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Natural human anti-Galα(1,3)Gal antibodies react with human mucin peptides

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We have recently demonstrated that both antibodies to Galα(1,3)Gal, and the Galα(1,3)Gal binding lectin (IB4), bind a synthetic peptide (DAHWESWL), there being a similar recognition of carbohydrate and peptide structures. We now report that the anti-Galα(1,3)Gal antibodies and IB4 lectin also react with peptides encoded by mucin genes (MUC 1, 3, 4)-sequences known to be rich in serine, threonine and proline. This activity was demonstrated (1) by the ability of mucin derived peptides to block the reaction of anti-Galα(1,3)Gal antibodies and IB4 lectin with a Galα(1,3)Gal$^+$ pig endothelial cell line; the reactions were specific and did not occur with a random peptide containing the same sequences or with other mucin peptides; (2) by the fact that anti-mucin1 antibodies could react with the Galα(1,3)Gal expressed after transfection of COS cells (Galα(1,3)Gal$^-$, Muc1$^-$) with cDNA encoding the pig α,3galactosyltransferase; and (3) that the IB4 lectin and anti-Galα(1,3)Gal antibodies could react with mucin 1 found on the surface of human breast cancer cells. Thus natural occurring anti-Galα(1,3)Gal antibodies found in all human serum can react with self (Muc1) peptides expressed in large amounts on the surface of tumour cells but not on normal cells. The findings are of interest and serve to explain the previously reported findings that human cells can, at times, express Galα(1,3)Gal; such expression is an artefact, the reaction is due to the phenomenon described herein, i.e. that anti-Galα(1,3)Gal antibodies react with mucin peptides.

***Keywords*: natural antibodies, Galα(1,3)Gal, mucins, MUC1, peptides**

***Abbreviations*: HPLC, high performance liquid phase chromatography; HRP, horse radish peroxidase; mAb, monoclonal antibody; NHS, normal human serum; PBS, phosphate buffered saline; VNTR, variable number of tandem repeats**

Introduction

Recently, there have been two sets of observations exciting interest in human biology – one in cancer, the other in transplantation. In the first, much attention has been drawn to cancer mucins – wherein there is a major increase in the amount of mucins present particularly mucin 1 (MUC1) [1, 2], the finding of these on the tumour cell surface but not on normal cells [3, 4] and alteration in the pattern of glycosylation of the mucins, leading to the exposure of the protein core [5]; thus, monoclonal antibodies reacting with the protein core appear to be tumour specific. Studies of mucins have advanced rapidly and the cloning of the cDNAs encoding the protein core have defined seven different mucin genes (MUC1-7) [6]. All mucins described thus far have in the extracellular portion of the molecule, a region consisting of a variable number of tandem repeats (VNTR) with a high content of the amino acids serine, threonine and proline [6–8]. There are immunodominant epitopes of mucins, for example, the immunogenic region of MUC1 consists of the amino acids APDTR, and many murine monoclonal antibodies (mAbs) to either cancer or natural milk mucin react with this sequence [9, 10]. Of interest is the finding that the APDTR sequence stimulates the induction of non-MHC restricted cytotoxic T cells in humans [11] – the basis for the current immunization of humans with APDTR containing peptides.

A second and apparently unrelated observation is in the field of transplantation, particularly the concept of xeno-transplantation of pig organs to humans, wherein natural human IgM and IgG antibodies reacting with pig cells would give rise to antibody mediated hyperacute rejection of pig-to-human xenografts. A recent observation is that the majority (if not all) of these antibodies react with the Galα(1,3)Gal epitope present on glycosylated surface molecules of the pig, but absent in humans [12–14]. Pigs synthesize this carbohydrate epitope by the α(1,3)galactosyltransferase utilizing N-acetyl lactosamine as substrate [14]. Higher primates (human and Old World monkeys) do not make Galα(1,3)Gal as they lack a functional α(1,3)galactosyltransferase [12] due to the presence of multiple in-phase stop codons, insertions and deletions, leading to frame shifts within the α(1,3)galactosyltransferase genes, resulting in non-functional pseudogenes [15, 16]. All humans have natural antibodies to Galα(1,3)Gal [13, 14, 17] – presumably due to exposure and immunization with bacteria

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which carry α-linked Gal as part of the lipopolysaccharide. Notwithstanding the functional malformations in the human α(1,3)galactosyltransferase gene rendering it a pseudogene, it has been proposed that the α(1,3)galactosyltransferase gene can become functional in humans in certain cancer and auto-immune diseases, as anti-Galα(1,3)Gal antibodies have been found to react with breast cancer cell lines [18–20] and proteins which are targets of autoimmune antibodies [18]. We consider these reports to be artefactual, as will be described.

We recently reported that the IB4 lectin and anti-Galα(1,3)Gal antibodies could react with the peptide sequence DAHWESWL [21]. In the course of these studies, we found that IB4 and anti-Galα(1,3)Gal antibodies could react with other peptides – indeed those encoded by mucin genes, and we now show that MUC1 (and MUC3 and MUC4) peptides can structurally mimic the Galα(1,3)Gal carbohydrate epitope, leading to a cross reaction between naturally occurring anti-Galα(1,3)Gal antibodies and mucins of tumours. These studies have implications in xenotransplantation and cancer biology, and provide an explanation for the reaction of anti-Galα(1,3)Gal antibodies with human tissues.

Materials and methods

Cells

The pig EC cell line (PIEC) was obtained from Dr Ken Welsh (Churchill Hospital, Oxford, UK). COS cells were maintained in Dulbecco's modified Eagles Medium (DMEM) (Cytosystems Pty. Ltd., Castle Hill, NSW, Australia).

Antibodies, lectin and saccharides

Normal human serum (NHS) was obtained from ten healthy volunteers, pooled and heat inactivated at 56 °C for 30 min before use. Purified human anti-Galα(1,3)Gal IgG antibodies were isolated from NHS by absorption and elution of IgG on a Protein G Sepharose column (Pharmacia LKB Biotechnology, Sweden), followed by affinity chromatography on Galα(1,3)Galβ(1,4)GlcNAc coated glass beads (Syntesome, NJ). FITC conjugated sheep anti-human IgM, sheep anti-mouse IgG and horse radish peroxidase (HRP) conjugated sheep anti-human IgM or IgG were obtained from Silenus Laboratories Pty. Ltd., Australia. HRP conjugated streptavidin and anti-human Ig were obtained from Amersham International, UK. The BC2 mAb , which recognizes the APDTR epitope of human MUC1, is described elsewhere [10, 22]. The IB4 lectin from *Griffonia simplicifolia*, which binds to Galα(1,3)Gal [23], was obtained from Sigma, USA and was labelled with FITC or biotin [24, 25]. Melibiose (Galα(1,6)Glc) and glucose (Sigma) were $>99\%$ pure. The Galα(1,3)Gal disaccharide, either free or coupled to BSA, was obtained from Dextra Laboratories, UK.

Peptide synthesis

Peptides (Table 1) were synthesized using an Applied Biosystems Model 430A automated synthesizer (Applied Biosystems, USA), based on the standard Merrifield solid phase synthesis method [26]. All reagents for synthesis were purchased from Applied Biosystems. Crude peptides were purified by reversed phase high performance liquid phase chromatography (HPLC) (Waters Associates, USA) on a C-8-Aquapore RP-300 column using a gradient solvent system of 0.1% aqueous trifluoroacetic acid/acteonitrile. The purity of synthetic peptides was $>90\%$ as judged by HPLC analysis. Peptides were dissolved in phosphate buffered saline (PBS) prior to use in the serological assays.

Serological assays

Peptides or carbohydrates were examined cytofluorographically for their ability to inhibit antibody/lectin binding to the cell surface using a FACSCAN flow cytometer (Becton Dickinson, USA) [21]: 25 μl of inhibitor was mixed with 25 μl of antibody or lectin at the appropriate concentration and incubated at 22 °C for 3 h (peptides) or at 4 °C for 16 h (carbohydrates) [25], prior to addition of appropriate targets. An Elisa assay [21], using Galα(1,3)Gal-BSA coated plates, was used to calculate the relative affinity of the sugars or the peptides for both IB4 lectin and antibodies, and were defined as the molar concentration of sugar or the peptide giving 50% inhibition of the maximal binding of lectin or antibody.

Transfection studies

COS cells transfection experiments were performed using DEAE/Dextran [27] and cDNA clones encoding human MUC1 [28], or the porcine α(1,3)galactosyltransferase [29] As additional controls, COS cells were also transfected with the vector lacking an insert (mock transfections) or a cDNA clone encoding human CD48 [30]. Indirect immunofluorescence was performed on cell monolayers in 6 well tissue culture plates using fluoresceinated IB4 lectin (which binds only to Galα(1,3)Gal), or anti-MUC1 monoclonal antibodies 3E1.2 [4] and VA1 [31] or anti-CD48 mAb [30] and immunopurified sheep anti-mouse IgM or IgG to detect antibody binding.

Histological analysis

Fresh or formalin fixed human tissue was incubated with biotinylated IB4 lectin (at 100 $\mu g\,ml^{-1}$) for 40 min at 22 °C, washed, incubated with streptavidin-HRP for 1 h, followed by diaminobenzidine (Amersham International, UK) at 1.5 $mg\,ml^{-1}$ with 0.05% H_2O_2 for 5 min, prior to the removal of excess substrate by washing in running tap water for 3 min. The sections were counterstained with Haematoxylin, mounted and examined microscopically. Tissues staining was graded independently by two investigators and scored as: 0 (no staining) to 4 (very strong staining). Fresh or

Table 1. Sequences of peptides used in this study.

Peptide	*Sequence*	*Description*	*Residues*
Gal pep1	DAHWESWL		
CD48 pep1	YTFDQKIVEWDSRKSKC	Human CD48	
CD4 pep1	TECKHKKGKVVSGSKVLSY	Murine CD4	133–149
FcR pep1	RYHHYSSNFSIPKANHSHSGDYYCKGSL	MURINE FCRIIβ_2	144–157
TNF pep1	LSGVRFSAARTAHPLPQKH	MURINE TNFβ	
Muc pep1	CPAHGVTSAPDTRPAPGSTAP	Human Muc1 VNTR	13–32
Muc pep2	PAHGVTSAPDTRPAPGSTAP	Human Muc1 VNTR	13–32
Muc pep3	PDTRAPGSTAPPAHGVTSAPDTR	Human Muc1 VNTR	1–24
Muc pep4	APDTRPAPGSTAPPAH	Human Muc1 VNTR	A–1–15
Muc pep5	PAPGSTAPPAHGVTSA	Human Muc1 VNTR	5–20
Muc pep6	TGSGHASSTPGGEKETSAQRSSVP	Human Muc1 NH2 to VNTR	31–35
Muc pep7	RSSVPSSTEKNAVSMTSSVL	Human Muc1 NH2 to VNTR	51–70
Muc pep8	TGFNQYKTEAASRVNL	Human Muc1 COOH to VNTR	408–423
Muc pep9	KYPTTTPISTTTMVTPTPTPTGTQTPTTT	Human Muc2 VNTR	
Muc pep10	CHSTPSFTSSITTTETTS	Human Muc3 VNTR	
Muc pep11	TSSASTGHATPLPVTP	Human Muc4 VNTR	
Scram pep1	WEADLHWS	Scrambled Gal pep1	

formalin fixed human tissue was also incubated with purified human anti-Galα(1,3)Gal IgG antibodies or human serum depleted of anti-Galα(1,3)Gal antibodies, as above and detected with anti-human Ig-HRP.

Results

Our previous studies had shown that: (1) natural human antibodies of the IgM and IgG classes bound to a single carbohydrate epitope in the pig, Galα(1,3)Gal [13, 14, 25, 29]; IB4 lectin binds the same epitope [23]; (2) the antibodies and IB4 lectin could also bind to the synthetic peptide DAHWESWL [21]. During these studies and testing the specificity of peptide binding, it was apparent that the anti-Galα(1,3)Gal reagents (antibodies or IB4 lectin) could also bind to other peptides – notably those of the protein core of several mucins as described herein.

Blocking IB4 binding to Galα(1,3)Gal expressing pig cells by mucin derived peptides

Muc pep1, derived from the MUC1 VNTR, could block the binding of IB4 lectin to the cell surface of PIEC cells (Fig. 1); there was almost complete inhibition of binding in the range of 10–0.125 mM – indeed, the inhibition by the MUC1 VNTR peptide was greater than that found with the previously identified IB4 lectin binding peptide, Gal pep1 (Fig. 1a). When other peptides derived from the VNTR region of MUC 1 were examined (see Table 1 for sequences), complete inhibition (at 5 mM) was observed with Muc pep2 and Muc pep3 and partial inhibition with Muc pep4 and Muc pep5 (Fig. 1b). In addition Muc pep6, derived from sequence amino terminal to the VNTR, showed partial inhibition; by contrast Muc pep7, derived from the same region, did not (Fig. 1b). Muc pep8, derived from sequence carboxy terminal to the VNTR, also inhibited IB4 binding to PIEC cells (Fig. 1b). Peptides derived from the VNTR regions of other human mucin molecules showed variable results: Muc pep9, derived from the VNTR region of MUC 2, did not inhibit binding, whereas both Muc pep10 and Muc pep11, derived from the VNTR region of MUC3 and MUC 4 respectively, inhibited IB4 binding (Fig. 1b). The same trend of inhibition was noted when peptides were used at 10 mM and 2.5 mM (data not shown). Thus, peptides derived from the VNTR and elsewhere in MUC1 can inhibit the interaction of IB4 with its ligand, Galα(1,3)Gal. It should be noted that these peptides have been used to produce anti-mucin mAbs which specifically recognize the MUC1 peptide and there were no cross-reactions of the antibodies with other MUC peptides, although the sequences are similar [10].

Inhibition of binding of IB4 and anti-Galα(1,3)Gal antibodies to Galα(1,3)Gal by mucin peptides

Mucin peptides were also examined for their ability to block the binding of IB4 lectin and anti-Galα(1,3)Gal IgG to the oligosaccharide epitope Galα(1,3)Gal-BSA bound in a microplate using an Elisa test (Fig. 2). At a peptide concentration of 5 mM, complete inhibition of binding of IB4 was observed with peptides Muc pep1, Muc pep3, Muc pep4, Muc pep5, Muc pep6 and Muc pep11 (Fig. 2a). By contrast Muc pep7 and Muc pep9 gave no inhibition of binding. Gal pep1 inhibited as previously described [21]; the control peptides CD48 pep1, CD4 pep1 and TNF pep1 did not inhibit IB4 binding (Fig. 2a).

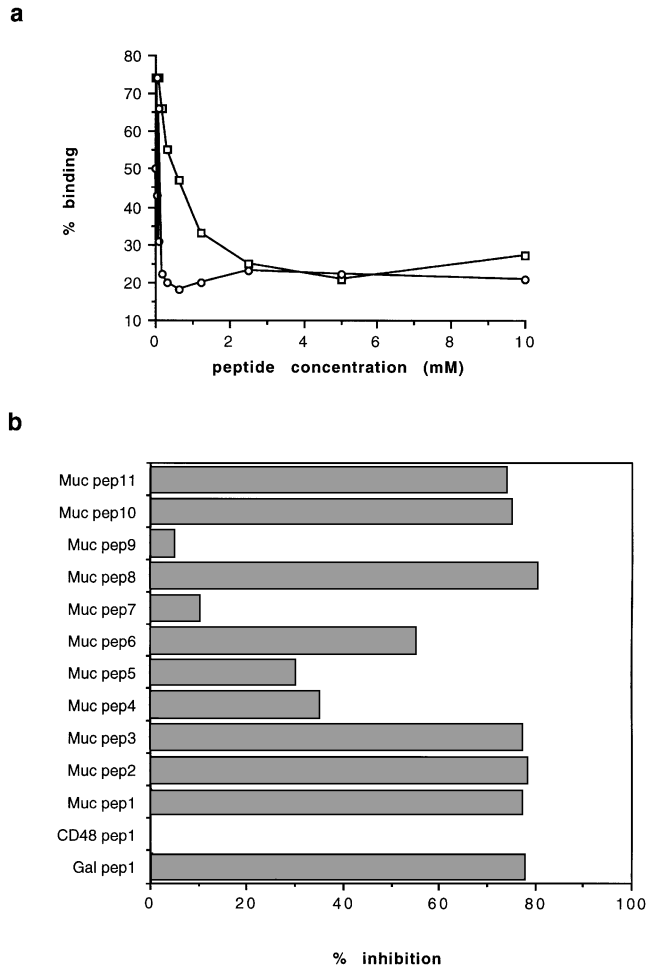

Figure 1. Peptide inhibition of IB4 lectin binding to pig endothelial cells. (a) Varying concentrations of peptides (shown in mM on horizontal axis) were examined for ability to inhibit the binding of IB4 lectin (1 μg ml^{-1}) to the PIEC cells using cytofluorographic analysis: Gal pep1 (□), Muc pep1 (○). (b) All peptides used at 5 mM, using cytofluorographic analysis. Inhibition calculated relative to no peptide control.

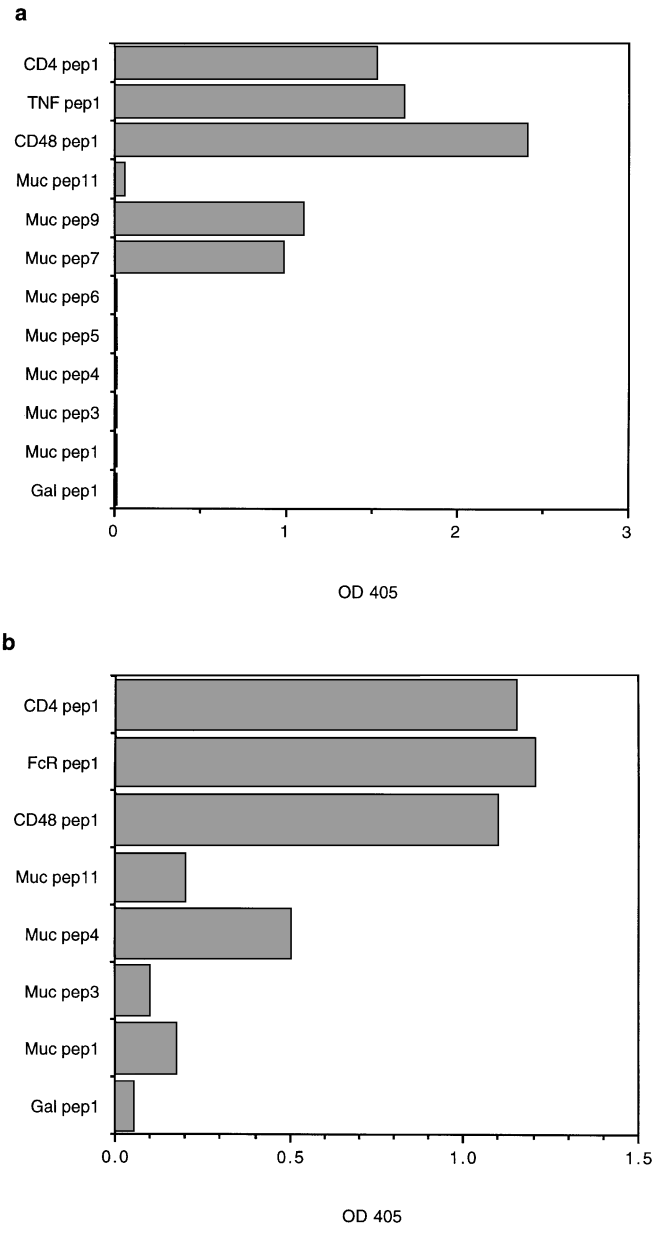

Figure 2. Inhibition of binding of IB4 lectin and anti-Gal*α*(1,3)Gal IgG to Gal*α*(1,3)Gal-BSA by mucin peptides. Peptides at 5mM were examined in an Elisa for inhibition of IB4 (16 μg ml^{-1}) (a) or anti-Gal*α*(1,3)Gal IgG antibodies (75 μg ml^{-1}) (b). Vertical axis shows peptides used, horizontal axis shows OD at 405 nm.

In accord with the inhibition results obtained previously with human antibodies [21], Gal pep1 inhibited the binding of the purified human anti-Galα(1,3)Gal IgG antibody (Fig. 2b). Three peptides tested from the VNTR region of human MUC: Muc pep1, Muc pep3 and Muc pep11 also inhibited the binding of anti-Galα(1,3)Gal IgG (Fig. 2b), with Muc pep4 showing partial inhibition. The three control peptides (CD48 pep1, CD4 pep1 and FcR pep1) had no effect (Fig. 2b).

Relative affinity of mucin peptides for Galα(1,3)Gal binding proteins

Elisa results were used to calculate the relative affinity of the mucin peptides for the IB4 lectin and for human anti-Galα(1,3)Gal antibodies, and to compare these with the affinity for α-galactosyl sugars. Biotinylated IB4 lectin and purified anti-Galα(1,3)Gal IgG antibodies were titred and for the affinity experiments were used at four times the concentration required to give 50% of the maximum OD_{405} reading; the concentration of peptide or sugar which inhibited the binding of antibody or lectin by 50% was calculated as a measurement of relative affinity (Table 2). The studies showed that 0.300 mM Gal pep 1 inhibited the binding of IB4 compared with < 0.030 mM Galα(1,3)Gal; 0.078 mM Muc pep1, 0.625 mM Muc pep3, 0.975 mM Muc pep4, 2.500 mM Muc pep5, 1.250 mM Muc pep6 and 0.156 mM Muc

Table 2. Relative affinity of peptides and sugars for IB4 lectin and pure anti-Gal*α*(1,3)Gal IgG.

Inhibitor	I^{a}_{50} *(mM)*	
	IB4[b]	*Anti-Gal*α(1,3)*Gal*[c]
Gal*α*(1,3)Gal	0.015	0.030
Glucose	> 20	> 20
Gal pep	0.300	10.000
CD48 pep1	> 20	> 20
Muc pep1	0.078	2.500
Muc pep3	0.625	3.750
Muc pep4	0.975	5.000
Muc pep5	2.500	ND[d]
Muc pep6	1.250	ND
Muc pep11	0.156	2.500
Scram pep1	> 20	> 20

[a] I_{50} is the concentration of peptide or sugar required to give 50% inhibition of antibody or lectin binding.
[b] Biotinylated IB4 lectin used at a final concentration of 6.25 $\mu g s ml^{-1}$.
[c] Anti-Gal*α*(1,3)Gal antibody (IgG) at a final concentration of 87 $\mu mgs ml^{-1}$.
[d] ND = not determined.

pep11 inhibited the binding of IB4. 0.030 mM Galα(1,3)Gal inhibited the binding of the anti-Galα(1,3)Gal IgG compared with 2.500 mM Muc pep1, 3.750 mM Muc pep3, 5.000 mM Muc pep4 and 2.500 mM Muc pep11 (Table 2). All the mucin peptides inhibited at a lower concentration than Gal pep1 (10.000 mM). Neither the CD48 pep1, Scram pep1 (a random peptide of Gal pep1 sequence), nor glucose had any effect on the binding of antibody or lectin. The differences obtained between the Elisa and the inhibition using cytofluorographic analysis is due to the differences in sensitivity of these two techniques.

Binding of IB4 lectin to COS cells expressing MUC1 on the cell surface

We have previously shown that COS cells, derived from Old World monkeys, do not react with the IB4 lectin (ie. they do not express Galα(1,3)Gal), and that the Galα(1,3)Gal epitope can be expressed on the surface of these cells only after transfection with the porcine or mouse α(1,3)galactosyltransferase cDNA clones [25, 29]. To examine whether expression of human MUC1 leads to IB4 binding, transfection of COS cells with the full length human MUC1 cDNA clone was performed. MUC1 could be detected on the COS cell surface by binding of the anti-MUC1 mAbs (Fig. 3a, b, Table 3); these cells were also reactive with the IB4 lectin (Fig. 3c, Table 3). Thus native MUC1 polypeptide encodes amino acid sequences to which IB4 lectin can bind. When COS cells transfected with the porcine α(1,3)galactosyltransferase cDNA clone were examined for anti-MUC1 mAb binding, weak staining was observed (Table 3). In contrast, no cell surface binding of COS cells was observed,

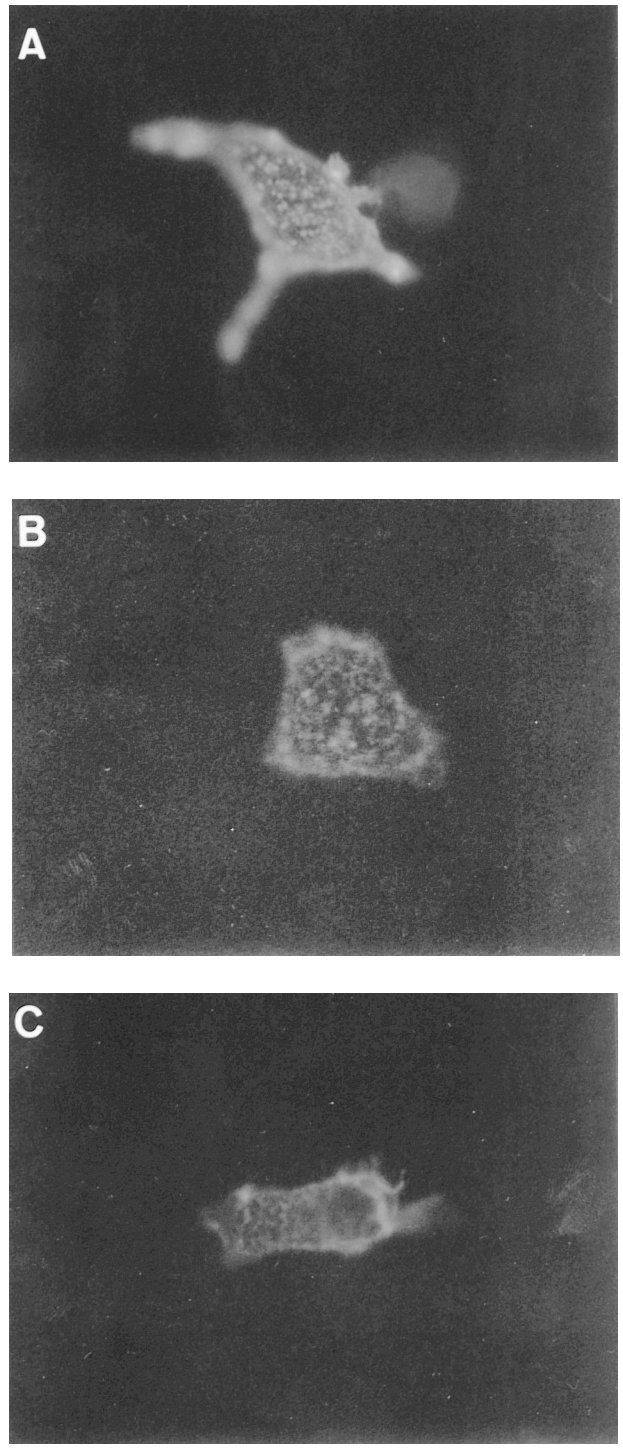

Figure 3. Cell surface staining of COS cell transfected with MUC1 cDNA clone. (A) stained with VA1 mAb. (B) stained with 3E1.2 mAb. (C) stained with IB4 lectin.

after transfection with either CD48 or vector and using the IB4 lectin or the anti-MUC1 mAbs (Table 3). COS cells transfected with the α(1,3)galactosyltransferase cDNA and particularly with the MUC1 cDNA clone, also reacted with natural human antibodies (Table 3).

Table 3. Cell surface staining of transfected COS cells.

Reagents	*COS cell transfected with cDNA encoding*			
	α1,3galactosyl-transferase	*MUC1*	*CD48*	*mock*[1]
IB4 lectin	++[b]	+	−	+
VA1		++	−	−
3E1.2	+	++	−	−
CD48	−	−	++	−
SAMG[c]	−	−	−	−
HuIg	+	+	−	−
SAHG[d]	−	−	−	−

[a] Mock transfected COS cells were transfected with vector alone.
[b] ++ = strong cell surface staining; + = weak cell surface staining, − = no cell surface staining.
[c] SAMG = sheep anti-mouse Ig alone.
[d] SAHG = sheep anti-human Ig alone.

Histological analysis of IB4 reactivity of human tissue

As IB4 and anti-Galα(1,3)Gal antibodies bind to mucin peptides, histological studies with biotinylated IB4 lectin on human normal or malignant tissues were performed. Using formalin fixed tissue, none of the normal breast tissues were reactive with the IB4 lectin, compared with three of 13 breast cancers which were reactive; five formalin-fixed colon cancer samples did not bind IB4-biotin. The results with fresh tissue samples showed stronger binding of the IB4 lectin–0/3 normal breast tissue samples tested bound IB4 lectin, whereas 4/4 the breast cancer tissues were positive. With colon cancer tissues 3/7 were reactive, compared to 1/6 of the normal tissues. Representative samples of the histological analysis of human malignant tissues are shown (Fig. 4). The IB4 lectin was not reactive with formalin fixed normal breast tissue (Fig. 4a), in contrast reactivity was observed with infiltrating ductal breast carcinoma tissue (Figs. 4b, c), where both cytoplasmic staining (Fig. 4b) and cell surface staining of the lumen and glandular cells (Fig. 4c) was clearly seen. The binding of the IB4 lectin was also demonstrated on fresh frozen human malignant tissue. Poorly differentiated, infiltrating ductal breast carcinoma tissue showed strong cytoplasmic staining with IB4-biotin (Fig. 4d), whereas staining of secretions in the lumen of glandular cells can be seen in a moderately differentiated, infiltrating ductal breast carcinoma tissue (Fig. 4e). There is also strong staining of secretions and the cytoplasm of frozen sample of colon cancer tissue (Fig. 4f). There was no correlation seen between the blood group of the individual and reactivity with the lectin.

Similar results to those obtained with the IB4 lectin were also found with purified human anti-Galα(1,3)Gal antibodies (Fig 5a). Strong cytoplasmic and cell surface staining was noted in most samples – in those with luminal secretion these were also positive (Fig 5a). It is important to note that the staining was almost completely eliminated by absorbing the human serum with Galα(1,3)Gal beads so it no longer contained anti-Galα(1,3)Gal antibodies (Fig 5b). Both of these studies clearly indicate that the staining of breast cancer was due to anti-Galα(1,3)Gal antibodies.

Discussion

There is now convincing data that, at the molecular level, peptides can mimic carbohydrates and interact specifically with the carbohydrate binding regions of both lectins and anti-carbohydrate antibodies [21, 32–37]. In our previous studies, we were able to show that the peptide sequence (DAHWESWL) could specifically interact with the Galα(1,3)Gal binding lectin IB4 and immunopurified anti-Galα(1,3)Gal human antibodies [21]. In the course of these studies, we examined a number of peptides, and observed that certain peptides derived from human mucins also reacted with the Galα(1,3)Gal recognizing proteins (antibodies and IB4 lectin) and the data are presented herein.

It is clear that both IB4 and anti-Galα(1,3)Gal antibodies could react with several mucin peptides (Figs. 1 and 2), derived from the serine, threonine and proline rich regions of the VNTR of MUC1, 3 and 4, with inhibition also observed with other non-VNTR MUC1 peptides. However, the specificity of the interaction was shown by the lack of reaction with several other serine, threonine, and proline containing peptides, in particular, Muc pep 9, derived from the VNTR of MUC2. No primary amino acid consensus sequence was found, nor a possible secondary structure elucidated to explain the findings. We note the tendency of these peptides to form β turns [38], and conclude there may be a common secondary structure which leads to reactivity with the anti-Galα(1,3)Gal antibodies and IB4 lectin. In accord with this, are our findings that the peptides had to be used in the liquid phase, as variable results were found when the peptides were examined in the solid phase – such as bound to the wells of microtitre plates, or when one end was tethered on a pin (data not shown): thus, fine peptide mapping studies could not be done.

The interaction of Galα(1,3)Gal recognizing proteins with MUC1 was next examined using COS cell transfection experiments. COS cells have previously been shown not to bind the IB4 lectin, and only do so after transfection with the α1,3galactosyltransferase [25, 29]. After transfection with the MUC1 cDNA, MUC1$^+$COS cells were found to bind IB4 (Fig. 3), demonstrating that the intact MUC1 molecule has peptide sequences that can mimic the Galα(1,3)Gal epitope. Furthermore, α1,3galactosyl-transferase cDNA transfected COS cells were shown to bind anti-MUC1 antibodies (Table 3).

Finally, as the IB4 lectin can react with either mucin peptides or the whole MUC1 molecule, it might be expected that it could also detect such mucin peptides on the surface

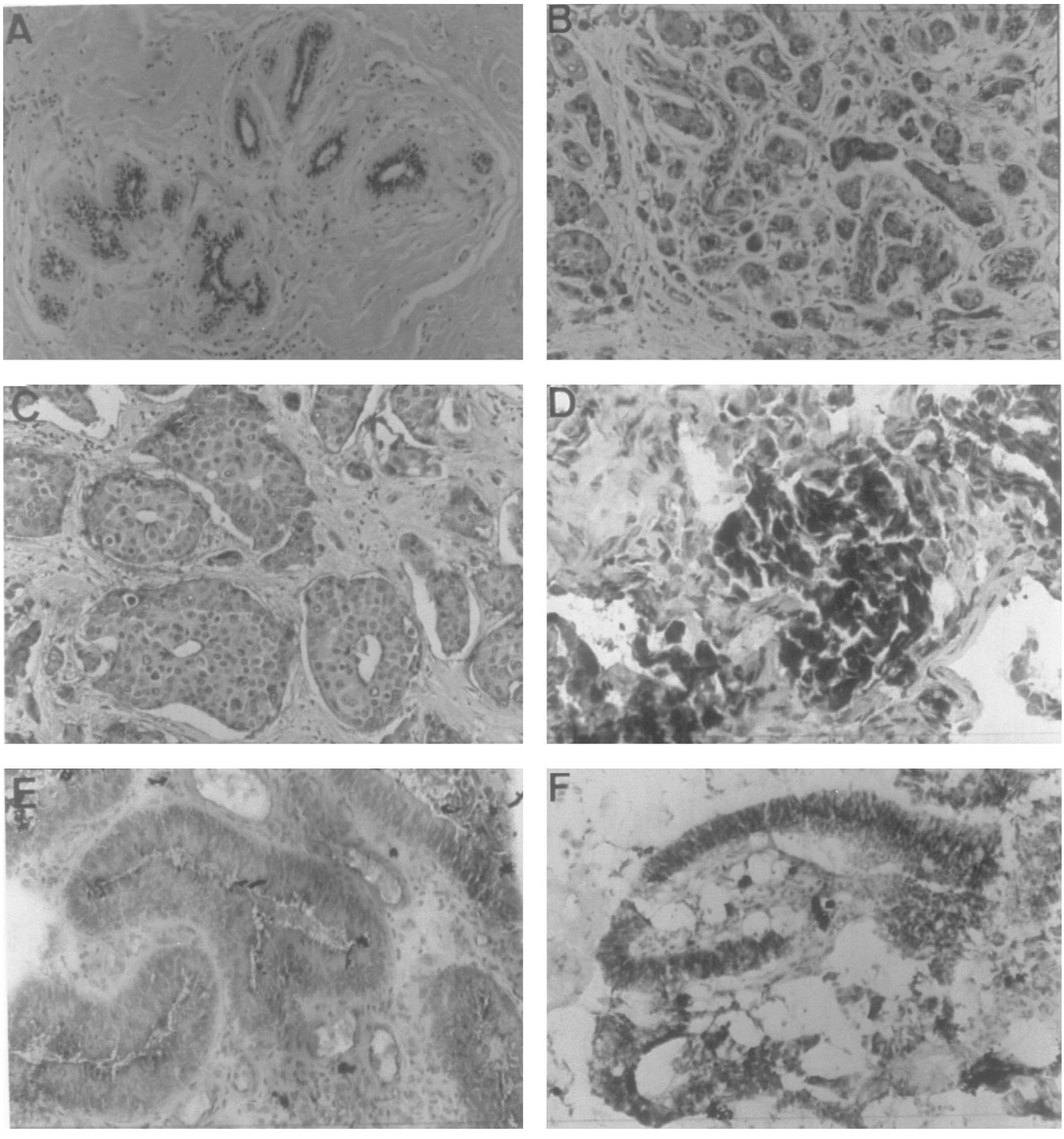

Figure 4. Histological analysis of malignant human tissue with IB4 lectin. Biotinylated IB4 lectin was used in an immunoperoxidase staining on formalin fixed and paraffin embedded tissue (A, B, C) or fresh frozen tissue (D, E, F). (A) normal breast. (B) moderately differentiated infiltrating breast carcinoma. (C) moderately differentiated infiltrating breast carcinoma. (D) poorly differentiated infiltrating breast carcinoma. (E) moderately differentiated infiltrating breast carcinoma. (F) adenocarcinoma of the colon. Original magnification × 200.

of tumour cells. We were able to demonstrate, in histological studies using an immunoperoxidase technique, that both the IB4 lectin and purified human anti-Galα(1,3)Gal antibodies are able to react with breast and colon cancer cells, but not with samples of normal tissue obtained from breast or colon (Figs. 4 and 5). The findings are in accord with the concept that in normal tissue the MUC1 protein core is masked by carbohydrate, only to become exposed

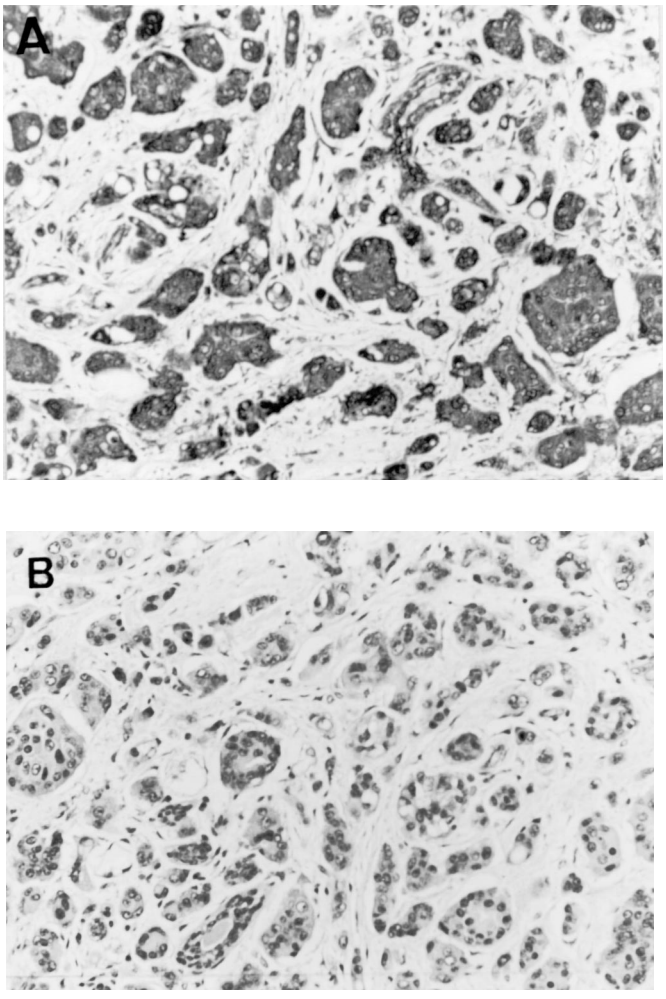

Figure 5. Histological analysis of malignant human tissue with anti-Galα(1,3)Gal antibodies. Human anti-Galα(1,3)Gal antibodies used in an immunoperoxidase staining on formalin fixed and paraffin embedded tissue (A), and tested with human serum depleted of anti-Galα(1,3)Gal antibodies.

in tumours due to defective or altered glycosylation [5]. Indeed a series of antibodies to MUC1 peptides have been described [39], which react with either peptide or deglycosylated mucin, but not with the fully glycosylated material – the IB4 lectin has the same effect as seen in the lack of reaction of the lectin with normal breast tissue, yet gave a strong reaction with malignant breast cancer cells (Fig. 4). Thus, human serum containing large amounts of soluble mucin (particularly in patients with adenocarcinomas) [1] would also contain anti-Galα(1,3)Gal antibodies. Whether this leads to immune complex formation, or could affect a potential immune response to mucins is unknown.

Our findings that Galα(1,3)Gal recognizing proteins react with mucins have implications for other studies. Castronova *et al.* [19] used cytofluorographic analysis with IB4 lectin and human anti-Galα(1,3)Gal antibodies to demonstrate the presence of Galα(1,3)Gal determinants on human carcinoma cell lines and on 50% of malignant breast carcinoma cells obtained by fine needle biopsy. Normal breast epithelial cells did not bind either to lectin or the antibodies [19], findings similar to those described here. In the same study, the presence of anti-Galα(1,3)Gal antibodies inhibited the attachment of malignant cells to human umbilical vein endothelium and also laminin, both of which are thought to be involved in the metastatic process, and suggested a possible role for anti-Galα(1,3)Gal antibodies in natural immunity to cancer [19]. In accord with these observations, Galili demonstrated that the human breast cancer cell line MCF-7 (which was shown to lack α1,3galactosyltransferase mRNA) was lysed by purified anti-Galα(1,3)Gal in an antibody-dependent cell-mediated cytolysis assay [18]. The explanation for these findings was that in the cancer cells, the human α(1,3)galactosyltransferase genes were 'derepressed' allowing for expression of the enzyme and synthesis of Galα(1,3)Gal. However, the lack of expression of the human α1,3galactosyltransferase genes is not because of gene repression mechanisms, but rather because of the presence of multiple mutations within the genes [15, 16]. It is difficult to see how these mutations could simultaneously revert to the wild type so regularly to allow for expression of the genes in the majority of cancer cells, particularly as no α1,3galactosyltransferase mRNA was present in these studies [18]. We consider a more likely explanation for the observations is that the anti-Galα(1,3)Gal antibodies directly cross react with deglycosylated mucins expressed on the cancer cells.

In addition to these observations, we note that the ability of the mucin peptides to inhibit the reaction of anti-Galα(1,3)Gal antibodies with porcine endothelial cell lines, suggested they could have some therapeutic benefit for the prevention of hyperacute rejection of pig organs by the antibodies. We are currently examining this possibility.

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