

Surface immunoglobulin on B lymphocytes as a potential target for specific peptide ligands in chronic lymphocytic leukaemia

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Summary. With the aim of producing unique targets for malignant cells we have identified peptide ligands for the clonal surface immunoglobulin isolated from the B cells of a chronic lymphocytic leukaemia (CLL) patient. The peptides were identified from random-peptide phage-display libraries. The obtained ligands bound specifically to the surface of the target lymphocytes as well as to clonal immunoglobulin in

lysate from the same cells. Peptide-based antigen mimotopes may have a future use in targeted therapy of CLL and other B-cell-derived malignancies displaying surface immunoglobulin.

Keywords: chronic lymphocytic leukaemia, surface immunoglobulin, random peptide phage display.

Chronic lymphocytic leukaemia (CLL) is characterized by proliferation of a single B- or T-cell clone, with the former being the most frequent (around 95%). B-cell CLL (B-CLL) has clonal immunoglobulin in the cytoplasm (Pianezze *et al.*, 1987) as well as on the cell surface (Aisenberg & Bloch, 1972), although in lower amounts than in normal B cells (Dighiero *et al.*, 1980). Surface immunoglobulin is a hallmark of B cells and constitutes an obvious homing moiety for cell-specific targeting. Individual B-cell clones differ from one another because of the variable sequences within the antigen-binding site of the expressed immunoglobulin, providing a feature to differentiate between malignant and normal B cells.

The dilemma of chemotherapy is how to achieve the preferential killing of malignant cells without affecting normal cells. Most conventional therapy is targeted towards dividing cells; however, not all malignant cells multiply fast enough for this. Normal rapidly dividing cells such as gastrointestinal mucosa and haematopoietic cells are also affected, impairing the effect of the treatment. A property or a molecule unique for the malignant cells is rarely present (if ever), although it would be an obvious target for chemotherapy.

Attempts to generate B-cell-targeting molecules have so far primarily focused on monoclonal antibodies directed

against cell-surface antigens such as CD5 (Hertler *et al.*, 1989) and CD19 (Grossbard *et al.*, 1992). However, these markers are neither exclusively nor uniformly expressed on tumour cells, thus limiting their use as targets. Other targets such as chronic lymphocytic leukaemia antigen (cLLa) may have a more disease-restricted expression (Faguet & Agee, 1997). Monoclonal antibodies raised against the idiotype of the surface immunoglobulin (Cachia *et al.*, 1989) circumvent the specificity problems, although the cumbersome task of producing enough of anti-idiotype antibodies for each patient has prevented the widespread use of the technique.

In order to obtain simple chemical handles that can be used to differentiate between malignant and normal B cells, we decided to exploit the unique binding specificity of the surface immunoglobulin expressed on clonal B-CLL cells to select peptide ligands from random-peptide phage-display libraries. Such libraries display a large number of random peptides (10^7 – 10^9) expressed in fusion with coat protein pIII or pVIII on the surface of filamentous bacteriophages, and the peptides can be selected by binding to target molecules. Immunoglobulins are known to be good targets for affinity selection (panning) of random-peptide phage-display libraries (Bonnycastle *et al.*, 1996). In a previous study, we utilized the method for the identification of patient-specific peptides able to bind to M components and myeloma cells from patients with multiple myeloma (Szecsei *et al.*, 1999). In the present work, we demonstrate binding of peptide-displaying phages to patient-specific surface immunoglobulin on neoplastic B cells using a similar

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technique. Such binders may be used in the design of tumour-specific targeting agents for monitoring and treatment of CLL.

PATIENTS AND METHODS

Subjects. Eleven patients diagnosed with CLL were investigated. White blood cell counts at the time of sampling were $98\text{--}232 \times 10^9/\text{l}$ with 80–99% lymphocytes. Patient 2345 served as the test subject, the rest as controls. Normal cells were obtained from laboratory personnel.

Antibodies. Mouse anti-M13 and its horseradish peroxidase (HRP) conjugate were obtained from Amersham Pharmacia (Buckinghamshire, UK). Phycoerythrin (PE)-conjugated mouse anti-CD19, rabbit anti-human immunoglobulin γ -, α -, μ - and δ -heavy and κ - and λ -light chains, and HRP-conjugated goat anti-rabbit immunoglobulins were obtained from Dako (Glostrup, Denmark).

Lymphocyte preparation. Mononuclear cells from peripheral blood samples were isolated by centrifugation through a Ficoll cushion (Ficoll-Paque Plus, Amersham Pharmacia). The cells were washed twice in 0.01 mol/l sodium phosphate, 0.15 mol/l sodium chloride, pH 7.4 (phosphate-buffered saline; PBS), resuspended in fetal calf serum with 5% dimethyl sulphoxide (DMSO) and stored at -80°C in aliquots of 2×10^7 cells until further use.

Cell lysis. Frozen peripheral mononuclear blood cells were thawed and pelleted by centrifugation. The pellet was lysed overnight at 4°C after addition of an equal volume of PBS containing 2% Tween 20 and a cocktail of protease inhibitors (2 mmol/l ethylenediaminetetraacetic acid (EDTA), 2 mmol/l phenylmethylsulphonyl fluoride and 20 mmol/l iodoacetamide). Insoluble cell debris was removed by centrifugation and the supernatant was aliquoted and stored at -20°C .

Isolation of B-cell-associated immunoglobulin. Immunoglobulin in the cell lysate (2×10^7 cells) was incubated for 4 h at room temperature in a slurry of 50% v/v protein A sepharose CL-4B (Amersham Pharmacia) pre-equilibrated with PBS. The resin was washed with several volumes of PBS and bound immunoglobulin was eluted with 0.1 mol/l glycine-HCl, pH 2.3, and neutralized by addition of 2 mol/l Tris-HCl, pH 9.0.

Panning on B-cell-associated immunoglobulin. A mixture of two phagemid libraries displaying constrained and unconstrained random nonamer peptides in fusion with pVIII coat protein was used for this work (Felici *et al*, 1991; Luzzago *et al*, 1993). Three rounds of panning were carried out essentially as described previously (Felici *et al*, 1996). In short, protein A purified immunoglobulin was covalently immobilized on M450 tosyl-activated paramagnetic beads (Dynal, Oslo, Norway) and incubated with 2×10^{11} library phages overnight. Unbound phages were removed by extensive washing and the remaining phages were eluted with 0.1 mol/l glycine-HCl, pH 2.3, and neutralized by addition of 2 mol/l Tris-HCl, pH 9.0. The eluted phages were propagated in *Escherichia coli* strain XL1 Blue (Stratagene, La Jolla, CA, USA) using M13K07 for phage rescue and carried on to the next panning round. From the

amplified pool of the third panning phages, monoclonal plaques were randomly selected for further analysis.

DNA sequencing. The sequence of the random peptide insert was deduced from positive clones by BigDye Terminator cycle sequencing with standard M13–40 primer on an Applied Biosystems 877/377 system (Foster City, CA, USA) on single- or double-stranded purified DNA (QIAprep, Qiagen, Hilden, Germany).

Fluorescein isothiocyanate labelling of mouse anti-M13. Labelling of monoclonal mouse anti-M13 antibody took place for 1 h in the dark at room temperature with fluorescein isothiocyanate (FITC) isomer 1 (Sigma, St Louis, MO, USA) dissolved in DMSO (60 μg FITC per mg antibody). Unreacted FITC was removed by gel filtration using a Sephadex G-25M column (Amersham Pharmacia). The quality of the labelling was assessed by calculation of the F/P ratio [$3.1 \times A_{495}/(A_{280} - 0.31 \times A_{495})$]. Antibodies with an F/P ratio within the range 3–10 were tested on a flow cytometer for the ability to stain beads coated with M13 bacteriophage.

Flow cytometric analysis. Thawed peripheral mononuclear blood cells were washed and resuspended at a density of 10^7 cells/ml in PBS containing 10% newborn calf serum and 1 mmol/l EDTA. All subsequent washings and incubations were carried out in this buffer on ice. Approximately 5×10^5 cells were incubated with phage dilutions, washed twice and incubated for 1 h with 100 μl of FITC-conjugated anti-M13 (1:50 dilution) and PE-conjugated anti-CD19 (1:10 dilution). Cells were washed twice and resuspended in 300 μl washing buffer and analysed on a FACScan flow cytometer using the CELLQUEST software (Becton Dickinson, Franklin Lakes, NJ, USA). Forward and right-angle scatter thresholds were set by inspection of the cytogram to exclude dead cells and debris. The lymphocyte population was identified by light-scattering properties and gated to avoid monocytes. The fluorescence of FITC anti-M13 phage and PE anti-CD19 was analysed simultaneously and 1.5×10^4 cells were analysed in each distribution.

Blotting. Two-microlitre samples of cell extracts (approximately 4×10^5 cells) were separated by agarose gel electrophoresis as described previously (Jeppsson *et al*, 1979) and transferred to polyvinylidene difluoride membranes (Hybond-P, Amersham Pharmacia) by capillary blotting. Excess binding sites were blocked with 5% skimmed milk, 0.1% (v/v) Tween 20 in PBS, and the membranes were then incubated with phages or 1:3000 dilutions of chain-specific rabbit anti-human immunoglobulin in blocking buffer for 1 h at room temperature. Bound phages and chain-specific antibodies were detected using chemiluminescence (ECL Plus, Amersham Pharmacia) after a 1-h incubation with HRP-conjugated anti-M13 or goat anti-rabbit immunoglobulin.

RESULTS

Obtained binding phages

Twenty-four phage clones were picked randomly after three rounds of affinity selection on protein A purified B-cell-associated immunoglobulin from CLL patient 2345.

Table I. Amino acid sequence of isolated phagotopes.

Name	Amino acid sequence
M5*	LVRSTGQFV
M1*	LVSPSGSWT
M14*	ALRPSGEWL
M10*	AIMASGQWL
M8*	QILASGRWL
M3	RRPSHAMAR
M2	DNNRPANSM
M18	LQDRLRFAT
M20	PLSGDKSST

Amino acid sequences of peptide inserts displayed by phages binding specifically to B cells from CLL patient 2345. Phage clones marked with an asterisk had constrained inserts.

Twenty-two of these were sequenced after they had tested positive for binding to B cells from CLL 2345 using flow cytometry as exemplified below. Nine different peptide sequences (Table I), representing the random inserts in the pVIII coat proteins of the monoclonal phages, were identified. Comparison of the deduced amino acid sequences revealed that some peptide inserts possessed no apparent sequence homology, whereas others had a recognizable consensus sequence. All the phage clones with different peptide inserts (Table I) bound specifically to the cells from patient CLL 2345, used for biopanning. One of the phage clones (M1) containing a constrained peptide insert LVSPSGSWT was selected for further investigation.

Binding to B-CLL cells

The specific binding of clone M1 to the surface of B cells from patient 2345 was investigated by flow cytometric analysis. Non-permeabilized cells from four CLL patients

including CLL 2345 and three controls (CLL 8318, CLL 2493 and CLL 1802) and a normal individual were examined. The CD19⁺ cells from patient CLL 2345 were also positive for binding FITC antiphage (Fig 1B), whereas CD19⁺ cells from the normal individual (data not shown) and three CLL controls, exemplified by CLL (1802), were not stained (Fig 1C). The specificity of the M1 phage towards CLL 2345 B cells was further emphasized by the inability of the CLL 2345 B cells to bind phages without peptide insert (Fig 1A). The binding of M1 to CLL 2345 B cells was dose dependent (Fig 2) and approximately 5×10^9 colony-forming units (cfu) per ml gave a signal above the background level. A steep increase in fluorescence emission could be seen at higher phage concentrations, but no plateau was reached with the maximum concentration of 10^{11} cfu/ml. No staining of B cells from CLL 2345 was observed with M13K07 helper phage even in higher concentrations (10^{13} cfu/ml).

Binding to clonal immunoglobulin

To investigate whether the specific binding of M1 phages to B cells from patient 2345 was the result of interaction with the surface immunoglobulin, we performed an agarose gel electrophoresis of B-cell lysate from the CLL patient 2345 and 10 CLL controls as well as dilutions of normal serum.

Blots of the gel probed with a set of chain-specific antibodies revealed monoclonal or oligoclonal immunoglobulin (Ig) bands in the lysate from all of the CLL patients, as exemplified for μ -heavy chains in Fig 3B. Patient 2345 had a single band of mixed μ/δ and κ phenotype, the remaining three patients had oligoclonal bands of mixed μ/γ and λ phenotype. Parallel probing of blots revealed that the phage M1 reacted with the band corresponding to the mobility of the monoclonal band from CLL 2345 B cells (lane 1, Fig 3A and B), whereas no binding to the CLL control lysates could be detected (lanes 2–4, Fig 3A and B). In accordance with the flow cytometric analysis, binding was not observed with wild-type or other phages (data not shown). Furthermore, the M1 phages did not bind to a

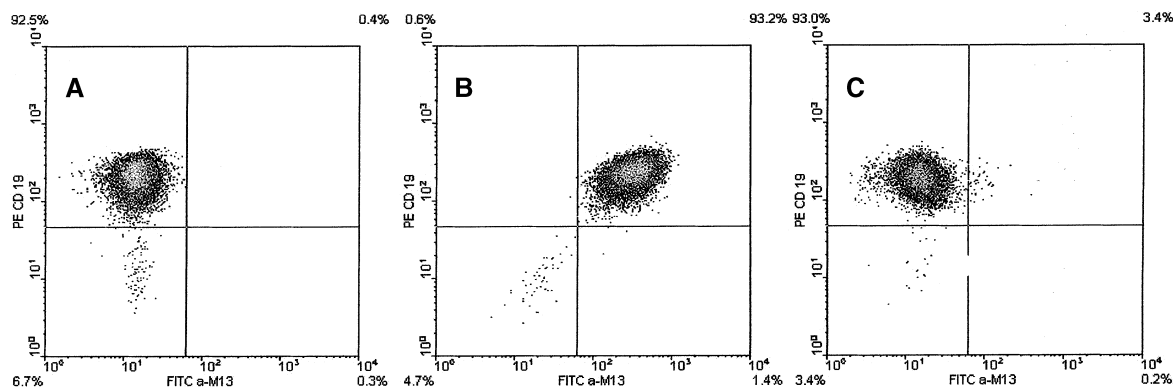


Fig 1. Specificity of phage clone M1 binding by flow cytometric analysis. Non-permeabilized mononuclear cells from CLL patient 2345 were incubated with helper phage M13K07 (without insert) (A) or M1 phages (peptide insert LVSPSGSWT, obtained by panning on B-cell-associated immunoglobulin obtained from patient CLL 2345) (B) and control CLL patient 1802 were incubated with M1 phages (C). In all experiments 6.5×10^{10} cfu were added and bound phages were detected with FITC anti-M13 and the co-presence of CD19 with PE anti-CD19.

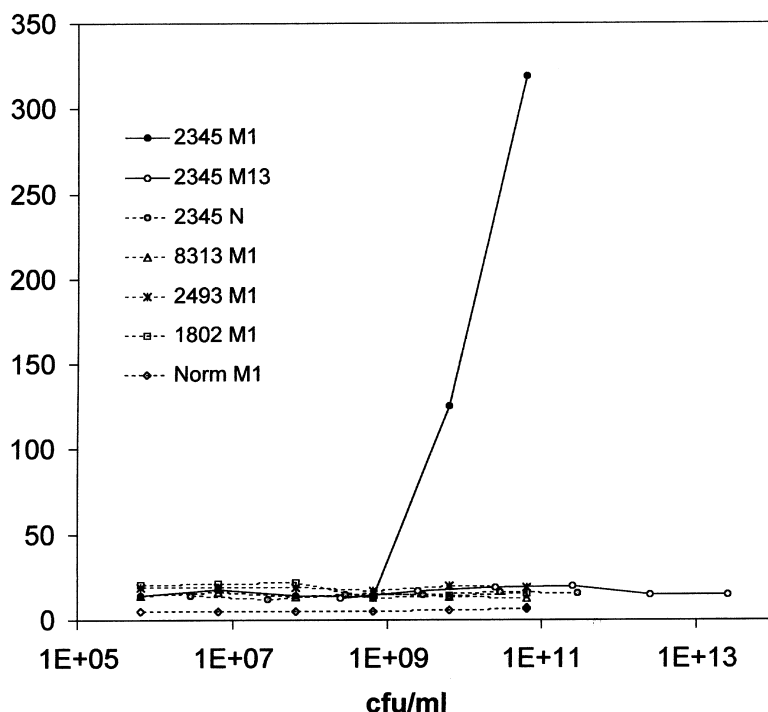


Fig 2. The concentration-dependent binding of M1 phage (with the constrained LVSPSGSWT peptide insert obtained by panning on B-cell-associated immunoglobulin obtained from patient CLL 2345) to B-CLL cells from patient 2345. The following phages were added to cells, at dilutions ranging from 10^6 to 10^{13} cfu/ml: M1 phage (M1), M13K07 phage without insert (M13) or an irrelevant library phage (N). Cells from three CLL patients (1802, 2493 and 8313) and a normal subject (Norm) were included as negative controls. Bound phages were detected with a monoclonal FITC-conjugated mouse anti-M13 phage. The mean value of fluorescence at 520 nm for the B-cell samples was calculated and depicted versus phage concentration (cfu/ml).

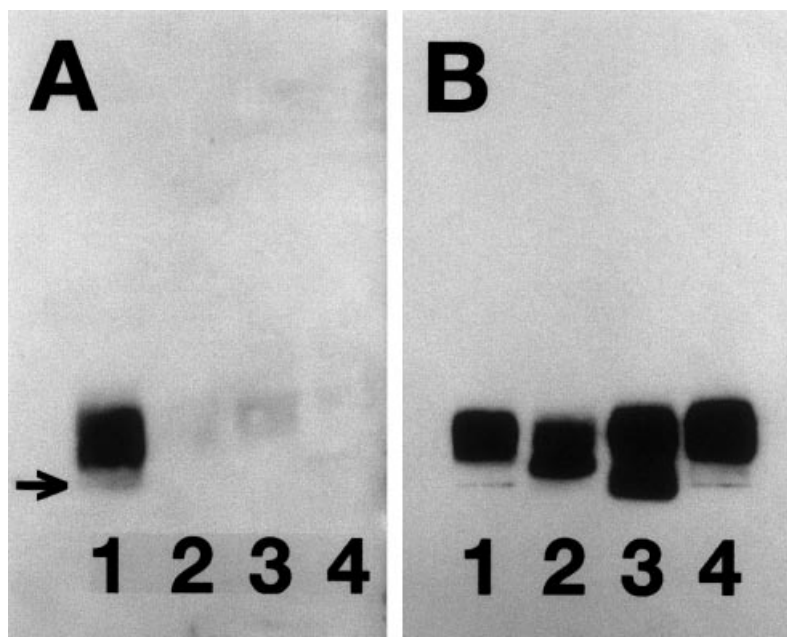


Fig 3. Specific binding of phage to clonal immunoglobulin. Blot on polyvinylidene difluoride membranes of an agarose gel electrophoresis. Two-microlitre extracts of CLL cells from patient 2345 (lane 1) and three CLL control subjects (lane 2–4) were applied (arrow). Membranes were probed with: (A) bacteriophage MK1 with the constrained LVSPSGSWT peptide insert obtained by panning on B-cell-associated immunoglobulin obtained from patient CLL 2345; (B) rabbit antihuman μ -chain antibody. Binding of phages and antibodies were visualized by incubation with HRP-labelled anti-M13 antibodies or goat anti-rabbit immunoglobulin and luminol-based chemiluminescent light detection on radiographic film with the cathode on top.

10^{-2} – 10^{-5} serial dilution of normal serum (12.2 g/l IgG, 3.4 g/l IgA and 5.4 g/l IgM).

DISCUSSION

The results presented in this paper describe a way to identify patient-specific B-CLL cell handles using phage-display random-peptide libraries. We selected phages that bound to the surface of B cells from a patient with CLL. These

phages also bound specifically to monoclonal IgM in a B-cell lysate from this patient. Therefore, we conclude that the cellular binding results from an interaction between the phages and the surface immunoglobulin. The inability of the phages to stain B cells from other CLL patients precludes the possibility that the phages was selected as a result of affinity for a common B-cell marker and also that the binding was due to a general polyreactive nature of the surface immunoglobulin (Ramsland *et al*, 1999). It is most

likely that the phage bind to the antigen-binding site of the immunoglobulin. If the phages interacted with a subspecies of immunoglobulin, none of the 10 control CLL cells should present this type of immunoglobulin. We find this most unlikely, especially since no reaction was observed with normal serum that should contain all immunoglobulin subtypes in ample amounts.

Protein A from *Staphylococcus aureus* has been reported to react with surface immunoglobulin from some B-CLL cells (Romagnani *et al*, 1982). In order to use protein A for the isolation of the immunoglobulin, initial tests must be performed to establish reactivity toward protein A. Lysis of the B cells will give access to the cytoplasmic pool of immunoglobulin. Since B-CLL cells express low amounts of surface immunoglobulin, this procedure increases the amount of target and reduces the risk of selecting phages binding to other surface molecules on the B cells. In case of non-reactivity toward protein A, an alternative catching strategy, for example through chain specific antibodies, should be employed.

Serum immunoglobulin attached to the B-CLL cells via interaction with Fc receptors (Hattori *et al*, 1980) would also be isolated by the protein A column. However, the dominance of monoclonal immunoglobulin bands in electrophoretic analysis of B-cell lysate observed in this and other studies (Plesner *et al*, 1982) suggests that serum is not a major contributor to the total amount of B-cell-associated immunoglobulin.

Assuming that successful isolation of B-CLL cell-specific peptide binders is not limited to a few CLL patients, peptides may become useful tools in the monitoring and treatment of CLL. In an earlier study, focusing on multiple myeloma, we found peptides binding to myeloma cells in a patient-specific manner (Szecsi *et al*, 1999). Using these peptides to detect M components in blood samples, we observed a 200-fold increase in sensitivity compared with traditional electrophoresis. A similar approach may be used with CLL where the presence of paraproteins in the blood is not unusual (Hansen *et al*, 1994). Indirect proof, such as coinciding light and heavy chain phenotype, have suggested a common clonal origin of the paraprotein and B-CLL surface immunoglobulin (Deegan *et al*, 1984). Specific peptide binders to clonal immunoglobulin would support this connection. Using flow cytometry we have shown that a peptide ligand can bind to B-CLL cells (Fig 1), and this approach could be used for the detection of residual leukaemic cells in blood samples or bone marrow.

High-dose chemotherapy followed by autologous stem cell transplantation for treatment of CLL has increased in use in recent years, but one problem with the approach is that the transplant may be contaminated with tumour cells. For this reason, *ex vivo* antileukaemic treatment ('purging') of remission marrow was developed and studies using both CD34⁺ selection and negative B-cell depletion look promising (Dreger *et al*, 2000). The results presented here suggest that B-CLL cell-specific peptide binders could be of use in *ex vivo* depletion of leukaemic cells. The peptides can be isolated because of a specific affinity for the variable region of surface immunoglobulin from the clonal

B-CLL cells, and this enables a selective removal of malignant cells, leaving the normal B cells unaffected. Furthermore, flow cytometric analysis (Fig 1) showed that the phage-displayed peptide ligand bound to practically all of the B-CLL cell population. This indicates that virtually all B-CLL cells carry the immunoglobulin target, which facilitates an effective purification of the transplanted bone marrow.

With the objective of killing the leukaemic cells in B-CLL patients, peptide ligands could be conjugated with radioactive isotopes or cytotoxins. Cells that engage in a specific binding reaction with the homing agent would be brought into close contact with, and perhaps internalize, the isotope or the toxin, resulting in selective cell death of the malignant B cells. The majority of work within targeted immunotherapy has been carried out using monoclonal antibodies (Vitetta & Thorpe, 1992), and although the success of using this approach in patients with CLL has been limited, new antibodies including CAMPATH-1H and rituximab look promising (Rai, 1999).

The peptide itself may have a potential to eliminate malignant cells. Synthetic peptides, identified by phage-display libraries, have in one case been shown to induce apoptosis *in vitro* in a human B-cell lymphoma cell line (Renschler *et al*, 1994). Peptide handles identified from phage-display random-peptide libraries have several features that make them preferable to antibodies (Lam, 1993). The total time required for identifying and producing a peptide ligand is less than a month for a fraction of the cost, compared with 3–4 months for creating anti-idiotypic antibodies (Kwak *et al*, 1992). Since the molecular mass of the peptide is several orders of magnitude smaller than antibodies, large molar excess of peptide conjugate can be easily achieved. Furthermore, peptides are less immunogenic than antibodies, diminishing the likelihood of provoking a host-antibody reaction and, thus, omitting the need for humanizing antibodies. As the panning procedure identifies several different and unrelated peptide sequences, as demonstrated in Table I, treatment can be continued, even if an immunogenic response does occur, by switching to a peptide handle with a different primary sequence unknown to the immune system. The disadvantage of the peptide handle is of course that the panning procedure has to be performed for each individual patient, but once peptides have been identified and synthesized they last throughout the duration of treatment. Ongoing studies will show whether the approach will have its place in the clinical treatment of chronic lymphocytic leukaemia.

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