Eur. J. Biochem. **270**, 4497–4506 (2003) © FEBS 2003 doi:10.1046/j.1432-1033.2003.03843.x

Identification of CD4 and transferrin receptor antibodies by CXCR4 antibody-guided Pathfinder selection

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To generate human antibodies against CXCR4, a seven-transmembrane chemokine receptor and a principal coreceptor for HIV-1, several rounds of Pathfinder and Step-back selection from a large phage display antibody library were performed on Jurkat cells. A mAb against CXCR4 or biotinyated phage antibodies were used as guide molecules. Over 100 pan-Jurkat-cell-positive antibodies were characterized, but none were CXCR4 specific. However, several antibodies against CD4 and the transferrin receptor were identified. Our results indicate that, although Pathfinder and Step-back selection can be used to select phage antibodies on whole cells, the successful selection of certain targets is still complex and limited. The reason is probably, in part, due to the inaccessibility of the targeted extracellular structures and the range of the horseradish peroxidase-labeled guide molecule. Refinements of these techniques are required to improve target specificity and selectivity.

Keywords: CXCR4; Pathfinder selection; phage display; scFv; transferrin receptor.

Naïve and nonimmune antibody phage libraries are a powerful tool that enables the selection and identification of antibodies to purified antigens [1–3]. In some cases, in which potential therapeutic or research antibodies against cell surface antigens are required, phage screening is performed on whole cells or cell membranes. However, cell based screening is often difficult because of the much greater antigen complexity, lower antigen concentration and antigen inaccessibility. Some selection methods using whole cells have been developed to allow isolation of antibodies against certain cell surface antigens [4–8]. One strategy, called Pathfinder, has been shown to be more efficient for whole cell selection [9]. In brief, an existing ligand or antibody against a target molecule on cells is used to guide selection and recovery of only those phage antibodies (PhAbs) that bind close to the target antigen. In this way, the epitopes on the target recognized by the selected PhAbs will be different from the epitope recognized by the cell-surface-bound guide molecule. A Step-back selection was also developed to isolate antibodies against the particular epitope occupied by the original guide molecule by performing a second round of Pathfinder selection using the output phage from the first round as a guide [10]. The combined techniques provide a means of relatively specific selection of antibodies with the desired binding characteristics on intact cells.

The chemokine receptor CXCR4, a seven-transmembrane G-protein-coupled receptor, has been the focus of much interest and examination. This is because of not only its important role and its sole ligand stromal-cell-derived factor-1 (SDF-1) in lymphocyte trafficking, hematopoiesis, and developmental processes [11], but also the crucial part it plays as a coreceptor in T-tropic HIV infection and may play as a chemokine/chemokine receptor cofactor in the metastasis of breast cancer [12,13]. CXCR4, as a member of the serpentine family of G-protein-coupled chemokine receptors, is generally functional in its native conformation, and purification from the cell membrane typically results in loss of its native conformation. It has also been reported that distinct conformations of CXCR4 exist between cell types, which can be differentially recognized by CXCR4 mAbs [14]. The conformational heterogeneity of CXCR4 explains the cell-type-dependent ability of CXCR4 mAb to block the chemotaxis to SDF-1 and inhibit HIV-1 infection. To date, only one CXCR4 mouse mAb, 12G5, has been extensively studied. Its inhibition of HIV-1 infection is dependent on the cell type and virus strain. No human mAbs against CXCR4 have been reported [15–17]. In this study, we sought to identify human antibodies that would preferentially recognize native conformational epitopes of CXCR4 and potentially neutralize X4-tropic HIV-1 entry as a first step toward developing therapeutic mAbs against CXCR4 for passive immunotherapy of HIV-1 infection. We reasoned that the most effective way to identify such antibodies would be selection on CXCR4-expressing intact cells with a large nonimmune phage library. The Pathfinder and Step-back PhAb selection strategies were used to pan our large nonimmune 1.5×10^{10} phage display human single chain (scFv) library by targeting the CXCR4 receptor on Jurkat cells using the mouse mAb 12G5 as the guide

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Abbreviations: FACS, fluorescence-activated cell sorter; HRP, horseradish peroxidase; PBL, primary peripheral blood lymphocyte; PhAb, phage antibody; scFv, single-chain antibody variable fragment; SDF-1, stromal cell-derived factor-1; TfR, human transferrin receptor.

(Received 10 July 2003, revised 11 September 2003, accepted 19 September 2003)

molecule. Extensive characterization of selected scFvs showed that none of them were CXCR4 specific, although antibodies against CD4 and transferrin receptor (TfR, CD71) were identified. Some of the limitations of these selection technologies are discussed.

Materials and methods

Cell lines and cell culture

The following cell lines, obtained from the American Type Culture Collection (ATCC), were used: Jurkat E6-1 (human acute T lymphocyte leukemia), U937 (human promyleoid cell line), HL-60 (human acute promyelocytic leukemia), Raji (human Burkitt's lymphoma), KG1a (human acute myelogenous leukemia) and Cf2Th (dog normal thymus). The following cell lines were obtained from the National Institutes of Health AIDS Research and Reference Reagent Program: Hut78 (human T-cell line), CEMX174 (hybrid human B-cell-T-cell line) and HeLa (human cervical carcinoma). All of the above cell lines were maintained according to the supplier's recommendations. Primary peripheral blood lymphocytes (PBLs) were purified on Ficoll-Hypaque gradients and kept with interleukin 2 (100 $U \cdot mL^{-1}$) and phytohemagglutinin (5 $\mu g \cdot mL^{-1}$) in RPMI 1640 containing 10% fetal bovine serum and 100 IU penicillin/streptomycin. CHO-TRVb [human transferrin receptor (TfR)1-deficient Chinese hamster ovary] and CHO-TRVB-1 (human TfR1 stably transfected and expressing CHO-TRVb) cell lines were kindly provided by Dr T. McGraw (Cornell University, New York, NY, USA). They were cultured as described previously [18]. All adherent cell lines were detached with 5 mM EDTA in $NaCl/P_i$ before use. The stable cell lines expressing CXCR4 were kindly provided by Dr Babcock (Dana-Farber Cancer Institute, Boston, MA, USA) and cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum containing 0.5 $mg \cdot mL^{-1}$ G418.

Antibodies and reagents

The following purified antibodies were used in this study: mouse mAb against CXCR4, 12G5 (R & D Systems, Minneapolis, MN, USA); mouse mAb against CD4 (BD PharMingen, San Jose, CA, USA); TfR (CD71) antibody M-A712 (BD PharMingen); fluorescein isothiocyanate-conjugated goat anti-(mouse IgG) Ig (Pierce, Rockford, IL, USA); mouse IgG2a isotype control and phycoerythrin-conjugated goat anti-(mouse IgG) Ig (BD PharMingen); mouse mAb against c-myc, 9e10 (Santa Cruz Biotech, Santa Cruz, CA, USA); mouse mAb against M13 and the horseradish peroxidase (HRP)-labeled version (Amersham Pharmacia Biotech Inc.); HRP-labeled anti-mouse IgG (Pierce). Other reagents were: HRP–streptavidin (Sigma); streptavidin-coated magnetic beads (Daynal, Oslo, Norway); NHS-LC-biotin (Pierce); tyramine (Sigma); Ficoll-Hypaque (Pharmacia); *Escherichia coli* TG1 and helper phage VCS M13 (Stratagen, La Jolla, CA, USA); TMB substrate and stop buffer (KPL, Gaithersburg, MD, USA); CHAPSO (Anatrace, Maumee, OH, USA); anti-His_6–agarose conjugate and Protein A/G–agarose (Santa Cruz Biotech); [^{35}S]methionine and cysteine (Pekin-Elmer Life Sciences); SuperSignal chemiluminescent substrate kit (Pierce).

Pathfinder and Step-back selections from the PhAb library

Exponentially growing Jurkat cells $1–2 \times 10^6$ were incubated with 10^{12} phage prepared from a 1.5×10^{10} nonimmune human scFv phage display library [19] in the presence or absence of 5 μg mouse CXCR4 mAb 12G5 in 3% (v/v) nonfat milk/$NaCl/P_i$ in a total volume of 100 μL at 4 °C for 4 h. Cells were washed three times with 2 mL milk/$NaCl/P_i$ by centrifugation (1500 ***g***). HRP–anti-(mouse IgG) Ig (1 : 200) in 100 μL milk/$NaCl/P_i$ was added to the cells and incubated for 2 h at room temperature. The cells were washed as before. Biotinylated tyramine buffer was prepared and incubated with cells for 10 min at room temperature in the presence of 0.03% H_2O_2 as described [9]. Phage bound to the cells were released into the supernatant by lysing cells with 100 μL 0.5% Triton X-100/$NaCl/P_i$ for 5 min on ice and centrifuging for 5 min at 12 000 ***g***. Biotinylated phage were captured by adding 20 μL preblocked streptavidin-coated magnetic beads to the supernatant and rotating for 15 min at room temperature. (The beads were blocked for 1 h at room temperature in milk/$NaCl/P_i$ before use.) The beads were pelleted using a magnet (designed for a 1.5-mL tube) and washed 10 times in 1 mL milk/$NaCl/P_i$ containing 0.1% Tween 20 (milk/$NaCl/P_i$/Tween). Captured biotinylated phage were eluted by addition of 100 μL 0.1 M triethylamine and incubation for 20 min, then neutralized with 50 μL 1 M Tris/HCl, pH 7.4. Half of the eluted biotinylated phage were used to infect an exponentially growing culture of *E. coli* TG1 for amplification and preparation of PhAbs for further rounds of selection. Phage titration and selection of single clones for ELISA screening were as described previously [20]. Three rounds of Pathfinder selection were performed. After each round, the other half of the recovered biotinylated phage with no amplification were used as guide molecules to conduct a further Step-back selection. In brief, biotinylated phage were incubated with Jurkat cells in the presence of 10^{12} PhAbs prepared from the unselected phage scFv library. After 8–12 h of incubation at 4 °C, cells were washed and HRP–streptavidin was added. The mixture was incubated for 2 h at room temperature and then treated with biotinylated tyramine as described above. The beads were used to directly infect *E. coli* TG1, and single colonies were picked out randomly for subsequent screening by ELISA.

Cell-based phage ELISA

Cell ELISA with phage scFvs was performed, with modifications, as described previously [8]. A suspension of cells (2×10^5 per well) in 100 μL $NaCl/P_i$ were centrifuged in U-bottomed 96-well plates at 200–500 ***g*** for 5 min. Cells were preblocked in 1% BSA/$NaCl/P_i$ for 30 min at room temperature. The phage scFvs of individual clones ($\approx 5 \times 10^{10}$ phage particles) were obtained as described previously [20]. Phage scFvs were preblocked in 3% BSA/$NaCl/P_i$ (v/v) and then added to preblocked cells and incubated for 1 h at room temperature. The cells were washed twice with 200 μL washing buffer [4% (v/v) fetal bovine serum in $NaCl/P_i$] at

4 °C. The cells were then resuspended in 100 μL washing buffer containing HRP-conjugated anti-M13 Ig (1 : 5000) and incubated for 30 min at room temperature. Finally, the cells were washed three times in washing buffer and resuspended in 100 μL TMB substrate buffer. They were then incubated for 10 min. The incubation was stopped with 100 μL stop buffer. Absorbance was read at 450 nm. All assays were performed in duplicate.

*Bst*NI fingerprint analysis and sequence analysis

The scFv inserts of individual clones were amplified by PCR, using pel B (5′-GAAATACCTATTGCCTACGG CAGCCGCTGG-3′) as a forward primer and M13-pIII (5′-CTTATTAGCGTTTGCCATTTTTTCATAAT-3′) as a reverse primer. The amplification products were digested with the restriction enzyme *Bst*NI, which makes frequent cuts in the human γ heavy-chain [21]. The fingerprints were analyzed on 2% agarose gel. The same primers as above were used as sequencing primers. The sequences were analyzed and assembled with the program SEQUENCHER (Gene Codes Corporation, Ann Arbor, MI, USA). The family assignments were analyzed by the program DNAPLOT (http://www.dnaplot.de/).

Flow cytometry assay with phage scFvs

Cells (10^6) were preblocked in 1% BSA/NaCl/P_i for 30 min at room temperature and allowed to bind to preblocked phage ($\approx 10^{11}$ phage particles in 3%/BSA/NaCl/P_i for 30 min) at 4 °C for 1 h. Bound phage were detected using anti-M13 Ig (1 : 100 diluted in 0.5% BSA/NaCl/P_i) and phycoerythrin-labeled goat anti-(mouse IgG) Ig. After each incubation step, cells were spun down and washed three times with NaCl/P_i containing 1% BSA and 0.1% sodium azide. Finally, cells were suspended in 300 μL NaCl/P_i and freshly analyzed using a Becton–Dickinson FACScan with CELLQUEST software.

Expression and purification of soluble scFvs

scFv-coding DNA fragments were subcloned into *E. coli* expression vector pSyn1 to express scFvs tagged with C-terminal c-myc and His_6 [19,22]. For expression, XL1-Blue were transformed and cultured in 1 L 2 × YT medium (Stratagene, La Jolla, CA, USA) containing 100 μg·mL^{-1} ampicillin and 0.1% glucose to a A_{600}-value of 0.9. Soluble scFv expression was induced with 0.5 M isopropyl thio-β-D-galactoside at 30 °C for 4 h. The bacterial pellets were harvested and sonicated. Clear supernatants were obtained by centrifugation at 30000 ***g*** for 20 min. For purification of His_6-tagged scFvs, immobilized metal affinity chromatography was applied as described previously [19]. The final purified scFvs were dialyzed in NaCl/P_i, their purity was assessed by SDS/PAGE, and their concentration was determined with a protein assay kit (Bio-Rad).

Flow cytometry assay using mAbs or soluble scFvs

Sample cells (1×10^6) were incubated with 10 μg·mL^{-1} isotype control or mAb or 20 μg·mL^{-1} scFv in a final volume of 50 μL NaCl/P_i/0.5% BSA/0.1% sodium azide at 4 °C for 1 h. For detection of mouse mAbs, phycoerythrin-labeled anti-mouse IgG was used as secondary antibody. For detection of scFvs, c-myc antibody 9e10 was incubated with the cells and then phycoerythrin-labeled anti-mouse Ig. After each incubation step, cells were washed twice in NaCl/P_i/0.5% BSA/0.1% sodium azide. Samples were analyzed using a Becton–Dickinson FACScan with CELLQUEST software.

Metabolic labeling of cells

Cells were grown to exponential phase. The growth medium was removed, and 10^7 cells were washed once with NaCl/P_i by centrifugation. RPMI 1640 lacking cysteine and methionine and supplemented with dialyzed 10% fetal bovine serum, 100 IU penicillin/streptomycin, and 50 μCi (^{35}S)methionine and (^{35}S)cysteine was added, and the cells were incubated for 24 h. After incubation, the metabolically labeled cells were harvested and washed extensively. Approximately 5×10^6 cells were solubilized in CHAPSO-containing buffer. The procedure was as described previously [23]. The lysates were cleared by centrifugation at 14 000 ***g*** for 1 h at 4 °C and kept at 4 °C for immunoprecipitation.

Immunoprecipitation with scFvs or CD71 mAb and immunoprecipitation/Western blot

Anti-His_6-conjugated agarose was used to precipitate scFv-binding proteins. Protein A/G–agarose was used for the immunoprecipitation by mAb. The agarose beads were conjugated with scFvs or mAbs by incubating them with 20 μg soluble scFvs or 5 μg mAbs for 1 h at 4 °C. The cleared radiolabeled lysates were incubated with 20 μL antibody-conjugated agarose beads and rotated for 4 h overnight at 4 °C. After incubation, the agarose beads were washed five times in solubilization buffer and resuspended in 20 μL 2 × SDS sample buffer. Samples were incubated at 37 °C for 1 h and run on 10% SDS/polyacrylamide gels (SDS/PAGE). Gels were fixed in 20% methanol/10% acetic acid for 30 min. Fixed gels were dried for 1 h and visualized by autoradiography on Kodak Biomax MR film. For immunoprecipitation/Western blot, 2×10^7 exponential phase cells were harvested and lysed with CHAPSO buffer and immunoprecipitated with scFv 92 (see below) or CD71 mAb. The precipitates were subjected to SDS/PAGE and blotted with CD71 mAb and then HRP-labeled anti-mouse IgG. The signal was detected with a SuperSignal chemiluminescent substrate kit.

Results

CXCR4 mAb-guided Pathfinder selection

A Pathfinder selection was carried out using CXCR4 mAb 12G5 as the guide molecule, and three rounds of selection were performed on CXCR4-positive Jurkat cells. PhAbs (2×10^{12}) prepared from a large nonimmune human scFv phage library were used for the selection. The mAb 12G5 was indirectly conjugated with HRP using HRP-labeled anti-mouse IgG. It was used at a saturating concentration of 50 μg·mL^{-1} [14]. PhAbs binding to the cells in close

Table 1. Summary of results from Pathfinder selections. NT, Not tested.

Round of pathfinder selection	CXCR4 mAb (12G5)	Number of output phage	Clones positive for Jurkat cells	Number of positive clones for CXCR4	Number of positive clones for CD4	Number of positive clones for TfR
1	+	5.0×10^3	0/72	NT	NT	
	–	4.0×10^3	0/72	NT	NT	
2	+	3.3×10^4	59/96	0	18 (3 unique)	1
	–	3.6×10^3	NT	NT	NT	
3	+	1.5×10^6	53/96	0	NT	1
	–	1.0×10^4	NT	NT	NT	

proximity to HRP were biotinylated when biotinylated tyramine was added. Streptavidin-coated beads were used to retrieve the biotinlyated PhAbs. The number of PhAbs recovered from the first round selection was 5.0×10^3 in the presence of the guide mAb 12G5, and 4.0×10^3 in the absence of 12G5. This suggests that the presence of 12G5 did not result in an increase in phage recovery after the first round selection. The number of recovered PhAbs rose to 3.3×10^4 (after the second round) and 1.5×10^6 (after the third round). In the absence of 12G5, the equivalent values were 3.6×10^3 and 1×10^4, respectively (Table 1). These results indicate that PhAb recovery was driven by the presence of the mAb guide but was only detected after the second and third rounds of selection.

Step-back selection with biotinylated PhAbs recovered from Pathfinder selection

For isolation of scFvs with the same binding site as 12G5, a Step-back selection was performed with biotinylated PhAbs directly recovered from the proximity selection without amplification, which should include a high proportion of antibodies that bind close to, but not at, the 12G5-binding site. These biotinylated PhAbs were conjugated with HRP using HRP–streptavidin. The same amount of PhAbs from the unselected library as the Pathfinder selection was added to the cells. A new population of PhAbs, a proportion of which should bind to the 12G5-binding site, were biotinylated as described above. Three Step-back selections were carried out in the presence of biotinylated PhAbs recovered from one, two or three rounds of the Pathfinder selection. As a control, Step-back selections were also performed in the absence of biotinylated PhAbs. The number of PhAbs recovered from each selection were $1.0–2.6 \times 10^5$, and there was no significant difference as compared to control groups. Thus, in contrast with Pathfinder selection, Step-back selections with biotinylated PhAbs as guide molecules did not show any enrichment of output phage.

Identification of Jurkat cells that bind PhAbs

Clones from each round of Pathfinder and Step-back selection in the presence of guide molecules (mAb 12G5 for Pathfinder, biotinlyated PhAbs for Step-back) were screened by ELISA to identify Jurkat cell-positive PhAbs (Table 1); 293T cells were used as negative controls. For Pathfinder selection, none of the 72 clones picked at random from the first round output phage were Jurkat cell-positive. However, 62% (59/96) and 55% (53/96) of the output phage from the second and third round selection, respectively, were Jurkat cell-positive. For the three rounds of Step-back selection with input biotinylated phages from first, second or third round of Pathfinder selection, the Jurkat cell-positive percentages of randomly picked clones were 0 (0/48), 14 (10/72), and 8 (4/48), respectively (Table 2). The percentages that were positive for the second and third rounds of Step-back selection were much lower than that observed after the second and third rounds of Pathfinder selection. Clones that bound to Jurkat cells at least three times more strongly than to 293T cells (evaluated from the A_{450} readings) were scored as positive.

Identification of CD4-expressing and CXCR4-expressing cells that bind PhAbs

A total of 59 Jurkat-positive clones from the second round of Pathfinder selection were further analyzed by PhAb ELISA for binding to CD4 using the CD4-stable transfected cell line NIH.3T3-CD4 and its parent cells NIH.3T3. Eighteen clones recognized NIH.3T3-CD4 but not NIH.3T3 cells. Similar results were obtained using the PhAb fluorescence-activated cell sorter (FACS) assay. The result of one representative,

Table 2. Summary of results from Step-back selections. NT, Not tested.

Step-back selection	Source of biotinylated phage	Biotinylated phage	Number of output phage	Clones positive for Jurkat cells	Number of positive clones for CXCR4
1	1st Pathfinder	+	2.0×10^5	0/48	NT
		–	1.8×10^5	NT	NT
2	2nd Pathfinder	+	1.0×10^5	10/72	0
		–	2.1×10^5	NT	NT
3	3rd Pathfinder	+	2.6×10^5	4/48	0
		–	2.4×10^5	NT	NT

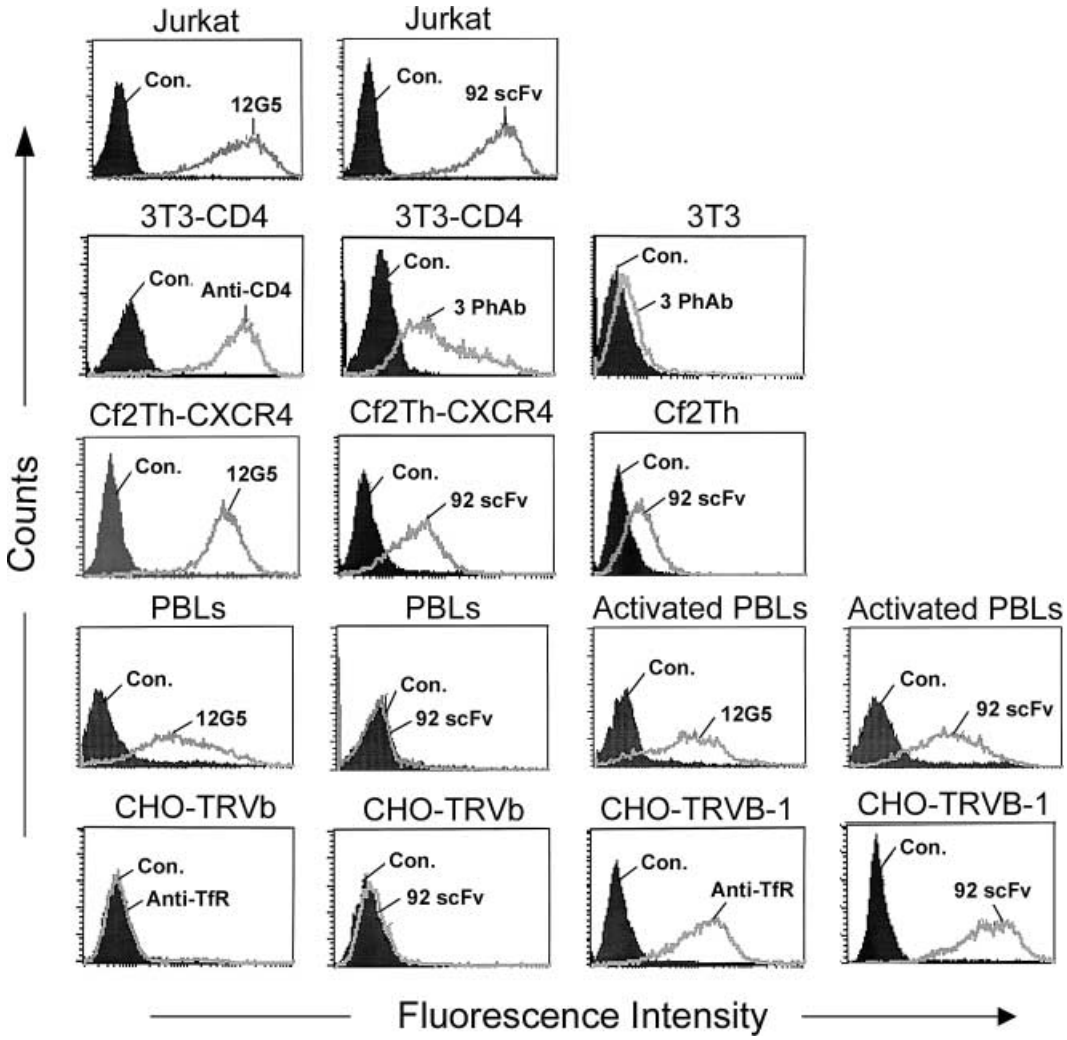

Fig. 1. Characterization of scFvs by flow cytometry analysis on different cell types. For staining with mouse mAb 12G5, anti-TfR M-A712 or anti-CD4, the second antibody was fluorescein isothiocyanate-conjugated goat anti-(mouse IgG) Ig; the control was stained with mouse isotype control. For staining with scFv 92, the second antibody was c-myc mAb followed by phycoerythrin-conjugated goat anti-(mouse IgG) Ig; the scFv A8 was used as a control. For staining with PhAbs (clone 3 shown as a representative), the bound phage was detected with M13 mAb followed by phycoerythrin-conjugated goat anti-(mouse IgG) Ig; the control was stained with 3T3-CD4 cell-negative PhAbs.

clone 3, is shown in Fig. 1. A total of 126 Jurkat cell-positive clones were further analyzed for CXCR4 binding by using cf2Th-CXCR4 (CXCR4-stable transfected cf2Th) and its parent cells cf2Th. Only two clones bound to cf2Th-CXCR4 more strongly than to cf2Th cells. FACS assay with either PhAbs or soluble scFv further confirmed this results (Fig. 1). One clone, designated 18, was from the second round of Pathfinder selection, and the other, designated 92, was from the third round of Pathfinder selection.

Analysis of clone diversity

The diversity of the 59 Jurkat cell-positive clones from the second round of Pathfinder selection was analyzed by *Bst*NI fingerprint and DNA sequencing. Six unique restriction patterns (fingerprints) were observed (data not shown), indicating the recovery of several different antibodies. Most clones belonged to one of two major groups. One group consisted of 31 clones with unknown binding specificity. The other consisted of 18 clones that were CD4 positive. Sequencing of these 18 clones revealed three unique clones with similar V_H and V_L sequences. This suggests that they may originate from one or two closely related B-cell clones and hence may be directed against the same epitope (Table 3). Clone 18, which showed stronger binding to cf2Th-CXCR4 than to cf2Th cells, had a distinctive fingerprint. The other nine clones, which were not further characterized with regard to binding specificity, also had three different fingerprints (data not shown). The only genetic analysis performed on third round Pathfinder clones was for clone 92. A comparison of the DNA sequences of clones 92 and 18 showed that they have very similar VH but different VL segments. 'Promiscuous' light-chains resulting in similar specificity if paired with the same V_H have been reported on several occasions. Therefore this result suggests that clones 92 and 18 may also recognize the same epitope (Table 3).

Table 3. Amino-acid sequence analysis of anti-CD4 and anti-TfR V_H and V_L genes. Numbering and small letters defined as previously [35]. Positions with different amino acids between the group of anti-CD4 or anti-TfR scFvs are represented by bold small capital lettering. JH: CD4-#3 = JH3, CD4-#53 = JH3a, CD4-#55 = JH3a, TfR#18 = JH4, TfR#92 = JH4. JL: CD4-#3 = Jλ3, CD4-#53 = Jλ3, CD4-#55 = Jλ3b, TFR#18 = Jλ3, TFR#92 = Jλ3.

	V_H genes			
	VH1 FR1	VH CDR1	VH FR2	VH CDR2
	1 2 3		4	5 6
Sample	12345678901234567890123456789O	1ab2345	67890123456789	012abc3456789012345
CD4-#3 VH3	**E**VQLVQSGGGVVQPGRSLRLSCAASAFS**F**S	**T**--YD**M**H	WVRQAPGKGLEWVA	GISY--DGYNKYY**A**DSVKG
CD4-#53 VH3	**Q**VQLVQSGGGVVQPGRSLRISCAASAFS**S**S	**S**--YD**I**H	WVRQAPGKGLEWVA	GISY--DGYNKYY**G**DSVKG
CD4-#55 VH3	**Q**VQLVQSGGGVVQPGRSLRISCAASAFS**S**S	**S**--YD**I**H	WVRQAPGKGLEWVA	GISY--DGYNKYY**G**DSVKG
TfR#18 VH3	EVQLVESGGGLVQPGGSLRLSCTTSGFT**F**R	R--HAMS	WVRQAPGKGLEWVS	GIGG--SGDTTYYADSVKG
TfR#92 VH3	EVQLVESGGGLVQPGGSLRLSCTTSGFT**L**R	R--HAMS	WVRQAPGKGLEWVS	GIGG--SGDTTYYADSVKG

	VH FR3	VH CDR3	VH FR4
	7 8 9	0	1
Sample	67890123456789012abc345678901234	567890abcdefghijk12	34567890123
CD4-#3	RFTISRDN**S**KNTVDLQ**I**N**N**LR**P**EDT**A**V YYCAR	**A**R GNAGTYEAF------DL	WGQGT**M**VTVSS
CD4-#53	RFTISRDN**P**KNTVDLQ**M**N**S**LR**A**EDT**S**M YYCAR	--GNAGTYEAF------DL	WGQGT**T**VTVSS
CD4-#55	RFTISRDN**P**KNTVDLQ**M**N**S**LR**A**EDT**S**M YYCAR	--GNAGTYEAF------DL	WGQGT**T**VTVSS
TfR#18	RFTISRDNSKSTLYLQMNSL**G**ADDTAIYYCAK	AKDGLPFYDFWSGFF--DY	WGQGTLVTVSS
TfR#92	RFTISRDNSKSTLYLQMNSL**R**ADDTAIYYCAK	AKDGLPFYDFWSGFF--DY	WGQGTLVTVSS

	V_L genes			
	VL FR1	VL CDR1	VL FR2	VL CDR2
	1 2	3	4	5
Sample	123456789012345678901 23	45678901abcdef234	567890123456789	0123456ab
CD4-#3 Vl6	NFMLTQPHSVSESPGKT**V**TISCT	**R**SSGSIA**N**N-----**F**VQ	WYQQRPGSAPTTVIY	EDNQRPS--
CD4-#53 Vl6	NFMLTQPHSVSESPGKT**I**TISCT	**V**SSGSIA**S**N-----**F**VQ	WYQQRPGSAPTTVIY	EDNQRPS--
CD4-#55 Vl6	NFMLTQPHSVSESPGKT**V**TISCT	**R**SSGSIA**S**N-----**Y**VQ	WYQQRPGSAPTTVIY	EDNQRPS--
TfR#18 Vl8	**QTVVTQEPSFSVSPGGTVTLTC**G	**LNSGSV**S**TSY**----**YPS**	WYQQ**T**PGQAP**RTL**V**F**	**STNT**RSS--
TfR#92 Vl3	**YELTQPPSVSVAPGKTARITCG**G	**NNIGSK**S--------**VH**	WYQQ**K**PGQAP**VLV**V**Y**	**EDRG**RPS--

Table 3. (Continued).

Sample	VL FR3 6 7 8 78901234567890123456789	VL CDR3 9 012345abcdef67	VL FR4 0 890123456a7
CD4-#3	GVPDRFSGSIDSSNSASLTISGLKTEDEADYYC	QSYDSSIH----WV	FGGGTKLTVLG
CD4-#53	GVPDRFSGSIDTSSNSASLTISGLKTEDEADYYC	QSYDRTKS----WV	FGGGTKLTVLG
CD4-#55	GVPDRFSGSIDTSTNSASLTISGLKTEDEADYYC	QSYDSTIN----WV	FGGGTKLTVLG
TfR#18	GVPDRFSGS--ILGNKAALTITGAQADDESDYYC	MLYLGDGS----WV	FGGGTKLTVLG
TfR#92	GIPERFSGS--NSGNTATLTISRVEAGDEADFYC	QVWDSSSDHA--WV	FGGGTKLTVLG

Further characterization of scFvs 18 and 92

To further characterize the binding specificity of scFvs 18 and 92, CXCR4-expressing cell lines, including CEMX174, U937, Raji, HL60, Hut78, KG1a, and HeLa, and freshly isolated nonactivated PBLs and phytohemagglutinin/interleukin 2-activated PBLs were stained with soluble scFvs 18 and 92. The cells were also stained with CXCR4 mAb 12G5 for positive control and analyzed by FACS. scFvs 18 and 92 bound to all the CXCR4-expressing cell lines and activated PBLs tested, although the binding patterns of positive cells percentage and fluorescence intensity are not completely consistent with 12G5 (data not shown). In addition, clones 18 and 92 did not bind to nonactivated PBLs, in contrast with 12G5 (Fig. 1).

Following on from this result, we tested whether CXCR4 could be immunoprecipitated by scFvs. The staining patterns of these two scFvs were essentially the same on all cell types tested. Therefore, we used clone 92 for the following analysis. Two cell lines, Jurkat and CEMX174, were radiolabeled with (^{35}S)cysteine and (^{35}S)methionine and then solubilized with CHAPSO-containing buffer (CHAPSO is the best detergent for maintaining the native conformation of CXCR4 [23]). Cell lysates were precipitated with soluble scFvs 92 and a control scFv A8 (against chemokine receptor CCR5; our unpublished data) through its C-terminal His_6 tag using anti-His6–agarose beads. Unexpectedly, a band corresponding to the molecular mass of CXCR4 was not

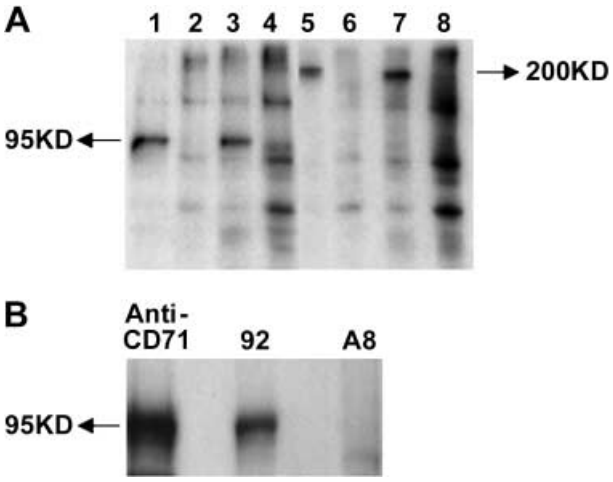

Fig. 2. Characterization of scFv 92 by immunoprecipitation and Western blot. (A) Immunoprecipitation by scFv 92 from radiolabeled cell lysates. Jurkat and CEMX174 cells were radiolabeled and lysed. Cleared cell lysates were precipitated with scFv 92 or control scFv A8 using anti-His–agarose. The immunoprecipitates were subjected to SDS/PAGE (10% gels) and visualized by autoradiography. Lanes 1–4 were run under reducing conditions; lanes 5–8 were run under non-reducing conditions. Lanes 1, 2, 5, 6, Jurkat cell lysates; lanes 3, 4, 7, 8, CEMX174 cell lysates; lanes 1, 3, 5, 7, scFv 92; lanes 2, 4, 6, 8, scFv A8. (B) Western blot of TfR with TfR mAb. Immunoprecipitations from nonradiolabeled Jurkat cells lysates using scFv 92, control scFV A8 or TfR mAb were subjected to reducing SDS/PAGE (10% gels), transferred to nitrocellulose membrane, detected with TfR mAb and HRP–anti-mouse IgG.

precipitated by scFv 92. Instead, a 95-kDa protein under reducing conditions and a 200-kDa protein under nonreducing conditions were precipitated by scFv 92 but not by the control scFv A8 (Fig. 2A).

It is well known that TfR is a lymphocyte activation marker with an apparent molecular mass of 95/200 kDa under reducing/nonreducing conditions and selectively expressed on activated PBLs but not on nonactivated PBLs [24,25]. Taken together, these results suggest that scFvs 18 and 92, initially identified as probable CXCR4 antibodies, may actually recognize CXCR4-expressing cells through TfR. To determine the identity of the scFv 92 recognizing protein and TfR, immunoprecipitate of scFv 92 was blotted using the TfR mAb M-A712 in Western blotting. Figure 2B shows that the precipitate of scFv 92, but not the control scFv, reacted with the TfR mAb. Furthermore, scFv 92 specifically recognized TfR-expressing CHO-TRVB-1 but not TfR-deficient CHO-TRVb in the FACS assay (Fig. 1). These results show that scFvs 18 and 92 are directed against TfR, but not CXCR4.

Discussion

In this study, the Pathfinder and Step-back guided selection methods were employed using a CXCR4 mAb and biotinylated PhAbs recovered from Pathfinder selection, respectively, to isolate scFvs against CXCR4 because of our interest in developing neutralizing antibodies against CXCR4 for potential use in inhibition of HIV-1 infection. Pathfinder selection resulted in enrichment of Jurkat cell-positive clones after the second and third rounds of panning. However, none of the recovered clones analyzed were positive for CXCR4. Step-back selection failed to show enrichment in the number of output phage during the three rounds of panning, and again none of the Jurkat cell-positive clones were specific for CXCR4. Thus, using the experimental conditions described in this study, these techniques did not lead to the identification of anti-CXCR4 scFvs, although three unique anti-CD4 and two unique anti-TfR clones were recovered.

Pathfinder selection was designed for targeted recovery of binding molecules from phage libraries. The targeted recovery of PhAbs is achieved by using guide molecules, such as antibodies and natural ligands, which are conjugated directly or indirectly to HRP. The PhAbs that bind to the target antigen can be directly biotinylated *in situ* by the addition of biotinylated tyramine and easily recovered using streptavidin-coated beads. Because the effective range of biotinylated tyramine is limited (a range of $\approx$15–20 nm, equivalent to 3–4 protein diameters), only PhAbs that bind in close proximity to the target antigen are selectively biotinylated. It has been reported that these techniques are much more efficient than standard cell surface selection for obtaining PhAbs of the desired specificity from a phage library. Although these selection methods have not been widely used, they have been successfully applied to the selection of antibodies against CEA, selectin, and, in particular, a seven-transmembrane chemokine receptor (CCR5) on whole cells [9,10]. In theory, the combined techniques provide an attractive means of targeting specific cell surface molecules and overcoming the binding of PhAbs to irrelevant antigens on cells.

In this report, experiments were designed to isolate scFvs against CXCR4 from a large nonimmune phage display antibody library using Pathfinder selection on whole cells. Jurkat cells were used as the target cells because they express a high level of CXCR4 receptors on the cell surface (163 521 $\pm$ 35 875 binding sites per cell for its ligand SDF-1α and $\approx$120 000 per cell for its antibody 12G5 [17,26]). We performed three rounds of Pathfinder selection and three rounds of Step-back selection, and over 500 clones were screened for Jurkat cell-binding activity. A total of 126 clones were identified as Jurkat cell-positive. However, none were active against CXCR4. This result is in marked contrast with a previous report in which CCR5-specific antibodies were identified by the same selection strategy [10]. CXCR4 and CCR5 are the major HIV-1 coreceptors and chemokine receptors. They belong to the rhodopsin class of the G-protein-coupled receptor superfamily characterized by a conserved transmembrane structure comprising seven α-helices. Their extracellular structures comprise both a N-terminal domain and three extracellular loops. Their glycosylation patterns are not identical but similar: CXCR4 has two potential N-linked glycosylation sites [27] whereas CCR5 does not appear to possess any N-linked glycosylation modifications, but rather O-linked glycosylation modifications [28]. Therefore, their extracellular structures and glycosylation patterns do not appear to be the reason for our failure to isolate CXCR4-specific PhAbs. Although the first 3D structure of a G-protein-coupled receptor, bovine rhodopsin, has been solved, and theoretical models of CXCR4 and CCR5 are available, modeling of appropriate conformational spaces of the N-terminus and extracellular regions is still challenging in the 3D structural modeling of G-protein-coupled receptors because of limited homology with known structures and the limitations of current loop modeling techniques [29–31]. A more complex tertiary folding structure and fewer immunodominant amino-acid sequences may exist for CXCR4, which would explain why isolation of PhAbs against CXCR4 is more difficult. Of note, Jurkat cells were used as target cells in our study because of their high level of CXCR4 expression, whereas in the previous report on CCR5, $CD4^+$ peripheral blood mononuclear cells were used as target cells. It is possible that antigen inaccessibility through steric hindrance caused by the presence of other proteins may differ for these two cell types, with greater inaccessibility existing on Jurkat cells, sufficient to prevent the selection of antibodies specific for CXCR4. Moreover, the guide molecule used in our study is a mAb, whereas macrophage inflammatory protein 1α, a ligand for CCR5, was used as the guide molecule in the previous CCR5 study. The mAb is much larger than the ligand and this may also contribute to the steric hindrance. In addition, it is unlikely that the size and genetic complexity of the nonimmune library used in this study limited our ability to isolate CXCR4 scFvs when other successful applications of this library have been achieved [19,32].

Although we were not able to isolate CXCR4 antibody by Pathfinder selection, we identified 3 unique clones as CD4 antibodies. CD4 is a single-chain molecule composed of four immunoglobulin-like extracellular domains of 370 amino acids, a transmembrane region (25 amino acids)

and a cytoplasmic tail (38 amino acids). CD4 and the chemokine receptor CXCR4 were preferentially localized on the microvilli. These molecules tend to be found in homogeneous microclusters which are often closely associated (≈ 10 nm apart) [33]. Although the level of CD4 expression is ≈1000 per antibody-binding site on Jurkat cells and is lower than that of CXCR4, the larger extracellular domain of CD4 and its close proximity to CXCR4 on the cell membrane may be the reason for the selection of CD4 PhAbs by Pathfinder selection guided by the CXCR4 mAb 12G5. In addition, two scFv clones bound to Cf2Th-CXCR4 cells more strongly than to the parent cells, and they were preliminarily isolated as probable anti-CXCR4 clones. However, both clones were shown to be TfR antibodies by further immunoprecipitation, immunoprecipitation/Western blotting, and FACS analysis. Human TfR is a homodimeric type II transmembrane protein with a short, N-terminal cytoplasmic region (1–67 amino acids), a single-transmembrane pass (68–88 amino acids), and a large extracellular portion (89–760 amino acids). TfR is expressed by dividing cells, but not nondividing cells such as resting lymphocytes [24,25]. For some reason, Cf2Th-CXCR4 cells express a higher level of TfR than the parent cells, and this contributed to the intitial misidentification of the two scFvs as anti-CXCR4. It is noteworthy that radiolabeled immunoprecipitation with scFvs using anti-His–agarose beads is a feasible and reliable method for characterization of scFv with unknown binding specificity. We tried several other ways but failed; for example, anti-(c-myc)–agarose did not work for scFv precipitation (data not shown).

In summary, despite the potential advantages of the Pathfinder and Step-back selection methods, we were not successful in selecting antibodies against a seven-transmembrane receptor, CXCR4, on whole cells, although successful selection of a similar receptor, CCR5, has been reported with the same strategy. The isolated scFvs in our study were mainly active against dominant or accessible cell surface antigens. Taking our negative results together with another report of the unsuccessful selection of PhAbs against the human seven-transmembrane somatostatin receptor [34], we conclude that these guided selection techniques require better understanding and further experimental development to become widely acceptable and generally useful for isolating PhAbs against known cell surface molecules. The location of the HRP-labeled guide molecules in relation to the overall surface topography of the cell and the target molecule is probably critical to the success or failure of these techniques. Smaller guide molecules may be a better choice for certain complex surface proteins such as seven-transmembrane receptors. A systematic study of these effects is now needed. In addition, as the requirements for targeted screening strategies vary from case to case, it is clear that more specific selection techniques need to be developed.

Acknowledgements

We thank Wen Yuan, Akikazu Murakami (Department of Cancer Immunology & AIDS, Dana Farber Cancer Institute, Boston), and Michael Farzan (Department of Microbiology and Molecular Genetics, Brigham & Women's Hospital, Boston) for helpful discussions and technical assistance.

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