The monoclonal antibody HCM31 specifically recognises the Sd\textsuperscript{a} tetrasaccharide in goblet cell mucin

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1. Introduction

The gastrointestinal mucus covering the mucosal surface is considered a major factor in the gastrointestinal defense mechanism against various infectants such as microorganisms, viruses and parasites [1]. Mucin, a highly O-glycosylated glycoprotein with a high molecular mass, is a major component of the gastrointestinal mucus and plays an important role in the mucus barrier covering mucosal surfaces. Mucins are located site-specifically in the gastrointestinal mucosa according to the distinct type of core protein [2–4] and attached carbohydrates [5,6]. For the precise characterization of individual mucins on a biochemical and physiological basis, many anti-mucin monoclonal antibodies (mAbs) reacting with specific types of carbohydrate chains of gastrointestinal mucins have been developed in our laboratory, and their properties have been characterized histochemically and biochemically. Histochemi-
tical studies show that distinct types of mucins have specific stain-
ability with mAbs [7–9]. The epitope of an mAb, HIK1083, which
stains mucins from mucus neck cells and pyloric gland cells, has
been determined as a peripheral α-linked GlcNAc on the mucin oli-
gosaccharides [9]. Recently, gastric mucins with this epitope on
their oligosaccharides have been shown to act as a natural antibi-
otic protecting the host from Helicobacter pylori [10] and to play an
essential role in preventing gastric cancer [11]. Thus, epitope anal-
ysis of an mAb that reacts with a specific oligosaccharide chain
bound to the mucin molecules is needed to clarify the biological
function of the particular oligosaccharides.

The sialomucins with specific sialylated oligosaccharide deter-
minants are considered to have multiple biological functions. The
sialyl 6-sulfo Lewis x determinant on cell-surface-associated sia-
locmun is expressed on the high endothelial venules in lymph
nodes as a major ligand for L-selectin in order to allow lymphocyte
homing [12]. Increasing serum levels of sialomucin having the sial-
yl Lewis A determinant are correlated with increasing tumor metastasis [1]. In gastrointestinal mucosa, sialomucins as mucus components are secreted by the epithelial cells and have diverse sialylated oligosaccharide structures. However, the distribution and the function of individual gastrointestinal sialomucins having distinct types of sialylated oligosaccharides are poorly understood. Appropriate mAbs are needed to distinguish the specific types of sialomucins.

Recently, we developed an mAb, HCM31, which reacts with
sialylated oligosaccharides of rat small intestinal mucins [13].
Although HCM31 only partially stains the jejunal goblet cells in
normal rat, HCM31-positive goblet cells increased remarkably
during the processes of regeneration from mucosal damage caused by
the administration of an antineoplastic chemotherapy drug [14]
and nonsteroidal anti-inflammatory drugs [15]. Furthermore,
HCM31-positive goblet cells were found to increase remarkably
after infection with the intestinal nematode Nippostrongylus brasili-
ensis (N.b) [16]. HCM31-reactive sialomucins are therefore consid-
ered to play an important role in the physiological and pathological
changes in the gastrointestinal mucosa.

In this study, to characterize the epitope recognized by HCM31,
the oligosaccharides reacting with this mAb were obtained from
small intestinal mucins of N.b-infected rats and their structures
were analyzed by tandem mass spectrometry (MS/MS) and NMR
spectroscopy. The oligosaccharides obtained from uninfected rats
were also analyzed to verify specific expression of the epitope of
HCM31 after N.b infection. In this paper, the unique epitope se-
quence containing a sialic acid residue and the histochemical dis-
tribution of the sialomucins recognized by this mAb in human
normal and cancer gastrointestinal tract are presented.

2. Results

2.1. Studies of antigenic determinant of HCM31 by the modification of mucin

To characterize the epitope of HCM31, an mAb developed using
human colonic mucin as an antigen, periodate oxidation and tryp-
sin digestion of the purified rat mucin were performed to degrade
the carbohydrate and peptide moieties, respectively, and then the
residual antigenic activity was tested by ELISA. Periodate oxidation
reduced the antigenic activity to HCM31, whereas trypsin diges-
tion did not affect the reactivity of this mAb (data not shown).
These results indicate that the carbohydrate moieties of the mucin
were involved in the epitope of HCM31.

Fig. 1 shows the immunohistochemical observations of rat jeju-
nal mucosa stained with HCM31. Only a small number of goblet
cells were stained on uninfected rat jejunum (Fig. 1a). In contrast,
The inhibition assay indicated that IA1 and IA2 significantly reacted with HCM31 (Fig. 2d), whereas UA1 did not react with HCM31, but UA2 did (Fig. 2c). The reactivity of IA2 was higher than that of UA2. These findings indicated that the oligosaccharides reacting with HCM31 were acidic; this result is consistent with the immunohistochemical examination using neuraminidase treatment (Fig. 1).

UA1 and IA1 were chosen for further analyses because IA1 reacted with HCM31 whereas UA1 did not (Fig. 2). UA1 and IA1 were further purified by two steps of normal-phase HPLC using TSK-Gel Amide-80 columns. By the first-step HPLC, eight major fractions, designated as UA1-1 to -8 and IA1-1 to -8, were obtained from uninfected (Fig. 3a; upper panel) and infected rats (Fig. 3a; lower panel), respectively. The inhibition assay showed that fractions IA1-5 and IA1-8 (Fig. 3b) and IA1-6 and IA1-7 (data not shown) significantly reacted with HCM31, whereas fractions IA1-2 and IA1-3 (Fig. 3b) and IA1-1 and IA1-4 (data not shown) did not. None of UA1-1 to -8 reacted with this mAb (data not shown).

The oligosaccharides fractionated by the first-step HPLC were analyzed by MALDI-TOF/MS in negative mode (Table 1). The masses of the oligosaccharides were distributed from 675 to 1746, corresponding to tri- to nona-saccharides. The sugar compositions of all the tested oligosaccharides were assigned to the appropriate acidic oligosaccharide–alditols, bearing either a sulfate or a sialic acid residue, as well as having GalNAc-ol at the reducing terminus on the basis of their masses. Because all HCM31-reactive fractions (IA1-5, -6, -7 and -8 in infected rat) contained sialylated oligosaccharides, these data support the idea that HCM31 reacted with sialylated carbohydrate sequence (Fig. 1). Fig. 4a shows MS spectra of UA1-5 and IA1-5. Two oligosaccharides (m/z [M–H]⁻ of 1097 and 1284) were detected in IA1-5, but not in UA1-5, as also shown in Table 1. Similarly, three oligosaccharides (m/z [M–H]⁻ of 1486, 1535 and 1592) were detected in IA1-8, but not in UA1-8 (Fig. 4b, Table 1).

Because it was expected that HCM31-reactive oligosaccharides were induced by N. b infection, each of IA1-5 and IA1-8 was further purified by the second-step HPLC and characterized. Fraction IA1-5 separated into five fractions, designated IA1-5a, -5b, -5c, -5d and -5e (Fig. 5a; lower panel), corresponding to m/z [M–H]⁻ of 1121, 1097, 1186, 1284 and 1186, respectively (Table 2). Fraction IA1-8 was separated into two fractions, designated IA1-8a and -8b (Fig. 5b; lower panel), corresponding to m/z [M–H]⁻ of 1072 and 1186, respectively (Table 2). Fraction IA1-8b was further purified by the second-step HPLC and characterized. Fraction IA1-8b separated into two fractions, designated IA1-8b1 and -8b2 (Fig. 6b; lower panel), corresponding to m/z [M–H]⁻ of 1072 and 1186, respectively (Table 3). Fraction IA1-8b2 was further purified by the second-step HPLC and characterized. Fraction IA1-8b2 separated into two fractions, designated IA1-8b2a and -8b2b (Fig. 6b; lower panel), corresponding to m/z [M–H]⁻ of 1072 and 1186, respectively (Table 3).
rides assigned as IA1-5d and IA1-8b, respectively (Fig. 5), this result (Fig. 6). Because UA1-5 and IA1-8b significantly reacted with HCM31, purified HCM31-reactive oligosaccharides, IA1-5d and IA1-8b, were analyzed by MS/MS, amino sugar analysis and NMR spectroscopy.

**Table 1**

Differences of mucin oligosaccharide separated by the first-step HPLC between uninfected and N.b-infected rats: identified by MALDI-TOF/MS. Oligosaccharides detected only in the infected rats are noted in bold text.

| Fraction | [M-H] (m/z) | Expected composition of oligosaccharide-alditols | Uninfected (U) | Infected (I) |
|----------|-------------|-------------------------------------------------|----------------|--------------|
| A1-1     | 675         | (NeuAc)(Hex)GalNAc-ol                            | +              | +            |
| A1-2     | 813         | (SO_3H)(dHex)(Hex)GalNAc-ol                      | +              | +            |
| A1-3     | 878         | (NeuAc)(Hex)GalNAc-ol                            | +              |              |
| A1-4     | 975         | (SO_3H)(dHex)(Hex)GalNAc-ol                      | +              | +            |
| A1-5     | 1097        | (NeuGc)(dHex)(Hex)GalNAc-ol                      |                |              |
| A1-6     | 1186        | (NeuAc)(dHex)(Hex)GalNAc-ol                      | +              |              |
| A1-7     | 1178        | (SO_3H)(dHex)(Hex)GalNAc-ol                      |                |              |
| A1-8     | 1486        | (SO_3H)(dHex)(Hex)GalNAc-ol                      |                |              |

* The compositions were determined by MS/MS analysis in terms of whether they contained NeuAc or NeuGc.

To obtain further structural information for the epitope of HCM31, purified HCM31-reactive oligosaccharides, IA1-5d and IA1-8b, were analyzed by MS/MS, amino sugar analysis and NMR spectroscopy. MS/MS analyses of the oligosaccharides were performed using positive mode after methylesterification of the sialic acid residue.

### Table 2

Oligosaccharides separated by the second-step HPLC from small intestine in N.b-infected rats: identified by MALDI-TOF/MS.

| Fraction | [M-H] (m/z) | Expected composition of oligosaccharide-alditols |
|----------|-------------|-----------------------------------------------|
| IA1-5a   | 1121        | (SO_3H)(dHex)(Hex)GalNAc-ol                    |
| IA1-5b   | 1097        | (NeuGc)(Hex)GalNAc-ol                          |
| IA1-5c   | 1186        | (NeuAc)(dHex)(Hex)GalNAc-ol                    |
| IA1-5d   | 1284        | (NeuAc)(Hex)GalNAc-ol                          |
| IA1-5e   | 1186        | (NeuAc)(dHex)(Hex)GalNAc-ol                    |
| IA1-8a   | 1486        | (SO_3H)(dHex)(Hex)GalNAc-ol                    |
| IA1-8b   | 1592        | (NeuAc)(dHex)(Hex)GalNAc-ol                    |
| IA1-8c   | 1535        | (SO_3H)(dHex)(Hex)GalNAc-ol                    |

2.3. Structural analysis of sialylated oligosaccharides recognized by HCM31

To obtain further structural information for the epitope of HCM31, purified HCM31-reactive oligosaccharides, IA1-5d and IA1-8b, were analyzed by MS/MS, amino sugar analysis and NMR spectroscopy. MS/MS analyses of the oligosaccharides were performed using positive mode after methylesterification of the sialic acid residue.

2.3.1. Structural analysis of IA1-5d

Amino sugar analysis of IA1-5d showed that the molar ratio of GalNAc-ol:GalNAc and GlcNAc was 1.0:1.0:2.1. Fig. 7a shows the fragmentation spectrum of IA1-5d ([M-H] - 1322 as [M+Na]+) by MS/MS analysis. In this spectrum, the core 4 structure was identified by the diagnostic ion Y2 at m/z 652. The series of Bi ions at m/z 591 (HexNAc-HexNAc), 693 (NeuAc-Hex,HexNAc or NeuAc-[HexNAc]2) and 896 (NeuAc-[HexNAc]2) were identified by the fragment ions, and Y1+Y2 at m/z 916 (NeuAc-Hex,HexNAc-GalNAc-ol) allowed reconstruction of the sequence as follows: NeuAc-GalNAc-GlcNAc-GalNAc-ol illustrated in Fig. 7a. Fig. 8a shows the one-dimensional^1 H NMR spectrum of IA1-5d ([M-H] - 0.15 mg). In the spectrum, β-anomeric resonances (4.69, 4.56, 4.52
and 4.51 ppm) were recognized as one residue of the β-linked GalNAc, two residues of the β-linked GlcNAc, and one residue of the β-linked Gal, respectively, by their coupling to a high-field H-2 resonance and the pattern of the cross-peaks in the TOCSY spectrum (data not shown). The carbohydrate composition of IA1-5d obtained from the NMR spectrum agreed with that expected by the data obtained from the molecular mass and amino sugar analyses. From the 13C chemical shifts of the heteronuclear multiple-quantum coherence spectroscopy (HMQC) spectra of IA1-5d (Table 3), no substitution could be observed on one of the two β-linked GlcNAc residues, indicating that this GlcNAc residue is present at the non-reducing terminal in this structure. Another β-linked GlcNAc is attached at position 4 (3.73 ppm), which could be proved by the lower field changes in the HMQC spectrum (+4.0 ppm) of GlcNAc compared with that of the standard β-methylated GlcNAc [9].

Table 3

| Sugar                  | IA1-5d | IA1-8b | Standards
|------------------------|--------|--------|-----------
| 1H        | 13C    | 1H        | 13C     | 13C   |
| GalNAc-ol          | 3.58/3.84 | 62.7 | 3.58/3.83 | 62.7 | 61.5 |
| Position 1          | 4.24   | 51.4 | 4.24 | 51.3 | 51.5 |
| Position 2          | 3.94*  | 76.6* | 3.93* | 77.0* | 68.4 |
| Position 3          | 3.47   | 69.1 | 3.45 | 69.3 | 69.4 |
| Position 4          | 4.20   | 67.7 | 4.17 | 67.9 | 69.8 |
| Position 5          | 3.66/3.86* | 71.0* | 3.66/3.86* | 71.1* | 63.2 |
| Position 6          | 2.04   | 22.2 | 2.06 | 22.2 | 21.7 |
| Ac-CH3              | 2.04   | 22.2 | 2.06 | 22.2 | 21.7 |
| β-GlcNAc3a          | 4.56   | 102.3 | 4.53 | 101.5 | 101.9 |
| Position 1          | 3.71   | 55.7 | 3.76 | 55.1 | 55.4 |
| Position 2          | 3.55   | 73.4 | 3.63 | 72.3 | 73.9 |
| Position 3          | 3.44   | 69.9 | 3.73* | 73.9* | 69.9 |
| Position 5          | 3.42   | 75.6 | 3.44 | 75.0 | 75.8 |
| Position 6          | 3.71/3.91 | 61.0 | 3.76/3.97 | 61.0 | 60.7 |
| Ac-CH3              | 2.00   | 22.0 | 2.02 | 21.9 | 21.9 |
| β-Gal4,3a           | 4.52   | 101.5 | 4.52 | 102.7 | 101.9 |
| Position 1          | 3.72   | 54.8 | 3.76 | 55.1 | 55.4 |
| Position 2          | 3.66   | 74.6 | 3.67 | 74.6 | 73.9 |
| Position 3          | 3.73*  | 73.9* | 3.72* | 73.9* | 69.9 |
| Position 4          | 3.56   | 74.7 | 3.55 | 74.9 | 75.8 |
| Position 5          | 3.76/3.96 | 60.4 | 3.73/3.95 | 60.4 | 60.7 |
| Ac-CH3              | 2.02   | 22.1 | 2.02 | 22.2 | 21.9 |
| β-Gal4,6a           | 4.52   | 100.1 | 100.1 | 100.1 | 103.7 |
| Position 1          | 3.72   | 54.8 | 3.76 | 55.1 | 55.4 |
| Position 2          | 3.66   | 74.6 | 3.67 | 74.6 | 73.9 |
| Position 3          | 3.73*  | 73.9* | 3.72* | 73.9* | 69.9 |
| Position 4          | 3.56   | 74.7 | 3.55 | 74.9 | 75.8 |
| Position 5          | 3.76/3.96 | 60.4 | 3.73/3.95 | 60.4 | 60.7 |
| Ac-CH3              | 2.02   | 22.1 | 2.02 | 22.2 | 21.9 |
| α-Fuc2,4a           | 5.17   | 99.3 | 99.4 |
| Position 1          | 3.74   | 67.9 | 67.8 |
| Position 2          | 4.22   | 66.4 | 66.4 |
| Position 5          | 1.19   | 15.2 | 15.2 |
| β-GalNAc            | 4.69   | 102.7 | 102.6 | 99.0 |
| Position 2          | 3.87   | 52.2 | 3.88 | 52.2 | 52.0 |
| Position 3          | 3.64   | 71.3 | 3.64 | 71.1 | 70.5 |
| Position 4          | 3.86   | 67.9 | 3.86 | 67.7 | 67.5 |
| Ac-CH3              | 1.97   | 22.5 | 1.97 | 22.5 | 22.3 |
| α-NeuAc             | 1.99   | 22.3 | 1.99 | 22.3 | 22.2 |

A superscript at a monosaccharide residue indicates to which position of the adjacent monosaccharide it is glycosidically linked. Two superscripts map out the pathway from the residue toward the GalNAc-ol residue.

Standards are α- and β-methyl derivatives of each component sugar except GalNAc-ol.

Fig. 7. MS/MS spectra of oligosaccharides, IA1-5d (a) and IA1-8b (b). Precursor ions are indicated by an asterisk. Fragmentation uses the Domon and Costello nomenclature [37].

D. Tsubokawa et al. / FEBS Open Bio 2 (2012) 223–233
presented the C6 side of the reducing-end GalNAc-ol residue. Finally, it was proposed that oligosaccharide IA1-5d had the following structure:

\[
\text{GalNAc}\beta 1-4\ 
\text{NeuAc}\alpha 2-3\text{Gal}\beta 1-4\text{GlcNAc}\beta 1-6-\text{GalNAc}\text{-ol}
\]
\[
\text{GlcNAc}\beta 1-3
\]

2.3.2. Structural analysis of IA1-8b

Fig. 7b shows the fragmentation spectrum of IA1-8b (m/z 1630 as [M+Na]+). This oligosaccharide has the same branch having NeuAc as IA1-5d because the series of Bi ions at m/z 591, 693 and 896 and Y1/a/Y3/b ion at m/z 916 were also detected. The existence of Y2/a/Y2/b or Y2/b/Y1/a ion at 449 (HexNAc-GalNAc-ol), but not at 408 (Hex-GalNAc-ol), indicated that this structure is based on a core 3 or 4 structure. The B3/a ion at 534 indicated a fragment of (dHex)Hex-HexNAc. From these fragment ions and data of amino sugar analysis (GalNAc-ol:GalNAc:GlcNAc, 1.0:1.0:2.0), the structure was estimated to be NeuAc-\{(GalNAc)-Gal-GlcNAc-\}Fuc-Gal-GlcNAc-ol. From these fragment ions and data of amino sugar analysis (GalNAc-ol:GalNAc:GlcNAc, 1.0:1.0:2.0), the structure was estimated to be NeuAc-\{(GalNAc)-Gal-GlcNAc-\}Fuc-Gal-GlcNAc-ol. Fig. 8b shows the one-dimensional 1H NMR spectrum of IA1-8b (\(\approx 0.2\) mg). \(\beta\)-anomeric resonances (4.69, 4.62, 4.53, 4.52 and 4.51 ppm) were recognized as one residue of the \(\beta\)-linked GalNAc, one of two residues of the \(\beta\)-linked Gal, two residues of the \(\beta\)-linked GlcNAc, and one residue of another \(\beta\)-linked Gal, respectively, by their coupling to a high-field H-2 resonance and the TOCSY spectrum. One lower-field \(\alpha\)-anomeric resonance was also recognized as one residue of the \(\alpha\)-linked Fuc by a method similar to that described above. The Fuc residue attached to the one \(\beta\)-linked Gal residue at position 2 (3.57 ppm), which could be proved by the lower-field changes in the HMOC spectrum (+5.6 ppm) of Gal [9]. The glycosylation shifts were observed at positions 3 and 6, positions 3 and 4, and position 4 of the GalNAc-ol, another \(\beta\)-linked Gal and two residues of the \(\beta\)-linked GlcNAc, respectively, on the basis of the 13C chemical shift assessment (Table 3). The structure was confirmed by the milder periodate oxidation: two fragments, corresponding to NeuAc-(GalNAc)-Gal-GlcNAc-O–CH2–CHO and Fuc-Gal-GlcNAc-O–CH(CHO)–CH(NHCOCH3)–CH2–CHO, were obtained from IA1-8b. The structure of IA1-8b was estimated as follows:

\[
\text{GalNAc}\beta 1-4\ 
\text{NeuAc}\alpha 2-3\text{Gal}\beta 1-4\text{GlcNAc}\beta 1-6-\text{GalNAc}\text{-ol}
\]
\[
\text{Fuc}\alpha 1-2\text{Gal}\beta 1-4\text{GlcNAc}\beta 1-3
\]

2.4. Structural analysis of other sialylated oligosaccharides

Because IA1-6 and IA1-7 also reacted with HCM31, MS/MS analyses of sialylated oligosaccharides contained in these fractions were achieved after the first-step HPLC. The fragmentation spectrum of IA1-6 (m/z 1427 as [M+Na]+) included the series of Bi ions at m/z 591, 693 and 896 and Y1 ion at m/z 916 (data not shown), which indicated the presence of the sequence, NeuAc-\{(HexNAc)Gal-GlcNAc-GalNAc-ol. Y1 ions at m/z 408 and 449 indicated that this oligosaccharide had a core 2 structure. Therefore, the
AI-6 may include the oligosaccharide Fuc-Gal-[NeuAc-HexNAc-Gal]-GalNAc-o1, having core 2 and a terminal sequence, NeuAcx2-3(GalNAc)-Gal[Gal1-4-GlcNAcy]. Although AI-7 reacted with HCM31, the MS/MS data did not allow reconstruction of the sequence owing to the low intensity of the fragment ions. MS/MS analyses of sialylated oligosaccharides in UA1-1 to -8 and AI-1-1 to -4, none of which reacts with HCM31, were also achieved (Table S1). The fragmentation spectra for the sialylated oligosaccharides showed that all oligosaccharides contained Y ion at m/z 408, which indicated the presence of a core 1 or 2 structure, but not at 591 (HexNAc-HexNAc) and 896 (HexNAc-HexHexNAc). These results suggest that the HCM31-unreactive sialylated oligosaccharide fractions contain neither the core 4 structure nor NeuAc-(GalNAc)-Gal-GlcNAc- sequence. This result strongly suggests that the core 4 oligosaccharides having a tetrasaccharide sequence, NeuAcr2-3GalNAcβ1-4Galβ1-4GlcNAcy- (Sdα antigen), include the epitope of HCM31.

2.5. Comparison of antigenic specificities of HCM31 with anti-Sdα and anti-GM2 monoclonal antibodies

Because the Sdα antigen, NeuAcr2-3(GalNAcβ1-4)Galβ1-4GlcNAcy-, has a common terminal trisaccharide sequence with GM2 ganglioside, NeuAcr2-3(GalNAcβ1-4)Galβ1-4Glcβ-Cer, the antigenic specificity of HCM31 was compared with previously established mAbs, GMB28 [18] and KM694 [19,20]. GMB28 specifically reacts with GM2 ganglioside. KM694 has been shown to detect Sdα active carbohydrate epitopes, in addition to GM2 ganglioside, efficiently. We found that KM694 bound to the mucin obtained from N.binfected rats as well as HCM31 did, and the reactivity of KM694 to infected mucin was higher than that obtained from non-infected rats (Fig. S1a). A commercial GM2 ganglioside was recognized by GMB28 and KM694, but not by HCM31 (Fig. S1b). The antigenic specificities of these mAbs are summarized in Table S2.

2.6. Immunohistochemical study of human gastrointestinal tract with HCM31

Fig. 9 shows the immunohistochemical reactivity of HCM31 with various sections of human gastrointestinal mucosa. There were no epithelial cells stained with HCM31 in the epithelia of the esophagus, gastric fundus (data not shown), gastric pylorus (Fig. 9a) and duodenum (Fig. 9b). The bronchial mucosa was also negative with this mAb (data not shown). In the upper part of crypt charides were obtained from the small intestinal mucin of 3. Discussion

In the present study, two HCM31-reactive sialylated oligosaccharides were obtained from the small intestinal mucin of N.b-infected rats. These were completely characterized as core 4 hexa- and octa-saccharides both having Sdα blood group antigen, NeuAcr2-3(GalNAcβ1-4)Galβ1-4GlcNAcy-. To our knowledge, the core 4 oligosaccharides having the Sdα antigen have not been described previously. This study indicates that the Sdα tetrasaccharide sequence is necessary as an epitope determinant of HCM31 for the following reasons. (a) The two HCM31-reactive oligosaccharides, IA1-5d and IA1-8b, contain this common tetrasaccharide sequence. (b) The reduction in the reactivity with HCM31 of the mucin by the neuraminidase treatment showed that HCM31 recognized the sialylated oligosaccharides (Fig. 1). (c) Because HCM31 did not react with GM2 ganglioside, NeuAcr2-3(GalNAcβ1-4)Galβ1-4Glcβ-Cer (Fig. S1), the GlcNAcy residue on the Sdα antigen is a necessary part of the epitope. This idea is also supported by the fact that HCM31 did not react with IA1-2 and -3 (Fig. 3b), which seems to include the Sdα-like trisaccharide sequence, NeuAcr2-3(GalNAcβ1-4)Galβ1-. Without the GlcNAcy residue (Table S1) [21]. (d) HCM31 did not react with the oligosaccharide fractions IA1-1 to -4 obtained from N.b-infected animals, and all the UA1 fractions, IA1-1 to -8, obtained from the uninfected animals, fractionated by the first-step HPLC. The MS/MS analysis showed that the fragment ions derived from the Sdα antigen could not be detected in these fractions (Table S1). (e) The sialylated oligosaccharide, Fuc-Gal-[NeuAc-Gal-GlcNAc]-GalNAc-o1, in UA1-5 of uninfected rat mucin seemed to include NeuAcr2-3Galβ1-4GlcNAcy- sequence (Table S1). Thus, the GalNAcy residue linked to Gal in the NeuAcr2-3Galβ1-4GlcNAcy sequence via β1-4 linkage could be required for sufficient reaction with HCM31.

Both IA1-5d and IA1-8b have a core 4 structure. However, the core 4 structure may be unnecessary as an epitope determinant of HCM31. Although HCM31 was developed using human colonic mucin as an antigen [13], the Sdα antigens are expressed on core 3 oligosaccharides, but not on core 4 oligosaccharides, in human colonic mucin [22]. Therefore, HCM31 may also recognize core 3 oligosaccharide having Sdα antigen. Furthermore, HCM31 may recognize core 2 oligosaccharide having the Sdα antigen in fraction IA1-6 from the infected rat mucin. Thus, the type of core structure seems not to relate to the reactivity with HCM31. In conclusion, we propose that HCM31 recognizes the tetrasaccharide sequence, NeuAcr2-3(GalNAcβ1-4)Galβ1-4GlcNAcy-.

Some mAbs against Sdα antigen, CT1 and CT2, were developed by Conzelmann and Lefrancois [23], which react with a terminal trisaccharide sequence, NeuAcr2-3(GalNAcβ1-4)Galβ1-. Previously reported KM694 [19] and KM531 [24] react with both GM2 ganglioside and a neolacto-series Sdα glycolipid, which share oligosaccharide NeuAcr2-3(GalNAcβ1-4)Galβ structures at their non-reducing terminals. Therefore, the epitope determinant of KM694 and KM531 seems to be the trisaccharide sequence, the same as that of CT1 and CT2. In the present study, we showed that KM694 also recognized the mucins (Fig. S1). On the other hand, HCM31, developed using human colonic mucin as an immunogen, reacted with rat goblet cell mucins having Sdα, but not GM2. The difference of antigenic activities is assumed to be attributable to their epitope saccharide sequences. HCM31 and KM694 probably recognize the terminal tetrasaccharide and trisaccharide without inner GlcNAcy, respectively, of the Sdα tetrascarachide attached to the mucin. As an anti-GM2 mAb, GMB28, did not react with the mucins (Fig. S1), the Glcβ residue on GM2 seems to be a necessary part of the epitope for this mAb. Although the biological function of Sdα tetrasaccharide on mucin remains unknown, HCM31 will contribute to clarify this issue.

The Sdα antigen expressed as a glycolipid on chief cells disappeared along with the malignant changes in human gastric mucosa [20]. Kawamura et al. [19] reported that metastasis of human gastrointestinal cancer cells was reduced by expression of the Sdα antigen on their surface. In the present study, HCM31 did not stain the epithelial cells on the human gastric mucosa. The tissue fixation method used in our study might not fix the membrane glycolipids including Sdα antigen. Meanwhile, HCM31 extensively stained the secretory granules of goblet cells in sigmoid colon and rectum (Fig. 9). Furthermore, the stainability with HCM31 dis-
HCM31 might be useful as a negative marker for colonic cancer. This is consistent with the data that Sda antigen on core 3-type mucin oligosaccharide [22] and β1-4-N-acetylgalactosamine-transferase (β1-4GalNAcT), responsible for the Sda antigen biosynthesis, are co-expressed on human colonic mucosa, but this expression declines along with malignant changes [25]. However, it is still unknown how the secretory-type mucin having this antigen acts on the gastrointestinal mucosa. HCM31 seems to be useful for examining the function of secretory-type mucin with Sda antigen.

Recently, we reported that goblet cell sialomucins that reacted with HCM31 in the rat jejunal mucosa increased up to 16 days after N.b infection, the time when the worms were expelled from the rats [16]. No similar change could be observed by another mAb, PGM34, which extensively stains goblet cells in the rat small intestine, and recognizes sulfated oligosaccharide [26]. Karlsson et al. [21] reported that sialylated tetra- and penta-saccharides having Sda-like trisaccharide, NeuAc/NeuGcα2-3(GalNAcb1-4)Galβ-, were expressed in small intestinal mucin of N.b-infected rats. In the present study, NeuAc/NeuGc-(HexNac-)Gal-[GlcNac-]GalNAC-ol appeared in A1 fraction by N.b infection in addition to Sda-containing oligosaccharides. Thus, sialomucins with Sda antigen-related oligosaccharides are associated with worm expulsion. The present study also showed that the core 4 mucin-type oligosaccharides having sialic acid with or without Sda sequence are expressed upon infection (Fig. 5 and Table S1). β1-3GlcNAc-transferase (β1-3GlcNAcT) and β1-6GlcNAc-transferase (β1-6GlcNAcT) are needed for the synthesis of core 4 glycan [27]. Therefore, expression of these transferases may increase with infection, but further studies are needed to confirm these changes.

Bio-Gel A-1.5m was obtained from Bio-Rad Laboratories (Richmond, CA, USA). Dowex-50 was purchased from Dow Chemical Company (Midland, MI, USA). TOYOPEARL QAE-550C resin and TSKgel Amide-80 column were purchased from Tosoh Co. Inc. (Tokyo, Japan). A graphitized carbon column (GL-Pak Carbograph, 150 mg/3 ml) was obtained from GL-Science (Tokyo, Japan). The mAb, HCM31 (antibody subclass IgG1 κ), was prepared as previously described [13]. The anti-GM2 mAb, GMB28, was kindly provided by Drs. I. Kawashima and K. Ogura (Tokyo Metropolitan Institute of Medical Science). The anti-Sda mAb, KM694, was kindly provided by Tokyo Research Park, Kyowa Hakko Kirin Co. Ltd. (Machida, Japan).

4. Materials and methods

4.1. Reagents

N.b was maintained in our laboratory as previously described [16]. Eight-week-old male Wistar rats (weighing 200–250 g, SLC, Shizuoka, Japan) were used. The rats were inoculated subcutaneously with the third-stage larvae (L3) of N.b (2000 L3/rat). The rats were sacrificed after 24 h of fasting at 16 days after the inoculation, and their small intestines were removed. The small intestines were also obtained from uninfected rats. The small intestines were successively cut open, washed with saline and then lyophilized followed by weighing. Mucins were extracted from the pulverized small intestine using 2% Triton X-100 in 50 mM Tris–HCl (pH 7.2), isolated with a Bio-Gel A-1.5 m column and precipitated with ethanol as described previously [28]. This study was conducted in accordance with the guidelines of the Animal Laboratory Center of Kitasato University School of Medicine.
4.3. Preparation and purification of oligosaccharides from the rat intestinal mucin

Alkaline borohydride treatment of the purified rat small intestinal mucin was carried out according to the method of Carlson [29] with 0.05 M NaOH in 1.0 M NaBH₄ at 50 °C for 24 h. After acidification, the reaction mixture was applied onto a column of Dowex-50 (H⁺-form) to remove sodium and the peptides. The passed fractions were evaporated and borate was removed by repeated evaporation with acidic methanol. After being redissolved in water, the oligosaccharide solution was subsequently applied onto an anion-exchange column of TOYOPEARL QAE-550C. The column was washed with distilled water and eluted with a linear gradient of 0–0.6 M NaOAc. The effluent was monitored by hexose content [30]. The fractions were collected and desalted on a graphitized carbon column using 25% acetonitrile for the neutral oligosaccharides or 25% acetonitrile containing 0.05% trifluoroacetic acid for the acidic ones as eluting solvent [31].

4.4. Normal-phase HPLC

Two-step normal-phase HPLC using TSKgel Amide-80 columns was employed. In the first step, the columns (7.8 mm × 300 mm × 2 columns) were equilibrated with 68% (v/v) CH₃CN in 2.5 mM NaH₂PO₄, and the gradient was initiated after injection and decreased to 55% CH₃CN over 30 min at a flow rate of 2.0 ml/min. In the second step (4.6 mm × 250 mm × 2 columns), the fractions obtained from the first-step HPLC were rechromatographed under isocratic conditions of 74% CH₃CN for 1 h at a flow rate of 1.0 ml/min. UV absorption of the effluent solution was monitored at 210 nm. For the removal of NaH₂PO₄, the fractions were chromatographed using a graphitized carbon column as described above.

4.5. Amino sugar analysis

Amino sugar analysis was performed as previously described [32] after the oligosaccharides were hydrolyzed with 6 M HCl at 98 °C for 4 h using the Waters’ Pico-Tag Workstation.

4.6. Mass spectrometry

The molecular masses of the oligosaccharides were analyzed by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF/MS) using an autoflex III TOF/TOF instrument (Bruker Daltonik GmbH, Bremen, Germany) in reflector mode by summarizing 1000 signal spectra (5 × 200 shots) with a 50 Hz laser applying the following instrumental settings: ion source 1: 19.00 kV, ion source 2: 16.60 kV, lens: 8.55 kV, reflector 1: 21.00 kV, reflector 2: 9.70 kV, reflector detector: 1400 V, suppression up to 400 Da by deflection. Then, MS/MS spectra were further measured with an Autoflex III TOF/TOF instrument in LIFT (MS/MS) mode using the following instrumental settings: ion source 1: 6.00 kV, ion source 2: 5.30 kV, lens: 3.00 kV, reflector 1: 27.00 kV, reflector 2: 11.65 kV, lift 1: 19.00 kV, lift 2: 4.20 kV, reflector detector: 1400 V. Each sample was mixed with an equal volume of 2.5-dihydroxy benzolic acid dissolved in distilled water/CH₂CN (1:1, v/v) at 10 mg/ml as the matrix solutions [33]. Two μl of this mixture was then applied to a stainless steel target plate and air-dried at room temperature before the target was introduced into the spectrometer.

4.7. Methylesterification of sialylated oligosaccharides

Sialylated oligosaccharides were methylesterified for MS/MS analysis in accordance with the procedure by Powell and Harvey [34]. The oligosaccharides were dissolved in 1 μl of dimethyl sulf-oxide, mixed with 1 μl of methyl iodide and allowed to react for 2 h at room temperature. Unreacted methyl iodide was removed and dried under a gentle stream of nitrogen.

4.8. NMR spectroscopy

The NMR spectra were obtained using Varian INOVA 600 or Varian NMR System 600 NMR spectrometer (Varian Associates, Palo Alto, CA, USA) equipped with a1H/15N–31P pulse field gradient (PGF) indirect-detecting probe. Standard pulse sequences were used throughout. The proton NMR spectrum was assigned through PGF multiple-quantum-correlation spectroscopy and one-dimensional HOHAHA spectroscopy. The 13C assignments were made from an HMQC spectrum obtained with carbon decoupling. The lyophilized powder of the purified oligosaccharides was dissolved in deuterium oxide (D₂O) and evaporated to exchange the unstable 1H with 2H. The evaporation and dissolution were repeated five times and the sample was finally dissolved in 0.04 or 0.15 ml ofD₂O and then subjected to NMR spectroscopy using nano-probe or Shigemi tube, respectively. Chemical shifts of the reduced oligosaccharides were identified with reference to those described by Kamerling and Vliegenthart [17]. NMR spectra of α-methyl-N-acetylneuraminic acid (Nagara Science, Gifu, Japan) and β-para-nitrophenyl-N-acetylglactosamine (Glycosynth, Cheshire, England) were utilized as references for chemical shift assignment. The NMR spectral data of the standard α and β methylated monosaccharides as well as those of GalNAc-o1 reported by Ishihara et al. [9] were also utilized as references for the chemical shift assignment.

4.9. Mild periodate oxidation of oligosaccharides

To cleave the C–C bond of the reducing terminal GalNAc-o1 at positions C4 and C5, mild periodate oxidation of oligosaccharides was performed according to the methods of Chai et al. [35]. The oligosaccharides (approx. 10 μg) were oxidized with sodium periodate in imidazole buffer, pH 6.5, at 0 °C for 5 min. After excess periodate was destroyed by incubation with butane-2,3-diol at 0°C for 40 min, the oligosaccharides were purified with a column of graphitized carbon.

4.10. ELISA and competitive ELISA

The microtiter plates were coated with 2–200 ng of the purified mucin and kept overnight at 4 °C followed by blocking with 2% skimmed milk [8]. In case of using 2–200 ng of GM2 ganglioside (Alexis Corporation, Lausen, Switzerland) as antigen, the coated wells were blocked with 1% BSA for overnight. After the wells were washed, a definite amount of HCM31, KM694 or GMB28 was added to each well followed by incubation at ambient temperature for 1 h. The wells were successively incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulins (Dako, Kyoto, Japan) and 2,2′-azino bis-[3-ethylbenzthiazolone-6-sulfonate] (ABTS)/H₂O₂ solution (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA), and the color was allowed to develop. The wells were washed three times with PBS containing 0.05% Tween-20 for mucin or PBS for GM2 between each process. The UV absorption was measured at 405 nm (reference at 492 nm) at 30 min thereafter using a Bio-Rad Model 550 microplate reader.

A competitive ELISA was applied to detect the reactivity of HCM31 with the oligosaccharide fractions. The microtiter plates coated with 100 ng of the purified mucin followed by blocking with 2% skim milk were prepared as previously mentioned. At the same time, PBS solutions of the oligosaccharide fraction, each containing 0.15–10 μg or 0.15–75 nmol per well as the hexose
basis or the GalNac-OI basis, respectively, were pre-incubated with a definite amount of HCM31 for 2 h at ambient temperature. Instead of the sugar-containing solution, PBS was pre-incubated with HCM31 as the negative control. The pre-incubated mixtures were then added to the antigen-coated wells and incubated for 1 h. The remaining ELISA steps were the same as already described.

4.11. Periodate treatment and trypsin digestion of mucins

Periodate treatment was performed by exposing the mucin coated on the microtiter wells to 0.1–2.5 mM NaOAc in 50 mM sodium acetate, pH 4.5, for 1 h at room temperature. Trypsin digestion was performed by exposing the mucin coated on the microtiter wells to trypsin for 1 h at 37 °C. Trypsin at 2.5 mg/ml in 10 mM Tris–HCl, pH 8.0, containing 2 mM CaCl₂ was used with two-fold serial dilution. Each of the remaining ELISA steps was the same as already described.

4.12. Immunohistochemistry

Human tissues (20% formalin-fixed, routinely processed, and paraffin-embedded) were selected from the surgical pathology files of the Department of Laboratory Medicine, Shinshu University Hospital, Matsumoto, Japan. The following histologically normal tissues were selected: bronchi (n = 6), esophagus (n = 5), gastric fundic mucosa (n = 6), gastric pyloric mucosa (n = 6), duodenum (n = 6), cecum (n = 4), sigmoid colon (n = 4) and rectum (n = 1). The cancer tissues of cecum (n = 5), sigmoid colon (n = 4) and rectum (n = 1) were also selected. In addition, the proximal half of rat small intestine, defined as the jejunum, was resected and immediately fixed for 3 h in freshly prepared Carnoy's solution according to the method described previously [36]. After fixation, the jejunum was routinely processed and embedded in paraffin.

From these specimens, 3 μm paraffin sections were prepared for immunostaining with HCM31. Immunohistochemical staining was performed using the immuno-enzyme polymer method (Histofine Simple Stain MAX PO Multi, Nichirei Biosciences, Tokyo, Japan) with 3,3-diaminobenzidine as the chromagen. Briefly, endogenous peroxidase activity was blocked with 0.3% H₂O₂, then the specimens were sequentially incubated with the mAb, HRP-conjugated immunoenzyme polymer, and 0.02% 3,3-diaminobenzidine in 50 mM Tris–HCl, pH 7.6, containing 0.005% H₂O₂. Counterstaining was performed with hematoxylin.

4.13. Sialidase treatment and de-O-acetylation for the specimens

Enzymatic treatment was performed by exposing the specimens for immunostaining to 0.1 units of S2-3,6,8-sialidase from Arthrobacter ureafaciens (Nacalai Tesque, Kyoto, Japan) in 10 mM sodium phosphate, pH 5.0, for 4 h at 37 °C.

De-O-acetylation treatment of sialic acid was performed by exposing the specimens to 0.2 M NaOH at 37 °C for 2 h.

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Supplementary Material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fob.2012.07.006.

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