[CANCER RESEARCH 63, 5213–5217, September 1, 2003]

*Advances in Brief*

# Selection of Chronic Lymphocytic Leukemia Binding Peptides[1]

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## Abstract

**To provide cell-binding ligands for *ex vivo* gene therapy and chronic lymphocytic leukemia (CLL)-targeting ligands for *in vivo* drug and gene therapy, we selected 44 20-mer peptides from peptide-presenting phage libraries by panning against primary patient CLL cancer cells. Twenty-nine of the selected peptides were assayed for cell binding. Eight of the selected peptides bound CLL cells, B cells, T cells, and monocyte cells, 12 bound only CLL cells and B cells, and 1 peptide bound only B cells. However, eight of the selected peptides were CLL specific. When two of the peptides were tested out of the context of phage, the synthetic peptides were able to bind cells and functionally retarget adenovirus to increase *ex vivo* gene delivery to primary CLL cells. These data demonstrate the ability to identify lead cancer-targeting peptides by selection of phage libraries against primary human cancers cells.**

## Introduction

CLL[3] is the most common adult leukemia in the United States and Western countries, accounting for 30% of leukemias (1). CLL is identified by the accumulation of lymphocytes in the marrow and lymphoid organs and in the peripheral blood. In 95% of cases, these CLL tumor cells are derived from the B-lymphocyte lineage (B-CLL; Ref. 1). Although most patients remain largely asymptomatic, the disease may progress to cause lethal marrow failure or other complications. Several chemotherapeutic agents have been tested for CLL (reviewed in Ref. 2). Gene therapy is a complementary approach to the treatment of CLL because, unlike most cancer chemotherapeutic agents, this approach can bypass the requirement that target cells be dividing for therapeutic effects (3).

Whether gene therapy of CLL is pursued *in vivo* or *in vitro*, both approaches necessitate that genes be effectively delivered to cells. Although this is not a problem for many cancers, CLL cells can be relatively difficult to transfect or transduce *in vitro* or *in vivo* by some of the most popular gene delivery methods (*e.g.*, electroporation, liposomes, or retroviral vectors; Ref. 3). For example, although adenoviral vectors can deliver genes to many tumors, this vector is ~100–1000-fold less efficient for transduction of B-CLL cells when compared with permissive cells (3). In our hands, Ad5 vectors transduce only ~4% of primary patient CLL cells even when multiplicities of infection of 15,000 to 1 are used (4). This poor transduction of CLL by adenovirus appears to be attributable to the fact that B-CLL cells do not express high levels of the receptors for adenovirus (3, 4).

One strategy to circumvent this problem is to identify new CLL-binding ligands to redirect vectors to CLL cells. For *ex vivo* applications, the ligands for CLL need to mediate cell binding but do not necessarily have to be CLL specific. By contrast, effective and safe *in vivo* CLL drug or gene therapy will require that CLL-binding ligands have a high degree of cell specificity to maximize destruction of cancer cells while avoiding damage of nontumor cells throughout the body. Given the need for new ligands for CLL drug and gene therapy, we have applied peptide-presenting phage library technology (5, 6) to identify these needed CLL ligands. In this work, we demonstrate the ability to select both CLL-binding and CLL-specific peptide ligands by selection of peptide libraries against primary patient B-CLL cells. In addition, we demonstrate the direct application of two of these peptides to retarget fluorophores and adenoviral gene therapy vectors to CLL cells for *ex vivo* applications.

## Materials and Methods

**Cells.** Primary B-CLL samples were collected from the peripheral blood of patients with high leukocyte counts. PBMCs were isolated from healthy donors, after informed consent and Institutional Review Board approval at MD Anderson Cancer Center (Houston, TX). Patient samples were stored frozen in DMSO and 10% fetal bovine serum in liquid nitrogen. The diagnosis of CLL was based on the revised guidelines of a National Cancer Institute-sponsored working group. Approximately 90% of patient PBMCs were $CD5^+$, $CD19^+$ B-CLL cancer cells mixed with 10% or less of normal PBMC nontarget cells. Autologous B-cell lines (EBV-Immortalized Cell Line) from CLL patients were generated by transformation with EBV, using a standard protocol (7), by infecting PBMCs with the B95-8 supernatant in cyclosporin A (Novartis, East Hanover, NJ).

**Phage Library Selection.** The ON543 20-mer peptide library was generously provided by Steve Cwirla and Bill Dower (Affymax, Palo Alto, CA). Peptide selection was performed as described by Barry *et al.* (5) with modifications to select nonadherent cells. Briefly, PBMCs from CLL patients were thawed and washed twice in serum-free RPMI medium. Cell surface receptors were cleared by incubation for 2 h in serum-free medium at 37°C. Ten library-equivalents of ON543 were diluted into 2 ml of HBSS-BSA (HBSS containing 0.1% BSA) containing Complete protease inhibitors (Roche) and 10 $\mu$M chloroquine. The cell were incubated for 1 h with the phage at 4°C, 25°C, or 37°C as indicated in Table 1. Selection at 4°C tends to bias for peptides that bind the surface of the cell, whereas incubation at 37°C tends to bias toward peptides that both bind and internalize (5). CD19+ cells were than isolated with MACS CD19 beads on an MS+ separation column according to the manufacturer's protocol (Miltenyl). The CD19 cells on the MACS beads were then washed six times with 5 ml of HBSS-BSA by serial centrifugation and removal of the supernatant. Cell surface phage were recovered by two acid elutions using 2 ml of 0.1 N HCl (pH 2.2.) for 5 min as described by Barry *et al.* (5). The acid fraction was neutralized.

The more tightly associated phage or internalized phage were recovered by hypotonic lysis of the cells in 30 mM Tris (pH 8) on ice for 30 min combined with brief vortexing. The resulting acid or cell-associated fractions of phage were then amplified by infection into F′-positive bacteria as described by

Received 3/6/03; revised 6/10/03; accepted 7/7/03.

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[1] This work was supported by grants to M. A. B. from the Leukemia Research Foundation and from the National Cancer Institute of the NIH (R01 CA93942). This work was also supported in part by a grant to M. A. from the Chronic Lymphocytic Leukemia Foundation and by a grant to M. K. B. from the National Cancer Institute of the NIH (R01 CA78792).

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[3] The abbreviations used are: CLL, chronic lymphocytic leukemia; B-CLL, CLL of B-lymphocyte lineage; Ad5, adenovirus type 5; PBMC, peripheral blood mononuclear cell; PE, phycoerythrin; GFP, green fluorescent protein; SMCC, succinimidyl-4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate.

Table 1 *CLL-selected peptides*

| Selection target[a] | Clone incidence[b]/condition[c] 4 A | 4 C | 25 A | 25 C | 37 A | 37 C | Clone[d] | Sequence[e] |
|---|---|---|---|---|---|---|---|---|
| Patient 16 | 4/4 | | | | | | 1-1 | GVSKRGLQCHDFISCSGVPW |
| | | 9/10 | 3/4 | | 9/10 | 1/10 | 1-2 | NQSIPKVAGDSKVFCWWCAL |
| | | | | | 1/10 | | 1-3 | QSTPPTKHLTIPRHLRNTLI |
| | | 1/10 | | 4/10 | | | 1-4 | DMSFQLVTPFLKALPTGWRG |
| | | | | 4/10 | | | 1-5 | GGHGRVLWPDGWFSLVGISP |
| | | | 1/4 | 2/10 | | 9/10 | 1-6 | QIMMGPSLGYYMPSESIFAY |
| Patient 25 | 10/10 | | | | | | 4-1[f] | KPWMTWQWVVEKSSTEGFRT |
| | | 6/10 | | | | | 4-2 | GDELGWNWVTIWPKTLSSRV |
| | | | | | 5/10 | | 4-3 | MTARPDPMMSTSTTVNTVLI |
| | | | | | | 3/10 | 4-4 | LTSPPKEARPSTTVGKSGRE |
| | | | | | | 2/10 | 4-5 | MGTRWQGDGESQHASVGSGS |
| | 2/5 | 5/5 | | | | | 4*-1[f,g] | KPWMTWQWVVEKSSTEGFRT |
| | 3/5 | | | | | | 4*-2[f,g] | GDELGWNWVTIWPKTLSSRV |
| | | | | | 4/4 | | 4*-3[g] | ASSWKSFWHPNPVGMTPASS |
| | | | | | | 1/4 | 4*-4[f,g] | LTSPPKEARPSTTVGKSGRE |
| | | | | | | 3/4 | 4*-5[g] | MARTVTANVPGMGEGMVVVP |
| Patients | | 1/10 | | | | | 2-1 | LAVTRPSPNKYATVNKSAQA |
| R1, patient 16 | | 3/10 | | | | | 2-2 | RTSTGPEARDAWMWYWKPPA |
| R2, patient 25 | | 1/10 | | | | | 2-3 | FGWTWEDSSSNSLYDMSFPH |
| R3, patient 42 | | 1/10 | | | | | 2-4 | GARSPIRESFGNTVPFDTRF |
| R4, patient 19 | | 1/10 | | | | | 2-5 | TGQGEREHREGRYTNSEGSD |
| R5, patient 49 | | 1/10 | | | | | 2-6 | VQTREAGGMYGGPDWWWEWS |
| | | 1/10 | | | | | 2-7 | ARAFSYWSVTDTERVKFFVP |
| | | 1/10 | | | | | 2-8 | LQIGEGVELVMARPIDSDSG |
| | | | | 3/9 | | | 2-9 | MGSRSAVGDFESAEGSRRP |
| | | | | 1/9 | | | 2-10 | MARMSTSEVPPVRIATSHSR |
| | | | | 3/9 | | | 2-11 | ISWDIWRWWYTSEDRDAGSA |
| | | | | 1/9 | | | 2-12 | EFGHGSPYETRQHSQARRWL |
| | | | | 1/9 | | | 2-13 | MAQKWGGGQALQGYHIGANA |
| | | | | | | 1/10 | 2-14 | VWGMTTSDHQRKTERLDSPE |
| | | | | | | 1/10 | 2-15 | GTFLHSRSQTSAEERAGGLG |
| | | | | | | 4/10 | 2-17 | GWWQFWDWSARRQDRGEALL |
| | | | | | | 1/10 | 2-18 | MAMGGKPERPADSDNVQVRG |
| | | | | | | 1/10 | 2-19 | QTIWRGHAASVNDSTTVSRT |
| | | | | | | 1/10 | 2-20 | MTSAQTSEKLKAETDRHTAE |
| Patients | 4/5 | 3/5 | | | 2/4 | 1/5 | 3a-1 | GAGNHPDAVSYPDILVKPRL |
| R1, patient 25 | 1/5 | 1/5 | | | 1/4 | 2/5 | 3a-2 | TAYTTGIRWSSQLQRLQHAS |
| R2, patient 16 | | 1/5 | | | | | 3a-3 | TDAMLGATVREHLEAMALVG |
| R3, patient 18 | | | | | 1/4 | | 3a-4 | MPHLDRVLWKPSGIRDLVSE |
| R4, patient 43 | | | | | | 1/5 | 3a-5 | VTGAVWMVLEESSHVLL |
| | | | | | | 1/5 | 3a-6 | EYRVENRIQPIRAYSGSLNR |
| Patients | 6/9 | | | | | | 3b-1 | KPWMTWQWVVEKSSTEGFRT |
| R1, patient 25 | 1/9 | | | | 2/9 | | 3b-2[f] | GAGNHPDAVSYPDILVKPRL |
| R2, patient 16 | | 1/3 | | | 1/9 | 2/10 | 3b-3[f] | TAYTTGIRWSSQLQRLQHAS |
| R3, patient 18 | 1/9 | | | | 1/9 | | 3b-4 | TQGRMAFYQTSVVLSSADST |
| R4, patient 38 | | | | | 3/9 | 3/10 | 3b-5 | WGTKPTTQWRKPQLQEEVRP |
| R5, patient 43 | | | | | | 3/10 | 3b-6 | MGRTVQSGDGTPAQTQPSVN |

[a] Selection target refers to the patient or patients used for selection. Where one patient number is indicated, only that one patient's cells were used at every round of selection. Where multiple patients are shown, cells from different patients were used at the indicated rounds of selection (*e.g.*, R1 = round 1).

[b] Selection clone incidence indicates the fraction of sequenced phage from a given selection that corresponded to this clone. This fraction is shown in the columns to the left of each clone. Fractions for each selection are in one column and add to 1.

[c] Condition refers to the temperature of phage binding (4 = 4°C, 25 = 25°C, 37 = 37°C) and whether this peptide was selected by recovery of phage from the cells by acid elution (A) or from the cell-associated fraction (C).

[d] Clone refers to the phage clone's identification number.

[e] Sequence refers to the displayed peptide sequence inferred from DNA sequencing of the modified *pIII* gene.

[f] This same peptide was selected by an alternative selection with a different identification number.

[g] Selection against CLL 25 was performed for five rounds to yield the 4-1 through 4-5 peptides. Selection was continued after round 5 with the addition of a preclearing step by adsorbing phage to nontarget cells before applying them to the CLL cells to attempt to isolate more CLL-specific peptides. This clearing in rounds 5–10 produced peptides 4*-1 through 4*-5. Peptides 4*-1, 4*-2, and 4*-4 were the same as peptides 4-1, 4-2, and 4-4, indicating that this clearing strategy was applied too late in selection to rescue specific peptides from the preselected promiscuous peptides.

Barry *et al.* (5). The resulting population of enriched cell-binding phage was then recycled onto CLL cells for the next round of selection as described above. If clearing was performed, the phage population was first incubated with nontarget cells for 1 h, the cells were centrifuged, and the supernatant was then applied to the target CLL cells. Clearing with autologous EBV-immortalized cell line from patient 25 was applied after round 5 on CLL 25 to generate peptides 4*-1 through 4*-5. Clearing was also applied in rounds 2–5 in the selections against multiple patients cells. In both cases, clearing did not appear to enhance selectivity. For CLL 25, this failure was likely attributable to the application of clearing too late, such that few CLL-specific peptides remained in the population by round 6. For the multiple patient selections, variation in receptors at each round on the different CLL cells likely confounded selection regardless of clearing.

**Phage Binding Comparison.** Each phage clone was grown up, and purified phage were prepared as described by Barry *et al.* (5). The negative control "random" phage was a peptide-presenting phage clone picked at random from the ON543 library. Each phage was diluted in a master solution for binding to all cell samples at a concentration of $10^{10}$/ml of HBSS-BSA-chloroquine. One ml of phage was then combined with $10^6$ target cells and incubated for 2 h at the indicated temperature. The cells were then washed three times with HBSS-BSA and stained with a 1:100 dilution of anti-M13 antibody (Amersham Pharmacia) for 30 min; the cells were then incubated with goat anti-mouse immunoglobulin-FITC conjugate for 30 min. The cells were washed, incubated with CD19-PE for 30 min, washed again, and analyzed on a Becton Dickinson FACScan flow cytometer.

**Synthetic Peptide Binding.** Fluorescent peptides were generated by cross-linking of each cysteine-labeled peptide to AlexaFluor 488-maleimide (Molecular Probes). Each peptide was then dialyzed overnight in 1-kDa cutoff dialysis tubes (Spectrapor) to remove excess dye. Fluorescent peptides were bound to $10^6$ target cells at the indicated concentrations in 1 ml of HBSS-

BSA-chloroquine for 1 h at 37°C. The cells were washed and visualized on the flow cytometer after counterstaining with CD19-PE antibody.

**Peptide Retargeting of Adenoviral Vectors.** CsCl-purified replication-defective Ad5-expressing GFP was conjugated with peptides in a manner analogous to that described by O'Riordan *et al.* (8) with the exception that the bifunctional cross-linker SMCC ($M_r$ 344; 11.6 Å bridge; Pierce Chemical) was used. Each was at a ratio of $10^3$–$10^4$ cross-linkers per virus particle to first cross-link free amines of the virus to the *N*-hydroxysuccinimde group of the reagent. The conjugated virus was then purified from the free cross-linker by gel filtration. The maleimide groups of the cross-linker on the virus were then reacted with the COOH-terminal cysteines on 1 $\mu$g of the indicated labeled peptide, and the complexes were purified again. The indicated conjugates were then incubated with the indicated target cells for 24 h, and the cells were washed and then cultured for 24 h before assay for GFP/CD19 by flow cytometry.

## Results

**Selection of Peptide-presenting Phage Libraries against Primary Human B-CLL Cells.** The 20-mer peptide-presenting phage library ON543 (5) was selected *in vitro* against CLL cells from patients with B-cell-derived CLL. Peptide selection was performed against whole PBMCs from the patients where 90% of the cells were CLL cells. Selection was performed as described by Barry *et al.* (5) with the exception that the target CLL cells in suspension were purified from non-target PBMCs by use of magnetic beads loaded with CD19 antibody (Fig. 1). This bead protocol captures not only CLL cells, but also normal B cells, which influenced the specificity of selected peptides. Phage panning and amplification were repeated for five to nine rounds, at which point the DNA from single phage colonies was sequenced to assess whether a finite set of peptides had been selected of the repertoire of $\sim 10^8$ peptides in the library.

Peptides were selected against CLL cells by panning the library on the cells at 4°C, 25°C, or 37°C (Table 1). Phage were selected against a single patient's cells in every round of selection (patients 16 and 25) or were selected against a different patient at each round (patients 25, 16, 18, and 43 or patients 25, 16, 18, 38, and 48; Table 1). Phage were recovered from the cells by acid elution to isolate peptides bound to the surface of the cells and by direct lysis of the cells to obtain peptides that were more tightly bound or that were internalized (Ref. 5; Fig. 1). Sequencing of phage clones after four to five rounds of selection demonstrated that each selection isolated a different set of peptides from the $10^8$ peptides in the library (Table 1). No overt amino acid consensus was observed between the selected 20-mer peptides. This was consistent with previous selections using libraries with long peptides, where consensus is rarely observed (5). In most cases, each selection generated a nonoverlapping set of peptides, with the exception of selection of peptides 3a-1, 3a-2, 3b-1, and 4-1, which were selected independently in two different selections. When 29 of the selected peptides on phage were tested for cell binding by flow cytometry, all demonstrated binding to CLL cells better than that mediated by control peptide, but at variable levels (Fig. 2). The peptides fell into three classes of binding specificity. Eight peptides (4*-5, 3b-6, 2-17, 2-9, 2-18, 2-2, 2-12, and 4-1) were promiscuous and bound not only CLL cells, but also normal B cells, T cells, and monocytes from PBMCs (Fig. 2*A*). Thirteen peptides were relatively specific for B cells (peptides 2-11, 2-14, 4-4, 4-, 4-3, 2-20, 4*-3, 2-1, 3b-5, 2-7, 3b-1, 3b-4, and 3a-1) as demonstrated by their binding to both normal B cells and the B-cell-derived CLL cells, but with low binding to T cells and monocytes. A final set eight peptides were relatively specific for CLL cells (peptides 1-5, 1-1, 1-6, 1-4, 1-3, 1-2, 4-2, and 3a-2) and bound poorly to normal B cells, T cells, and monocytes.

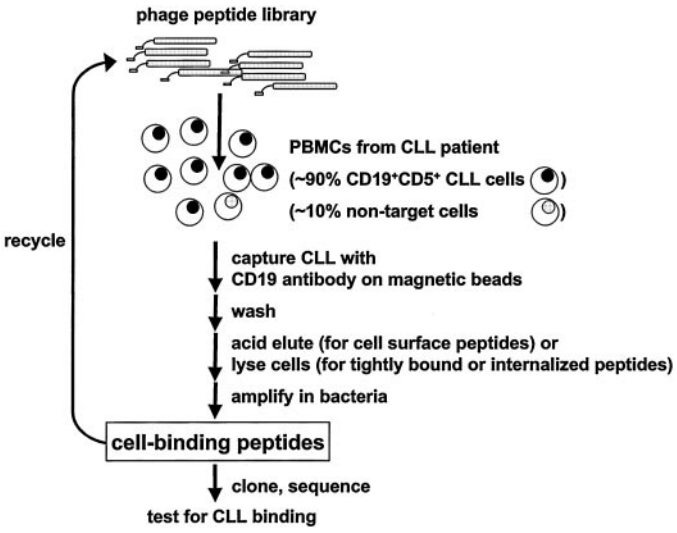


Fig. 1. Diagram of CLL selection protocol.

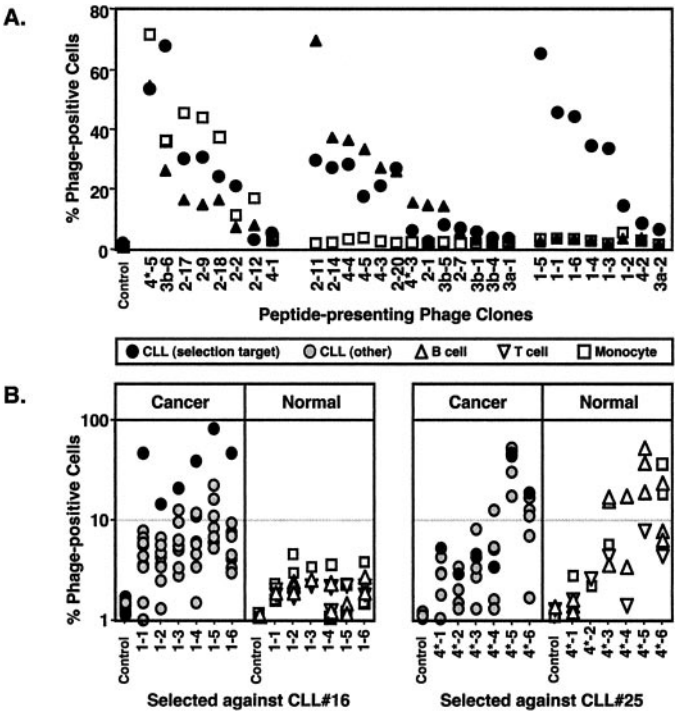


Fig. 2. Binding of CLL-selected phage to CLL and non-CLL cells. The indicated peptide-presenting phage clones were bound on the indicated cell types, and phage binding was quantitated by flow cytometry using anti-M13 antibody. The control is a peptide-presenting phage picked at random from the ON543 phage library. *A*, phage binding to CLL and non-CLL cells by 30 of the selected phage from Table 1. Binding for CLL cells is shown for the best binding to a panel of 7–10 different CLL samples. Patient-to-patient variation is seen in *panel B*. B-cell binding shows the highest binding of the phage to autologous or allogeneic EBV-transformed B cells or to normal, nontransformed allogeneic B cells. T cells/monocytes show the highest binding to normal allogeneic T cells or monocytes. B-cell, T-cell, and monocyte binding was determined by flow cytometry and counterstaining with CD19, CD3, or CD14 antibodies combined with the anti-M13 antibody. *B*, phage binding by peptides selected on CLL cells from patients 16 and 25. Each *point* represents one cell sample from one CLL patient. Binding was highest on the selection target CLL 16 for 1-1 to 1-6. Binding of 1-1 through 1-6 to allogeneic CLL samples was statistically significant relative to control peptide ($n = 9$; $P < 0.05$; data not shown). Only 4*-1 and 4*-6 bound their selection target, CLL 25, best. 4*-2, 4*-3, and 4*-4 bound CLL 59 best, whereas 4*-5 bound CLL 62 best.

**Heterogeneity in CLL Cells from Different Patients Affects Both Peptide Selection and Peptide Binding.** The ability of all of the selected peptides to bind CLL cells better than control phage demonstrated that each selection succeeded in isolating legitimate cell-binding peptides from the phage library. Although all peptides bound CLL cells, there was wide variation in cell specificity across the panel of peptides. This variation appeared to be related to which

CLL cells were used as the target for selection. For example, selection against CLL 16 generated peptides 1-1 through 1-6, all of which were relatively specific for CLL cells (Fig. 2). By contrast, selection of the same peptide library by the same protocol against CLL 25 generated an entirely different set of peptides (4-1 through 4-5, 4*-3, and 4*-5), all of which were promiscuous and bound all cell types tested (Fig. 2). Similar effects were observed when CLLs from more than one patient were used for selection at each round. Although different peptides were selected on different CLL samples, the selected peptides were nonetheless able to cross-bind at various levels on other CLL samples. These observations suggest that variations in receptor repertoire or receptor density on different patient samples fundamentally affected the specificity and binding characteristics of the peptides selected from the library.

**Testing Selected Peptides out of the Context of Phage.** Not all phage-selected peptides will function correctly when applied out of the context of the phage proteins on which they are displayed. Therefore, one critical test for phage-selected peptides is their ability to function as synthetic peptide ligands. Peptide 1-5 was tested as a candidate CLL-"specific" peptide that might be useful for targeting applications. Peptide 4*-5 was tested as a promiscuous peptide with potential use for *ex vivo* gain of function transduction. Binding was compared with that of peptide DU, a promiscuous cell-binding peptide selected against human prostate carcinoma (9). Both 1-5 and 4*-5 bound CLL cells more strongly than the DU peptide, and both bound with the same specificity to CLL and non-CLL cells as observed on phage (Fig. 3*A*).

**Retargeting of Adenoviral Gene Therapy Vectors with CLL-selected Peptides.** Peptides 1-5 and 4*-5 were next tested as lead ligands for their ability to retarget adenoviral vectors to increase transduction of patient CLL cells for *ex vivo* gene therapy. The synthetic peptides were covalently conjugated to adenovirus expressing GFP (Ad5-GFP) by use of a bifunctional chemical cross-linker to tether cysteine-labeled peptides to primary amines on the virus (8). Cell targeting was then tested by comparing transduction of control virus and peptides *versus* the CLL-selected peptides on CLL cells from patients 25 and 58 (Fig. 3*B*). Unmodified Ad5-GFP mediated 0.3–1.1% transduction of the CD5, CD19-positive CLL cells. Conjugation of the virus with the cross-linker alone (Ad5-SMCC) or with peptide DU mediated similar low-level transduction. In contrast, peptide 4*-5 mediated a 2–3-fold increase in transduction, and peptide 1-5 mediated larger increases. These data demonstrate proof of principle for functional application of phage-selected peptides to increase delivery of small molecules and gene therapy vectors to primary CLL cells *in vitro*. The levels of transduction mediated by these lead peptides will likely be increased once their affinity is optimized by mutant library selection (5, 10). Nonetheless, this work demonstrates the ability to select lead peptide ligands against primary cancer cells to identify peptides that can bind and functionally delivery therapeutic agents to the target cells.

## Discussion

Peptide-presenting phage libraries were originally used to select peptides against single proteins (11–15). Subsequent work adapted this technology for direct selection on intact cells in culture (5) and by selection against vasculature targeting *in vivo* (6). In most cases, phage display has been used to select peptides against noncancer cells (reviewed in Ref. 9). In other cases, peptides selected against normal vasculature have generated peptides that could fortuitously be used to



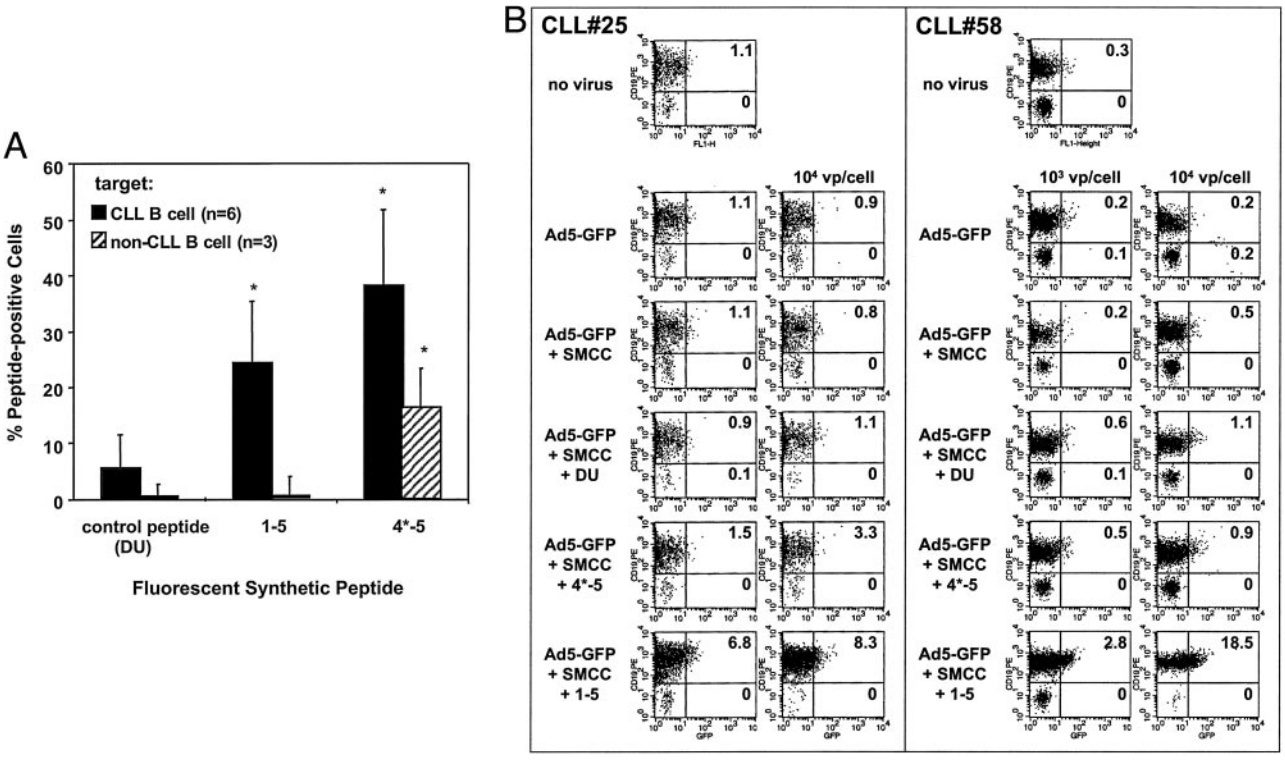


Fig. 3. Testing of synthetic peptides derived from CLL-selected peptides. *A*, binding of fluorescent peptides. The indicated peptides were synthesized with COOH-terminal cysteines and were conjugated to AlexaFluor 488-maleimide. The fluorescent peptides were incubated at 5 $\mu$M with only allogeneic CLL cells and non-CLL B cells for 1 h at 37°C; cells were then washed and analyzed for green fluorescence and CD19-PE staining. Control peptide DU was selected from the ON543 library on human prostate carcinoma cells and binds a variety of cell types. Peptides selected against any cell always have higher binding to cells than irrelevant peptides that do not bind a substrate. *B*, retargeting of a adenoviral gene therapy vector by CLL-selected peptides and control peptides. Ad5-GFP was applied to the CLL cells of patients 25 and 58 as an unmodified vector, a SMCC cross-linked vector without peptide, or as a vector cross-linked to the indicated peptide. *no virus* indicates untransduced cells. Cells were transduced with 1000 particles/cell (20 plaque-forming units/cell) or 10,000 particles/cell (200 plaque-forming units/cell) and analyzed by flow cytometry 48 h later. The percentages of GFP-positive CD19-negative or -positive cells are shown in the *right quadrants* of each scatter plot. Neither CLL 25 nor CLL 58 was the selection target for 1-5. CLL 25 was the selection target for 4*-5.

target the neovasculature of tumors *in vivo* (16). The first example of peptide selection specifically for cancer involved selection against prostate cancer cell lines (17). Subsequent work has selected peptide libraries against breast cancer cell lines (18) or against specific cancer-associated proteins (19). In this work, we selected CLL-binding and CLL-specific peptides by use of peptide-presenting phage libraries by direct panning on primary cancer cells. To our knowledge, this is the first example of peptide selection against primary cancer cells from a large set of patients. This work using heterogeneous primary cancer cells rather than cell lines has revealed how normal cancer cell heterogeneity can fundamentally affect the ability to both identify and apply peptide ligands when using clinical targets.

Functional testing of 29 of the CLL-selected candidate ligands identified three classes of peptides: (*a*) promiscuous peptides that bound many cells, (*b*) peptides that appeared B-cell-specific in binding both B cells and B-cell-derived CLL cells, and (*c*) peptides that were relatively CLL specific. Selection of peptides that bind CLL cells, but not B cells or other PBMCs was fortuitous. Selection of peptides that bound both normal B cells and B-cell-derived CLL cells was not surprising because these cells are quite similar and the selection protocol copurified both cell types on the CD19 antibody beads. Although these results were expected, the effects that different patient samples had on the character of selected ligands was unexpected. This effect may be attributable to differences in the repertoire of receptors present on each sample as well as to differences in receptor density from patient to patient. This observation indicates that one cannot rely on selection against a single patient or a single cell line to produce cell-targeting peptides. This also suggests that peptide selection against clonal tumor cell lines may not generate peptides that can address the heterogeneity that exists from patient to patient or that likely exists in the diverse population of cancer cells in any one patient. These data also suggest that selection against different patient cells at each round is a poor strategy because this appears to focus selection on common cell receptors rather than cancer cell-specific ones. This is perhaps not surprising because the receptors present in one round on one patient may not be present in high enough levels on the next patient to carry that peptide forward to subsequent rounds. In contrast, ubiquitous cell surface proteins are present on every cell in every round of panning, and there is therefore an inherent selection advantage for isolating promiscuous peptides over cell-specific ones.

Within individual selections, it was unclear how peptide recovery by acid elution or cell lysis affected the character of the peptides. Likewise, temperature effects were unclear as well. However, when the selection parameters of the 10 peptides with highest binding were compared, differences could be inferred. Of the top 10 peptides, 6 were selected at 37°C, 3 were selected at 25°C, and 1 was selected at 4°C. Of this same group, nine were selected from the cell-associated fraction, and only one was selected in the acid fraction. These data suggest that better peptides may be isolated by selection at 37°C. More importantly, there appears to be a marked advantage for selecting peptides from the cell-associated fraction rather than the more traditional acid elution fraction. These data are consistent with previous results (5).

This work provides proof of principle for the direct application of phage-selected peptides to increase drug or vector delivery to CLL cells. When applied as synthetic peptides out of the context of phage, peptides 1-5 and 4*-5 were able to "deliver" a small molecule fluorophore to the CLL cells *in vitro*. Furthermore, both peptides were able to increase transduction by adenovirus from 3- to 80-fold on primary human CLL cells. Although transduction was increased, the absolute percentage of transduction increase was modest. This result was not surprising given that most peptides selected from phage libraries have affinities in the micromolar range (20). Therefore, phage-selected peptides should always be considered as lead ligands whose activities will likely be enhanced when they are affinity-matured by use of mutant peptide libraries based on the sequences of these lead ligands (20).

We are currently screening the full set of 44 peptides with the goal of applying multiple peptides for targeting CLL cells to maximize the ability to address CLL cells with a range of receptor repertoires. Many of these peptides could be useful for *ex vivo* applications on CLL and other cell types to increase transduction because cell specificity is not required in culture. Lead peptides, such as 1-5, that are relatively CLL specific may have utility for both *ex vivo* and *in vivo* drug or gene therapy vector targeting. The subset of B-cell-specific lead peptides may have utility for targeting normal B cells or malignant B cells *in vivo*. Work is under way to screen the full panel of selected peptides for CLL applications as well as for applications against normal B cells, B-acute lymphoblastic leukemia, non-Hodgkin's lymphoma, and multiple myeloma.

## Acknowledgments

We would like to thank Mary E. Barry for excellent technical assistance.

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