factor I and II (IGF-I/II) on human breast cancer cell lines (HBCCL). *J Cell Biology* 1990, **111**, 472a.
17. Pollak M, Richard M. Suramin blockade of insulin like growth factor I-stimulated proliferation of human osteosarcoma cells. *J Natl Cancer Inst* 1990, **82**, 1349–1352.
18. Osborne CK, Arteaga CL. Autocrine and paracrine growth regulation of breast cancer: clinical implications. *Breast Cancer Res Treat* 1990, **15**, 3–11.
19. Lippman ME, Dickson LB, Bates S, *et al.* Autocrine and paracrine growth regulation of human breast cancer. *Breast Cancer Res Treat* 1986, **7**, 59–70.
20. Karey KP, Sirbasku DA. Differential responsiveness of human breast cancer cell lines MCF-7 and T47-D to growth factors and 17-β estradiol. *Cancer Res* 1988, **48**, 4083–4092.
21. Huff KK, Kaufman D, Gabbay KH, Spencer E, Lippman ME, Dickson RB. Secretion of an insulin-like growth factor I related protein by human breast cancer cells. *Cancer Res* 1986, **46**, 4613–4619.
22. Furlanetto RW, Di Carlo JN. Somatomedin-C receptors and growth effects in human breast cells maintained in long-term tissue culture. *Cancer Res* 1984, **44**, 2122–2128.
23. Lin PH, Wharton W. Purification of plasmalemma membrane from transformed cell lines and the characterization of their binding properties to EGF. *J Cell Biol* 1985, **101**, 297a.
24. Elgin RG, Busby WH, Clemmons DR. An insulin-like growth factor (IGF) binding protein enhances the biologic response to IGF-I. *Proc Natl Acad Sci USA* 1987, **84**, 3254–3258.
25. Yee D, Favoni RE, Lupu R, *et al.* The insulin-like growth factor binding protein BP-25 is expressed by human breast cancer cells. *Biochem Biophys Res Commun* 1989, **158**, 38–44.
26. Yee D, Favoni RE, Lippman ME, Powell DR. Identification of insulin-like binding proteins in breast cancer cells. *Breast Cancer Res Treat* 1991, **18**, 3–10.
27. Yee D, Rosen N, Favoni RE, Cullen KJ. The insulin-like growth factors, their receptors and their binding proteins in human breast cancer. In Lippman ME, Dickson R, eds. *Regulatory Mechanisms in Breast Cancer*. Boston, Kluwer Academic Publishers, 1991, 93–106.
28. Rohlik QT, Adams D, Kull FC Jr, Jacobs S. An antibody to the receptor for the receptor for insulin-like growth factor I inhibits the growth of MCF-7 cells in a tissue culture. *Biochem Biophys Res Commun* 1987, **149**, 276–281.
29. Arteaga CC, Kitten L, Coronado E, Jacobs F, Osborne CK. Blockade of the type I somatomedin receptor inhibits growth of estrogen receptor negative human breast cancer cells in athymic mice. *Proc 70th Annual Meeting Endocr Soc* 1988, **191**, 683.
30. Vignon F, Prebois C, Rochefort H. Inhibition of breast cancer growth by Suramin. *J Natl Cancer Inst* 1992, **84**, 38–42.

Acknowledgements—This study was partially supported by a grant awarded by the Italian Association for Cancer Research (A.I.R.C.). The authors are grateful to Dr Douglas Noonan for critical reading and Miss Patrizia Orcamo for editorial assistance and revision of the manuscript.

Eur J Cancer, Vol. 29A, No. 2, pp. 230–237, 1993.
Printed in Great Britain

0964–1947/93 $5.00 + 0.00

Fine Specificity of Antibody Recognition of Carcinoma-associated Epithelial Mucins: Antibody Binding to Synthetic Peptide Epitopes

Shaun Briggs, Michael R. Price and Saul J.B. Tendler

The protein core of polymorphic epithelial mucins consists predominantly of a repeating 20 amino acid peptide motif. Many monoclonal antibodies reactive with breast carcinomas recognise determinants located within the mucin protein core, and epitope mapping techniques have demonstrated that these antibodies bind to epitopes of three, four or five amino acids within the hydrophilic sequence, P D T R P A P. Each of these mucin core-reactive antibodies map to epitopes containing the central arginine residue. The fine specificity of a panel of anti-mucin antibodies binding to the tetrameric peptides P D T R or R P A P (synthesised on the heads of polyethylene pins) was examined by systematically replacing each amino acid in turn with all other 19 natural amino acids, and then testing these analogues for antibody binding. We have (i) identified those amino acids in epitopes which are essential for antibody binding, (ii) shown that for each epitope there is a hierarchy of residues required for immune recognition—certain amino acids may be replaced with little or no loss of antibody binding, while the presence of others is essential, and (iii) concluded that antibody specificity is further regulated by the residue(s) flanking an epitope motif which may impose conformational constraints upon the presentation of the epitope to an antibody.
Eur J Cancer, Vol. 29A, No. 2, pp. 230–237, 1993.

INTRODUCTION

HUMAN EPITHELIAL mucins identified using monoclonal antibodies are widely expressed molecules which are associated with secretory epithelia and with carcinomas of the breast and ovary [1–3]. These high molecular weight glycoproteins are extensively glycosylated with oligosaccharides attached in O-linkage to serine and threonine residues of the single chain polypeptide core [4,5]. Furthermore, the complexity of their banding pattern in electrophoretic gels is attributable to a genetic polymorphism which has been defined at both the protein and DNA level [6, 7].

Many murine monoclonal antibodies produced against human

milk fat globule membranes (HMFG) or human breast carcinoma cells or tumour extracts react with epithelial mucins,and these reagents have proved to have clinical utility [8,9]—mucins released from a developing tumour are detectable in patients' circulation and they are particularly elevated in metastatic disease [9–12]. It has also been found that there is a correlation between levels of circulating mucins and the response to treatment, so that immunoassays to quantitate these products are being developed to assist in patient management.

Progress in defining the nature of human epithelial mucins encoded by the MUC-1 gene [4], has been rapid since it was determined that the mucin protein core is composed largely of a highly conserved tandem repeat of a 20 amino acid sequence [13]. Several of the established anti-HMFG and anti-carcinoma monoclonal antibodies have been shown to recognise epitopes within the protein core and it has been feasible to identify short sequences of three to five amino acids which constitute the minimum antibody binding sites [14–17]. The present investigation extends these observations and provides a basis to explore the factors involved in the immune recognition of peptide determinants by a number of monoclonal antibodies which have potential and proven clinical utility in the diagnosis and therapy of human breast cancer.

MATERIALS AND METHODS

Murine monoclonal antibodies

Monoclonal antibody NCRC-11 (IgM) was prepared using spleen cells from a Balb/c mouse immunised against dissociated breast carcinoma cells [18]. Antibodies C595(IgG3) and 789/91 (IgM) were prepared using spleen cells from donor Balb/c mice immunised against epithelial mucin antigen preparations isolated from normal urine [19].

The following monoclonal antibodies were also used: HMFG-1 (IgG1) and HMFG-2 (IgG1) which were both prepared by immunisation with HMFG membranes [1]; SM-3 (IgG1) which was prepared against deglycosylated human milk mucin [20]; Ca2 (IgG1) which was prepared by immunisation with the human urinary mucin antigen isolated by immunoadsorbent chromatography using immobilised Ca1 (IgM) monoclonal antibodies [21] and EMA clone E29 (IgG2a) which was prepared against HMFG membranes [22].

Epitope mapping

Peptides bound at the C-terminus to polyethylene pin supports, were synthesised according to the procedure described by Geysen *et al.* [23] using an Epitope Mapping Kit (Cambridge Research Biochemicals, Cambridge, U.K.). The pins were secured in a plastic holder so that their format was that of a standard 96-well microtitre plate. Each peptide (with the exception of the 400 individual dipeptides) was synthesised in replicates of two or four. Two tetrapeptides, P L A Q and G L A Q, were also prepared so that an anti-P L A Q monoclonal antibody could be utilised as a control for the synthetic procedure and for the enzyme-linked immunosorbent (ELISA) assay.

A set of five plates containing all possible combinations of dipeptides synthesised on the heads of the plastic pins was obtained from Cambridge Research Biochemicals .

Correspondence to M.R. Price.
S. Briggs and M.R. Price are at the Cancer Research Campaign Laboratories, and S.J.B. Tendler is at the Department of Pharmaceutical Sciences, University of Nottingham, Nottingham NG7 2RD, U.K.
Revised 22 June 1992; accepted 20 July 1992.

ELISA assay

Antibodies in hybridoma supernatants or as purified antibodies (10 μg/ml) were tested for their ability to bind to the immobilised peptides using conventional ELISA techniques. Each pin was first incubated in 200 μl of blocking buffer [1% bovine serum albumin (BSA), 1% ovalbumin in phosphate buffered saline (PBS), pH 7.3] for 1 h at 20°C to minimise non-specific binding. The plates were left overnight at 4°C with each antibody at 175 μl/well/pin. After four 10-min washes with PBS containing 0.1% (v/v) Tween 20, each plate was incubated for 1 h at 25°C with rabbit anti-mouse Ig–peroxidase conjugate (DAKO, Copenhagen, Denmark) at a dilution of 1/1000 in blocking buffer. The plates then received a further four 10-min washes. The substrate solution (50 mg azino-di-3-ethylbenzothiazoline-sulphonate in 100 ml 0.1 mol/l citrate-phosphate, pH 4.0 with 120 vol. hydrogen peroxide added at 0.3 μl/ml immediately before use) was added at 150 μl/well and the absorbance at 405 nm in each well was measured using an Anthos 2001 ELISA Plate Reader (Denley Instruments, U.K.).

In the ELISA assays, the pins may be taken out of the substrate solution at any stage and the development of colour measured. In this investigation, absorbance measurements were recorded at various stages in colour development with values of up to 1.5 taken for wells with maximum colour development. The profiles of absorbance values for any set of pins with any particular antibody were essentially equivalent and not dependent upon the time of pin removal from the substrate solution, indicating that tests were performed within the linear range of the assays.

After each assay, bound antibodies were removed from the pins by sonication in a 0.1 mol/l phosphate buffer containing 1% sodium dodecylsulphate and 0.1% mercaptoethanol. The plates were then washed in warm water, then in a boiling methanol bath, followed by air drying and storage at room temperature in desiccators.

Immunisation with purified mucin

Balb/c mice were immunised by three intraperitoneal injections of purified mucin (approximately 50 μg) isolated from normal urine by affinity chromatography [19]. The initial immunogen was prepared in Freund's Complete Adjuvant and thereafter in Freund's Incomplete Adjuvant. 1 week after the final injection, the mice were exsanguinated and the sera were collected and stored in small aliquots at −20°C.

Prediction calculations

Prediction (hydropathicity) calculations were performed on a 60 amino acid polypeptide with a sequence corresponding to three repeat units of the P(1-20) peptide. Calculations were carried out using the University of Wisconsin Genetic Computer Group Package [24] mounted on the S.E.R.C. SEQNET Data Base (located at Daresbury, U.K.).

RESULTS

Pepscan procedure for epitope mapping

A panel of eight monoclonal antibodies reactive with the peptide core of epithelial mucins was employed. These antibodies were originally prepared against a variety of immunogens, each of which contained mucin material in a purified form, or associated with cells, or with HMFG membranes. The minimum sequences of amino acids recognised by 18 anti-mucin core antibodies (including the eight antibodies analysed in the present investigation) are detailed in Table 1. These results were orig-

Table 1. *Summary of the epitopes or minimum antibody binding peptides for a series of anti-mucin monoclonal antibodies*

Monoclonal antibody	Epitope	References
BC1, BC2,BC3	APDTR	[16]
SM-3*	PDTRP	[14,15]
HMFG-1*	PDTR	[15]
HMFG-2*, EMA*, M8, RINA 9/22, RINA 5/2	DTR	[14,15,17]
BrE-2, BrE-3	TRP	[17]
onc-M15	TRPA	[14]
Ca2*	TRPAP	[15]
NCRC-11*	RPA	[15]
C595, 789/91*, F36/22	RPAP	[14,17,Fig. 1]
Repeat sequence (1–10) of MUC-1 mucin (11–20)	PDTRPAPGST– –APPAHGVTSA	[4,13]

*Antibodies which have been included in subsequent tests on the fine specificity of antibody binding.

inally obtained using the Pepscan procedure which is illustrated by data shown in Fig. 1, by which the epitope for the new IgM antibody, 789/91 has been identified. For this experiment, a series of 15 heptamers, with sequences based upon that of the 20 amino acid repeat sequence of the epithelial mucin core [13], was synthesised on the heads of polyethylene pins. Each heptamer overlapped its neighbour in six residues. Figure 1 depicts the reactivity of the antibody, 789/91, with the heptamers in an ELISA assay. Clearly, all heptamers which react with positively with the 789/91 antibody contain the sequence R P A P which is thus concluded to be its epitope.

For each of the 18 anti-mucin core antibodies detailed in Table 1 and Fig. 1, their epitopes involve some three, four or five residues which are located within the domain, A P D T R P A P. In addition, the arginine residue is included in all of the epitopes for the 18 antibodies which have been subjected to epitope mapping using the Pepscan approach [14–17], Fig. 1.

Omission peptides for epitope mapping

In order to confirm the results of the Pepscan approach for epitope mapping, an alternative strategy was employed involving the synthesis of a series of solid phase-linked peptides with sequences related to S A P D T R P A P G S. However, with this series, each amino acid was systematically omitted from the sequence. Again, these peptides were then tested for their capacity to bind the antibodies with recognised epitopes within the P D T R P region. As shown in Fig. 2, the binding of HMFG-1, HMFG-2, EMA and SM-3 was lost when aspartic acid, threonine or arginine was omitted from the sequence and restored when this motif was present indicating that D T R is common to the epitopes for these antibodies. With HMFG-1, when the first proline in the P D T R motif was omitted, HMFG-1 binding was also lost although HMFG-2 and EMA were able to bind to this peptide (S A D T R P A P G S), Fig. 2. These findings support the initial Pepscan epitope mapping results for HMFG-1, HMFG-2 and EMA (Table 1). However, the antibody SM-3, which types to the P D T R P motif using the Pepscan approach (Table 1) appeared to require only the tetrameric motif, P D T R for binding using the omission peptides. This could indicate that the final proline in the P D T R P sequence was one of those residues which are selectively replaceable (with alanine, in the case of the S A P D T R A P G S peptide, Fig. 2).

Antibodies C595, NCRC-11, 789/91and Ca2 recognising epitopes in the R P A P region were examined for their reactivity with the omission peptides. The findings were confirmatory of those obtained using the Pepscan approach.

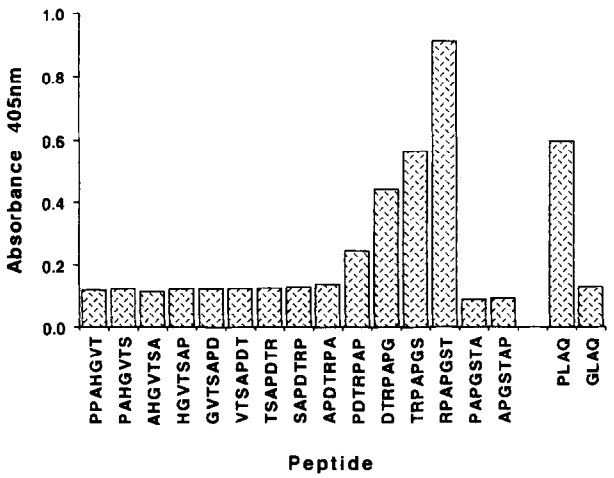

Fig. 1. Identification of the epitope for the antibody, 789/91, by Pepscan analysis. The binding of the antibody with a series of overlapping heptamers with sequences based upon that of the epithelial mucin core repeat motif was analysed by ELISA. The binding of an anti- P L A Q antibody with the positive and negative control peptides, P L A Q and G L A Q, is shown to the right of the figure.

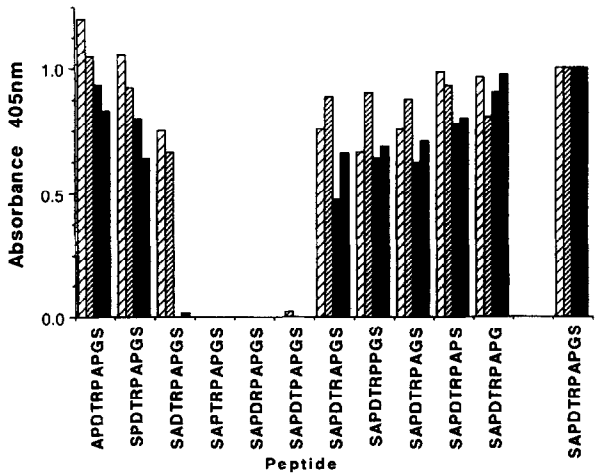

Fig. 2. Epitope mapping of anti-HMFG antibodies using a series of omission peptides. Peptides with sequences related to S A P D T R P A P G S were synthesised. With each analogue, a single amino acid was omitted from the sequence and all peptides were tested for their capacity to bind the antibodies EMA ▨, HMFG-2 ▨, SM-3 ▩ and HMFG-1 ■, respectively, in an ELISA test. Absorbance values for antibody binding to each peptide are normalised relative to the value obtained with the parent peptide, S A P D T R P A P G S (shown to the right of the figure), which was assigned a value of unity.

Replacement net analysis of the P D T R epitope region

Eight of the antibodies in Table 1 were employed to explore further the factors influencing the recognition of small peptide domains by monoclonal antibodies.

The sequence P D T R appears to be an immunodominant region of the P D T R P A P domain (Table 1) and, using the Pepscan mapping procedure, several antibodies, including HMFG-2, M8 and EMA, map to the short sequence of D T R (Table 1). Initial experiments were designed to examine the fine specificity of antibodies binding to this trimeric sequence. Preliminary experiments upon the D T R trimer, synthesised as a tethered peptide indicated that it failed to bind HMFG-2 and EMA antibodies which was somewhat at variance with the Pepscan mapping tests. However, when a series of tetrapeptides (rather than trimers) based upon the P D T R sequence was synthesised, significant HMFG-2 antibody binding was obtained. This would indicate a role for the initial proline in providing the appropriate conformational presentation of the D T R epitope for recognition by HMFG-2.

The strategy for analysing the interaction of antibodies with their epitopes at the level of individual amino acids was as follows: comparative measurements were made of the binding of antibodies to a series of tethered P D T R-related tetrapeptides in which each of the four residues in turn was systematically replaced by the other natural 19 amino acids (i.e. peptides X D T R, P X T R, P D X R and P D T X, with X indicating each of the 20 residues—the so-called replacement net of peptides). Thus, this generated a series of 80 peptides which were synthesised in duplicate and tested for their capacity to bind antibodies reactive with the P D T R sequence.

Therefore, as shown in Table 2, when HMFG-1 was tested against the X D T R peptides, antibody binding was only evident when proline was at position one, emphasising the importance of this residue for binding (unique residues such as this proline at position 1 are described as critical residues within an epitope). Similarly, the aspartic acid and arginine residues at positions two and four in the sequence were of major importance of HMFG-1 binding (although selective replacements were permissible), while the threonine at position three could be replaced with most other residues with little or no loss in antibody binding, and even with increased binding upon substitution with leucine, methionine and glutamine (Table 2).

With HMFG-2, there was a different profile of binding to the 80 tetramers. Even though the epitope for HMFG-2 had been identified as D T R (Table 1), the nature of the residue at position one flanking the aspartic acid at position two, had a profound effect upon antibody binding. Thus, as shown in Table 2, the binding of HMFG-2 to the X D T R peptides was evident with the tetramer P D T R, and to lesser extents with L D T R and R D T R. The aspartic acid at position two may be classed as a critical residue since its replacement resulted in complete loss in antibody binding activity (Table 2). Conversely, the threonine and arginine residues at positions three and four, respectively, in the tetrapeptide, were selectively replaceable (Table 2). Indeed, substitution of threonine at position three with glutamine and serine in P D X R peptides and substitution of arginine with phenylalanine or leucine in P D T X peptides resulted in increased HMFG-2 antibody binding (Table 2). Comparably, the reactivity of HMFG-1 with the P D X R peptides was particularly enhanced when threonine was replaced with glutamine (Table 2).

When the antibody EMA was tested against the series of P D T R-related peptides, no binding was observed in spite of the fact that its epitope had been identified as D T R (Table 1). These findings suggest that while the Pepscan procedure (illustrated in Fig. 1) may identify the minimum antibody binding sequence within an epitope, this sequence may have to be presented to its antibody within the appropriate context of flanking residue(s) as was the case with HMFG-2 and its dependence upon the initial proline for antibody binding (Table 2). In addition, the antibody SM-3 which maps to the epitope P D T R P failed to bind any of the P D T R replacement net peptides (Table 2) which would indicate that the proline in position five is critical for antibody binding. This is somewhat at variance with the data in Fig. 2 showing the binding of SM-3 to the omission peptides which suggests that the final proline is replaceable with alanine. Further tests involving the synthesis of a series of synthetic pentamers based upon the P D T R P sequence confirmed the requirement for the presence of *both* prolines in the epitope for maximal binding.

Replacement net analysis of the R P A P epitope

Within the sequence of residues of P D T R P A P recognised by the panel of antibodies, the R P A P domain also appears to be immunodominant and is recognised in full or in part by four antibodies, Ca2, NCRC-11,C595 and 789/91, selected in this investigation. Analysis of the interaction of these antibodies with their epitopes at the level of individual amino acids was achieved by comparison of antibody binding to a replacement net of R P A P-related tetrapeptides.

As shown in Table 2, in which C595 antibody binding to X P A P was evaluated, replacement of the arginine residue at position one with any other residue resulted in a loss of antibody binding. Comparably, replacement of the proline at position four with any other residue prevented antibody binding. Conversely, other residues in the R P A P sequence (R X A P and R P X P, Table 2) could be selectively replaced and still allow C595 antibody to bind. Effectively, the proline at position two can be replaced by most residues, with the exception of the relatively large aromatic residues phenylalanine, tryptophan and tyrosine, as well as aspartic acid, and C595 still binds to a significant extent. With the R P X P series of peptides, C595 antibody binding was still evident to several of the peptides, although to a lesser extent than with the R X A P series.

The antibodies 789/91, NCRC-11 and Ca2 were also tested on the replacement net of R P A P peptides. Although the full epitope domain determined for Ca2 in the Pepscan epitope mapping tests with heptamers was determined to be the pentapeptide T R P A P (Table 1, [15]), the antibody bound sufficiently to the R P A P replacement net peptides to allow its fine specificity to be examined. This is in contrast with the findings with HMFG-2 (Table 2) which indicated that not only did the epitope need to be presented to an incoming antibody, but there was a requirement for at least one flanking amino acid residue to be present. In addition, with EMA, this antibody only recognised its D T R epitope in the context of synthetic heptamers [15] or 10-mers or11-mers (Fig. 2) but not trimers or tetramers (Table 2).

Table 2 shows that the arginine at position one in the R P A P tetramer is essential for the binding of all antibodies. However, with each of the antibodies tested, except C595, all the remaining residues (proline, alanine and proline at positions two, three and four, respectively) can be selectively replaced with alternative residues and still permit antibodies to bind. As already indicated, C595 requires the presence of the proline at position four in the tetramer to bind.

Table 2. Antibody binding to the replacement net peptides of epitope sequences, P D T R and R P A P

Monoclonal antibody	Target peptide	Relative absorbance values at 405 nm for antibody binding to peptide substituted at residue X with: A	C	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y
HMFG-1	XDTR	—*	—	—	—	—	—	—	—	—	—	—	—	*10*†	—	—	—	—	—	—	—
	PXTR	—	—	*10*	7	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	PDXR	5	—	—	6	5	6	—	10	—	14	12	6	10	17	8	10	*10*	8	—	—
	PDTX	—	—	3	—	—	—	—	—	—	—	—	—	—	—	*10*	—	—	—	—	—
HMFG-2	XDTR	3	—	3	4	—	—	—	—	—	5	—	—	*10*	—	5	—	—	—	—	—
	PXTR	—	—	*10*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	PDXR	4	—	4	4	4	5	4	7	7	8	5	—	—	19	7	12	*10*	5	—	—
	PDTX	—	—	—	—	13	3	5	—	4	17	4	—	—	3	*10*	5	8	8	6	9
EMA	XDTR	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	PXTR	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	PDXR	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	PDTX	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
SM3	XDTR	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	PXTR	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	PDXR	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	PDTX	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
C595	XPAP	—	—	—	—	—	—	—	—	—	—	—	—	—	—	*10*†	—	—	—	—	—
	RXAP	11	4	—	6	—	6	5	7	6	6	9	6	*10*	9	3	7	8	7	—	—
	RPXP	*10*	—	—	—	—	—	—	—	—	—	—	—	9	—	—	5	—	4	—	—
	RPAX	—	—	3	—	—	—	—	—	—	—	—	—	*10*	—	—	—	—	—	—	—
789/91	XPAP	—	—	—	—	—	—	—	—	—	—	—	—	—	—	*10*	—	—	—	—	—
	RXAP	10	—	3	7	4	5	—	4	—	—	6	—	*10*	7	—	4	5	5	—	—
	RPXP	*10*	—	—	5	—	—	—	—	—	—	6	—	5	6	—	—	4	3	—	—
	RPAX	6	—	6	7	—	8	—	—	—	—	7	5	*10*	7	—	6	—	—	—	—
NCRC-11	XPAP	—	—	—	—	—	—	—	—	—	—	—	—	—	—	*10*	—	—	—	—	—
	RXAP	10	—	10	10	4	6	—	5	—	4	6	—	*10*	6	—	12	11	4	—	—
	RPXP	*10*	6	13	13	—	7	—	9	—	7	11	10	12	12	4	14	14	12	—	—
	RPAX	7	4	7	7	3	7	—	7	—	6	5	7	*10*	7	4	6	7	5	—	5
Ca2	XPAP	—	—	—	—	—	—	—	—	—	—	—	—	—	—	*10*	—	—	—	—	—
	RXAP	14	4	12	9	4	6	—	4	—	—	4	6	*10*	—	—	15	9	4	—	—
	RPXP	*10*	6	16	16	—	4	9	15	—	12	6	16	17	18	—	16	29	20	8	15
	RPAX	12	6	6	14	—	12	—	7	—	8	8	8	*10*	10	—	8	7	7	—	—

*Absorbance values of less than 30% of that given with the parent peptides P D T R or R P A P are indicated by —.

†Absorbance values for antibody binding against each target peptide are normalised relative to the value obtained with the parent P D T R or R P A P sequence (italicised) which in each case, has been assigned a value of *10*.

Analysis of antibody reactivity with tethered dipeptides

In order to examine further the critical nature of the individual residues in their binding to monoclonal antibodies, several antibodies reactive with peptide epitopes were screened for binding to a panel of 400 different dipeptides prepared with all combinations of the 20 natural amino acids. The reactivity of the antibody NCRC-11 was the most informative, although the pattern of binding of the other R P A P-reactive antibodies was similar. As shown in Table 3, the 28 most prominent reactions are identified, and of these, the 15 most intense reactions are also noted. The most striking feature of these results is that 19 of the possible 39 arginine-containing dipeptides were among the high NCRC-11 antibody binding dipeptides. Of the 15 "best" reactions, 11 of these dipeptides involved arginine. Conversely, those non-arginine-containing dipeptides which bound NCRC-11 antibody showed no striking pattern of reactivity or common residues, although alanine, which is a component of the NCRC-11 epitope, is relatively well represented among the reactive dipeptides (Table 3).

The approach using tethered dipeptides is essentially qualitat-

Table 3. Binding of NCRC-11 monoclonal antibody to tethered dipeptides

	Binding of NCRC-11 antibody to dipeptide*																		
	XA	XC	XD	XE	XF	XG	XH	XI	XK	XL	XM	XN	XP	XQ	XR	XS	XT	XV	XW	XY
AX	AA	—	—	—	—	—	—	—	—	—	—	—	—	—	AR	—	—	—	—	—
CX	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
DX	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
EX	—	—	—	—	—	—	—	—	—	—	—	—	—	—	*ER*	—	—	—	—	—
FX	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
GX	GA	—	—	—	—	GG	—	—	—	—	—	—	—	—	—	—	—	—	—	—
HX	HA	—	—	—	—	*HG*	—	—	—	—	—	—	HP	—	HR	—	—	—	—	—
IX	—	—	—	—	—	—	*IH*	—	—	—	—	—	—	—	—	—	—	—	—	—
KX	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
LX	—	—	—	—	—	—	—	—	—	*LL*	—	—	—	—	*LR*	—	—	—	—	—
MX	*MA*	—	—	—	—	—	—	—	—	—	—	—	—	—	*MR*	—	—	—	—	—
NX	—	—	—	—	—	—	—	—	—	—	—	—	—	—	*NR*	—	—	—	—	—
PX	—	—	—	—	—	—	—	—	—	—	—	—	—	—	*PR*	—	—	—	—	—
QX	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
RX	*RA*	—	RD	*RE*	RF	*RG*	—	—	—	—	*RM*	*RN*	—	—	—	*RS*	RT	RV	RW	—
SX	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
TX	—	—	—	—	—	—	—	—	—	—	—	—	—	—	TR	—	—	—	—	—
VX	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
WX	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
YX	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

*400 different dipeptides were examined for their capacity to bind the NCRC-11 monoclonal antibody. 28 peptides displaying the greatest reactivity with NCRC-11 are shown in the matrix and the best 15, showing the most intense reactivity, are italicised.

ive and permits identification of significant features of an epitope which may contribute to antibody recognition and binding. The ELISA signals with the best binding dipeptides are less (20 to 30%) than those obtained with the "complete" epitopes identified using the Pepscan approach. In our experience with a number of antibodies, the technique is not always informative although in the example presented, the findings highlight the critical nature of the arginine residue in the recognition of the R P A P domain by monoclonal antibody NCRC-11. However, the results also suggest that other fortuitous combinations of amino acids as dipeptides may also bind antibody.

Immunodominance of the epitope domain A P D T R P A P

The inference from the summary of epitope mapping data in Table 1 is that the domain A P D T R P A P is immunodominant in mice treated with either purified mucin material or mucin containing preparations such as HMFG membranes. This could be due to the differential accessibility of this domain in the intact mucin so that it may preferentially stimulate elements of the B-cell repertoire. This may be achieved by the differential glycosylation of distinct serine and threonine residues of the mucin molecule, leading to masking of selected regions of the protein core. Alternatively, the physico-chemical properties of the constituent amino acids may dictate that certain regions of the repeated sequence are buried within aspects of secondary structure [15]. It was attempted to analyse this problem, at least in part, by utilising purified urinary mucin as the immunogen. The resulting antiserum (from three mice) was examined for reactivity with the overlapping synthetic heptamers previously employed for epitope mapping (Fig. 1). As shown in Table 4, this antiserum (at a dilution of 1/100) displayed a peak of reactivity with the P D T R P A P peptide (peptide 10). Normal

Table 4. Immunodominance of the epitope domain PDTRPAP

		Absorbance with		Prediction calculations*
Number	Peptide	Anti-mucin serum	Normal mouse serum	Hydrophilicity [25]
1	PPA*H*GVT	0.54	0.06	0.21
2	PAHGVTS	0.22	0.02	0.10
3	AHG*V*TSA	0.06	0.04	−0.39
4	HGV*T*SAP	0.14	0.04	0.10
5	GVT*S*APD	0.06	0.02	0.14
6	VTS*A*PDT	0.05	0.04	0.19
7	TSA*P*DTR	0.18	0.06	1.43
8	SAP*D*TRP	0.26	0.10	1.56
9	APD*T*RPA	0.73	0.06	1.19
10	PDT*R*PAP	1.18	0.10	1.67
11	DTR*P*APG	0.53	0.05	1.50
12	TRP*A*PGS	0.58	0.10	1.11
13	RPA*P*GST	0.55	0.09	1.11
14	PAP*G*STA	0.03	0.00	0.21
15	APG*S*TAP	0.02	0.06	0.21

*Hydrophilicity calculations for each residue in the repeat motif of the mucin core peptide were averaged over a window of seven amino acids. The predicted value for each residue is aligned with the relevant central residue (italicised) in each of the synthetic heptamers.

mouse serum (1/100), by comparison, showed only background binding to the heptamers. There was a sharp loss in reactivity of the antiserum with peptide 14 (P A P G S T A) compared with the preceding peptide 13 (R P A P G S T) and this correponds exactly with the loss of the arginine residue in peptide 14 (Table 4). This would be in accord with the important role of this residue as a critical amino acid in a number of epitopes (Tables 2 and 3).

It is of interest that peptide one and, to a lesser extent peptide two, were weakly reactive with the anti-mucin antiserum so that the production of antibodies against regions of the protein core other than P D T R P A P is feasible but as yet no monoclonal reagents have been produced.

As illustrated in Table 4, prediction calculations show a high degree of concordance with the experimental findings. Peptides seven to 13, which respectively contain the residues P, D, T, R, P, A and P as their central residue, are the most hydrophilic with values greater than unity. With several predictive calculations (including algorithms for hydrophilicity, surface probability and antigenicity), there is a marked cut-off at the interface where the arginine residue, in the immunodominant sequence, is lost from the heptamers (i.e. between peptides 13 and 14) which further emphasises the importance of this residue.

DISCUSSION

The findings presented show that many established anti-mucin antibodies recognise determinants of three, four or five amino acids within a hydrophilic region of seven amino acids (P D T R P A P) in the repeat sequence of the MUC-1 mucin protein core. That this region is immunodominant in mice has been further confirmed by examination of the reactivity of anti-mucin antiserum with the overlapping synthetic heptamers. Each of these findings (including the reactivity of NCRC-11 with the tethered dipeptides) highlight the importance of the central arginine residue in the immunodominant motif. Remarkably, this is a common residue in all of protein core-related epitopes defined by 18 individual anti-mucin antibodies and clearly contributes to the immunogenic character of the native mucin.

While individual antibodies have epitopes which map to determinants with as few as three amino acids, it is evident that appropriate presentation of such small determinants to an incoming antibody is required for recognition and binding. The data would indicate that flanking residues can have an important role in this respect and such secondary constraints illustrate differences in the fine specificity of antibodies which apparently recognise the same epitope sequence (e.g. compare the reactivity of HMFG-2 with that of EMA—Tables 1, 2 and Fig. 2; also compared the reactivities of C595 and 789/91 with synthetic peptides—Tables 1, 2). These considerations would also provide an explanation as to why an antibody which recognises as few as three or even two residues may also display a descrete and characteristic profile of reactivity with secretory epithelia and breast carcinomas in conventional immunohistological tests [18, 19].

The 18 anti-core antibodies detailed in Table 1 can be divided into two major categories on the basis of the epitopes they define. Antibodies prepared against human milk-derived materials (BC1, BC2, BC3, SM3, HMFG-1, HMFG-2, EMA and M8) or breast glycoprotein (RINA 9/22, RINA 5/2) all require the presence of the sequence D T R within their epitope. Conversely, the majority of the remaining antibodies have the trimer R P A within their epitopes and these were produced by immunisation with purified mucins (Ca2, C595, 789/91, F36/22) or against tumour tissue (NCRC-11) or are products of mixed immunisations (onc-M15). More examples of anti-mucin antibodies are required to be evaluated to determine whether this apparent segregation of antibodies reflects a separation of the immunodominant region into two sub-loci.

The reactivity of anti-mucin antibodies with the tethered dipeptides suggested that this approach has some application for the design of ligands which may mimic native epitopes (in that they bind antibody), but the procedure does not necessarily lead to accurate identification of the epitope. With the example shown in Table 3, it was possible to confirm the importance of the arginine residue in the epitope for antibody NCRC-11.

The involvement of carbohydrates in the full expression of the epitope motifs is presently unknown. In the mucin glycoproteins, the carbohydrates are attached in *O*-linkage to serine or threonine residues via the linkage sugar *N*-acetylgalactosamine. The immunodominant region is flanked by doublets of serine and threonine. Glycosylation of these residues will undoubtedly limit the accessibility of the immunodominant region to approaching antibodies, as well as imposing constraints upon secondary structure in the protein core. Indeed, aberrant or incomplete glycosylation of mucins in tumours may result in the greater exposure of the determinants in the protein core. Antibodies which react with "unmasked" epitopes in the protein core may then display a degree of preferential reactivity with tumours. This is considered to account for the enhanced tumour reactivity of SM-3 which recognises the pentameric motif P D T R P [4,14,17,20].

While the effect of glycoslyation of threonine at position three in unknown, it is interesting to note that this residue shows a high degree of replaceability for binding of antibodies HMGF-1 and HMFG-2, as well as SM-3 (Briggs, unpublished findings—using analogues of the pentameric sequence P D T R P). Hence, this "vacancy" in the epitope at the threonine residue could be accommodated by the linkage sugar which, itself, could contribute to the epitope.

The findings obtained upon the nature of monoclonal antibody-defined epitopes using the approaches described in this report are being consolidated with the results from parallel studies using alternative strategies for the analysis of antigenic peptides. Using high field nuclear magnetic resonance spectroscopy, a type I β turn has been identified spanning the P D T R region, and the side chains of the aspartic acid and arginine residues have been found to interact to form a salt bridge, thus stabilising the turn region [26,27]. Future studies are directed to integrate all information with a view to developing a model which describes the immune recognition of epithelial mucins by tumour-reactive monoclonal antibodies.

Finally, it is emphasised that with the replacement net approach, subtle differences in specificity between antibodies may result in major differences in the profiles of antibody binding to the series of synthetic epitope analogues produced using this stragegy (Tables 1, 2). In a preliminary comparative study of HMFG-1 and HMFG-2 reactivity, the differences in antibody reactivity have been interpreted with respect to the recognition of a salt bridge between the aspartic acid and arginine side-chains [28]. Further structural studies are underway to provide a more complete description of the interaction of antibodies with their peptide epitopes in epithelial mucins.

1. Burchell J, Durbin H, Taylor-Papadimitriou J. Complexity of expression of antigenic determinants recognised by monoclonal

antibodies HMFG-1 and HMFG-2, in normal and malignant human mammary epithelial cells. *J Immunol* 1983, **131**, 508–513.
2. Hilkens J, Buijs F, Hilgers J, *et al.* Monoclonal antibodies against human milk-fat globule membranes detecting differentiation antigens of the mammary gland and its tumour. *Int J Cancer* 1984, **34**, 197–206.
3. Price MR, Edwards S, Owainati A, *et al.* Multiple epitopes on a human breast carcinoma associated antigen. *Int J Cancer* 1985, **36**, 567–574.
4. Gendler S, Spicer AP, Lalani E-N, *et al.* Structure and biology of a carcinoma-associated mucin, MUC1. *Am Rev Respir Dis* 1991, **144**, S42–S47.
5. Hilkens J. Biochemistry and function of mucins in malignant disease. In Hilgers J, Zotter S, eds. *Cancer Reviews*. Copenhagen, Munksgaard, 1988, Vol. 11–12, 25–54.
6. Swallow DM, Griffiths B, Bramwell M, Wiseman G, Burchell J. Detection of the urinary "PUM" polymorphism by the tumour binding monoclonal antibodies Ca1, Ca2, Ca3, HMFG-1 and HMFG-2. *Disease Markers* 1986, **4**, 247–254.
7. Gendler S, Burchell J, Duhig T, *et al.* Cloning of partial cDNA encoding differentiation and tumour-associated mucin glycoproteins expressed by human epithelium. *Proc Natl Acad Sci USA* 1987, **64**, 6060–6064.
8. Price MR. High molecular weight epithelial mucins as markers in breast cancer. *Eur J Cancer Clin Oncol* 1988, **24**, 1799–1804.
9. Kenemans P, Bast RC, Yemeda CA, Price MR, Hilgers J. Mucins and mucin-like antigens as circulating tumour markers. In Hilgers J and Zotter S, eds. *Cancer Reviews*. Copenhagen, Munksgaard, 1988, Vol. 11–12, 119–144.
10. Kufe D, Hayes D, Abe M. Monoclonal antibody assays for breast cancer. In Kupchik HZ, ed. *Cancer Diagnosis* In Vitro *Using Monoclonal Antibodies*. New York, M. Dekker, 1988, Vol. 39, 67–100.
11. Price MR, Clarke AJ, Robertson JFR, O'Sullivan C, Baldwin RW, Blamey RW. Detection of polymorphic epithelial mucins in the serum of systemic breast cancer patients using the monoclonal antibody, NCRC-11. *Cancer Immunol Immunother* 1990, **31**, 269–272.
12. Gion M, Mione R, Nascimben O, *et al.* The tumour associated antigen CA15.3 in primary breast cancer. Evaluation in 667 cases. *Br J Cancer* 1991, **63**, 809–813.
13. Gendler S, Taylor-Papadimitriou J, Duhig T, Rothbard J, Burchell J. A highly immunogenic region of a human polymorphic epithelial mucin expressed by carcinomas is made up of tandem repeats. *J Biol Chem* 1988, **263**, 12820–12823.
14. Burchell J, Taylor-Papadimitriou J, Boshell M, Gendler S, Duhig T. A short sequence, within the amino acid tandem repeat of a cancer-associated mucin, contains immunodominant epitopes. *Int J Cancer* 1989, **44**, 691–696.
15. Price MR, Hudecz F, O'Sullivan C, Baldwin RW, Edwards PM, Tendler SJB. Immunological and structural features of the protein core of human polymorphic epithelial mucin. *Mol Immunol* 1990, **62**, 795–802.
16. Xing PX, Reynolds K, Pietersz GA, McKenzie IFC. Effect of variations in peptide sequence on anti-human milk fat globule membrane antibody reactions. *Immunology* 1991, **72**, 304–311.
17. Taylor-Papadimitriou J. Report on the first international workshop on carcinoma-associated mucins. *Int J Cancer* 1991, **49**, 1–5.
18. Ellis IO, Robins RA, Elston CW, Blamey RW, Ferry B, Baldwin RW. A monoclonal antibody, NCRC-11, raised to human breast carcinoma. I. Production and immunohistological characterization. *Histopathology* 1984, **8**, 501–506.
19. Price MR, Pugh JA, Hudecz F, *et al.* C595—a monoclonal antibody against the protein core of human urinary epithelial mucin commonly expressed in breast carcinomas. *Br J Cancer* 1990, **61**, 681–686.
20. Burchell J, Gendler S, Taylor-Papadimitriou J, *et al.* Development and characterization of breast cancer reactive monoclonal antibodies directed to the core protein of the human milk mucin. *Cancer Res* 1987, **47**, 5476–5482.
21. Bramwell ME, Ghosh AK, Smith WD, Wiseman S, Spriggs A, Harris H. Ca2 and Ca3. New monoclonal antibodies as tumour markers in serous effusions. *Cancer* 1985, **56**, 105–110.
22. Heyderman E, Strudley I, Powell G, Richardson TC, Mason D. A new monoclonal antibody to epithelial membrane antigen (EMA)—E29. A comparison of its immunocytochemical reactivity with polyclonal antibodies and with another monoclonal antibody, HMFG-2. *Br J Cancer* 1985, **52**, 355–361.
23. Geysen HM, Rodda SJ, Mason TJ, Tribbick G, Schoofs PG. Strategies for epitope design using peptide synthesis. *J Immunol Meth* 1987, **102**, 259–274.
24. Devereux J, Haeberli P, Smithies O. A comprehensive sequence analysis programs for the VAX. *Nucl Acids Res* 1984, **12**, 387–395.
25. Kyte J,Doolittle RF. A simple method for displaying the hydropathic character of proteins. *J Mol Biol* 1982, **157**, 105–132.
26. Tendler SJB. Elements of secondary structure in a human epithelial mucin core peptide fragment. *Biochem J* 1990, **267**, 733–737.
27. Scanlon MJ, Morley S, Jackson DE, Price MR, Tendler SJB. Structural and computational investigations on the conformation of antigenic fragments of human polymorphic epithelial mucins. *Biochem J* 1991, **285**, 137–144.
28. Briggs S, Price MR, Tendler SJB. Immune recognition of linear epitopes in peptides fragments of epithelial mucins. *Immunology* 1991, **73**, 505–507.

Acknowledgements—These studies were supported by the Cancer Research Campaign. The skilful technical assitance of Mr M. Sekowski is gratefully acknowledged. Thanks are expressed to the following for supplying samples of their antibodies: to Drs J. Taylor-Papadimitriou and J. Burchell for HMFG-1, HMFG-2 and SM-3, and to Dr M. Bramwell For Ca2.