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Establishment of monoclonal antibodies against carbohydrate moiety of gastric mucins distributed in the different sites and layers of rat gastric mucosa

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Eight monoclonal antibodies (MAbs), designated RGM21 ~ RGM42, were generated against mucin purified from the rat gastric mucosa. By applying ELISA, all of these MAbs were proved to react not only with the purified mucin, but also with the oligosaccharide mixture obtained from the antigenic mucin by alkaline borohydride treatment. Treatment of the mucin-attached ELISA well with trypsin, sodium periodate or galactose oxidase prior to the addition of the MAb was applied to characterize these MAbs. Histochemical observation indicated that all these MAbs were able to stain the formalin fixed-paraffin embedded sections of the rat gastroduodenal mucosa. Although each of these MAbs reacted with distinct mucus-producing cells localized in particular regions of the gastroduodenal mucosa, their staining specificity could generally be classified into four groups. These MAbs might be useful for estimating the physiological and pathological changes of mucins in the gastric mucosa.

Keywords: gastric mucin; monoclonal antibody

Introduction

It has been established that a pH gradient, around pH 7 at the surface of the epithelium to approximately pH 2 at the luminal surface, is formed in the gastric mucus gel layer covering the mucosal surface [1, 2], and consequently, the layer is considered to protect the mucosa from acid, pepsin and other damaging agents [3–5]. In the mammalian gastric mucosa, mucus-secreting cells have been mainly classified into two types, namely the surface mucous cells and the gland mucous cells [6, 7]. The mucus accumulated in and/or secreted from these two types of cells in a single tissue section are individually characterized by a dual histochemical staining method [8]; a combination of galactose oxidase-cold thionin Schiff (GOCTS) staining and paradoxical concanavalin A staining (PCS) [9]. It has been demonstrated that these two types of mucus cooperatively construct a stable mucus gel layer, and it is postulated that these two types of mucus have distinct roles in the physiology of the gastric mucosal defence mechanism [10]. A major and important component of mucus is mucin, which is a highly-glycosylated and high molecular weight glycoprotein. It is also noted that mucins present in the antrum of the gastric mucosa are different from those in the corpus region [11]. Although specific sugar residues are considered to be responsible for the biochemical and histochemical diversity of mucins present in the different layers and sites of the gastric mucosa [12], details still remain to be elucidated. For further investigation of the functional role of mucins present in the particular site and layer of gastric mucosa, development of monoclonal antibodies (MAb) are necessary in order to recognize and discriminate the particular oligosaccharide structure attached to the specific type of mucin.

In this study, eight MAbs were raised by immunizing with mucins purified from rat gastric mucosa. These antibodies revealed intense immunoreactions confined to

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the mucin purified from rat gastric mucosa and reacted with the carbohydrate moieties of the mucin molecule.

Materials and methods

ISOLATION OF MUCIN

Female SD rats weighing 250–300 g (SLC Shizuoka, Japan) were killed by exsanguination from the carotid artery after light anesthetization. The stomachs were excised and the luminal surface was washed in ice-cold phosphate buffered saline (PBS). The obtained mucosa was separated into the antrum and corpus regions for the source of A-mucin and C-mucin, respectively. Alternatively, the whole gastric mucosa was utilized as A + C-mucin. Each specimen was extensively lyophilized and pulverized. The extraction and isolation of the mucin from each powdered specimen was performed according to the method of Ohara *et al.* [11] with some modifications. The powdered specimens were suspended in 50 mM Tris-HCl buffer, pH 7.2, heated in a boiling water bath for 5 min and then cooled in an ice bath. After the addition of an equal volume of the Tris-HCl buffer containing 20 mM Na_2EDTA, 2 mM phenylmethyl-sulphoniumfluoride, 20 mM *N*-ethylmaleimide and 5.8 μM pepstatin, as protease inhibitors, the suspension was homogenized using a Polytron tissue homogenizer (model PT 20; Kinematica GmbH, Luzern, Switzerland). After the addition of Triton X-100 up to 2% concentration, the homogenate was solubilized at 37 °C for 1 h with gentle shaking and centrifuged at 10 000 rpm at 4 °C for 30 min. The obtained supernatant was applied onto a Bio-Gel A-1.5 m column (Bio-Rad Laboratories, Richmond, CA) pre-equilibrated with 0.5 M NaCl containing protease inhibitors as previously described, and the column was eluted with the same solution. The void volume fractions (Fr. 1) containing mucin were pooled. For further purification of the mucin, the resulting pooled solution was subjected to duplicate operations of CsCl equilibrium density gradient centrifugation. The starting density of CsCl was 1.4 g ml^{-1} and guanidine chloride (Nacalai Tesque, Inc., Kyoto Japan) was added to make up a 3 M solution. Centrifugations were done at 1.5×10^5 **g** at 10 °C for 85 h (Model 72P, using RPS-40T rotor; Hitachi, Ltd., Tokyo, Japan). The profiles of hexose and protein distribution in the macromolecular materials of the second CsCl density gradient centrifugation showed a very similar pattern, indicating negligible contamination of non-glycosylated protein in the mucin fractions. Peak densities of the purified mucins were 1.35–1.40 g ml^{-1}. The sugar composition (mole/mole of GalNAc-ol) of themucins obtained after alkaline borohydride reduction followed by gel filtration as noted below were as follows: C-mucin; fucose(Fuc) 2.5, galactose(Gal) 5.6, *N*-acetylgalactosamine(GalNAc) 0.6, *N*-acetylglucosamine(GlcNAc) 6.0, *N*-acetylneuraminic acid(NANA) 0.2, mannose and glucose, not detected, A-mucin; Fuc 3.0, Gal 4.5, GalNAc 1.80, GlcNAc 4.5, NANA 0.03; A + C-mucin, Fuc 2.6, Gal 5.4, GalNAc 0.8, GlcNAc5.7, NANA 0.2. The compositions of amino acids of the peptide moiety were similar among the purified mucin preparations and distributed in the following range (mole % of total amino acids); Asx 5.9–7.6, Glx 8.7–9.7, Ser 14.5–16.0, Gly 8.7–9.5, His 4.6–5.4, Arg 1.5–2.1, Thr 15.4–17.9, Ala 4.9–6.7, Pro 10.4–11.4, Tyr 0.8–1.5, Val 4.8–5.2, Met 0.8–1.1, Ile 2.6–2.9, Leu 4.6–5.6, Phe 2.1–2.7, Lys 2.4–3.0. The density and chemical composition of the purified mucins coincide well with that of the typical rat gastric mucin obtained from previous studies in our laboratory [11, 13]. The purified products were used for mouse immunizations and screening of the MAb.

LIBERATION OF OLIGOSACCHARIDES FROM MUCIN

Alkaline borohydride treatment of the purified A + C-mucin was carried out using a slight modification of the procedure of Carlson [14], with 50 mM NaOH in 1.0 M $NaBH_4$ at 50 °C for 16 h. The reaction mixture was then cooled and acidified by the dropwise addition of acetic acid (final pH 4). The resultant solution was applied to a column (1.0 × 60 cm) of Toyopearl HW-50S (Tosoh Corporation, Tokyo, Japan) pre-equilibrated with 0.1 N acetic acid. The column was eluted with the same solution and the eluent was fractionated. The fractions corresponding to oligosaccharides were monitored by hexose measurement and were pooled and evaporated to dryness in vacuo.

PRODUCTION OF MONOCLONAL ANTIBODIES

The MAbs were produced according to the method of Köhler and Milstein [15] using the modification described by Groth *et al.* [16]. Female BALB/c mice were immunized intraperitoneally seven times at 4 week intervals with 100 μg of the purified A-mucin or C-mucin after being emulsified with adjuvant. Freund's complete adjuvant (Difco Laboratories, Detroit, MI) and Freund's incomplete adjuvant (Difco) were used during the first and subsequent immunization times, respectively. On the third day after the boosting, 7×10^7 cells of the splenic lymphocytes from an immunized mouse were fused with 7×10^6 cells of Sp2/0-Ag14 or in some cases F0 mouse myeloma cells using polyethylene glycol 4000 (E. Merck, FRG). Fused cells were seeded in eight 96-well tissue culture plates, and hybridomas were selected in the hybridoma medium (80% RPMI-1640 medium, 20% fetal calf serum) containing hypoxanthine, aminopterin and thymidine (HAT) (Flow Laboratories, Irvine, Scotland). The culture supernatants of multiplying hybridomas were examined for binding activity with the purified A + C-mucin by ELISA. The hybridoma, which showed reactivity with the mucin, was subsequently cloned using the limiting dilution method. Analysis of the antibody

subclass was performed by ELISA using an isotyping kit (PharMingen, San Diego, CA). ELISA and immunohistochemical staining were done using the culture supernatants. To inhibit the growth of contaminating microorganisms, gentamicin was added to the medium at a concentration of 40 mg l^{-1} throughout the experiments.

ELISA AND COMPETITIVE ELISA

Each well of a microtiter plate (Corning, NY) was coated with 100 ng of the A + C-mucin (100 μl per well) in 0.05 M carbonate-bicarbonate buffer, pH 9.6, and kept overnight at 4 °C. The wells were then washed three times with PBS containing 0.05% Tween-20 (Kanto Chemical Co. Inc., Tokyo, Japan), filled with 2% skimmed milk (Difco) in PBS, and incubated for 1 h to avoid any nonspecific adsorption of antibodies. After the wells were washed three times, 100 μl of the monoclonal hybridoma medium was added to each well followed by incubation at ambient temperature for 1 h. The wells were washed three times and 100 μl of horseradish peroxidase (HRP) conjugated goat anti-mouse immunoglobulins (Tago, Inc., Burlingame, CA) was added and incubated for 1 h. After washing the wells, 100 μl of 2,2′-azino-di[3-ethylbenzthiazoline sulfonate(6)] (ABTS)-H_2O_2 solution prepared from a kit (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) was added and the color allowed to develop. The optical density at 415 nm was measured using a MTP-120 microplate reader (Corona Electric Co., Ltd., Ibaragi, Japan).

A competitive ELISA was applied to detect the reactivity of the MAb with the mucin-derived oligosaccharides. The microtiter plate coated with the A + C-mucin followed by blocking with 2% skimmed milk was prepared as previously mentioned. At the same time, the PBS solution of the twofold serial dilution of oligosaccharides (maximum concentration; 200 μg of hexose per well) was pre-incubated with a definite amount of the monoclonal hybridoma medium at ambient temperature for 2 h. The preincubated mixtures were then added to the antigen-coated wells and incubated for 1 h. The remaining steps of the ELISA were the same as already described.

CHARACTERIZATION OF MONOCLONAL ANTIBODY BY THE MODIFICATION OF MUCIN COATED ON THE ELISA PLATE

The MAb reacting with the mucin was characterized by the ELISA using the following procedures.

Trypsin digestion

The microtiter wells coated with the purified mucin antigen were exposed at 37 °C for 1 h to a proteolytic treatment with twofold serial dilution of 2.5 mg ml^{-1} trypsin (Type XIII; Sigma, St Louis, MO), in 10 mM Tris-HCl, pH 8, containing 2 mM $CaCl_2$. Control wells were incubated with the same buffer without the enzyme. After treatment with trypsin, the wells were washed and blocked with 2% skimmed milk. The remaining steps of the ELISA were the same as already described.

Sodium periodate treatment

The method described by Woodward *et al.* [17] was applied in this study. The microtiter wells coated with the A + C-mucin were exposed to periodate as follows. A 100 μl aliquot of the solution containing 0.1–2.5 mM of $NaIO_4$ (Kanto Chemical) in 50 mM sodium acetate buffer, pH 4.5, was added to each well and kept for 1 h at room temperature in the dark. Following a brief rinse with the same buffer without periodate, the wells were blocked with 2% skimmed milk. The remaining steps of the ELISA were the same as already noted.

Galactose oxidase treatment

The mucin antigen was treated with galactose oxidase according to the GOCTS procedure described by Ota *et al.* [8]. The microtiter wells, each coated with the A + C-mucin and dipped in 50 mM potassium phosphate buffer (pH 7.0), were incubated at 37 °C for 4 h with potassium phosphate buffer solution of the twofold serial dilution of galactose oxidase (Sigma, maximum concentration; 2 U per well). Prior to the reaction, the enzyme preparation had been incubated at 50 °C for 30 min to inactivate the contaminating proteases [18]. Control wells were incubated without the addition of the enzyme. After the enzyme treatment, the wells were washed and blocked with 2% skimmed milk. The remaining steps of the ELISA were the same as described.

CHEMICAL ANALYSIS

Hexose was measured according to the phenol-sulfuric acid method of Dubois *et al.* [19] with galactose as the standard. The analysis of sugars consisting of purified mucin was performed on a Model GC7A gas chromatograph (Shimadzu Corporation, Kyoto, Japan) after preparation of their trimethylsilyl derivatives, as described by Sweeley *et al.* [20]. Protein was detected by the protein-dye binding method using the Bio-Rad protein assay kit (Bio-Rad). The amino acid composition was determined using a Pico-Tag™ amino acid analyzer (Waters, Milford, MA) after hydrolysis of the material by the Waters' Workstation in 6 N HCl at 110 °C for 22 h.

IMMUNOHISTOCHEMICAL STAINING

Gastric mucosa including duodenum were obtained from male SD rats weighing approximately 250 g. 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 Kit™ (Nichirei Corporation, Japan). Briefly, endogenous peroxidase activity was blocked with 0.3% H_2O_2 and the tissue was sequentially incubated with 10% 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, Japan) in 50 mM Tris-HCl, pH 7.6, containing 0.005% H_2O_2. The counter stain was performed with Hematoxylin. The culture supernatant of Sp2/0-Ag14 myeloma cells was used as the negative control.

Results

Screening and selection of monoclonal antibodies

Spleen cells from the BALB/c mice that had been immunized with C-mucin or A-mucin that exhibited an immune response to A + C-mucin were fused with Sp2/0-Ag14 myeloma cells. The culture supernatants of proliferating hybridomas showed significant binding activity to the microtiter wells coated with A + C-mucin. Each of the positive hybridomas was cloned using the limiting dilution method and the MAb produced by the clone was designated as RGM plus serial number. As shown in Fig. 1, all of the eight selected antibodies could be detectable by the ELISA pretreated with more than 1–10 ng of the purified mucin per well. The isotype of the selected antibodies was identified as either IgM, κ or IgM, λ by means of ELISA using the isotyping kit (Table 1).

Studies of antigenic determinant by the modification of mucin

In order to characterize the epitope of the established MAbs, the peptide and carbohydrate moieties of the purified mucin attached to the microtiter wells were degraded by trypsin digestion and periodate oxidation, respectively, and the residual antigenic activity was then tested by ELISA. Trypsin digestion did not affect the reactivity of any of the selected antibodies (Table 2), whereas sodium periodate treatment completely destroyed the antigenic activity of all MAbs except RGM41, when used at a concentration of 1.25 mM (Table 2).

Reactivity of the oligosaccharides obtained from the mucin with MAbs

To investigate the reactivity of MAb with oligosaccharides, a mixture of oligosaccharides was prepared from the purified A + C-mucin by alkaline borohydride reduction. The oligosaccharides were examined for antigenic activity to the MAbs by competitive ELISA as described in Materials and methods. As shown in Table 2, mucin-derived oligosaccarides inhibited the reaction of all MAbs with the purified mucin on the ELISA plate. The summarized results in Table 2 indicate that carbohydrate moieties of the mucin are involved in the epitope of all the eight MAbs selected in this study.

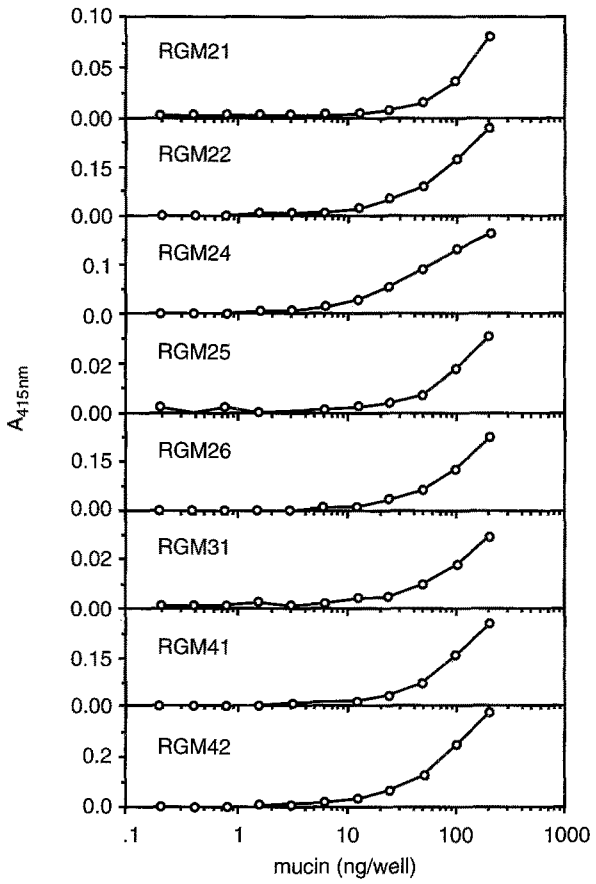

Figure 1. Reactivity of monoclonal antibodies (MAbs) with the mucin (A + C-mucin) prepared from the whole gastric mucosa of SD rat. Two-fold serial dilutions of A + C-mucin were coated on microtiter plates, and the antibody assay was carried out with the monoclonal hybridoma medium and horseradish peroxidase conjugated goat anti-mouse immunoglobulins (Tago, Inc.).

Effect of galactose oxidase treatment of antigenic mucin on the binding activity of MAb

To demonstrate whether the carbohydrate structure positive to the GOCTS reaction is or is not involved in the epitope of selected MAbs, the mucin antigen attached to the well was treated with galactose oxidase and followed by ELISA. As shown in Table 2, antigenicity to RGM21 declined with galactose oxidase treatment in a concentration dependent manner for this enzyme. A slight reduction in the MAb binding could also be observed in the case of RGM31. The reactions of other MAbs on the ELISA plate, however, were not positive to the pretreatment of the well with this enzyme.

Immunohistochemical studies on the antigenic distribution

To survey the antigenic mucin distribution in the rat gastroduodenal mucosa, an immunohistochemical

Table 1. Newly established anti-rat gastric mucin monoclonal antibodies from this study. The A-mucin and C-mucin were prepared from antrum and corpus regions of rat gastric mucosa, respectively. Isotype of each antibody was examined by ELISA using isotyping kit (PharMingen).

Code	*Immunogen*	*Myeloma*	*Isotype*
RGM21	C-mucin	Sp2/0-Ag14	IgM, κ
RGM22	C-mucin	Sp2/0-Ag14	IgM, κ
RGM24	C-mucin	Sp2/0-Ag14	IgM, λ
RGM25	C-mucin	Sp2/0-Ag14	IgM, κ
RGM26	C-mucin	Sp2/0-Ag14	IgM, κ
RGM31	C-mucin	F0	IgM, λ
RGM41	A-mucin	Sp2/0-Ag14	IgM, κ
RGM42	A-mucin	Sp2/0-Ag14	IgM, κ

technique was applied to the formalin-fixed sections of the rat gastroduedenal mucosa. As summarized in Table 3, antigen to RGM21, 24, and 31 could only be detected in the surface epithelial layer of both the corpus and antrum of the stomach and the villus epithelium of the duodenum. Immunohistochemical reactions of these three MAbs were distinct from antral surface mucous cells and duodenal goblet cells. RGM21 and RGM31 reacted slightly with antral surface epithelial, while RGM24 strongly reacted with this area of the antral mucosa. The strongest reaction to duodenal goblet cells was achieved by RGM31 while the reaction of RGM21 was very weak for this type of cell. RGM22, RGM25, RGM26 and RGM42 had common staining specificity to rat gastroduodenal mucosa. These MAbs reacted strongly with the antral surface epithelial cells, but did not entirely react with either the surface mucous cells of the corpus or the villus epithelial and the goblet cells of the duodenum. Of the four MAbs, RGM22, RGM25 and RGM42 had a strong reaction to the pyloric gland cells of the deep antral region as well as a faint reaction with mucous neck cells of the deep corpus region, while RGM26 showed a weak or no reaction to these regions, respectively. Different from the other seven MAbs, RGM41 reacted selectively and strongly with the mucous neck cells of the corpus, the pyloric gland cells of the antrum and Brunner's gland cells of the duodenum. Immunohistochemical observation of rat gastric corpus and antral mucosae stained with four typical MAbs of different types is shown in Fig. 2.

Discussion

It has been histologically characterized that there are more than two types of mucus secreting cells each producing and secreting distinct types of mucin in the mammalian gastric mucosa [18]. Mucins present on the surface epithelial cells of the corpus and antrum are specifically stained blue with the GOCTS method [8]. The gastric gland type mucous cells present in the deep layer of the gastric mucosa, including mucous neck cells of the corpus and pyloric gland cells of antrum, are specifically stained brown with the PCS method [8]. In addition, the high-iron diamine (HID) staining method indicates the localization of sulfated mucins in the isthmus region of the rat corpus mucosa [21]. Recently, combined with Carnoy's fixation method, Ota *et al.* demonstrated that the surface mucus gel layer of the human stomach, which is known to be involved in the gastric defense mechanism, consists of the GOCTS positive and PCS positive mucins having an alternating laminated array, and postulated that the gland mucous cell-derived mucins contribute to form a stable mucus gel layer as well as the surface mucous cell derived-mucin [10].

Recently, we devised a series of mucus scraping methods to separate the mucus gel layer and the surface epithelial layer from the remaining deep mucosa of the rat stomach [22]. By utilizing these methods, we have demonstrated the effects of various agents on the accumulation and/or secretion of mucin present in the specific sites of the rat gastric mucosa. The

Table 2. Reactivity of the monoclonal antibodies with the modified mucins and with the mucin-derived oligosaccharides. The mucin coated on microtiter plates was exposed to trypsin, sodium periodate or galactose oxidase solution, and antigenic activity for each antibody was assessed by the ELISA method. Antigenic activity of oligosaccharides was assessed by the competitive ELISA as described under Materials and Methods.

MoAb Code	*Reactivity with modified antigen*			*Competitive ELISA mucin-derived oligosaccharides*
	Trypsin	*Periodate*	*Galactose oxidase*	
RGM21	Unaltered	Reduced	Reduced	Inhibited
RGM22	Unaltered	Reduced	Unaltered	Inhibited
RGM24	Unaltered	Reduced	Unaltered	Inhibited
RGM25	Unaltered	Reduced	Unaltered	Inhibited
RGM26	Unaltered	Reduced	Unaltered	Inhibited
RGM31	Unaltered	Reducd	Reduced	Inhibited
RGM41	Unaltered	Unaltered	Unaltered	Inhibited
RGM42	Unaltered	Reduced	Unaltered	Inhibited

Table 3. Immunohistochemical reactivity of the monoclonal antibodies with SD rat stomach and duodenum.

MoAb Code	*Corpus*		*Antrum*		*Duodenum*		
	SMC	*MNC*	*SMC*	*PGC*	*Villus*	*Goblet*	*Brunner's*
RGM21	+++	–	t	–	+++	t	–
RGM22	t	t	+++	+++	–	–	++
RGM24	+++	–	+++	–	+++	+	–
RGM25	–	–	+++	+++	–	–	++
RGM26	–	–	+++	+	–	–	+
RGM31	+++	–	t	–	+++	++	+
RGM41	–	+++	–	+++	–	–	+++
RGM42	t	t	+++	+	–	–	t

The figures in the Table indicate staining intensity, namely: –, negative; t, <10% positive cells; +, <30% positive cells; ++, <60% positive cells; +++, ≥60% positive cells. Abbreviations: SMC, surface mucous cell; MNC, mucous neck cell; PGC, pyloric gland cell; villus, villus epithelial cell; goblet, goblet cell; Brunner's, Brunner's gland cell.

administration of an acid secretagogue tetragastrin caused an increase of mucin in the mucus gel as well as the surface epithelial layer of the corpus region [23]. The M_1 muscarinic receptor antagonist pirenzepine accumulated mucin at the deep corpus layer [24]. These observations strongly indicate the presence of a distinct control mechanism by which the biosynthesis and accumulation of mucin at the specific region and layer of the gastric mucosa takes place. It seems, therefore, very important to devise a highly sensitive and highly specific method such as the establishment of a monoclonal antibody to detect the gastric mucin of different origin.

All eight MAbs selected in this study recognized the carbohydrate moieties of the rat gastric mucin. From the immunohistochemical characterization using rat gastroduodenal mucosal sections, these MAbs could be classified into four groups, each possibly recognizing the particular carbohydrate chains attached to the mucins localized in the different sites and layers, although each of these MAbs did not have the same detailed features.

RGM21 and RGM31 are included in the first group. These MAbs strongly react with the surface mucous cells of the corpus and the villus epithelial cells, while partly recognizing the antral surface mucous cells. Pretreatment of the antigenic mucin coated-well with galactose oxidase reduced the subsequent reaction of these MAbs, indicating that peripheral Gal or GalNAc residues are involved in the epitope of these MAbs [25, 26]. In our previous report, a MAb, RGM11, whose reaction was susceptible to the galactose oxidase pretreatment, was shown to have very similar immunohistochemical characteristics to these MAbs [27]. Moreover, Seraclone™ anti-Lewis b MAb (Biotest AG, FRG) stained the rat gastric mucosal section cognate with RGM21 including the staining specificity to the cardiac mucosa localized at the oral edge of the corpus region (unpublished observation). Ota *et al.* observed that RGM31 reacted with the red blood cells in the fixed section of blood type H individuals (unpublished observation). These results indicate that RGM21 and RGM31 might react with a carbohydrate chain classified as Lewis b or H which is specifically present in the surface epithelial cell-derived mucin of the corpus and duodenum.

RGM24 strongly reacts not only with the surface mucous cells of the corpus and duodenum but also with the antral surface mucous cells, and on this particular point, immunohistochemically differs from RGM21 and RGM31. From the inhibition assay using the mucin derived-oligosaccharides fractionated by Toyopearl HW-50S column chromatography, RGM24 reacted with oligosaccharides of much higher molecular weight than those that reacted with RGM21 (unpublished observation).

RGM22, RGM25, RGM26 and RGM42 are included in the third group and have common characteristics concerning immunohistochemical specificities. These MAbs strongly reacted with the entire mucosal cells of the antral region but rarely reacted with the corpus mucous cells. Ota *et al.* observed that RGM22, RGM26 and RGM42 reacted with the red blood cells in the fixed section of blood type A individuals (unpublished observation). Ohara *et al.* demonstrated that the carbohydrate composition of mucin obtained from the antral mucosa of the rat stomach differs from that of the corpus region, especially in the presence of *N*-acetylgalactosamine residues not located in the peptide linkage region of the oligosaccharide chains of the antral mucin [11]. It is, therefore, conceivable that the *N*-acetylgalactosamine residue in the antral mucous cells might react with these MAbs of the third group. Different from the other three MAbs, RGM25 did not react with any red blood cells of the ABH type. This MAb could not stain the cardiac mucosa, while the other three MAbs reacted strongly with the surface mucous cells of this narrow area. These

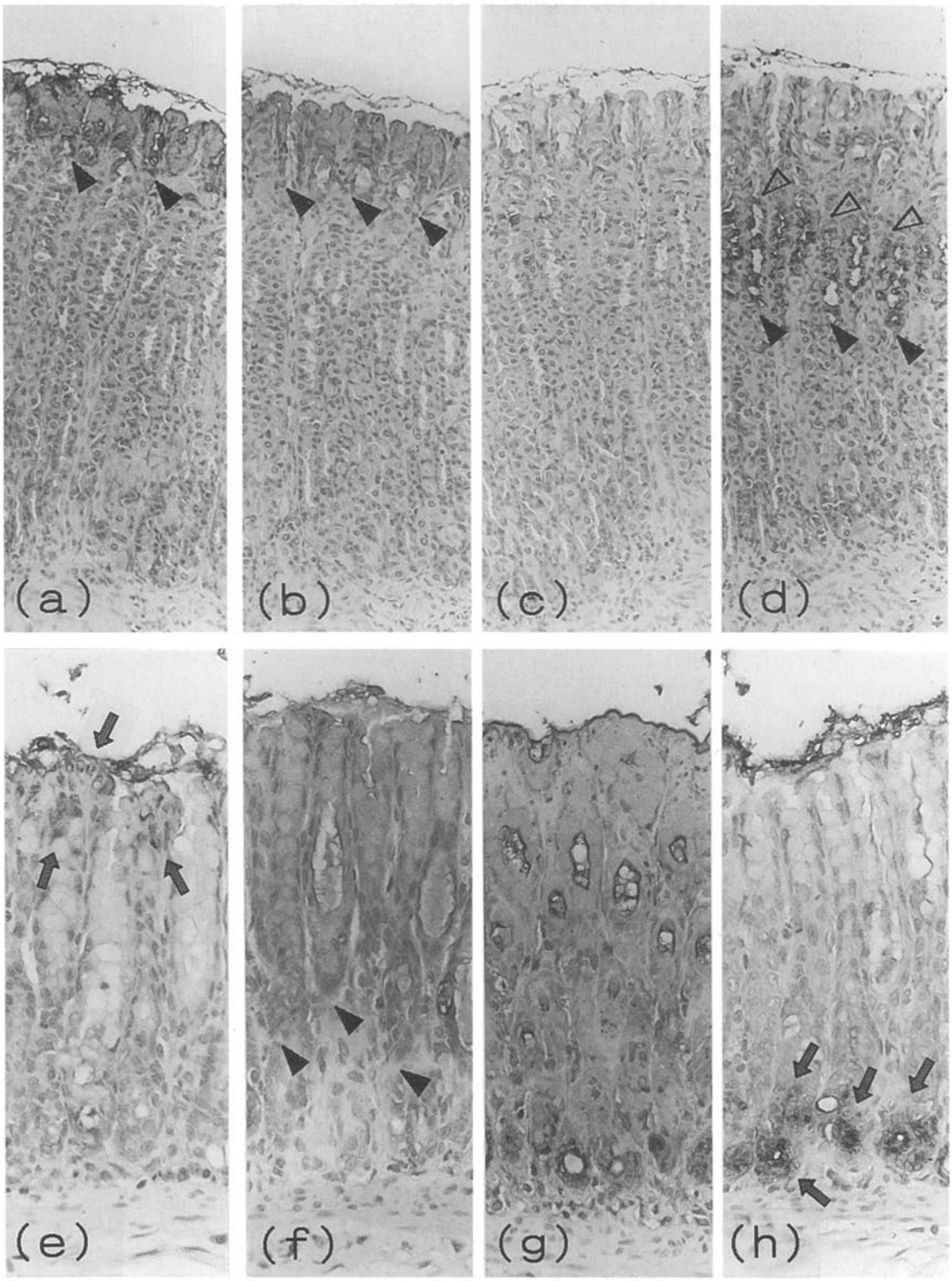

Figure 2. Light microscopic observations of SD rat gastric corpus (a–d, magnification, × 180) and antral (e–h, magnification, × 270) mucosae stained with RGM21 (a, e), RGM24 (b, f), RGM22 (c, g), and RGM41 (d, h). Open arrowheads and closed arrowheads indicate the upper edge and lower edge of the stained area, respectively. Arrows indicate the positively stained cells. The counterstain was performed with Hematoxylin.

observations indicate that RGM25 might react with an oligosaccharide different from that of the other MAbs included in the same group.

Different from other MAbs, only RGM41 recognizes the gland mucous cell-derived mucin localized in the mucous neck cells of the corpus, pyloric gland cells and Brunner's gland cells of the duodenum. These types of cells or mucin derived from these cells have been generally recognized by the PCS method or by a lectin, *Griffonia simplicifolia*-II (GS-II) [28]. So far reactivity of RGM41 is almost similar to these conventional staining methods. In an earlier study done at our laboratory, Hotta *et al.* demonstrated that human gastric mucin, which had been sequentially oxidized and reduced according to the PCS sequence, effectively bound to a concanavalin A-bonded Sepharose, and from the carbohydrate composition of the partially degraded mucin, they concluded that the GlcNAc residues, which remained and were unmasked at the non-reducing terminal of the mucin oligosaccharides, might be the ligand to the ConA in the PCS reaction [29]. At the present time, it remains to be elucidated whether the ligands to ConA in the PCS sequence are identical to the epitope sugar residue for RGM41. In relation to this, it is interesting that the epitope sugar structure of RGM41 is solely resistant to the periodate oxidation among the present MAbs.

We have established eight distinct MAbs each reacting with the carbohydrate moieties of the rat gastroduodenal mucins, and these can be classified into at least four groups. From the point of gastric physiology and pathology, especially regarding the mucosal protective mechanism, it is important to obtain more evidence on the quantitative and qualitative changes of mucin localized in the distinct sites and layers of the gastric mucosa. The eight MAbs described above should be useful tools to more selectively and more precisely recognize specific mucin species in the gastroduodenal mucosa than other tools such as antimucin antisera and lectins [30]. Further studies on the structural analyses of the epitope carbohydrates will provide us more information on this issue.

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