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Production of Monoclonal Antibodies Recognising the Peptide Core of MUC2 Intestinal Mucin

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A peptide based on the tandem repeat sequence of MUC2 mucin was used to produce a series of monoclonal antibodies (MAb). The fine specificity of these antibodies and their implications for MUC2 expression are presented. Three of the MAbs, 996/1, 996/7 and 995/25, were specific to the MUC2p and failed to bind to peptides based on the MUC1,3,4 tandem repeat sequences whereas three others, 994/152, 994/91 and 996/36, cross reacted with the MUC2p and the MUC3 tandem repeat peptide but not the MUC1 and MUC4 peptides. An antigen, affinity purified from a colorectal tumour on one of the MUC2p-specific MAbs, 996/1, was shown to be a high molecular weight polydisperse, mucin-like antigen. Two of the MAbs, 996/1 and 994/152, recognised MUC2 in tissue sections, although the fine specificity varied between the two MAbs, with 994/152 strongly staining gastric, ileum and kidney epithelia, and MAb 996/1 intensely staining colon, liver and prostate tissues. These antibodies also stained a colorectal cell line, and MAb 994/152 also stained a gastric and an ovarian cell line. Six of the MAbs were used to stain colorectal tumour and adjacent 'normal' colonic mucosa sections. All six stained normal mucosa, but only two of the MAbs, 996/1 and 994/91, stained tumour tissue. The staining probably reflects exposure of cryptic epitopes due to varying levels of glycosylation in different tissues. These anti-MUC2p MAbs may help in determining the normal role of MUC2 mucin and how it is subverted in malignancy.

Key words: MUC2, intestinal mucin, colorectal cancer
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INTRODUCTION

MUCINS ARE a family of highly glycosylated, large molecular weight (> 200 kDa) glycoproteins which are associated with many epithelial cells. Five mucin genes have now been cloned and designated MUC1, MUC2, MUC3 [1], MUC4 and MUC5. Each gene codes for a protein containing a variable number of tandem repeats of 20 (MUC1), 23 (MUC2), 17 (MUC3), 16 (MUC4) and 8 (MUC5) amino acids, but there is no significant homology between these tandem repeats [2–6].

The MUC1 gene is expressed in a variety of cancers including breast, pancreatic and ovarian cancer, and although the MUC1 expressed on the cancers and normal cells contains the same tandem repeat core peptide sequence, glycosylation differences do exist [7, 8] exposing normally cryptic peptide epitopes [9]. Synthetic peptides based on the cryptic MUC1 epitope have been used to generate specific cell-mediated immune responses against MUC1-encoded mucin expressed at the cell surface. Immunisation also caused rejection of MUC1 transfected mouse tumours and prolonged survival of mice [10]. Other workers have isolated MUC1-specific cytotoxic T lymphocytes (CTL) from human cancer patients which are specifically inhibited by the monoclonal antibody (MAb) SM3, an antibody which is reactive with part of the cryptic epitope of the MUC1 protein [11, 12]. Moreover the CTL only kill cells expressing mucins carrying the SM3 epitope. MAbs to the cryptic epitope of MUC1 have been useful in the prognosis of breast cancer [13] and for the detection of MUC1 in sera of breast and ovarian cancer patients.

More recently, the polypeptide structure of the intestinal mucins has been determined. cDNAs have been isolated that encode two types of human intestinal apomucins. These cDNA clones encode 23 amino acid tandem repeats (MUC2, 3, 14) or 17 amino acid tandem repeats (MUC3, 4). Both types of tandem repeats are rich in threonine. The MUC2-type tandem repeat are also rich in proline, whereas the MUC3-type tandem repeats are rich in serine. The messages for the intestinal mucins are large and polydisperse, and both mucin genes are polymorphic. The MUC2 gene (chromosome 11p15; 15) is distinct from the MUC3 gene (chromosome 7; 4), and both MUC2 and MUC3 genes are distinct from the MUC1 gene (chromosome 1) which encodes mammary mucin. It remains to be seen if MUC2 may also have potential as a tumour vaccine or if MAbs to this mucin may be useful in prognosis or disease monitoring.

Animal models for colon cancer metastases have shown that a human colon carcinoma cell line with high liver metastasising ability produced 2-fold more metabolically labelled intracellular MUC2, and secreted 4–5-fold more MUC2 into the culture medium compared to a poorly metastatic parental cell line [16]. Variants of a human colon cell line selected for high or low MUC2 production also yielded metastases after caecal growth, and colonised the liver after splenic portal injection in proportion to their ability to produce MUC2 [16].

More recently it has been shown that colonic cell lines showing

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high MUC2 production demonstrate increased adherence to basement membrane proteins and invade a reconstituted basement membrane to a greater extent than their counterpart cell lines showing low MUC2 production. Adherence of the low MUC2 secreting cell lines to various matrix proteins was potentiated by the addition of purified human colon cancer mucin. High MUC2 producing cell lines secreted more proteolytically active type IV collagenase than the low MUC2 producing cell lines, and collagenase activity was further stimulated by purified MUC2. These results suggest that MUC2 may play a role in adherence, invasion and proteolytic digestion, all important steps in the metastatic cascade [17].

Earlier studies have shown that patients with mucinous adenocarcinoma of the colon have a poor prognosis [18–22]. Whereas many authors report a lower survival for patients with mucinous cancer compared to 'non-mucinous' tumours, others have failed to find a significant correlation between this histological pattern and prognosis [22]. Some studies suggest that only tumours which produce large amounts of mucin may be associated with a poor prognosis [20]. This controversy may relate in part to lack of adequate quantification of cancer mucin production in most histochemical studies. In addition to mucinous colon cancer, intraglandular mucin may be present to a variable extent in non-mucinous, well or moderately well differentiated colonic adenocarcinomas.

The isolation of MAbs recognising the peptide core of the intestinal mucins may help in defining a better screen for the influence of intestinal mucin production on colorectal tumour prognosis. Previous attempts to produce MAbs to intestinal mucin have been complicated both by the difficulty in purifying these heavily glycosylated molecules, and the fact that subsequent immunisations resulted in anti-carbohydrate and not anti-peptide MAbs. However, identification of the peptide sequence of the MUC2 mucin has suggested an alternative method of production of anti-mucin MAbs. In this investigation, the mucin sequence was analysed for potential B cell epitopes and then that sequence was synthesised as a small peptide which was linked to a protein carrier. Subsequent immunisation and fusion of mouse splenocytes with a mouse myeloma resulted in seven MAbs which recognise mucin peptide and whose characterisation is described in this report.

MATERIALS AND METHODS

Identification of the core amino acid sequence of the protein core of the MUC2 mucin provided an alternative method for the production of MAbs for the detection and quantification of mucin expression, namely, the production of anti-MUC2 synthetic peptides monoclonal antibodies.

Identification of an immunogenic peptide

A sequence of 69 amino acids corresponding to three complete repeat units of the 23 residue sequence was analysed for potential B cell epitopes and structural features using the S.E.R.C. SEQNET Database (Daresbury, U.K.) and the University of Wisconsin genetic computer package [23]. Values for hydropathicity for the central 23 residue repeat and flanking residues, calculated over a window of seven residues, are presented in Fig. 1a. The sequence from residue 12 in the repeat through to residue 2 in the following repeat, was identified as a region of potential interest with regard to potential B cell epitopes. This region is the major hydrophillic region (Fig. 1a) with highest values for antigenic index (Fig. 1b). Predictions of secondary structure [24, 25] suggested the presence of a major turn region centering around residue 19 and it was concluded that this sequence would be an appropriate region for synthesis for preparation of anti-MUC2 peptide antibodies. The mucin peptide (MUC2p) was, therefore, synthesised using standard t-Boc solid phase synthesis on an Applied Biosystems synthesiser, and analysed for secondary structure by CD spectroscopy confirming distinct secondary folding even in a peptide of this size. The collected data indicated that this peptide should display a strongly immunogenic character.

Fusion

Four animals were immunised with MUC2p linked to keyhole limpet haemacyanin (KLH) (200 μg) by intraperitoneal injection on days 0, 14 and 28, initially in the presence of Freund's complete adjuvant, and then in Freund's incomplete adjuvant. Four animals were also immunised with MUC2p-KLH on days 0, 14 and 28 in the presence of Quil A adjuvant. The mice receiving the MUC2p-KLH in the presence of Freund's adjuvant produced the highest titre of antibody (titre of 1/20 000; Fig. 2). These animals were, therefore, selected for cell fusion. Splenocytes were fused with NSO at a ratio of either 7:1 or 15:1 and grown for 14 days in the RPMI supplemented with either 3% mouse ascites fluid or 10% fetal calf serum (FCS) (Table 1). The fusion efficiencies were superior in the fusions with the lower splenocytes to NSO ratio ($P < 0.001$) and in the fusions fed with medium containing mouse ascites. The latter was particularly obvious in the fusions involving high numbers of splenocyte when the fusion efficiency rose from 5 to 25% in the presence of mouse ascites ($P < 0.001$). The number of

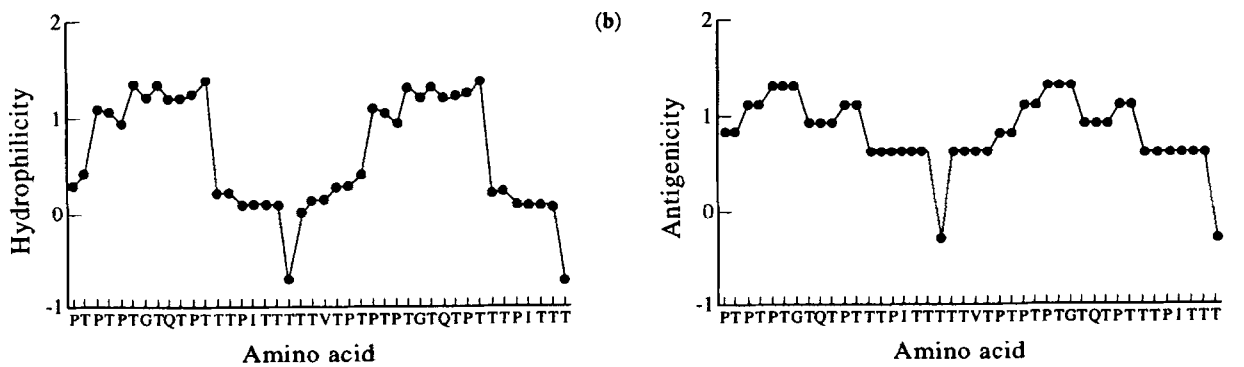

Fig. 1. Analysis of the core structure of the MUC2 protein for potential B cell epitopes by predicting both areas of (a) hydropathicity and (b) secondary structure according to the S.E.R.C. SEQNET Database and using the University of Wisconsin genetic computer package [23].

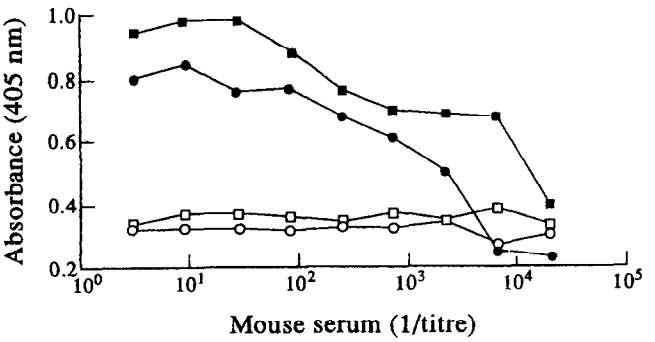

Fig. 2. Binding of serial titrations of a serum pool of six mice immunised with MUC2p-KLH in the presence of either Freund's adjuvant (■, □) or Quil A adjuvant (●, ○) to MUC2p-BSA (closed symbols) and BSA (open symbols) as assayed by ELISA. The absorbance represents the mean of values from quadruplicate wells the variance was always less than 5%.

hybridomas secreting antibodies which bound to MUC2p was also higher in the fusions supplemented with mouse ascites as were the number of stable MAbs which were isolated following cloning.

The specificity of the MAbs were measured by ELISA or radioimmunoassay (RIA) against purified antigens and cell lines, and by immunohistochemistry of frozen sections of tumour and normal tissues.

ELISA

Microtitre plates were coated with either mucin peptide conjugated to bovine serum albumin (BSA) or KLH at 5 μg/ml overnight, or antigen [carcinoembryonic antigen (CEA) C14gp200, MUC1 and MUC2] by overnight incubation at 37°C or by plating out 5×10^4 cells and leaving them overnight at 37°C prior to fixing with 0.02% glutaraldehyde. All the plates were blocked with 50 μl of BSA [1% in phosphate buffered saline (PBS)] for 1 h at room temperature. Serial dilutions of mouse serum (1/3–1/30 000), culture supernatant (50 μl) or purified MAbs (5 μg/ml) were added and incubated at room temperature for 1 h. After washing with PBS containing 0.1% (v/v) Tween 20, bound antibody was detected with rabbit anti-mouse peroxidase (1/400; Dakopatts, Denmark) and the assays developed with ABTS substrate solution (50 mg 2,2'-azino-bis-(3-ethyl-benzthiazoline-6-sulphon-ate) in 100 ml 0.1 M citrate phosphate, pH 4.0, with 120 vol hydrogen peroxide added at 0.3 μl/ml immediately before use. Absorbance was measured in each well at 405 nm.

Radioisotopic anti-globulin test

Binding of monoclonal antibodies to 996/1 and 994/152 antigen preparations were assayed as described previously [26]. Briefly, affinity purified 996/1 or 994/152 affinity purified mucin was dried on to Terasaki microtitre plates. Monoclonal antibodies binding specifically to these plates were detected with ^{125}I-labelled, affinity purified F(ab')$_2$ fragments of rabbit anti-mouse Ig. Separate wells were counted in an LKB Compugamma counter.

Immunoblotting

MUC2 mucin [26] was diluted 1:1 in SDS–PAGE reducing sample buffer and then applied to a 7.5% polyacrylamide gel, with a 4% stacking gel, using an LKB Midget Gel Electrophoresis Apparatus. Electrophoresis was performed at 300 V for 50 min using the discontinuous buffer system of Laemmli [27].

Electroblotting on to nitrocellulose membranes was performed as described by Towbin and colleagues [28] using the Biorad Transblot Apparatus for 20 h at 50 V and 200 mA in 25 mM Tris, 192 mM glycine buffer, pH 8.3, containing 20% methanol. Immunostaining of antigen with 996/1 MAb was performed as described previously [29].

Immunocytochemistry

Antibody binding to frozen sections of normal postmortem tissues, to colorectal tumours and normal colonic mucosa taken at the resection margin was examined by standard immunoperoxidase staining [30]. Endogenous peroxidase was inhibited by incubation with 0.3% H_2O_2 in 0.1% NaN_3, followed by blocking with human serum (10%), containing rabbit serum (0.1%) and BSA (1%) in PBS. After incubating sections with MAb and washing, bound antibody was stained with peroxidase-conjugated rabbit anti-(mouse immunoglobulin) and diaminobenzidene (1 mg/ml), and sections were finally counterstained with haematoxylin and mounted.

Statistical analysis of data

All results are presented as mean of quadruplicate samples performed in at least two separate assays. The results have been analysed for significance by Student's *t*-tests.

Cell lines

All cell line were grown in RPMI containing 10% fetal calf serum. If possible, tumour cell lines freshly derived from tumour tissues were chosen as they are more representative of *in vivo* tumour expression. PT01 was derived from a freshly disaggregated recurrent colorectal cancer, Pan1 a biopsy of a pancreatic tumour, and 59M was derived from a poorly differentiated ovarian tumour. MKN45 was derived from an advanced gastric tumour [31] and MCF7 from a pleural effusion of a breast carcinoma [32]. NSO is a mouse myeloma.

Monoclonal antibodies

MAbs 365 (IgG1-anti-CEA1), 228, 337 (IgG2as-anti-CEA2 and 3), 692/29 (IgG3-anti-Lewis$^{y/b}$1) 692/33, 692/43 (IgG1s-anti-

Table 1. Fusion efficiencies of splenocytes derived from mice immunised with mucin peptide KLH

Fusion number	No. of splenocytes fused	No. of NSO fused	No. of wells plated	Serum supplement	No. of hybridomas	Fusion efficiency	No. of positives	Number of MAbs
994	2×10^8	3×10^7	392	Mouse ascites	232	56	18	3
995	2×10^8	3×10^7	192	FCS	97	50	15	1
996	2×10^8	1.25×10^7	192	Mouse ascites	46	23	6	3
997	2×10^8	1.25×10^7	192	FCS	14	5	3	0

FCS, fetal calf serum; NSO, mouse myeloma.

Table 2. Binding of the monoclonal antibodies produced by immunisation with MUC2p-KLH to peptides based on the tandem repeat sequences of MUC1–4

Monoclonal antibody	Peptide	994/91	994/152	995/23	996/1	996/7	996/36	Anti-MUC1.1	Anti-MUC3.1	Anti-MUC4.1	Anti-p53
MUC2p-BSA	KVTPTPTPTGTQTPT	0.62	0.68	0.61	0.77	0.56	0.67	nd	nd	nd	nd
MUC1p-BSA	KCAPDTRPAPG	0.15	0.17	0.15	0.19	0.16	0.15	0.45	nd	nd	nd
MUC3p-BSA	KTTTETTSHSTPSF	0.34	0.49	0.12	0.15	0.11	0.32	nd	0.69	nd	nd
MUC4p-BSA	KTDTSSASTGHA	0.14	0.16	0.12	0.15	0.14	0.13	nd	nd	0.75	nd
p53p-BSA	KEVVRRCPHHE	0.16	0.17	0.16	0.16	0.16	0.15	nd	nd	nd	0.68

nd, not determined; BSA, bovine serum albumin.

Lewis$^{y/b}$2 and 3), GL-013 (IgG1-anti-mucin 1) and NCRC11, HMFG2 (IgMs-anti-MUC1.1 and 1.2) SM3 and 595 (IgG1 and IgG3-anti-MUC1.3 and 1.4) have been reported previously [9, 13, 30, 33–35]. Anti-MUC3.1 and anti-MUC4.1 are monoclonal antibodies which recognise peptides based upon the tandem repeat sequences of MUC3 and MUC4, respectively. Anti-p53 is antiserum raised by immunisation of Balb/c mice with the sequence of 172-180 of the human p53 gene.

Antigen preparations

Y hapten-bearing glycoproteins (C14gp200) were prepared from the sputum of a Y-hapten secretor using a C14 antibody immunoadsorbent column [36]. CEA was purified from hepatic metastases from a colonic adenocarcinoma [37] and high molecular mass (> 400 kDa) epithelial mucins (MUC1) were isolated from normal urine using NCRC11 antibody immunoadsorbent column [29].

MUC2 mucin was isolated from colorectal tumours and purified by immunoadsorbent chromatography using Sepharose linked 996/1 or 994/152 MAbs [26].

RESULTS

A peptide based on the tandem repeat sequence of MUC2 mucin was used to produce a series of MAbs. The fine specificity of these antibodies and their implications for MUC2 expression are presented.

Peptide specificity

Seven MAbs, 994/76, 994/91, 994/152, 995/23, 996/1, 996/7, 996/36, were produced which recognised MUC2p linked to BSA (Table 2). Two of these MAbs, 994/76 and 996/7, proved to be unstable and have, therefore, not been fully characterised. Three, 994/152, 994/91 and 996/36, also cross react with a peptide based on the MUC3 tandem repeat (Table 2). However, none of them recognise a peptide representing a MUC1 or a MUC4 tandem repeat or with a peptide based on a sequence of p53 (Table 2).

Although the antibodies bound well to peptides, it was important to determine if they could recognise the peptide sequence in native mucin.

Antigen specificity

Table 3 shows that the MAbs 994/76, 994/91, 994/152, 995/23 and 996/1 all recognise MUC2 purified by affinity chromatography on either 994/152 or 996/1 MAb columns, suggesting that they all recognise a similar antigen. None of the MAbs bound to MUC1 mucin or to the colorectal antigens, CEA and C14gp200 antigen. The antigen affinity purified on MAb 996/1 column was identified as a high molecular weight, mucin-like, polydisperse protein by western blotting (Fig. 3).

Cell lines

Binding of six of the MAbs to cell lines from different tissue origins and in freshly derived cell lines for colorectal, pancreatic and ovarian, and to a well established breast and a gastric cell line were measured by ELISA (Fig. 4). The two MAbs 994/152 and 996/1 stained both of the gastrointestinal cell lines, weakly stained the pancreatic cell line, and MAb 994/152 also stained the ovarian cell line. All of the other anti-MUC2p MAbs failed to show significant binding to any of the cell lines and none of the MAbs stained the breast cell line. In contrast, the MAb recognising the MUC1 mucin stained the breast, the colorectal and the pancreatic cell line but not the other cell lines. 996/1 and

Table 3. Binding of monoclonal antibodies recognising MUC2p to MUC2 mucin, MUC1 mucin, CEA and C14gp200 antigen by ELISA or RIA

MAb	MUC2* (cpm)	MUC1† (OD)	CEA‡ (OD)	C14gp200§ (OD)
994/76	2355	0.20	0.21	0.23
994/91	2306	0.28	0.18	0.24
994/152	2833	0.32	0.25	0.26
995/23	2290	0.24	0.21	0.28
996/1	3407	0.24	0.20	0.29
996/7	nd	0.44	0.23	0.32
996/36	42	0.25	0.22	0.28
Anti-CEA3	nd	nd	0.65	nd
Anti-CEA2	nd	nd	0.70	nd
Anti-CEA1	nd	0.22	0.59	nd
Anti-Lewis$^{y/b}$1	nd	0.33	0.17	0.70
Anti-Lewis$^{y/b}$3	nd	nd	nd	0.74
Anti-Lewis$^{y/b}$2	nd	nd	nd	0.73
Anti-MUC1.3	nd	1.14	nd	nd
Anti-MUC1.4	nd	0.80	nd	nd
Anti-MUC1.2	nd	0.85	nd	nd
Anti-MUC1.1	nd	1.25	0.21	nd

*MUC2 is a high molecular weight mucin purified on 994/152 or 996/1 MAb affinity column. †MUC1 is a high molecular weight mucin purified on a NCRC11 affinity column. ‡Carcinoembryonic antigen (CEA) was purified from colorectal tumour tissue by the method of Krupey *et al.*, 1972 [37]. §C14gp200 was purified from sputum by the method of Price *et al.*, 1986 [36]. All results are mean of quadruplicate wells, the standard deviations have been omitted for clarity but were always less than 5%. nd, not determined.

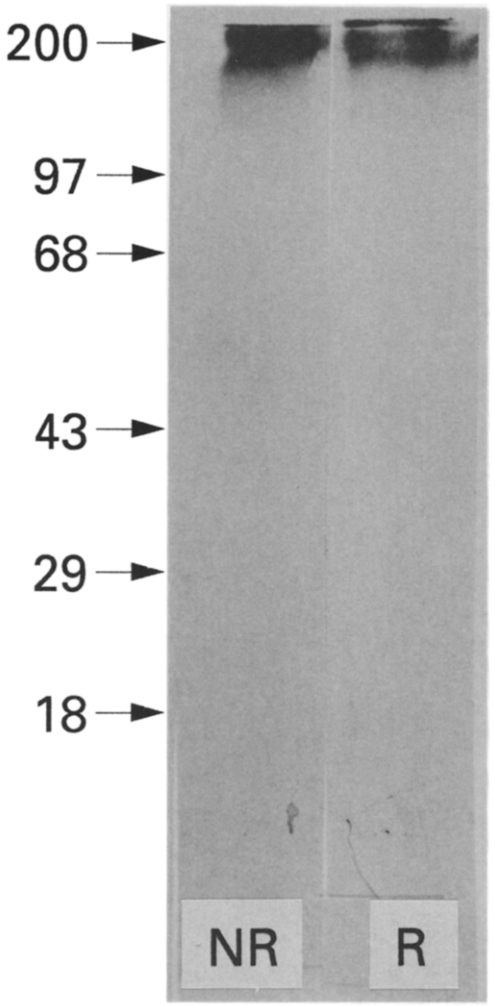

Fig. 3. SDS–PAGE western blot analysis of 996/1 MAb defined antigen isolated from colorectal tumour. The nitrocellulose sheet in both lanes is probed with MAb 996/1 and in lane NR is non-reduced and in lane R reduced. The centre bands are molecular weight standards in kDa.

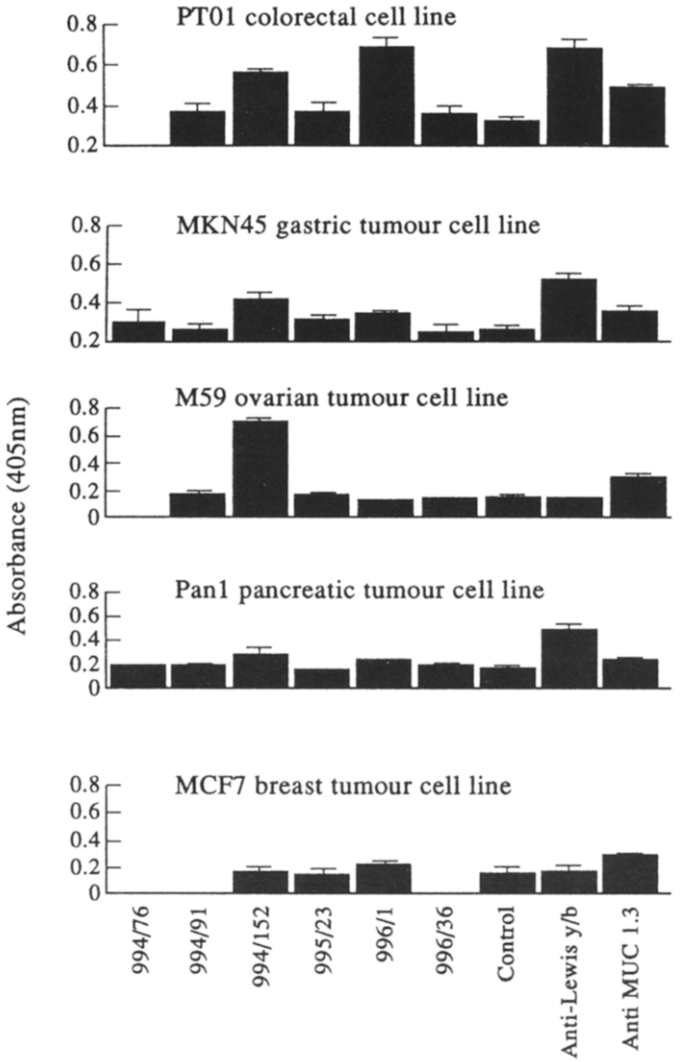

Fig. 4. Binding of the anti-MUC2p MAbs (10 μg/ml) to tumour cell lines grown as monolayers on microtitre plates and fixed with glutaraldehyde prior to ELISA assay. Control antibodies include 692/43 (anti-Lewis$^{y/b}$ hapten), NSO myeloma supernatant and 595 (anti-MUC1). The absorbance values represent the mean and standard deviation of quadruplicate wells.

994/152 were, therefore, the most useful at recognising mucin expressed by tumour cell lines.

Tumour specificity

Table 4 and Fig. 5 show the staining of the anti-MUC2p MAbs to colorectal tumour and adjacent mucosal tissues. The staining was all intracellular, implying that the antibodies recognise immature mucin but not mature secreted mucin.

All of the monoclonal antibodies but 996/36 stained 252 mucosa, however, only MAb 996/1 stained 258 mucosa strongly, whereas the other MAbs showed weaker staining. Only MAb 996/1 consistently recognised the tumour tissues, staining 5/6 of the tumours with moderate to strong intensity, although the staining was heterogeneous in two of these tumours. MAb 994/91 also stained 3/6 of the tumour tissues, but the staining was weak and heterogenous. The other MAbs failed to stain the tumour tissues. The epitopes recognised by 996/1 and 994/91 appear to be exposed on mucin expressed by tumours and adjacent mucosa. However, the epitopes recognised by the other MUC2p MAbs were either masked or destroyed in frozen tumour sections.

Normal tissue specificity

Table 5 shows the staining of the monoclonal antibodies to postmortem tissues. Only weak staining of goblet cells within the colon was observed with the MAbs 994/76, 994/91, 995/23, 996/7 and 996/36, suggesting these epitopes are not well recognised on frozen normal tissues. However, 994/152 and 996/1 MAbs both showed distinctive staining patterns. They stained goblet cells within the digestive tract, although 996/1 MAb showed the strongest staining to colon tissue, whereas 994/152 MAb stained stomach tissue most intensively. MAb 994/152 showed definite staining of lung and kidney tissue whereas weaker staining was observed with 996/1 MAb. Liver hepatocytes were stained by both MAbs, with MAb 996/1 showing the most intense staining. Spleen, ovary and prostate tissues stained very weakly or failed to stain with either monoclonal antibody. However, although MAb 994/152 also failed to stain prostate tissue, MAb 996/1 strongly stained this tissue.

Table 4. Binding of the anti-MUC2p MAbs to colorectal tumour and normal mucosal tissues by immunohistochemistry

MAb	258 mucosa*	252 mucosa	259 tumour	252 tumour	249 tumour	255 tumour	257 tumour	254 tumour
994/91	+	2+	−	+	+/−	−	+s	−
994/152	+/−	2+	−	−	−	−	−	−
994/7	+/−	+	−	−	−	−	−	−
995/23	+/−	2+	−	−	−	−	−	−
996/1	2+	2+	−	2+/−	2+/−	+	2+	+/−
996/36	+	−	−	−	−	−	+/−	−
Control†	−	−	−	−	−	−	−	−

Staining is described as 2+ strong, + moderate +/− weak and − negative. 2+/− is strong heterogeneous staining. *258 and 252 mucosa are taken from the resection margins of colorectal tumours 258 and 252. †The negative control was supernatant from the parent myeloma cell line.

DISCUSSION

Heterogeneity among mucin glycoproteins in the intestine has been suggested by histochemical, immunohistochemical and biochemical studies, and seems to involve both the carbohydrate and the protein moiety of the mucin molecule. It is only recently that the carbohydrates, which are multiple cancer associated antigenic epitopes, have been identified. These include core region carbohydrates such as Tn, sialosyl Tn, or T antigens, or peripheral and backbone region carbohydrates such as extended and/or polyfucosylated Le^x or Le^y antigens or sialyted Le^x antigens [38]. Until the recent cloning of the MUC2 and MUC3 genes [3, 4], very little was known about the peptide structure of these mucins. It is now becoming clear that both the MUC2 and the MUC3 mucins are variably expressed by colonic tumours but their role in tumorigenesis remains to be elucidated. Production of MAbs which recognise the peptide backbone would greatly facilitate these studies.

Analysis of the mucin peptide structure suggested that amino acids 12–25 of the 23 amino acid repeat structure would be an area with potential B cell epitopes. This was confirmed as this peptide proved to be extremely immunogenic, inducing titres in excess of 1/19 000 when linked to a T cell carrier and immunised in the presence of Freund's adjuvant. The success of this immunisation was attributed to the unique degree of secondary structure observed in this small peptide. This is in contrast with most short peptides which have a flexible structure and do not imitate the conformation that is induced when the same peptide is part of a larger molecule. The addition of mouse ascites to the culture medium post fusion helped in the stabilisation of the antigen specific hybridomas, presumably by providing the correct milieu of cytokines.

The immunisation schedule used in this study was also unusual as peptides are usually used to expand specific B cell clones following a primary immunisation with antigen [39]. The peptide is very immunogenic as it resulted in seven monoclonal antibodies, four of these specifically recognising the MUC2 peptide but not peptides based on the MUC1, MUC3 or MUC4 tandem repeat sequences. The antigen affinity purified on a 996/1 column from colonic tumours was a polydisperse high molecular weight protein, properties which are characteristic of mucins. These results would suggest that, like MUC1, peptides based on the tandem repeat amino acid sequence of MUC2 may have potential as tumour vaccines. The 12–25 peptide used in this study is currently being tested for its immunogenicity in severe combined immunodeficiency mice (SCID) which have been repopulated with lymphocytes from normal or tumour bearing donors.

Two of the MAbs, 996/1 and 994/152, stained tissue sections although the fine specificity varied between these two MAbs, with 994/152 strongly staining gastric, ileum and kidney epithelia and 996/1 MAb intensely staining colon, liver and prostate tissues. In contrast to the anti-MUC2 antibodies produced by Xing and colleagues [40], staining with MAbs 996/1 and 994/152 did not appear restricted to the digestive tract as strong staining of lung, liver, kidney and prostate tissues was observed. Strong to moderate staining of gastric mucosa was also observed. In agreement with all other studies normal ovary and uterine tissues were negative [39–41]. Whether this implies that MUC2 expression is not restricted to the gastrointestinal tract or suggests that the MAbs are binding to other antigens than MUC2 in tissue sections is unresolved. However, the peptide specificity of 996/1 makes the latter possibility unlikely. 994/152 did cross react with the MUC3 peptide but MUC3 MAbs failed to bind to the 994/152 antigen purified from colorectal tumours (unpublished results).

Binding by the MAbs to tumour tissue sections or on tumour cell lines gave differing results. All five MAbs tested bound to one or other of the normal mucosal tissues, but only MAbs 994/91 and 996/1 stained colonic tumour sections. In contrast, MAbs 994/152 and MAbs 996/1 stained the tumour cell lines. The staining of the mucosa at the resection margin of tumour 252 showed intense staining with all of the monoclonal antibodies whereas in the mucosa from tumour 258 and from the postmortem donor, only positive staining with MAbs 996/1 and 994/152 was seen. This suggests field changes throughout the mucosa of patient 252. These differences in recognition may be related to differential glycosylation of MUC2 between different tissues which may restrict access of the MAbs to the peptide core. As the peptide core of the colonic mucin is so rich in threonine, which is a site for potential glycosylation, it is perhaps surprising that any MAbs recognising core peptide can have access to the glycosylated molecule. However, 996/1 MAb consistently stains colonic tissues, tumour cell lines, mucin peptides and recognises a high molecular weight polydisperse, mucin-like, tumour antigen.

Antisera to native and deglycosylated intestinal mucin have shown alterations in the expression of mucin in malignant tissue, including loss of subcellular compartmentalisation, increased intensity of staining and disappearance of staining. In addition, *de novo* expression of an epitope on deglycosylated intestinal mucin was observed in one of five breast carcinomas and three of five ovarian mucinous cystadenocarcinomas [41]. Similar results have been seen with the anti-MUC2 MAbs produced in this study. Of particular interest is the staining of the ovarian

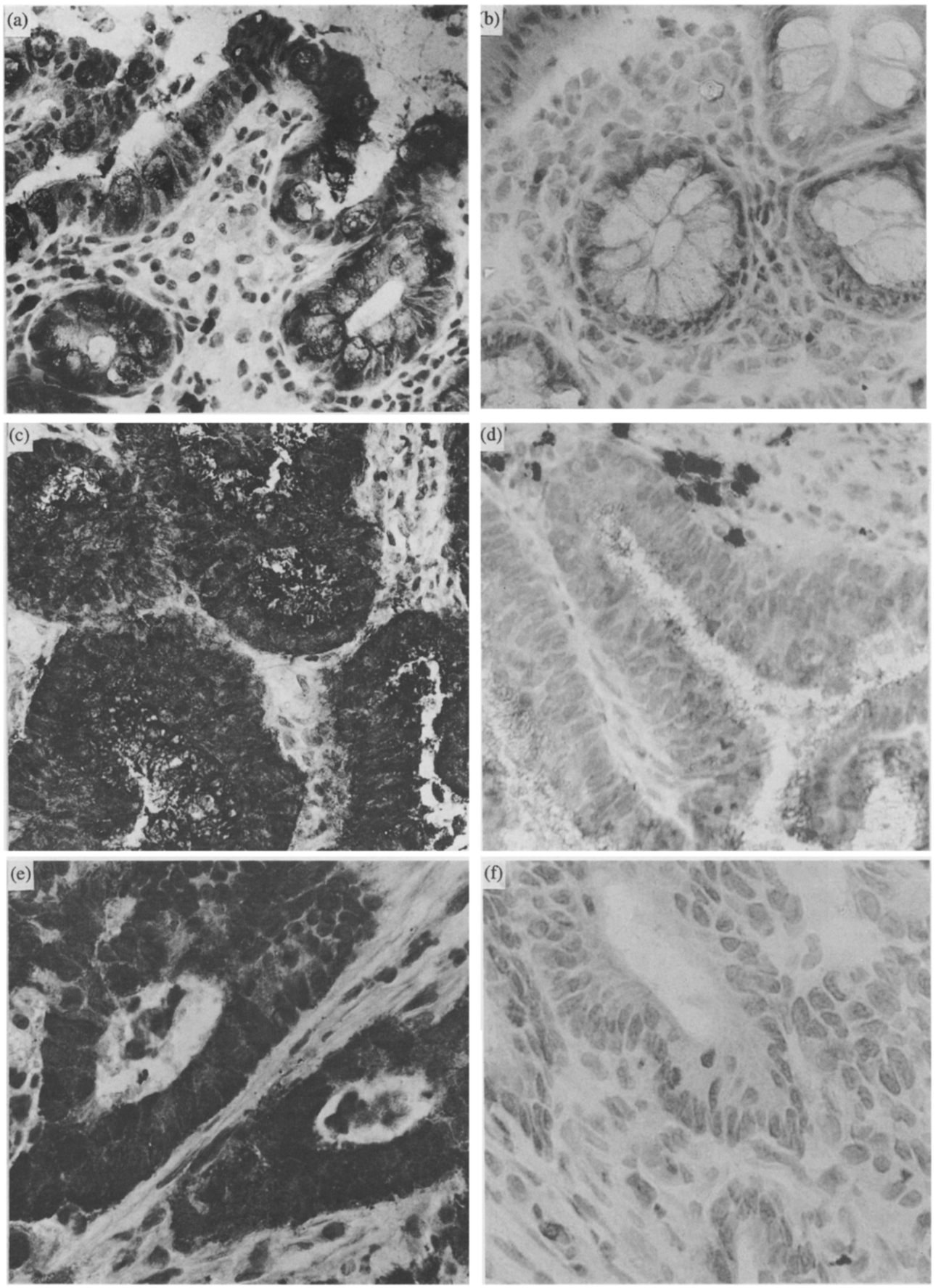

Fig. 5. Immunohistochemistry staining of: normal colon mucosa with (a) MAb 996/1 and (b) control IgG1; colon tumour 252T with (c) MAb 996/1 or (d) control IgG1; and colorectal tumour 257T with (e) MAb 996/1 and (f) control IgG1. Magnification ×40.

Table 5. Immunohistochemical staining of frozen tissue sections from postmortem specimens

	Anti-MUC2 monoclonal antibodies						
Postmortem tissues	994/76	994/91	994/152	995/23	996/1	996/7	996/36
Stomach	−	−	2+	+/−	+	−	−
Ileum	−	−	2+	−	+	−	−
Colon	+/−	+/−	+	−	2+	+/−	+/−
Lung	nd	nd	+	+	+/−	−	−
Liver	−	−	+	−	2+	−	−
Kidney	−	−	2+	−	+	−	−
Spleen	−	−	+/−	−	−	−	−
Prostate	nd	nd	+/−	+/−	2+	nd	nd
Ovary	nd	nd	−	−	−	nd	nd
Uterus	nd	nd	+/−	+/−	−	nd	nd

Staining is described as 2+ strong, + moderate, +/− weak and − negative. 2+/− is strong heterogeneous staining. nd, not determined.

cell line with MAb 994/152 which suggests that ovarian tumours react with anti-colonic mucin antibodies. This is perhaps not so surprising as ovarian tissue is derived from fetal gut during embryogenesis.

Elucidating the potential role of the MUC2 gene in malignancy is of prime importance as it may play a key role in the metastatic process. The production and characterisation of the MAb 996/1 should greatly facilitate these studies in humans.

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Folate Binding Protein Distribution in Normal Tissues and Biological Fluids From Ovarian Carcinoma Patients as Detected by the Monoclonal Antibodies MOv18 and MOv19

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Folate-binding proteins (FBP), which are molecules relevant in folate metabolism, are overexpressed in ovarian carcinomas, as detected by the monoclonal antibodies (MAb) MOv18 and MOv19, which recognise two different epitopes of the gp38/FBP. In this paper, features of the FBP such as the distribution on normal tissues and the release in biological fluids of normal and tumour origin have been investigated. Immunohistochemical analyses on frozen sections of normal tissues showed the presence of the gp38/FBP on some epithelia. The reactivity of both the MAb on Fallopian tubes was intense and comparable to that observed on ovary carcinoma sections. The kidney, bronchial glands, alveolar epithelium of the lung, oesophagus, stomach, pancreas, breast and thyroid showed different levels of staining. By MOv18/MOv19 double-determinant immunoradiometric assay (DDIRMA), the gp38/FBP was found in soluble form in ascitic fluid, serum and urine of nude mice in which the human ovary carcinoma cell line IGROV1 grew as ascitic carcinomatosis. In human biological fluids, the gp38/FBP was detected in ascites of 60% of ovarian carcinoma patients, and in 29% of those with other carcinomas, but not in patients with non-epithelial tumours or with other non-tumoral pathologies. The mean serum arbitrary units (a.u.)/ml values of ovary carcinoma patients were significantly different to those of healthy donors or patients with endometriosis ($P < 0.005$ and $P < 0.01$, respectively), but not when compared to the sera of lung carcinoma patients. In addition, the sensitivity of DDIRMA was poor, since only 24% of the ovary carcinoma patients were positive with this assay. When a restricted number of cases selected for the presence of tumour cells in the ascites was examined, the percentage of DDIRMA-positive sera and ascites rose to 41 and 94%, respectively. In the urine, a strong reactivity was observed in the samples of both normal and tumour origin.
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