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Structures of the Sugar Chains of Recombinant Macrophage Colony-Stimulating Factor Produced in Chinese Hamster Ovary Cells[1]

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The structures of the *N*- and *O*-linked sugar chains of recombinant human macrophage colony-stimulating factor (rhM-CSF) from Chinese hamster ovary (CHO) cells were studied. rhM-CSF is a homodimeric glycoprotein. Sugar composition analysis revealed that rhM-CSF contained 4.1 mol *N*-acetylgalactosamine, 10.3 mol *N*-acetylglucosamine, 5.0 mol mannose, 10.0 mol galactose, 1.4 mol fucose, and 11.8 mol sialic acid per mol of the monomer. The *N*- and *O*-linked sugar chains liberated by hydrazinolysis were *N*-acetylated, and the reducing-end sugar residues were tagged with 2-aminopyridine. The pyridylamino (PA-) sugar chains thus obtained were purified by HPLC. The structures of the PA-sugar chains were analyzed by a combination of reversed-phase and size-fractionation HPLC, and exoglycosidase digestions, from which the structures of the rhM-CSF sugar chains were estimated to be as follows: monosialo biantennary sugar chain (9 mol%), monosialo fucosylbiantennary sugar chain (10 mol%), disialo biantennary sugar chain (30 mol%), disialo fucosylbiantennary sugar chain (28 mol%), disialo triantennary sugar chain (7 mol%), trisialo triantennary sugar chain (11 mol%), and trisialo fucosyltriantennary sugar chain (5 mol%) for the *N*-linked sugar chains, and asialo (27 mol%), monosialo (51 mol%), and disialo (22 mol%) Galβ1-3GalNAc for the *O*-linked sugar chains. Sialic acid residues were linked to the *N*-linked sugar chains through an α2-3 linkage.

Key words: glycoprotein, macrophage colony-stimulating factor, structure, sugar chain.

Macrophage colony-stimulating factor (M-CSF), a hematopoietic growth factor that promotes the growth and differentiation of monocytes and macrophages, is also necessary for the survival of the mature cells (*1*). Recombinant human M-CSF (rhM-CSF) expressed in Chinese hamster ovary (CHO) cells stimulates macrophage effector functions such as microbicidal (*2*), tumoricidal (*3*), and cholesterol-lowering activities (*4*), and has been used in clinical studies. rhM-CSF is a 90-kDa disulfide-bridged homodimer. The protein is *N*- and *O*-glycosylated. The monomer consists of 223 amino acid residues containing two potential sites for *N*-glycosylation, at Asn 122 and Asn 140 (*5*). For clinical use of glycoproteins obtained by means of recombinant DNA techniques, detailed knowledge of the sugar chain structures is essential, because they can influence the biological properties of the protein such as its pharmacokinetics, stability, and antigenicity. The structures of the sugar chains were estimated from the molecular ions as analyzed by mass spectrometry of glycopeptides (*5*). However, the detailed structures have not yet been reported.

We have established a line of CHO cells, ATCC CRL-11275, which express much rhM-CSF, and we have reported the preparation and pyridylamination of both *N*- and *O*-linked sugar chains released from glycoproteins under the same hydrazinolysis conditions (*6, 7*). Here, this method is applied to analyze the structures of *N*- and *O*-linked sugar chains of rhM-CSF from CHO cells ATCC CRL-11275.

MATERIALS AND METHODS

Materials—rhM-CSF was purified to homogeneity from serum-free medium conditioned with CHO cells, ATCC CRL-11275, and was desalted by gel filtration. *Arthrobacter ureafaciens* sialidase was purchased from Nacalai Tesque (Osaka), Newcastle disease virus (NDV) sialidase and *Streptococcus pneumoniae* β-galactosidase from Boehringer Mannheim (Mannheim), bovine epididymis α-L-fucosidase and jack bean β-*N*-acetylhexosaminidase from Sigma (St. Louis), *Aspergillus* sp. β-galactosidase from Toyobo (Tokyo), and *Xanthomonas manihotis* β-galactosidase from Bio Labs (New England). PA-Glc, PA-Gal, PA-GlcNAc, PA-GalNAc, and PA-maltose were prepared as reported (*8*). A mixture of PA-isomaltooligosaccharides (DP = 3-22) was purchased from Takara Biomedicals (Kyo-

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Abbreviations: CHO, Chinese hamster ovary; Fuc, L-fucose; Gal, D-galactose; GalNAc, *N*-acetyl-D-galactosamine; Glc, D-glucose; GlcNAc, *N*-acetyl-D-glucosamine; Hex, hexose; HexNAc, *N*-acetylhexosamine; HPLC, high-performance liquid chromatography; Man, D-mannose; NDV, Newcastle disease virus; Neu5Ac, *N*-acetylneuraminic acid; Neu5Gc, *N*-glycolylneuraminic acid; PA-, pyridylamino; rhM-CSF, recombinant human macrophage colony-stimulating factor.

TABLE I. **Structures of authentic PA-sugar chains used in this study.**

SD	Structure
1	Galβ1-3GalNAc—PA
2	Neu5Acα2-6 Galβ1-3GalNAc—PA
3	Neu5Acα2-3 Galβ1-3GalNAc—PA
4	Neu5Acα2-6 Neu5Acα2-3 Galβ1-3GalNAc—PA
5	Manα1-6 (Manα1-3) Manβ1-4GlcNAcβ1-4GlcNAc—PA
6	Manα1-6 GlcNAcβ1-2Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc—PA
7	GlcNAcβ1-2Manα1-6 Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc—PA
8	GlcNAcβ1-2Manα1-6 GlcNAcβ1-2Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc—PA
9	GlcNAcβ1-6 Manα1-6 GlcNAcβ1-2Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc—PA
10	GlcNAcβ1-6 GlcNAcβ1-2 Manα1-6 Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc—PA
11	GlcNAcβ1-6 GlcNAcβ1-2 Manα1-6 GlcNAcβ1-2Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc—PA
12	Galβ1-4GlcNAcβ1-2Manα1-6 Galβ1-4GlcNAcβ1-2Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc—PA
13	Galβ1-4GlcNAcβ1-2Manα1-6 Galβ1-4GlcNAcβ1-2Manα1-3 Fucα1-6 Manβ1-4GlcNAcβ1-4GlcNAc—PA
14	Galβ1-4GlcNAcβ1-6 Galβ1-4GlcNAcβ1-2 Manα1-6 Galβ1-4GlcNAcβ1-2 Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc—PA
15	Galβ1-4GlcNAcβ1-6 Galβ1-4GlcNAcβ1-2 Manα1-6 Galβ1-4GlcNAcβ1-2 Manα1-3 Fucα1-6 Manβ1-4GlcNAcβ1-4GlcNAc—PA
16	Neu5Acα2-3 { Galβ1-4GlcNAcβ1-2Manα1-6 / Galβ1-4GlcNAcβ1-2Manα1-3 } Manβ1-4GlcNAcβ1-4GlcNAc—PA
17	Neu5Acα2-3 { Galβ1-4GlcNAcβ1-2Manα1-6 / Galβ1-4GlcNAcβ1-2Manα1-3 } Fucα1-6 Manβ1-4GlcNAcβ1-4GlcNAc—PA
18	Neu5Acα2-3 Galβ1-4GlcNAcβ1-2Manα1-6 Neu5Acα2-3 Galβ1-4GlcNAcβ1-2Manα1-3 Manβ1-4GlcNAcβ1-4GlcNAc—PA
19	Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-6 Neu5Acα2-3Galβ1-4GlcNAcβ1-2Manα1-3 Fucα1-6 Manβ1-4GlcNAcβ1-4GlcNAc—PA

to). The structures and abbreviations of authentic PA-sugar chains used in this study are shown in Table I. SD5-11 were purchased from Takara Biomedicals. SD1 was prepared by pyridylamination of Galβ1-3GalNAc (*8*). SD2-4 were prepared from human blood clotting factor IX (*9*), SD12, 13, and 16-19 from interferon-γ produced by the human myelomonocyte cell line HBL-38 (*10*), and SD14 and 15 from human erythropoietin expressed in CHO cells (*11*).

Sugar Composition Analysis—rhM-CSF (20 μg) was hydrolyzed with 2.5 M trifluoroacetic acid at 100°C for 4 h for neutral sugars, with 4 M hydrochloric acid at 100°C for 6 h for amino sugars, or with 0.025 M sulfuric acid at 80°C for 1 h for sialic acids, in evacuated sealed tubes. Each hydrolysate was analyzed by high-pH anion-exchange chromatography with a CarboPac PA-1 column (0.46×25 cm) (Dionex, Sunnyvale, CA) using a pulsed amperometric detector (*12*).

Preparation of PA-Sugar Chains from rhM-CSF—*N*- and *O*-linked sugar chains were released from 3 mg of rhM-CSF by hydrazinolysis (60°C, 50 h), and the products were *N*-acetylated (*6*). The sugar chains were pyridylaminated as described previously (*8*). The PA-sugar chains were purified by gel filtration on an HW-40F column (1.1×40 cm) (Tosoh, Tokyo) after most of excess reagents had been removed.

Preparation of Desialylated PA-Sugar Chains—Half the amount of the PA-sugar chains was treated with 0.45 unit of *Arthrobacter* sialidase in 30 μl of 140 mM ammonium acetate buffer, pH 5.0, at 37°C for 16 h. The reaction was terminated by heating the solution at 100°C for 3 min.

Exoglycosidase Digestion of PA-Sugar Chains—PA-sugar chains (100 pmol) were digested with 10 milliunits of α-L-fucosidase in 50 μl of 40 mM citrate-phosphate buffer, pH 6.0; with 1 milliunit of *Streptococcus* β-galactosidase in 10 μl of 50 mM sodium acetate buffer, pH 6.0; with 6 units of *Xanthomonas* β-galactosidase in 10 μl of 50 mM sodium acetate buffer, pH 4.5; with 13 units of *Aspergillus* β-galactosidase in 10 μl of 50 mM ammonium acetate buffer, pH 5.0; with 25 milliunits of β-*N*-acetylhexosaminidase in 10 μl of 50 mM citrate-phosphate buffer, pH 5.0; with 2 milliunits of NDV sialidase in 100 μl of 50 mM ammonium acetate buffer, pH 6.5; or with 2.5 milliunits of *Arthrobacter* sialidase in 50 μl of 100 mM ammonium acetate buffer, pH 5.0. The digestions were carried out at 37°C for 16 h except for the NDV sialidase digestion, which was performed at 37°C for 2 h. The enzymatic reaction was terminated by heating the solution at 100°C for 3 min.

High-Performance Liquid Chromatography—A Beckman System Gold 126 chromatograph equipped with a Shimadzu RF-550 fluorescence spectrometer was used.

Reversed-phase HPLC was performed on a Cosmosil 5C18-P column (0.46×15 cm) (Nacalai Tesque) at a flow rate of 1.5 ml/min at 25°C. PA-sugar chains were detected by fluorescence. Excitation and emission wavelengths were 310 and 380 nm, respectively, for elution condition RP-1; and 320 and 400 nm, respectively, for elution conditions RP-2 and RP-3.

Elution condition RP-1 (this condition was applied to desialylated PA-*O*-linked sugar chains): Ammonium acetate buffer (0.1 M, pH 6.0) containing 0.01% 1-butanol was used as an eluent. After injecting a sample, the 1-butanol concentration was increased linearly from 0.01 to 0.52% in 51 min and then to 1.0% in 12 min (*7*).

Elution condition RP-2 (this condition was applied to desialylated PA-*N*-linked sugar chains): Ammonium acetate buffer (0.1 M, pH 4.0) containing 0.03% 1-butanol was used as an eluent. After injecting a sample, the 1-butanol concentration was increased linearly from 0.03 to 0.40% in 90 min (*8*).

Elution condition RP-3 (this condition was applied to sialo PA-sugar chains): The column was equilibrated with 0.1 M acetic acid. After injecting a sample, the 1-butanol concentration was increased linearly from 0 to 0.6% in 60 min, then to 1.0% in 10 min (*7*).

Size-fractionation HPLC was performed on a Shodex NH2P-50 (0.46×5 cm) (Showa Denko, Tokyo) at a flow rate of 0.6 ml/min at 25°C (*13*). Excitation and emission wavelengths were 310 nm and 380 nm, respectively.

Elution condition SF-1 (this condition was applied to desialylated PA-sugar chains): Solvent A was 0.3% (v/v) acetic acid in a 20 : 80 (v/v) mixture of acetonitrile : water adjusted to pH 7.0 with aqueous ammonia. Solvent B was 0.3% (v/v) acetic acid in a 93 : 7 (v/v) mixture of acetonitrile : water adjusted to pH 7.0 with aqueous ammonia. The column was equilibrated with a 3 : 97 (v/v) mixture of Solvent A:B. After injecting a sample, the proportion of Solvent A was increased linearly to 33% in 1 min, then to 71% in 34 min.

Elution condition SF-2 (this condition was applied to sialo PA-sugar chains): Solvent C was 3% (v/v) acetic acid in a 20 : 80 (v/v) mixture of acetonitrile:water adjusted to pH 7.0 with triethylamine. Solvent D was 3% (v/v) acetic acid in a 93 : 7 (v/v) mixture of acetonitrile : water adjusted to pH 7.0 with triethylamine. The column was equilibrated with a 3 : 97 (v/v) mixture of Solvent C:D. Gradient elution was done as in elution condition SF-1.

Anion-exchange HPLC was carried out on a MonoQ HR 5/5 column (Pharmacia, Uppsala) (*8*).

Reducing-End Analysis of PA-Sugar Chains—Reducing-end residues of PA-sugar chains (each 100 pmol) were quantified by HPLC with a TSKgel Sugar AXI column (0.46×15 cm) (Tosoh) after hydrolysis with 4 M hydrochloric acid at 100°C for 8 h in evacuated sealed tubes followed by *N*-acetylation (*14*). The linkage position of the reducing-end residue of a PA-disaccharide (100 pmol) was analyzed by Smith degradation as reported previously (*15*).

Linkage Position Analysis of Sialic Acid Residues of Sialo PA-Sugar Chains—The linkage positions of sialic acid residues were analyzed by HPLC in combination with sequential exoglycosidase digestion, using a GlycoSEQ™ *N*-complex sequencing kit (Takara Biomedicals) according to the manufacturer's instructions. Briefly, sialo PA-sugar chains (each 100 pmol) were incubated with 1 milliunit of *Streptococcus* β-galactosidase and 66 milliunits of β-*N*-acetylhexosaminidase, and with or without 20 milliunits of α-L-fucosidase in 50 mM citrate-phosphate buffer, pH 5.3, at 37°C for 4 h. After stopping the digestions by heating the solutions, a mixture of 4 milliunits of *Arthrobacter* sialidase and 2 milliunits of *Streptococcus* β-galactosidase was added to the reaction mixture, and the solution was incubated at 37°C for 3 h. The reactions were terminated by heating the solutions, and a portion of the digest was analyzed by reversed-phase HPLC.

RESULTS AND DISCUSSION

Sugar Composition of rhM-CSF—The sugar composition analysis of rhM-CSF is shown in Table II. Most sialic acid was *N*-acetylneuraminic acid. *N*-Glycolylneuraminic acid, which is a constituent of Hanganutziu-Deicher antigens, made up only 0.2% of the total sialic acid—compared with approximately 3% of the total sialic acid in human tissue plasminogen activator, human chimeric plasminogen activator, human erythropoietin, and human follitropin expressed in CHO cells (*16*).

Reducing-End Analysis of PA-Sugar Chains from rhM-CSF—Reducing-end residues of the PA-sugar chains were PA-GlcNAc (1.5 mol/mol of monomeric rhM-CSF), PA-GalNAc (2.0 mol/mol), and PA-Gal (2.1 mol/mol). PA-Gal was probably a by-product of *O*-linked sugar chains (Galβ1-3GalNAc-Ser/Thr type) produced during liberation as reported previously (*6*). PA-Glc, PA-Xyl, and PA-Fuc were not detected, indicating that Xyl-Glc-*O*-Ser-, Fuc-*O*-Ser-, and glycosaminoglycan-type sugar chains were not linked to the glycoprotein. These results suggested that rhM-CSF had 1.5 mol *N*-linked sugar chains and 4.1 mol *O*-linked sugar chains per mol of the monomer. Possible *N*-glycosylation sites in the amino acid sequence deduced from the cDNA nucleotide sequence are Asn122 and Asn140 (*5*).

Anion-Exchange HPLC Analysis of PA-Sugar Chains from rhM-CSF—The molar ratio of fractions appearing at the elution positions of authentic asialo, monosialo, disialo, and trisialo PA-sugar chains was 28:49:21:2. After the PA-sugar chains had been digested with *Arthrobacter* sialidase, only one peak was observed at the position of the

TABLE II. **Sugar composition of rhM-CSF.**

Sugar	mol/mol of monomeric rhM-CSF[a]
GalN	4.1 ±0.6
GlcN	10.3 ±1.5
Man	5.0 ±0.4
Gal	10.0 ±0.8
Fuc	1.4 ±0.2
Neu5Ac	11.8 ±1.4
Neu5Gc	0.025±0.004

[a]Average of nine experiments±SD.

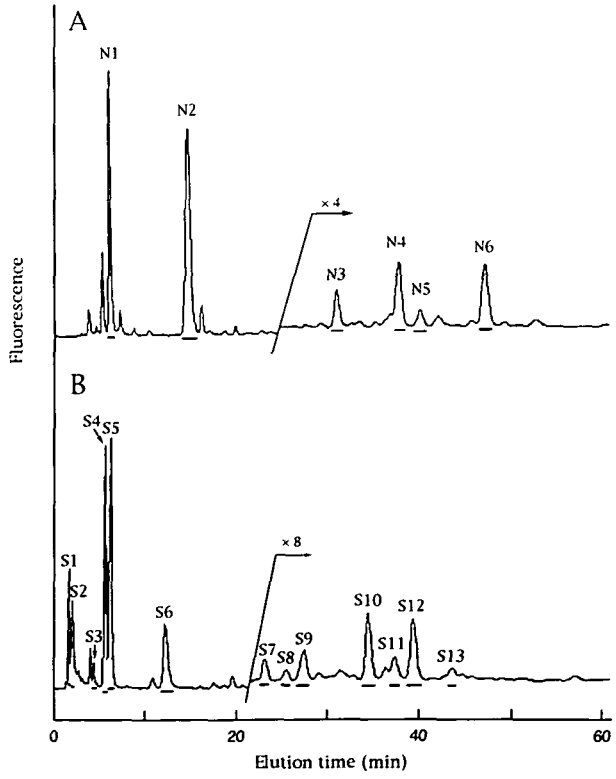

Fig. 1. **Separation by reversed-phase HPLC of the digests of PA-sugar chains with sialidase (A) and untreated PA-sugar chains (B) from rhM-CSF.** Elution conditions RP-1 (A) and 3 (B) were used.

TABLE III. **HPLC and reducing-end analysis of Fractions N1 and N2.** Elution condition RP-1 was used for reversed-phase HPLC, and elution condition SF-1 for size-fractionation HPLC.

PA-sugar chain	*Aspergillus* β-galactosidase	HPLC		Reducing-end PA-sugar
		Reversed-phase[a]	Size-fractionation[b]	
N1	−	0.43	1.2	PA-Gal
N2	−	1.00	1.7	PA-GalNAc
	+	1.17	0.9	
SD1		1	1.7	
PA-Gal		0.43	1.1	
PA-GalNAc		1.16	0.9	

[a]Elution time relative to SD1. [b]Glucose units.

TABLE IV. **HPLC analysis of the sequential exoglycosidase digests of Fractions N3-6, and reducing-end analysis.** Elution condition RP-2 was used for reversed-phase HPLC, and elution condition SF-1 for size-fractionation HPLC.

PA-sugar chain	Exoglycosidase	HPLC		Reducing-end PA-sugar
		Reversed-phase[a]	Size-fractionation[b]	
N3	No treatment	0.66	9.1	PA-GlcNAc
	Streptococcus β-galactosidase	0.56	6.9	
	β-*N*-Acetylhexosaminidase	0.64	4.4	
N4	No treatment	1.01	7.7	PA-GlcNAc
	Streptococcus β-galactosidase	0.83	6.2	
	β-*N*-Acetylhexosaminidase	0.64	4.4	
N5	No treatment	0.94	9.4	PA-GlcNAc
	α-L-Fucosidase	0.68	9.1	
	Streptococcus β-galactosidase	0.56	6.9	
	β-*N*-Acetylhexosaminidase	0.65	4.4	
N6	No treatment	1.32	8.0	PA-GlcNAc
	α-L-Fucosidase	1.01	7.7	
	Streptococcus β-galactosidase	0.83	6.2	
	β-*N*-Acetylhexosaminidase	0.64	4.4	
SD5		0.64	4.4	
SD8		0.84	6.2	
SD11		0.57	6.9	
SD12		1	7.7	
SD13		1.31	8.1	
SD14		0.67	9.1	
SD15		0.94	9.4	

[a]Elution time relative to SD12. [b]Glucose units.

authentic neutral PA-sugar chains (data not shown). These results indicate that the negative charges of the PA-sugar chains were due to sialic acid residues.

Purification of Desialylated PA-Sugar Chains from rhM-CSF—The desialylated PA-sugar chains were separated by reversed-phase HPLC (Fig. 1A). Size-fractionation HPLC and reducing-end analysis of the fractions indicated that the molecular sizes of fractions eluted before 24 min were smaller than 2 glucose units, and that the reducing-end residues were PA-GalNAc or PA-Gal, while the molecular sizes of fractions eluted after 24 min were larger than 7 glucose units, and the reducing-end residues were PA-GlcNAc (data not shown). From these results, we concluded that the former and latter fractions were PA-*O*-linked and PA-*N*-linked sugar chains, respectively. The first two main fractions (N1 and N2) comprised 73% of the amount of the PA-*O*-linked sugar chains, and the other four main fractions (N3-N6) comprised 63% of the amount of the PA-*N*-linked sugar chains. Each of these six fractions gave a single peak when analyzed by size-fractionation HPLC (data not shown).

Structural Analysis of Desialylated PA-Sugar Chains (Fractions N1-N6)—To identify the PA-sugar chains, reversed-phase and size-fractionation HPLC (a two-dimensional sugar map) were used.

PA-O-Linked sugar chains: Fraction N1 was eluted at the positions of PA-Gal by the two kinds of HPLC and also on a TSKgel Sugar AXI column (Table III), indicating that it was PA-Gal, which is probably a by-product of *O*-linked sugar chains (*6*).

Fraction N2, with PA-GalNAc at the reducing-end, was eluted at the positions of SD1 by the two kinds of HPLC, and the digest with *Aspergillus* β-galactosidase at the positions of PA-GalNAc (Table III). These results indicated that Fraction N2 was Galβ1-3GalNAc-PA.

Structural analysis of Fractions N1 and N2 thus suggested that most *O*-linked sugar chains of rhM-CSF were of the Galβ1-3GalNAc type.

PA-N-linked sugar chains: Fractions N3-N6, with PA-GlcNAc at the reducing-ends, were susceptible to digestion with *Streptococcus* β-galactosidase, which specifically hydrolyzes Galβ1-4GlcNAc (*17*), but were resistant to digestion with *Xanthomonas* β-galactosidase, which hydrolyzes Galβ1-3GlcNAc (*18*) (data not shown).

Fraction N3 was eluted at the positions of SD14, and the digest with *Streptococcus* β-galactosidase at the positions of SD11. When the product was further digested with β-*N*-acetylhexosaminidase, a new peak appeared at the positions of SD5 (Table IV). These results indicated that Fraction N3 was a PA-triantennary sugar chain (Table V).

Fraction N4 was eluted at the positions of SD12, and the digest with *Streptococcus* β-galactosidase at the positions of SD8. By further digestion with β-*N*-acetylhexosaminidase, the product was eluted at the positions of SD5 (Table IV). These results indicated that Fraction N4 was a PA-biantennary sugar chain (Table V).

Fraction N5 was eluted at the positions of SD15. After the fraction had been digested with α-L-fucosidase, a new peak appeared at the positions of Fraction N3. Further sequential digestion with exoglycosidases gave the same

TABLE V. **Proposed structures of desialylated sugar chains of rhM-CSF.**

Fraction	Structure	Relative amount[a]
O-linked sugar chain		
N1	Gal	51%
N2	Galβ1—3GalNAc	49%
N-linked sugar chain		
N3	Galβ1-4GlcNAcβ1-6 Galβ1-4GlcNAcβ1-2Manα1-6 Manβ1-4GlcNAcβ1—4GlcNAc Galβ1-4GlcNAcβ1-2Manα1-3	18%
N4	Galβ1-4GlcNAcβ1-2Manα1-6 Manβ1-4GlcNAcβ1—4GlcNAc Galβ1-4GlcNAcβ1-2Manα1-3	35%
N5	Galβ1-4GlcNAcβ1-6 Galβ1-4GlcNAcβ1-2Manα1-6 Fucα1-6 Manβ1-4GlcNAcβ1—4GlcNAc Galβ1-4GlcNAcβ1-2Manα1-3	8%
N6	Galβ1-4GlcNAcβ1-2Manα1-6 Fucα1-6 Manβ1-4GlcNAcβ1—4GlcNAc Galβ1-4GlcNAcβ1-2Manα1-3	39%

[a]Relative amounts of *O*- and *N*-linked sugar chains are expressed separately, based on the peak area observed by reversed-phase HPLC.

results as those obtained for Fraction N3 (Table IV). These results suggested that Fraction N5 was a PA-fucosyltriantennary sugar chain (Table V).

Fraction N6 was eluted at the positions of SD13. After the fraction had been digested with α-L-fucosidase, a new peak appeared at the positions of Fraction N4. Further sequential digestion with exoglycosidases gave the same results as those obtained for Fraction N4 (Table IV). These results indicated that Fraction N6 was a PA-fucosylbiantennary sugar chain (Table V).

Analysis of Linkage Positions of Sialic Acid Residues— The PA-sugar chains from rhM-CSF were purified by reversed-phase HPLC and the fractions indicated by bars (Fig. 1B) were collected. The first six main fractions (S1–S6) comprised 86% of the amount of PA-*O*-linked sugar chains, and the other seven main fractions (S7–S13) comprised 79% of the amount of PA-*N*-linked sugar chains, based on the reducing-end analysis of each PA-sugar chain (data not shown).

PA-O-linked sugar chains: Fractions S1 and S2 were eluted at the same elution positions as Fractions N1 and N2, respectively, by reversed-phase, size-fractionation HPLC and anion-exchange HPLC. These results indicated that Fractions S1 and S2 were PA-Gal and Galβ1-3GalNAc-PA, respectively (data not shown).

Fraction S3 was eluted at the positions of SD2 (Table VI). NDV sialidase specific for Siaα2-3Gal (*19*) failed to digest this fraction; however, the digest with *Arthrobacter* sialidase, which hydrolyzes Siaα2-3/6Gal, was eluted at the positions of Fraction N1 (SD1) (Table VI). These results indicated that Fraction S3 was Galβ1-3(Neu5Acα2-6)-GalNAc-PA.

Fraction S4 was eluted at the position of monosialo PA-sugar chains by anion-exchange HPLC, and the digest with *Arthrobacter* sialidase at the positions of Fraction N1 (PA-Gal) (Table VI). The linkage position of the sialic acid residue was analyzed by the Smith degradation method (*15*). The hydrolysate of the fluorogenic oxidation-reduction product derived from Fraction S4 was eluted at the position of PA-threose on HPLC (data not shown), indicating that the sialic acid residue was linked to C-3 of the PA-galactose residue. These results indicated that Fraction S4 was Neu5Acα2-3Gal-PA.

Fraction S5 was eluted at the positions of SD3, and the digest with NDV sialidase at the positions of Fraction N1 (SD1) (Table VI). These results indicated that Fraction S5

TABLE VI. **HPLC analysis of the sialidase digests of Fractions S3-6.** Elution condition RP-3 was used for reversed-phase HPLC, and elution condition SF-2 for size-fractionation HPLC.

PA-sugar chain	Sialidase	HPLC		Number of sialic acid residues[c]
		Reversed-phase[a]	Size-fractionation[b]	
S3	No treatment	0.70	3.8	1
	Arthrobacter sialidase	0.31	1.6	0
S4	No treatment	0.89	2.8	1
	Arthrobacter sialidase	0.25	1.1	0
S5	No treatment	0.99	3.1	1
	Newcastle sialidase	0.31	1.6	0
S6	No treatment	2.05	6.4	2
	Newcastle sialidase	0.70	3.8	1
	Arthrobacter sialidase	0.31	1.6	0
N1 (PA-Gal)		0.25	1.1	
N2 (SD1)		0.31	1.6	
SD2		0.70	3.8	
SD3		1	3.0	
SD4		2.04	6.4	

[a]Elution time relative to SD3. [b]Glucose units. [c]Determined by anion-exchange HPLC.

TABLE VII. **HPLC analysis of the sialidase digests of Fractions S7, S8-3, S9-1, S9-2, S10, S11, S12, and S13.** Elution condition RP-3 was used for reversed-phase HPLC, and elution condition SF-2 for size-fractionation HPLC.

PA-sugar chain	Sialidase	HPLC		Number of sialic acid residues[c]
		Reversed-phase[a]	Size-fractionation[b]	
S7	–	0.99	9.4	1
	+	0.60	7.3	0
S8-3	–	1.09	14.1	2
	+	0.39	9.0	0
S9-1	–	1.17	9.9	1
	+	0.75	7.6	0
S9-2	–	1.17	14.0	2
	+	0.39	9.0	0
S10	–	1.48	12.0	2
	+	0.60	7.5	0
S11	–	1.60	17.9	3
	+	0.39	9.0	0
S12	–	1.69	12.3	2
	+	0.74	7.8	0
S13	–	1.87	18.3	3
	+	0.58	9.3	0
N4 (SD12)		0.59	7.4	
N6 (SD13)		0.74	7.7	
N3 (SD14)		0.39	8.9	
N5 (SD15)		0.58	9.3	
SD16		1	9.3	
SD17		1.18	9.7	
SD18		1.47	11.8	
SD19		1.69	12.2	

[a]Elution time relative to SD16. [b]Glucose units. [c]Determined by anion-exchange HPLC.

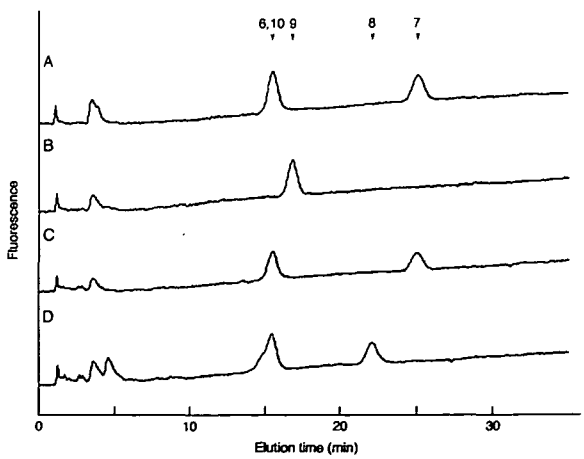

Fig. 2. **Reversed-phase HPLC analysis of the sequential exoglycosidase digests of Fractions S7 (A), S8-3 (B), S9-1 (C), and S9-2 (D).** Elution condition RP-2 was used. The numbered arrowheads indicate the elution positions of authentic PA-sugar chains (Table I).

TABLE VIII. **Proposed structures for sugar chains of rhM-CSF.**

Fraction	Structure	Relative amount[a]
O-linked sugar chain		
S1	Gal	20%
S2	Galβ1–3GalNAc	10%
S3	Neu5Acα2–6 Galβ1–3GalNAc	2%
S4	Neu5Acα2–3Gal	24%
S5	Neu5Acα2–3Galβ1–3GalNAc	30%
S6	Neu5Acα2–6 Neu5Acα2–3Galβ1–3GalNAc	14%
N-linked sugar chain		
S7	Neu5Acα2-3 Galβ1–4GlcNAcβ1–2Manα1–6 Galβ1–4GlcNAcβ1–2Manα1–3 Manβ1–4GlcNAcβ1–4GlcNAc	4%
	Galβ1–4GlcNAcβ1–2Manα1–6 Neu5Acα2-3 Galβ1–4GlcNAcβ1–2Manα1–3 Manβ1–4GlcNAcβ1–4GlcNAc	5%
S8-3	Neu5Acα2–3Galβ1–4GlcNAcβ1–6 Galβ1–4GlcNAcβ1–2Manα1–6 Neu5Acα2–3 Galβ1–4GlcNAcβ1–2Manα1–3 Manβ1–4GlcNAcβ1–4GlcNAc	2%
S9-1	Neu5Acα2–3Galβ1–4GlcNAcβ1–2Manα1–6 Fucα1–6 Galβ1–4GlcNAcβ1–2Manα1–3 Manβ1–4GlcNAcβ1–4GlcNAc	4%
	Galβ1–4GlcNAcβ1–2Manα1–6 Fucα1–6 Neu5Acα2–3Galβ1–4GlcNAcβ1–2Manα1–3 Manβ1–4GlcNAcβ1–4GlcNAc	6%
S9-2	Galβ1–4GlcNAcβ1–6 Neu5Acα2–3 Galβ1–4GlcNAcβ1–2Manα1–6 Neu5Acα2–3 Galβ1–4GlcNAcβ1–2Manα1–3 Manβ1–4GlcNAcβ1–4GlcNAc	2%
	Neu5Acα2–3Galβ1–4GlcNAcβ1–6 Neu5Acα2–3 Galβ1–4GlcNAcβ1–2Manα1–6 Galβ1–4GlcNAcβ1–2Manα1–3 Manβ1–4GlcNAcβ1–4GlcNAc	3%
S10	Neu5Acα2-3 Galβ1–4GlcNAcβ1–2Manα1–6 Neu5Acα2-3 Galβ1–4GlcNAcβ1–2Manα1–3 Manβ1–4GlcNAcβ1–4GlcNAc	30%
S11	Neu5Acα2–3Galβ1–4GlcNAcβ1–6 Neu5Acα2–3 Galβ1–4GlcNAcβ1–2Manα1–6 Neu5Acα2–3 Galβ1–4GlcNAcβ1–2Manα1–3 Manβ1–4GlcNAcβ1–4GlcNAc	11%
S12	Neu5Acα2–3Galβ1–4GlcNAcβ1–2Manα1–6 Fucα1–6 Neu5Acα2–3Galβ1–4GlcNAcβ1–2Manα1–3 Manβ1–4GlcNAcβ1–4GlcNAc	28%
S13	Neu5Acα2–3Galβ1–4GlcNAcβ1–6 Neu5Acα2–3 Galβ1–4GlcNAcβ1–2Manα1–6 Fucα1–6 Neu5Acα2–3 Galβ1–4GlcNAcβ1–2Manα1–3 Manβ1–4GlcNAcβ1–4GlcNAc	5%

[a] Relative amounts of *O*- and *N*-linked sugar chains are expressed separately, based on the peak area observed by reversed-phase HPLC.

was Neu5Acα2-3Galβ1-3GalNAc-PA.

Fraction S6 was eluted at the positions of SD4 (Table VI), and the digests with NDV and *Arthrobacter* sialidase at the positions of Fraction S3 (SD2) and Fraction N2 (SD1), respectively (Table VI). These results indicated that Fraction S6 was Neu5Acα2-3Galβ1-3(Neu5Acα2-6)GalNAc-PA.

PA-N-linked sugar chains: The digests of Fractions S7-S13 with NDV sialidase were eluted at the position of neutral PA-sugar chains by anion-exchange HPLC. These results indicated that sialic acid residues in these fractions were linked through an α2-3 linkage.

Fraction S7 was eluted at the positions of SD16, and the digest of Fraction S7 with NDV sialidase at the positions of Fraction N4 (SD12) (Table VII). The linkage position of the sialic acid residue was analyzed as follows. After digestion of Fraction S7 with *Streptococcus* β-galactosidase and β-*N*-acetylhexosaminidase, the product was digested with NDV sialidase and *Streptococcus* β-galactosidase. The digest was eluted at the positions of SD6 and 7 (Fig. 2A). These results indicated that Fraction S7 was a mixture of two monosialo PA-biantennary sugar chains in which one sialic acid residue was linked to the Manα1-3 or Manα1-6 branch (Table VIII).

Fraction S8 was separated into three further fractions, S8-1, S8-2, and S8-3, by size-fractionation HPLC (Fig. 3A). The reducing-end residue of Fraction S8-1, with a molecular size of 3.6 glucose units, was PA-GalNAc, indicating that this fraction was a PA-*O*-linked sugar chain. Fraction S8-1 was not analyzed further because its amount represented less than 1% of the total PA-*O*-linked sugar chains. Fraction S8-2 was a disialo PA-sugar chain. Desialylated Fraction S8-2 had a molecular size of 7.9 glucose units and PA-ManNAc as the reducing-end residue, which is produced by epimerization of a reducing-end GlcNAc residue of the *N*-linked sugar chain (data not shown). These results indicated that Fraction S8-2 was probably derived from Fraction S12. Fraction S8-2 was not analyzed further because its amount represented less than 1% of the total PA-*N*-linked sugar chains. Fraction S8-3 was a disialo PA-sugar chain. Desialylated Fraction S8-3 was eluted at the positions of Fraction N3 (SD14) (Table VII). Linkage position analysis of the sialic acid residues (Fig. 2B) indicated that one sialic acid residue was linked to the C-2 branch of the Manα1-3 residue, and the other to the C-6 branch of the Manα1-6 residue (Table VIII).

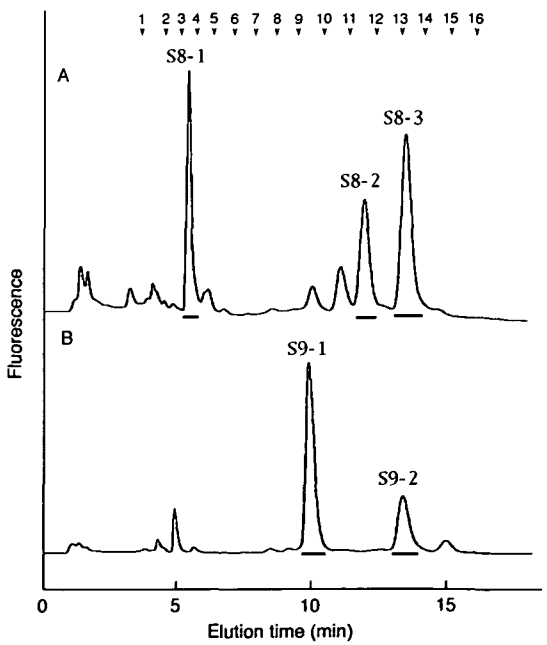

Fig. 3. **Separation by size-fractionation HPLC of Fractions S8 and S9 isolated by reversed-phase HPLC.** A, Fraction S8; B, Fraction S9. Elution condition SF-2 was used. The numbered arrowheads indicate the elution positions of PA-isomaltooligosaccharides.

Fraction S9 was separated into two fractions, S9-1 and S9-2, by size-fractionation HPLC (Fig. 3B). Fraction S9-1 was eluted at the positions of SD17, and when desialylated at the positions of Fraction N6 (SD13) (Table VII). Linkage position analysis of the sialic acid residue (Fig. 2C) indicated that this fraction was a mixture of two monosialo PA-fucosylbiantennary sugar chains (Table VIII). Fraction S9-2 was a disialo PA-sugar chain, and the desialylated Fraction S9-2 was eluted at the positions of Fraction N3 (SD14) (Table VII). Linkage position analysis of the sialic acid residues (Fig. 2D) indicated that Fraction S9-2 was a mixture of two disialo PA-triantennary sugar chains (Table VIII).

Fraction S10 was eluted at the positions of SD18, and when desialylated at the positions of Fraction N4 (SD12) (Table VII). These results indicated that Fraction S10 was a disialo PA-biantennary sugar chain (Table VIII).

Fraction S11 was eluted at the position of trisialo PA-sugar chains, and when desialylated at the positions of Fraction N3 (SD14) (Table VII). These results indicated that Fraction S11 was a trisialo PA-triantennary sugar chain (Table VIII).

Fraction S12 was eluted at the positions of SD19, and when desialylated at the positions of Fraction N6 (SD13) (Table VII). These results indicated that Fraction S12 was a disialo PA-fucosylbiantennary sugar chain (Table VIII).

Fraction S13 was eluted at the position of trisialo PA-sugar chains, and when desialylated at the positions of Fraction N5 (SD15) (Table VII). These results indicated that Fraction S13 was a trisialo PA-fucosyltriantennary sugar chain (Table VIII).

Structures of N- and O-Linked Sugar Chains of rhM-CSF—PA-Gal (Fractions N1 and S1) was presumably derived from Galβ1-3GalNAc-Ser/Thr and Galβ1-3(Neu5Acα2-6)GalNAc-Ser/Thr, and Neu5Acα2-3Gal-PA (Fraction S4) from Neu5Acα2-3Galβ1-3GalNAc-Ser/Thr and Neu5Acα2-3Galβ1-3(Neu5Acα2-6)GalNAc-Ser/Thr as by-products generated by β-elimination (*6*). Our findings indicate that rhM-CSF has mainly Galβ1-3GalNAc type *O*-linked sugar chains, and that its major *N*-linked sugar chains are of the sialo biantennary and sialo triantennary types, with or without fucose residues. These results are compatible with the preliminary results obtained from molecular ions (*5*).

The structures of the *N*- and *O*-linked sugar chains of rhM-CSF from CHO cells are found in native human glycoproteins and recombinant glycoproteins derived from CHO cells such as erythropoietin (*20, 21*), granulocyte colony stimulating factor (*22*), interleukin-6 (*23*), interferon-β (*24*), and interferon-γ (*25, 26*).

REFERENCES

1. Wong, G.G., Temple, P.A., Leary, A.C., Witek-Giannotti, J.S., Yang, Y.-C., Ciarletta, A.B., Chung, M., Murtha, P., Kriz, R., Kaufman, R.J., Ferenz, C.R., Sibley, B.S., Turner, K.J., Hewick, R.M., Clark, S.C., Yanai, N., Yokota, H., Yamada, M., Saito, M., Motoyoshi, K., and Takaku, F. (1987) Human CSF-1: Molecular cloning and expression of 4-kb cDNA encoding the human urinary protein. *Science* **235**, 1504-1508
2. Karbassi, A., Becker, J.M., Forster, J.S., and Moore, R.N. (1987) Enhanced killing of *Candida albicans* by murine macrophages treated with macrophage colony-stimulating factor: Evidence for augmented expression of mannose receptors. *J. Immunol.* **139**, 417-421
3. Wing, E.J., Waheed, A., Shadduck, R.K., Nagle, L.S., and Stephenson, K. (1982) Effect of colony stimulating factor on murine macrophages. *J. Clin. Invest.* **69**, 270-279
4. Motoyoshi, K. and Takaku, F. (1989) Serum cholesterol-lowering activity of human monocytic colony-stimulating factor. *Lancet* **2**, 326-327
5. Huberty, M.C., Vath, J.E., Yu, W., and Martin, S.A. (1993) Site-specific carbohydrate identification in recombinant proteins using MALD-TOF MS. *Anal. Chem.* **65**, 2791-2800
6. Kuraya, N. and Hase, S. (1992) Release of *O*-linked sugar chains from glycoproteins with anhydrous hydrazine and pyridylamination of the sugar chains with improved reaction conditions. *J. Biochem.* **112**, 122-126
7. Kuraya, N. and Hase, S. (1996) Analysis of pyridylaminated *O*-linked sugar chains by two-dimensional sugar mapping. *Anal. Biochem.* **233**, 205-211
8. Hase, S. (1994) High-performance liquid chromatography of pyridylaminated saccharides in *Methods in Enzymology* (Lennarz, J.W. and Hart, G.W.) Vol. 230, pp. 225-237, Academic Press, New York
9. Kuraya, N., Omichi, K., Nishimura, H., Iwanaga, S., and Hase, S. (1993) Structural analysis of *O*-linked sugar chains in human blood clotting factor IX. *J. Biochem.* **114**, 763-765
10. Yamamoto, S., Hase, S., Fukuda, S., Sano, O., and Ikenaka, T. (1989) Structures of the sugar chains of interferon-γ produced by human myelomonocyte cell line HBL-38. *J. Biochem.* **105**, 547-555
11. Sasaki, H., Bothner, B., Dell, A., and Fukuda, M. (1987) Carbohydrate structure of erythropoietin expressed in Chinese hamster ovary cells by a human erythropoietin cDNA. *J. Biol. Chem.* **262**, 12059-12076
12. Lee, Y.C. (1990) High-performance anion-exchange chromatography for carbohydrate analysis. *Anal. Biochem.* **189**, 151-162
13. Makino, Y., Kuraya, N., Omichi, K., and Hase, S. (1996) Classification of sugar chains of glycoproteins by analyzing reducing end oligosaccharides obtained by partial acid hydrolysis. *Anal. Biochem.* **238**, 54-59
14. Hase, S., Hatanaka, K., Ochiai, K., and Shimizu, H. (1992) Improved method for the component sugar analysis of glycoproteins by pyridylamino sugars purified with immobilized boronic acid. *Biosci. Biotech. Biochem.* **56**, 1676-1677
15. Omichi, K. and Hase, S. (1995) Determination of the linkage positions of reducing-end residues of oligosaccharides by partial periodate oxidation of pyridylaminated derivatives. *Anal. Biochem.* **227**, 404-407
16. Hokke, C.H., Bergwerff, A.A., Dedem, G.W.K.V., Oostrum, J.V., Kamerling, J.P., and Vliegenthart, J.F.G. (1990) Sialylated carbohydrate chains of recombinant human glycoproteins expressed in Chinese hamster ovary cells contain traces of *N*-glycolylneuraminic acid. *FEBS Lett.* **275**, 9-14
17. Paulson, J.C., Prieds, J.-P., Glasgow, L.R., and Hill, R.L. (1978) Sialyl- and fucosyltransferases in the biosynthesis of asparaginyl-linked oligosaccharides in glycoproteins. *J. Biol. Chem.* **253**, 5617-5624
18. Wong-Madden, S.T. and Landry, D. (1995) Purification and characterization of novel glycosidases from the bacterial genus *Xanthomonas*. *Glycobiology* **5**, 19-28
19. Paulson, J.C., Weinstein, J., Dorland, L., Halbeek, H.V., and Vliegenthart, J.F.G. (1982) Newcastle disease virus contains a linkage-specific glycoprotein sialidase. *J. Biol. Chem.* **257**, 12734-12738
20. Takeuchi, M., Takasaki, S., Miyazaki, H., Kato, T., Hoshi, S., Kochibe, N., and Kobata, A. (1988) Comparative study of the asparagine-linked sugar chains of human erythropoietins purified from urine and the culture medium of recombinant Chinese hamster ovary cells. *J. Biol. Chem.* **263**, 3657-3663
21. Inoue, N., Takeuchi, M., Asano, K., Shimizu, R., Takasaki, S., and Kobata, A. (1993) Structures of mucin-type sugar chains on human erythropoietins purified from urine and the culture medium of recombinant Chinese hamster ovary cells. *Arch. Biochem. Biophys.* **301**, 375-378
22. Oheda, M., Hase, S., Ono, M., and Ikenaka, T. (1988) Structures of the sugar chains of recombinant human granulocyte-colony-stimulating factor produced by Chinese hamster ovary cells. *J. Biochem.* **103**, 544-546
23. Orita, T., Oheda, M., Hasegawa, M., Kuboniwa, H., Esaki, K., and Ochi, N. (1994) Polypeptide and carbohydrate structure of recombinant human interleukin-6 produced in Chinese hamster ovary cells. *J. Biochem.* **115**, 345-350
24. Conradt, H.S., Egge, H., Peter-Katalinic, J., Reiser, W., Siklosi, T., and Schaper, K. (1987) Structure of the carbohydrate moiety of human interferon-β secreted by a recombinant Chinese hamster ovary cell line. *J. Biol. Chem.* **262**, 14600-14605
25. Mutsaers, J.H.G.M., Kamerling, J.P., Devos, R., Guisez, Y., Fiers, W., and Vliegenthart, J.F.G. (1986) Structural studies of carbohydrate chains of human γ-interferon. *Eur. J. Biochem.* **156**, 651-654
26. Yamamoto, S., Hase, S., Yamauchi, H., Tanimoto, T., and Ikenaka, T. (1989) Studies on the sugar chains of interferon-γ from human peripheral-blood lymphocytes. *J. Biochem.* **105**, 1034-1039