Monoclonal Antibody Recognizing a Core Epitope on Mucin

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ABSTRACT: Monoclonal antibody TH1 (IgM) was prepared by immunizing mice with deglycosylated (TFMSA-treated) cystic fibrosis mucin. TH1 reacted strongly with TFMSA treated cystic fibrosis mucin but not with the fully glycosylated mucin, indicating reactivity with a core mucin epitope. TH1 showed no reactivity with ovine mucin (98% of glycans as sialyl-Tn) but reacted strongly with desialylated ovine mucin, indicating the epitope for this mab was the Tn-antigen (O-linked GalNAc). However, TH1 showed no reactivity with Tn-positive red blood cells, and the binding of TH1 was not inhibited by GalNAc at 2.5 mg/ml, illustrating the importance of the peptide sequence to which the GalNAc is attached. TH1 stained the majority of cancers of the colon, lung, stomach, ovary, breast, and cervix, and the cellular distribution of this antigen in normal tissue suggested reactivity with immature mucin. This antibody appears to be a useful reagent for the detection of immature mucin.

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INTRODUCTION

Mucins are high molecular weight glycoproteins containing up to 80% by weight O-linked carbohydrate, and are characterised by containing a variable number of tandem repeats (VNTR) that contains numerous glycosylation sites [8]. They are located at the epithelial surface or secreted to form mucus. The molecules are believed to have a protective role in normal tissue and may also express carbohydrate structures involved in cell adhesion [8]. In tumours, increased mucin synthesis and secretion, and blocked or neosynthesis of O-linked carbohydrate chains can occur, resulting in the exposure of cryptic carbohydrate or protein epitopes [4,19,40]. Monoclonal antibodies recognising such structures will therefore be of value in the immunohistochemical detection of mucin. In this paper we describe the production of a monoclonal antibody (TH1) to a novel core epitope on mucin by immunising with partially deglycosylated cystic fibrosis mucin.

MATERIALS AND METHODS

Purification and Deglycosylation of Mucin

Mucin was prepared as described [43]. Briefly, mucus from a patient with cystic fibrosis was extracted with 4M guanidinium chloride containing protease inhibitors and purified by two-stage density gradient centrifugation [2]. The purified mucins, in 6M guanidinium chloride/0.1M Tris-HCl pH 8.0, were reduced with 10 mM DTT for 5 hours at 37 °C.

Iodoacetimide was added to a final concentration of 25 mM and left in the dark overnight at room temperature. The reduced and alkylated mucins were dialysed extensively against distilled water and lyophilised. Reduced mucins were then digested with neuraminidase and treated twice with trifluoromethanesulphonic acid (TFMSA)/anisole for 4 hours at 4 ºC and finally treated with periodate [15]. This preparation of deglycosylated respiratory mucin will subsequently be described as DR-mucin. Mucin from a patient with papillary serous cystadenocarcinoma of the ovary (OV-Mucin) was purified as described [4].

Monoclonal Antibody Production

A BALB/c female mouse (8 weeks old) was injected by sc and im routes with DR-mucin emulsified in Freund's Complete Adjuvant (1 mg/mL). Further injections were given one and two months later, except the mucin was emulsified in Freund's Incomplete Adjuvant (FIA) and injected by ip and im routes. After a further month, the mouse was injected iv with DR-mucin in PBS and im with DR-mucin in FIA. The spleen cells were fused with Ag8 cells four days later.

Hybridomas were screened by ELISA on DR-mucin or BSA as a negative control. One mab, TH1, showed specificity for DR-mucin, and this was cloned by limiting dilution. The subclass of the mab was determined by dual-determinant ELISA using subclass-specific antibodies to capture mab and anti-mouse Ig-peroxidase (AmRad, Melbourne, Australia) to detect bound mab.

Peptides, Glycoproteins, Carbohydrates and Monoclonal Antibodies

The peptides, antibodies and lectins used in this study are shown in Table 1. Peptides correspoding to the VNTR regions of 5 mucin genes (MUC1–5) were used in this study. The MUC1, MUC2, and T4N1 peptides were synthesized using an Applied Biosystems Model 430A automated peptide synthesizer (Forster

City, CA, USA) [21]. MUC1 corresponds to the 20 amino acid MUC1 repeat plus the first four amino acids of the next repeat [14]; MUC2 corresponds to the first 23 amino acid MUC2 repeat plus the first four amino acids of the next repeat [16], with KY added to the N-terminal for conjugation; T4N1 corresponds to the N-terminus of mouse CD4 [3], and was used as a control. The MUC3, MUC4, and MUC5 peptides were prepared using the "tea bag" method [22]. MUC3 represent the 17 amino acid MUC3 repeat plus the first five amino acids of the next repeat [17]. MUC4 represent the 16 amino acid MUC4 repeat plus the first 3 amino acids of the next repeat [38]. MUC5 represents two 8 amino acid MUC5C repeats plus the first 3 amino acids of the next repeat [1]. Peptides have lysine attached to the N-terminus for conjugation purposes. The peptides were purified by reversed-phase HPLC on a Deltapak-C18 column (Nihon Waters Ltd, Tokyo, Japan), with a gradient of acetonitrile in 0.1% TFA. Peptides were conjugated to keyhole limpet hemocyanin (KLH) (Pierce, USA) using glutaraldehyde [49].

Glycoproteins used were bovine submaxillary mucin (BSM), porcine stomach mucin (PSM), bovine serum albumen (BSA) (Sigma Chemical Co., MO, USA), and transferrin (Boehringer-Mannheim, Germany). Ovine submaxillary mucin (OSM) was kindly donated by Dr Rod Williams, Monash University, Australia. Asialo-mucins were prepared by incubation of 2 mg/ml mucin in PBS with an equal volume of 1 U/ml *Vibrio cholerae* neuraminidase (Hoechst-Behring, Marburg, Germany) for 3 hours at 37 ºC. All monosaccharides used in this study were purchased from the Sigma Chemical Co., MO, USA.

Cell Culture

Human mucin-producing tumour cell lines were maintained as described [28]. Cell lines used were COLO316 (ovary), T47D (breast), ZR75-1 (breast), LS174T (colon), and LIM1899 (colon). COLO316 and LIM1899 were gifts from Dr. Theonne de Kretser, Peter McCallum Cancer Institute, Melbourne, Australia and Dr.

Bob Whitehead, Ludwig Institute for Cancer Research, Melbourne, Australia, while other cell lines were purchased from ATCC, USA. Culture supernatant was clarified by centrifugation, while cells were harvested from the flasks with a cell scraper. Cells were homogenised in an equal volume of 0.5M sucrose, 1 mM PMSF, 0.02% EDTA in phosphate buffer pH 7.2.

ELISA

All assays were performed in duplicate, with the coefficient of variation of duplicates being < 10% in all cases. The monoclonal antibodies (mabs) and biotinylated lectins used in ELISA are shown in Table 1.

Table 1
Peptides, antibodies and lectins used in this study

PEPTIDES

Name	Sequence	Reference
MUC1	PDTRPAPGSTAPPAHGVTSAPDTR	[14]
MUC2	KYPTTTPISTTTMVTPTPTPTGTQTQTPTTT	[16]
MUC3	KSHSTPSFTSSITTTETTSHSTP	[17]
MUC4	KTSSSASTGHATPLPVTDTSS	[38]
MUC5	KSAPTTSTTSAPTTSTTSAP	[1]
T4N1	KTLVLGKEQESAELPCECY	[3]

ANTIBODIES

Name	Isotype	Specificity	Reference
TH1	IgM	Tn	-
3C2	IgM	sialyl-Tn	[10]
BC2	IgG1	MUC1 VNTR	[28]
BC3	IgM	MUC1 VNTR	[28]
4F1	IgM	MUC2 VNTR	[9]
4E7	IgM	CEA	[11]
CC3	IgG1	Lewis-a	[5]
43	IgM	H-glycan	[5]
401/21	IgG1	gliadin	[41]
FM1	IgM	control	unpublished

LECTINS

Name	Source	Specificity	Reference
SSA	*S.sieboldiana* (elderberry)	O-GalNAc±Gal	[7]
PNA	*A.hypogaea* (peanut)	Galß1,3GalNAc	[47]
WGA	*T.vulgaris* (wheat germ)	GlcNAc$_2$,GalNAc	[47]
		NeuAc	[46]
HPA	*H.pomatia* (garden snail)	GalNAc	[47]
LEA	*L.esculentum* (tomato)	GlcNAc$_3$	[31]
SBA	*G.max* (soybean)	GalNAc	[47]
SNA	*S.nigra* (elderberry)	NeuAcα2,6GalNAc	[46]
MAA	*M.amurensis*	NeuAcα2,3Gal	[46]

[a] CC3 and 43 donated by Professor Ian McKenzie; 401/21, BC2, and BC3 provided by Medical Innovations Ltd, Sydney, Australia; SSA provided by Dr. Hiroshi Harada, Honen Corporation, Japan; SNA-digoxigenin and MAA-digoxigenin purchased from Boehringer-Mannheim, Germany; all other lectins (biotin labelled) purchased from Sigma Chemical Co., MO, USA.

Indirect ELISA. Antigens (50 ml/well) were coated onto a Falcon flexible assay plate (Becton Dickinson, USA) by incubating overnight at room temperature (RT) in 0.1M carbonate buffer pH 9.6. Plates were blocked for 1 hour at RT with 2% (w/v) BSA in PBS (75 ml/well), and incubated overnight with mab in phosphate buffered saline containing 0.05% Tween-20 (PBS-Tween). Bound mab was detected using anti-mouse Ig-peroxidase (Silenus, Australia) and ABTS substrate [5]. Plates were washed three times with PBS-Tween between each incubation, and checkerboard titrations of antigen and mab were performed. Alternatively, plates were blocked with Blocking Reagent (Boehringer-Mannheim, IN, USA, cat.no.1142372) and probed with biotin or digoxigenin-labelled lectins followed by detection with streptavidin peroxidase (Amersham, UK) or anti-DIG-peroxidase (Boehringer-Mannheim, Germany) [6].

Inhibition ELISA. Optimal concentrations of antigens and mabs were determined by checkerboard ELISA, and inhibition ELISA was performed [6]. Briefly, mabs at twice the required concentration were incubated for three hours at RT with an equal volume of glycoprotein, peptide or carbohydrate solution in PBS-Tween. "No inhibition" (mab plus PBS-Tween) and "total inhibition" (PBS-Tween, no mab) incubations were also performed. Subsequently, 50 ml was transferred to a DR-mucin-coated plate and the assay was completed as above. Inhibition was calculated as described [28].

Dual-Determinant ELISA. BC2 was purified on Protein-A agarose [12], while TH1 and BC3 were purified by precipitation with 8% polyethylene glycol [33]. TH1 and BC3 were biotinylated as described [35]. Microtitre plates were coated with BC2 (anti-MUC1 VNTR) or TH1 by an overnight incubation at 4 ug/ml in carbonate pH 9.6 at RT. Plates were blocked with bovine serum albumen (BSA) as described above, and DR-mucin or ovarian tumour mucin in PBS-Tween were added for 3 hours at RT. After washing the plates, biotinylated BC3 (anti-MUC1 VNTR) or TH1 were added for 1 hour at RT, followed by streptavidin peroxidase for 1 hour at RT. ABTS was used as substrate.

Reactivity with Tn-Positive Red Blood Cells

Tn-positive red blood cells or normal red blood cells from Red Cross blood donors were treated for 1 hour at 37 ºC with 2 volumes of 0.1 U/ml *Clostridium perfringens* neuraminidase (Type X, Sigma Chemical Co, MO, USA), and subsequently washed three times and resuspended in PBS. A 5% suspension of untreated or neuraminidase-treated cells was incubated for 30 minutes at room temperature with 2 volumes of TH1 culture supernatant or Tn-reactive lectins from *Salvia sclarea* (Gamma Biologicals, USA) or *Dolichos biflorus* (purified from seeds in house). Tubes were then centrifuged at 150g and read microscopically as described [5]. Alternatively, an indirect test using anti-mouse IgM was also performed as described [5].

Immunohistochemistry Specimens and Analysis

A total of 111 tissue specimens were stained with TH1. Formalin fixed, paraffin embedded specimens were selected from files of the Anatomical Pathology Departments, The Prince Charles Hospital and Royal Brisbane Hospital. For each case, representative blocks were selected and sections 5 μm thick were cut and mounted on glass slides for immunochemical staining. Selected specimens included 20 cases of non-small cell lung carcinoma, 12 cases of colonic adenocarcinoma, 10 cases of gastric adenocarcinoma, 11 cases of breast carcinoma, 17 ovarian tumours and 16 cases of carcinoma of the uterine cervix. Normal tissue from the edge of the resection margins was also present in many of these specimens. In addition, a further 5 specimens from each site containing only normal epithelium were stained with TH1.

Specimens were stained with a standard streptavidin-biotin peroxidase method with DAB as the chromogen [39]. Folowing dewaxing,

rehydration and quenching of endogenous peroxidase with 4% hydrogen peroxide in 0.1% sodium azide, the sections were sequentially incubated with TH1 (1:300) for 1 hour, biotinylated rabbit anti-mouse sera (Dako, Carpenteria, CA) (1:500) for 30 mins and Streptavidin-biotin (Zymed, South San Francisco, CA) (1:4000) for 30 mins. Sections of normal colon were stained with each batch as positive controls. Negative controls were performed by substituting either 5% normal rabbit serum or FM1 diluted 1:300 for TH1. Staining patterns were evaluated with regard to the intensity, distribution and localisation of staining in tissues and cells. In tumour tissue, the distribution of staining was recorded as being either absent (−), or present in less than 25% (+), 25–50% (++), 51–75% (+++) or more than 75% of tumour cells (++++). Cellular localisation of staining was categorised as membranous, cytoplasmic or both.

RESULTS

Lectin and Antibody Reactivity with DR-Mucin

DR-mucin used for immunisation showed weak reactivity by indirect ELISA with the MUC1 core protein reactive antibody BC2 and stronger reactivity with murine polyclonal antisera to KLH conjugates of MUC4 and MUC5 VNTR peptides, but not the MUC2 core protein reactive antibody 4F1 or the carbohydrate reactive antibodies 3C2 (sialyl-Tn), 4E7 (CEA), CC3 (Lewis[a]), and 43 (H-antigen) (not shown). The GalNAc-reactive lectins from wheat germ (WGA), garden snail (HPA), and soybean (SBA) also reacted with DR-mucin, while lectins reacting with other carbohydrate structures (PNA, SNA, MAA) showed no reactivity (not shown).

Production of DR-Mucin Reactive Monoclonal Antibody

Monoclonal antibody TH1 (IgM) was chosen after the fusion, as this reacted with DR-mucin but not native mucin or BSA (not shown).

Control mabs 401/21 and FM1 were negative in all cases. Checkerboard titration showed that the reactivity of TH1 with DR-mucin was concentration dependent (not shown).

Reactivity of TH1 with Mucins and Glycoproteins by Indirect ELISA

The reactivity of TH1 with 2.5 mg/ml native and desialylated ovine submaxillary mucin (OSM), bovine submaxillary mucin (BSM), porcine stomach mucin (PSM), and native and TFMSA-treated cystic fibrosis (CF) mucin was determined by indirect ELISA. TH1 reacted with DR-mucin (OD405nm = 1.32) but not with native CF-mucin. TH1 also reacted with desialylated-OSM (OD405nm = 0.75) but not with native OSM, BSM, or PSM. The reactivity of TH1 with asialo-OSM and DR mucin was concentration dependent (Figure 1a).

Reactivity of TH1 with Mucin Synthetic Peptides in Solid Phase ELISA

In order to determine if TH1 reacted with a peptide epitope on the variable number of tandem repeat (VNTR) region of MUC1–MUC5, the reactivity with synthetic peptides was evaluated. TH1 showed no reactivity with peptides corresponding the the VNTR region of the MUC1, MUC2, MUC3, MUC4, or MUC5 mucins, or with KLH conjugates of these peptides (not shown). DR-mucin, included as a positive control, showed strong reactivity with TH1 (not shown). In contrast, BC2 (anti-MUC1 VNTR) reacted with free MUC1 peptide (MUC1-KLH not tested), while 4F1 (anti-MUC2 VNTR) reacted strongly with free MUC2 peptide and MUC2-KLH not shown).

Inhibition ELISA

TH1 binding to DR-mucin was inhibited strongly by culture supernatant from the human tumour cell lines T47D (breast), ZR75-1 (breast), and LS174T (colon), but not by supernatant from the COLO316 (ovary) or LIM1899 (colon) human tumour cell lines (Table 2). In addition,
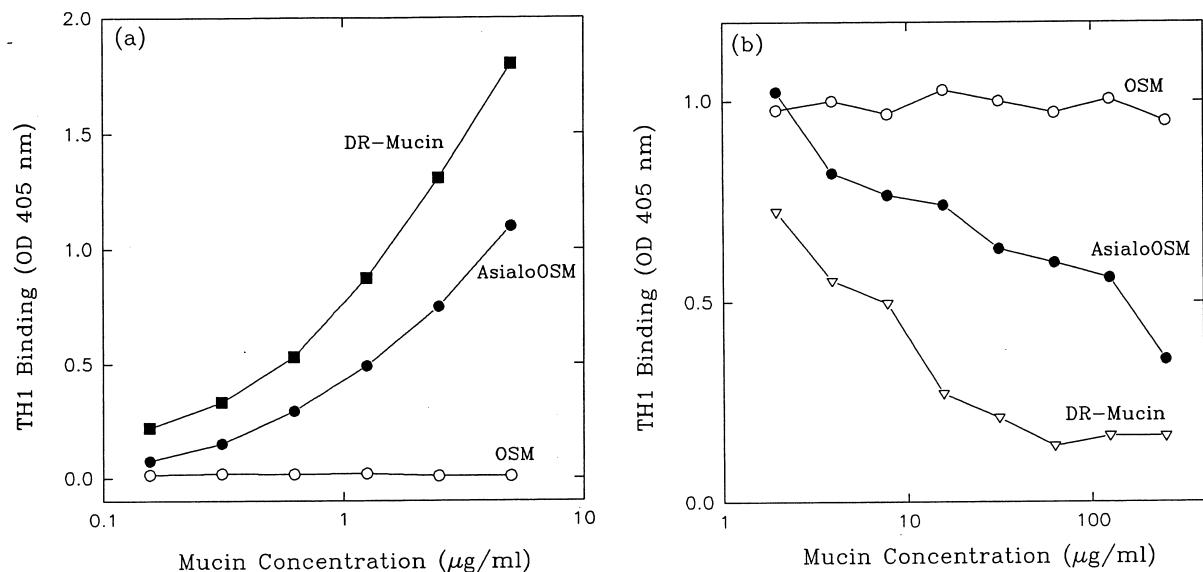
Fig. 1. Reactivity of TH1 with DR-Mucin, Native OSM and Asialo-OSM by (a) Solid Phase ELISA and (b) Inhibition ELISA. Assays were performed as described in the text. For inhibition ELISA, optimum mab and coated antigen concentrations were determined by solid phase checkerboard ELISA. TH1 hybridoma culture supernatant was used at 1/128 and DR-mucin was coated onto the assay plate at 2.5 mg/ml by incubation ON at RT in 0.1M carbonate pH 9.6.

TH1 was also inhibited strongly by an extract of LS174T cells but not ZR75-1 cells. TH1 binding to DR-mucin was not affected by mucin derived synthetic peptides (MUC1–5 at 0.5 mg/ml) or monosaccharides commonly found in mucins (GalNAc, GlcNAc, Gal, Fuc, NeuAc at 2.5 mg/ml). In contrast, the reactivity of a polyclonal antisera (raised against TFMSA-treated cervical mucin) with DR mucin was inhibited by GalNAc (not shown). Desialylation of OSM led to strong inhibition of TH1, and this was concentration dependent (Figure 1b).

In contrast, 3C2 (anti-sialyl-Tn) binding to ovine mucin was inhibited by OSM and BSM, but not the other antigens listed above, while BC2 (anti-MUC1 peptide) binding to COLO316 derived MUC1 was inhibited by the MUC1 peptide, supernatants from the COLO316, T47D, ZR75-1, and LS174T cell lines, ZR75-1 cell extract, and DR-mucin, but not the other antigens listed above (Table 2).

Dual-Determinant ELISA

DR-mucin reacted strongly in the TH1-TH1 ELISA, while mucin purified from the ascites of a patient with ovarian cancer (OV-mucin) did not react in this assay (Figure 2). In contrast, the OV-mucin reacted strongly in the BC2-BC3 (MUC1) ELISA, while DR-mucin showed a lower reactivity. DR-mucin or OV-mucin did not react in the BC2-TH1 ELISA (Figure 2). Similar results were observed with a panel of 24 ascites collected from patients with ovarian cancer (not shown). All reacted strongly in the BC2-BC3 (MUC1) ELISA, but only 1/24 samples showed very weak reactivity in the TH1-TH1 ELISA. In addition, none of these samples reacted in the BC2-TH1 or TH1-BC3 ELISA (not shown). The same profile of reactivity was observed after neuraminidase treatment of ascites prior to assay, with reactivity being increased in positive samples but the reactivity of negative samples was unaffected (not shown).

Table 2
Inhibition of antibody binding

Inhibitor	Concentration	Percentage Inhibition		
		TH1	BC2	3C2
Mucin Peptides				
MUC1 peptide	0.5 mg/ml	9	98	5
MUC2 peptide	0.5 mg/ml	8	0	6
MUC3 peptide	0.5 mg/ml	0	0	0
MUC4 peptide	0.5 mg/ml	2	0	4
MUC5 peptide	0.5 mg/ml	0	0	0
T4N1 peptide	0.5 mg/ml	0	0	5
Monosaccharides				
GlcNAc	1 mg/ml	0	0	3
GalNAc	1 mg/ml	0	3	3
Galactose	1 mg/ml	0	0	10
Glucose	1 mg/ml	3	0	0
Fucose	1 mg/ml	0	0	4
Sialic Acid	1 mg/ml	6	0	15
Human Tumour Cell Lines				
COLO316 S/N	neat	3	96	25
T47D S/N	neat	70	84	25
LIM1899 S/N	neat	27	0	0
ZR75-1 S/N	neat	70	83	32
LS174T S/N	neat	74	83	47
LS174T XT	neat	63	38	40
ZR75-1 XT	neat	23	69	7
Glycoproteins				
BSM	0.5 mg/ml	0	0	86
Asialo-BSM	0.5 mg/ml	39	0	ND
PSM	0.5 mg/ml	35	0	7
Asialo-PSM	0.5 mg/ml	41	0	ND
OSM	0.5 mg/ml	9	0	100
Asialo-OSM	0.5 mg/ml	85	0	ND
BSA	1 mg/ml	3	0	13
Transferrin	1 mg/ml	9	0	8
DR-mucin	0.5 mg/ml	94	99	12

[a] peptide sequences are shown in Table 1.
[b] abbreviations used: GlcNAc, N-acetyl glucosamine; GalNAc, N-acetyl galactosamine; S/N, culture supernatant; XT, cell extract; BSM, bovine submaxillary mucin; PSM, porcine stomach mucin; OSM, ovine submaxillary mucin; BSA, bovine serum albumen; DR-mucin, deglycosylated (TFMSA-treated) respiratory mucin; ND, not determined.
[c] Optimum mab and coated antigen concentrations were determined by solid phase checkerboard ELISA. Mabs were used at 1/128 (TH1 culture supernatant), 1/150 (3C2 culture supernatant), and 10 ng/ml (purified BC2). Antigens coated onto the assay plate were 2.5 mg/ml DR-mucin (for TH1), 0.05 mg/ml OSM (for 3C2), and COLO316 culture supernatant at 1/1000 (for BC2). DR-mucin and OSM were coated by incubation ON at RT in 0.1M carbonate pH 9.6, while COLO316 was coated overnight in vacuo at 37 ºC.
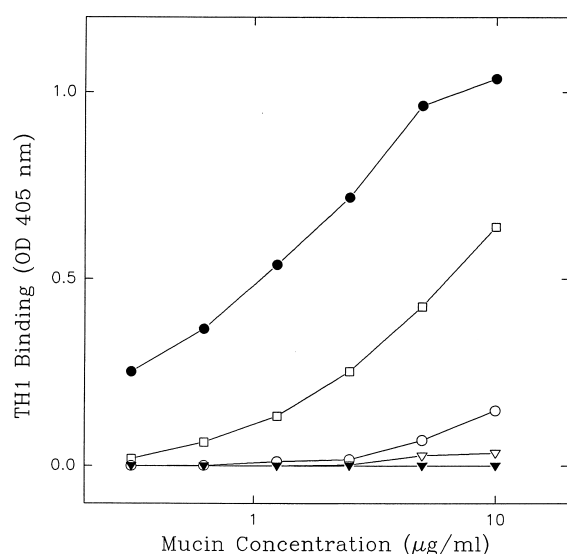
Fig. 2. Reactivity of deglycosylated respiratory (DR) mucin and ovarian tumour (OV) mucin in TH1-TH1, BC2-BC3, and BC2-TH1 sandwich ELISAs. Assays were performed as described in the text. Curves shown are: BC2-BC3 assay with OV-mucin (●) and DR-mucin (O); BC2-TH1 assay with OV-mucin (▼) and DR-mucin (∇); TH1-TH1 assay with OV-mucin (▼) and DR-mucin ( ).

TH1 Reactivity with Red Blood Cells

TH1 did not agglutinate untreated or neuraminidase-treated red blood cells, while *Dolichos biflorus* and *Salvia sclarea* lectins reacted strongly with Tn-positive cells.

Reactivity of Antibodies with Non-Malignant Tissue by Immunoperoxidase Staining

TH1 reacted with some epithelial components of all normal tissues tested. In all cell types which expressed the epitope, staining was granular and confined to the cytoplasmic areas around secretory vacuoles. Large secretory vacuoles, present in many cells, were uniformly negative. In normal colon, TH1 antigen expression was typified by diffuse cytoplasmic staining of the apical region of surface epithelial cells (Figure 3a), although antigen was not detected within goblet cell mucin droplets (Figure 3b). Cells present in the crypts were negative (Figures 3a and 3b). In normal gastric mucosa TH1 staining revealed strong staining of the surface epithelium and mucous glands. In lung tissue, TH1 did not stain alveoli but some staining was observed in bronchial epithelium and mucinous glands. In bronchial epithelium, diffuse cytoplasmic staining was found in the apical region of some columnar cells but staining was not observed in secretory vacuoles of goblet cells. Granular cytoplasmic staining was present in epithelial cells of normal premenopausal breast tissue (Figure 3c). Diffuse cytoplasmic staining was present throughout all suprabasal layers of the cervical squamous epithelium, and squamous metaplastic tissue. Occasional cells ($< 10\%$) in normal glandular epithelium of the endocervix were positive. In these cells staining was granular and confined to perinuclear regions of the cytoplasm. A majority of the cells present in two out of three benign ovarian tumours were stained with TH1.

Reactivity of Antibodies with Malignant Tumours by Immunoperoxidase Staining

Expression of the epitope defined by TH1 in malignant tumours is summarised in Table 3. The majority of carcinomas from all sites tested were positive with TH1. Furthermore, in most cases, the epitope was detected in $> 75\%$ of tumour cells present in each specimen. In general, the staining was of a similar intensity and cellular distribution as noted in non-malignant tissue. However, in poorly differentiated tumour cells granular cytoplasmic staining was not restricted to a subcellular compartment as was the case in non-malignant epithelium. Secretory vacuoles present in well differentiated adenocarcinoma cells were always negative (Figure 3d). Neither membrane or extracellular antigen was detected.

DISCUSSION

Distinctive features of the mucin genes isolated are the size of the molecules (up to 1
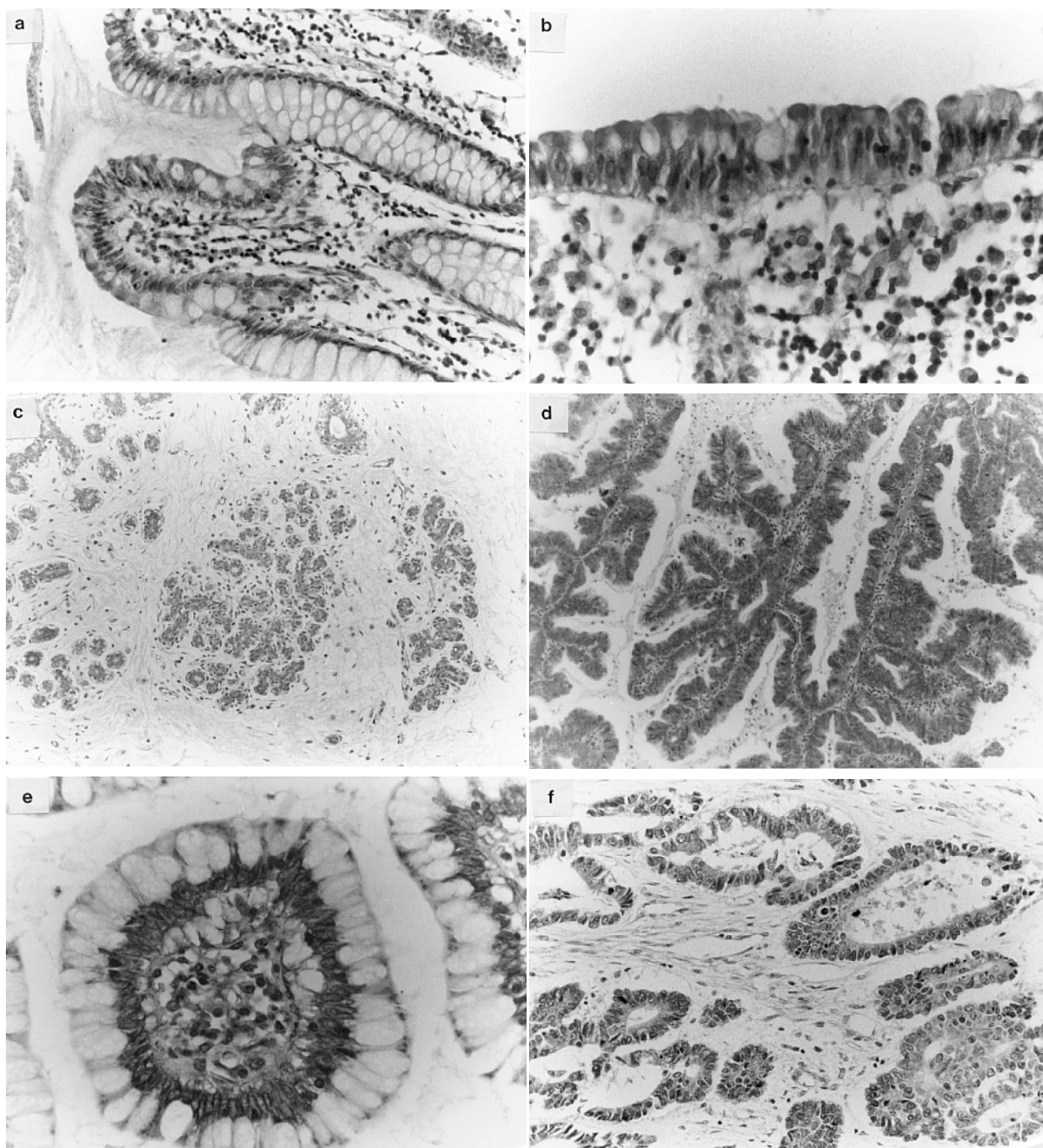
Fig. 3. Immunohistochemical staining with TH1. (a) Normal colonic epithelium stained with TH1. Only the surface epithelial cells are stained (original magnification x200); (b) Surface colonic epithelial cells at higher power. Cytoplasmic staining is present, however secretory vacuoles are negative (original magnification x400); (c) Normal breast epithelium stained with TH1 (original magnification x100); (d) Mucinous cystadenocarcinoma of the ovary stained with TH1. All tumour cells show cytoplasmic staining (original magnification x200); (e) High-power view of mucinous cystadenocarcinoma of the ovary with prominent cytoplasmic vacuolation. TH1 staining is limited to cytoplasmic areas surrounding secretory vacuoles (original magnification x400); (f) Colonic adenocarcinoma showing diffuse cytoplasmic staining with TH1 (original magnification x200).

million daltons) and the variable number of tandem repeats (VNTR) that contains numerous O-linked glycosylation sites [8]. These molecules have been reported to have roles in bacterial binding, protection of epithelial cells and the elimination of toxins and pathogens [8]. Changes in expression and glycosylation have also been shown to have important roles in tumour cells where overexpression of otherwise cryptic epitopes has been observed [4,19,40]. In this study, a monoclonal antibody (TH1) recognising a novel core epitope on mucin is described.

TH1 reacted strongly with TFMSA-treated cystic fibrosis mucin but showed no reactivity with the untreated mucin, indicating a specificity for a core mucin epitope. TH1 was suspected to react with the Tn-antigen (GalNAcα1-O-Ser/Thr), since it showed no reactivity with OSM [more than 98% of glycans as sialyl-Tn (NeuAcα2,6GalNAcα1-O-Ser/Thr)] [26] and strong reactivity with desialylated OSM. Similarly, desialylation of BSM (53% of glycans as sialyl Tn) [45] led to reactivity with TH1 in the inhibition ELISA. However, TH1 binding to DR-mucin was not inhibited by GalNAc at 2.5 mg/ml (11.3 mM), and the antibody showed no

reactivity with Tn-positive red blood cells (RBC). This is in contrast to other Tn-reactive antibodies (10F4, 15D3a, and 91S8) that have been reported to be inhibited by GalNAc at 8.3–14.3 mM (50% inhibition) [23]. These differences may be explained by TH1 binding being dependent on the conformation of the peptide to which the GalNAc is attached, as reported using mab MLS128 which reacts to GalNAc attached to a cluster structure of Ser-Thr-Thr [32]. However, unlike TH1, MLS128 agglutinated Tn erythrocytes [34].

Homology between non-VNTR regions of human MUC2, human MUC5 and bovine and porcine submaxillary mucins has been reported [18,30]. A mucin preparation similar to that used for the preparation of TH1 has been shown to contain high amounts of the MUC5AC mucin [44], and the DR-mucin used in this study reacted with MUC4 and MUC5 specific antisera but not with the 4F1 mab specific for the MUC2 mucin. Sandwich ELISA showed that TH1 reactivity was associated primarily with a mucin other than MUC1, since the ovarian tumour mucins were detected strongly in the assay using MUC1 core-protein specific mabs (BC2-BC3) but not detected in the BC2-TH1, TH1-BC3 or TH1-TH1

Table 3
Immunohistochemical staining for TH1 in tumour tissues

Tumour type and Site	Negative	+	++	+++	++++	Total +ve/Total tested
Adenocarcinoma						
Colon	2	3	2	1	4	10/12
Lung	1	0	0	0	11	11/12
Stomach	2	1	0	1	6	8/10
Ovary						
mucinous	0	0	0	5	1	6/6
non-mucinous	3	1	1	1	2	5/8
benign	1	0	0	0	2	2/3
Breast						
ductal	2	0	1	0	4	5/7
lobular	1	0	1	0	2	3/4
Cervix	0	0	0	3	4	7/7
Squamous cell carcinoma						
Lung	0	0	3	0	5	8/8
Cervix	4	0	1	3	1	5/9

[a] + = 1–25% of tumour cells positive; ++ = 26–50% positive; +++ = 51–75% positive; ++++ = 76–100% positive.

ELISAs. Similarly, DR-mucin was detected strongly in the TH1-TH1 ELISA, but showed no reactivity in the BC2-TH1 (MUC1 capture ELISA). Furthermore, the spectrum of tissue reactivity was different to that reported for MUC1-reactive mabs such as BC2. TH1 Reacted strongly with gastrointestinal and mucinous tumours, while BC2 has been reported to show stronger reactivity with non-mucinous ovarian tumours and breast tumours [29].

The observations from immunohistochemical staining of both normal and malignant tissues are consistent with TH1 recognizing a core epitope on mucin. Cellular distribution in normal gastro-intestinal and respiratory tissues was similar to that described for antigens recognized by polyclonal antibodies prepared against deglycosylated LS174T mucin [48] and deglycosylated sputum from a cystic fibrosis patient [36]. The peri-nuclear granular staining probably represents detection of the protein core in the endoplasmic reticulum prior to the completion of glycosylation. The lack of reactivity with mucin droplets of goblet cells and luminal secretions suggests low reactivity with the mature mucin in normal cells. The observation that not all cells of a given type showed equal expression of either epitope is consistent with the findings of other studies utilising various antibodies to colonic and/or respiratory mucins [13,36,37,48]. These findings suggest either phenotypic or stage dependent maturational differences may exist in mucin production patterns of morphologically similar cells.

Detection of the TH1 epitope in colonic, gastric, lung and ovarian cancers demonstrates continued mucin production by at least some carcinomas derived from these organs. The expression of TH1 in some malignancies was greater than the expression by corresponding non-malignant cells. Increased expression in cancers could reflect either increased production and/or neoglycosylation of mucin. The loss of compartmentalization of granular cytoplasmic staining is consistent with the loss of polarity that occurs following malignant transformation of

epithelial cells. Similarly, the higher expression in mucinous than non-mucinous ovarian carcinomas is consistent with the phenotypes of these tumours.

Elevated Tn-antigen expression has been reported previously in a variety of carcinomas [20,24,25,27,42]. The specificity of this antibody for Tn expressed on specific mucin core proteins should make it a useful reagent for the immunohistochemical detection of this epitope in epithelial cancers and normal cells.

Acknowledgments

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