glycopeptides. Analysis of glycopeptides is not yet widely implemented outside specialist glycobiology laboratories, but it is clear from both the present study and the increasing volume of publication in this area that glycoproteomic strategies are now sufficiently mature to allow practical site-specific characterization of the oligosaccharide profiles of even highly heterogeneous glycoproteins (Harazono *et al.*, 2005; Imre *et al.*, 2005; Tajiri *et al.*, 2005; Chalabi *et al.*, 2006). Our present results justify the use of MS for relative quantitation of oligosaccharides, and highlight the fact that glycopeptide MS will be a key interfacing technique in proteomics and functional glycomics in the future.

METHODS

Glycoprotein samples. The blood samples were obtained from three healthy Japanese donors (A, B and C) with the permission from the Medical Ethics Committee of Osaka University Graduate School of Medicine. Transferrin and IgG were purified from individual serum by immunoaffinity with rabbit polyclonal antibody against human transferrin, and by protein G affinity chromatography, respectively, and then lyophilized. The purity of distributed samples was validated by SDS polyacrylamide gel electrophoresis under reducing conditions. Each 1 mg sample from six specimens (A, B and C for transferrin and IgG) in total was delivered to 26 laboratories at ambient temperature. Twenty of these laboratories submitted results. The stability of these materials during transport was guaranteed by a test incubation, during which neither degradation nor modification of protein and glycan moieties was observed after one week of storage at 37 °C. In most laboratories, a 100 μg sample was used for each analysis.

Release of oligosaccharides. A majority of laboratories employed the method of oligosaccharide release from enzymatic digests of glycoproteins, typically as follows. Proteins (0.5 mg of transferrin or IgG) were dissolved in 0.5 mL of 6 M guanidium hydrochloride, 0.25 M Tris-HCl, pH 8.5 and reduced with 5 mg of dithiothreitol under N_2 at room temperature for 3 h. Then, 9 mg of iodoacetamide were added to the solution, followed by incubation in the dark at room temperature for 30 min for carboxyamidomethylation. The reagents were removed by a gel fitration column, NAP-5 (GE Healthcare, Piscataway, NJ), equilibrated with 0.05 N HCl, and the recovered proteins were lyophilized. The alkylated proteins were dissolved in 50 mM ammonium hydrogen carbonate, pH 8.0, and digested with 10 μg of trypsin at 37 °C for 3 h. Subsequently, 20 U of PNGase F (*N*-glycanase F) (Roche, Mannheim, Germany) were added to the solution, followed by incubation at 37 °C for 12 h to release *N*-linked oligosaccharides from glycopeptides. The solution was then passed through a solid phase extraction Sep-Pak Light C18 cartridge (Waters, Millford, MA), and the oligosaccharides in the pass-through fraction were recovered and lyophilized.

When oligosaccharides were released directly from glycoproteins, the in-solution or

in-gel release method was used. For in-solution release, the 0.5 mg protein samples were dissolved in 400 μL of water and 40 μL of 10x denaturing solution (5% SDS and 10% β-mercaptoethanol). The sample was then denatured at 100 °C for 5 min. After cooling, a 40 μL of reaction buffer (0.5 M sodium phosphate, pH 7.5) containing 10% Nonidet P-40 were added. The sample was mixed and incubated with PNGase F at 37 °C for 12 h. The digested sample solution was run through a C18 cartridge to remove the detergent, and then lyophilized (Sheeley and Reinhold, 1998). The sample was desalted on a graphitized carbon column or by the normal phase extraction method (Wada *et al.*, 2004).

For in-gel release (Royl *et al.*, 2006), 80μg samples were reduced with 0.5M dithiothreitol for 10 min at 70 °C, alkylated with 100mM iodacetamide for 30 min at room temperature, then run over three lanes on 10% SDS-PAGE gels and visualised with Coomassie blue. Protein bands were excised, cut into ~1mm^3, frozen for 2h at -20 °C, then washed with alternating 1mL acetonitrile and 1mL 20mM $NaHCO_3$ pH 7 (5 washes, 30min each) and the gel pieces lyophilized. *N*-linked glycans were released *in situ* with 100U/mL peptide-N-glycanase F with overnight incubation. Glycans were extracted by washing with 3 x 0.2mL water, 1 x 0.2mL acetonitrile, 1x 0.2mL water, 1 x 0.2mL acetonitrile. (30min each). Samples were lyophilized ready for MS or fluorescent labeling.

Fluorescence labeling and chromatography of oligosaccharides. For fluorescence detection, the oligosaccharides were subjected to reductive amination at the reducing end with 2-aminopyridine (Natsuka and Hase, 1998), 2-aminobenzamide (Bigge *et al.*, 1995) or 2-aminobenzoate (anumula and Dhume, 1998). The typical procedure using 2-aminobenzoate was as follows. To the lyophilized oligosaccharides, a solution (200 μL) of 2-aminobenzoate and sodium cyanoborohydride, freshly prepared by dissolution of both reagents (30 mg each) in methanol (1 mL) containing sodium acetate and 2% boric acid, was added. The mixture was kept at 80 °C for 1 h. After cooling, the solution was applied to a column of Sephadex LH-20 (1 x 30 cm) equilibrated with 50% methanol. Earlier eluted fractions showing fluorescence at 410 nm with 335 nm-wavelength irradiation were collected and evaporated to dryness. The residue was dissolved in water (100 μL), and a portion (10 μL) was analyzed by HPLC. Separation was done at 50°C with a polymer-based Asahi Shodex NH2P-50 4E column (Showa Denko, Tokyo; 4.6 x 250 mm) using a linear gradient formed by 2% acetic acid in acetonitrile (solvent A) and 5% acetic acid in water containing 3% triethylamine (solvent B). The column was initially equilibrated and eluted with 70% solvent A for 2 min, at which point solvent B was increased to 95% over 80 min and kept at this composition for a further 100 min. The flow rate was 1.0 ml/min throughout the analysis. Detection was performed by fluorometry with λ_{ex} = 350 nm and λ_{em} = 425 nm.

The oligosaccharides derivatized with 2-aminopyridine were analyzed by multidimensional chromatography (Takahashi, 1996). In some laboratories, the separated oligosaccharide were analyzed by MALDI TOF MS for structure verification.

MALDI MS of permethylated oligosaccharides. Permethylation was performed by the solid sodium hydroxide technique (Dell *et al.*, 1993; Lemoine *et al.*, 1996; Ciucanu and Costello, 2003). Briefly five pellets (approximately 1g) of NaOH were ground in a dry mortar to obtain a fine powder. This should be done as quickly as possible to minimize absorption of moisture from the atmosphere. The NaOH powder was mixed with 4 mL of anhydrous dimethyl sulfoxide (DMSO). The oligosaccharide sample released from glycoproteins was dried in a glass tube. Approximately 1 mL of the slurry was added to the sample followed by 0.5 mL of iodomethane. The sample was agitated at room temperature for 10 minutes. The reaction was then terminated by addition of 2 reaction volumes of water. Subsequently, 1mL of chloroform was added, and the mixture was vortexed for 30 sec and centrifuged at 3000 x g to facilitate partitioning. The top aqueous layer was removed and the chloroform layer was then washed 3 additional times with 4 mL of water. The chloroform was evaporated to obtain a dried permethylated sample. One laboratory used the capillary permethylation method as described previously (Kang *et al.*, 2005).

For MALDI MS, the dried permethylated sample was resuspended in 10 μL of pure methanol The sample was mixed with an equal volume of DHB matrix solution at 20 mg/mL in 80% methanol and then spotted onto the MALDI plate. To attain good ion statistics the spectra presented were generated from several sub-spectra of 100 laser shots. The peak height of the $[M+Na]^+$ monoisotopic ions or the integrated peak area for their entire isotopic cluster was measured for relative quantitation.

Reproducibility of the quantitation was examined in one laboratory (lab-17) as follows. Sample B IgG (0.3 mg) was divided into three portions, and each sample was separately subjected to permethylation according to the procedures described above. The MALDI spectrum was acquired with a Voyager DE Pro mass spectrometer (Applied Biosystems, Foster City, CA) in reflectron mode. The signals from a total of 500 shots at 10 different laser irradiation spots were averaged, and the measurement was repeated five times.

The oligosaccharides derivatized at the reducing end or those with intact non-reducing hydroxyls were analyzed by MALDI MS in a few laboratories, among which a simple on-target derivatization with phenylhydrazine was carried out in lab-10 (Lattová *et al.*, 2006).

Analysis of oligosaccharides by LC/ESI MS or LC/ESI MS/MS. The alditol forms of oligosaccharides were analyzed by LC/ESI MS (Karlsson *et al.*, 2004). Typically, the enzymatically released oligosaccharides were converted into alditols by incubation in 20 μL of 0.5 M sodium borohydride/20 mM potassium hydroxide solution at 50 °C for two hours. The resulting solutions were neutralized by addition of 1 mL of glacial acetic acid, desalted and dried. Borate was removed by repeated addition and evaporation of 50 μL of 1% acetic acid in methanol. Oligosaccharide samples were dissolved in water and subjected to negative ion LC/MS employing a graphitized carbon column (Hypercarb, Thermo Electron;

0.2 x 150 mm) using a linear gradient formed by 5 mM ammonium acetate / 2% acetonitrile (solvent A) and 5mM ammonium acetate / 80% acetonitrile (solvent B) (Kawasaki, 1999). The deprotonated $[M-H]^-$ ions were measured and the peak areas of the multiply charged ions corresponding to one specific component were summed up manually for relative quantitation data.

MS of glycopeptides. Glycoproteins were reduced and alkylated, and then digested with trypsin as described above. Resulting peptide/glycopeptide mixtures were analyzed with LC/MS(/MS) or MALDI MS (Huddleston *et al.*, 1993; Wada *et al.*, 2004; Harazono *et al.*, 2005). In either case, protonated peptides were monitored for detection. In LC/MS, glycopeptide profiles can be inferred from a low CID energy MS survey while the molecular weight contribution of the core peptide can usually be inferred from the MS/MS data. Relative quantitation was carried out in the same way as LC/MS of oligosaccharides. For MALDI MS, glycopeptides were enriched from an enzymatic digest and the resulting glycopeptide mixture was mixed with DHB matrix solution at 10 mg/mL in 0.1% trifluoroacetic acid/50% acetonitrile and analyzed in linear mode (Wada *et al.*, 2004). Relative quantitation was based on the intensities (heights) of the signals.

Data presentation. The relative abundances of the glycoforms identified were reported by each participating laboratory.

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Figure caption

Figure 1

Relative quantities of transferrin oligosaccharides from sample A.

The amounts of all glycoforms in total oligosaccharides are presented. Abbreviations are those presented in Table 1. (a) The laboratory numbers for the data are not the same as the numbers given to the authors. (*) The error bars representing standard deviation (SD) from repeated measurements by lab-12 (n=7) and lab-16 (n=2) show intra-assay variance. The SDs of lab-12 were quite small. (b) Comparison of the quantitation by different methods. The data from different laboratories were averaged: chromatography (n=4), a combination of permethylation and MALDI MS (n=7), and LC/ESI MS (n=2). Lab-5 was excluded from the calculation (see text). The error bars representing SD indicate inter-laboratory, or inter-assay, variance.

Figure 2

Relative quantities of IgG oligosaccharides from sample B.

Three bars on the left, for each laboratory, show the relative abundance of differently galactosylated species as a percentage of the total. That for monogalactosylated biantennary oligosaccharide bearing bisecting GlcNAc represents the content of total oligosaccharides identified. (a) The laboratory numbers for the data are not the same as the numbers given to the authors. (*) The error bars representing standard deviation (SD) from repeated measurements by lab-12 (n=7) and lab-16 (n=2) show intra-assay variance. The SDs of lab-12 were quite small. (b) Comparison of the quantitation by different methods. The data from different laboratories were averaged: chromatography (n=4), a combination of permethylation and MALDI MS (n=5), and LC/ESI MS (n=2). Lab-4 was excluded from the calculation. The error bars representing SD indicate inter-laboratory, or inter-assay, variance.

Figure 3

MALDI mass spectrum of permethylated oligosaccharides from sample A transferrin.

Oligosaccharides released by PNGase F were permethylated, and analyzed by MALDI TOF MS in positive ion and reflectron mode. The signals from polyhexose as an internal calibrant are indicated by asterisks. The mass spectrum was provided by lab-7.

Figure 4

MALDI mass spectra of permethylated oligosaccharides from sample B IgG.

The mass spectrum was provided by lab-8.

Figure 5

Mass spectrometric analysis of glycopeptides for site-specific glycan profiling of sample A

transferrin.

(a) Relative abundances of the oligosaccharides at each *N*-glycosylation site measured by RP-LC/ESI MS/MS (lab-15) or MALDI linear TOF MS of tryptic glycopeptides (lab-17). (*) The error bars representing standard deviation (SD) from repeated measurements by lab-17 (n=5) show intra-assay variance. (b, c) MALDI mass spectra of tryptic glycopeptides. The glycopeptides containing Asn-432 (b) or Asn-630 (c) were isolated by reversed phase chromatography and analyzed by MALDI linear TOF MS (lab-17). The ions indicated by asterisks are derived from the glycosidic cleavage during measurements.

Figure 1a (Wada)

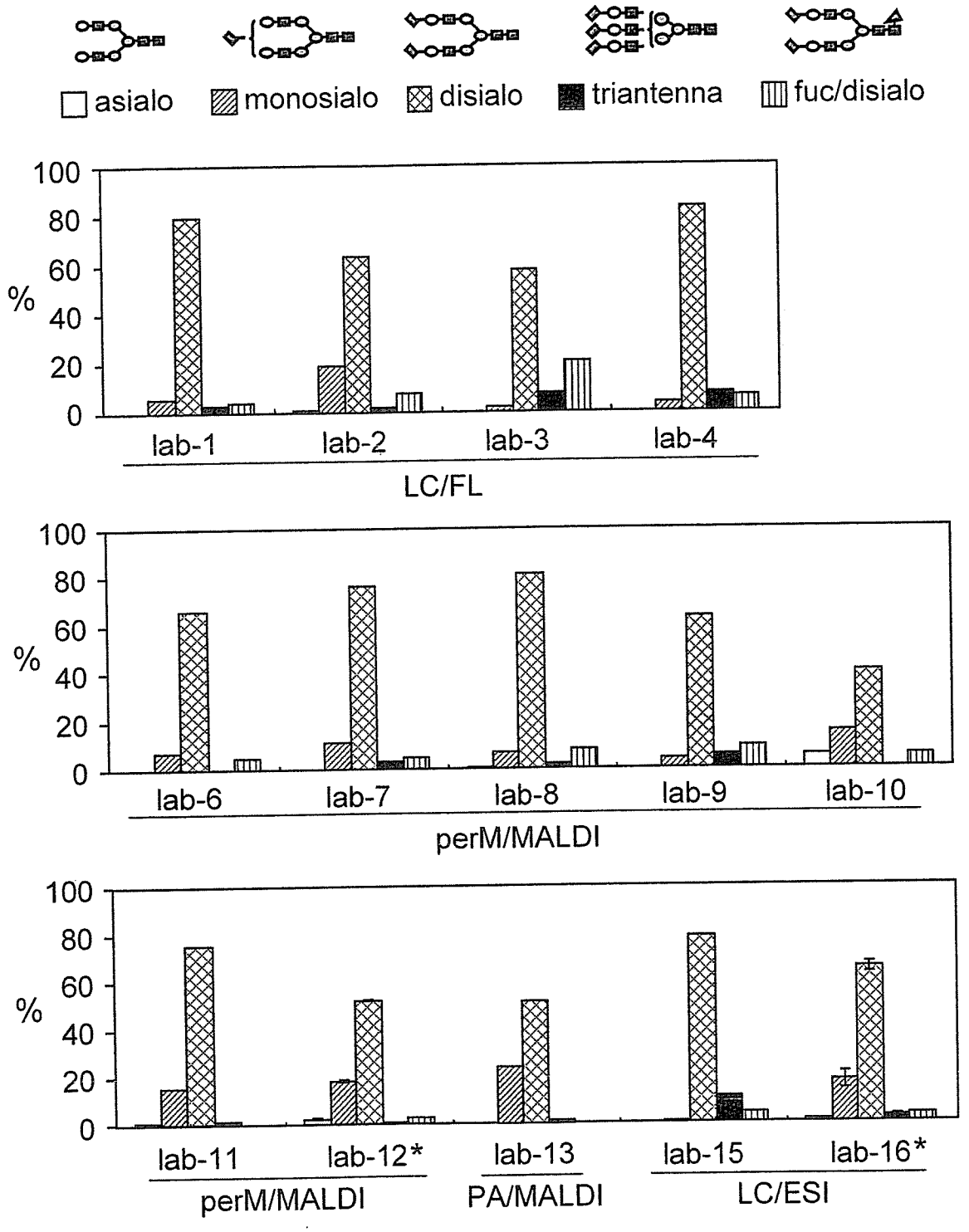


Figure 1b (Wada)

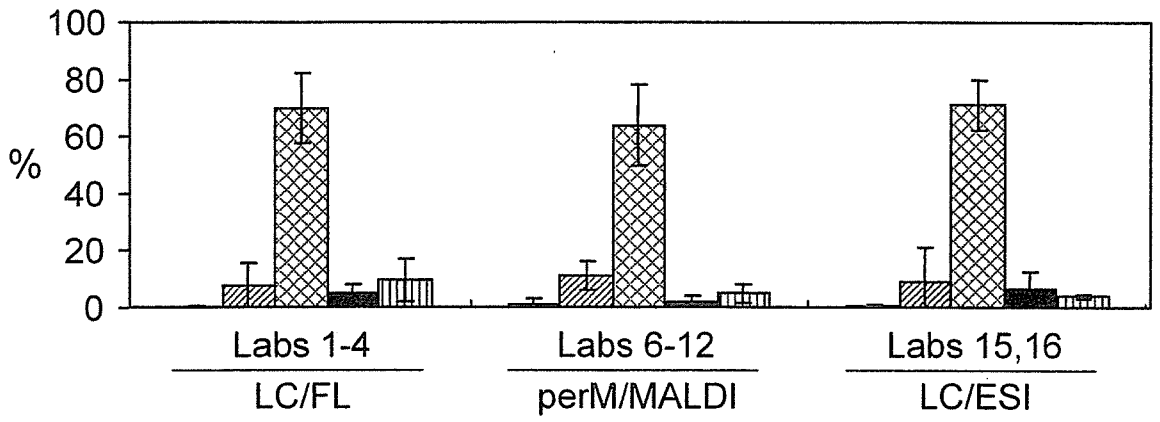


Figure 2a (Wada)

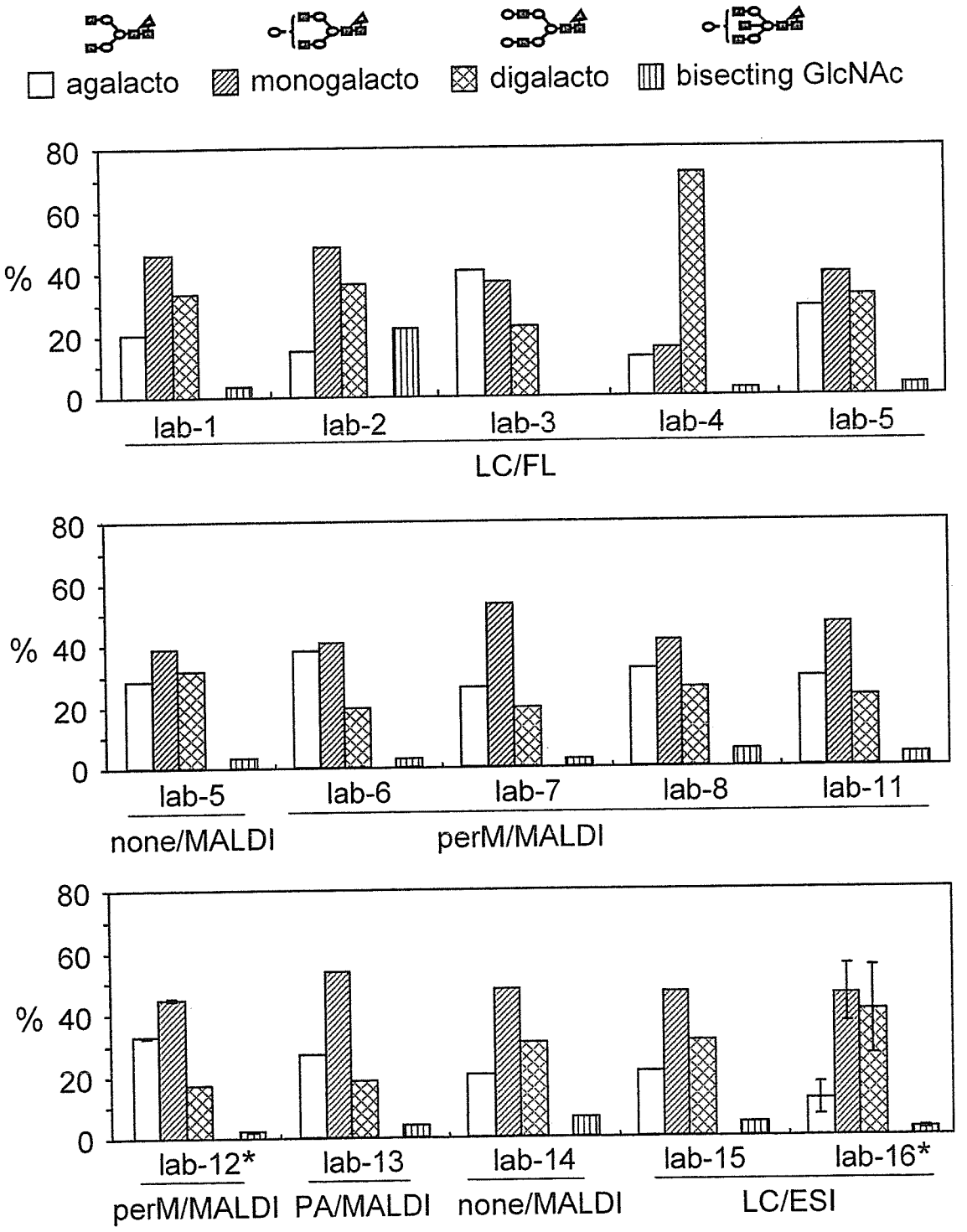


Figure 2b (Wada)

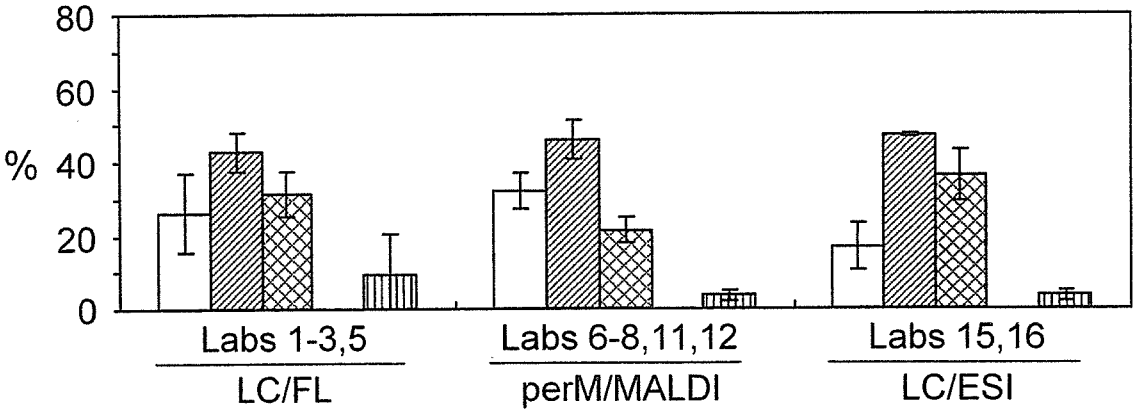


Figure 3 (Wada)

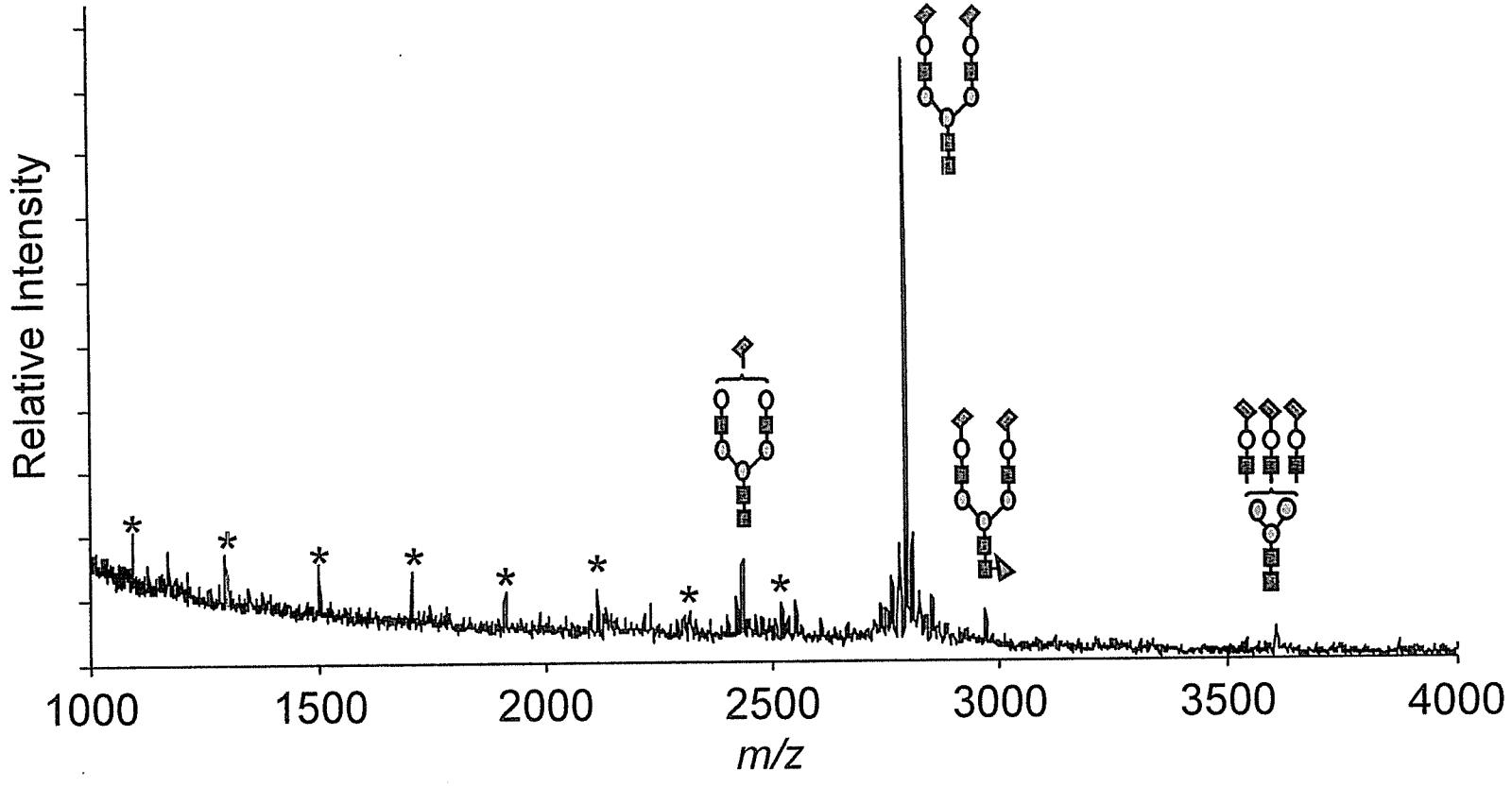


Figure 4 (Wada)

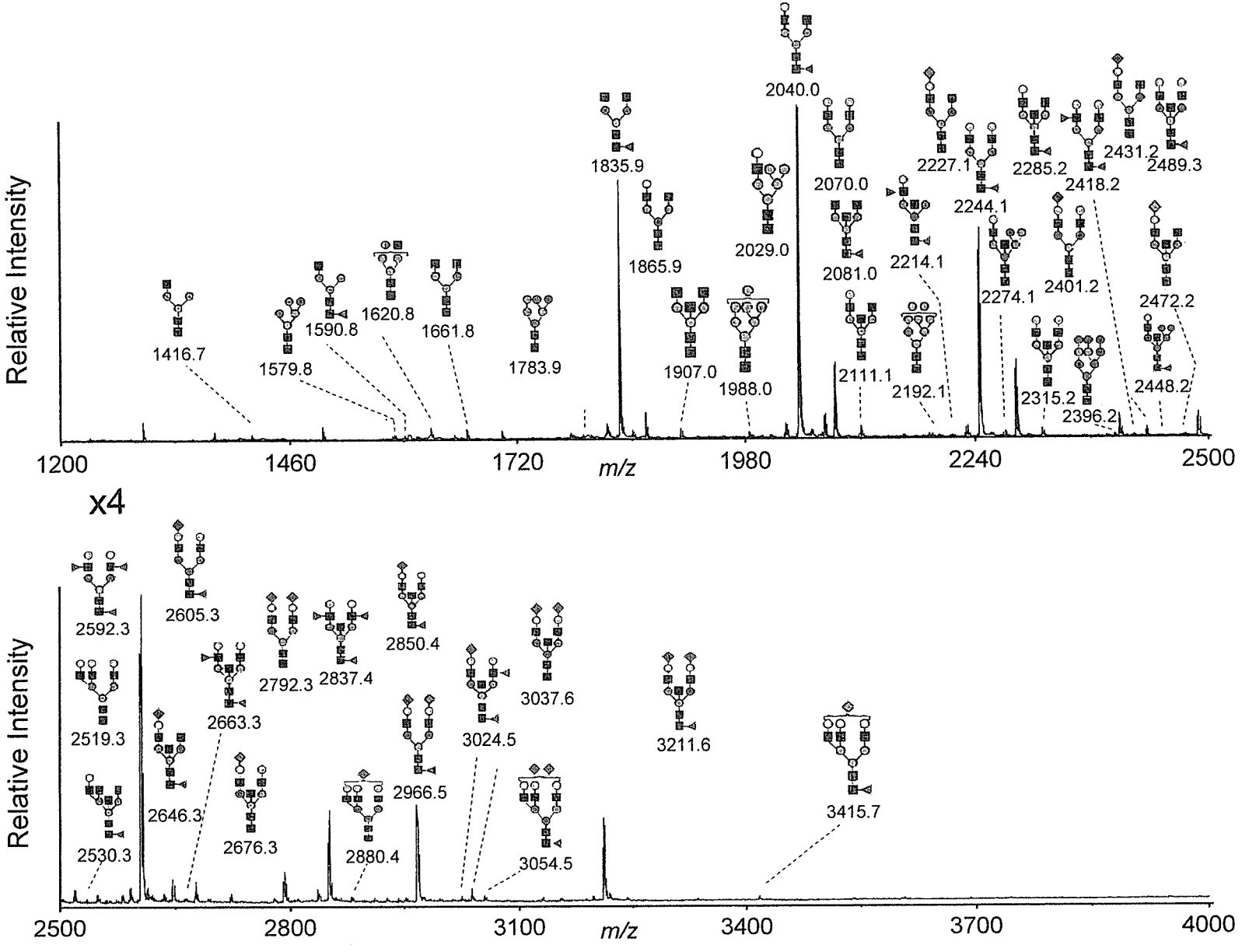
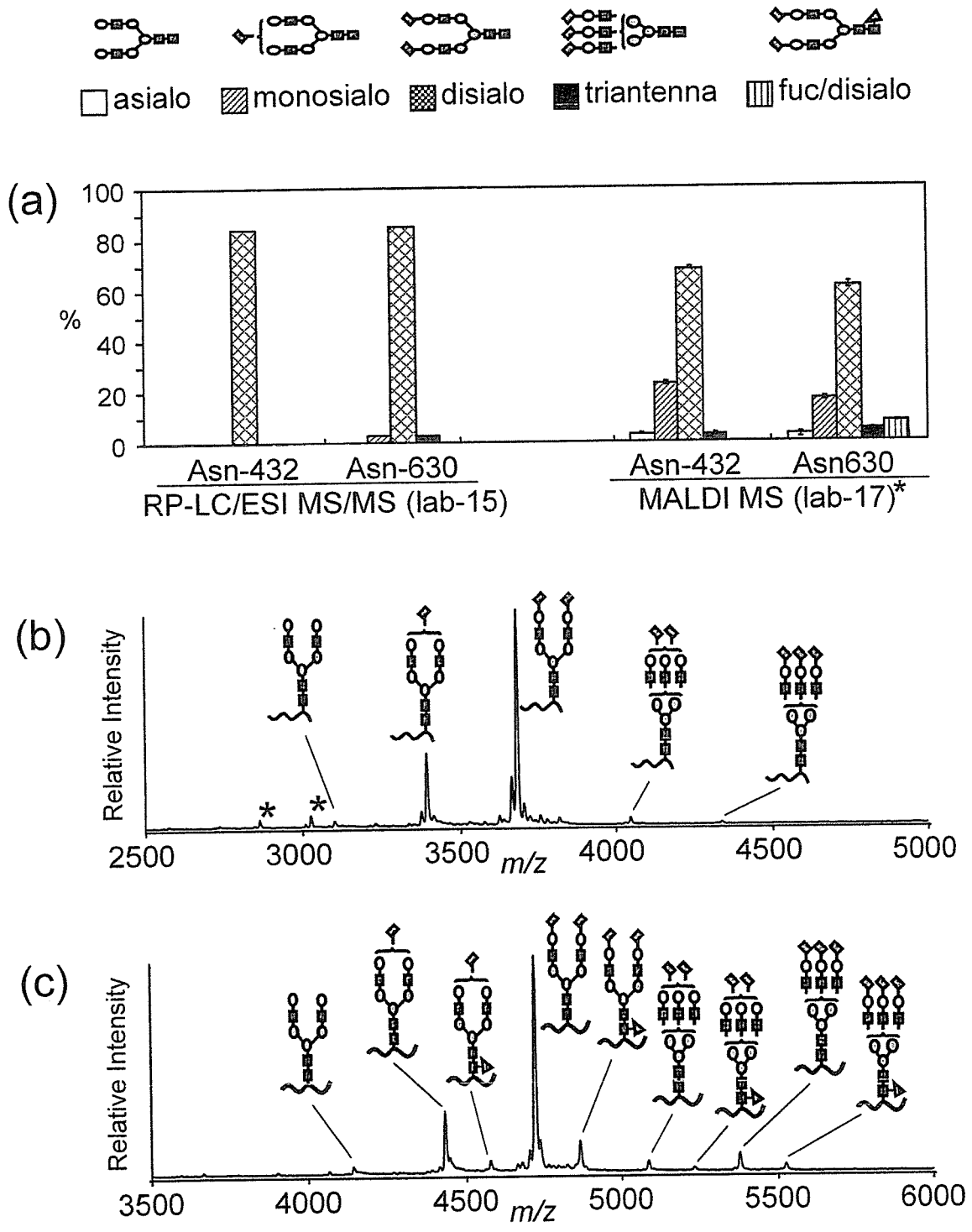


Figure 5 (Wada)



ARTICLES

薬の名前
ステムを知れば薬がわかる

Stems used in drug names : For the better understanding of pharmacological actions of drugs

第5回

国立医薬品食品衛生研究所
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はじめに

本連載では，これまで化学薬品のステムについて紹介してきたが，今回から数回に分けて，生物薬品のステムを紹介する。生物薬品の第1回目は，生物薬品の一般名の命名に関する基本的ルールを紹介するとともに，サイトカイン類のステムについて紹介する。

生物薬品の国際一般名(INN)は，化学薬品と同様にWHOのINN委員会で決定される。生物薬品でも多くの場合，医薬品の分類ごとにステムが与えられ，ステムを用いて一般名が命名される。例えば，「**som-**」は成長ホルモンに関連する医薬品，「**-stim**」はコロニー刺激因子類，「**-mab**」はモノクローナル抗体などである。**表1**に，生物薬品の主な分類とステムの例を示した。一方，表1に示したインスリン類やインターフェロン類などにはステムがなく，学術用語と同じ「**insulin**」，「**interferon**」が命名に用いられている。命名ルールには統一されていない部分もあるが，本連載では，「**insulin**」や「**interferon**」もステムとして扱うことにする。

医薬品の分類をさらに小分類に分ける必要がある場合は，ステムから派生した**サブステム(sub-stem)**を用いる。**表2**に，インターロイキン類のサブステムの例を示

表1　生物薬品の主な分類とステムの例

ステムのあるもの(Groups with respective stems)	ステム
成長ホルモン類(growth hormones)	som-
ホルモン放出促進／抑制ペプチド (hormone-release stimulating/inhibiting peptides)	-relin/relix
サイトカイン／インターロイキン類 (cytokines/interleukins)	-kin
コロニー刺激因子類(colony stimulating factors)	-stim
エリスロポエチン類(erythropoietin type blood factors)	-poetin
モノクローナル抗体類(monoclonal antibodies)	-mab
成長因子類(growth factors)	-ermin
酵素類(enzymes)	-ase
血液凝固因子類(blood coagulation factors)	-cog
血液凝固カスケード阻害剤 (blood coagulation cascade inhibitors)	-cogin
ペプチド，糖ペプチド類 (peptides and glycopeptides)	-tide
受容体分子類 (receptor molecules, native or modified)	-cept (pre-stem)*1
ヒルジン誘導体類(hirudin derivatives)	-irudin
ヘパリン誘導体類(heparin derivatives)	-parin
ステムのないもの(Groups without INN stems)	
インスリン類(insulins)	insulin
インターフェロン類(interferons)	interferon

*1：暫定ステム

した。

同一のステムに属するペプチドあるいはタンパク質性医薬品でアミノ酸配列が異なることを示す場合には，ステムに接頭語あるいは接尾語を付加してアミノ酸配列の違いを区別している。例えば，インターロイキン-2の場合，ステムは「**-leukin**」であるが，Celmo**leukin**(セルモ**ロイキン**)とTece**leukin**(テセ**ロイキン**)は，N末端のメチオニン残基の有無が異なる。また，インスリン類の場合は，アミノ酸配列の違いを2語式(two-word name)の命名をして区別している。例えば，**Insulin** Aspart(**インスリン** アスパルト)は，**Insulin**(**インスリン**)のアミノ酸残基1カ所がアスパラギンに置換した誘導体である。

糖タンパク質や糖ペプチド医薬品で，アミノ酸配列は同一であるが糖鎖部分の構造が違うことを示す場合には，ギリシャ文字を略さずに記載したアルファ，ベータ，ガンマ(alfa，beta，gamma)等を用いた2語式の命名で糖鎖構造の違いを区別している。例えば，「**-poetin**」はエリスロポエチン類のステムであるが，糖鎖の異なるものは，E**poetin** Alfa，E**poetin** Beta，E**poetin** Gamma等，命名されている。

しかし，例外的な命名ルールとして，インターフェロン類では糖鎖の違いではなく，インターフェロンの小分類を区別するためにギリシャ文字が用いられている。インターフェロンの名称については，ステム31「インターフェロン」の項で詳しく説明する。

なお，JANでは，遺伝子組換え技術を用いて製造された生物薬品の正名にはINNの後に括弧書きで(遺伝子組換え)，英名では(Genetical Recombination)と記載し，遺伝子組換えであることを明示するが，本連載では本文中では記載を省略した。

ステム29 「-stim」:コロニー刺激因子類

「**-stim**」は，コロニー刺激因子(colony stimulating factor, CSF)類に共通のステムである。コロニー刺激因子とは，骨髄細胞に作用して，半固形培地で血液細胞のコロニー形成を促進する造血因子の総称であり，サイトカインの1種である。形成されるコロニーの種類によってさらにサブステムに分類される。

表2　インターロイキン類のサブステム

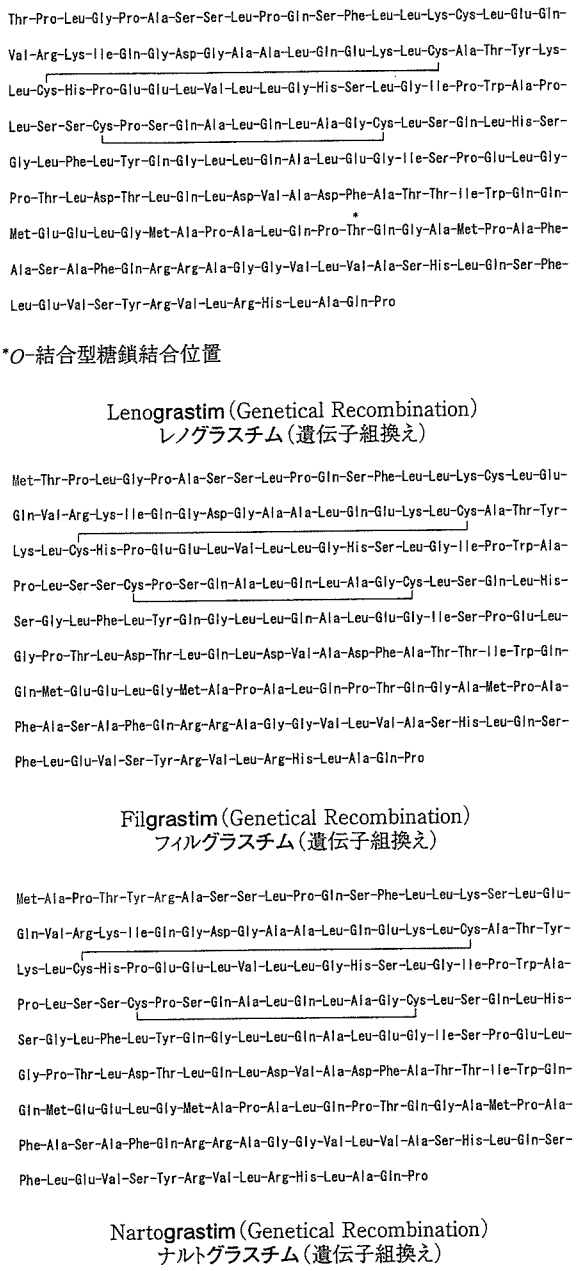
インターロイキン類(-kin)	サブステム	INN
インターロイキン-1(IL-1)	-nakin	
インターロイキン-1α(IL-1α)	-onakin	Pifonakin(ピホナキン)
インターロイキン-1β(IL-1β)	-benakin	Mobenakin(モベナキン)
インターロイキン-2(IL-2)	-leukin	Adargileukin Alfa Aldesleukin Celmoleukin(セルモロイキン) Denileukin Diftitox Pegaldesleukin Teceleukin(テセロイキン) Tucotuzumab Celmoleukin
インターロイキン-3(IL-3)	-plestim	Daniplestim Muplestim
インターロイキン-4(IL-4)	-trakin	Binetrakin
インターロイキン-6(IL-6)	-exakin	Atexakin Alfa
インターロイキン-8(IL-8)	-octakin	Emoctakin
インターロイキン-10(IL-10)	-decakin	Ilodecakin
インターロイキン-11(IL-11)	-elvekin	Oprelvekin(オプレルベキン)
インターロイキン-12(IL-12)	-dodekin	Edodekin Alfa
インターロイキン-13(IL-13)	-tredekin	Cintredekin Besudotox
ニューロトロピン(インターロイキン-78, Brain derived neurotropic factor)	-neurin	Abrineurin
インターロイキン-1受容体アンタゴニスト	-nakinra	Anakinra
インターロイキン-4受容体アンタゴニスト	-kinra	Pitrakinra

(1)「-grastim」：顆粒球コロニー刺激因子類

「**-grastim**」は，顆粒球コロニー刺激因子(granurocyte-colony stimulating factor, G-CSF)類を示すサブステムである。G-CSFは顆粒球(好中球)の前駆細胞に特異的に作用してその増殖，分化を促進してコロニー形成を誘導する作用を有する。天然のヒトG-CSFは174個のアミノ酸残基からなり，Thr133に*O*-結合型糖鎖を有する分子量約20,000の糖タンパク質である。

ステム「**-grastim**」を持ち，現在，日本で承認されている医薬品には，Leno**grastim**(レノ**グラスチム**)，Fil**grastim**(フィル**グラスチム**)，Narto**grastim**(ナルト**グラスチム**)の3品目がある(**図1**)。これらの医薬品は主にがん化学療法後の好中球減少症治療薬として用いられているほか，造血幹細胞の末梢血中への動員や造血幹細胞移植時の好中球数の増加促進にも用いられる。今後，日局への収載が予定されている医薬品である。

Leno**grastim**(レノ**グラスチム**)はCHO細胞で製造された遺伝子組換えヒトG-CSFで，天然のものと同様に174個のアミノ酸残基からなり，*O*-結合型糖鎖を有する糖タンパク質である。Fil**grastim**(フィル**グラスチム**)は大



*O-結合型糖鎖結合位置

図1　顆粒球コロニー刺激因子類を示すステム「-grastim」を持つ医薬品

腸菌で製造された遺伝子組換えヒトG-CSFで，N末端にメチオニンが1残基付加したアミノ酸175個からなるタンパク質である。また，Nartograstim(ナルトグラスチム)は大腸菌で製造されたヒトG-CSF誘導体で，N末端にメチオニンが1残基付加しているほか，アミノ酸残基5カ所が置換されているアミノ酸175個からなるタンパク質である。天然型G-CSFと比べて高い比活性を示す。なお，図1には天然型と異なるアミノ酸残基を赤文字で示した。

これらの他にINNに登録されている医薬品には以下のものがある。

Pegfilgrastim

Pegnartograstim

これらは，それぞれFilgrastim(フィルグラスチム)，Nartograstim(ナルトグラスチム)にポリエチレングリコールを結合した修飾タンパク質である。「Peg-」はポリエチレングリコール(PEG)が結合していることを意味する接頭語である。PEGによる修飾(PEG化)はDDS(Drug delivery system)の手法のひとつで，タンパク質性医薬品の体内での安定性の向上，血中消失半減期の延長や抗原性の低下を目的として行われる。欧米ではすでに持続性を高めたPegfilgrastimが承認されているが，日本ではまだ実用化されていない。

(2)「-gramostim」：顆粒球マクロファージコロニー刺激因子類

「-gramostim」は，顆粒球マクロファージコロニー刺激因子(granurocyte macrophage colony stimulating factor, GM-CSF)類を示すサブステムである。GM-CSFは，顆粒球(好中球)，マクロファージ，好酸球またはこれらの混合コロニー形成を誘導する作用を持つ。ヒトGM-CSFは127個のアミノ酸残基からなる分子量約18,000～24,000の糖タンパク質である。

ステム「-gramostim」を持つINNは以下のものがある。

Molgramostim

Ecogramostim

Regramostim

Sargramostim(サルグラモスチム)

Molgramostimは大腸菌で製造した遺伝子組換えヒトGM-CSF，Ecogramostimは大腸菌で製造したヒトGM-CSFでN末端にメチオニン残基が付加したもの，Regramostimは CHO細胞で製造したヒトGM-CSFで糖鎖が結合しているものである。Sargramostim(サルグラモスチム)はヒトGM-CSFの23番目のアルギニンをロイシンに置換したGM-CSF誘導体で，遺伝子組換えにより酵母で製造した糖タンパク質である。米国では化学療法後の白血球増加薬として承認されている。JANに登録され，クローン病患者の治療薬として臨床開発中である。

(3)「-mostim」：マクロファージコロニー刺激因子類

「-mostim」は，マクロファージコロニー刺激因子