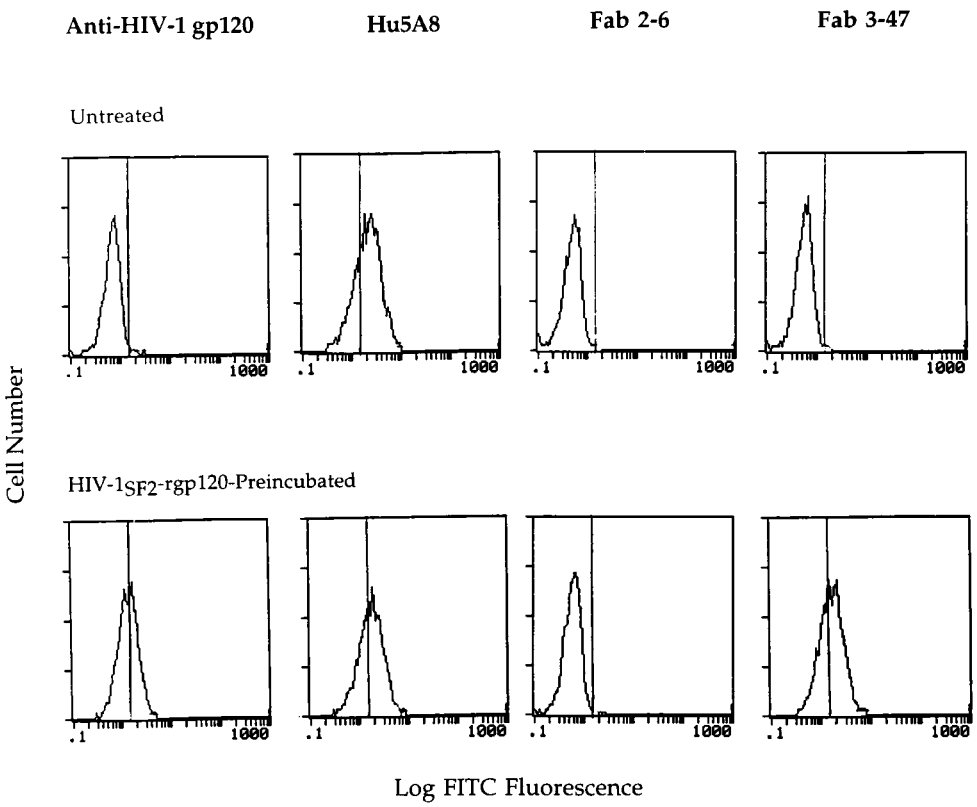

FIG. 3. Fab clone 3-47 reactivity with cell surface-expressed CD4 on untreated and HIV-1_{SF2} rgp120-pretreated H9 cells. The H9 cells were incubated with medium alone or with HIV-1_{SF2} rgp120 and then with the indicated antibodies or Fab fragments. Bound antibodies were detected by incubating these cells with a FITC-conjugated goat anti-human IgG, F(ab′)$_2$. The cells were analyzed by flow cytometry. The data are also expressed as the percent HIV-1 rgp120-preincubated cells specifically bound by antibody: Hu5A8, 65%; anti-HIV-1 gp120, 46%; Fab clone 3-47, 60%.

library over human rsCD4/HIV-1 envelope complexes, recognized cell surface-expressed CD4 complexed with recombinant HIV-1 envelope protein. As shown in Fig. 3, one of these human rsCD4-specific Fab clones (Fab clone 3-47) bound to H9 cells after these cells had been incubated for 1 h at 37°C with HIV-1_{SF2} rgp120. In fact, Fab clone 3-47 exhibited the same binding specificity on primary cells, recognizing freshly isolated human PBL only after preincubation of these cells with HIV-1_{SF2} rgp120 (Fig. 4).

Fab clone 3-47 binds to H9 cells preincubated with HIV-1 virions. On the basis of these observed binding characteristics, we postulated that Fab clone 3-47 recognized an epitope of CD4 which is not exposed on the native cell surface-expressed molecule but is revealed on the cell surface after HIV-1 envelope protein binding. While we had demonstrated that this CD4 epitope was exposed upon binding of a recombinant form of the HIV-1 envelope protein, it was important to show that the exposure of the same epitope could be induced upon binding of HIV-1 particles to cell surface-expressed CD4. H9 cells were incubated overnight at 37°C with HIV-1_{MN} virions in the presence or absence of purified Fabs. Bound Fabs were detected by incubating these cells with a FITC-labelled goat anti-human IgG, F(ab′)$_2$. As demonstrated in Fig. 5, Fab clone 3-47 specifically bound to the virus-preincubated cells.

Fab clone 3-47 does not recognize native HIV-1 envelope proteins expressed on the surface of chronically infected H9 cells. While the above observations suggested that Fab clone 3-47 recognized a conformationally altered CD4 molecule induced upon HIV-1 binding, it was important to confirm that this Fab, generated from an HIV-1-infected human, did not react with the HIV-1 envelope protein. Although this Fab clone did not recognize HIV-1_{SF2} rgp120, as determined by ELISA, it was possible that the Fab recognized cell surface-expressed HIV envelope proteins. The CD4 molecule is downmodulated off the surface of cells productively infected with HIV-1 (7). Therefore, we reasoned that Fab clone 3-47, if specific for the CD4 molecule, should not bind to cells chronically infected with HIV-1. By flow cytometry, we demonstrated that Fab clone 3-47 does not recognize native HIV-1 envelope proteins expressed on the surface of chronically infected H9 cells (data not shown).

Fab clone 3-47 immunoprecipitates cellular CD4. Since Fab clone 3-47 had been selected by panning the combinatorial Fab library over human rsCD4/HIV-1_{SF2} rgp120 complexes, it was possible that this Fab was specific for a neoepitope defined by domains of both human CD4 and HIV-1 envelope protein. To rule out this possibility, we attempted to identify a form of cellular CD4 which is recognized by Fab clone 3-47.

Since Fab clone 3-47 had been generated from an individual immunized with rsCD4, which probably assumes a tertiary structure different from that of membrane-expressed CD4, we were interested in determining whether the epitope recognized by Fab clone 3-47 was exposed on detergent-solubilized cellular CD4. As shown in Fig. 6, Fab clone 3-47 specifically immunoprecipitated a 55-kDa protein, corresponding to the molecular mass of cellular CD4, from H9 cells. Thus, although Fab 3-47 bound to cell surface-expressed CD4 only following HIV-1 envelope binding, this Fab did recognize solubilized cellular CD4 in the absence of added HIV-1 gp120.

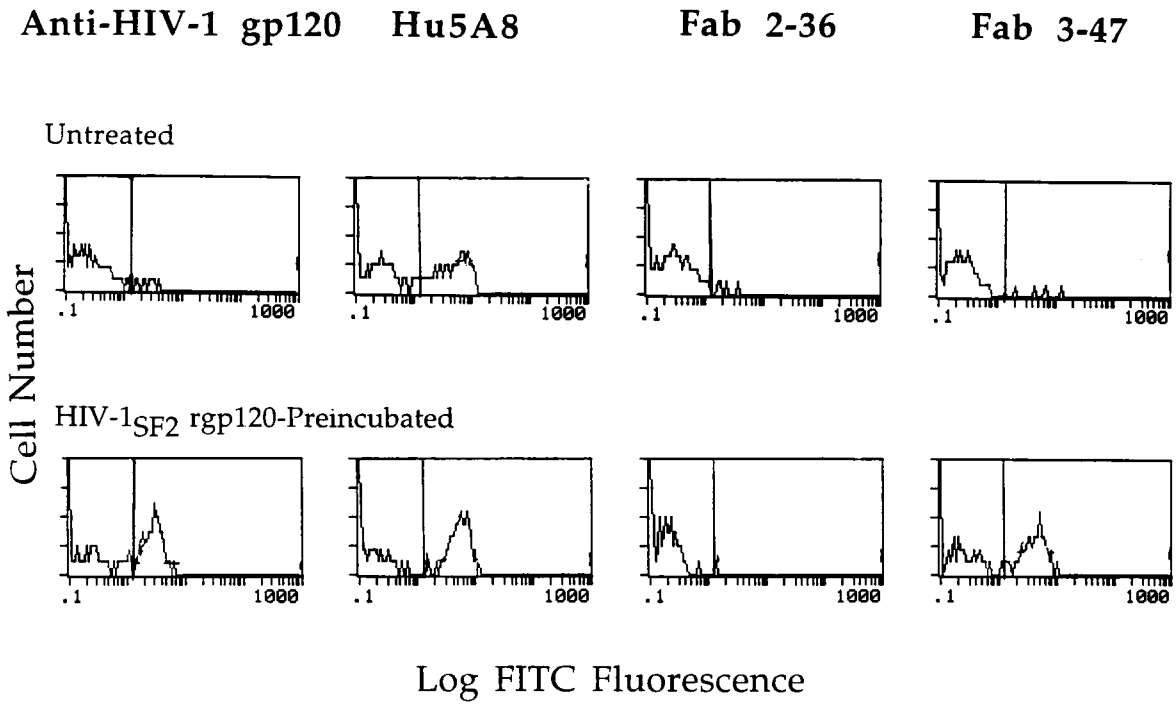

FIG. 4. Reactivity of Fab clone 3-47 with cell surface-expressed CD4 on untreated and HIV-1_{SF2} rgp120-pretreated human PBL. Cells were incubated with either medium alone or HIV-1_{SF2} rgp120, followed by the indicated primary antibodies or Fab preparations. Bound antibodies were detected with a FITC-labelled secondary antibody. The cells were analyzed by flow cytometry. The data are also expressed as the percent HIV-1 rgp120-preincubated cells specifically bound by antibody: Hu5A8, 58%; anti-HIV-1 gp120, 49%; Fab 3-47, 54%.

Competition studies do not identify the domain of CD4 recognized by Fab clone 3-47. Having confirmed that Fab clone 3-47 was CD4 specific, we were interested in mapping the location of this epitope within the four-domain structure of CD4. Therefore, we performed competition studies between Fab clone 3-47 and a panel of monoclonal antibodies specific for different domains of CD4. Human PBL were pulsed with HIV-1 recombinant envelope protein and incubated sequentially with Fab clone 3-47 followed by a CD4-specific murine monoclonal antibody. Fab clone 3-47 did not compete with monoclonal antibodies specific for CD4 domains 2, 3, and 4 for binding to HIV-1_{SF2} rgp120-preincubated human PBL (Fig. 7).

DISCUSSION

To access and characterize novel CD4-specific monoclonal antibodies, we have constructed a recombinant Fab library from the bone marrow of a human rsCD4-immunized, HIV-1-infected individual. Although the production of hybridomas has proven useful in the generation of murine monoclonal antibodies, this technology has not been perfected for the synthesis of human monoclonal antibodies. The construction of combinatorial Fab libraries is an important alternative to the generation of hybridomas, providing a powerful tool for the selection of numerous antibody clones with high affinity for an antigen of interest. This approach has proven valuable in the characterization of antibody responses to various antigens (2, 22, 26).

The pCOMB3 phagemid has become the favored cloning vehicle for the production of combinatorial Fab libraries. This vector directs the periplasmic expression of an Ig light-chain fragment and a protein consisting of Ig heavy-chain sequences fused to the membrane-spanning region of a bacteriophage coat protein. This fusion protein allows Fab molecules to be incorporated onto the surface of M13 bacteriophage particles which are produced after these transformed cells are infected with an M13 helper phage. One can select for a subpopulation of clones with high affinity for an antigen of interest from such libraries by performing repeated rounds of bacteriophage panning over this antigen.

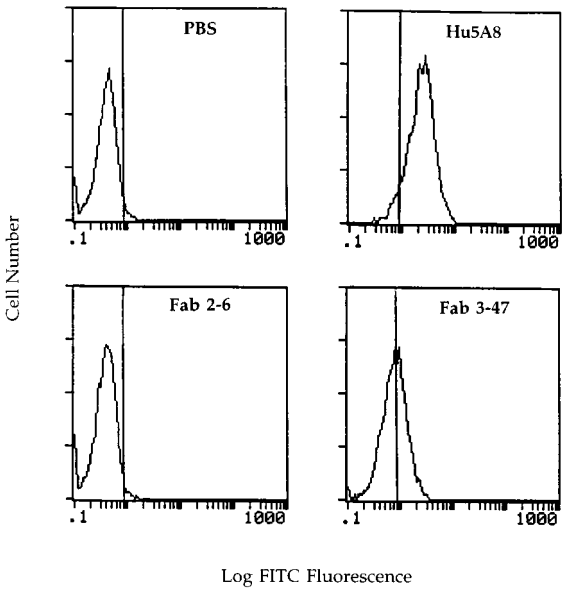

FIG. 5. Fab clone 3-47 reactivity with H9 cells preincubated with HIV-1 virions. H9 cells were incubated with live HIV-1_{MN}. The cells were then washed with PBS and incubated with the indicated monoclonal antibodies or Fab clones. Bound antibodies were detected with a FITC-labelled secondary antibody, and the cells were analyzed by flow cytometry. The data are also expressed as the percent virus-preincubated cells bound by antibody: Hu5A8, 86%; Fab 3-47, 52%.

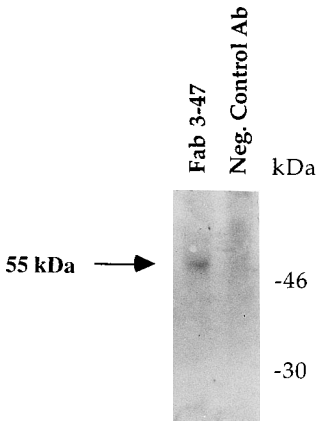

FIG. 6. Reactivity of Fab clone 3-47 with solubilized cellular CD4. H9 cells were labelled with Sulfo-NHS biotin. These cells were then lysed in Triton X-100 lysis buffer. Cellular proteins were immunoprecipitated with the indicated antibody or Fab clone. The humanized CD8-specific antibody M-T807 was used as a negative control antibody (Neg. Control Ab) in these experiments. Immunoprecipitated proteins were transferred to nitrocellulose and probed with horseradish peroxidase-conjugated avidin. Biotinylated proteins were then detected by chemiluminescence.

In addition to allowing for the rapid generation of numerous antigen-specific Fab clones, the production of large Fab libraries in the pCOMB3 vector increases the probability of accessing antibodies represented at a low frequency in the Ig repertoire of an individual. Although human rsCD4-specific antibodies were elicited in this study by immunization of a human with a recombinant self protein, the titer of these antibodies was lower than might be expected following immunization with a foreign antigen. Because of the low frequency of B cells secreting CD4-specific antibodies in this immunized individual, a monoclonal antibody with the specificity of Fab clone 3-47 may never have been identified by B-cell immortalization and/or fusion techniques. We were interested in determining whether antibodies specific for a conformationally altered form of the CD4 molecule, induced upon HIV binding, were represented in the Ig repertoire of this human rsCD4-immunized individual. To optimize the likelihood of accessing Fab fragments which demonstrate a greater affinity for CD4 complexed with the HIV envelope protein than for CD4 alone, we panned this recombinant Fab library over complexes of the HIV-1 envelope protein and human rsCD4. The generation of Fab clone 3-47 from this panned library demonstrates the power of this cloning technology in gaining access to high-affinity, low-frequency antibodies.

Ig heavy- and light-chain fragments combine randomly in the pCOMB3 vector. Therefore, one potential drawback of using the recombinant Fab cloning technology to characterize the antibody specificities existing in an individual is that Fab specificities which do not exist in the immunized subject may be represented in the library. Thus, we cannot be certain that the CD4-specific Fab clone 3-47 is truly representative of the antibody specificities present in the Ig repertoire of this human rsCD4-immunized individual. However, others have reported that Fabs with high affinity for a given antigen can be accessed

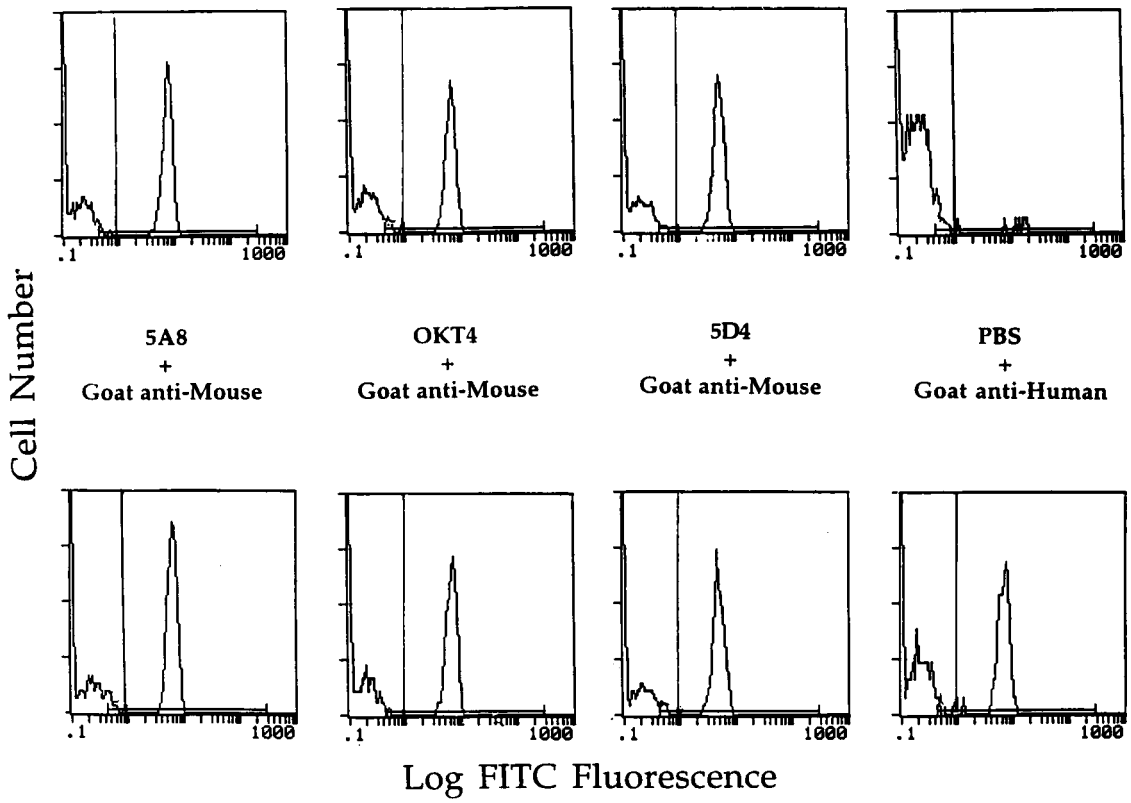

FIG. 7. Cross competition between Fab clone 3-47 and a panel of CD4-specific monoclonal antibodies for binding to HIV-1_{SF2} rgp120-preincubated human PBL. Freshly isolated human PBL were incubated with HIV-1 $rgp120_{SF2}$. These cells were then incubated with PBS (top four panels) or Fab clone 3-47 (bottom four panels). Finally, the indicated monoclonal antibodies were incubated with these cells. Bound antibodies were detected with the indicated FITC-conjugated secondary antibodies. These samples were analyzed by flow cytometry.

from combinatorial libraries only if a measurable antibody response against this antigen is elicited in the immunized individual (18, 20). These studies argue against the probability of accessing Fab clones consisting of artifactual heavy- and light-chain combinations. Moreover, Fab clones with binding specificities similar to that of clone 3-47 were represented at a high frequency in the panned library generated from this individual (unpublished results). These observations suggest that B cells secreting an antibody with the same specificity as Fab clone 3-47 were probably present in this immunized individual at the time of library construction.

We believe that this antibody specificity was raised upon human rsCD4 immunization of this HIV-1-infected individual. Although CD4-specific autoantibodies have been reported to exist in the serum of approximately 10% of AIDS patients (15), the serum antibodies of this human rsCD4-immunized individual demonstrated reactivity with human rsCD4 only after immunization (unpublished results). This observation suggests that Fab clone 3-47 is not representative of an autoantibody produced by this patient prior to rsCD4 immunization. The elicitation of this antibody specificity by immunization with a recombinant soluble form of a self protein suggests that the rsCD4 molecule assumes a tertiary structure different from that of cell surface-expressed CD4.

The binding specificity of Fab clone 3-47 provides compelling evidence that HIV-1 induces a conformational change in cell surface-expressed CD4. This conformational change is not induced upon the binding of other CD4 domain one ligands, since antibodies specific for this domain do not induce the exposure of the Fab 3-47 epitope (data not shown). Previous studies have raised the possibility that a conformational change in the HIV receptor occurs following virus binding. Healey et al. (11) described an antibody raised in a mouse by human rsCD4 immunization that exhibited a greater affinity for human rsCD4–HIV-1 rgp120 complexes than for human rsCD4 alone. Monoclonal antibodies with a similar binding specificity have also been generated by immunizing mice with human rsCD4–HIV-1 rgp120 complexes (6, 8). While these studies demonstrated that recombinant gp120 binding to recombinant CD4 can induce rsCD4 conformational alterations, they did not address whether such changes occur in cell surface-expressed CD4 following HIV binding.

On the basis of our knowledge of cell surface receptors which undergo conformational changes upon ligand binding, it is interesting to speculate on the physical nature of the CD4 conformational change defined by Fab clone 3-47. Ligand-induced alterations in the oligomeric state of their respective receptors have been described for numerous receptors, including the platelet-derived growth factor receptor (12). Such changes are often accompanied by conformational alterations in these receptors. Although X-ray crystallography studies of rsCD4 have not indicated that this molecule tends to oligomerize (5, 16, 23), proteins which bind to cell surface-expressed CD4, such as the tyrosine kinase lck, may facilitate the formation of CD4 multimers. Direct evidence for the existence of CD4 oligomers was recently provided by the observation that the interaction of major histocompatibility complex class II-expressing cells with CD4 is dependent on the expression of CD4 oligomers on the cell membrane (21a). It is possible that the binding of HIV to CD4-positive cells alters the oligomeric state of CD4 on the cell membrane. The conformationally altered form of cell surface-expressed CD4 which is recognized by Fab clone 3-47 may represent an intermediate or final configuration of CD4 as it undergoes such alterations in its oligomeric state. Alternatively, this HIV-induced CD4 conformational change may reflect changes in the interaction of CD4 with other cell surface proteins, such as the T-cell receptor.

Numerous growth factor-induced conformational changes in receptor molecules have been shown to result in the activation of signal transduction cascades. Since the CD4 molecule is known to play a critical role in T-cell receptor-mediated signal transduction and cellular activation (21), it is possible that this HIV-induced conformational change alters the activation state of a cell during viral entry. In fact, it has been demonstrated that HIV binding to cell surface-expressed CD4 results in the translocation of NF-κB to the nucleus of a cell (4). This cellular activation was shown to be a direct consequence of virus binding to CD4 and was not dependent on virus replication. It is possible that the virus-induced CD4 conformational change described in the present article plays a role in such signalling through the CD4 molecule.

These studies indicate that the CD4 receptor undergoes a conformational change upon HIV-1 binding. The elucidation of the consequences of this conformational change on the host cell and its importance in postbinding steps of HIV infection may provide novel targets for the design of antiviral therapeutic agents.

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REFERENCES

1. **Arthos, J., K. C. Deen, M. A. Chaikin, J. A. Fornwald, G. Sathe, Q. J. Sattentau, P. R. Clapham, R. A. Weiss, J. S. McDougal, C. Pietropaolo, R. Axel, A. Truneh, P. J. Maddon, and R. W. Sweet.** 1989. Identification of the residues in human CD4 critical for the binding of HIV. Cell **57:**469–481.
2. **Barbas, C. F., J. E. Crowe, D. Cababa, T. M. Jones, S. L. Zebedee, B. R. Murphy, R. M. Chanock, and D. R. Burton.** 1992. Human monoclonal Fab fragments derived from a combinatorial library bind to respiratory syncytial virus F glycoprotein and neutralize infectivity. Proc. Natl. Acad. Sci. USA **89:**10164–10168.
3. **Benkirane, M., P. Corbeau, V. Housset, and C. Devaux.** 1993. An antibody that binds the immunoglobulin CDR3-like region of the CD4 molecule inhibits provirus transcription in HIV-infected T cells. EMBO J. **12:**4909–4921.
4. **Benkirane, M., K. Jeang, and C. Devaux.** 1994. The cytoplasmic domain of CD4 plays a critical role during the early stages of HIV infection in T-cells. EMBO J. **13:**5559–5569.
5. **Brady, R. L., E. J. Dodson, G. G. Dodson, G. Lange, S. J. Davis, A. F. Williams, and A. N. Barclay.** 1993. Crystal structure of domains 3 and 4 of rat CD4: relation to the NH_2-terminal domains. Science **260:**979–983.
6. **Celada, F., C. Cambiaggi, J. Maccari, S. Burastero, T. Gregory, E. Patzer, J. Porter, C. McDanal, and T. Matthews.** 1990. Antibody raised against soluble CD4-rgp120 complex recognizes the CD4 moiety and blocks membrane fusion without inhibiting CD4-gp120 binding. J. Exp. Med. **172:**1143–1150.
7. **Dalgeish, A. G., P. C. Beverly, P. R. Clapham, D. H. Crawford, M. F. Greaves, and R. A. Weiss.** 1984. The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. Nature (London) **312:** 763–767.
8. **Gershoni, J. M., G. Denisova, D. Raviv, N. I. Smorodinsky, and D. Buyaner.** 1993. HIV binding to its receptor creates specific epitopes for the CD4/gp120 complex. FASEB J. **7:**1185–1187.
9. **Golding, H., R. Blumenthal, J. Manischewitz, D. R. Littman, and D. S. Dimitrov.** 1993. Cell fusion mediated by interaction of a hybrid CD4.CD8

molecule with the human immunodeficiency virus type 1 envelope glycoprotein does occur after a long lag time. J. Virol. **67:**6469–6475.

10. **Hasunuma, T., H. Tsubota, M. Watanabe, Z. Chen, C. Lord, L. Burkly, J. Daley, and N. L. Letvin.** 1992. Regions of the CD4 molecule not involved in virus binding or syncytia formation are required for HIV-1 infection of lymphocytes. J. Immunol. **148:**1841–1846.
11. **Healey, D., L. Dianda, J. P. Moore, J. S. McDougal, M. J. Moore, P. Estess, D. Buck, P. D. Kwong, P. C. Beverly, and Q. J. Sattentau.** 1990. Novel anti-CD4 monoclonal antibodies separate human immunodeficiency virus infection and fusion of CD4+ cells from virus binding. J. Exp. Med. **172:** 1233–1242.
12. **Heldin, C. H., A. Ernlund, C. Rorsman, and L. Ronnstrand.** 1989. Dimerization of B-type platelet-derived growth factor receptors occurs after ligand binding and is closely associated with receptor kinase activation. J. Biol. Chem. **264:**8905–8912.
13. **Helseth, E., M. Kowalski, D. Gabuzda, U. Olshevsky, W. Haseltine, and J. Sodroski.** 1990. Rapid complementation assays measuring replicative potential of human immunodeficiency virus type 1 envelope glycoprotein mutants. J. Virol. **64:**2416–2420.
14. **Klatzmann, D., E. Champagne, S. Chamaret, J. Gruest, D. Guetard, T. Hercend, J. C. Gluckman, and L. Montagnier.** 1984. T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV. Nature (London) **312:**767–768.
15. **Kowalski, M., B. Ardman, L. Basiripour, Y. C. Lu, D. Blohm, W. Haseltine, and J. Sodroski.** 1989. Antibodies to CD4 in individuals infected with human immunodeficiency virus type 1. Proc. Natl. Acad. Sci. USA **86:**3346–3350.
16. **Kwong, P. D., S. E. Ryu, W. A. Hendrickson, R. Axel, R. M. Sweet, G. F. Wasserman, P. Hensley, and R. W. Sweet.** 1990. Molecular characteristics of recombinant human CD4 as deduced from polymorphic crystals. Proc. Natl. Acad. Sci. USA **87:**6423–6427.
17. **Maddon, P. J., D. R. Littman, M. Godfrey, D. E. Maddon, L. Chess, and R. Axel.** 1985. The isolation and nucleotide sequence of a cDNA encoding the T cell surface protein T4: a new member of the immunoglobulin gene family. Cell **42:**93–104.
18. **Marks, J. D., H. R. Hoogenboom, T. P. Bonnert, J. McCafferty, A. D. Griffiths, and G. Winter.** 1991. By-passing immunization. Human antibodies from V-gene libraries displayed on phage. J. Mol. Biol. **222:**581–597.
19. **Moore, J. P., Q. J. Sattentau, P. J. Klasse, and L. C. Burkly.** 1992. A monoclonal antibody to CD4 domain 2 blocks soluble CD4-induced conformational changes in the envelope glycoproteins of human immunodeficiency virus type 1 (HIV-1) and HIV-1 infection of $CD4^+$ cells. J. Virol. **66:**4784–4793.
20. **Persson, M. A., R. H. Caothien, and D. R. Burton.** 1991. Generation of diverse high-affinity human monoclonal antibodies by repertoire cloning. Proc. Natl. Acad. Sci. USA **88:**2432–2436.
21. **Portoles, P., J. Rojo, A. Golby, M. Bonneville, S. Gromkowski, L. Greenbaum, C. A. Janeway, Jr., D. B. Murphy, and K. Bottomly.** 1989. Monoclonal antibodies to murine CD3 epsilon define distinct epitopes, one of which may interact with CD4 during T cell activation. J. Immunol. **142:**4169–4175.

21a. **Reinherz, E.** Personal communication.

22. **Roben, P., J. P. Moore, M. Thali, J. Sodroski, C. F. Barbas, and D. R. Burton.** 1994. Recognition properties of a panel of human recombinant Fab fragments to the CD4 binding site of gp120 show differing abilities to neutralize human immunodeficiency virus type 1. J. Virol. **68:**4821–4828.
23. **Wang, J., Y. Yan, T. P. J. Garrett, J. Liu, D. W. Rodgers, R. L. Garlick, G. E. Tarr, Y. Husain, E. L. Reinherz, and S. C. Harrison.** 1990. Atomic structure of a fragment of human CD4 containing two immunoglobulin-like domains. Nature (London) **348:**411.
24. **Watanabe, M., J. E. Boyson, C. I. Lord, and N. L. Letvin.** 1992. Chimpanzees immunized with recombinant soluble CD4 develop anti-self CD4 antibody responses with anti-human immunodeficiency virus activity. Proc. Natl. Acad. Sci. USA **89:**5103–5107.
25. **Watanabe, M., Z. W. Chen, H. Tsubota, C. I. Lord, C. G. Levine, and N. L. Letvin.** 1991. Soluble human CD4 elicits an antibody response in rhesus monkeys that inhibits simian immunodeficiency virus replication. Proc. Natl. Acad. Sci. USA **88:**120–124.
26. **Williamson, R. A., R. Burioni, P. P. Sanna, L. J. Partridge, C. F. Barbas, and D. R. Burton.** 1993. Human monoclonal antibodies against a plethora of viral pathogens from single combinatorial libraries. Proc. Natl. Acad. Sci. USA **90:**4141–4145.