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Heteroclitic Recognition of Combinatorial TX^1TX^2T Peptide Mixtures by Mucin-2 Protein Specific Monoclonal Antibody

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Abstract: The mucin-2 (MUC2) glycoprotein secreted by the epithelial cells of human colon may be abnormally under-glycosylated in the case of cancer. Monoclonal antibody (mAb) 994 raised against the immunogenic part of the protein core, recognizes malignant human colon tissues as well as pentapeptides with TX^1TX^2T motif present in MUC2. Using a combinatorial approach and ELISA experiments it was found that mAb 994 is able to recognize peptides of the sub-library $TQTX^2T$ very strongly, and to some extent also peptides from $TETX^2T$, $TLTX^2T$ and $TVTX^2T$ sub-libraries. Binding studies with peptides corresponding to the $TQTX^2T$ and $TETX^2T$ sub-libraries showed that mAb 994 recognized only six peptides (IC_{50} = 9–208 μmol dm^{-3}) from the 19 compounds of the $TQTX^2T$ sub-library and only three peptides (IC_{50} = 3500–16700 μmol dm^{-3}) from the 'second-best' $TETX^2T$ sub-library. The most pronounced mAb binding occurred when Gln was in position X^1 and it was much weaker in the case of Glu, Val or Leu. As for X^2 amino acids, the presence of Pro, Ala can provide a strong, while Tyr, Trp, Phe and Ser a weaker, peptide–antibody interaction. Data from this study suggest that pentapeptide TQTPT, whose sequence is present in the native protein, is bound most strongly. However, almost identical binding properties were observed with peptide TQTAT, whose sequence is not present in the protein. Apart from this, some other 'heteroclitic' peptides were found with a different rank in the binding-hierarchy. Based on these peptides artificial compounds can be prepared as potential candidates for vaccine development. Results of this study also provide a rationale for understanding the molecular background of the heteroclitic nature of the MUC2 protein core specific mAb 994. Copyright © 2003 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: mucin 2 glycoprotein; synthetic epitope peptides; combinatorial mixture; antibody recognition of peptide libraries

INTRODUCTION

Gastrointestinal cancer is a common cause of death worldwide [1]. In the absence of metastasis most cancers would be cured by surgery, chemotherapy and radiation therapy [2]. For diagnosis of the disease and/or monitoring the effect of medical treatment there is a need to identify tumour specific marker molecules.

Mucins are high molecular weight glycoproteins (>200 000 Da) providing a protective layer against chemical and enzyme attack at the outer surface of epithelial cells. In carcinomas mucins may be abnormally overexpressed and/or deficient in glycosylation [3]. Because of these features mucins of tumour tissues may serve as markers of the

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disease [4] and might enable a renaissance in clinical diagnosis [2].

The mucin-2 (MUC2) glycoprotein secreted mainly by the epithelial cells of human colon is heavily O-glycosylated at the side chain of Thr and/or Ser residues. The protein core consists of a variable number of tandem repeats with the consensus amino acid sequence of ^{1}PTTTPITTTTTVTPTPTPTGTQT^{23} [5]. Previously several monoclonal antibodies (mAbs) have been developed in mice using a synthetic peptide (K^{12}VTPTPTPTGTQTPT^{25}) derived from the repeat region of MUC2 and conjugated to KLH (keyhole limpet haemocyanin) carrier [4]. Some of these antibodies (such as mAb 994 and 996, both belonging to the IgG1 subclass of antibodies) have recognized samples from human tumour tissues of the colon, as well as soluble tumour derived immunoaffinity purified MUC2 mucin [7]. For several years the polypeptide chain related antigenic structure of MUC2 mucin has been studied. During the course of these investigations an epitope (^{18}PTGTQ^{22}) recognized by MUC2 protein specific mAb 996 was localized using synthetic overlapping peptides [8,9]. It was found that the other monoclonal antibody, mAb 994, exhibits different epitope specificity. The binding studies performed with a set of synthetic *N*- or *C*-terminally truncated peptides covering the 12–25 region showed that mAb 994 bound three peptides with different affinities: ^{21}TQTPT^{25} ($IC_{50} = 4 \pm 1$ μmol dm^{-3}), ^{19}TGTQT^{23} ($IC_{50} = 65 \pm 15$ μmol dm^{-3}) and ^{13}TPTPT^{17} ($IC_{50} = 180 \pm 20$ μmol dm^{-3}). Interestingly these peptides comprise a common motif $TX^{1}TX^{2}T$ [10].

The phenomenon of the lack of absolute specificity of immune recognition frequently referred to as heteroclitic recognition (heteros 'other' and klinein 'to incline'). It was documented first in antibody binding to antigens (reviewed by Berzofsky [11]; Van Regenmortel *et al.* [12]), and much later also in the context of T cell recognition of MHC presented epitope peptides [13,14]. Several studies have been reported in which antibodies bind not only to the immunizing antigen but also to other structurally related antigens (cross-reacting antigens) with even higher affinity. The heteroclitic antibody recognition was first described by Mäkelä [15]. The serum antibody produced by mice immunized with T2 phage had an extremely poor binding to the immunogen (surviving PFU of T2 was 256), but had an extremely high inactivating effect for the closely related T6 or T4 phages (surviving PFU was 5). In another study the heteroclitic nature of monoclonal antibodies has been demonstrated. Monoclonal IgG antibodies raised against dinitrophenylated (DNP-) proteins were capable of binding not only the DNP (IC_{50} value is 250 ng cm^{-3}), but also the trinitro phenyl ($IC_{50} = 1.6$ μg cm^{-3}) and the 4-hydroxy-3-nitro-phenyl ($IC_{50} = 150$ ng cm^{-3}) moieties in their ε-aminocaproic acid conjugate [16]. IgM and IgG monoclonal antibodies of CBA and CBH mice against 2-furyl-4-ethoxymethylene-oxazol-5-on were also found to be heteroclitic: they had a 4–6 times higher affinity for 2-styryl-4-ethoxymethylene-oxazol-5-on than for the immunizing furyl oxazolone hapten [17]. For the identification of epitopes on insulin recognized by monoclonal IgM and IgG antibodies raised against bovine insulin, Rathjen and Underwood utilized a number of insulin variants from different species. These polypeptides differed only in a few amino acid residues. Three of the bovine insulin specific antibodies had heterospecific reactivity, e.g. mAb1 (IgM, with $IC_{50} \sim 1$ μg cm^{-3} for bovine insulin) recognized sheep insulin ($IC_{50} \sim 5$ ng cm^{-3}) better. Since sheep insulin differs only in a single amino acid residue, this finding indicated that glycine in position 9 of the A chain is responsible for improved antibody recognition [18].

Strangely enough, only a limited number of studies have been performed to understand the structural/molecular basis of the heteroclitic antibody recognition. The heteroclicity of an antibody fragment against 4-hydroxy-3-nitrophenyl acetic acid (NP) was studied. X-ray crystallographic data showed that improved binding of NP specific Fab to iodinated NP is due to the enhanced stability in the iodinated ring [19]. The heteroclitic binding of a mAb specific for hen egg-white lysozyme (D11.15) to avian egg-white lysozymes was investigated by x-ray crystallography. The differences in lysozyme binding were interpreted by the altered steric accommodation of different amino acids at positions 113 and 121 of lysozyme within the interface of the immune complex [20].

The different reactivity profile of mAb 994 towards human tumour tissues [6] as well as to truncated mucin-2 peptides [10] led to the investigating the molecular background of the heteroclitic binding of the antibody towards multiple peptides with the $TX^{1}TX^{2}T$ motif. Therefore a systematic study was initiated to investigate the effect of amino acids X^{1} and X^{2} on the antibody recognition of $TX^{1}TX^{2}T$ pentapeptides. For this purpose nearly all variants of $TX^{1}TX^{2}T$ peptides were generated by a combinatorial approach using proteinogenic amino acids,

except Cys. First 19 sub-libraries were prepared corresponding to the TATX^{2}T, TDTX^{2}T,..., TYTX^{2}T sequences using portioning-mixing solid phase synthesis [21]. The antibody binding properties of these peptide mixtures were studied in the competitive ELISA assay. In the second set of experiments, individual peptides were synthesized corresponding to the best two sub-libraries and the mAb 994 binding characteristics of these compounds were determined.

In this paper the synthesis and the monoclonal antibody binding hierarchy of TX^{1}TX^{2}T peptides are reported. The data show that besides peptides corresponding to the native mucin-2 protein sequence (^{12}VTPTPTPTGTQTPT^{25}) several other pentameric compounds are recognized by mAb 994 with similar efficacy. This observation might be important not only for the better understanding of the heteroclitic nature of MUC2 antibody recognition, but also to initiate further studies to design synthetic peptide antigens with a non-native sequence for immunization against malignant diseases associated with aberrantly expressed MUC2 glycoprotein. It might be important to note that this study also provides for the first time comparative data on the reliability and efficacy of the combinatorial approach in establishing the relative order among pentapeptide antigens using the same binding assay.

MATERIALS AND METHODS

Materials

Solvents (dichloromethane [DCM], *N*,*N*-dimethylformamide [DMF], diethyl ether) were from Reanal (Budapest, Hungary). Other chemicals (*N*,*N*′-diisopropylcarbodiimide [DIC], *N*,*N*-diisopropylethylamine [DIEA], HF, 1-hydroxybenzotriazole [HOBt], *p*-cresol, trifluoroacetic acid [TFA], TFMSA) were analytical grade products from Fluka AG (Buchs, Switzerland). Boc-Thr(Bzl) resin (capacity: 0.68 mmol g^{-1}) was prepared using Boc-Thr(Bzl) and Bio-Beads S-X1 chloromethylated polystyrene resin containing 1% divinyl benzene (200–400 mesh, 1.26 mmol Cl g^{-1}; Bio-Rad Laboratories, Richmond, CA, USA) by anhydrous KF according to Horiki *et al.* [22]. Protected amino acid derivatives: Boc-Ala, Boc-Asn, Boc-Asp(OBzl), Boc-Arg(Tos), Boc-Gln, Boc-Glu(OBzl), Boc-Gly, Boc-His(Tos), Boc-Ile, Boc-Leu, Boc-Lys(Z), Boc-Met, Boc-Phe, Boc-Pro, Boc-Ser(Bzl), Boc-Thr(Bzl), Boc-Trp, Boc-Tyr(Bzl) and Boc-Val were purchased from Sigma-Aldrich (Budapest, Hungary). The HF apparatus was a product from the Peptide Institute, Inc. (Minoh-Shi, Osaka, Japan).

Combinatorial Peptide Synthesis

The TX^{1}TX^{2}T sub-libraries were synthesized by the portioning–mixing method [20] using Boc/Bzl chemistry. Nineteen Boc-Thr(Bzl)-resin samples (50 mg, 34 μmol each) in plastic tubes equipped with teflon frits using a vacuum manifold apparatus (Shimadzu Scientific Research Inc., Tokyo, Japan) were swollen in DCM (3 ml) for 10 min. Then TFA/DCM (1 : 2, v/v; 2 × 3 ml) mixture was added to the filtered resins for 2 min and 30 min. The resin samples were washed successively with 3 ml of DCM, MeOH, DCM, MeOH and DCM. Neutralization was performed by adding DIEA/DCM (1 : 9, v/v; 2 × 3 ml) mixture to the resin sample for 2 + 8 min. After washing with 3 × 3 ml DCM, one of the 19 Boc-amino acid derivatives after activation was added to the resin sample as follows: a 5-fold molar excess Boc-amino acid derivative (170 μmol) dissolved in DCM/DMF (2 : 1, v/v; 2 ml) was mixed with DIC (27 μl, 170 μmol) and HOBt (25 mg, 187 μmol), added to the resin samples and incubated for 2 h at room temperature. After washing of DMF, DCM and MeOH (3 × 3 ml), the effectiveness of coupling was monitored by ninhydrin [23] or isatin assay [24]. The protected dipeptidyl resin samples from each tube were suspended in DCM (3 ml) and poured into a common tube. Then the combined and thoroughly mixed resin was prepared, deprotected as above and reacted with Boc-Thr(Bzl) (999 mg, 3.23 mmol) in DCM/DMF (2 : 1, v/v; 10 ml) mixture using the protocol described above. The tripeptidyl (TX^{2}T) resin mixture was suspended in DCM/DMF (2 : 1, v/v; 10 ml). The mixture was shaken for 10 min and, to avoid sedimentation, the slurry was quickly divided into 19 equal volumes (0.5 ml of each). The next two synthetic steps (coupling of one of the 19 protected amino acids and then Boc-Thr(Bzl)) were performed in a parallel synthesis format in the individual tubes.

After the removal of the N^{α}-Boc-group, the side-chain protected peptides were cleaved off from the resin. Two cleavage methods were applied. Method A: the resin was treated with 3 ml anhydrous HF containing 5% (m/v) *p*-cresol, at 0 °C for 1 h. After removal of HF *in vacuo* the crude product was precipitated with diethyl ether, filtered and the peptides were extracted with 10% aqueous acetic acid. The solution was diluted with distilled water and freeze-dried. Method B: the resin was

stirred with TFMSA:TFA mixture (1:9, v/v) at room temperature for 1 h. The reaction mixture was filtered into diethyl ether (25 ml). The precipitate was allowed to stand overnight at −20 °C, then collected by centrifugation, washed twice with diethyl ether and dried over KOH *in vacuo*, dissolved with distilled water (0.5 ml) and freeze-dried.

The peptide content of the crude TX^1TX^2T sub-libraries were determined by (i) measurement of nitrogen content after Kjeldahl digestion and distillation (ii) spectrophotometric *N*-terminal threonine content analysis using ninhydrin reagent and calculation with the aid of a calibration curve taken from free threonine [25]. The peptide content of each sub-library was determined at least by two measurements.

The Synthesis of Individual Pentapeptides

Individual peptides corresponding to $TQTX^2T$ and $TETX^2T$ sub-libraries were prepared in a parallel synthesis format using the same equipment and protocol as described in the previous paragraph.

In each experiment sample of Boc-Thr(Bzl)-resin (50 mg, 34 μmol) was swollen and treated with reagents in 2–3 ml solutions. At the end of the syntheses peptides from the $TETX^2T$ sub-library were produced with the TFMSA/TFA cleavage (method B), peptides corresponding to $TQTX^2T$ sub-library were cleaved by HF (method A) as described above.

Purification and Analysis of the Individual Peptides

All pentapeptides were purified by RP-HPLC on a C_{18} column (Phenomenex, 5 μm, 300 Å, 250 × 10 mm) using a linear gradient of 5% to 35% of eluent B (Eluent A: 0.1% TFA in water, Eluent B: 0.1% TFA in 80% acetonitrile/water) over 30 min at a flow rate of 4 ml min^{-1} at room temperature. The eluent was monitored by UV detection at $\lambda = 214$ and $\lambda = 254$ nm.

The homogeneity of the purified peptides was checked by RP-HPLC on a C_{18} column (Phenomenex, 5 μm, 300 Å, 250 × 4.6 mm) using a linear gradient of 0 to 30% of eluent B (eluents see above) over 30 min at a flow rate of 1 ml min^{-1} at room temperature. The eluent was detected by UV absorbance at $\lambda = 214$ and $\lambda = 254$ nm.

Amino Acid Analysis

The amino acid composition of the peptides was verified by amino acid analysis. Amino acid analyses of individual peptides were performed using a Beckman Model 6300 analyser (Fullerton, CA, USA). Hydrolysis of samples was performed in 6 M HCl in sealed and evacuated tubes at 110 °C for 24 h.

Mass Spectrometry

The composition of the $TQTX^2T$ sub-library was analysed by ESI-FTICR mass spectrometry [25,26]. The Fourier transform-ICR spectrometer was a Bruker Daltonics APEX II® instrument (Bremen, Germany) equipped with a 7.0 Tesla actively shielded superconducting magnet (Magnex, Oxford, UK) using a Bruker Apollo ESI source with an API1600 ESI/APCI Control Unit, and a UNIX based Silicon Graphics O2 workstation data system. Details of the instrumental conditions of FTICR-MS were as previously reported [27]. Acquisition of the spectra was performed with Bruker Daltonics software XMass (version 5.0.10) and corresponding programs for mass calculation, data calibration and processing. The samples were dissolved in a solution of 3% acetic acid in 50% methanol/water. Ionization was made with a flow rate of 2 μl min^{-1}.

The relative molecular mass of individual peptides was determined by MALDI-TOF mass spectrometry [25]. The spectra were recorded using a Bruker Biflex linear MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a SCOUT-48 ion source and sample stage at a digitizing rate of 500 MHz [28]. Sample preparation was carried out with α-cyano-4-hydroxi-cinnamic acid. The spectra were recorded at an acceleration voltage of 20 kV. Evaluation of the mass spectra was carried out with the Bruker XMass software (Bruker Daltonics, version 4.0.1) on a Sparc-1 workstation.

Inhibition ELISA Immunoassay

Synthetic peptides in solution were tested for their capacity to inhibit 994 antibody binding to the target antigen [K^{12}VTPTPTPTGTQTPT^{25}-OH]-BSA conjugate [9]. Conjugate at 1 μg cm^{-3} in PBS (pH 7.3) containing 0.02% NaN_3 was added into a 3912 FALCON III flexible PVC microtitre plate (96 well, well capacity = 300 μl; Becton Dickinson, Oxnard, CA) at 50 μl per well and air dried overnight. The wells were washed with a washing buffer consisting of PBS (pH 7.3) and then incubated with PBS (pH 7.3) containing 1% BSA for 60 min at room temperature in order to block non-specific adsorption sites. After washing the microtitre plate with PBS (pH 7.3) the 994 antibody at 1 μg cm^{-3}

concentration, 30 µl to each well, or washing buffer alone in negative controls was added to the wells to which 30 µl of the test peptide or peptide sub-library solution had been added. In the case of the sub-libraries a concentration range between 0.064 and 20 mg cm^{-3} was used. In the experiments with the individual peptides the highest concentration (1.756 mmol dm^{-3}) was calculated according to the participation rates of peptides in the stock solution of sub-libraries (roughly 1 mg cm^{-3} concentration each) using the average molecular mass (569.18 g mol^{-1}) of all (19) TQTX^{2}T peptides. Consequently, solutions of single peptides were added in a concentration range between 0.00056 and 1.756 mmol dm^{-3}. In the case of TETXT peptides a higher concentration by an order of magnitude (17.56 mmol dm^{-3}) was used as well. After incubation for 2 h at room temperature the wells were washed four times with PBS buffer (pH 7.3). Horseradish peroxidase (HPO)-linked rabbit anti-mouse Ig (Dako, High Wycombe, UK) at a dilution of 1/1000 (in PBS, pH 7.3, containing 0.1% BSA) was dispensed at 50 µl per well, incubated for 1 h at room temperature, and the plate was again thoroughly washed. The ABTS substrate solution (50 mg 2,2′-azino-bis-(3-ethylbenzthiazoline)-6-sulphonate in 100 ml of 0.1 M citrate-phosphate buffer, pH 4.0, containing 0.01% hydrogen peroxide) was added at 50 µl per well. Absorbance in each well was measured at $\lambda =$ 405 nm using an ELISA reader (Labsystems iEMS Reader MF, Helsinki, Finland). In each case, the amount of added peptide required to inhibit antibody binding by 50% (IC_{50} values) was calculated as a measure of antigenic potency. The IC_{50} values of peptides and sub-libraries were determined by 2–5 parallel measurements.

RESULTS

Synthesis and Antibody Binding of muc2 Peptide Sub-libraries Corresponding to the TX^{1}TX^{2}T Sequences

For studying the role of the X^1 and X^2 amino acids of the TX^{1}TX^{2}T epitope motif of tumour-associated gastrointestinal MUC2 in antibody recognition, 19 peptide sub-libraries were prepared. These mixtures were produced by combinatorial peptide synthesis using the portioning–mixing strategy [21] (as outlined in Figure 1) and Boc/Bzl technique. Each library contained threonine in positions 1, 3, 5, one pre-selected amino acid in position X^1 and all proteinogenic amino acids, except cysteine, in position X^2. The peptide content of the mixtures was determined by two independent methods. The results of the nitrogen content analyses by the Kjeldahl method in accord with those of *N*-terminal threonine content determination using ninhydrin assay showed that the preparations contain roughly similar amounts of peptides representing 59% ± 12% (by weight) of the sample. Non-peptide components of the samples were predominantly water and salts originating from the cleavage procedures. It should be noted that no detailed analysis was carried out to identify the salt composition.

All 19 TX^{1}TX^{2}T sub-libraries were investigated for their ability to inhibit the binding of the mAb 994 monoclonal antibody to the BSA-[K^{12}VTPTPTPTGTQTPT^{25}] target antigen in competitive ELISA studies. In the design of these comparative experiments the peptide content of the mixtures was considered. The binding capacity of mixtures was characterized by IC_{50} values calculated as described in the Materials and Methods section. Data are summarized in Figure 1. It was found that peptides of only four sub-libraries of the 19 bound to the mAb 994. Among these the TQTX^{2}T sub-library was the most active with an IC_{50} value of 5.4×10^{-3} mg cm^{-3}. A further three sub-libraries (TVTX^{2}T, TLTX^{2}T, TETX^{2}T) were also markedly recognized by the antibody, but the corresponding IC_{50} values show much lower levels of interaction ($IC_{50} = 2.1$, 2.5 and 3.3×10^{-1} mg cm^{-3}, respectively). The lack of mAb 994 binding observed with the remaining 15 TX^{1}TX^{2}T sub-libraries is demonstrated by data with the TGTX^{2}T sub-library shown in Figure 2.

The best-recognized sub-library, TQTX^{2}T was further characterized by MS methods to verify the presence of all components. Since the MALDI-TOF-MS analysis failed to resolve the complex mixture of 19 components, one of the most advanced methods in the field was used. The results of the ESI-FTICR-MS analysis, published in detail, recently demonstrated that all 19 peptides are present in the TQTX^{2}T sub-library with a relative ion abundance distribution approximately reflecting a roughly equimolar ratio of components [25]. Thus, the indistinguishable isomeric pentapeptides, TQTIT and TQTLT, were observed with approximately twofold greater abundance compared with that of the other components. Furthermore, the ESI-FTICR spectrum readily provides unequivocal resolution

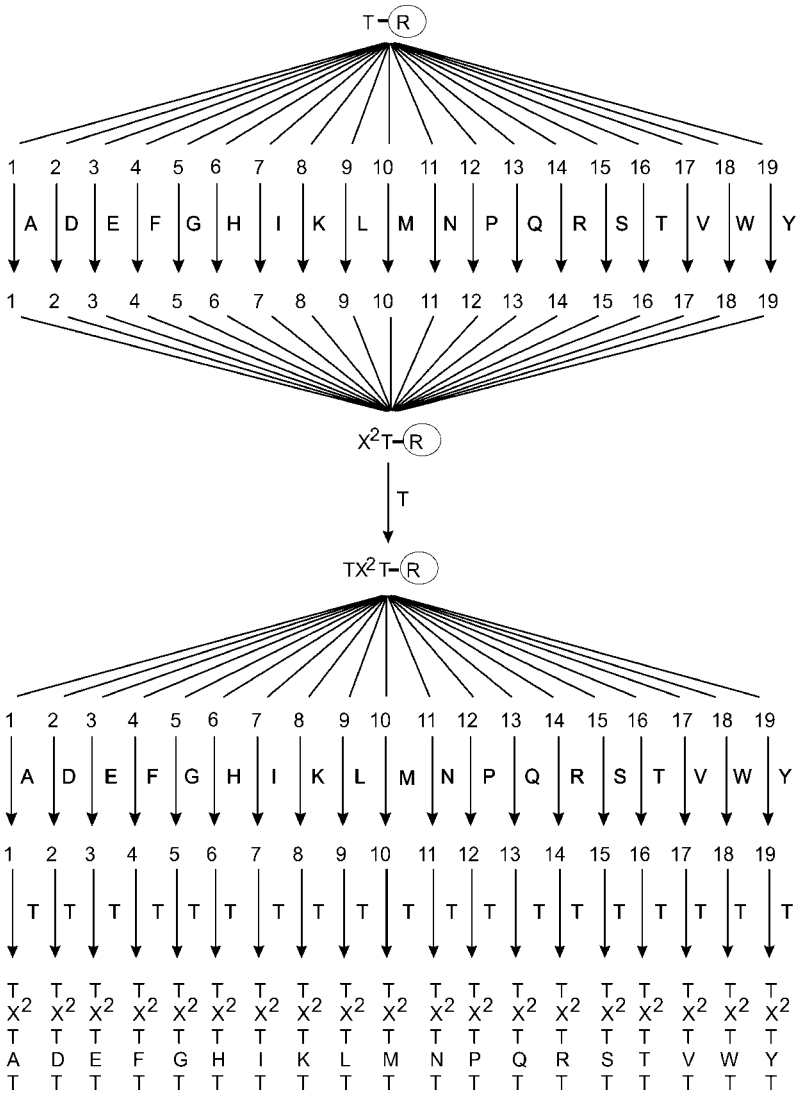

Figure 1 Outline of the synthesis strategy of the 19 TX^1TX^2T sub-libraries using the portioning–mixing method according to Furka *et al.* [21].

and identification of the 'isobaric' peptides TQTKT and TQTQT ($\Delta m = 0.0364$ Da).

Synthesis and Antibody Binding of Individual Peptides Corresponding to the $TQTX^2T$ and $TQTX^2T$ Sequences

Based on the antibody binding results with the TX^1TX^2T combinatorial mixtures two sub-libraries were selected for further studies. Individual peptides corresponding to the 'best-binder' $TQTX^2T$ and one of the 'second-best binder' sub-libraries ($TETX^2T$) were prepared using the parallel strategy and Boc/Bzl technique. As before Cys-containing variants were omitted. As an example, a mass spectrometry spectrum and HPLC chromatogram of a pentapeptide (TQTPT) are presented in Figure 3.

The characteristic data of all peptides including the results of mass spectrometry, amino acid analysis and of analytical RP-HPLC for $TQTX^2T$ [24] and $TETX^2T$ peptides proved the expected primary structure (Tables 1 and 2). The purity of all peptide preparations was greater than 95%.

The effect of the amino acid in position X^2 on the antibody binding using all 19 components of the 'best-binder' sub-library ($TQTX^2T$) was investigated. The binding capacity of individual peptides was characterized by the IC_{50} value and determined in the competitive ELISA immunoassays. The results

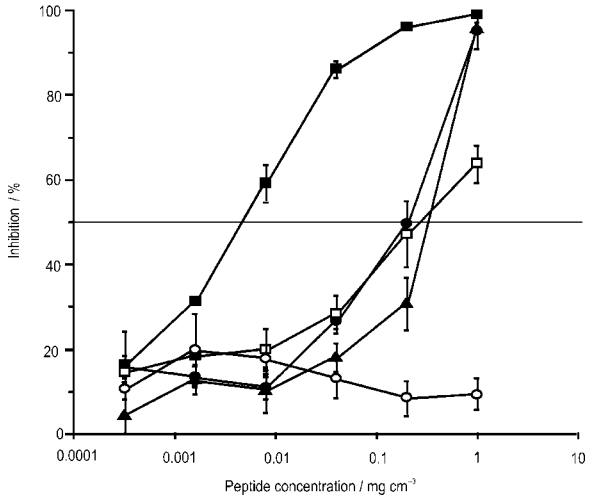

Figure 2 Inhibition of 994 antibody binding to [K[12]VTP-TPTPTGTQTPT[25]-OH]-BSA target antigen with the TX[1]TX[2]T sub-libraries: TQTX[2]T (■), TETX[2]T (●), TLTX[2]T (□), TVTX[2]T (▲), TGTX[2]T (○) in an ELISA competition assay.

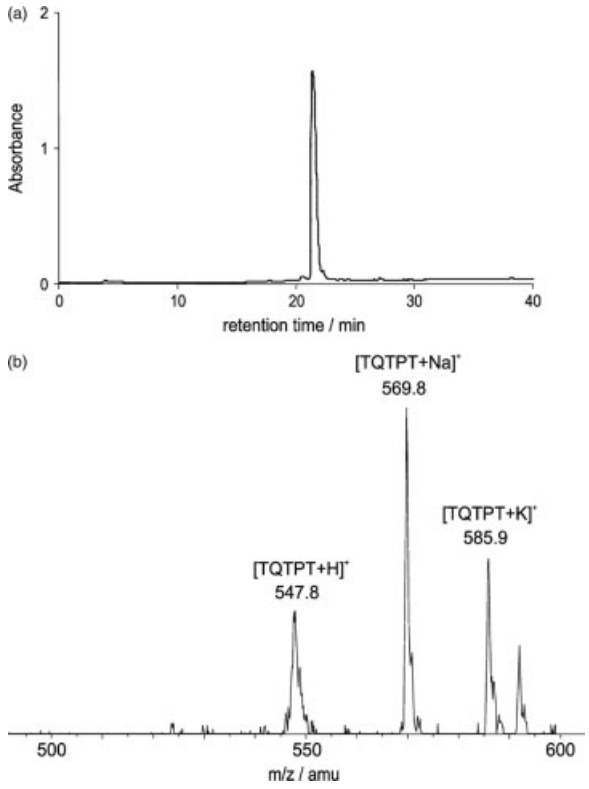

Figure 3 RP-HPLC chromatogram (a) and MALDI-TOF-MS spectrum (b) of the purified TQTPT peptide. Conditions are described in the Materials and Methods section.

of this experiment show (Figure 4, Table 3) that only a limited number of TQTX[2]T exhibit binding

Table 1 Characteristics of Synthetic Peptides Corresponding to the TQTX[2]T Sub-library

Peptide	Amino acid composition[a] measured (calculated)	[MH⁺] mass[b]		R_t[c] min
		Found	Calc.	
TQTAT	T 3.1 (3), E 0.9 (1), A 1.0 (1)	521.6	521.5	18.0
TQTDT	n.t.[d]	565.6	565.6	10.0
TQTET	T 3.0 (3), E 2.0 (2)	579.6	579.6	18.0
TQTFT	T 3.0 (3), E 1.0 (1), F 1.0 (1)	597.0	597.7	20.0
TQTGT	T 3.2 (3), E 0.9 (1), G 0.9 (1)	507.5	507.5	15.0
TQTHT	n.t.[d]	586.9	587.6	16.0
TQTIT	T 3.1 (3), E 1.2 (1), I 1.1 (1)	563.6	563.6	18.0
TQTKT	n.t.[d]	578.6	578.6	12.5
TQTLT	n.t.[d]	563.6	563.6	38.0
TQTMT	n.t.[d]	581.6	581.6	23.5
TQTNT	n.t.[d]	564.6	564.6	12.0
TQTPT	T 3.0 (3), E 1.2 (1), P 1.0 (1)	547.6	547.6	21.5
TQTQT	n.t.[d]	578.6	578.6	12.5
TQTRT	n.t.[d]	606.0	606.7	28.5
TQTST	n.t.[d]	537.6	537.6	19.0
TQTTT	n.t.[d]	551.6	551.6	27.0
TQTVT	n.t.[d]	549.7	549.6	22.0
TQTWT	n.t.[d]	636.7	636.7	33.5
TQTYT	T 2.97 (3), E 1.1 (1), Y 1.2 (1)	613.6	613.6	25.0

[a] Amino acid analysis: asparagine and glutamine were measured as aspartic acid and glutamic acid, respectively.
[b] Molecular mass measured by MALDI-TOF-MS.
[c] Phenomenex column, 300 Å, 5 µm 4.6 × 250 mm, flow rate: 1 cm^3 min^{-1}, A eluent: 0.1% TFA/water, B eluent: 0.1% TFA/MeCN–water 80–20 (v/v), gradient elution: 0–5 min 0% B, 5–35 min 0–30% B eluent.
[d] Not tested.

to mAb 994. The IC_{50} values corresponding to the six binder peptides fall into the range 3–208 µmol dm^{-3}. It was found that peptide TQTPT representing a pentamer sequence of the polypeptide core of the native mucin-2 has the highest capacity to inhibit the binding of mAb 994 to the target antigen (IC_{50} = 3 µmol dm^{-3}). It is worth noting that peptide K[12]VTPTPTPTGTQTPT[25] derived from the repeat unit of the MUC2 protein and used in its conjugated form as a target antigen in ELISA, had similar binding properties (IC_{50} = 2.5 µmol dm^{-3}). Interestingly, it was also observed that other pentapeptides (TQTX[2]T, where X = A, Y, W, F or S) whose sequences are not present in mucin-2 glycoprotein were also recognized by the monoclonal antibody (Figure 4). Based on IC_{50} values, the following order of antibody binding capacity was established: TQTAT > TQTYT > TQTWT > TQTFT > TQTST. It should be noted that peptide TQTAT has

Table 2 Characteristics of Synthetic Peptides Corresponding to the TETX^{2}T Sub-library

Peptide	Amino acid composition[a] measured (calculated)	[MH$^+$] mass[b]		R_t[c] min
		Found	Calc.	
TETAT	T 2.9 (3), E 1.0 (1), A 1.1 (1)	521.9	522.5	5.7
TETDT	T 2.7 (3), E 1.2 (1), D 1.1 (1)	565.9	566.5	5.5
TETET	T 2.9 (3), E 2.1 (1)	579.3	580.6	7.4
TETFT	n.t.[d]	597.9	598.6	20.0
TETGT	T 3.3 (3), E 0.9 (1), G 0.8 (1)	507.9	508.5	6.1
TETHT	T 2.9 (3), E 1.2 (1), H 0.9 (1)	588.0	588.6	5.2
TETIT	T 2.8 (3), E 1.2 (1), I 1.0 (1)	563.9	564.6	18.0
TETKT	T 2.8 (3), E 1.3 (1), K 1.0 (1)	579.0	579.6	5.5
TETLT	T 2.8 (3), E 1.2 (1), L 1.1 (1)	563.9	564.6	21.0
TETMT	T 2.8 (3), E 1.2 (1), M 0.7 (1)	581.8	582.6	16.5
TETNT	T 2.8 (3), E 1.2 (1), D 1.0 (1)	565.0	565.6	5.0
TETPT	T 2.9 (3), E 1.2 (1), P 1.0 (1)	547.9	548.6	15.2
TETQT	T 2.7 (3), E 2.3 (2)	578.9	579.6	6.6
TETRT	T 2.9 (3), E 1.2 (1), R 0.9 (1)	607.2	607.6	7.8
TETST	T 2.7 (3), E 1.1 (1), S 1.3 (1)	538.0	538.6	4.0
TETTT	T 3.8 (4), E 1.2 (1)	552.0	552.6	6.1
TETVT	T 2.6 (3), E 1.2 (1), V 1.2 (1)	550.0	550.6	16.0
TETWT	n.t.	637.0	637.7	21.1
TETYT	T 3.2 (3), E 0.9 (1), Y 0.9 (1)	613.9	614.6	17.3

[a] Amino acid analysis: asparagine and glutamine were measured as aspartic acid and glutamic acid, respectively.
[b] Molecular mass measured by MALDI-TOF-MS.
[c] Phenomenex column, 300 Å, 5 µm 4.6 × 250 mm, flow rate: 1 cm^3·min^{-1}, A eluent: 0.1% TFA/water, B eluent: 0.1% TFA/MeCN–water 80–20 (v/v), gradient elution: 0–5 min 0% B, 5–35 min 0–30% B eluent.
[d] Not tested.

an IC_{50} value of 11 µmol dm^{-3}, which represents almost the same binding activity as peptide TQTPT from the protein sequence (3 µmol dm^{-3}) (Table 3). Other peptides (e.g. TQTGT in Figure 4) did not inhibit the binding of the mAb 994 to the target antigen (IC_{50} > 10 000 µmol dm^{-3}). In a control experiment an equimolar mixture of ten TQTX^{2}T pentapeptides was also tested. This mixture containing all five binder pentapeptides exhibited a low IC_{50} value (2 µmol dm^{-3}) which was comparable to that of the peptide TQTPT.

Since other three sub-libraries (TETX^{2}T, TVTX^{2}T and TLTX^{2}T) also showed remarkable immunoreactivity (Figure 3), one of those (TETX^{2}T) was selected for further studies and the corresponding 19 individual peptides were prepared and characterized (Table 2).

In binding experiments performed with mAb 994 it was found that three peptides (TETPT, TETAT and TETYT) were capable of inhibiting the attachment of mAb994 to the target antigen (Table 3). Two of the three compounds (TETPT and TETAT) had lower, but similar, IC_{50} values in the 3500–4700 µmol dm^{-3} range. Peptide TETYT exhibited a weak antibody inhibition (IC_{50} = 16 700 µmol dm^{-3}). No other

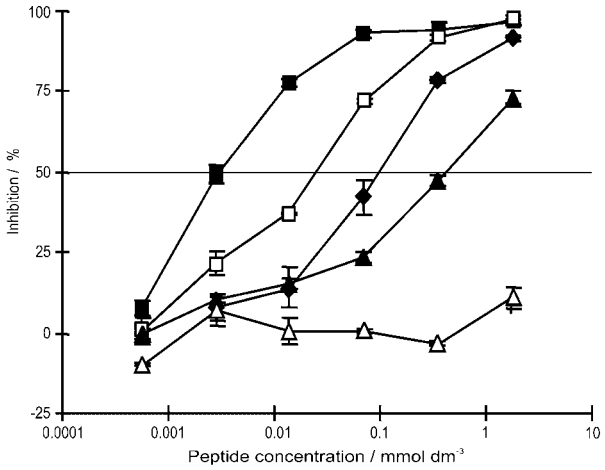

Figure 4 Inhibition of 994 antibody binding to [K^{12}VTPTP-TPTGTQTPT^{25}-OH]-BSA target antigen with pentapeptides TQTPT (■), TQTAT (□), TQTYT (♦), TQTFT (▲) and TQTET (Δ) corresponding to the TQTX^{2}T sub-library in an ELISA competition assay.

Table 3 Binding of Peptides Corresponding to the Selected TQTX^{2}T and TETX^{2}T Sub-libraries to 994 Antibody

X^2 Amino acid	IC_{50} value (µmol dm^{-3})[a] of	
	TQTX^{2}T peptide	TETX^{2}T peptide
P	3 ± 0	3500 ± 140
A	11 ± 4	4700 ± 290
Y	40 ± 15	16700 ± 820
W	70 ± 22	>100 000
F	88 ± 13	>100 000
S	208 ± 61	>100 000
K[b]	>10 000	>100 000

[a] IC_{50} value is the peptide concentration required for 50% inhibition of 994 antibody binding to BSA-[K^{12}VTPTPTPTGTQTPT^{25}] target antigen calculated from ELISA experiments. Each peptide was tested in 2–5 replica assays. The figures demonstrate the averages of these results.
[b] Similar IC_{50} values were obtained for X^2 = D, E, G, H, I, L, M, N, Q, R, T and V containing peptides.

peptides were capable of interacting with the monoclonal antibody. The binding properties of peptide TQTPT from the 'best-binder' mixture were compared with those of peptide TETPT, which represents the most efficiently recognized compound from the 'second best-binder' sub-library (Figure 5). It was found that the difference between the IC_{50} values of these compounds was approximately two orders of magnitude. Similarly, the efficiency of the antibody binding between the TQTX^{2}T and TETX^{2}T sub-libraries was also approximately two orders of magnitude (Figure 2). Detailed comparison of the sequence of binder-pentapeptides from both TQTX^{2}T and TETX^{2}T sub-libraries reveals that these compounds possess the same set of amino acid residues in their fourth position. All three pairs of peptides reacted with the antibody with the following order: TQTPT vs TETPT > TQTAT vs TETAT > TQTYT vs TETYT (Figure 5, Table 3).

DISCUSSION

Earlier findings indicated that the monoclonal antibody specific to MUC2 tumour associated glycoprotein is able to react with three peptides derived from the tandem repeat unit of the protein core. These peptides possess Thr at positions 1, 3 and 5 and could be described by the TX^{1}TX^{2}T motif. Based on this observation the effect of X^1 and X^2 amino acids of the TX^{1}TX^{2}T epitope motif in the antibody recognition was analysed. To achieve this a combinatorial synthesis and competitive antibody binding assay were used.

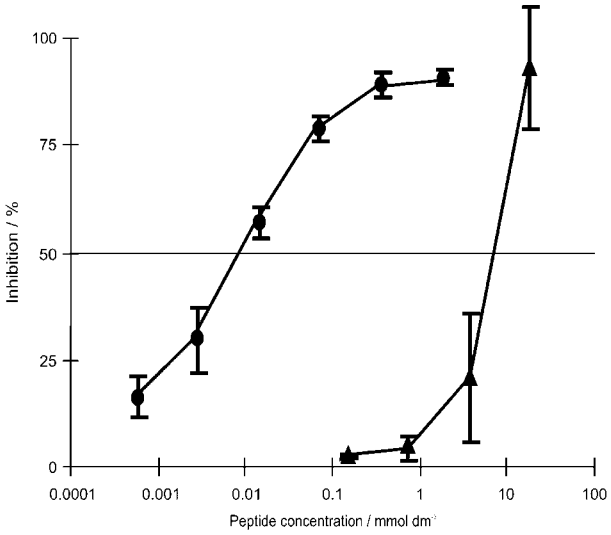

Figure 5 Inhibition of 994 antibody binding to [K^{12}VTPTP-TPTGTQTPT^{25}-OH]-BSA target antigen with pentapeptides TQTPT (●) and TETPT (▲) in an ELISA competition assay.

It was found that the most pronounced monoclonal antibody binding occurs when Gln is in position X^1 and it is much weaker in the case of Glu, Val or Leu. As for X^2 amino acids, the presence of Pro, Ala can provide a strong, while Tyr, Trp, Phe and to a less extent Ser, a weaker peptide–antibody interaction. Perhaps it is worth noting that none of the binder peptide contains a charged amino acid residue at position X^2.

These results provide not only a rationale for understanding the heteroclitic nature of this mucin-2 protein core specific monoclonal antibody, but could also demonstrate the reliability and efficacy of the combinatorial approach in establishing relative order among pentapeptide antigens using the same binding assay. Our data clearly document that the best binder peptide (TQTPT) was responsible for the antibody binding of the peptide library (TQTX^{2}T).

Data from this study suggest that pentapeptide TQTPT, whose sequence is present in the native protein, is bound most strongly. However, almost identical binding properties were observed with peptide TQTAT, whose sequence is not present in the native protein. Apart from this, some 'heteroclitic' peptides were found ordered in binding-hierarchy. These peptides can be considered as potential candidates for the design of artificial antigens. Further studies are in progress to understand the way of accommodation/orientation of amino acid residues at X^1 positions within the antibody–peptide interface.

These findings and perhaps the approach applied may be useful for therapeutic vaccine design [28–30]. Namely heteroclitic immunization with the aid of 'artificial' cross-reacting antigens may induce tumour specific immune responses [13,14].

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