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The sugar chain structures of carcinoembryonic antigens and related normal antigens

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Abstract : Glycoproteins, cross-reacting with carcinoembryonic antigen (CEA), were found in normal human feces (NFA-2) and meconium (NCA-2). These three antigens are considered as the same gene products. Comparative study of their sugar chain structures, however, revealed that they have different sets of N-linked sugar chains reflecting the developmental and malignant transformational changes of the sugar chains of colon glycoproteins. High mannose type sugar chains and C2,4-branched biantennary sugar chains were found only in CEA. The branching structures of the complex type sugar chains of the three antigens are almost the same. However, Type 1 chain is detected in the outer chain moieties of NFA-2 and NCA-2 but not in those of CEA. All three antigens contain sulfated and sialylated sugar chains. The acidic sugar chains of NFA-2 and NCA-2 contain the Galβ1→3(HSO_3^-→6)GlcNAc and the Neu5Acα2→3Galβ1→3GlcNAc groups. In contrast, the Galβ1→4(HSO_3^- →6)GlcNAc and the Neu5Acα2→6Galβ1→4GlcNAc groups are detected in CEA.

INTRODUCTION

Carcinoembryonic antigen (CEA) was first reported in 1965 by Gold and Freedman (1) as a glycoprotein which is present in the glycocalyx of adenocarcinoma cells of the human digestive tract as well as in fetal gastrointestinal tissues. CEA has been recognized as the most widely used tumor marker, the serum level of which is used for monitoring the prognosis of patients with colon, breast or lung adenocarcinoma after surgery (2). Glycoproteins cross-reacting with anti-CEA antibodies were found in normal human feces (3), meconium (4), lung and spleen (5) as well as hematopoietic cells including granulocytes (6) and monocytes (7).

The primary structure of the polypeptide of CEA was elucidated by Oikawa et al (8) by cloning the cDNA of CEA. The peptide portion of CEA is composed of 668 amino acid residues, which can be divided into five domains : an N-terminal domain (domain N) of 108 amino acid residues, three homologous repetitive domains I, II and III, and a hydrophobic C-terminal domain (domain M) composed of 26 amino acid residues. Each of the three repetitive domains, which are composed of 178 amino acid residues, could be divided into A and B sub-domains. Based on this structural evidence, Barnett and Zimmermann (9) proposed the nomenclature A1-B1, A2-B2 and A3-B3 for domains I, II and III, respectively as shown in Fig.1. After the precursor polypeptide of CEA was translated, its domain M is replaced by a glycosylphosphatidylinositol, which anchors CEA molecule to the plasma membrane of epithelial cells (10).

Successful cloning of the cDNA of CEA has opened the door to resolve the genomic sequences which encode the CEA-related antigens in normal tissues. It was found that the largest cross-reacting antigen in normal adult feces, which was named normal fecal antigen (NFA-2) (11), is the same gene product as CEA (12, 13). The non-specific cross-reacitng antigen-2 (NCA-2), which was isolated from meconium (4), is also considered to have the same amino acid sequence (14).
It has been known from the early stage of CEA research that CEA contains large amount of sugars. Actually, twenty eight potential N-glycosylation sites are included in the amino acid sequence of CEA (Fig.1). Because NFA-2, NCA-2 and CEA are respectively produced by colon epithelial cells of normal

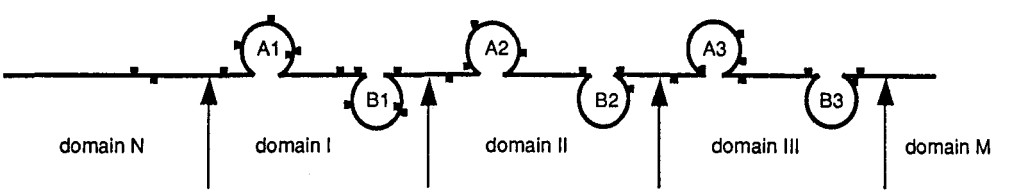

Fig.1. Schematic model of CEA. Black triangles indicate the sugar chains.

adults, those of fetus at their last differentiation stage, and those in malignant states, comparative study of their sugar chain structures was expected to give a useful information as to the developmental and malignant transformational changes of the sugar chains of colon glycoproteins. It was also expected that the information may contribute to improve the diagnostic value of CEA if any difference in the sugar chains of CEA and CEA-related antigens in normal tissues are found.

PURIFICATION OF CEA, NCA-2 AND NFA-2 SAMPLES

CEA samples were purified from liver metastases of colon carcinoma obtained from three patients (TY. MY and TT) by a series of procedures reported previously (15). All three donors were blood type A, secretor. The three samples gave a broad single band stainable with both Coomassie brilliant blue and periodic acid-Schiff reagent after SDS-polyacrylamide gel electrophoresis. The molecular weights of all three samples were approximately 180,000. All of them gave a single precipitin line by immunoelectrophoresis with use of goat anti-human CEA antisera. These three CEA samples were named CEA-TY, CEA-MY and CEA-TT, respectively.

An NCA-2 sample was purified from a pooled meconium collected from more than 20 neonates of various blood types. After treatment with 0.6M perchloric acid to precipitate the protein, the antigens with CEA activity were adsorbed to goat anti-CEA adsorbent and eluted with 0.175M glycine-HCl buffer, pH 2.3. NCA-2 in the eluate was further purified by serial chromatography on a Sepharose 6B and a Sephadex G-200 column as reported previously (16). NCA-2, thus obtained, gave a broad single band of molecular weight of 170,000 upon SDS-polyacrylamide gel electrophoresis, which is stainable with either Coomassie brilliant blue or periodic acid-Schiff reagent.

NFA-2 samples were purified from feces of four healthy individuals (YK, AM, KT and KM) by perchloric acid extraction followed by affinity chromaography on an immunoadsorbent like in the case of NCA-2 preparation. The antigenic samples were then further purified by concanavalin A (Con A)-Sepharose column chromatography and gel filtration as reported by Koga et al (15). All of these four donors happened to be blood type Lewis b^+, secretor. These four NFA-2 samples were named NFA-2YK, NFA-2AM, NFA-2KT and NFA-2KM, respectively.

PRELIMINARY STUDIES OF THE SUGAR CHAINS OF CEA, NCA-2 AND NFA-2

Analysis of the monosaccharide compositions of CEA, NCA-2 and NFA-2 revealed that they all contain large amounts of glucosamine but almost negligible amount of galactosamine. These data indicated that only N-linked sugar chains are included in these samples. Therefore, the antigenic samples were subjected to hydrazinolysis (17) in order to release all of their sugar chains as oligosaccharides from their polypeptide backbones. Released oligosaccharides were then labeled by NaB^3H_4 reduction after *N*-acetylation. Based on the radioactivities incorporated, the amounts of the oligosaccharides released from 1 mol of CEA, NCA-2 and NFA-2 were calculated to be 24-26, 27 and 24-27 mol, respectively (Table I).

Table I. Characteristics of *N*-linked sugar chains released from NFA-2, CEA and NCA-2.

	NFA-2				CEA			NCA-2
	KM	AM	KT	YK	TY	MY	TT	
Number of oligosaccharides (mol/mol)	25	27	24	25	26	25	24	27
Neutral oligosaccharides (%)	50	52	51	42	34	42	28	42
Sialylated oligosaccharides (%)	18	12	11	20	18	48	65	50
Sulfated oligosaccharides (%)	32	36	38	38	48	10	7	8
High mannose type (%)	0	0	0	0	8	10	7	0
Complex type (%)	100	100	100	100	92	90	93	100

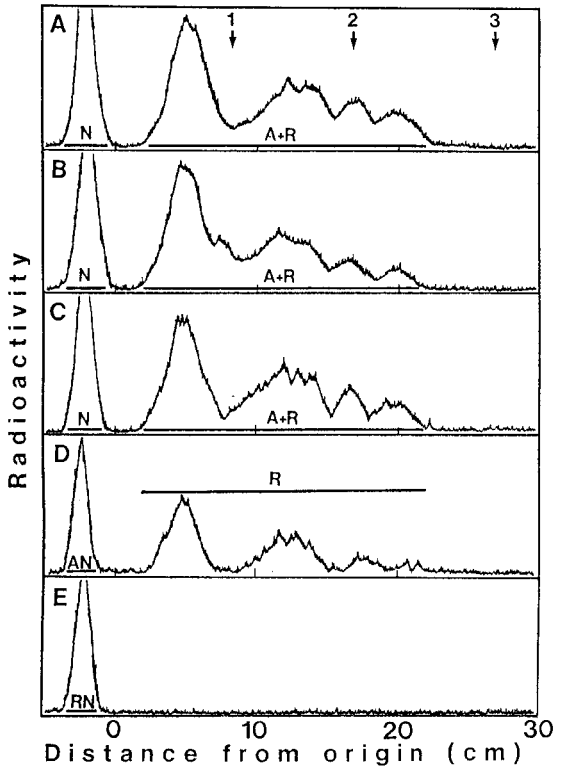

Fig.2. Paper electrophoresis of the radioactive oligosaccharide fractions released from CEA and its related antigens by hydrazinolysis followed by NaB^3H_4 reduction. *Arrows*, migration positions of authentic oligosaccharides : 1, Neu5Acα2→6Galβ1→4GlcNAcβ1→ 2Manα1→3 or 6(Galβ1→4GlcNAcβ1→ 2Manα1→6 or 3) Manβ1→ 4GlcNAcβ1→ 4(Fucα1→6)$GlcNAc_{OT}$; 2, Neu5Acα2→ 6Galβ1→ 4GlcNAcβ1→2Manα1→ 6(Neu5Acα2→ 6Galβ1→4GlcNAcβ1→ 2Manα1→3)Manβ1→4GlcNAcβ1→ 4(Fucα1→6)$GlcNAc_{OT}$. A, B and C, oligosaccharide fractions from CEA-TY, NCA-2 and NFA-2KM ; D, fraction A+R in (C) digested with *Arthrobacter* sialidase; E, fraction R in (D) after methanolysis.

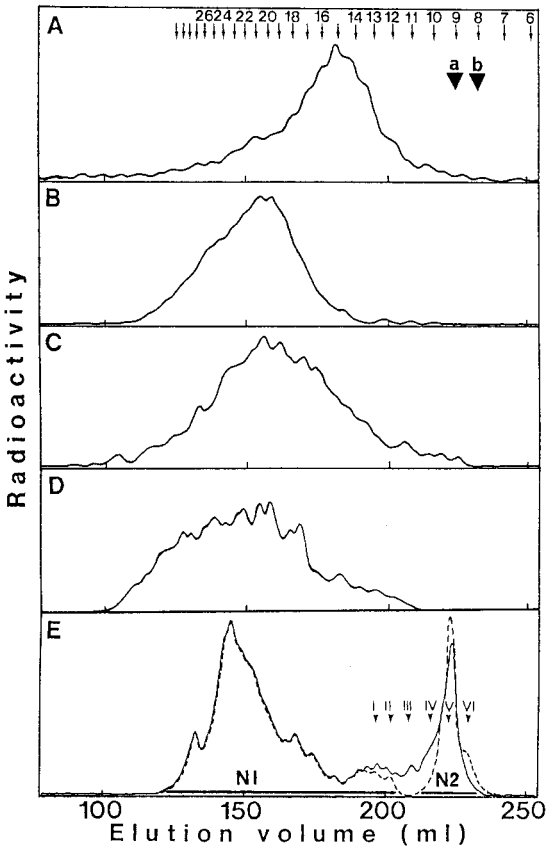

Fig.3. Bio-Gel P-4 column chromatography of neutral oligosaccharide fractions. Arrows at the top indicate the elution positions of glucose oligomers (numbers indicate the glucose units). Black triangles indicate the elution positions of authentic $Man_5 \cdot GlcNAc \cdot GlcNAc_{OT}$(a) and $Man_4 \cdot GlcNAc \cdot GlcNAc_{OT}$(b). A, B and C, elution patterns of fractions N, AN and RN in Fig.2 C; D, fraction N from NCA-2 in Fig.2 ; E, solid line and dotted line indicate the fraction N from CEA-TY before and after *Aspergillus* α-mannosidase I digestion, respectively.

Upon paper electrophoresis, the tritium-labeled oligosaccharide fractions obtained from the eight antigenic samples were separated into a neutral (N) and several acidic fractions. In Fig.2A, B and C, radioelectrophoretograms of the oligosaccharide fractions from CEA-TY, NCA-2 and NFA-2KM are shown as representative data. Parts of the acidic fractions (A+R) were converted to neutral oligosaccharides by *Arthrobacter* sialidase treatment (fraction AN in Fig.2D : the data obtained from NFA-2KM is shown as an example.). The remaining acidic oligosaccharides shown as fraction R in Fig.2D were completely resistant to repeated sialidase digestion but were converted to neutral oligosaccharides by methanolysis (fraction RN in Fig.2E). These results indicated that the oligosaccharides in fraction R probably contain sulfate.

Bio-Gel P-4 column chromatography of fractions N, AN and RN from the eight antigenic samples revealed that all of them were mixtures of extremely heterogeneous oligosaccharides : data of the fractions N, AN and RN from NFA-2KM and of the fractions N from NCA-2 and CEA-TY are shown as solid lines in Fig.3A,B,C,D and E, respectively. When incubated with *Aspergillus* α-mannosidase I, which cleaves only the Manα1→2Man linkage (18), a small part of the oligosaccharides in the fractions N from the three CEA samples were converted to two radioactive components with the same mobilities as authentic Manα1→6(Manα1→3)Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4$GlcNAc_{OT}$(Man_5·

GlcNAc·GlcNAc$_{OT}$) and Manα1→3Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4GlcNAc$_{OT}$ (Man4·GlcNAc·GlcNAc$_{OT}$), respectively (fraction N2 shown by dotted line in Fig.3E). By comparing the elution profiles of the solid line and the dotted line in Fig.3E, it was confirmed that the two radioacitve oligosaccharides in fraction N2 were converted from Man9~5·GlcNAc·GlcNAc$_{OT}$. In contrast, no degradation was observed by the α-mannosidase digestion of fractions N from NCA-2 and NFA-2 samples. Therefore, a series of high mannose type sugar chains should be included in the fractions N from the three CEA samples, but not in those from NCA-2 and NFA-2 samples (Table I).

When the α-mannosidase resistant neutral oligosaccharides (corresponding to the fraction N1 of the dotted line in Fig.3E) from the three CEA samples, the fractions N from NCA-2 and NFA-2 samples, and the fractions AN and RN from all of the eight antigenic samples were incubated with a mixture of *Streptococcus* 6646K β-galactosidase (will be called hereafter as 6646K β-galactosidase) and jack bean β-*N*-acetylhexosaminidase, only small portions of them were converted to two radioactive oligosaccharides with the same mobilites as authentic Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→4(Fucα1→6) GlcNAc$_{OT}$ (Man3·GlcNAc·Fuc· GlcNAc$_{OT}$) and Manα1→6(Manα1→3)Manβ1→4GlcNAcβ1→ 4GlcNAc$_{OT}$(Man3.GlcNAc.GlcNAc$_{OT}$). Upon digestion with a mixture of bovine epididymis α-fucosidase, 6646K β-galactosidase and jack bean β-*N*-acetylhexosaminidase, however, all of them were converted to Man3·GlcNAc·GlcNAc$_{OT}$. Therefore, the oligosaccharides in these neutral fractions should be complex type oligosaccharides with fucosylated and non-fucosylated trimannosyl cores and various combination of the (Galβ1→GlcNAcβ1→)n groups and their fucosylated forms in their outer chain moieties.

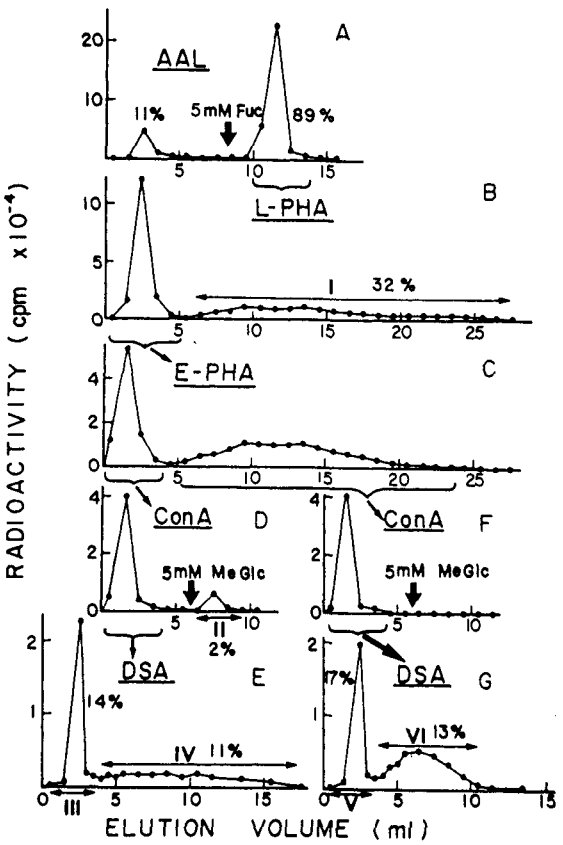

Fig.4. Serial immobilized lectin column chromatography of fractions N+AN+RN from NCA-2.
AAL, *Aleuria aurantia* lectin;
L-PHA, phytohaemagglutinin-L4;
E-PHA, phytohaemagglutinin-E4;
ConA, concanavalin A;
DSA, *Datura stramonium* agglutinin.

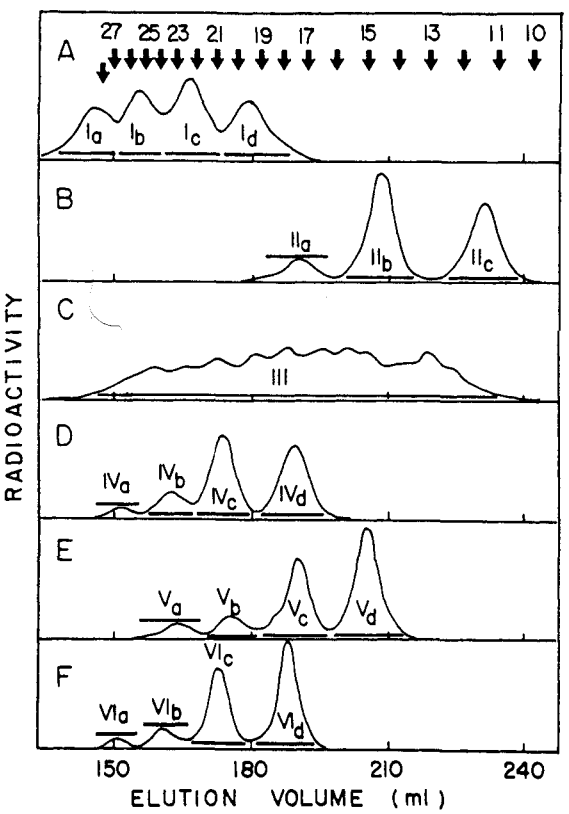

Fig.5. Bio-Gel P-4 column chromatography of oligosaccharide fractions I ~ VI in Fig.4. Arrows are the same as in Fig.3. Panels A, B, C, D, E and F represent the elution patterns of fractions I, II, III, IV, V and VI, respectively.

STRUCTURES OF THE DEFUCOSYLATED OLIGOSACCHARIDES.

In order to elucidate the defucosylated backbone structures of the complex type sugar chains, the oligosaccharides in the pooled fractions N, AN and RN from NCA-2,CEA and NFA-2 samples were dissolved in 0.01N HCl and heated at 100°C for 90 min. α-Fucosyl residues in the outer chain moieties of the complex type oligosaccharides were removed by this treatment, while almost all of other glycosidic linkages including the Fucα1→6GlcNAc$_{OT}$ group remained intact (19). It was also confirmed that a part of the GlcNAc$_{OT}$ residues is de-*N*-acetylated by this treatment. After the defucosylated oligosaccharide fractions had been re-*N*-acetylated, they were subjected to affinity chromatography with use of an *Aleuria aurantia* lectin (AAL)-Sepharose column. All of them were separated into a pass through fraction (AAL^-) and a fraction bound to the column, which was then eluted with the buffer containing 5mM L-fucose (AAL^+). The molar ratio of fractions AAL^- and AAL^+ obtained from NCA-2, CEA and NFA-2 samples were approximately 1:9. The two fractions from NCA-2 were further fractionated by a serial affinity chromatography with use of four immobilized lectin columns (Fig.4). Although the data of the fraction AAL^+ are shown in the figure, the fraction AAL^- gave almost the same fractionation pattern as AAL^+. The six fractions, thus obtained, were then subjected to Bio-Gel P-4 column chromatography. As shown in Fig.5, all of them were separated into several peaks. The structures of oligosaccharides in each peak were determined as summarized in Fig.6 by combination of endo-β-galactosidase digestion, sequential exoglycosidase digestion and methylation analysis. Since the details of the results to reach to their structural assignments were reported in detail in the orginal paper (20), they are not repeated here.

The defucosylated complex-type oligosaccharide fractions from the three CEA samples and four NFA-2 samples also contain the bisected and non-bisected sugar chains with fucosylated and non-fucosylated trimannosyl cores as shown in Fig.6. However, only a trace amount of the Galβ1→3GlcNAc group was included in the outer chain moieties of CEA samples, because most of them were converted to trimannosyl cores by incubation with a mixture of diplococcal β-galactosidase and jack bean β-*N*-acetylhexosaminidase. On the contrary, 75% of the oligosaccharides from NFA-2 samples were not converted to the trimannosyl cores by incubation with the same enzymes mixture, indicating that the Galβ1→3GlcNAc group is enriched in their outer chain moieties. The molar ratio of the bisected oligosaccharides in both CEA and NFA-2 are 40%, and mono-, bi-, tri- and tetraantennary oligosaccharides were similarly detected in both antigens. An important evidence is that approximately 5% of C-2 and 4 substituted abnormal biantennary sugar chains were detected in CEA but not in NFA-2 and NCA-2 (Table II). Because this C-2, 4 substituted biantennary sugar chains were also detected in hepatoma γ-glutamyltranspeptidase (21) and choriocarcinoma human chorionic gonadotropin (22), alteration in the substrate specificity of *N*-acetylglucosaminyltransferase IV may be a widely occurring phenomenon in malignant cells.

STRUCTURES OF THE OUTER CHAIN MOIETIES OF THE COMPLEX TYPE SUGAR CHAINS OF CEA AND ITS RELATED ANTIGENS

In fractions N, AN and RN, some of the oligosaccharides in Fig.6 should occur as fucosyl derivatives. In order to elucidate the structures of the outer chain moieties of the complex type sugar chains of NFA-2, NCA-2 and CEA samples, the pooled fractions of N, AN and RN in Fig.2 obtained from eight antigenic samples were subjected to sequential glycosidase digestion as summarized in Table III. First of all, the neutral oligosaccharide fractions were digested with endo-β-galactosidase, and the released oligosaccharides were labeled by NaB^3H_4 reduction. The fragments released from the outer chains of the oligosaccharides (*peaks b* in Fig.7) were separated from the fragments containing the trimannosyl core (*peak a* in Fig.7) by Bio-Gel P-4 column chromatography. Based on the specific activity of the NaB^3H_4 used, the amount of oligosaccharides in the *peaks b* was calculated. Structural study of the oligosaccharides included in *peaks b* by sequential immobilized lectin column chromatography and sequential exoglycosidase digestion revealed that they have the structures as listed in Table III. Since the details of the analytical procedures were reported in a previous paper (20), they are not repeated here.

The *peak a* in Fig.7 contains complex type oligosaccharides in which the Galβ1→3GlcNAcβ1→ and the Galβ1→4GlcNAcβ1→ groups and their fucosylated forms remained linked to the trimannosyl cores together with the remaining β-*N*-acetylglucosaminyl residues. In order to elucidate the structures of these outer chains, which were resistant to endo-β-galactosidase digestion, all of the *peak a* was subjected to sequential exoglycosidase digestion shown as steps 2-9 in Table III, and the amount of the galactose residues released at each step was determined by an HPLC method after NaB^3H_4 reduction (23). After treatment with each step, the fragment containing the trimannosyl cores were recovered by Bio-Gel P-4 column chromatography, and then subjected to the next step digestion.

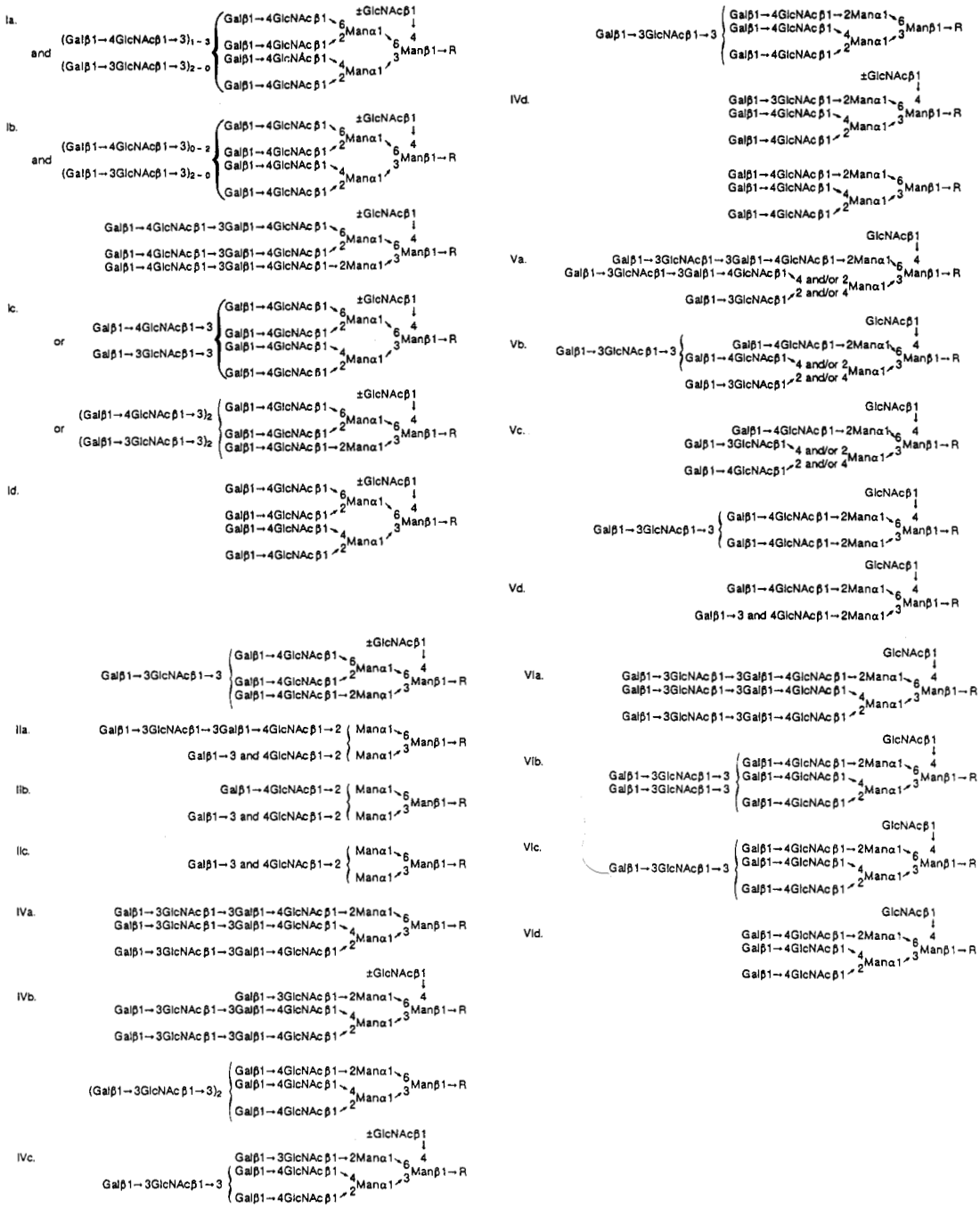

Fig.6. Structures proposed for oligosaccharides included in radioactive peaks in Fig.5. R represents 4GlcNAcβ1→4(Fucα1→6)GlcNAc$_{OT}$. In the case of fraction AAL$^-$, R represents 4GlcNAcβ1→4GlcNAc$_{OT}$. Because of the complex nature, the structures of oligosaccharides in fraction III are not included.

The step 2 specifically hydrolyzed the Galβ1→4GlcNAcβ1→Man groups. Since 6646K β-galactosidase cleaves the Galβ1→3GlcNAc linkage as well as the Galβ1→4GlcNAc linkage, the galactose residues of the Galβ1→3GlcNAcβ1→Man groups were then released by the step 3 treatment. *Bacillus* α-fucosidase cleaves specifically the Fucα1→2Gal linkage (24). The galactose residues of the newly

Table II. Characteristics of complex type sugar chains of NFA-2, CEA and NCA-2

Complex type sugar chains		NFA-2	CEA	NCA-2
		% molar ratio		
Bisected oligosaccharides		40	40	80
Oligosaccharides containing repeating structures		15	15	60
Oligosaccharides containing Type 1 chains in the backbone		75	trace	34
Branching structures				
monoantennary	C-2	4	3	2
biantennary	C-2, C-2	30	25	14
	C-2,4	0	6	0
triantennary	C-2, C-2,4	25	23	41
	C-2, C-2,6	16	14	16
tetraantennary	C-2,4, C-2,6	25	29	27

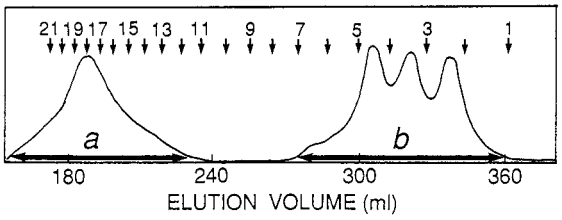

Fig.7. Bio-Gel P-4 column chromatography of fraction N+AN+RN after endo-β-galactsidase digestion. Arrows are the same as in Fig.3.

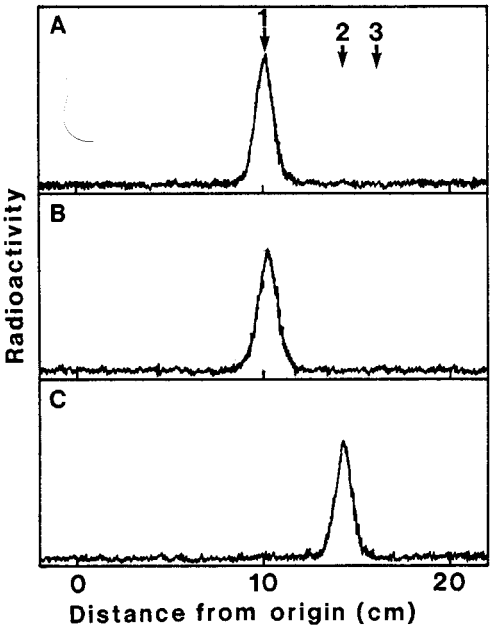

Fig.8. Cellulose thin layer chromatography of the acidic fragments obtained from the fraction R from NFA-2KM by hydrazinolysis-nitrous acid treatment. Arrows indicate the migration positions of authentic sulfated sugars : 1, Galβ1→4(HSO_3^-→6)2,5-anhydromannitol ; 2, (HSO_3^-→6)2,5-anhydromannitol; 3, (HSO_3^-→3)2,5-anhydromannitol. A, the radioactive acidic fragment obtained from the fraction R by hydrazinolysis-nitrous acid treatment ; B, the fragment in (A) after diplococcal β-galactosidase digestion ; C, the fragment in (A) after 6646K β-galactosidase digestion.

exposed Galβ1→4GlcNAcβ1→Man groups were cleaved by the step 4. The galactose residues of the Galβ1→3GlcNAcβ1→Man groups newly exposed by *Bacillus* α-fucosidase digestion were then cleaved by the step 5 treatment. Since *Bacillus* α-fucosidase cannot cleave the Fucα1→2Gal linkages of the Fucα1→2Galβ1→3 and 4(Fucα1→4 and 3)GlcNAc groups, these tetrasaccharides linked to the α-mannosyl residues of the trimannosyl cores remained intact. By treatment with almond α-fucosidase I, the Fucα1→3GlcNAc linkages in the Galβ1→4(Fucα1→3)GlcNAcβ1→Man and the Fucα1→2Galβ1→ 4(Fucα1→3)GlcNAcβ1→Man groups and the Fucα1→4GlcNAc linkages in the

Table III. The results of sequential endo- and exoglycosidase digestion, which were used to elucidate the structures of the outer chain moieties of complex type oligosaccharides released from NFA-2, CEA and NCA-2.

Step	Enzymes in the reaction mixture	Groups to be hydrolyzed	NFA-2	CEA			NCA-2
				TY	MY	TT	
			% molar ratio				
1.	endo-β-galacosidase	Galβ1→3GlcNAcβ1→3Gal (Type 1)	7	0	0	0	5
		Galβ1→4GlcNAcβ1→3Gal (Type 2)	0	7	11	6	3
		Fucα1→2Galβ1→3GlcNAcβ1→3Gal (Type 1H)	0	0	0	0	7
2.	diplococcal β-galactosidase +jack bean β-HexNAc'ase	Galβ1→4GlcNAcβ1→ (Type 2)	27	28	37	33	42
3.	6646k β-galactosidase +jack bean β-HexNAc'ase	Galβ1→3GlcNAcβ1→ (Type 1)	20	3	0	4	17
4.	Bacillus α-fucosidase +diplococcal β-galactosidase +jack bean β-HexNAc'ase	Fucα1→2Galβ1→4GlcNAcβ1→ (Type 2H)	5	7	0	7	8
5.	6646k β-galactosidase +jack bean β-HexNAc'ase	Fucα1→2Galβ1→3GlcNAcβ1→ (Type 1H)	5	0	0	0	6
6.	almond α-fucosidase +diplococcal β-galactosidase +jack bean β-HexNAc'ase	Fucα1↘3 Galβ1→4GlcNAcβ1→ (X)	11	26	34	41	9
7.	6646k β-galactosidase +jack bean β-HexNAc'ase	Fucα1↘4 Galβ1→3GlcNAcβ1→ (Le^a)	12	2	11	0	0
8.	Bacillus α-fucosidase +diplococcal β-galactosidase +jack bean β-HexNAc'ase	Fucα1↘3 Fucα1→2Galβ1→4GlcNAcβ1→ (Y)	0	25	7	6	1
9.	6646k β-galactosidase +jack bean β-HexNAc'ase	Fucα1↘4 Fucα1→2Galβ1→3GlcNAcβ1→ (Le^b)	11	2	0	3	0

Galβ1→3(Fucα1→4) GlcNAcβ1→Man and the Fucα1→2Glcβ1→3(Fucα1→4)GlcNAcβ1→Man groups were cleaved. The galactose residues of the Galβ1→4GlcNAcβ1→Man groups newly exposed by the α-fucosidase treatment were released by diplococcal β-galactosidase digestion in the step 6. The galactose residues of the newly exposed Galβ1→3GlcNAcβ1→Man groups were then released by the step 7 treatment. At this stage, the Fucα1→2Galβ1→4(Fucα1→3)GlcNAcβ1→Man and the Fucα1→2Galβ1→3(Fucα1→4) GlcNAcβ1→Man groups were converted to the Fucα1→2Galβ1→4GlcNAcβ1→Man and the Fucα1→ 2Galβ1→3GlcNAcβ1→Man groups, respectively. The amounts of these groups could be estimated by the amounts of the galactose released by diplococcal β-galactosidase (the step 8) and 6646K β-galactosidase (the step 9) after *Bacillus* α-fucosidase digestion, respectively.

In Table III, the amount in percent molar ratio and the structure of the outer chain hydrolyzed by each step of the glycosidase digestion are summarized. Structures of the outer chain moieties of NFA-2 and NCA-2 are very similar. The only prominent difference is the absence of the Galβ1→3(Fucα1→4) GlcNAc (Le^a antigenic determinant) and the Fucα1→2Galβ1→3(Fucα1→4)GlcNAc (Le^b antigenic determinant) groups in NCA-2. This evidence meets with the reported fact that Lewis enzyme responsible for the formation of the Fucα1→4GlcNAc group is expressed in colon epithelial cells after birth and the Lewis antigens are immuno-histochemically detected in the digestive tract after birth but not at fetal stage. Another interesting evidence is that Y antigenic determinant is detected in the sugar chains of NCA-2 but not in those of NFA-2 samples.

Several striking differences were found in the outer chain moieties of the sugar chains of CEA and NFA-2. The most remarkable one is in the content of the Galβ1→3GlcNAc group (Type 1 chain) as already described. Approximately half of the outer chains of NFA-2 contain Type 1 chain which were distrib-

uted in 75% of the complex type sugar chains (Table II), while only a trace amount of the chain was detected in the three CEA samples. This result indicated that the expression of GlcNAc : β1→3 galactosyl transferase in the colon epithelial cells is strongly suppressed by malignant transformation.

SULFATED OLIGOSACCHARIDS

The sulfate linkages of the oligosaccharides in the fractions R were determined by the hydrazinolysis-nitrous acid deamination method by Edge and Spiro (25). After fractions R were heated in anhydrous hydrazine at 100°C for 24hrs, the de-*N*-acetylated oligosaccharides were deaminated and partially hydrolyzed by treatment with 0.2M $NaNO_2$ in 0.5N acetic acid at room temperature for 2hrs. After the oligosaccharide fragments were labeled by reduction with NaB^3H_4, the radioactive sulfated fragments were separated from neutral fragments by paper electrophoresis. The radioactive acidic fragments from NFA-2, NCA-2 and CEA samples all migrated at the position of sulfated disaccharides. The radioactive acidic fragments were extracted from paper, spotted on a cellulose plate and then subjected to a thin layer chromatography with use of Fisher-Nöbel solvent.

The radioactive sulfated disaccharides from NFA-2 and NCA-2 migrated to the same position as authentic Galβ1→4(HSO_3^-→6)2,5-anhydromannitol (Fig.8A). In contrast to the authentic sample, however, the acidic sample did not bind to an RCA-I-agarose column and not converted to (HSO_3^- →6)2,5-anhydromannitol by diplococcal β-galactosidase digestion (Fig.8B). Upon digestion with 6646K β-galactosidase, the acidic sample was converted to (HSO_3^-→6)2,5-anhydromannitol but not to (HSO_3^- →3)2,5-anhydromannitol (Fig.8C). Because both the Galβ1→3GlcNAc and the Galβ1→4GlcNAc groups were detected in the outer chain moieties of the complex-type sugar chains of NFA-2 and NCA-2, the above results indicated that the acidic fragment in Fig.8A should be Galβ1→3(HSO_3^-→6)2,5-anhydromannitol. Therefore, the sulfate residues of the oligosaccharides in the fractions R from NFA-2 and NCA-2 should exclusively occur as the Galβ1→3(HSO_3^-→6)GlcNAc group.

Analysis of the oligosaccharides in the fractions R from CEA samples by the cellulose plate thin layer chromatography also gave a single peak with the same mobility as authentic Galβ1→4(HSO_3^-→6)2,5-anhydromannitol (data not shown). However, the sulfated disaccharide bound to an RCA-I agarose column and was converted to ($HSO3^-$→6)2,5-anhydromannitol by digestion with diplococcal β-galactosidase. These results indicated that the sulfate residues of the oligosaccharides from CEA samples should exclusively occur as the Galβ1→4(HSO_3^-→6)GlcNAc group.

SIALYL LINKAGES OF THE COMPLEX TYPE SUGAR CHAINS

Same portions of the acidic oligosacccharides from the four NFA-2 samples and the NCA-2 sample were converted to neutral oligosaccharides by *Salmonella* sialidase digestion and by *Arthrobacter* sialidase degestion. Since *Salmonella* sialidase hydrolyzes the Neu5Acα2→3Gal linkage but not the Neu5Acα2→6Gal linkage, all sialic acid residues of the five antigens should occur as the Neu5Acα2→3Gal group. Furthermore, the acidic oligosaccharide fractions from the five antigens completely passed through a column containing *Macckia amurensis* lectin (MAL)-Sepharose. Because the lectin specifically interacts with the Neu5Acα2→3Galβ1→4GlcNAc group (26), the result indicated that all sialic acid residues of NFA-2 and NCA-2 occur as the Neu5Acα2→3Galβ1→3GlcNAc group. In contrast to the five antigens, the acidic oligosaccharide fractions from the three CEA samples were mostly resistant to *Salmonella* sialidase digestion. Furthermore, the sialylated oligosaccharide fractions strongly bound to a column containing TJA-I-Sepharose, but mostly passed through a MAL-Sepharose column. These results and methylation analysis performed previously (27) indicated that the sialic acid residues of CEA mainly occur as the Neu5Acα2→6Galβ1→4 GlcNAc group.

CONCLUDING REMARKS

This study revealed that the N-linked sugar chains of CEA and its related normal antigens are extremely heterogeneous. Actually, the complicated elution patterns of neutral oligosaccharides as shown in Fig.3 have not been reported in the past, except for rat intestinal aminopeptidase N (28). We have recenty investigated the N-linked sugar chains of human intestinal alkaline phosphatase (T. Endo and A. Kobata, unpublished data) and dipeptidylpeptidase IV (K. Yamashita and Y. Matsuda, unpublished data), and found very heterogeneous sugar chains containing blood group determinants, sialic acid and sulfate residues. Therefore, complicated structures with blood group antigenic determinants could be a common characteristic of the N-linked sugar chains of mammalian intestinal glycoproteins.
As reported in many other tumor glycoproteins, CEA contains high mannose type sugar chains. However their amounts are rather small as summarized in Table I, and complex type sugar chains are the major constituents of CEA.

Kuroki et al. (29) reported that large amount of CEA-related antigens are produced by normal colon mucosae maintained in an organ culture. However, the antigens are not retained in the mucosae unlike cancerous tissues. Approximately 5mg of NFA-2 was usually obtained from the perchloric acid extract of normal adult feces evacuated in a day. Matsuoka et al. (30) recently found that additional CEA-related antigen amounting to 10 times of the perchloric acid extract can be released from the precipitate of the feces by treatment with phosphatidylinositol-specific phospholipase C. Comparative study of this CEA-related antigens and NFA-2 revealed that the glycosylinositol-phosphate moiety and a small portion of C-terminal of CEA peptide is missing in NFA-2 sample. These results indicated that normal colon mucosae produce a large amount of CEA, like in the case of cancerous tissues. Most of the CEA will peel off from colon as a membrane bound state together with the epithelial cells extruded from the apical area of crypts of colon mucosa. A small part of these CEA molecules will be released as NFA-2 by some enzymatic reaction in the digestive tract. Accordingly, CEA could no more be considered as tumor specific product.
The prominent structural differences found in the sugar chains of CEA and NFA-2 might be effectively used for the improvement of the diagnostic value of CEA in the future.

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