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Development and characterization of an antibody directed to an *α*-*N*-acetyl-D-galactosamine glycosylated MUC2 peptide

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In an attempt to raise anti-Tn antibodies, an *α*-*N*-acetyl-D-galactosamine glycosylated peptide based on the tandem repeat of the intestinal mucin MUC2 was used as an immunogen. The MUC2 peptide (PTTTPISTTTMVTPTPTPTC) was glycosylated *in vitro* using concentrated *α*-*N*-acetylgalactosaminyltransferases activity from porcine submaxillary glands which resulted in the incorporation of 8–9 mol of GalNAc. Rabbits and mice developed specific anti-MUC2-GalNAc glycopeptide antibodies and no detectable anti-Tn antibodies. Anti-glycopeptide antibodies did not show reactivity with the unglycosylated MUC2 peptide or with other GalNAc glycosylated peptides. A mouse monoclonal antibody (PMH1) representative of the observed immune response was generated and its immunohistological reactivity analysed in normal tissues. PMH1 reacted similarly to other anti-MUC2 peptide antibodies. However, in some cells the staining was not restricted to the supranuclear area but extended to the entire cytoplasm. In addition, PMH1 reacted with purified colonic mucin by Western blot analysis suggesting that PMH1 reacted with some glycoforms of MUC2. The present work presents a useful approach for development of anti-mucin antibodies directed to different glycoforms of individual mucins.

***Keywords*: Tn-antigen, MUC2, mucin, glycopeptide, carbohydrate antigen, *O*-glycosylation, monoclonal antibody**

Introduction

The simple mucin-type carbohydrate antigens Tn, sialyl-Tn, and T are regarded as general carcinoma-associated antigens [1] and a number of investigators are developing tumour vaccines based on these carbohydrate structures [2–7]. Simple mucin-type carbohydrate antigens are of particular interest because it appears that the cellular immune system may recognize these carbohydrates [1–3, 8, 9]. Tn and sialyl-Tn antigens are also found on the HIV envelope glycoprotein, gp120, and they constitute potent immunoneutralization epitopes which appear to be a general phenomenon for different HIV isolates [10]. Thus the Tn antigen is also considered as a potential target for HIV therapy and vaccines.

Development of a vaccine for the stimulation of anti-Tn antibodies has previously focused on using glycoprotein antigens such as glycosidase-treated glycophorin A and desialylated ovine submaxillary mucin (AOSM) or synthetic haptens such as Tn and T hapten neoglycoproteins [2–4, 6, 11]. Partially desialylated ovine submaxillary mucin was used as a vaccine in colorectal cancer patients showing that anti-Tn and sialyl-Tn antibodies can be stimulated [4].

The specificity of anti-Tn antibodies is generally believed to be restricted to the GalNAc-α1-O-Ser/Thr epitope with the monosaccharide GalNAc showing inhibitory effect [11, 12]. Nakada *et al.* [13, 14] have provided evidence indicating that some anti-Tn antibodies may require a cluster of GalNAc α1-O-Ser/Thr. In addition, three anti-Tn antibodies, raised against partially deglycosylated intestinal mucin peptides have shown different tissue reactivity [15]. Thus, some anti-Tn antibodies may require more complex epitopes than the GalNAc-Ser/Thr structure.

In order to stimulate Tn immunoreactivity it may be of advantage to use Tn-antigens that occur naturally such as glycopeptides derived from mucins. Mucins are high molecular weight glycoproteins characterized by a high degree of O-linked glycosylation [16]. The intestinal mucin, MUC2, contains a high degree of serine and threonine

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residues which are potentially *O*-glycosylated and therefore could be used as a Tn-carrier [17].

In the present study a synthetic peptide with amino acid sequence based on the intestinal mucin MUC2 tandem repeat was used as acceptor substrate for preparative *in vitro* *O*-glycosylation using UDP-GalNAc:polypeptide *N*-acetylgalactosaminyltransferase (GalNAc-transferase). The immune response to GalNAc-MUC2 glycopeptide was analysed in rabbits and mice and found to show specificity for the MUC2 glycopeptide and not to the peptide or to GalNAc alone. A mouse monoclonal antibody (PMH1) representative of the observed immune response was produced and its immunohistological reactivity analysed in human normal tissues. The present study presents a useful approach for development of anti-mucin antibodies directed to different glycoforms of individual mucins.

Materials and methods

Synthetic peptides

Synthetic peptides based on the tandem repeat sequence of MUC1 (APPAHGVTSAPDTRPAPGC [18]) and MUC2 (PTTTPISTTTMVTPTPTPTC [17]) were synthesized by Carlbiotech (Copenhagen, Denmark). The peptides were purified by high performance liquid chromatography (HPLC) and their structure confirmed by amino acid analysis and mass spectrometry (MS).

GalNAc-transferases

A concentrated GalNAc-transferase preparation was obtained from porcine submaxillary glands using Cibacron Blue 3GA (Sigma, St Louis, MO, USA) and S-Sepharose (Pharmacia, Uppsala, Sweden) chromatography as previously described [19]. Production and purification of recombinant human GalNAc-transferases: GalNAc-T1, -T2 and -T3 was performed as previously described [20, 21].

In vitro glycosylation

Terminal glycosylation of 1 mg of MUC2 peptide and the control MUC1 peptide was achieved with a reaction mixture consisting of 25 mM Tris-HCl (pH 7.2), 0.25% Triton X-100, 5 mM $MnCl_2$, 5 mM CDP-choline, 5 mM 2-β mercaptoethanol, 10 mM UDP-[^{14}C]-GalNAc (300 cpm nmol^{-1}), 0.5 mM peptide and 5 mU ml^{-1} of concentrated porcine submaxillary gland enzyme. The mixture was incubated overnight at 37 °C followed by purification with C-18 reverse phase chromatography using 0.1% trifluoroacetic acid in deionized water and a gradient from 0 to 80% acetonitrile.

Partial glycosylation of peptides using the recombinant enzymes GalNAc-T1, T2 and T3 was performed using 100 µg peptide and 0.5 mU ml^{-1} enzyme under the same conditions described above but incubated for 1 h. Purification of the different glycoforms of the glycopeptide was achieved by C-18 reverse phase HPLC under the conditions described above.

Structure determination of glycopeptides

Peptides were sequenced automatically by Edman degradation (Applied Biosystems 470) and the phenylthiohydantoin derivates were analysed by on line HPLC.

Matrix-assisted laser desorption/ionization mass spectrometry was performed as follows: samples were dissolved in 0.1% trifluoroacetic acid to a concentration of approximately 0.05 µg µl^{-1}. One µl of the sample solution was applied to a stainless steel probe tip precoated with 1 µ1 of matrix solution (α-cyano-4-hydroxycinnamic acid dissolved in acetone, 15 µg µl^{-1}) and washed thoroughly before introduction into the mass spectrometer. All mass spectra were obtained as previously described [19].

Immunochemical characterization of GalNAc-MUC2 glycopeptide

Enzyme linked immune sorbent assay (ELISA) was performed using Maxisorp plates (Nunc, Roskilde, Denmark) coated with peptides or peptide conjugates diluted in phosphate buffered saline (PBS), pH 7.2, overnight at 4 °C or 2 h at room temperature. Plates were blocked with 5% bovine serum albumin (BSA) in PBS followed by incubation for 1 h with monoclonal antibodies HB-Tn1 (anti-Tn) and HB-STn1 (anti Sialyl-Tn) (Dako, Glostrup, Denmark) both diluted 1:200 in PBS. Plates were then incubated for 1 h with peroxidase conjugated rabbit anti-mouse serum (Dako, Glostrup, Denmark) diluted 1:200 before development with 0.4 mg ml^{-1} of 1,2-phenylendiamin (OPD) tablets (Dako, Glostrup, Denmark) in 0.1 M citric acid-phosphate buffer (pH 5.0) using 1 µl of 30% H_2O_2 per ml of development solution. A peroxidase conjugated anti-GalNAc specific lectin – Vicia Villosa (VVA) (Sigma, St Louis, MO, USA) was also used diluted 1:1000 in PBS and developed as described above.

The inhibition ELISA was carried out similarly with the exception that the primary antibody was incubated with the inhibitor for 1 h before it was added to the coated and blocked ELISA plate.

Production of antibodies to GalNAc-MUC2 glycopeptide

The terminally glycosylated MUC2 peptide and the unglycosylated MUC2 peptide were coupled to Maleimide-activated keyhole limpet haemocyanin (KLH) through the C-terminal Cysteine (Pierce, Rockford, IL, USA) by mixing 1 mg of peptide with 1 mg activated KLH and incubating for 1 h at room temperature. The coupled peptides were dialysed overnight at 4 °C against PBS.

Rabbits were immunized with the KLH-coupled GalNAc-MUC2 glycopeptide. Control rabbits were immunized with the KLH-coupled unglycosylated MUC2 peptide.

Immunizations were performed with an interval of 3 weeks using 100 μg of immunogen mixed with Freund's adjuvant. Sera from rabbits were collected 10 days after each immunization.

Female 8 to 12 week old BALB/c mice were immunized with KLH-coupled GalNAc-MUC2 glycopeptide. Control mice were immunized with the KLH-coupled unglycosylated MUC2 peptide. Immunizations were performed intraperitoneally with an interval of 2 weeks using 50 μg of immunogen mixed with Freund's adjuvant. Blood samples from the mice were collected 10 days after the third immunization and sera tested by ELISA. Three days after the fourth immunization, spleen cells from one mouse were fused with NS1 myeloma cells. The screening strategy included the GalNAc-MUC2 peptide and the following controls: unglycosylated MUC2 peptide, asialo-ovine submaxillary mucin (AOSM) as well as the glycopeptide GalNAc-MUC1. One hybridoma PMH1 (IgM) specific to the GalNAC-MUC2 peptide was cloned by limiting dilution at least three times [22].

Chemical synthesis of a GalNAc-MUC2 peptide analogue (Syn-Tn-MUC2)

The glycopeptide syn-Tn-MUC2:

P(GalNAc)T(GalNAc)T(GalNAc)TPI(GalNAc)S(GalNAc)

T(GalNAc)TM

was synthesized using the solid phase strategy and then purified by reversed phase HPLC [23]. The structure of syn-Tn-MUC2 was confirmed by ^{1}H NMR and MS and by amino acid sequence analysis.

Purification, deglycosylation and immunochemical characterization of mucins

Colon cell line LS174T xenografts were a kind gift of Dr Francisco Real and Dr Carmen de Bolós (IMIM – Barcelona, Spain). The whole procedure was carried out at 4 °C. Samples from LS174T xenografts were homogenized in 10 mM Tris(hydroxymethyl)aminomethane (Tris) hydrochloride, pH 7.6, 150 mM NaCl containing 0.02% NaN_3 and 1 mM phenyl methyl sulfonyl fluoride, and centrifuged for 20 minutes at $7200 \times \mathbf{g}$ [24]. The pellet was rehomogenized and supernatants were combined. Supernatants were precipitated by adding perchloric acid to a concentration of 100 mM as previously described [25]. After centrifugation at $2000 \times \mathbf{g}$ the supernatants were neutralized with 1 M Tris to pH 7.0 and dialysed/concentrated against 100 mM Tris-HCl, pH 7.0, in a Microprodicon MPDC-20 using a 25 000 molecular weight cut-off membrane (Spectrum, Houston, USA). Concentrated samples were purified by gel filtration on either S-400 HR column or Superose 6 column (SMART system) (Pharmacia, Uppsala, Sweden). Void volume fraction was characterized immunochemically by ELISA using MAbs PMH1, HB-Tn1 and HB-STn1. Mucin extracts were enzymatically desialylated by incubation with neuraminidase from *Clostridium perfringens* type IV (Sigma, St Louis, MO, USA) diluted in 0.1 M sodium acetate buffer, pH 5.5, to a final concentration of 0.1 U ml^{-1}, for 2 h at 37 °C. The mucin was also deglycosylated by trifluoromethanesulfonic acid (TFMSA) as described by Sojar and Bahl [26].

SDS-PAGE and Western blot

SDS-PAGE was carried out using pre-casted 4–15% gradient polyacrylamide gels and blotting to nitrocellulose was performed with the Pharmacia Phastsystem (Pharmacia, Uppsals, Sweden) using a 25 mM Tris, 192 mM Glycine, 20% Methanol, pH 8.3, transfer buffer. The Blots were blocked in 5% BSA in Tris buffered saline (TBS), pH 7.6, followed by incubation for 1 h with PMH1, HB-Tn 1 and HB-STn1. After washing three times with TBS the membrane was incubated for 1 h with alkaline phosphatase conjugated rabbit anti-mouse serum (DAKO, Glostrup, Denmark) diluted 1:500 in TBS. The membrane was washed three times with TBS and developed with 10 ml Tris buffer (0.1 M, pH 9.5), 10 mM $MgCl_2$, 2.5 mg 5-bromo-4-chloro indoxyl phosphate in 0.1 ml *N*,*N*-dimethylformamide, 3 mg nitroblue tetrazolim (Sigma, St Louis, MO, USA) and 2.5 mg levamisole [27].

Immunohistology with MAb PMH1

Normal specimens were collected from patients with diseases not affecting the organs under study or from young healthy volunteers (normal labial salivary glands). The samples were immediately embedded in Tissue-Tek (Miles Scientific, Elkhart, IN, USA), quick-frozen in isopentane precooled on dry ice, and stored at −80 °C until use. Sections were cut at a thickness of 5 μm and mounted on gelatin coated slides followed by acetone fixation. Tissue samples were also fixed in formalin buffered with PBS and embedded in paraffin. Sections were cut at a thickness of 4 μm and mounted on gelatin coated slides.

Indirect immunofluorescence staining: samples from normal labial salivary glands, gastric normal mucosa and normal colonic mucosa were analysed. Frozen slides fixed in acetone were tested with MAbs PMH1 and 1E3 (anti-Tn; Clausen and Hakomori, unpublished) used as undiluted hybridoma supernatants. Incubation was performed overnight at 4 °C, washed three times for 5 min in PBS, pH 7.2, and incubated with fluorescein isothiocyanate-conjugated rabbit anti-mouse immunoglobulins (Code F-261, Dako, Glostrup, Denmark) for 1 h at room temperature. Slides were examined in a Zeiss fluorescence microscope using epiillumination. The microscope was equipped with FITC interference filters and a 75 W Zenon lamp. For control of the staining, primary antibody was replaced with PBS or Mabs of other specificities but with the same immunoglobulin isotype as the tested antibody.

Avidin biotin peroxidase staining: samples from lung, breast, uterus, ovary, kidney, liver, spleen, stomach (antrum and body and gastric mucosa with intestinal metaplasia), gallbladder, pancreas, small intestine and colon were also stained by the avidin biotin complex (ABC) method [28]. All paraffin sections were dewaxed. Sections designated for neuraminidase treatment were washed three times in PBS and incubated with neuraminidase from *Clostridium perfringens* type VI (Sigma, St Louis, MO, USA) diluted in 0.1 M sodium acetate buffer, pH 5.5, to a final concentration of 0.1 U ml^{-1}. Slides were incubated for 2 h at 37 °C followed by washing in cold water. All the sections were then treated with 0.5% H_2O_2 in methanol for 30 min followed by incubation for 20 min with normal non-immune serum to eliminate nonspecific staining. Sections were washed and incubated with MAbs PMH1 and 1E3 overnight at 4 °C, followed by incubation with a 1 : 200 diluted biotin-labelled secondary antibody for 30 min and with avidin-biotin-peroxidase complex for 1 h. Sections were developed with 0.05% 3,3′ diaminobenzidintetrahydrochloride freshly prepared in 0.05 M Tris-HCl buffer at pH 7.6 containing 0.1% H_2O_2, counterstained with haematoxylin, dehydrated and mounted. Normal non-immune serum and secondary antibody were diluted in TBS, pH 7.6, containing 0.1% BSA.

Results

Production and characterization of GalNAc-MUC2 glycopeptide

Mass spectrometry showed that 8 to 9 mol of GalNAc were incorporated per mole of MUC2 peptide using concentrated porcine submaxillary gland GalNAc-transferase activity. The sites of *O*-glycosylation were determined by amino acid sequencing comparing the unglycosylated peptide to the terminally glycosylated peptide (Figure 1). Glycosylated serine and threonine are defined by 'empty' cycles in the amino acid sequencing. However, in the present case, the uniform glycosylation, GalNAc-Ser/Thr, resulted in pseudo-peaks eluting earlier than the corresponding serine and threonine phenylthiohydantoin (PTH) amino acid derivatives [29]. Glycosylated PTH-threonine resulted in a peak corresponding to the mobility of PTH-glutamine and glycosylated serine resulted in an apparent PTH-aspartic acid. Although this analysis is complicated by significant carry-over between cycles, it indicated that most, if not all, serine and threonine sites in the peptide were used as acceptors. The two threonines, residues 3 and 4, and the single serine, residue 7, were the least-used sites. The low yield in the C-terminal end precludes quantification in this region.

Immunochemical characterization of GalNAc-MUC2 glycopeptide

Anti-Tn monoclonal antibody HB-Tn1 and the lectin VVA reacted with GalNAc glycosylated MUC2 peptide in ELISA showing that the glycopeptide was a potent Tn antigen (Figure 2). The control antibody directed to sialyl-Tn did not react with GalNAc-MUC2 peptide (not shown). The anti-Tn antibody reacted equally well with GalNAc-MUC2 glycopeptide coated directly to ELISA plates or when coupled through the carboxy-terminal cysteine group to maleimide activated KLH or BSA (not shown).

GalNAc-MUC2 glycopeptide as an immunogen and production of MAb PMH1

In order to evaluate if the GalNAc-MUC2 glycopeptide could elicit anti-Tn antibodies, rabbits and mice were immunized with the glycopeptide coupled to KLH. As illustrated in Figure 3, the KLH conjugated GalNAc-MUC2 glycopeptide was highly immunogenic and gave rise to antibodies specific for the glycopeptide, which did not react with the unglycosylated peptide in rabbits. Control rabbits immunized with unglycosylated peptide produced antibodies which did not react with the glycopeptide. The antisera from rabbits immunized with the GalNAc-MUC2 did not react with the control Tn antigen in AOSM.

Analysis of sera from immunized mice revealed the same immunoreactivity pattern as observed in rabbits (not shown). One hydbridoma, designated PMH1, producing anti-GalNAc-MUC2 IgM antibody, was cloned and characterized. MAb PMH1 reacted with the glycosylated GalNAc-MUC2 peptide but did not react with the unglycosylated peptide and AOSM (Figure 4A). MAb PMH1 also showed no reactivity with a different GalNAc-glycosylated glycopeptide (GalNAc-MUC1) (Figure 4B).

Characterization of PMH1 epitope

The lack of reactivity of PMH1 with the chemically synthesized GalNAc-MUC2 glycopeptide analogue (Syn-Tn-MUC2) covering amino acids 1–10 with GalNAc on all serine and threonine residues suggested that the antibody defined an epitope at the carboxyl-terminal end of the glycopeptide (Figure 4B).

The MUC2 tandem repeat sequence was a good substrate for three recombinant GalNAc-transferases, GalNAc-T1, -T2 and -T3 [20, 21]. In order to determine the number of moles of GalNAc incorporation required to generate the PMH1 epitope, a partially glycosylated MUC2 peptide was produced by using GalNAc-T2 and the product purified by reverse phase HPLC. Fractions were analysed by MS and found to represent incorporation of 1, 2 or more mol of GalNAc per mol of MUC2. Figure 5 shows the mass spectra of unglycosylated peptide and the glycopeptide with 1 mol of GalNAc incorporated. MAb PMH1 reacted with all GalNAc-MUC2 glycopeptides including the one containing only 1 mol GalNAc incorporated (Figure 6). Attempts to define an initial preferred site of glycosylation by GalNAc-T2 were unsuccessful.

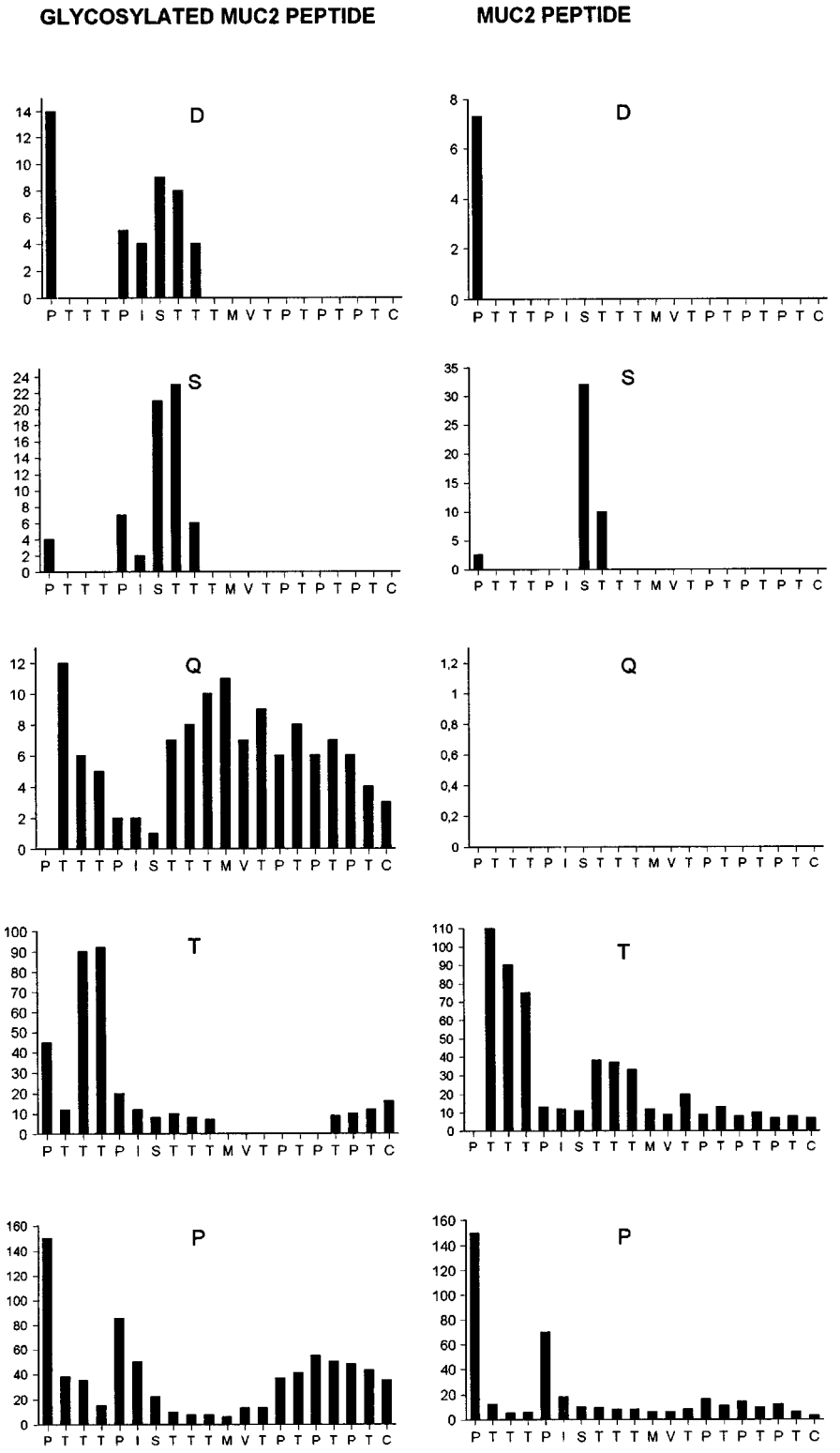

Figure 1. Amino acid sequencing of terminally glycosylated MUC2 peptide (left) and MUC2 unglycosylated peptide (right). The abscissa depicts the amino acid corresponding to the sequencing cycle and the ordinate depicts the amount in pmol of the amino acid PTH derivatives detected at the elution point of D, S, Q, T and P. Note: Glycosylation of threonine resulted in appearance of a peak corresponding to PTH-glutamine and glycosylation of serine resulted in a peak corresponding to PTH-aspartic acid.

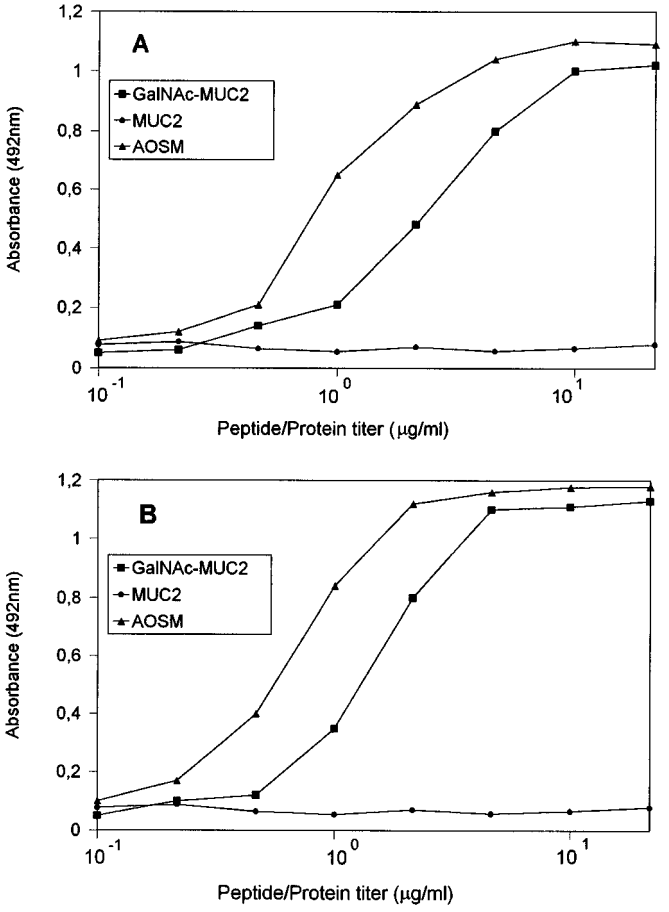

Figure 2. Immunochemical characterization of GalNAc glycopeptide. Panel A: ELISA using antibody HB-Tn1 (diluted 1 : 200) against the terminally GalNAc glycosylated MUC2 peptide, the unglycosylated MUC2 peptide and AOSM. Panel B: ELISA using lectin VVA (diluted 1 : 1000) against the same antigens as panel A.

MAb PMH1 reacts with high molecular weight mucin

Native, TFMSA deglycosylated and neuraminidase-treated mucin, extracted from LS174T xenografts, were analysed by SDS-PAGE and Western Blotting with PMH1, HB-Tn1 and HB-STn1 (Figure 7). MAb PMH1 reacted strongly with TFMSA deglycosylated and neuraminidase-treated mucin. Native mucin was weakly reactive. No reactivity was detected with AOSM. Antibodies against Tn and Sialyl-Tn were used as controls in order to monitor the extent of deglycosylation and degradation of the samples. The difference of intensity of reactivity with antibodies PMH1 and HB-Tn1 between the TFMSA deglycosylated sample and the neuraminidase-treated sample observed by Western Blotting was not observed when the samples were tested in ELISA with the same antibodies (Figure 8).

Immunohistological analysis of PMH1 epitope

PMH1 immunohistological staining is summarized in Table 1. PMH1 showed strong staining of goblet cells throughout the cytoplasm in normal colon (Figure 9). PMH1 also stained goblet cells of the small intestine with a supranuclear staining pattern.

Few cells in normal gastric mucosa were weakly labelled with a weak supranuclear staining suggestive of Golgi localization. Acinar cells of salivary glands were strongly stained, but similarly to normal gastric mucosa, only with a supranuclear localization. Intestinal metaplasia of the stomach was immunoreactive with MAb PMH1, with staining of vacuoles and mucin in goblet cells. Neuraminidase treatment of the sections enhanced the immunoreactivity.

The reactivity of the MAb PMH1 was compared to the reactivity of the anti-Tn antibody 1E3 in colon and labial salivary glands. As shown in Figure 9, the anti-Tn antibody, contrary to PMH1, did not stain the vacuoles of colonic goblet cells. Both antibodies stained acinar cells of salivary glands in the Golgi area.

Discussion

In order to stimulate an anti-Tn immune response it may be of advantage to use Tn-antigens that occur naturally such as glycopeptides derived from mucins. Aiming to prepare a potent Tn immunogen, we biosynthetically-produced a GalNAc glycosylated glycopeptide based on the tandem repeat sequence of intestinal mucin MUC2.

The MUC2 tandem repeat was chosen because of its high density of potential *O*-glycosylation sites and because it is an excellent substrate for *in vitro O*-glycosylation [17, 20]. The biosynthetically-glycosylated GalNAc-MUC2 was a potent antigen for anti-Tn antibodies and VVA lectin indicating that it could also be a potent Tn immunogen. Surprisingly, however, the GalNAc-MUC2 glycopeptide did not elicit a Tn-antibody response in rabbits or mice. The immune response elicited by the GalNAc-MUC2 glycopeptide was specifically directed towards the glycopeptide. No reactivity was observed with the unglycosylated peptide or with the generally used Tn-antigen asialo-ovine submaxillary mucin. Immunization with the unglycosylated peptide yielded antiserum that did not react with glycosylated peptide but was specific for the peptide core.

A monoclonal antibody, PMH1, with specificity representative of the polyclonal response, was generated for further studies. MAb PMH1 reacted specifically with the MUC2 peptide sequence with a minimum of 1 mol of GalNAc attached. The method of conjugation and the orientation of a peptide at the carrier protein can influence the immune response [30, 31]. In the present work the MUC2 peptide and the GalNAc-MUC2 glycopeptide were coupled to KLH through the C-terminal cysteine. Analysis of reactivity with the synthetic glycopeptide syn-Tn-MUC2 derived from the N-terminal sequence of the repeat with GalNAc at all sites showed that PMH1 did not react, suggesting that the epitope is located in the C-terminal region, -VTPTPTPT-. Attempts to clarify the specific site of

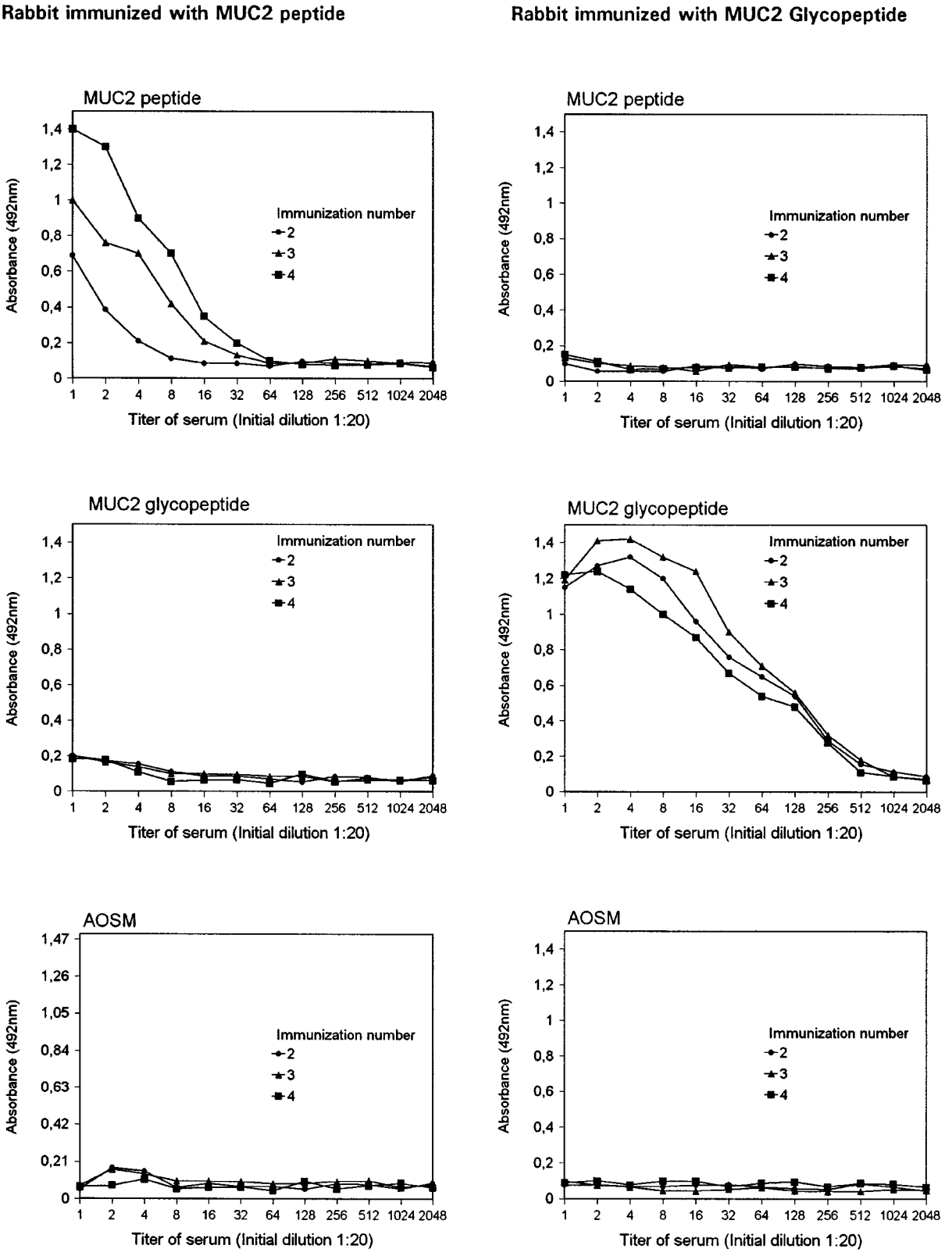

Figure 3. ELISA analysis of rabbits' immune response to MUC2 peptide (left) and GalNAc-MUC2 glycopeptide (right). Rabbit sera were tested on unglycosylated MUC2 peptide (10 $\mu g\,ml^{-1}$), GalNAc-MUC2 glycopeptide (10 $\mu g\,ml^{-1}$) and AOSM (10 $\mu g\,ml^{-1}$). Reactivities are shown for sera collected after second, third and fourth immunization. Immunization was performed at 3 week intervals.

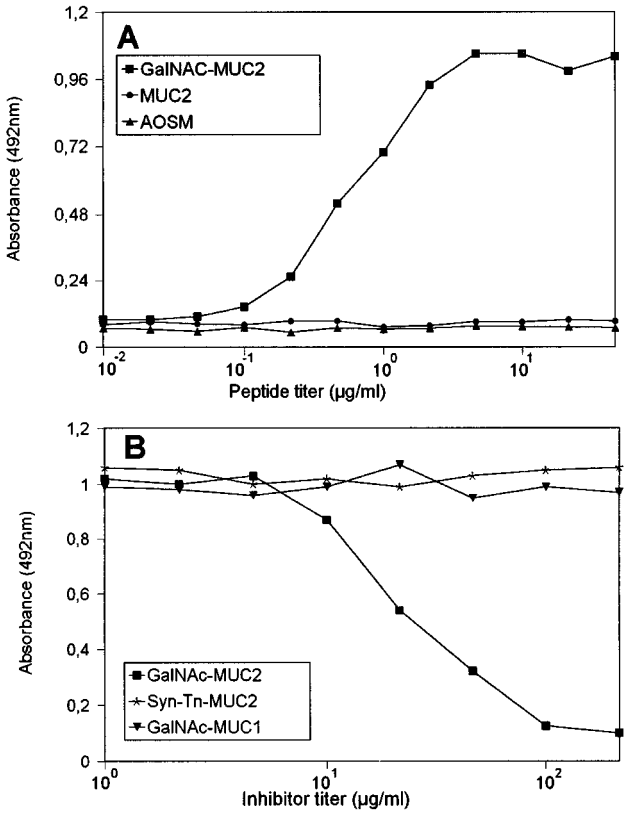

Figure 4. Characterization of PMH1 specificity by ELISA. Panel A: PMH1 with the GalNAc-MUC2 glycopeptide, unglycosylated MUC2 peptide and AOSM. Panel B: PMH1 in an inhibition ELISA with three glycopeptides. The plate was coated with glycosylated GalNAc-MUC2 peptide. Inhibition of PMH1 reactivity with the coated antigen was tested by preincubating PMH1 with the inhibitors as described in Material and Methods. The only peptide that inhibited the reaction of PMH1 was GalNAc-MUC2 itself. The chemically synthesized glycopeptide Syn-GalNAc-MUC2 and the glycopeptide GalNAc-MUC1 did not inhibit the reactivity of PMH1.

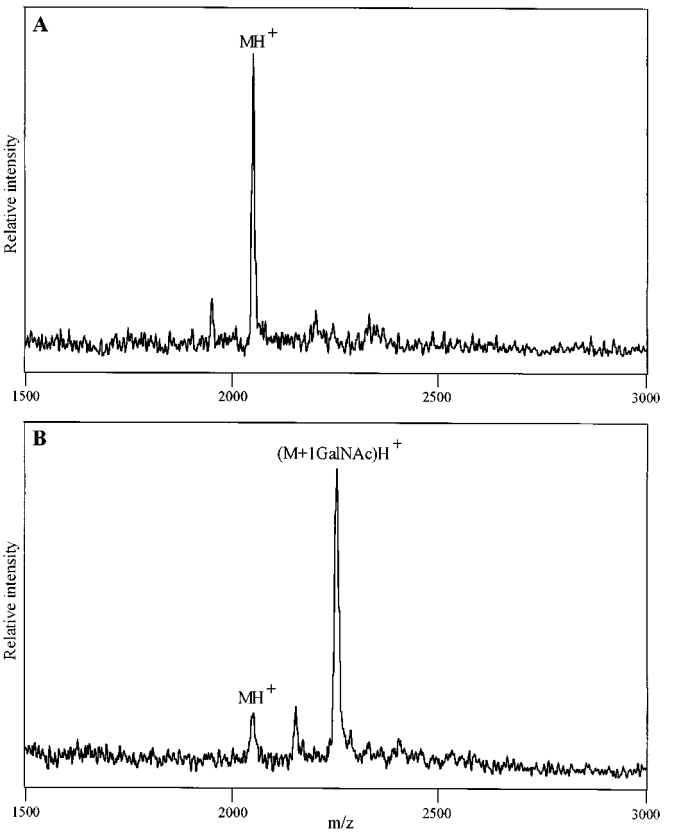

Figure 5. Mass spectrometry. Panel A: mass spectrum of peptide MUC2 (MH^+). Panel B: mass spectrum of glycopeptide MUC2 with 1 mol of GalNAc ($M + 1$ GalNAc)H^+.

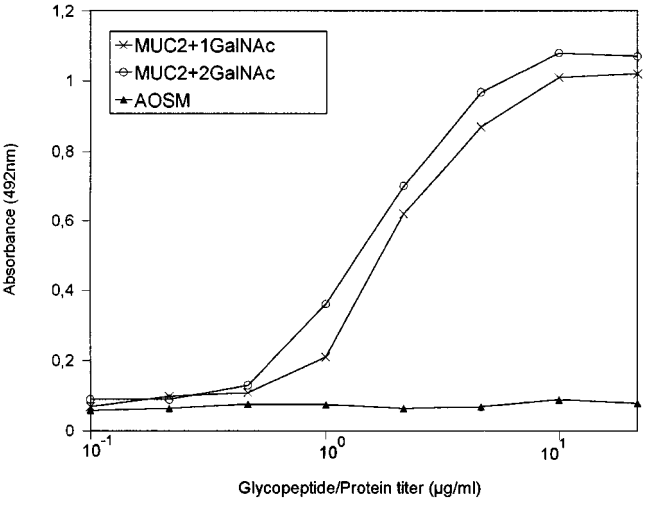

Figure 6. ELISA with MAb PMH1 against glycopeptides MUC2 + 1 mol GalNAc, MUC2 + 2 mol GalNAc, and AOSM.

GalNAc attachment required for antibody binding failed, due to difficulties with amino acid sequencing. Interestingly, the MUC2 peptide glycoform with a single GalNAc incorporated was not reactive with the anti-Tn antibody. This lack of reactivity may be due to low sensitivity of the assay or to the fact that antibody binding may require more than one GalNAc residue attached to a serine or threonine residue. Nakada *et al.* described an antibody with a similar property [13]. PMH1 was found to react with native purified colonic mucin although TFMSA treatment indicated that partial deglycosylation increased immunoreactivity. Reactivity with mucin extracts from a colonic cell line as well as findings that the immunohistological staining pattern of PMH1 in human tissues correlated with previous studies of MUC2 expression strongly suggest that PMH1 specifically defines glycoforms of MUC2.

Immunohistologically, PMH1 showed a similar tissue distribution to that previously described for anti-MUC2 peptide antibodies. Intestinal and colonic goblet cells were strongly stained. Acinar cells from labial salivary glands were also stained. These results are in agreement with previous studies using antibodies and *in situ* hybridization

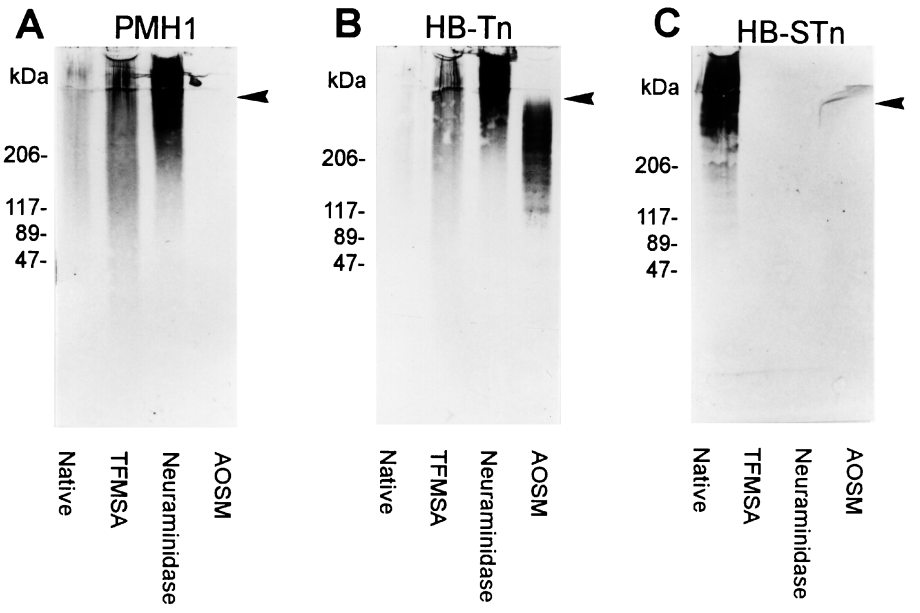

Figure 7. Immunoreactivity with mucin extracts from colonic cell line LS174T by Western blotting. Reactivity of MAbs PMH1 (A), HB-Tn1 (B), and HB-STn1 (C) with: native, TFMSA deglycosylated, neuraminidase treated mucin extract from LS174T cell line xenograft and AOSM. Prestained high range SDS-PAGE molecular weight markers (Bio-Rad) were used. Arrowhead indicates the start of the running gel.

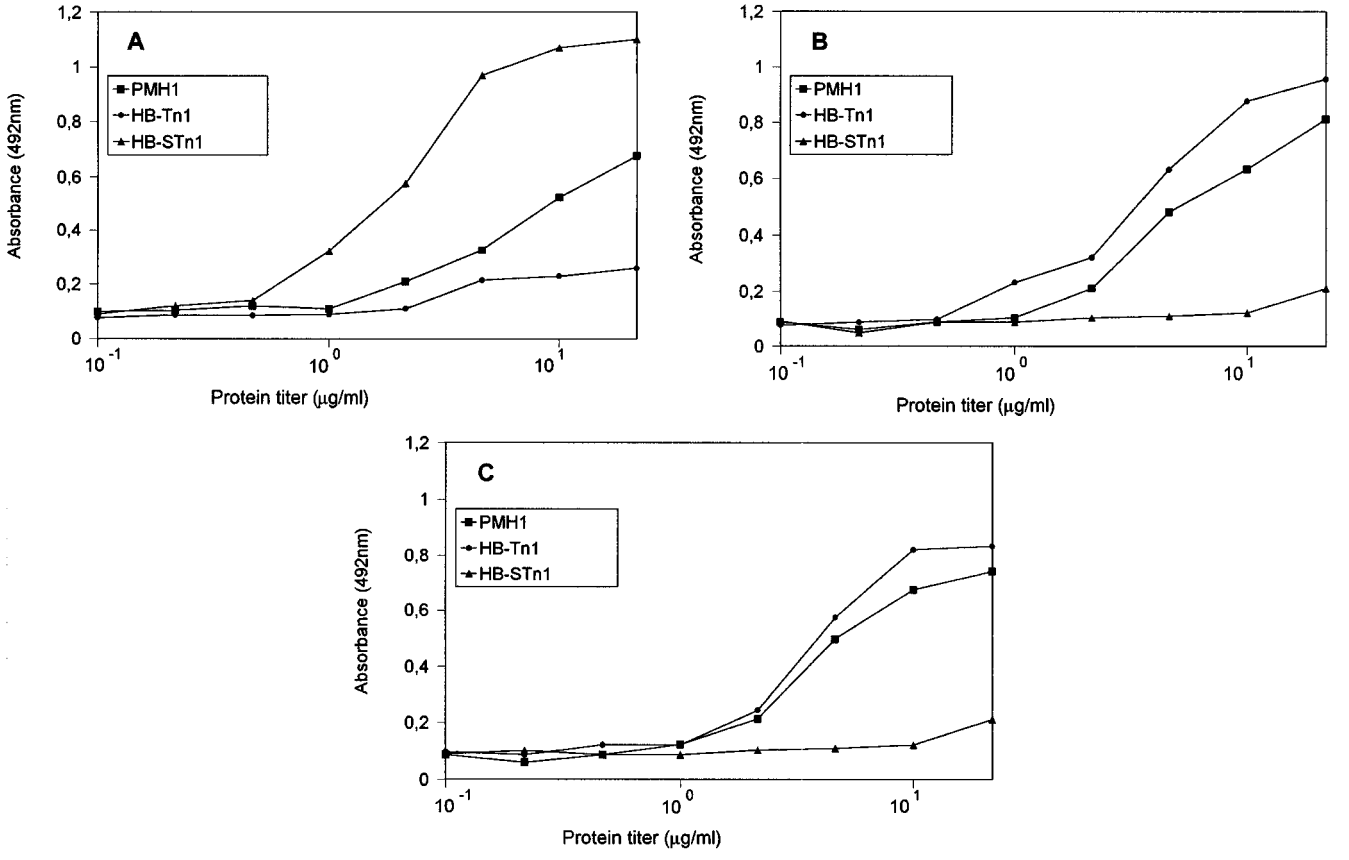

Figure 8. Immunoreactivity of mucin extracts in ELISA. Reactivity of native (A), TFMSA deglycosylated (B) and neuraminidase treated (C) mucin extract from LS174T cell line xenograft in ELISA using MAbs PMH1, HB-Tn and HB-STn.

Table 1. Immunoreactivity of monoclonal antibody PMH1 with several tissues

	MAb PMH1 immunoreactivity and staining pattern*	
Non gastrointestinal tissues		
Breast ($n = 2$)	−	
Bronchus ($n = 2$)	+	Supranuclear staining of bronchial cells.
Kidney ($n = 1$)	+	Apical membrane staining of tubular cells
Liver ($n = 1$)	−	
Ovary ($n = 1$)	−	
Spleen ($n = 1$)	−	
Uterus ($n = 2$)	−	
Gastrointestinal tissues		
Salivary gland ($n = 5$)	+	Supranuclear staining of mucus acinar cels from labial salivary gland.
Stomach-body ($n = 5$)	+	Supranuclear staining of foveolar epithelium.
Stomach-antrum ($n = 5$)	+	Supranuclear staining of foveolar epithelium.
	+	Diffuse cytoplasmic staining of cells of the pyloric glands.
Stomach-intestinal metaplasia ($n = 5$)	+ +	Staining of vacuoles and mucus in goblet cells.
	+	Supranuclear staining of columnar cells.
Gallbladder ($n = 1$)	+	Supranuclear staining of epithelial cells.
Pancreas ($n = 1$)	+	Supranuclear staining of acinar cells and duct cells.
Small intestine ($n = 2$)	+ +	Supranuclear staining of goblet cells.
Colon ($n = 3$)	+ + + +	Staining of vacuoles and mucus in goblet cells.

\+ less than 25% of positive cells, + + between 25 and 50%, + + + between 50 and 75%, + + + + more than 75% of positive cells.

[32, 33]. Immunoreactivity was also observed in gastric mucosa, ciliated bronchial cells in the lung, gallbladder epithelial cells, pancreatic cells and tubular cells of kidney, as has been previously described [33–35].

Monoclonal antibody PMH1 showed a supranuclear staining (Golgi-area) of goblet cells in the small intestine and staining of vacuoles and mucus in the colonic goblet cells. This pattern of reactivity was different from that of the anti-Tn antibodies, which characteristically do not stain intestinal and colonic cells [36]. Similar results were found in gastric intestinal metaplasia, where PMH1 showed staining of mucous vacuoles of goblet cells, while Tn-antibodies revealed staining confined to the Golgi area of the goblet cells. The cytolocalization of PMH1 staining of intestinal goblet cells differed from the supra- and peri-nuclear staining pattern of anti-MUC2 peptide antibodies [32–34]. We have not been able to establish to what degree glycosylation beyond the initial GalNAc will interfere with PMH1 binding. However, the findings that sections treated with neuraminidase, as well as deglycosylated mucin samples in Western blots are more reactive than native mucin suggest that sialic acid may partially block the PMH1 epitope. Furthermore, PMH1 staining was restricted to the supranuclear area of salivary gland acinar cells and of normal stomach epithelium cells contrary to staining of vacuoles and mucin in colon goblet cells. These findings suggest that PMH1 antibody is inhibited by glycosylation patterns seen in salivary glands and gastric cells, whereas the glycosylation in colonic goblet cells do not inhibit the reactivity of the antibody. This may be explained by differences in the structures of O-glycans in different cells [37, 38].

Antibodies directed to glycopeptide epitopes involving *O*-glycosylation have been described previously. Antibody FDC-6 identifies oncofetal fibronectin with its epitope being elucidated as a six amino acid peptide with *O*-glycosylation of a single threonine. This antibody appears to react with the glycosylated peptide sequence regardless of the glycan structure, but not with the unglycosylated peptide or carbohydrates alone [39]. Another antibody that can be compared to PMH1 is MAb BW835 which has recently been described by Hanish *et al.* [40]. MAb BW835 recognizes an epitope that comprises the Thomsen-Friedenreich (T antigen) disaccharide linked to threonine within the VTSA motif of the MUC1 tandem repeat [40]. Several studies have focused on the influence of the carbohydrate moiety on the conformation adopted by a peptide [41–47]. Most likely PMH1 reacts with a conformational peptide epitope induced by *O*-glycosylation. Future studies should address the epitope more precisely using overlapping peptides and different carbohydrate residues.

It is difficult to analyse mucins either chemically or immunochemically. The high molecular weight and resistance to proteolytic digestion have hampered the determination of the glycosylation sites within mucins tandem repeats. Antibodies directed to specific mucin core proteins are good tools for studying mucin expression in cells and tissues.

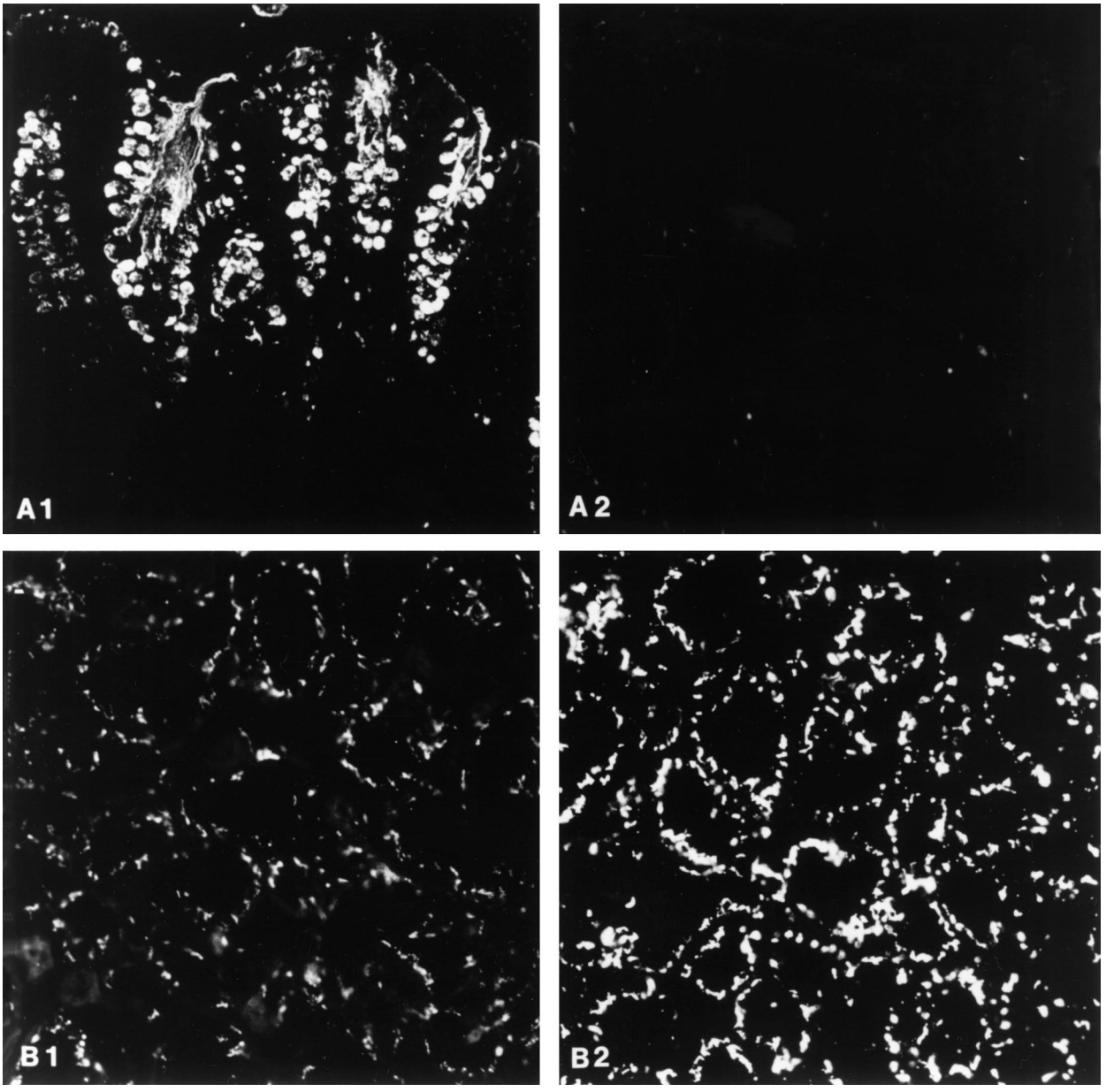

Figure 9. Immunofluorescence stained sections with MAb PMH1 and MAb 1E3 (anti-Tn)
Panel A (1) PMH1 staining of vacuoles and mucus in colonic goblet cels.
(2) Neighbouring section showed no staining with 1E3.
Panel B (1) Golgi staining with MAb PMH1 of labial salivary glands.
(2) Neighbouring section showing stronger Golgi staining with 1E3.

Antibodies raised to native mucins generally only give rise to anti-carbohydrate antibodies, and antibodies established to the protein core of mucins have usually been generated against deglycosylated mucins or mucins from tumour cells with incomplete glycosylation. The strategy introduced here, using glycosylated peptides covering mucin tandem repeats to generate antibodies which are immunoreactive with glycoforms of mucins, may be helpful for production of antibodies that can be used for the identification of mucins in their glycosylated forms, for example in body fluids.

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