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Research Article

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Development of Anti-Human Colonic Mucin Monoclonal Antibodies

Characterization of Multiple Colonic Mucin Species

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Abstract

Structural relationships between colonic mucin species were assessed using a library of monoclonal antibodies (MAbs) directed against purified human colonic mucin (HCM). After immunization of mice with purified mucin from normal human colonic mucosa, 14% of 1,920 fusion products screened were positive for anti-HCM activity in a solid-phase assay. Patterns of selective binding by hybridomas to six discrete HCM species (I–VI) separated by DEAE-cellulose chromatography suggested the presence of both shared and species-specific antigenic determinants among HCM species I–VI. 23 anti-HCMs MAbs (7 IgM, 7 IgG_1, and 9 IgG_2), demonstrating a range of anti-HCM species specificities, were produced and used to study structural relationships between mucin species. Binding of various mucin species by individual anti-HCM MAbs was shown by competitive solid-phase radioimmunoassay to reflect the presence of identical epitopes on the different species. Adsorption of HCM species on a variety of affinity resins prepared with anti-HCM MAbs demonstrated that binding to multiple mucin species by a single MAb was related to intrinsic structural determinants. Four anti-HCM MAbs recognized protease-sensitive antigenic structures, which suggests that they may be directed to core HCM proteins. 12 of the anti-HCM MAbs were shown by solid-phase assay to recognize either complete (n = 5) or partial (n = 7) isolated colonic mucin oligosaccharide side chains of defined structure. Collectively, these data show the presence of both shared and unique antigenic structural determinants among colonic mucin species. Chromatographic heterogeneity of mucin glycoproteins seems to be related to the existence of biologically significant subclasses in the normal human colon.

Introduction

The abundant representation of mucin glycoproteins throughout the human colon suggests that they play an important role in colonic function (1–4). The importance of mucin in normal colonic function is underscored by the apparent association of alterations in mucin content with a variety of disease processes (5–11). However, insights into the functional properties of colonic mucin glycoprotein and the role of alterations in the pathophysiology of disease states have been limited by incomplete knowledge of the composition and structure of normal mucin. Early studies in a variety of animals suggested that colonic mucin was composed of a single, polydisperse, high molecular weight glycoprotein (12–14). More recently a number of workers have suggested that there may be subpopulations within purified colonic mucin from both experimental animals and humans (7, 15–18). Despite the increasing appreciation of the extensive heterogeneity of colonic mucin, the structural basis of this heterogeneity and its biological importance remain uncertain. Many studies have used incompletely characterized preparations or indirect structural methods, and demonstration of related functional consequences has been lacking (9, 13, 19–22).

Through our studies, we have attempted to expand our knowledge of the structure and function of normal human colonic mucin (HCM).[1] Purified normal HCM isolated from mucosa of fresh surgical specimens was found to contain at least five distinct mucin species as well as a sixth heterogeneous component recovered in a high salt wash after DEAE-cellulose chromatography (5). Each HCM species (I–VI) appeared to have a distinctive hexose, hexosamine, and amino acid composition, which suggests that the chromatographically defined entities might differ in their fundamental structures. Similar analysis of colonic mucin from patients with ulcerative colitis demonstrated a selective decrease in one HCM species (IV). Although the basis for this deficit remains uncertain, subsequent studies have confirmed a selective reduction of species IV in specific association with this disease process (7). Beyond its possible significance in the pathogenesis of ulcerative colitis, the apparent alteration in one HCM species independent of other components of HCM supports the concept that HCM subclasses may represent functionally distinct substances.

The structural relationship between HCM species remains unclear. Preliminary studies in this laboratory have focused on oligosaccharide side chain analysis of the most abundant HCM species (III, IV, and V). These studies have shown the presence of a complex array of at least 21 neutral and acidic oligosaccharide side chains which range in size from two to 12 sugar residues (23). Three different HCM species (III, IV, and V) were found to contain many common oligosaccharides. However, some oligosaccharides were identified only in individual components. Despite determination of the structures of some of the components of the complex mixture of oligosaccharide side chains attached to the HCM species, the fundamental structural relationships between HCM species require further evaluation.

In this report, we describe the production and characterization of a library of monoclonal antibodies (MAbs) directed against HCM and their use in the study of the structural relationships between HCM species. In an accompanying paper, we describe related studies using MAbs with defined anti-HCM

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1. *Abbreviations used in this paper:* HCM, human colonic mucin; MAbs, monoclonal antibodies.

specificity to examine colonic goblet cell function and the cellular basis of HCM heterogeneity.

Methods

Isolation, purification, and fractionation of human colonic mucin, mucin species, and oligosaccharides

Human colonic mucin was prepared from mucosal scrapings of fresh surgical specimens of normal human sigmoid colon from patients undergoing resection for diverticulosis. Pure mucin was isolated from materials solubilized by sonication using sequential Sepharose 4B column chromatography and CsCl density centrifugation as previously described (5). Individual mucin species I–VI were separated on DEAE-cellulose column chromatography. Neutral and acidic oligosaccharides were isolated from HCM species III, IV, and V by sequential chromatography after alkaline degradation (23). Oligosaccharide structures were determined by combination of permethylation analysis and sequential glycosidase digestion (23).

Preparation of anti-human colonic mucin monoclonal antibodies (anti-HCM MAbs)

Immunization. Primary immunization of Balb/c mice (Charles River Breeding Laboratories, Wilmington, MA) was carried out with unfractionated pure human colonic mucin (100 μg) by intravenous injection. Secondary and tertiary immunizations identical in route and amount were accomplished at subsequent 3–5-wk intervals. All animals received further doses of antigen on days 4 and 3 before fusion.

Fusion technique and production of monoclonal antibodies. Splenocytes from immunized animals were prepared and fused with P2-NS1/1-Ag 4-1 (NS1) myeloma cells essentially as described (24–26). Hybrids were selected by use of medium containing hypoxanthine/aminopterin/thymidine (HAT) on days 3, 4, and 5 after fusion. Medium from surviving hybrids was assessed for anti-HCM activity after transferring to 24-well culture plates (Costar, Cambridge, MA). Positive primary hybridomas were grown to confluence in Dulbecco's modified Eagle's medium supplemented with 20% fetal calf serum in 60-mm dishes and assessed for activity against individual HCM species I–VI. Hybrids with a range of anti-HCM species specificities were double-cloned at limiting dilution in complete medium on 3T3 monolayers previously treated with mitomycin C (1 μg/ml). After the second cloning cycle, larger amounts of MAbs were obtained by inoculating Balb/c mice intraperitoneally (10^5 cells per animal) 1 wk after priming with pristane and subsequently collecting ascitic fluid.

Purification and characterization of MAbs. For isotypic analysis of anti-HCM MAbs, medium supernatant from double-cloned anti-HCM hybridomas was incubated first with HCM-coated polystyrene beads and then with ^{125}I-labeled goat anti-mouse IgG, IgG_2, IgA, or IgM, and subsequently bound radioactivity was determined. Alternatively, isotype determinations were carried out using a commercially available peroxidase-linked immunoassay kit (Catalog No. 100-036; Boehringer Mannheim Diagnostics, Inc., Houston, TX).

IgM anti-HCM MAbs were purified from ascitic fluid by gel exclusion chromatography on Sepharose 4B. IgG MAbs were purified from ascitic fluid by chromatography on *Staphylococcus* A-Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) using previously published methods (27). Purity of MAbs was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by staining with Coomassie Blue (28).

Assay of anti-HCM and anti-HCM species I–VI activities

Anti-HCM and anti-HCM species I–VI activities of culture supernatants and purified MAbs were assessed using solid-phase sandwich radioimmunoassay (RIA). Polystyrene beads (0.25 in. [6.35 mm]: Precision Ball Co., Chicago, IL) were coated with purified HCM (see above) by incubation of 50 μg of HCM/10 ml of bicarbonate buffer (pH 9.2)/40 beads overnight at room temperature with gentle shaking. Before use in binding assays, beads were incubated for 1 h in buffer containing either spent NS1 medium or 10% bovine serum albumin (BSA) to saturate sites of nonspecific binding. Coated beads were incubated with test sample at 37°C for 60 min. After washing three times with water, the sandwich was completed by incubation with ^{125}I-labeled sheep anti-mouse Ig Fab (100,000 cpm per assay mixture; specific activity, 7.3 μCi/mg; New England Nuclear, Boston, MA) in 150 μl of buffer containing 0.01 M Tris-HCl (pH 7.5), EDTA at 2.0 μg/ml, and thimerisol at 37°C for 60 min. Bound radioactivity was measured after extensive washing with distilled water. Intra-assay variation was found to be limited to a SD±8% when individual samples were assayed in multiples (n = 6). Samples were assayed in duplicate and results were expressed as the mean value. Activity threefold greater than negative control (medium from NS1 cells or diluted serum from unimmunized animal) was considered positive. Positive samples were subsequently assayed for activity against individual mucin species I–VI by modification of the described methods; beads were coated with individual purified HCM species separated as described above. Individual mucin species appeared to bind to beads to an equivalent extent (±12% standard variation) as measured by disappearance of hexose from the supernatant.

Competitive RIAs were performed by two-stage solid-phase assay. Supernatant recovered after initial incubation of beads coated (as described above) with an antigen being assessed for its cross-reactivity (unfractionated mucin, individual mucin species, or isolated mucin species oligosaccharides) was transferred and incubated under comparable conditions with a second bead coated with the reference HCM species antigen. Controls included samples incubated with beads coated with either bovine serum albumin (negative control) or the reference HCM species or unfractionated HCM (positive controls) during the first phase incubation.

Structural determinants specified by MAbs were assessed in solid-phase sandwich assays using a panel of beads coated with individual isolated mucin species (III, IV, and V) oligosaccharides in assays similar to those described above. Isolated oligosaccharides (10–100 μg/ml) were incubated with polystyrene beads (2 beads/ml) in 0.1 M bicarbonate buffer, pH 9.2 (acidic oligosaccharides), or 0.1 M Nacacodylate, pH 6.5 (neutral oligosaccharides) in the presence of 0.1% Na azide (wt/vol) overnight at room temperature with gentle shaking. Binding of individual oligosaccharides was quantitated by addition of oligosaccharide radiolabeled at the reducing terminus during initial alkaline cleavage (23) (specific activity, $1.4–2.7 \times 10^6$ cpm/μm *N*-acetylgalactosaminitol). Final concentrations were adjusted to achieve a specific activity of 1.0×10^6 cpm/μm oligosaccharide. Maximum binding of oligosaccharides ranged from 0.1 to 1.4×10^{-9} M/bead. To standardize oligosaccharide binding studies to permit assessment of relative binding, we adjusted amounts of oligosaccharides used during the coating process to achieve final concentration of $1.0 \pm 0.1 \times 10^{-10}$ M oligosaccharide/bead. Before use, sites of nonspecific binding were blocked as above. Beads were stored up to 1 wk at 0–4°C. After incubation of the individual MAb with oligosaccharide-coated beads, bound radiolabel after incubation with the Fab probe was assessed as before. Supernatants from initial incubations were added to beads coated with intact mucin species to determine the effect of this prior adsorption on binding to the intact mucin glycoprotein.

Effect of protease treatment of HCM

Purified HCM was suspended (1 mg/ml) in 0.01 M Tris-HCl, pH 7.5 with 0.01 mg $CaCl_2$ and 0.01% Na azide (wt/vol) and digested with Pronase (1% wt/vol) (Sigma Chemical Co., St. Louis, MO) at 45°C overnight. Digestion was stopped by heat inactivation (80°C, 20 min) and beads were coated with the mixture as described above. Control assays included a HCM and Pronase mixture heat-inactivated at zero time. Pilot studies with intact HCM labeled at nonreducing termini as previously described (7) indicated equivalent binding to beads of radiolabel from the pronase digest and zero time control incubation. Treatment of 1.4×10^6 cpm ^{3}H-labeled HCM followed by incubation with 50 beads suspended in 0.1 M Na bicarbonate buffer, pH 9.2, resulted in a specific activity of $4.8 \pm 0.5 \times 10^2$ cpm/bead for the zero time control and $4.1 \pm 0.4 \times 10^2$ cpm/bead after overnight digestion. Efficacy of pronase treatment was confirmed by chromatography on Sepharose 4B as previously described (7); >85% of radiolabeled HCM was found to migrate in the included

volume after pronase digestion in contrast to <5% in the untreated material.

MAb affinity absorption of HCM species

Purified anti-HCM MAbs (3.3 mg) were individually coupled to CNBr-activated Sepharose 4B (1.0 g) using previously described methods (29). Coupling was ~70% efficient as determined by retention of tracer ^{125}I-MAbs radiolabeled using the Bolton-Hunter reagent (30). Affinity resin was packed in a Pasteur pipette and sample (200 μl) of individual mucin species (0.1 mg/ml 0.1 M Tris–HCl pH 8.0) applied at room temperature. The column was clamped for 30 min and then washed with 15 ml of application buffer, collecting 1.0 ml fraction. Mucin species content of eluant was assessed by coating polystyrene beads as described above with individual fractions followed by addition of anti-HCM MAbs ascites (either the same MAb conjugated to the affinity resin or one with an appropriate mucin species specificity) using conditions described above. Control chromatography was performed using uncoupled Sepharose 4B or resin conjugated with a non–cross-reacting MAb directed against a serum galactosyltransferase (29).

Results

Monoclonal antibodies were produced from mice immunized with normal human colonic mucin and characterized for their mucin species specificities. Human colonic mucin was found to be highly antigenic. Anti-HCM titers were routinely found to be >1:10^4 after an initial boost following a primary immunization. After fusion of splenocytes from immunized mice with a nonproducing myeloma line, 14% of 1,920 products had measurable anti-HCM activity in a solid-phase sandwich assay (12–21% among seven separate fusions). Results of determination of anti-HCM activity by solid-phase RIA of positive hybridomas from a representative fusion are depicted in Fig. 1 and indicate a broad range of activity among the positive fusion products. Comparable binding in solid-phase sandwich assays was observed when individual hybridomas were assayed using different preparations of HCM.

All hybridomas that demonstrated binding to the pure unfractionated HCM were subsequently characterized for their binding to individual mucin species [I–VI] separated by DEAE-cellulose chromatography (5). A wide range of patterns of HCM species specificities was encountered as demonstrated in Table I, which summarizes the characteristics of some positive hybrids obtained from three fusions. These primary fusion products included a group of anti-HCM hybridomas that appeared to recognize individual species II, III, IV, V, and VI. No hybridomas reacted uniquely with mucin species I. In addition to hybridomas that appeared to recognize determinants on a single mucin species, other fusion products recognized determinants common to two or more species, including in some instances all six species. Certain combinations were repeatedly encountered including hybridomas recognizing species III, and IV, IV, and V, III and VI; IV and VI; III, IV, and V; IV, V, and VI; III, IV, V, and VI; I, II, III, IV, and V; and I–VI. Finally, a number of fusion products were obtained which did not appear to react with any of the separated species in solid-phase sandwich assay despite initial demonstration of positivity against pure unfractionated HCM. Confirmation that these latter hybridomas were directed against HCM determinants and not contaminants was obtained by indirect immunofluorescent studies (31).

Efforts were directed at the production of MAbs exhibiting the range of HCM species specificities observed in initial hybridomas and summarized in Table I. Stable MAb-producing cultures could not be established from all representative initial hybridomas cultures. However, it was possible to develop a library of anti-HCM monoclonal antibodies, which included MAbs with many of the different mucin species recognition patterns found

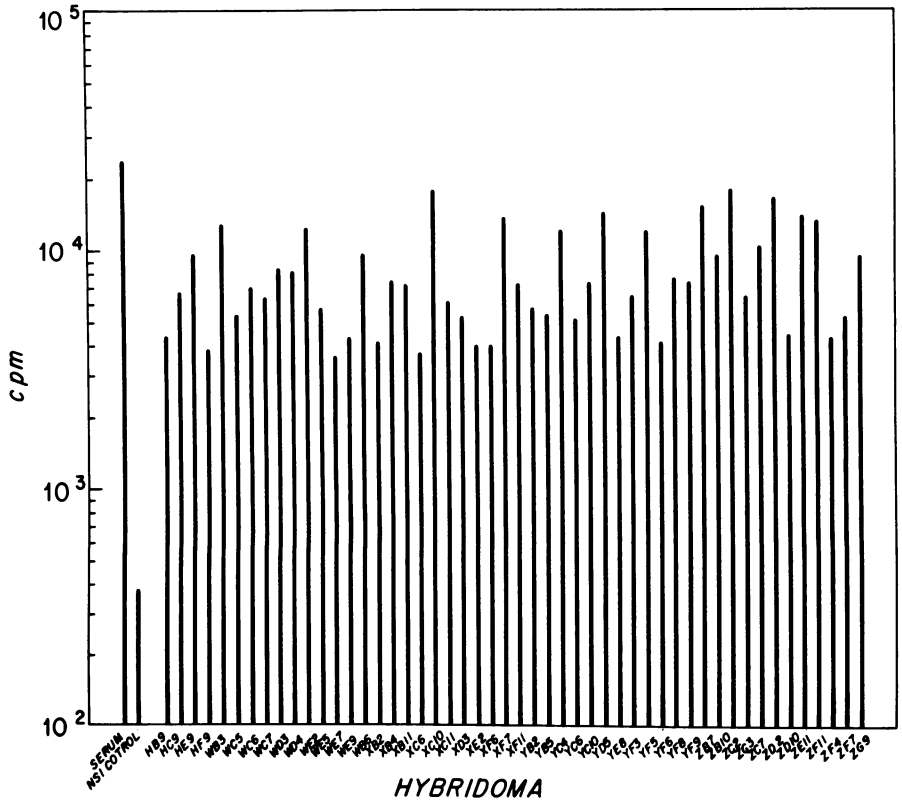

Figure 1. Binding of positive hybridomas to HCM. Spent supernatant was collected from confluent cultures after transfer to 24-well plates of fusion products derived from splenocytes of Balb/c mice immunized with pure unfractionated HCM as detailed in text. Solid-phase sandwich RIAs were performed by incubation of sample with polystyrene beads precoated with unfractionated purified human colonic mucin (50 μg/10 ml, pH 9.2) followed by incubation with ^{125}I-sheep anti-mouse Ig Fab as described in text. Figure shows results from individual positive hybridoma products of representative single fusion (49/240 wells).

Table I. Determination of Mucin Species Specificity of Representative Primary Anti-HCM Fusion Products

	Antigen‡							
		Mucin species						
Hybridoma*	HCM	I	II	III	IV	V	VI	Specificity§
ZG9	17,840	1,050	980	1,340	8,970	7,850	2,810	IV, V
WC7	7,750	980	2,600	1,170	2,230	1,470	1,950	0
WE9	5,140	4,750	3,980	9,570	8,710	6,410	2,100	I, II, III, IV, V
YF5	10,760	1,170	1,080	1,450	5,860	6,100	5,730	IV, V, VI
Q5	8,970	1,310	980	1,520	2,040	5,700	1,930	V
R35	7,680	930	890	4,870	1,990	1,240	1,420	III
S5	10,180	1,020	1,620	2,270	6,370	2,080	1,820	IV
R26	11,760	2,030	1,580	1,790	1,510	2,020	1,930	0
K21	8,940	1,620	2,130	5,350	4,100	4,290	3,870	III, IV, V, VI
K22	9,760	980	1,470	6,100	2,330	5,400	1,900	III, V
K27	17,737	1,270	990	1,010	1,460	1,720	1,110	0
L4	12,079	1,860	1,922	2,190	1,670	2,320	1,590	0
L16	8,830	827	791	2,060	5,850	1,840	820	IV
M3	17,650	1,150	1,450	1,620	2,100	2,320	1,740	0
M5	8,460	1,820	2,320	1,790	1,650	4,480	4,910	V, VI
M7	10,810	2,010	1,650	5,980	1,870	2,190	5,820	III, VI
M23	12,350	3,950	4,210	5,470	4,910	5,760	5,180	I, II, II, IV, V, VI
N25	7,170	880	990	1,070	1,490	2,310	6,320	VI
K23	8,150	780	890	930	4,100	5,050	4,680	IV, V, VI
HB9	4,310	1,190	2,020	3,990	5,840	5,180	1,460	III, IV, V
WC6	7,760	4,570	4,280	5,910	5,720	6,130	5,340	I, II, III, IV, V, VI
YB5	7,350	1,100	950	1,720	6,720	5,940	2,030	IV, V
ZB7	9,890	930	1,070	1,320	1,490	6,780	1,570	V
ZE11	10,270	1,230	1,540	940	1,340	1,060	910	0
YC10	10,810	970	870	1,240	5,660	1,700	1,050	IV
XC6	4,830	3,750	4,870	5,100	4,070	4,280	1,810	I, II, III, IV, V
HB6	6,970	1,040	3,980	1,220	920	1,130	1,250	II
ME2	8,050	1,090	1,860	1,540	5,170	2,030	5,720	IV, VI
HB3	13,000	1,450	1,230	7,210	6,860	7,140	1,860	III, IV, V
K52	19,000	780	940	1,130	1,340	990	1,270	0
ZG3	7,470	940	1,120	3,980	4,320	2,070	1,030	III, IV
Q33	9,890	2,210	5,370	1,920	7,610	1,860	1,620	II, IV
WG6	14,280	7,130	5,980	5,930	8,100	7,640	5,890	I, II, III, IV, V, VI
WE5	6,950	970	860	1,290	1,390	4,230	1,080	V
WE2	10,140	1,950	1,770	5,100	5,430	4,920	2,030	III, IV, V
XB2	6,280	1,650	1,430	2,000	7,280	6,810	9,120	IV, V, VI
ZB1	7,530	3,990	5,110	4,830	4,080	4,720	1,860	I, II, III, IV, V
N10	11,780	904	2,170	4,835	1,170	1,317	1,256	III
K26	12,500	2,110	2,580	6,310	12,710	5,170	2,120	III, IV, V
K23	5,410	1,270	4,130	1,480	5,260	2,240	1,890	II, IV
NS1	860	920	1,010	1,050	970	870	1,130	—
Serum (1:10^3)	22,282	6,940	8,720	7,540	8,140	5,970	6,900	I, II, III, IV, V, VI

* Supernatant from representative primary products of three separate fusions after achieving near confluent growth in 24-well plates. ‡ Polystyrene beads were coated with either unfractionated pure HCM or separated mucin species I–VI prepared as previously described (5) and binding in solid-phase sandwich RIA determined as detailed in Methods; expressed as cpm bound/bead; intraassay variation ±8% SD determined by multiple assay of individual samples (n = 6); values expressed as mean of duplicates. § HCM species-specificity binding derived from cpm representing >3 times NS1 control (values underlined).

in the primary fusion products. Repeat evaluation of the MAbs obtained after two cycles of cloning at limiting dilution demonstrated that their species specificities were identical to the original hybridomas. The mucin species specificities of anti-HCM MAbs produced from cell fusions as determined by solid-phase RIA are summarized in Table II. They include a broad spectrum of both HCM species-specific and HCM species-nonspecific antibodies. As demonstrated in Table II, analysis of anti-HCM

MAbs for isotypic class indicated that the library included both IgM and IgG (IgG_1, IgG_{2a}, IgG_{2b}) antibodies.

Competitive solid-phase RIAs were performed to determine the similarity of the determinants present on the different mucin species recognized by those anti-HCM MAbs that reacted with multiple mucin species. These studies assessed the reduction of MAb binding to one HCM species after prior incubation with beads coated by a different HCM species also recognized by that MAb. The results of a representative analysis of one multi-species specific MAb are demonstrated in Fig. 2. In this instance, prior incubation of MAb 4 (previously shown to bind isolated mucin species III, IV, and V; Table II) with mucin species III or IV effected a linear inhibition of binding to mucin V (Fig. 2 *C*). Conversely, species V caused reciprocal inhibition of binding to species III (Fig. 2 *A*) and IV (Fig. 2 *B*). No inhibition was observed when beads were preincubated with mucin species II, a component not recognized by MAb 4. Equivalent findings of inverse log-linear inhibition were obtained when the concentration of antigen on the first bead was fixed (50 μg HCM/10 ml per 20 beads) and the concentration of HCM species on second beads was varied (data not shown). These data suggest that MAb 4 recognizes the same epitope on mucin species III, IV, and V. In each instance, anti-HCM MAbs recognizing multiple species appeared to bind identical or closely related determinants on the different mucin species as evidenced by the reciprocal inhibition of binding by appropriate HCM species in competitive RIAs.

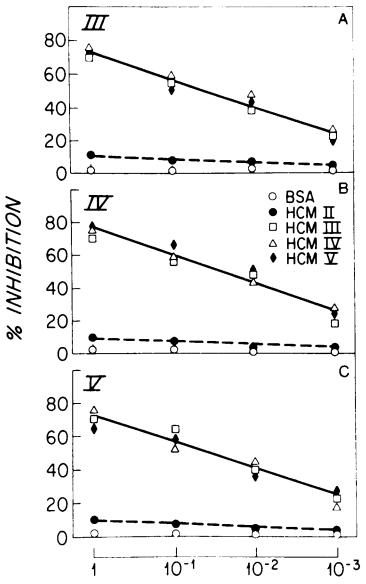

Figure 2. Competitive binding of HCM species to anti-HCM MAb 4. Representative series of competitive binding experiments evaluating the equivalence of antigenic determinants recognized on different mucin species by an anti-HCM MAb binding multiple species. Supernatant containing anti-HCM MAb 4 (specific binding to HCM species III, IV, V) was initially incubated (60 min at 37°C) with polystyrene beads coated with dilutions of individual species II (●), III (□), IV (▲), and V (♦) or bovine serum albumin control (○) (50 μg/10 ml per 20 beads). After this initial adsorption, medium was then transferred to a second set of beads coated with individual species III, IV, and V and the amount of residual MAb binding was determined after incubation (60 min at 37°C) by subsequent incubation with ^{125}I-labeled goat anti-mouse Ig as detailed in text. Inhibition was calculated as decline in amount of ^{125}I-labeled goat anti-mouse Ig bound to second bead relative to control (preincubation with bovine serum albumin). (*A*) Inhibition of binding to HCM species III. (*B*) Inhibition of binding to HCM species IV. (*C*) Inhibition of binding to HCM species V.

Table II. Characterization of Anti-HCM Monoclonal Antibodies

MAb*	Mucin specifies specificity	Isotype
1	IV, V	IgG_1
2	0	IgM
3	I, II, III, IV, V	IgG_{2B}
4	III, IV, V	IgG_{2B}
5	IV, V, VI	IgM
6	V	IgG_{2B}
7	III	IgM
8	IV, VI	IgG_1
9	0	IgG_{2A}
10	0	IgG_1
11	III, IV, V, VI	IgM
12	0	IgG_{2A}
13	III, IV	IgG_{2B}
14	IV	IgM
15	0	IgG_1
16	V, VI	IgM
17	IV, V	IgG_1
18	IV, V	IgG_{2B}
19	VI	IgG_1
20	I, II, III, IV, V, VI	IgM
21	III	IgG_{2B}
22	III, IV, V	IgG_1
23	II, VI	IgG_{2B}

* Monoclonal antibodies isolated from seven fusions after two cycles of cloning at limiting dilutions demonstrating binding to unfractionated pure human colonic mucin in solid-phase sandwich RIA. HCM species specificity determined by solid-phase sandwich assay using panel of polystyrene beads coated with separated mucin species as detailed in text and Table I. Isotypes of anti-HCM MAbs determined by sandwich RIA using heavy-chain specific ^{125}I-labeled goat anti-mouse Ig or peroxidase immunoassay.

Subsequently, adsorption chromatography was performed to determine whether apparently equivalent binding to determinants on different colonic mucin species by single anti-HCM MAbs reflected the presence of shared integral structural components or unappreciated cross-contamination of the mucin species preparations. Initial adsorption studies used resins conjugated with a MAb that bound multiple HCM species. As shown in Table III, Sepharose 4B conjugated with pure MAb 1 (recognizing HCM species IV and V by solid-phase RIA) adsorbed either mucin IV or mucin V. Adsorption of both mucin species IV and V by a single MAb was confirmed by the elimination of binding reactivity to the column eluant by monospecific MAbs directed against either mucin IV (MAb14) or mucin V (MAb6).

In a reciprocal study, affinity resin was prepared using a monospecies-specific anti-HCM MAb. As shown in Table IV, application of mucin species IV to Sepharose 4B conjugated with MAb 14 (specific for species IV by solid-phase RIA), resulted in effective adsorption of this substance alone as indicated by the reduction in binding to the eluant by the multispecies-specific MAb 1, which was not observed when HCM species V was also applied to the column.

Next, a HCM species was conjugated to Sepharose 4B to determine if adsorption of a multispecies-specific MAb resulted in parallel loss of binding activity to all species recognized by that MAb. In representative experiments, MAb 1 was applied to HCM V-Sepharose 4B. As indicated in Table V, the eluant from this column showed a 66% reduction in binding to both HCM V and HCM IV. In contrast, HCM species IV binding was not reduced when a mixture of the two monospecific MAbs

Table III. HCM Adsorption on a Multispecies-specific Anti-HCM MAb Affinity Resin: Sepharose 4B-MAb 1 [IV, V]

	Anti-MAb binding					
	MAb 1 [IV, V]‡		MAb 14 [IV]‡		MAb 6 [V]‡	
HCM species*	Pre-	Post-	Pre-	Post-	Pre-	Post-
		(*percent*)		(*percent*)		(*percent*)
IV	8,740	3,160 (36.2)	7,690	2,160 (28.1)	1,230	1,410 (114.6)
V	7,910	3,410 (43.1)	790	920 (116.4)	6,670	2,150 (32.2)
IV and V	10,730	4,380 (40.8)	6,910	1,850 (26.8)	7,320	3,140 (42.9)

* Separated HCM species applied to MAb-1 conjugated Sepharose 4B resin. ^{125}I-labeled goat anti-mouse Ig F(ab) bound to beads coated with samples pre- or post-absorption on MAb 1-Sepharose 4B resin after prior incubation with the designated MAb using conditions described in text; cpm bound/bead after subtraction of blank controls (140±30 cpm) of MAbs incubated with nonantigen-bearing BSA coated beads. ‡ HCM species specificity of anti-HCM MAb defined by solid-phase RIA as described in text and Tables I and II.

14 (recognizing IV only) and MAb 6 (recognizing V only) were applied to this same matrix. Collectively, these data suggest that MAb 1 binds an intrinsic and identical determinant on both species IV and V, which differs from the determinant on species IV recognized by MAb 14. Conversely, MAb 14 must bind a determinant of species IV that is not present on species V.

The actual structural determinants recognized by anti-HCM MAbs were studied by solid-phase sandwich RIA using beads coated with oligosaccharides of defined structure isolated from HCM species III, IV, and V. Each MAb was assessed for binding activity using a panel of 37 oligosaccharides isolated from mucin species III, IV, and V encompassing 21 discreet defined structures (23). Results of binding determinants of individual MAbs to each of the 21 structures are detailed in Table VI. In total, 12 MAbs showed significant binding to isolated oligosaccharides. Five recognized intact oligosaccharide side chain structures. These oligosaccharides appeared to represent the actual structural determinants recognized in the intact mucin species inasmuch as preincubation of MAbs with the oligosaccharide competitively inhibited binding to the intact mucin species. Thus, for example, as shown in Table VI, HCM MAb 1 bound to the oligosaccharide GalNacα(1-3)[Fucα(1-2)]Galβ(1-4)GlcNacβ(1-4)Galβ(1-3)GlcNacβ(1-3)Galβ(1-4)GlcNacβ(1-3)GalNac-ol but not to the other 20 different oligosaccharide structures studied. Note that this oligosaccharide was isolated from mucin species IV and V, which corresponds to the binding specificity of this MAb (see Table II). This anti-HCM MAb did not bind to closely related but smaller structures (e.g., Galβ(1-4), GlcNacβ(1-3)GalNac-ol), indicating specificity for the intact structure.

Seven anti-HCM MAbs bound more than one oligosaccharide side chain. In each instance, when a single MAb recognized more than one oligosaccharide, these different side chains contained common structural components. The structural determinants specified by these seven anti-HCM MAbs could be deduced from comparative analysis of the structures of the oligosaccharides bound. In a representative series of assays, MAb 4 bound a number of diverse oligosaccharides structures (Table VI). However, all of the oligosaccharides recognized by this MAb contained one trisaccharide, Galβ(1-4)GlcNacβ(1-3)Galβ(1-x), at or near the nonreducing terminus although this trisaccharide was not found as a discrete independent structure. Oligosaccharides lacking this trisaccharide were not recognized by MAb 4.

The structures of the apparent antigenic determinants of 12 anti-HCM MAbs derived from the data presented in Table VI are summarized in Table VII and include oligosaccharides containing two to nine carbohydrate residues. Binding specificity of these 12 MAbs to species III, IV, and V correlated with the presence of these oligosaccharide structures in the mucin species;

Table IV. HCM Adsorption on a Monospecies-specific Anti-HCM MAb Affinity Resin: Sepharose 4B-MAb 14 [IV]

	Anti-HCM MAb binding‡					
	MAb 1 [IV, V]§		MAb 14 [IV]§		MAb 6 [V]§	
HCM species*	Pre-	Post-	Pre-	Post-	Pre-	Post-
		(*percent*)		(*percent*)		(*percent*)
IV	8,320	3,060 (36.8)	9,170	3,140 (34.2)	1,230	1,020 (83.0
V	9,150	8,180 (89.4)	1,470	1,050 (71.4)	6,320	5,970 (93.5)
IV and V	11,610	6,130 (52.8)	7,460	2,190 (29.4)	7,400	6,370 (86.1)

* Separated HCM species applied to conjugated MAb 14-Sepharose 4B. ‡ ^{125}I-labeled goat anti-mouse Ig Fab bound to beads coated with samples pre- or post-adsorption on MAb 14 conjugated resin after prior incubation with the designated MAb using conditions described in text. cpm bound/bead after subtraction of blank controls as described in legend to Table III. § Species specificity of anti-HCM MAb defined by solid-phase RIA as described in text and Tables I and II.

Table V. Adsorption of Monospecies and Multispecies-specific Anti-HCM MAbs on Sepharose 4B-HCM V

	HCM species binding			
	Anti-HCM IV activity		Anti-HCM V activity	
Anti-HCM MAb*	Pre-	Post-	Pre-	Post-
		(*percent*)		(*percent*)
MAb 1 [IV, V]	9,210	2,140 (34.1)	8,140	2,860 (33.9)
MAb 14 [IV]	7,250	6,760 (93.2)	1,490	1,180 (79.2)
MAb 6 [V]	1,130	980 (86.7)	5,930	1,530 (25.8)
MAb 14 + MAb 6	8,170	7,140 (87.4)	7,370	3,210 (43.6)

* Anti-HCM MAb (supernatant from cultures) incubated with Sepharose 4b conjugated with purified HCM species V. Roman numerals in brackets indicate HCM species specificity determined by solid-phase assay as detailed in text and Table II. Solid-phase RIA or supernatant pre- or post-adsorption of anti-HCM MAbs with Sepharose 4B-HCM V resin using beads coated with either HCM species IV or V as described in Methods; cpm bound/bead after subtraction of blank control determined as described in legend to Table III.

e.g., MAb 5 specifically bound to oligosaccharides containing the structure NeuAcα(2–6)Galβ(1–4)GlcNacβ(1–3)[NeuAcα(2–6)]GalNac-ol, which was present in HCM species IV and V (but not III), mirroring the binding specificity of this MAb in initial solid-phase RIA. Not surprisingly, MAbs recognizing more limited structural determinants were among those exhibiting broader HCM species specificities in solid-phase RIA. This finding is illustrated by MAb 11, which appeared to recognize the limited oligosaccharide structure GalNacα(1–3)[Fucα(1–2)]Galβ(1–x) and was relatively nonspecific in its RIA-defined HCM species binding properties, recognizing species III, IV, and V (as well as VI). In a manner similar to MAb 4, the oligosaccharide GalNacα(1–3)[Fucα(1–2)]Galβ(1–x) specified by MAb 11 was not isolated as a discreet structure in any of the mucin species III, IV, and V but reflects a component common to more extended oligosaccharides which are themselves distinct, i.e., GalNacα(1–3)[Fucα(1–2)]Galβ(1–4)GlcNacβ(1–3)[Galβ(1–4)GlcNacβ(1–6)]Galβ(1–4)GlcNacβ(1–3)[NeuAcα(2–6)]GalNac-ol in HCM species III, GalNacα(1–3)[Fucα(1–2)]Galβ(1–3)GlcNacβ(1–4)Galβ(1–4)GlcNac-ol in HCM species IVandGalNacα(1–3)[Fucα(1–2)]Galβ(1–3)GlcNacβ(1–3)[NeuAcα-(2–6)]Galβ(1–4)GlcNacβ(1–3)[NeuAcα(2–6)]-GalNac-ol in HCM species V. It appears that the demonstration of binding to multiple HCM species by individual MAbs does not necessarily indicate the presence of identity of intact structures but can be related to recognition of limited determinants.

To provide insight into the nature of the structural determinants specified by 11 anti-HCM MAbs that did not bind the available isolated HCM oligosaccharides, the effect of nonspecific proteolytic digestion of HCM on subsequent binding was determined. Prolonged exposure of HCM to Pronase was found to effect a marked alteration in apparent size as judged by Sepharose 4B chromatography; >85% of material radiolabeled at nonreducing terminus appeared in the included volume following digestion. As noted in Table VIII, binding of four anti-HCM MAbs (2, 8, 10, 17) was significantly reduced after protease digestion of HCM, which suggests that these MAbs may be directed to components of the protein core. Note that protease digestion HCM appeared to bind to beads with an efficiency equivalent to the intact material, indicating that the impaired binding was not simply an effect of diminished adherence of the antigen to the bead. Furthermore, protease treatment had little effect on subsequent binding by those MAbs recognizing other antigenic structures (e.g., MAb 1; Table VIII). Alternatively diminished binding by some MAbs after protease treatment of the antigen may reflect a requirement for extended conformational features rather than recognition of a protein determinant per se. Binding to HCM by the 12 MAbs previously found to recognize oligosaccharide side chain structures was less affected by the protease (e.g., MAb 1 included for comparison in Table VIII). The nature of the structural determinants recognized by the remaining seven anti-HCM MAbs whose binding in solid-phase RIA was neither related to available oligosaccharides nor affected by protease treatment remains unknown.

Discussion

Previous work in this laboratory has suggested the presence of at least six structurally distinct species of mucin glycoprotein in the normal human colon (5, 7). The observation of a specific and selective reduction of mucin species IV in association with ulcerative colitis, independent of changes in other mucin components, suggests that distinctions made on the basis of chromatographic and compositional properties reflect biologically meaningful heterogeneity (5, 7, 32). However, these early studies were unable to assess the relatedness of the chromatographically defined mucin subclasses. Therefore, efforts reported here were undertaken to develop and characterize anti-HCM MAbs as probes for the further study of HCM structure and function. The results of the studies reported here have been augmented by conventional structural analysis of mucin oligosaccharides carried out in parallel (23).

HCM was found to be highly immunogenic in mice. Hybridomas showed a broad spectrum of relative selectivity for isolated HCM species, ranging from recognition of single HCM species to broad reactivity with all HCM species (Table I). These findings with uncloned fusion products indicate that each HCM species possesses "private" structural determinants as well as "common" structural features, which are shared to varying extent among the different species. At the same time they suggest that each species is distinct and not (antigenically) a subpopulation of another. It is of interest that some anti-HCM hybridomas recognized combinations of species with markedly divergent chromatographic elution properties (e.g., HCM species II and V), supporting the notion that separation into HCM species did not reflect arbitrary partition of a polydisperse substance.

These conclusions are further substantiated by the results of studies carried out with MAbs. Characterization of these stable reagents confirmed the presence of both shared and unique antigenic determinants among the HCM species. Various HCM species competitively inhibited binding of other species to multispecies-specific MAbs, supporting the conclusion that the different species contained some equivalent antigenic structures (Fig. 2). Although these findings could reflect the presence of cross-contamination between the mucin species recovered from ion exchange resin, this seems unlikely for several reasons. First, as noted above, some multispecies-specific MAbs were found to

Table VI. Binding of Anti-HCM MAbs to HCM Oligosaccharides

Structure*	Anti-HCM MAbs§ 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
Acidic oligosaccharides‡																							
1. NeuAcα(2–6)GalNAc-ol	410	350	290	450	310	210	340	660	490	520	460	530	610	450	360	510	490	650	280	350	340	560	420
	(16)	(5)	(12)	(16)	(15)	(8)	(12)	(17)	(18)	(15)	(12)	(17)	(10)	(9)	(15)	(11)	(20)	(17)	(7)	(3)	(18)	(14)	(5)
2. GlcNAcβ(1-3)GalNAc-ol 6 2 NeAcα	380	650	2,180	320	280	260	450	370	700	390	350	500	460	410	470	380	510	620	320	1,870	470	400	490
	(10)	(14)	(71)	(10)	(14)	(11)	(14)	(7)	(12)	(11)	(15)	(16)	(13)	(14)	(9)	(6)	(16)	(19)	(10)	(69)	(15)	(11)	(11)
3. Galβ(1–4)GlcNAcβ(1–3)GalNAc-ol 6 2 NeuAcα	520	710	1,040	610	250	290	570	490	540	370	540	560	390	460	380	410	530	340	280	350	520	560	480
	(14)	(8)	(25)	(16)	(11)	(7)	(17)	(12)	(11)	(7)	(11)	(13)	(10)	(21)	(12)	(15)	(17)	(11)	(0)	(3)	(12)	(14)	(9)
4. Galβ(1–4)GlcNAcβ(1–3)Galβ(1–4)GlcNAcβ(1–3)GalNAc-ol 6 2 NeuAcα	620	490	970	3,720	420	310	620	580	420	620	620	410	280	550	460	230	470	240	390	540	430	370	260
	(9)	(12)	(18)	(71)	(7)	(10)	(16)	(13)	(15)	(12)	(20)	(15)	(8)	(19)	(10)	(4)	(13)	(5)	(11)	(18)	(9)	(15)	(4)
5. Galβ(1–4)GlcNAcβ(1–3) Galβ(1–4)GlcNAcβ(1–3)GalNAc-ol 6 2 NeuAcα GlcNAcβ(1–6)	460	630	740	390	340	240	510	620	470	480	370	430	3,130	550	420	390	450	570	240	320	480	550	230
	(10)	(13)	(16)	(11)	(8)	(6)	(11)	(14)	(9)	(15)	(17)	(11)	(83)	(16)	(13)	(7)	(14)	(14)	(7)	(4)	(17)	(13)	(6)
6. GalNAcα(1–3)Galβ(1–4)GlcNAcβ(1–3) 2 1 Fucα Galβ(1–4)GlcNacβ(1–6) Galβ(1–4)GlcNAcβ(1–3)GalNAc-ol 6 2 NeuAcα	910	710	790	630	410	470	360	640	530	380	2,510	500	270	480	350	410	540	430	520	390	370	2,150	450
	(15)	(18)	(18)	(19)	(11)	(12)	(10)	(19)	(12)	(10)	(65)	(14)	(6)	(12)	(6)	(8)	(15)	(11)	(16)	(7)	(10)	(87)	(12)
7. GalNAcα(1–3)Galβ(1–3)GlcNAcβ(1–3)Galβ(1–4)GlcNAcβ(1–3)GalNAc-ol 2 6 6 1 2 2 Fucα NeuAcα NeuAcα	710	670	680	480	1,620	790	690	700	520	460	2,230	370	490	520	480	320	470	1,920	450	510	320	2,910	390
	(17)	(10)	(14)	(14)	(81)	(22)	(16)	(12)	(8)	(14)	(74)	(8)	(15)	(13)	(12)	(9)	(12)	(82)	(11)	(13)	(7)	(89)	(10)
8. GalNAcα(1–3)Galβ(1–3)GlcNAcβ(1–3) Galβ(1–4)GlcNAcβ(1–3)GalNAc-ol 6 2 NeuAcα Galβ(1–4)GlcNAcβ(1–6) 6 2 NeuAcα	390	480	580	420	660	300	410	540	670	500	660	450	410	600	570	450	410	370	290	640	570	360	430
	(14)	(11)	(17)	(9)	(15)	(8)	(13)	(10)	(17)	(15)	(13)	(11)	(14)	(17)	(11)	(10)	(11)	(4)	(3)	(13)	(12)	(2)	(11)
9. GalNAcα(1–3)Galβ(1–3)GlcNAcβ(1–3) 2 1 Fucα Galβ(1–4)GlcNAcβ(1–3)GalNAc-ol 6 2 NeuAcα GalNAcα(1–3)Galβ(1–3)GlcNAcβ(1–6) 6 2 NeuAcα	550	430	700	650	530	260	360	430	580	470	2,760	620	540	460	410	390	380	460	340	520	410	1,820	280
	(11)	(15)	(23)	(15)	(17)	(10)	(9)	(9)	(12)	(14)	(81)	(13)	(7)	(10)	(8)	(3)	(4)	(12)	(7)	(9)	(10)	(66)	(8)
10. Galβ(1–4)GlcNAcβ(1–3)GalNAc-ol 6 6 2 2 NeuAcα NeuAcα	640	350	590	540	920	1,470	550	610	380	620	380	490	360	590	360	410	420	420	470	320	340	480	370
	(16)	(13)	(10)	(15)	(27)	(68)	(17)	(18)	(15)	(12)	(11)	(8)	(20)	(17)	(6)	(11)	(10)	(75)	(20)	(11)	(9)	(14)	(9)

Neutral oligosaccharides																							
11. GlcNAcβ(1-3)GalNAc-ol	710 (12)	760 (17)	390 (12)	410 (18)	360 (10)	320 (12)	390 (13)	420 (8)	560 (12)	540 (17)	410 (18)	520 (15)	370 (12)	380 (16)	450 (14)	360 (12)	430 (8)	390 (10)	250 (4)	2,880 (78)	310 (5)	410 (12)	470 (14)
12. Galβ(1-4)GlcNAcβ(1-3)GalNAc-ol	540 (11)	710 (10)	470 (9)	760 (17)	470 (15)	380 (17)	450 (9)	520 (15)	600 (16)	380 (11)	590 (16)	650 (16)	510 (15)	470 (11)	320 (3)	470 (10)	340 (5)	410 (8)	280 (3)	470 (11)	520 (15)	530 (14)	520 (17)
13. GlcNAcβ(1-3)Galβ(1-4)GlcNAcβ(1-3)GalNAc-ol	880 (11)	570 (9)	410 (14)	840 (10)	260 (8)	350 (9)	430 (16)	490 (8)	420 (10)	510 (18)	470 (12)	460 (11)	380 (6)	390 (5)	380 (9)	420 (8)	470 (13)	490 (10)	330 (6)	460 (12)	430 (15)	350 (9)	540 (18)
14. Galβ(1-4)GlcNAcβ(1-3)Galβ(1-4)GlcNAcβ(1-3)GalNAc-ol	740 (15)	680 (18)	390 (11)	3,340 (63)	290 (5)	270 (16)	380 (14)	510 (11)	590 (15)	460 (10)	280 (6)	430 (13)	560 (4)	400 (17)	370 (9)	460 (12)	510 (15)	340 (4)	420 (10)	410 (11)	380 (8)	310 (5)	330 (6)
15. GalNAcα(1-3)Galβ(1-4)GlcNAcβ(1-3)Galβ(1-4)GlcNAcβ(1-3)GalNAc-ol 2 1 Fucα	810 (16)	490 (12)	560 (12)	2,390 (58)	480 (13)	360 (9)	540 (19)	360 (17)	620 (21)	570 (14)	2,420 (76)	380 (5)	420 (16)	410 (10)	340 (7)	280 (9)	510 (16)	640 (13)	390 (4)	440 (10)	370 (5)	2,360 (73)	260 (7)
16. GalNAcα(1-3)Galβ(1-4)GlcNAcβ(1-3) Galβ(1-4)GlcNAcβ(1-3)GalNAc-ol GlcNAcβ(1-6)	390 (11)	500 (21)	490 (11)	580 (15)	420 (14)	290 (10)	440 (9)	620 (13)	450 (15)	480 (19)	360 (11)	270 (5)	430 (6)	540 (12)	420 (11)	530 (10)	390 (17)	480 (17)	510 (15)	350 (4)	410 (10)	270 (8)	450 (12)
17. GalNAcβ(1-3)Galβ(1-4)GlcNacβ(1-3) 2 1 Fucα Galβ(1-4)GlcNAcβ(1-3)GalNAc-ol GalNAcα(1-3)Galβ(1-4)GlcNAcβ(1-6)	760 (14)	720 (15)	570 (17)	450 (17)	330 (11)	220 (7)	350 (16)	540 (8)	460 (14)	530 (10)	2,170 (68)	550 (8)	580 (12)	630 (15)	290 (2)	340 (12)	520 (18)	410 (16)	370 (3)	300 (9)	470 (11)	1,940 (70)	610 (18)
18. Galβ(1-4)GlcNAcβ(1-3) Galβ(1-4)GlcNAcβ(1-3)GalNAc-ol GlcNAcβ(1-6)	490 (12)	670 (13)	420 (16)	790 (15)	390 (16)	280 (8)	490 (13)	610 (17)	450 (18)	570 (12)	420 (10)	290 (3)	2,690 (74)	2,280 (86)	350 (10)	280 (6)	540 (15)	480 (11)	390 (13)	440 (8)	560 (14)	290 (3)	330 (10)
19. GalNAcα(1-3)Galβ(1-4)GlcNAcβ(1-3) Galβ(1-4)GlcNAcβ(1-3)GalNAc-ol GalNAcα(1-3)Galβ(1-4)GlcNAcβ(1-6)	630 (10)	510 (11)	640 (15)	370 (19)	340 (12)	370 (15)	530 (22)	650 (17)	520 (11)	390 (12)	360 (8)	370 (11)	570 (14)	610 (16)	520 (15)	370 (8)	460 (14)	290 (5)	380 (3)	410 (12)	330 (10)	250 (9)	560 (14)
20. GalNAcα(1-3)Galβ(1-4)GlcNAcβ(1-3)Galβ(1-4)GlcNAcβ(1-3)Galβ(1-4)GlcNAcβ(1-3)GalNAc-ol 2 1 Fucα	4,200 (76)	490 (13)	370 (13)	4,170 (57)	440 (17)	400 (17)	610 (15)	290 (10)	570 (23)	490 (17)	1,970 (61)	240 (9)	290 (5)	320 (10)	410 (15)	600 (10)	370 (3)	460 (18)	720 (14)	260 (17)	480 (5)	2,590 (66)	280 (11)
21. GalNAcα(1-3)Galβ(1-3)GlcNAcβ(1-3) Galβ(1-4)GlcNAcβ(1-3)GalNAc-ol Galβ(1-4)GlcNAcβ(1-6)	650 (11)	650 (14)	410 (18)	500 (12)	470 (16)	210 (6)	470 (14)	510 (18)	630 (15)	420 (11)	530 (18)	460 (8)	730 (42)	1,210 (27)	390 (5)	2,140 (91)	520 (13)	470 (16)	580 (7)	440 (14)	530 (17)	490 (10)	650 (21)

* Individual oligosaccharides isolated from human colonic mucin species by sequential chromatography and structures determined as previously described (23).

‡ Anti-HCM MAbs with properties detailed in text and legend to Table II. Individual MAbs were incubated at 37°C × 60 min beads coated with polystyrene beads coated with oligosaccharides followed by incubation with ^{125}I-labeled goat anti-mouse Ig as described under Methods. (%) = inhibition of MAb binding to HCM coated beads by prior adsorption with oligosaccharide coated beads in two-stage solid phase assay detailed under Methods.

§ Values are given in cpm. Numbers in parentheses = percent inhibition.

Table VII. Summary of Oligosaccharide Determinants of 12 Anti-HCM MAbs

MAb	HCM species specificity	Oligosaccharide*
1	IV, V	GalNAcα(1-3)[Fucα(1-2)]Galβ(1-4)GlcNAcβ(1-3)Galβ(1-4)GlcNAcβ(1-3)Galβ(1-4)GlcNAcβ(1-3)GalNAc-ol
3	I, II, III, IV, V	GlcNAcβ(1-3)[NeuAcα(2-6)GalNAc-ol
4	III, IV, V	Galβ(1-4)GlcNAcβ(1-3)Gal-
5	IV, V, VI	GalNAcα(1-3)[Fucα(1-2)]Galβ(1-4)GlcNAcβ(1-3)[NeuAcα(2-6)]Galβ(1-4)GlcNAcβ(1-3)GlcNAcβ(1-3)-[NeuAcα(2-6)]GalNac-ol
6	V	NeuAcα(2-6)Galβ(1-4)GlcNAcβ(1-3)[NeuAcα(2-6)]GalNAc-ol
11	II, IV, V, VI	GalNAcα(1-3)[Fucα(1-2)]Gal-
13	III, IV	Galβ(1-4)GlcNAcβ(1-3)[GlcNAcβ(1-6)]Galβ(1-4)GlcNAcβ(1-3)[±NeuAcα(2-6)]GalNAc-ol
14	IV	Galβ(1-4)GlcNAcβ(1-3)[GlcNAcβ(1-6)]Galβ(1-4)GlcNAcβ(1-3)GalNAc-ol
16	V, VI	GalNAcα(1-3)Galβ(1-4)GlcNAcβ(1-3)[Galβ(1-4)GlcNAcβ(1-6)]Galβ(1-4)GlcNAcβ(1-3)GalNAc-ol
18	IV, V	-[NeuAcα(2-6)]Galβ(1-4)GlcNacβ(1-3)[NeuAcα(2-6)]GalNac-ol
20	I, II, III, IV, V, VI	GlcNAcβ(1-2)GalNAc-ol
22	III, IV, V	GalNAcα(1-3)[Fucα(1-2)]Galβ(1-4)GlcNAc-

HCM subspecies determined by solid-phase RIA as described in text and Table II. * Oligosaccharide isolated from HCM species III, IV, V as described (23); specificity of MAbs determined as described in text and derived from data presented in Table VI.

bind to chromatographically remote mucin species without binding intermediate migrating components. Second, individual species could not be further dissociated into subpopulations as indicated by the uniform adsorption of each recognized mucin species on MAb affinity resins. Third, adsorption of a multi-species-specific MAb by one HCM species reduced binding to all recognized species in parallel (Table V). Collectively, these studies indicate that the determinants recognized by the MAb are in each instance integral structures of the mucin species bound. As a corollary, these data support the concept that the determinants on a particular mucin species recognized by different MAbs are distinct but reside on the same molecule.

A number of multispecies-specific MAbs were noted to recognize relatively circumscribed structural determinants (Table VI). Many of these MAbs were directed against di- or tri- saccharides present at or near the nonreducing termini of HCM oligosaccharides. Although binding to the various species did reflect the presence of these structures, they were not independent constituents but rather components of more extended and distinct structures.

Table VIII. Effect of Proteolytic Digestion of HCM on Anti-HCM MAb Binding

MAb*	HCM binding‡		
	Intact	Protease-treated	Percent
	cpm	*cpm*	
2	8,470±320	2,010±160	23.7
8	7,650±290	2,340±120	30.6
10	4,970±280	1,490±130	30.0
17	12,130±450	3,350±210	27.6
1	8,140±470	7,650±310	94.0

* Anti-HCM MAbs as designated and characterized in text and Table II. HCM (1.0 mg/ml) was incubated with Pronase (1% wt/vol) at 45°C; digestion was terminated at 0 time ("intact") or after overnight incubation ("protease treatment") by heat inactivation (60°C, 20 min).

‡ Binding of MAbs to polystyrene beads coated with either intact or protease digested HCM in solid-phase RIA as described in Methods. Expressed as cpm of ^{125}I-labeled goat anti-mouse Ig bound/bead; mean±SD.

These observations indicate that solid-phase binding studies using intact glycoproteins could lead to overestimation of the extent of biologically significant structural homology. These studies also indicate that reagents directed at smaller determinants may be insufficiently discriminating to distinguish biologically significant structural differences among glycoproteins. Indeed, several earlier studies have utilized probes directed to circumscribed nonreducing terminal structures to explore colonic mucin heterogeneity. These probes have included lectins that recognize terminal monosaccharides or disaccharides (18, 22, 33, 34), histochemical stains that bind peripheral charged residues (11, 30, 35, 36) or conventional antisera directed at limited peripheral carbohydrate determinants (17, 37, 38). The present results suggest that relatively nonspecific tools may both underestimate heterogeneity and overestimate structural homologies. These findings show the importance of defining the antigenic determinants recognized by MAbs before they can be meaningfully used to assess structural relationships.

The presence and nature of oligosaccharides common to different mucin species were shown by the identification of the antigenic determinants recognized by MAbs that bound multiple HCM species. Conversely, and perhaps more importantly, isolation of MAbs with defined antigenic determinants that recognized only a single HCM species confirms the structural relevance of the chromatographic distinctions made in separating these entities.

Note that several anti-HCM MAbs failed to bind separated HCM species despite recognition of unfractionated HCM. While it is possible that these MAbs are directed to contaminating non-mucin components, this seems unlikely in view of the avid staining of goblet cell mucin droplets found in related studies (31). This apparent paradox may reflect limitations of the assay

methodology used. Conformational presentation of the relevant structural determinant may be lacking on the solid phase used for sandwich RIA in these studies. Alternatively, the assay conditions used may not facilitate MAb binding because of the nature of the antigenic determinant or properties of the MAb itself. However, failure to demonstrate solid-phase binding to the separated species for these technical reasons is difficult to reconcile with the ability to effect binding to the unfractionated preparations. Alternatively, these MAbs may be directed to components of HCM which are not recovered from the DEAE-cellulose column used to separate HCM species. As previously reported, recovery of HCM after ion exchange chromatography is ≤84% and it is possible that minor mucin constituents recognized by these MAbs are lost during these procedures.

In summary, development and characterization of anti-HCM MAbs has confirmed the presence of structurally distinct subclasses of HCM that nonetheless share some common oligosaccharide side chains. While much further study will be necessary to define the structure of normal HCM species, these MAbs should prove particularly useful. Use of MAbs recognizing defined antigen determinants will permit assessment of the presence of these structures in uncharacterized mucin species from the colon as well as mucin from other sites. Further, the availability of MAbs that appear to recognize protein determinants should facilitate isolation and characterization of HCM peptides. It will be especially important to learn whether HCM species possess distinctive protein cores reflecting different gene products. Efforts are under way to exploit anti-HCM MAbs for efficient purification and quantitation of total HCM and component species. Finally, the demonstration of the presence of multiple and distinct HCM species raises the question of the functional and cellular significance of this heterogeneity. A companion report contains initial attempts to address the latter issue using anti-HCM MAbs and indirect immunofluorescence techniques to localize HCM species in goblet cells in situ (31).

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