Peripheral α -linked *N*-acetylglucosamine on the carbohydrate moiety of mucin derived from mammalian gastric gland mucous cells: epitope recognized by a newly characterized monoclonal antibody

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To obtain a tool to study the structural characterization and the detection of mucin derived from the gastric gland mucous cells, we developed a monoclonal antibody, designated HIK1083, against mucin purified from rat gastric mucosa. In an ELISA, HIK1083 reacted strongly with the mucin purified from a deep layer of the corpus and antrum but only slightly reacted with that obtained from the surface mucosal layer. The reaction of mucin and HIK1083 was inhibited by the oligosaccharides obtained by the alkaline borohydride reduction of antigenic mucin. Two purified oligosaccharide alditols reacting with the monoclonal antibody obtained from the antigenic mucin had one and two peripheral α -linked *N*-acetylglucosamine residues, respectively,

according to the evidence from NMR spectroscopy. Moreover, among the commercially available *p*-nitrophenyl derivatives of monosaccharides, only *p*-nitrophenyl-*N*-acetyl- α -D-glucosaminide inhibited the reaction of this monoclonal antibody and the antigenic mucin in a concentration-dependent manner. These results, as well as the immunohistochemical observations, indicate that α -linked *N*-acetylglucosamine residues are specifically attached to the peripheral region of the carbohydrate moiety of the mucin synthesized in and secreted from the gastric-gland-type cells, and indicate that the monoclonal antibody HIK1083 recognizes this structure.

INTRODUCTION

Gastric mucus covering the mucosal surface is considered to be a major factor in the gastric defence mechanism against various aggressive factors such as gastric acid and pepsin. Mucin, a highly glycosylated high-molecular-mass glycoprotein, is a major component of gastric mucus. Mucus-secreting cells of the mammalian gastric mucosa have been classified mainly into the surface mucous and the gland mucous cells [1,2]. Mucus from these two types of cells is suggested to have distinct roles in the physiology of the gastric mucosa [3], and the mucins of these two types of cells in a single tissue section are individually characterized by a combination of galactose oxidase–cold thionin Schiff (GOCTS) staining and paradoxical concanavalin A staining (PCS)[4]. For a more precise characterization of individual mucins on a biochemical and physiological basis, monoclonal antibodies (MAbs) which recognize the mucin in one cell type and not the other are likely to be necessitated. In a previous report, we described a MAb which specifically reacts with the surface mucous cell-derived mucin of rat gastric mucosa and noted the partial characterization of the epitope sugar [5].

In this study, a MAb was established that reacts with the mucin obtained from rat gastric gland mucous cells. This MAb has a unique character which recognizes the peripheral α -linked GlcNAc residue present in the carbohydrate moiety of the mucin molecule and reacts with oligosaccharides bearing a peripheral α -GlcNAc structure.

MATERIALS AND METHODS

Isolation of rat gastric mucin for immunization and ELISA

Female retired Sprague–Dawley (SD) rats weighing 250–300 g were killed by exsanguination from the carotid artery. The stomach and the duodenum were excised and washed in ice-cold PBS. The mucosal layers of the whole stomach and duodenum were then collected. Alternatively, the gastric mucosa of male SD rats weighing approx. 200 g were divided into the following four portions according to the method of Komuro et al. [6]: mucus gel layer, surface mucosa, deep corpus mucosa and deep antral mucosa. The obtained specimens were extensively lyophilized and pulverized. The extraction with a buffer solution containing Triton X-100 and isolation of the purified mucins by gel filtration followed by CsCl equilibrium density gradient centrifugation from the powdered specimens were performed as described previously [5]. Biochemical characterization of the mucin obtained from each of these layers has been described previously [7]. In some experiments, the gastric mucins purified from male Wistar rats weighing approx. 170 g were also utilized.

Isolation of partially purified pig gastric mucin

To obtain the preparation of an oligosaccharide mixture of pig gastric mucin, crude pig gastric mucin (Sigma Chemical Co.; Type II) was further purified by the following method. Crude mucin (100 g) was suspended in 500 ml of 0.02% NaN₃ and the

Abbreviations used: ABTS, 2,2'-azinobis-[3-ethyl-benzthiazoline-6-sulphonate (6)]; GS-II, *Griffonia simplicifolia*-II; HRP, horseradish peroxidase; MAb, monoclonal antibody; PNP, *p*-nitrophenyl; SD, Sprague–Dawley; CPC, cetylpyridinium chloride; PFG, pulse field gradient; PCS, paradoxical concanavalin A staining; ConA, concanavalin A; GalNAc-ol, *N*-acetylgalactosaminol; Fuc, fucose; HMQC, heteronuclear multiple-quantum coherence spectroscopy.

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pH was adjusted to 2.5 with the addition of conc. HCl. Then 1 g of pepsin was added to this suspension at 0, 24 and 48 h, and peptic digestion was allowed to continue up to 72 h at 37 °C. The reaction was terminated by neutralization of the reaction mixture with NaOH, followed by centrifugation at 10000 *g* at 4 °C for 30 min. To remove the contaminated mucopolysaccharides, 5 g of cetylpyridinium chloride (CPC) was added to the resultant supernatant, which was dissolved and the mixture allowed to stand overnight at room temperature. The insoluble complex formed with CPC was removed by centrifugation and the clear supernatant thus obtained was thoroughly dialysed against tap water. The soluble mucin was then fractionated by ethanol fractionation. The precipitate formed at a 33–50 % (v/v) ethanol concentration was collected by centrifugation at 10000 *g* at 4 °C for 30 min. After lyophilization of the precipitate, 20.8 g of partially purified pig gastric mucin was obtained as a colourless powder. The pig gastric mucin thus obtained was utilized as a source of the oligosaccharide–alditol mixture.

Preparation and purification of oligosaccharides from mucin

Alkaline borohydride treatment of the partially purified pig gastric mucin was carried out according to the method of Carlson [8] with 0.05 M NaOH in 1.0 M NaBH₄ at 50 °C for 24 h. The reaction mixture was then cooled and acidified by the dropwise addition of acetic acid (final pH = 4) and then evaporated. The resultant oligosaccharide mixture was dissolved in distilled water and applied to a column of Bio-Gel P-6 (3.4 cm × 100 cm; Bio-Rad Laboratories, Richmond, CA, U.S.A.). The column was eluted with distilled water and the effluent solution was monitored by hexose measurement [9]. An oligosaccharide fraction, Fr-4, from the Bio-Gel P-6 column was pooled, lyophilized and extensively separated by HPLC (Jasco HPLC system) using a reverse-phase column (7.8 mm × 300 mm × 2; TSK-Gel ODS-80TM, Tosoh Corporation, Tokyo, Japan) with distilled water at a flow rate of 1.2 ml/min. UV absorption of the effluent solution was monitored at 210 nm.

Carbohydrate composition analysis of mucin and oligosaccharide–alditols

The molar composition of oligosaccharide–alditols and the carbohydrate composition of mucins were determined by gas–liquid chromatography using a Shimadzu GC-7A (Shimadzu Corporation, Kyoto, Japan). Methanolysis, N-re-acetylation, trimethyl-silylation and chromatography were performed as described by Sweeley et al. [10].

NMR spectroscopy

NMR spectra were obtained on a Varian Unity 400 NMR spectrometer (Varian Associates, Palo Alto, CA, U.S.A.) equipped with an ¹H{¹⁵N-³¹P} pulse field gradient (PFG) indirect-detecting probe. Standard pulse sequences were used throughout. The proton NMR spectrum was assigned through a combination of a PFG multiple-quantum (MQ)-COSY and one-dimensional homonuclear Hartmann–Hahn (HOHAHA) spectroscopy. ¹³C assignments were made from a heteronuclear multiple-quantum coherence spectroscopy (HMQC) spectrum obtained with carbon decoupling. Some additional assignments and information on the sequence and linkage of the sugar residues were derived from a NOESY and a PFG-heteronuclear multiple-bond spectroscopy (HMBC) spectrum. NMR spectra of GalNAc-ol, α-methyl-fucoside, α- and β-methyl derivatives of GlcNAc, GalNAc and

Gal (all obtained from Sigma Chemical Co.) were utilized as references for chemical shift assignment. About 1 mg of lyophilized powder of purified oligosaccharide was dissolved in ²H₂O and evaporated to exchange unstable ¹H with ²H. The evaporation and the dissolution were repeated five times and finally the sample was dissolved into 0.5 ml of ²H₂O and then subjected to NMR spectroscopy.

MAB production

The MAb was produced as described previously [5]. A hybridoma, which showed reactivity with the mucin obtained from whole rat stomach, was generated by fusing the splenic lymphocytes of a mouse immunized with the rat gastric mucin and the cells of Sp2/0-Ag14 mouse myeloma, and subsequently cloned by the limiting dilution method. The resulting antibody (named HIK1083) was an IgM type as identified by ELISA using an isotyping kit (PharMingen, San Diego, CA, U.S.A.).

Competitive ELISA

A competitive ELISA was applied to detect the reactivity of the MAb with various sugar derivatives. The microtitre plate coated with 100 ng of the purified mucin followed by blocking with 2 % (w/v) skimmed milk was prepared [5]. At the same time, PBS solutions of the sugar derivatives, the eluate from the column of Bio-Gel P-6 (each containing 100 μg per well as hexose base), or the appropriately concentrated solution of the eluate from the column of ODS-80TM corresponding to 1.5 mg of oligosaccharide mixture at the start of the chromatography, were pre-incubated with a definite amount of the monoclonal hybridoma medium for 2 h at ambient temperature. Instead of the sugar-containing solution, PBS was added to the negative control wells. The pre-incubation mixture was then added to the antigen-coated wells and incubated for 1 h. The wells were successively incubated with horseradish peroxidase (HRP)-conjugated goat anti-(mouse immunoglobulins) (Tago, Inc., Burlingame, CA, U.S.A.) and 2,2'-azino bis-[3-ethylbenzthiazoline-6-sulphonate (6)] (ABTS)/H₂O₂ solution (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, U.S.A.), and the colour was allowed to develop.

Immunohistochemical staining

Various organs and glands including gastric mucosa were obtained from male SD rats weighing approx. 250 g. Stomachs of BALB/c mice, 5 mm × 20 mm strips of mucosa of cardiac, fundic and pyloric gland of Japanese White rabbit and pig stomachs were also obtained. After fixation in 50 mM phosphate buffer (pH 6.8) containing 4 % formaldehyde at 4 °C for 48 h, the materials were dehydrated through ethanol, cleared in xylene and embedded in paraffin. Paraffin sections, 4 μm thick, were processed by the avidin–biotin peroxidase method using a Histofine SAB-PO KitTM (Nichirei Corporation, Tokyo, Japan). Briefly, endogenous peroxidase activity was blocked with 0.3 % H₂O₂ and then the tissue was sequentially incubated with 10 % (v/v) rabbit normal serum (Nichirei Corp.), the monoclonal hybridoma medium, biotinylated rabbit anti-(mouse IgG + IgA + IgM) (H + L) (Nichirei Corp.), streptavidinylated HRP (Nichirei Corp.), and 0.02 % 3,3'-diaminobenzidine (Dojin, Kumamoto, Japan) in 50 mM Tris/HCl, pH 7.6, containing 0.005 % H₂O₂. The counterstain was performed with haematoxylin. The culture supernatant of Sp2/0-Ag14 myeloma cells was used as a negative control. Assessment of the immunohistochemical observation was made from at least three different individual tissues.

RESULTS

Reaction of HIK1083 with the purified mucin obtained from the various regions of rat gastric mucosa

As described in the Materials and methods section, HIK1083 reacted with the gastric mucin purified from the whole stomach of SD rats. To obtain more information about the specificity of this MAb for the mucin present in the different layers and the regions of rat gastric mucosa, the reaction of HIK1083 was examined with the purified gastric mucin isolated from the adherent mucus gel, the surface mucosal layer, and the deep corpus and antrum, and with the purified duodenal mucin. As shown in Figure 1, the most intensive reaction was achieved with the mucin obtained from the deep corpus and antral mucosa, while almost no reaction was observed between the surface mucous cell-derived mucin and HIK1083.

Figure 1 Reactivity of HIK1083 with the purified mucins from SD rats

Microplate wells were coated with mucins isolated from the adherent mucus gel (○), surface mucosal layer (●), deep corpus (△), deep antrum (▲) and with the duodenal mucin (+), successively incubated with 2% skimmed milk, HIK1083 culture supernatant, HRP-conjugated anti-(mouse immunoglobulins) and ABTS substrate. Data are expressed as values relative to the highest reactivity which was achieved by the deep corpus-derived mucin. Each binding value corresponds to the mean $\pm$ S.D. of a triplicate determination. This experiment is the representative of four similar observations including the study using purified Wistar rat mucin preparations.

Table 1 Immunohistochemical reactivity of HIK1083 with normal SD rat

—, Negative; +, presence of positive cells.

Tissue	Reactivity	Site
Alimentary tract		
Oesophagus	—	
Stomach		
Corpus	+	Mucous neck cell
Antrum	+	Pyloric gland cell
Small intestine		
Duodenum	+	Brunner's gland cell
Jejunum	—	
Ileum	—	
Large intestine	+	Cecal mucosal cell
Other organs		
Sublingual gland, parotid gland, thymus, lung, heart, liver, spleen, pancreas, kidney	All negative	

Table 2 Immunohistochemical reactivity of HIK1083 with normal stomach

Grading index from — to + + + based on percentage of positive cells. —, negative; +, < 30% positive cells; ++, < 60% positive cells; + + +, $\geq$ 60% positive cells.

		Reactivity			
		Mouse	Rat	Rabbit	Pig
Cardia	Surface mucous cell	+	—	—	—
	Cardiac gland cell	+ + +	+ + +	+ + +	+ + +
Corpus	Surface mucous cell	—	—	—	+
	Mucous neck cell	+ + +	+ + +	+ + +	+ + +
Antrum	Surface mucous cell	+	—	—	—
	Pyloric gland cell	+ + +	+ + +	+ + +	+ + +

Immunohistochemical reactivity of HIK1083

Tables 1 and 2 show the immunohistochemical reactivity of HIK1083 with various organs of the rat and the stomachs of four animal species, respectively. In the alimentary tract of a normal SD rat, mucous neck cells from the corpus, pyloric gland cells from the antrum and Brunner's gland cells from the duodenum were strongly stained with this MAb (Table 1 and Figure 2). Weak but significant staining could also be observed on the base of gland which was located in the distal end of the small intestine and cecum. Other organs of the rat examined so far were all negative to the staining with this MAb. Table 2 shows that HIK1083 reacts with the gland mucous cells including the mucous neck, the pyloric gland and Brunner's gland cells of all the mammalian stomachs and duodenum tested so far. Thus, it has been demonstrated that this MAb is not a species-specific antibody but is able to react with the mucus of the upper gastrointestinal tract from a variety of animals.

Reactivity of HIK1083 with the oligosaccharides obtained from the mucin

In order to study the characterization of the epitope of HIK1083, the reaction of this MAb with the oligosaccharides of antigenic mucin was examined. The oligosaccharides were prepared from the partially purified pig gastric mucin by the alkaline borohydride reduction and were fractionated on a Bio-Gel P-6 column. Fractions eluted from this column were examined for antigenic activity to HIK1083 by the competitive ELISA as described in the Materials and methods section. As shown in Figure 3, an oligosaccharide fraction, Fr-4, distributed from fraction numbers 51 to 60 from the column, most strongly inhibited the reaction of the MAb and the purified mucin on the ELISA plate. These results indicate that the oligosaccharides bearing a specific structure are responsible for the antigenic activity to this MAb. In some preparations, but not all of the oligosaccharide mixture, Fr-1 and Fr-5 showed a fairly strong reactivity with HIK1083. The reactive products obtained from Fr-1 were degraded to the lower-molecular-mass forms by further treatment with the alkaline borohydride reduction, indicating the remnants of undegraded mucin peptides bearing the oligosaccharide chains which could bind to this MAb. By further purification of the reactive products in Fr-5 by HPLC, we could obtain a HIK1083-reactive peak whose carbohydrate composition was noted as GlcNAc:galactitol (1:1, by molar ratio) by gas-liquid chromatography (results not shown), indicating the occurrence of successive degradation of oligosaccharides formed by the alkaline borohydride cleavage, the so called 'peeling reaction'. Oligosaccharide fraction Fr-4 was further purified by HPLC using a reversed-

Figure 3 Bio-Gel P-6 column chromatography of oligosaccharide mixture prepared from the partially purified pig gastric mucin by the alkaline borohydride reduction (upper panel) and the antigenic activity of oligosaccharide fractions (lower panel)

Upper panel: the hexose content (○) of each fraction was assessed by the phenol-sulphuric acid method of Dubois et al. [9]. External calibration standards: (A) Dextran T-500 (500 kDa), (B) maltohexaose, (C) glucose. Lower panel: the antigenic activity of oligosaccharide fractions, Fr-2 (○), Fr-3 (●) and Fr-4 (△), were examined by the competitive ELISA as described in the Materials and methods section. Data are expressed as a percentage of the reactivity in the negative control well and are means $\pm$ S.D. of three experiments. This experiment is representative of three similar observations.

obtained from the mixture of OP-2 and OP-5 in the ratio of 60 % and 40 %, respectively.

Reaction of various *p*-nitrophenyl (PNP) derivatives of monosaccharides with HIK1083 in the competitive ELISA

The commercially available PNP monosaccharides were investigated for antigenic activity to HIK1083 by the competitive ELISA using the mucin purified from the whole layer of rat gastric mucosa. All the monosaccharide derivatives, except α -linked GlcNAc, were negative to the reaction up to 100 μ g per well (Figure 6). Only PNP-*N*-acetyl- α -glucosaminide acted as an inhibitor in the ELISA reaction at 6–100 μ g per well. A similar result could be observed using methyl-*N*-acetyl- α -glucosaminide in the competitive ELISA in contrast to the respective β -anomer (results not shown).

Comparative study of HIK1083 and *Griffonia simplicifolia*-II (GS-II) lectin in the binding reaction to the mucin attached to the ELISA plate

It has been recognized that a lectin, GS-II, has a high affinity for the α - and β -*N*-acetylglucosaminyl residues present at the non-reducing terminal of the carbohydrate moiety of the glycoconjugates [12]. To study the similarity of HIK1083 MAb to GS-II lectin, the inhibitory activity of GS-II lectin was estimated based on the binding reaction of HIK1083 to the antigenic mucin attached to the ELISA well. As shown in Figure 7 (upper panel), pretreatment of the ELISA well with GS-II lectin inhibited the

Figure 4 HPLC of oligosaccharide fraction Fr-4 using a TSK-gel ODS-80TM column (upper panel) and the antigenic activity of two purified oligosaccharides examined by the competitive ELISA (lower panel)

Upper panel: absorbance at 210 nm was monitored by a UV-VIS spectrometer (Model 870-UV, JASCO Corporation, Japan). Lower panel: the antigenic activity of two purified oligosaccharides, OP-2 (●) and OP-5 (○), was examined by the competitive ELISA as described in the Materials and methods section. Data are expressed as a percentage of the reactivity in the negative control well and are means $\pm$ S.D. of three experiments. This experiment is representative of three similar observations.

binding reaction of HIK1083 to the mucin-coated well in a concentration-dependent manner. Alternatively, pretreatment with a serial dilution of HIK1083 reduced the binding reaction of HRP-labelled GS-II to the mucin-coated well in a concentration-dependent manner (results not shown). These results indicate that HIK1083 and GS-II lectin share a common epitope sugar in the mucin attached on the ELISA well.

Methyl- or PNP- α -GlcNAc are known to bind to GS-II six to eight times more strongly than their respective β -anomers [12]. For the comparison of HIK1083 with this lectin regarding the specificity toward α - and β -GlcNAc residues under similar conditions, the inhibitory activity of PNP- α - and β -GlcNAc on the mucin binding reaction of GS-II lectin was determined. As shown in Figure 7 (lower panel), PNP- α -GlcNAc inhibited the mucin binding reaction of this lectin about ten times more strongly than that of the respective β -anomer. These and the results of Figure 6 significantly indicate that HIK1083 binds in a highly specific manner to the α -linked GlcNAc residue, and this specificity is higher than that of the GS-II lectin known to have a similar specificity.

DISCUSSION

It has been histologically characterized that the surface epithelial cells and gland mucous cells of gastric mucosa produce a distinct type of mucin having a different stainability [4,13]. Recently we devised a series of mucus scraping methods to separate the mucous gel layer and the surface mucosal layer from the remaining deep mucosa of rat stomach [6]. By using these

Figure 5 One-dimensional ^1H NMR spectra of oligosaccharides OP-2 and OP-5

methods, we have been surveying the effects of various agents such as the acid secretagogue tetragastrin [14] and the M_1 muscarinic receptor antagonist pirenzepine [15] on the accumulation of mucin present in specific sites of rat gastric mucosa. This evidence strongly indicates that a distinct control mechanism underlies the biosynthesis and accumulation of a mucin present in a specific region and layer of the gastric mucosa. Moreover, it has recently been characterized that the peptide sequence of apomucin derived from the surface mucous cells of human stomach is MUC5, and differs from that of the gland-type mucous cell-derived mucin which was identified as MUC6. It seems, therefore, very important to devise a highly sensitive and highly specific method to detect mature gastric mucins of different origin [16,17]. In our previous study, we have established and partially characterized an MAb, RGM11, which reacts only with the surface mucous cell-derived mucin of rat stomach [5]. In the present study, we reported an MAb, HIK1083, which recognizes the mucous neck cell- and pyloric gland mucous cell-derived mucin.

As demonstrated in the text, the epitope of HIK1083 includes a peripheral α -linked GlcNAc residue due to the following reasons. (1) Among the PNP- or methyl- derivatives of various monosaccharides, only those of α -linked GlcNAc strongly inhibited the binding reaction of this MAb with the purified rat gastric mucin attached to the ELISA well. (2) HIK1083 reacted with the oligosaccharide mixture obtained from pig and rat gastric mucin by alkaline borohydride cleavage. Moreover, two oligosaccharides, OP-2 and OP-5, purified from pig mucin-derived oligosaccharides due to their inhibitory activity toward the binding of HIK1083 and the antigenic mucin, had two and one residues of peripheral GlcNAc, respectively, which attached to Gal residues via an α 1-4 linkage. (3) HIK1083 and GS-II lectin, which has been reported to react more strongly with α -linked GlcNAc-containing glycoconjugate than with the β -linked

counterpart, alternatively inhibited the binding reaction of the opponent with the gastric mucin on the ELISA well. These observations strongly indicate that the epitope of HIK1083 involves an α -linked GlcNAc residue attached to the peripheral region of the oligosaccharide moiety of the antigenic mucin molecule.

It has already been demonstrated that GlcNAc α 1-4 Gal residues are attached to the peripheral region of the O-linked oligosaccharides obtained from pig [11,18] and rat [11] gastrointestinal mucin. The structure of OP-5 was presented by Kochetkov et al. [18] and was obtained from the oligosaccharides of pig gastric mucin. The mixture of OP-2 and OP-5 was copurified from rat duodenal gland total oligosaccharides, and the structural analysis was performed using 500 MHz ^1H -NMR by Van Halbeek et al. [11]. They also showed these structures to be characteristic constituents of mucins obtained from rat and pig duodenal gland. Based on our present study, OP-2 and OP-5 were found in the pig gastric mucin-derived oligosaccharides, but OP-5 could not be found in the rat gastric oligosaccharides. Anyway, based on the immunohistochemical observation of this study, it is strongly indicated that O-linked sugar chains containing peripheral α -linked GlcNAc residues such as OP-2 and OP-5 are specifically present in the mucin of mammalian gastric and duodenal gland cells, i.e., mucous neck, pyloric gland and Brunner's gland cells.

Recently, Hanisch et al. established an MAb, 2B5, which reacts to mucus from deep gastric and duodenal glands, and reported the epitope sugar structure of 2B5 to be GlcNAc β 1-3Gal β 1-4GlcNAc β 1-6-(GalNAc)- [19]. 2B5 and HIK1083 have different epitope structures; however, the immunohistochemical properties of the two MAbs seem to be similar. In our more recent studies, we found some MAbs which specifically reacted with gastric gland-type mucin but not with PNP- α -GlcNAc (M. Kurihara, K. Ishihara, T. Nakano and K. Hotta, unpublished

Table 3 Chemical shifts of each sugar component in OP-2 and OP-5Standards are α and β methyl derivatives of each component sugar except GalNAc-ol.

Sugar	OP-2		OP-5		Standards ¹³ C
	¹ H	¹³ C	¹ H	¹³ C	
GalNAc-ol					
Position 1	3.73, 3.79	60.3	3.72, 3.78	61.0	61.5
Position 2	4.38	51.3	4.39	51.3	51.5
Position 3	4.06	77.0*	4.05	77.0*	68.4
Position 4	3.55	68.8	3.55	68.7	69.4
Position 5	4.24	67.3	4.24	67.4	69.8
Position 6	3.67, 3.91	70.4*	3.65, 3.91	70.5*	63.2
Ac-CH ₃	2.08	21.7	2.08	21.7	21.7
α -GlcNAc					
Position 1	4.84†	98.0†	4.84	97.8	98.0
Position 2	3.88†	53.9†	3.87	53.8	53.5
Position 3	3.78†	70.3†	3.78	70.3	71.0
Position 4	3.52†	69.5†	3.51	69.5	69.9
Position 5	4.15†	71.8†	4.14	71.8	71.6
Position 6	3.77, —†	59.9†	3.78, —	59.9	60.5
Ac-CH ₃	2.04†	21.8†	2.04	21.8	21.8
β -GlcNAc					
Position 1	4.55	101.2	4.53	101.3	101.9
Position 2	3.72	55.1	3.77	55.1	55.4
Position 3	3.68	72.4	3.65	72.4	73.9
Position 4	3.73	75.4*	3.74	75.5*	69.9
Position 5	3.57	74.7	3.45	75.1	75.8
Position 6	3.99, —	60.0	3.85, 3.96	60.2	60.7
Ac-CH ₃	2.03	21.8	2.03	21.9	21.9
β -Gal(4s)‡					
Position 1	4.50†	104.3, 103.2†	4.50	104.4	103.7
Position 2	3.58†	70.7†	3.57	70.6	70.7
Position 3	3.72†	71.8†	3.72	71.8	72.7
Position 4	3.95†	76.4*†	3.95	76.2*	68.6
β -Gal(2s)‡					
Position 1			4.51	100.2	103.7
Position 2			3.63	76.3*	70.7
Position 3			3.85	73.5	72.7
Position 4			3.86	69.0	68.6
α -Fuc					
Position 1			5.28	99.3	99.4
Position 2			3.75	68.1	67.8
Position 3			3.80	71.6	71.7
Position 4			3.79	69.5	69.5
Position 5			4.20	66.8	66.4
CH ₃			1.20	15.0	15.2

* Occurrence of glycosylation shift.

† Corresponding to two saccharide residues.

‡ 2s and 4s indicate the galactose residue substituted at position 2 and 4, respectively.

Figure 6 Inhibitory activity of PNP-monosaccharides on the mucin-binding reaction of HIK1083

The competitive ELISA described in the Materials and methods section was carried out using HIK1083 culture supernatant. All of the PNP-monosaccharides were purchased from Sigma, St. Louis, MO, U.S.A. Data are expressed as a percentage of the reactivity in the negative control well and are means $\pm$ S.D. of three experiments. This experiment is representative of three similar observations.

Figure 7 Comparative study of HIK1083 and GS-II lectin

Upper panel: inhibitory activity of GS-II lectin on the mucin-binding reaction of HIK1083. An ELISA plate was successively treated with 100 ng of the purified mucin per well (100 μ l/well), 2% skimmed milk, 2-fold serial dilution of GS-II lectin (E-Y Laboratories, Inc., San Mateo, CA, U.S.A.), HIK1083 culture supernatant, HRP-conjugated anti-mouse immunoglobulins and ABTS substrate. Data are expressed as a percentage of the reactivity in the negative control well and are means $\pm$ S.D. of three experiments. This experiment is representative of four similar observations. Lower panel: Inhibitory activity of PNP- α - and - β -GlcNAc on the mucin-binding reaction of GS-II lectin. The competitive ELISA described in the Materials and methods section was applied to the binding reaction using a definite amount of HRP-labelled GS-II lectin (E-Y Laboratories) which had been pre-incubated with a serial dilution of the respective PNP-GlcNAc derivative. The HRP-labelled GS-II lectin was previously titrated with 100 ng of rat gastric mucin per well, and the 3000-fold diluted lectin was used in this study. Data are expressed as a percentage of the reactivity in the negative control well and are means $\pm$ S.D. of three experiments. This experiment is representative of three similar observations.

work). These results may indicate that more than one species of oligosaccharide is specifically present in the gastric-gland-type mucins which can be recognized by distinct MABs.

The PCS method which has been histochemically applied to detect the gastric-gland-type mucus is based on the sequential treatment of a deparaffinated section with a periodate oxidation, a sodium borohydride reduction and an adhesion reaction with concanavalin A (ConA) lectin [20]. An earlier study in our laboratory demonstrated that human gastric mucin which had been treated according to the PCS sequence *in vitro* effectively bound to a ConA-Sepharose column [21]. From the carbohydrate composition of the partially degraded mucin, we concluded that the GlcNAc residues which remained and were unmasked at the non-reducing terminal of the mucin oligosaccharides might be responsible for the binding with the ConA column. At the

present time, it remains to be elucidated whether the ligands to ConA are the oligosaccharides bearing peripheral α -linked GlcNAc, such as OP-2 and OP-5 attached to the partially degraded mucin in the final step of the PCS reaction. More detailed experiments are needed to understand the mechanism by which the reaction of 'paradoxical' ConA binding occurs in the PCS sequence.

It has been established from histochemical studies that GS-II lectin is a valuable marker for identifying the gland mucous cell-derived mucus [22,23]. As shown in Figure 6 and Figure 7 (lower panel), HIK1083 reacts more selectively to an α -linked GlcNAc residue than GS-II. As for the reaction in the ELISA well, if GS-II reacts with the same ligand as that of the HIK1083 epitope in the lectin histochemistry of mucins, the latter MAb should render more appropriately and more precisely the histochemical staining of gland mucous cell-derived mucus.

In the human pathophysiological studies using light microscopy, the PCS method has been utilized to detect the appearance of gastric-gland-type cells during the metaplastic and carcinogenic changes in various organs such as the pancreas, lung, ovary and so on [24,25]. From our histochemical observations obtained so far, the specificity of HIK1083 staining could not be distinguished from that of the PCS sequence including the human cases (N. Nakamura, H. Ota, T. Katsuyama, K. Ishihara, M. Kurihara and K. Hotta, unpublished work). The PCS sequence is a rather complicated method compared with that of the immunohistochemical staining by HIK1083. Thus, this MAb should offer a very useful way to detect the gastric-gland-cell-type mucus in the various pathological specimens. Moreover, HIK1083, which is different from the PCS method, might be applied to the ELISA for the detection and quantification of the gastric-gland-type mucin in various body fluids such as serum and gastrointestinal secretions obtained under different physiological and pathological conditions. In summary, HIK1083 will be a very useful tool to recognize the specific mucin molecule and other glycoconjugates bearing the peripheral α -linked GlcNAc residues in immunochemical and immunohistochemical methods.

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