

Identification of patient-specific peptides for detection of M-proteins and myeloma cells

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Summary. We have taken advantage of the selection power of phage display technology to define specific peptide mimotopes that recognize individual M-proteins, isolated from patients with multiple myeloma. Preferred amino acid motifs of phages binding to M-proteins were identified in 6/9 patients investigated. Chemically synthesized peptides, corresponding to the phage-displayed peptide inserts, were used to verify the specificity of binding in competition assays. The peptides were able to bind to the M-proteins, as well as the myeloma cells, with high sensitivity and specificity. Employing simple

immunological techniques, <0.01 g/l of M-protein could be quantified, suggesting a novel way for monitoring minimal residual disease in the production of guidelines for adjusting or reintroducing conventional chemotherapy. The peptide mimotopes defined by this technology may be useful as tumour-specific targeting agents and as a tool for purging cells in autologous bone marrow transplantation.

Keywords: multiple myeloma, M-protein, phage display, peptides.

Multiple myeloma accounts for >10% of the haematological malignancies with an incidence peak at around 70 years of age and is characterized by neoplastic proliferation of a single plasma cell clone producing a monoclonal immunoglobulin, known as M-protein, paraprotein or M-component (Kyle, 1994). Therapy has been attempted for more than half a century with limited success, since approximately one-third of patients do not respond to chemotherapy and the remainder will eventually relapse if they do not succumb to other diseases (Singhal *et al.*, 1997). The M-protein can be observed after serum electrophoresis as a localized band predominantly in the γ area (Jeppsson *et al.*, 1979), and semi-quantitative estimation can be performed with densitometry (Kyle, 1994). These routine methods for monitoring the tumour load are rather crude, and immunochemical quantification is often hampered by the background signal of normal immunoglobulin and the lack of a suitable reference (Kyle, 1994). Staining of bone marrow cells with class or subclass-specific antibodies detects both tumour cells and normal cells that produce the same immunoglobulin type. More sophisticated methods have been described for the detection and quantification of circulating malignant cells (Bakkus *et al.*, 1996; Björkstrand *et al.*, 1995; Brown *et al.*,

1998; Corradini *et al.*, 1995; Cremer *et al.*, 1997; Dreyfus *et al.*, 1995; Shimazaki *et al.*, 1991; Yamada *et al.*, 1989; van Zaanen *et al.*, 1995).

This report presents a simple way to quantify the M-protein. As immunoglobulins, M-proteins have the potential ability to bind antigens. We have taken advantage of the recently developed method of random peptide phage display libraries to identify peptides that are bound by the myeloma immunoglobulin of individual patients. Such peptides provide a novel and simple method for specific monitoring of the M-protein level and provide potential handles for targeting of the malignant clones.

MATERIALS AND METHODS

Patients. We investigated nine patients with multiple myeloma (three females and six males with a median age of 69 years, range 42–83). The M-protein was of type IgG in six patients and of type IgA in three patients. The estimated median concentration of the M-protein at the time of sampling was 19.5 g/l (range 10–30). The Durie-Salmon stage at diagnosis was III in six cases and stage II in three cases. The median duration from diagnosis was 39 months (range 7–69) and all patients had received cytotoxic therapy prior to the investigation.

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M-proteins. The M-proteins were enriched from 0.5 ml serum by cation chromatography (Mono-S column, Pharmacia, Sweden) using a linear gradient of 0.01–0.6 M sodium acetate, pH 5.5. Fractions containing M-protein were pooled and neutralized with 1 M Tris base and stored frozen until further use.

Phage libraries. Two filamentous phage libraries were used for isolation of phages binding to M-proteins. One library had a random 6-mer peptides insert near the N-terminal of the pIII coat protein of the filamentous phage carrying a tetracycline resistance gene. The library contained approximal 2×10^8 original clones representing about 70% of all possible hexamer peptides (Scott & Smith, 1990). The other library, of unknown diversity, was of the same construction with a random 15-mer peptide insertion (Nishi *et al*, 1996).

Bacteria. Bacteriophages were propagated in *Escherichia coli* strains NF1829 MC1000/F'[lac^q, Tn5(kan^r)] in 2xYT medium with 20 µg/l of tetracycline.

Biopanning. Microtitre plates (MaxiSorb, Nunc, Denmark) were coated overnight with 5–10 µg of M-protein per well in 0.1 M sodium phosphate, 0.15 M sodium chloride, pH 7.4 (PBS); washed and blocked for 2 h with PBS containing 0.5% (v/v) Tween-20 (PBS-Tw). Bacteriophage from each library (10^{10-11} pfu) were allowed to bind to the wells for 2 h at room temperature followed by extensive washing with PBS-Tw. Bound phages were eluted with 0.1 M glycine/HCl, pH 2.2, and immediately neutralized with 2 M Tris base. The eluted phages were amplified in NF1829 and subjected to three or four rounds of biopannings as described by Scott & Smith (1990). Individual phage clones were tested for binding to M-proteins by enzyme-linked immunosorbent assay (ELISA). Clones that bound to a pool of human immunoglobulin were considered as unspecific and were discarded. The peptide insert sequences of phages only binding to M-protein were deduced by sequencing, using the dideoxynucleotide chain termination method with 5'-α-³⁵S ATP and T7 DNA polymerase.

Phage ELISA. M-protein (10 µg/ml in PBS) was immobilized on 96-well microtitre plates (MaxiSorb, Nunc, Denmark). Phages were allowed to bind to the blocked wells for 2 h at room temperature. Detection of binding phages was performed with a horseradish peroxidase conjugated antibody against bacteriophage M13 (Pharmacia, Uppsala, Sweden) using o-phenylenediamine as chromophore and recording of OD at 490 nm. Specificity of binding was validated by displacement of phages by peptides of different sequences and concentrations, and by lack of binding to a panel of non-target M-proteins.

M-protein ELISA. Microtitre plates were coated overnight with streptavidin (10 µg/ml in 0.1 M carbonate, pH 9.3). Biotinylated peptides were then immobilized for 1 h at room temperature (10 µg/ml in 50 mM Tris, pH 8.6/0.15 M NaCl/0.1% (v/v) Tween 20). Serum dilutions were then allowed to bind for 1 h at room temperature. Detection of bound immunoglobulins was performed with an alkaline phosphatase conjugated antibody against human immunoglobulin (DAKO, Glostrup, Denmark) using p-nitrophenyl phosphate as chromophore. Sera from normal individuals and other myeloma patients served as controls.

Peptide synthesis. Peptides corresponding to the inserts were synthesized using solid phase Fmoc-chemistry. Two flanking amino acids (Gly and Ala from the pIII protein of the bacteriophage) were included on the N- and C-terminus. An additional C-terminal cysteine was incorporated to provide polymeric peptides by conjugation to bovine serum albumin (BSA) activated using N-succinimidyl-3-[2-pyridyldithio]propionate (Pierce, Rockford, Ill.). Fluorescent peptides were obtained by N-terminal conjugation to fluorescein isothiocyanate (FITC) or rhodamine B isothiocyanate. Some peptides were N-terminally biotinylated during synthesis. All peptides were purified to >95% purity by reverse-phase high-pressure liquid chromatography and verified by mass spectroscopy.

Blotting. Agarose gel electrophoresis of serum proteins was performed as described by Jeppsson *et al* (1979). The serum proteins and M-protein were transferred to nitrocellulose by capillary blotting and excess binding sites were blocked with PBS-Tw. The membranes were incubated with phages and detected essentially as described for phage ELISA using chemoluminescence (ECL, Amersham, Buckinghamshire, U.K.) or diaminobenzidine as chromophore. In one case, binding of the relevant peptide was investigated employing a FITC-conjugate of the peptide and inspection in ultraviolet light.

Affinity chromatography. Synthetic peptides (15 mg) were immobilized through the C-terminal cysteine residue to 1 ml of thiopropyl-Sepharose 6B (Pharmacia, Uppsala, Sweden). 1 ml of patient serum was allowed to bind to the column for 10 min. The column was then washed with six volumes of 0.1 M sodium phosphate, 0.2 M sodium chloride, pH 6.9, and bound protein was subsequently eluted with 0.1 M glycine/HCl, pH 2.35. Absorbance measurement at 280 nm and electrophoresis with protein staining was performed on all eluted fractions.

Cytology. Bone marrow aspirate was washed in PBS and attached to glass slides by cytopsin centrifugation. Myeloma cells were visualized with FITC-labelled BSA peptide conjugate in 50 mM Tris/HCl, pH 8.6, containing 5% (v/v) normal rabbit serum and 0.05% (v/v) Tween 20. Simultaneous staining for cells expressing the same type of heavy chain as the myeloma protein was performed with goat anti-human immunoglobulin Cy3 conjugate (Amersham, Buckinghamshire, U.K.). Bone marrow from other patients and competition with unlabelled peptides served as controls.

RESULTS

Pannings

Patient sera were biopanned using both the 6-mer and the 15-mer libraries. Antigen mimotopes were readily identified for six of the nine investigated M-proteins (Table I). Positive clones were found in the 6-mer library for two patients (nos. 3 and 6) and in the 15-mer library for six patients (nos. 3, 4, 6, 7, 8 and 9).

Mimotopes

Some of the M-proteins were found to interact with several

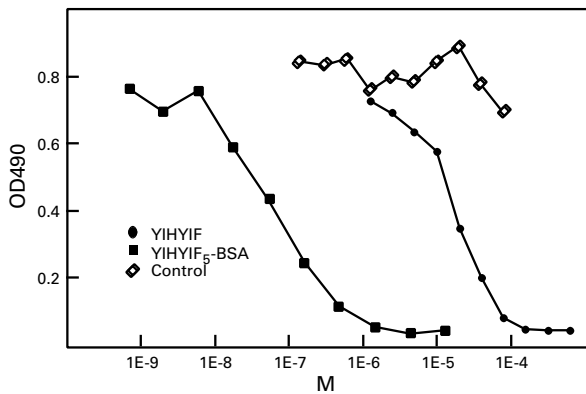


Fig 1. Binding strength of isolated peptide mimotopes. Competitive phage ELISA using mono- and pentavalent peptides to inhibit the binding of the isolated phage (#50) on M-protein from patient 6. The equivalent synthetic peptide competed with phage binding at a 50% inhibitory concentration of approximately 2.5×10^{-5} for the monovalent (YIHYIF) and 2.3×10^{-8} for the pentavalent (YIHYIF₅-BSA) peptide. A control peptide did not influence the binding.

peptides possessing no apparent homology based on the primary sequence, whereas other peptide binders clearly showed sequence homology. M-protein from patient 3 bound seven homologous 6-mer and three different 15-mer sequences (Table II). M-protein from patient 6 showed binding to five 6-mer sequences of which three showed sequence homology and a single 15-mer sequence (Table II).

Table I. Obtained unique peptide sequences by panning on M-proteins with pIII random peptide phage display libraries.

Patient	6-mer library	15-mer library
1	—	—
2	—	—
3	7	3
4	—	n.d.
5	—	—
6	5	1
7	—	n.d.
8	—	1
9	—	2

—, No clones found, n.d., number of individual positive sequences not determined.

Only a single 15-sequence was identified for patient 8, and two 15-mer sequences with no apparent homology were found to bind to M-protein from patient 9 (data not shown). Phages binding to patients 4 and 7 were not sequenced.

Specificity

Three of the patients (nos. 3, 6 and 9) were investigated in more detail. Positive phages obtained from the individual panning were subjected to phage ELISA in competition with synthetic peptides. Both free and BSA-conjugated peptides

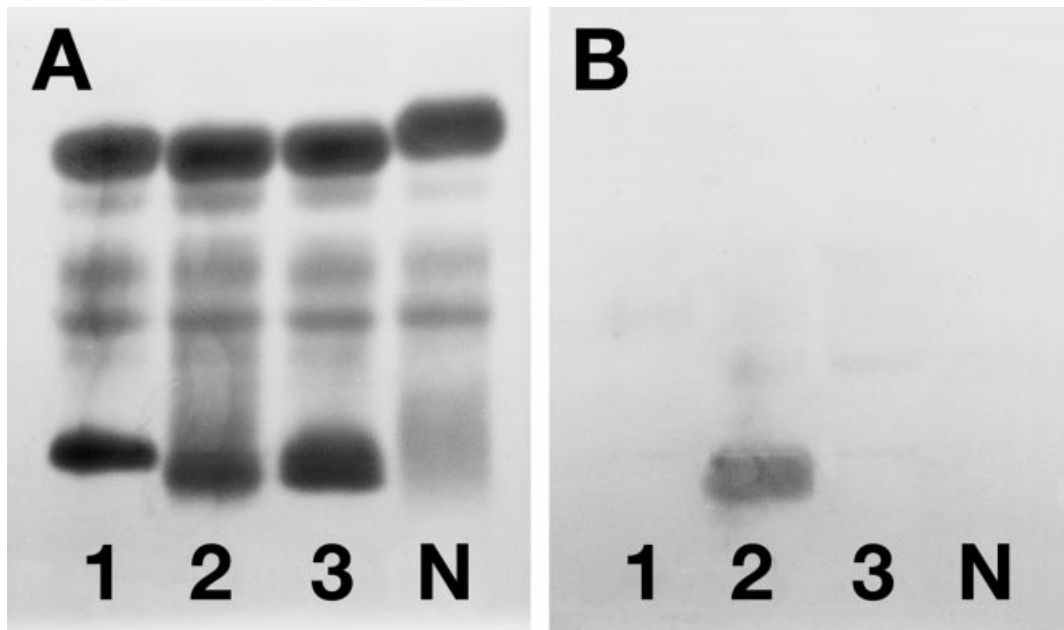


Fig 2. Specific binding of phage to the M-protein from which it was obtained. Panel A: Coomassie brilliant blue staining of agarose serum electrophoresis, patients 2 (lane 1), 6 (lane 2) and 9 (lane 3) and a normal individual control (lane N). Panel B: Blot on nitrocellulose of the same electrophoresis as in the left panel. The membrane was incubated with phage clone #50 containing the peptide insert YIHYIF obtained by panning with M-protein from patient 6 (lane 2). Bound phages were visualized by incubation with horseradish peroxidase labelled anti-M13 antibodies and luminol based chemiluminescent light detection on X-ray film. The phage bound only to the M-protein from which it was obtained, no reaction toward other serum proteins or M-proteins from other patients was observed. Cathode at top.

Table II. Unique mimotope peptide sequences obtained for patients 3 and 6.

Patient/clone	Sequence
3/#23	FDDARL
3/#11	FSDARL
3/#106	FSDMRL
3/#105	FVDVRL
3/#88	FTDIRL
3/#54	FNDYRL
3/#101	FSDTRL
3/#310	DPAFIFYHSTLFFNS
3/#318	GGHDGDPVLTGTLFY
3/#313	AVDPRMFYLLLRGA
6/#9	PIHYIF
6/#50	YIHYIF
6/#6	RIHYIF
6/#15	WREWFL
6/#16	WWAMKP
6/#602	LILSSGELLRHPRGA

showed a dose-dependent inhibition of the bound phages, whereas control peptides did not influence the binding (Fig 1). As an example, the binding of phages with peptide inserts YIHYIF and WREWFL (patient 6/#50 and #15) could be inhibited by either of the two corresponding BSA-conjugated synthetic peptides (data not shown). Binding specificity of the obtained phages was further evaluated by blotting of serum proteins separated by electrophoresis (Fig 2). Each of the phages investigated bound only to the M-protein used during its selection. As seen in Figs 2A and 2B, no binding was observed to other serum proteins or to M-proteins from other patients. Fluorescent synthetic peptides derived from the phage sequences gave similar results (data not shown). In accordance with these results, affinity chromatography of serum from patient 6 (Fig 3) shows selective binding of the M-protein to the immobilized peptide. Other serum components were not retained on the column (Fig 3B) nor were proteins and M-protein from another patient (Fig 3C).

Binding strength

The 50% inhibitory concentration for free peptides was 1×10^{-5} M for the free peptides and 1×10^{-7} M for BSA-conjugated peptides. An example is shown in Fig 1, where the phage clone 50 (peptide insert YIHYIF), binding to M-protein from patient 6, was competed with the corresponding synthetic peptide in free and in BSA-conjugated form. A control peptide showed no competition. In contrast, neither the free nor the BSA-conjugated peptide (patient 6/#50) was able to compete with the binding of the phage clone #952 isolated on M-protein from patient 9 (data not shown).

Sensitivity

The M-protein in serum from patient 6 diluted with a pool of normal sera and was clearly detectable at 5×10^{-5} dilution, using biotinylated peptide (YIHYIF) on streptavidin-coated ELISA-trays. This corresponded to a detection limit of about

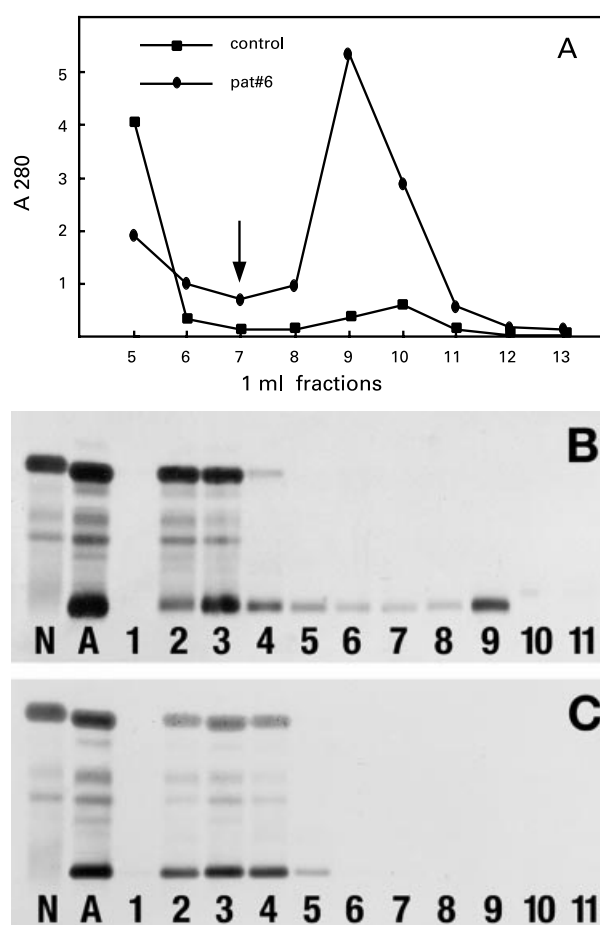


Fig 3. Ability of a peptide epitope to selectively remove an M-protein. Panel A: Absorbance at 280 nm of fraction from a 1 ml thiopropyl-Sepharose column conjugated with 15 mg of peptide YIHYIF (eqv. to clone #50) loaded with 1 ml serum of the corresponding patient 6 or a control patient 3. The elution of bound proteins at pH 2.35 took place from fraction number 8 (arrow). Panel B (patient 6) and panel C (control patient 3) show the Coomassie brilliant blue stained agarose serum electrophoresis of the eluted fraction. A normal serum is marked N and the applied serum is marked A. It is seen that the peptide is selective toward the M-protein from the patient it was isolated. The 1 ml column could remove about 9 mg of M-protein from 1 ml serum (approximately one third of the total amount).

0.01 g/l for this M-protein. Sera from another myeloma patient and several healthy individuals showed no reaction (Fig 4).

Cytology

The possibility for detection of myeloma cells with the obtained peptide mimotopes was examined using bone marrow from patient 6 and the FITC-labelled and BSA-conjugated peptide (FITC-YIHYIF₅-BSA). Direct labelling of myeloma cells from a cytospin was observed, although the cells stained with variable intensity (Fig 5A, green). Simultaneous staining for immunoglobulin G showed, as

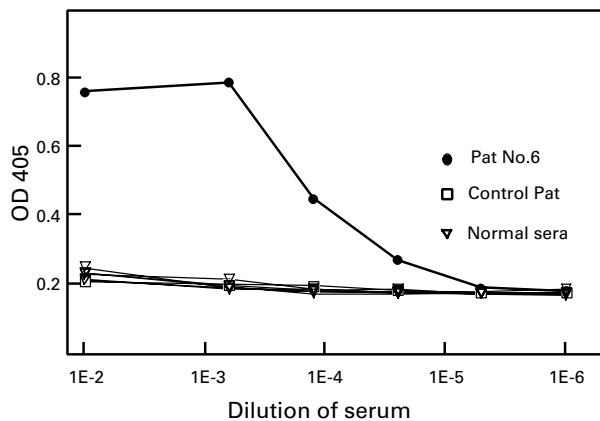


Fig 4. Detection of M-protein with peptide mimotopes. Serial dilution of M-protein from patient 6 monitored with mimotope M-protein ELISA using biotinylated peptide (YIHYIF) on streptavidin-coated trays. The M-protein could be detected in a 5×10^{-5} dilution, corresponding to about 10 mg/l. A control M-protein (patient 3; control pat) and several normal sera did not react.

expected, some cells reacting predominantly with immunoglobulin (Fig 5A, red). Other cells stained heavily with the peptide and faintly with anti-immunoglobulin. Co-incubation with the unlabelled peptide (1 mg/ml) completely prevented binding of the fluorescent peptide conjugate (not shown). Control bone marrow from another patient with M-protein of the same chain class showed only staining with anti-immunoglobulin (Fig 5B, red) and no staining with the peptide conjugate (Fig 5B, green).

DISCUSSION

Random peptide libraries express large numbers of different peptides (10^7 – 10^9), which can be selected by binding to antibodies, receptors or other target molecules (Parmley & Smith, 1988). Using M13 phage peptide libraries, random peptides can be expressed in fusion with filamentous bacteriophage coat protein pIII or pVIII. The phage library system has been used for identification of a variety of peptide ligands, especially antigen mimotopes (Clarkson & Wells, 1994; Scott & Craig, 1994).

Like other antibodies, M-proteins have variable regions that are potential antigen binders. In most cases the binding specificity of a M-protein is unknown, but several specific ligands have been reported (Merlini *et al.*, 1986). The antigen-binding groove of immunoglobulins can accommodate about six or seven amino acid residues, not necessarily occurring in a consecutive linear chain (Davies *et al.*, 1988). In the present study, only two of the M-proteins bound to 6-mer peptides, whereas six bound to 15-mer peptides. This may indicate that in many cases some secondary structure is required for adequate binding to M-proteins. Another possibility is that the binding strength of mimotopes were too low to survive the rather harsh panning conditions or simply that relevant antigenic mimotopes were not present in the libraries. Other libraries may provide more mimotopes. Since finishing this study we have used libraries which have encoded random peptide inserts in the pVIII major coat protein of the phages resulting in thousands of attachment points per phage. These libraries seem to allow selection for weaker binders due to multi-point binding.

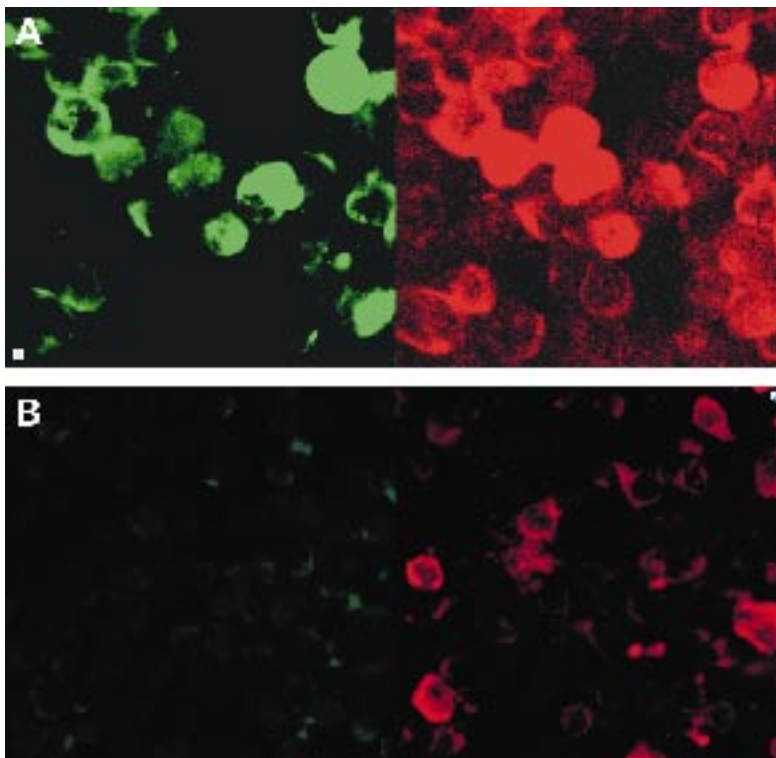


Fig 5. Detection of M-protein on bone marrow cells. Bone marrow cells from patient 6 (A) and control patient 8 (B) were attached to glass slides by cytopsin. The slides were stained with FITC-labelled pentavalent peptide YIHYIF (green) and with Cy3 goat anti-human immunoglobulin (red).

In this study we used M-protein enriched from serum. This step may be omitted since the relative ratio of the monoclonal M-protein compared with any other immunoglobulin greatly favours selection of mimotopes toward the M-protein. Furthermore, the total amount of immunoglobulin attached to the solid phase for panning is well below a microgram; this can easily be achieved by the direct coating of serum or with a catching antibody. In fact, the mimotope screening works with polyclonal antibodies where no purification is done; however, this increases the need for validating relevant clones.

Each M-protein was found to react with several peptides with different sequences. In spite of the lack of primary sequence homology, all of the peptides fit into the same antigen-binding pocket, since a given peptide from each of the themes inhibit the binding of a peptide from another theme. It should be noticed that the specificity is due to the nature of the immunoglobulin; many peptides, even without any apparent homology, may be accommodated in the binding groove with sufficient binding strength. We have conducted several other mimotope screenings toward conventional antibodies; sometimes the obtained peptides clearly had homology with the genuine antigen, at other times no apparent homology was seen. For our purpose, any of the peptides isolated for a given patient could be used, as long as the specificity was not hampered. For practical reasons, the shortest peptide with the highest avidity should be selected.

The M-proteins were readily isolated in affinity chromatography with immobilized peptide (Fig 3). The small amount of UV-absorbing material eluted in fractions 9–11 in the control experiment (Fig 3A) represents unspecific binding of non-stainable material (Fig 3C). Not surprisingly, the multivalent BSA conjugate showed two or three orders of magnitude stronger binding as compared to the free peptides. These binding strengths are comparable with many other antibody reactions, which can be monitored by immunological techniques.

Complete remission can be achieved in about 5% of myeloma cases with conventional chemotherapy (The Nordic Myeloma Study Group, 1996; Attal *et al*, 1996) and in 20–40% with high-dose chemotherapy combined with autologous or allogeneic stem cell support (Attal *et al*, 1996; Barlogie *et al*, 1997). However, virtually all patients relapse due to persistence of minimal residual disease undetectable with conventional techniques. Methods that are more sensitive are obviously needed for monitoring of these patients in order to resume treatment upon early signs of relapse.

Positive identification of an M-protein in agarose serum electrophoresis, with or without immunofixation, requires a concentration >0.5 g/l. Quantification of smaller amounts is virtually impossible due to the background levels of normal immunoglobulin (Kyle, 1994). The phage mimotopes provide a specific and apparently sensitive handle for a given M-protein, allowing for construction of a patient-specific M-protein ELISA. We could detect M-protein in serum at concentration at least 200 times lower than the detection limit in serum electrophoresis. Once a patient-specific

peptide ligand is obtained, the method provides a specific quantitative assay for monitoring the patient's M-protein throughout the treatment and post-treatment period. The measurement does not require isolation of cells and can be performed in any laboratory with standard equipment.

Several other methods have been used in an attempt to improve the monitoring of multiple myeloma. Flow cytometry and polymerase chain reaction (PCR) techniques both require bone marrow aspirates or the presence of circulation myeloma cells in the blood. Residual myeloma cells have been identified by flow cytometry. Light chain antibodies and propidium iodide were able to detect 0.1% contaminating myeloma cells (Shimazaki *et al*, 1991). Aneuploidy is, however, only present in 50–70% of myeloma patients (San Miguel *et al*, 1996). The combination of aberrant immune phenotype and DNA aneuploidy may enable the investigation of minimal residual disease in a higher proportion of myeloma patients (Almeida *et al*, 1999). PCR-based techniques using patient-specific probes to the complementary determining regions (CDR) of the immunoglobulin gene have been extensively used during the last years (Yamada *et al*, 1989; Bakkus *et al*, 1996; Corradini *et al*, 1995; Björkstrand *et al*, 1995; Cremer *et al*, 1997). With this sensitive method it is possible to detect one residual monoclonal cell per 10^5 – 10^6 cells in the bone marrow and blood and in stem cell collections (Corradini *et al*, 1995; Dreyfus *et al*, 1995). Quantitative PCR is, however, not easy to perform and it is not always possible to identify a unique nucleotide sequence. Circulating myeloma cells have been monitored by patient-specific biotinylated antisense oligonucleotides with *in situ* hybridization (Brown *et al*, 1998). This technique requires sequencing of each patient's rearranged immunoglobulin gene and quantification is limited to microscopically counting the percentage of positive cells.

The peptide ligands used here can, in addition to quantification of the M-protein, also be used to detect myeloma cells, although it is unclear whether binding takes place on the cell surface or in the cytoplasm. The general convention is that mature plasma cells do not expose surface immunoglobulins. However, some studies have demonstrated surface immunoglobulins in more than a third of multiple myeloma cases (Omedé *et al*, 1993; Ocqueteau *et al*, 1996; Knapp *et al*, 1974), whereas others studies could not detect any membrane-bound immunoglobulins (Miller *et al*, 1982). It may be that the cells that express surface immunoglobulins are the more immature and proliferating 'stem' myeloma cells (Sato *et al*, 1978). If so, the peptide ligands provide a potential tool for targeting treatment at these cells. Idiotypic monoclonal antibodies have been used with dramatic effect on B-cell lymphoma (Kwak *et al*, 1992; Renschler *et al*, 1994), and synthetic peptides identified by phage display libraries have in one case been shown to induce apoptosis in human B-cell lymphoma cells *in vitro* (Lam, 1993). If the peptide ligand itself is not sufficient for inducing programmed cell death, active toxic compounds may be coupled to the peptides, similar to the report of Brinkmann & Pastan (1995). They fused an immunoglobulin fragment to a truncated *Pseudomonas* exotoxin A, resulting in a selective cytotoxic agent. In multiple myeloma

the high concentration of circulating monoclonal immunoglobulin is likely to interfere with the binding of the peptide ligand to the cell surface. However, since peptides have a small molecular mass compared to immunoglobulin, a favourable molecular ratio could be attainable in patients that are brought in remission with conventional cytotoxic therapy. Peptide therapy might eradicate the residual myeloma cells in these patients. Furthermore, peptide mimotopes conjugated to a solid phase may also be useful by selectively removing myeloma cells from the bone marrow prior to autologous transplantation.

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