TABLE 3
Intra- and Interlaboratory Micronucleated Reticulocyte Data

Laboratory	Method	Compartment	Treatment	%MN-RET Average[a] ± SEM	%CV	Fold difference
L1	MeOH-AO	BM	Vehicle	0.15 ± 0.03	33.3	
			CP	3.35 ± 0.10	5.4	22.3
L9	MeOH-AO	BM	Vehicle	0.05 ± 0.05	173.2	
			CP	1.63 ± 0.27	28.4	32.6
L10	MeOH-AO	BM	Vehicle	0.03 ± 0.02	86.6	
			CP	2.33 ± 0.23	17.3	77.7
L11	MeOH-AO	BM	Vehicle	0.18 ± 0.03	31.5	
			CP	2.44 ± 0.20	13.9	13.6
Pooled[a] L1, 9, 10, 11			*Vehicle*	*0.10 ± 0.02*	*80.5*	
			CP	*2.44 ± 0.21*	*29.1*	*24.4*
L1	MeOH-AO	PB	Vehicle	0.05 ± 0.03	100.0	
			CP	1.77 ± 0.17	16.6	35.4
L9	MeOH-AO	PB	Vehicle	0.05 ± 0.00	0.0	
			CP	0.50 ± 0.03	10.0	10.0
L11	MeOH-AO	PB	Vehicle	0.18 ± 0.04	41.7	
			CP	1.42 ± 0.10	12.3	7.9
Pooled L1, 9, 11			*Vehicle*	*0.09 ± 0.03*	*85.6*	
			CP	*1.23 ± 0.20*	*48.2*	*13.7*
L5	SV-AO	PB	Vehicle	0.13 ± 0.03	43.3	
			CP	1.83 ± 0.15	13.7	14.1
L6	SV-AO	PB	Vehicle	0.12 ± 0.07	99.0	
			CP	1.77 ± 0.32	31.2	14.8
L7	SV-AO	PB	Vehicle	0.22 ± 0.14	113.8	
			CP	1.47 ± 0.27	31.7	6.7
Pooled L5, 6, 7			*Vehicle*	*0.16 ± 0.05*	*94.3*	
			CP	*1.69 ± 0.14*	*24.7*	*10.6*
L1	FCM	PB	Vehicle	0.12 ± 0.02	24.8	
			CP	0.99 ± 0.04	6.5	8.3
L2	FCM	PB	Vehicle	0.11 ± 0.02	31.5	
			CP	1.04 ± 0.04	6.7	9.5
L3	FCM	PB	Vehicle	0.11 ± 0.02	32.9	
			CP	1.11 ± 0.04	6.8	10.1
Pooled L1, 2, 3			*Vehicle*	*0.11 ± 0.01*	*26.5*	
			CP	*1.05 ± 0.03*	*7.6*	*9.5*

Note. Abbreviations: MN-RET = micronucleated reticulocyte; MeOH-AO = acridine orange staining of methanol-fixed smears; SV-AO = supravital staining using acridine orange–coated slides; FCM = flow cytometry; BM = bone marrow; PB = peripheral blood; CP = cyclophosphamide; SEM = standard error of the mean; %CV = percent coefficient of variance.

[a]Values are the mean of three separately coded, but identical, samples. By "Pooled" it is meant that like-method data from three or four laboratories were combined for these calculations.

Most laboratories detected a reduction in %RET for the CP-treated rat (see Table 2). However, this was somewhat variable across microscopy-based laboratories, especially when the MeOH-AO technique was used to evaluate bone marrow specimens. In two of the three laboratories that scored both bone marrow and peripheral blood, peripheral blood measurements demonstrated greater CP-associated reduction of %RETs than in bone marrow. Intra- and interlaboratory %CV values for the replicate RET analyses are presented in Table 2. Flow cytometric measurements were more consistent within and across laboratories than microscopic scoring. For instance, vehicle control specimens' %CV for pooled laboratory MeOH-AO/bone marrow data was 13.6%, while the corresponding blood-based analyses for flow cytometric, SV-AO, and MeOH-AO techniques were 1.93, 6.5, and 12.5%, respectively.

The interlaboratory %CV values for MN-RET determinations and the intralaboratory %CV values for the triplicate blinded analyses conducted within each laboratory are provided in Table 3. The flow cytometric analyses demonstrate superior intra- and interlaboratory consistency relative to both microscopy-based methods. %CV values for MN-RET measurements performed on vehicle control blood specimens pooled across like-method laboratories were 26.5, 94.3, and 85.6% for the flow cytometric, SV-AO, and MeOH-AO methods, respectively, and 80.5% for MeOH-AO scored bone marrow. Analogous %CV values for CP blood samples were 7.6, 24.7,

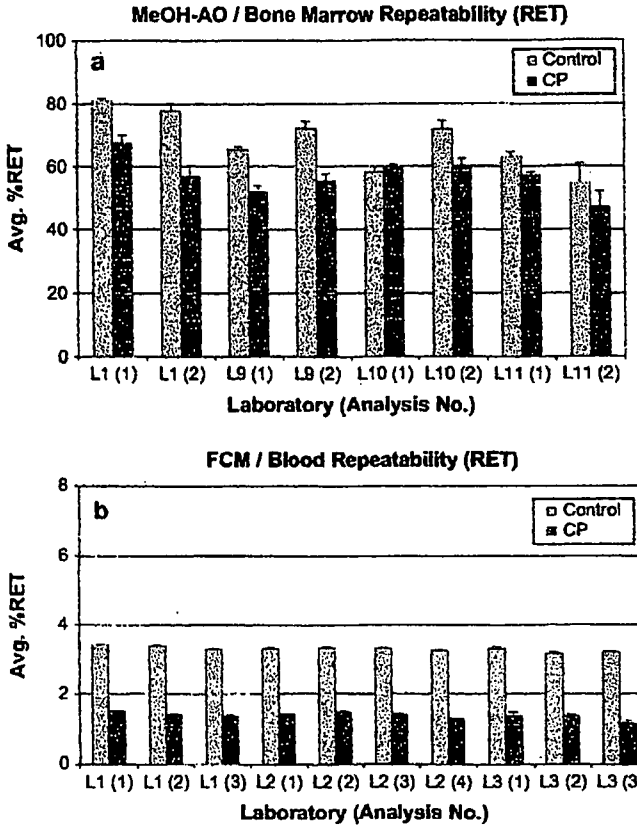

FIG. 3. Values are the mean of three identical, but separately coded, samples. Panel (a): The average frequency of bone marrow RETs (%RET) as measured by the standard MeOH-AO microscopy technique are graphed (with standard error of mean [SEM] bars). These data were collected on two separate occasions at each laboratory. Panel (b): The average frequency of peripheral blood RET as measured by the flow cytometric (FCM) technique are graphed (with SEM bars). These data were collected on three or four separate occasions.

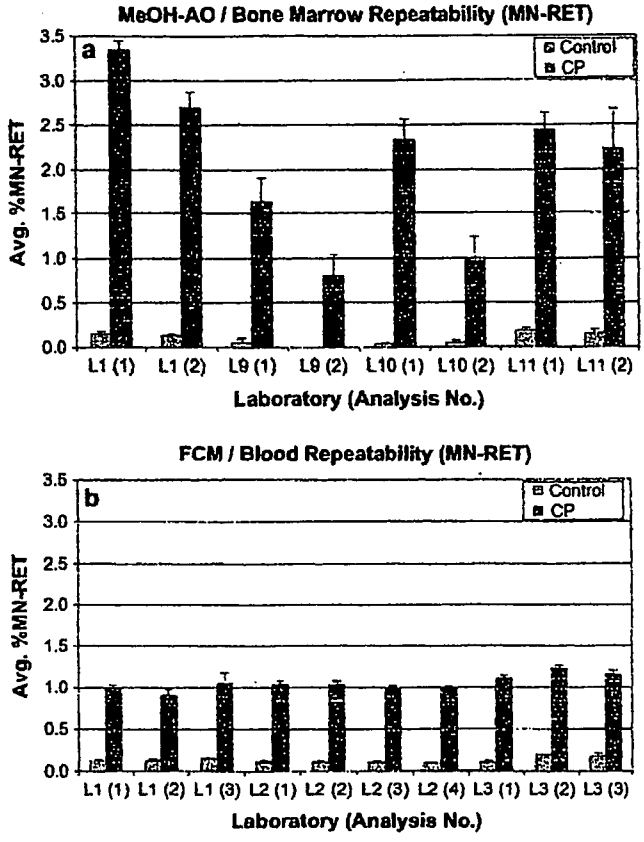

FIG. 4. Values are the mean of three identical, but separately coded, samples. Panel (a): The average frequency of bone marrow micronucleated RETs (%MN-RET) as measured by the standard MeOH-AO microscopy technique are graphed (with standard error of mean [SEM] bars). These data were collected on two separate occasions at each laboratory. Panel (b): The average frequency of peripheral blood MN-RET as measured by the flow cytometric (FCM) technique are graphed (with SEM bars). These data were collected on three or four separate occasions.

and 48.2%, respectively, and 29.1% for MeOH-AO scored bone marrow.

Fold difference values based on each laboratory's average MN-RET frequencies, as well as for like-method pooled data, are also presented (Table 3). It was somewhat surprising that the fold difference in MN-RETs between vehicle and CP-associated blood specimens, as well as absolute MN-RET frequencies, were no higher with the flow cytometric or SV-AO techniques than with the conventional MeOH-AO method as it has been reported that restriction of MN analysis to an immature RET cohort based on RNA content or CD71 expression levels could reduce, if not eliminate, the influence of the spleen's erythrophagocytotic activity (Abramsson-Zetterberg *et al.*, 1999; Hayashi *et al.*, 1992). Splenic activity and its effects on assay sensitivity for blood-based analyses have been investigated thoroughly, and these data are discussed in a companion paper that appears in this issue (MacGregor *et al.*).

Intralaboratory Variability Across Time

In addition to the inter- and intralaboratory analyses, an evaluation of scoring reproducibility over time was studied. This was accomplished by having flow cytometry laboratories analyze coded peripheral blood specimens on three or four different occasions, while triplicate vehicle and CP bone marrow slides were submitted to L9, L10, and L11 laboratories for analysis on two separate occasions. Reagents were prepared separately for each day of analysis. The resulting repeat-analysis RET data are presented in Figure 3 and demonstrate higher reproducibility for the multiple flow cytometric analyses compared to MeOH-AO.

As with RET enumeration, repeat-analysis MN-RET microscopy data were also quite variable. For instance, laboratories using the MeOH-AO method reported average CP-induced values that differed from their original mean reading by 19.4,

50.9, 58.4, and 8.6% (L1, L9, L10, and L11, respectively; see Fig. 4). Repeat-analysis MN-RET data generated by the flow cytometric technique were considerably more reproducible as average values were all within 11% of the originally reported mean frequencies. It should also be noted that the fourth flow cytometric analyses by L2 was performed more than 2 years after blood fixation, demonstrating this procedure's compatibility with long-term storage of fixed blood specimens.

CONCLUSIONS

Distribution of replicate bone marrow and blood specimens obtained from single rats that were first shown to exhibit steady-state spontaneous or genotoxicant-induced MN-RET frequencies were used to assess inter- and intralaboratory scoring variability using two widely used microscopic and one flow cytometric procedure. These results demonstrate that the quantification of MN-RETs benefits from an objective flow cytometry–based method of data acquisition. The flow cytometric method provides better reproducibility, and the high throughput capability allows interrogation of tens of thousands of RETs per specimen. Enhanced scoring precision is important as it is necessary to offset the spleen-dependent loss of dynamic range observed in peripheral blood relative to bone marrow—a phenomenon that was observed in this as well as other reports (MacGregor *et al.*, this issue; Wakata *et al.*, 1998). A recent report by Torous *et al.* (2006) delincates the consequential improvements to assay power when the number of cells scored per specimen is increased and supports this view.

Beyond overcoming lower genotoxicant-induced MN-RET frequencies in blood relative to bone marrow, further incentive for automating rat peripheral blood MN-RET measurements comes from a recent recommendation of the *In Vivo* MN Assay Expert Group of the International Working Group on Genetic Toxicology Testing (IWGT; Hayashi *et al.*, in press). Specifically, IWGT has recommended that a sufficient number of RETs should be scored to ensure that the MN-RET counting error is kept below the level of interanimal variability. This allows the sensitivity of the experiment to be limited by the variability of spontaneous MN-RET frequency among animals, rather than being limited by the statistical variation of count. Based on the flow cytometric scoring of 20,000 peripheral blood RETs from each of the 15 control animals from the three experiments reported in the MacGregor *et al.* companion paper in this issue (laboratory L2, the reference laboratory), we find that the mean incidence of MN-RET ± 1 SD is 0.11% ± 0.045. This is a 41% CV. Poisson distribution theory allows us to calculate that 6 MN-RETs per animal must be scored to limit counting error to this level of variation (SD of the Poisson count = $\sqrt{\text{absolute count}}$). At a spontaneous MN-RET frequency of approximately 0.1%, this means that an average of 6000 RETs per individual need to be scored for micronuclei in order to achieve a CV that is at or below the interanimal variance. This is a significantly higher number of RETs per animal than required to be scored under the current OECD MN assay guideline (which recommends scoring 2000 RETs per animal) and is difficult to achieve by manual microscopic scoring.

In conclusion, the data presented herein and in the companion paper that follows support the growing consensus that rat peripheral blood can be used to perform *in vivo* MN tests more effectively than the standard bone marrow–based assay. The ability of the described automated scoring procedure to greatly enhance the precision of MN-RET measurements overcomes the somewhat attenuated genotoxicant-induced frequencies observed in peripheral blood relative to bone marrow. This conclusion is supported by experiments described in the accompanying paper whereby intact and splenectomized rats were treated with diverse genotoxicants (MacGregor *et al.*, this issue).

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Comparison of the Methods for Profiling Glycoprotein Glycans: HUPO HGPI (Human Proteome Organisation Human Disease Glycomics/Proteome Initiative) Multi-institutional Study

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Mass spectrometry (MS) of glycoproteins is an emerging field in proteomics, poised to meet the technical demand for elucidation of the structural complexity and functions of the oligosaccharide components of molecules. Considering the divergence of the mass spectrometric methods employed for oligosaccharide analysis in recent publications, it is necessary to establish technical standards and demonstrate capabilities. In the present study of the HUPO HGPI (Human Proteome Organisation Human Disease Glycomics/Proteome Initiative), the same samples of transferrin and immunoglobulin-G were analyzed for *N*-linked oligosaccharides and their relative abundances in 20 laboratories, and the chromatographic and mass spectrometric analysis results were evaluated. In general, matrix-assisted laser desorption/ionization (MALDI) time-of-flight MS of permethylated oligosaccharide mixtures carried out in six laboratories yielded good quantitation, and the results can be correlated to those of chromatography of reductive amination derivatives. For underivatized oligosaccharide alditols, graphitized carbon-liquid chromatography (LC)/electrospray ionization (ESI) MS detecting deprotonated molecules in the negative ion mode provided acceptable quantitation. The variance of the results among these three methods was small. Detailed analyses of tryptic glycopeptides employing either nanoLC/ESI MS/MS or MALDI MS demonstrated the excellent capability to determine site-specific or subclass-specific glycan profiles in these samples. Taking into account the variety of MS technologies and options for distinct protocols used in this study, the results of this multi-institutional study indicate that MS-based analysis appears as the efficient method for identification and quantitation of oligosaccharides in glycomic studies and endorse the power of MS for glycopeptide characterization with high sensitivity in proteomic programs.

Glycosylation is a common post-translational modification providing a highly diverse structure variation to more than half of all secretory and cellular proteins (Apweiler, 1999). Several lines of evidence have indicated that attachment of a specific monosaccharide to core glycans or branches changes glycoprotein function, and the resulting transformation of cellular phenotypes is suggested to be involved in various biological or pathological processes such as cancer, infection, and reproduction (Taniguchi *et al.*, 2001; Hakomori, 2002; Helenius and Aebi, 2004). From a pharmacological point of view, glycosylation profoundly affects biological activity, function, clearance from the circulation, and crucially, the antigenicity of recombinant proteins (Brooks, 2004; Jefferis, 2005). Increasing knowledge of the biological significance of glycosylation has resulted from the development of analytical methods, among which mass spectrometry (MS) is an essential tool as it allows rapid and high sensitivity profiling and detailed characterization of heterogeneous glycan structures. In fact, in recent years, mass spectrometric analysis by soft ionization techniques, electrospray ionization (ESI)

or matrix-assisted laser desorption/ionization (MALDI), has been employed in most studies of oligosaccharide structure (Harvey, 1999; Dell and Morris, 2001; Mechref and Novotny, 2002; Zaia, 2004).

The capability of MS and data interpretation is based on various instrumental factors. For example, the physicochemical properties such as charge and internal energy of generated ions are largely dependent on the ionization methods employed. Different types of mass analyzers have specific time frames for detection and this affects the fragmentation patterns observed in the mass spectrum. Moreover, there are still other factors to consider with oligosaccharides such as derivatization, ion polarity and the nature of ion species derived from protonation and deprotonation, alkali-metal cations and different anion adduction. Although all of these factors affect, to varying extents, the relative intensities of the molecular ions for different oligosaccharide structures, it is also true that good quantitation can be expected with appropriately designed measurements, as already reported (Viseux *et al.*, 2001). While detailed reviews of current mass spectrometric techniques and analysis workflow are available, the emerging need for rapid and sensitive analysis of glycoprotein glycans makes it desirable to compare the performance of standard method(s). To this end, the HUPO HGPI (Human Proteome Organisation Human Disease Glycomics/Proteome Initiative) has formed a consortium with expertise in glycobiology, and has distributed *N*-glycosylated protein samples, transferrin and immunoglobulin-G (IgG) from healthy individuals to participating members for analysis. The released oligosaccharides were analyzed by MS or conventional chromatography, and in some cases, glycopeptides obtained by enzymatic proteolysis were also analyzed by MS. This report describes these analyses of glycoprotein glycans, focusing mainly on relative quantitative profiling of structural heterogeneity.

RESULTS

The laboratories enrolled in this study analyzed released oligosaccharides or glycopeptides, or both. The methods are summarized in Table 1. [The laboratory numbers designated in Table 1 and the succeeding figures are not the same as those given to the authors in the title.]

Human transferrin contains two *N*-linked complex type oligosaccharides at Asn-432 and Asn-630 (Spik *et al.*, 1975). Human IgG has a conserved *N*-linked glycosylation site, Asn-297, in the heavy chain of the Fc region, with variable glycosylation in the Fab depending on the presence or absence of the glycosylation motif in the variable region. The oligosaccharides attached within the Fc region are of a complex type (Takahashi *et al.*, 1987), showing higher heterogeneity than transferrin, and were the subject of this study.

For transferrin, the evaluation was primarily focused on the *N*-acetylneuraminic (sialic) acid levels. Sialic acids are major residues giving negative charge to the molecule and play a key role in various biological processes such as infection and cellular communication. The rather labile glycosidic linkage of sialic acid to the oligosaccharide chain can be cleaved during sample preparation or analysis thus making quantitation difficult. For IgG,

galactosylation levels were evaluated because of the possible alterations implicated in pathological conditions (Axford, 1999). In these respects, detection of minor glycans, specifically, the fucosylation and triantennary branching of the transferrin glycans and the bisecting *N*-acetylglucosamination of IgG glycans, was evaluated.

In addition, in the glycopeptide analysis, different oligosaccharide profiles at two glycosylation sites of transferrin and those of different IgG subclass molecules were identified.

Chromatographic analysis

Chromatography of reductively aminated oligosaccharides is generally accepted as a standard method of quantitation, in which the fluorescence correlates with the amounts of individual components. In the present study, five laboratories carried out this type of analysis utilizing 2-aminopyridine, 2-aminobenzamide or 2-aminobenzoate, as the labeling agent (Table 1), and the results were compared with those obtained by MS.

The chromatographic measurements of the oligosaccharides from sample A transferrin are summarized in Figure 1a (labs 1-4), in which the relative abundances of differently sialylated biantennary chains and trisialo-triantennary and fucosylated disialo-biantennary chains in the total oligosaccharides are presented. One laboratory (lab-1) discriminated the monosialo-binatennary isomers bearing a sialic acid at either antenna, and the values were summed for data presentation. In labs 1-4, the levels of monosialo- and disialo-biantennary, fucosylated disialo-biantennary and trisialo-triantennary chains were 7.6 ± 7.0, 70.8 ± 12.3, 5.1 ± 2.9 and 9.7 ± 7.5 (mean ± SD, %), respectively, apparently showing a considerable variance among reports. No laboratories detected significant amounts of the asialo-biantennary chain.

A majority of IgG oligosaccharides are of the fucosylated biantennary type, and are partially galactosylated. The contents of each differently galactosylated species among total fucosylated biantennary oligosaccharides from sample B IgG were calculated and are shown in Figure 2a (labs 1-5). Two laboratories (labs-1 and 5) discriminated the isomers with a galactose at either antenna, and the values were combined and represented by the monogalactosyl species. The most abundant structure was the monogalactosyl form, in three reports, while it was either agalactosyl or digalactosyl species in other laboratories. The number of galactose residues in a fucosylated biantennary oligosaccharide from this sample ranged from 0.82 (lab-3) to 1.59 (lab-4), and had a mean of 1.16 ± 0.28 for the five laboratories (Table 2). The relative abundance of the monogalactosylated / fucosylated species with *vs.* without bisecting *N*-acetylglucosamine (GlcNAc) ranged from 2.2 to 3.4 % in three laboratories (labs-1, 4 and 5), while others overestimated abundance or did not present the data due to insufficient separation of the oligosaccharides with or without bisecting GlcNAc.

MS of oligosaccharides

MS of oligosaccharides was carried out with MALDI or LC/ESI MS (Table 1). The former was employed by many laboratories, most of which derivatized by permethylation prior to analysis. Permethylation stabilizes the sialic acid residues by converting them to methyl esters, thus preventing sialic acid loss whilst also improving the efficiency of positive ion formation.

Transferrin

The results of sample A transferrin with attention to the sialylation of biantennary oligosaccharides and the minor glycans with triantennary branching or fucosylation are summarized in Figure 1a (labs 6-13, 15 and 16), and a typical MALDI time-of-flight (TOF) mass spectrum of permethylated oligosaccharides is shown in Figure 3. Standard deviations of the data provided by two laboratories, lab-12 (n=7) and lab-16 (n=2), were small especially for the combination of permethylation and MALDI MS (lab-12). The abundance of monosialylated species was 11.0 ± 5.1 % (mean ± SD) in seven laboratories using a combination of permethylation and MALDI MS, a little higher than the results obtained with chromatography (Figure 1b). Four laboratories had levels below 15% for the monosialylated chain, whereas the higher levels obtained by others (labs 10-12) were probably due to sialic acid loss during sample preparation. The (fully sialylated) triantennary oligosaccharide level ranged from 0 to 5.6 %, apparently lower than the chromatographic data, and two laboratories (labs-6 and 10) failed to detect this oligosaccharide. The fucosylated oligosaccharide in seven laboratories (labs 6-12) ranged from 0 to 9.2 %, comparable to the chromatographic results. One laboratory (lab-12) repeated the measurement for the same sample and reported good intra-assay CVs less than 5 % for either oligosaccharide. One laboratory applied pyridylaminated oligosaccharides to MALDI TOF MS, which showed significant sialic acid loss. LC/ESI MS was employed by two laboratories (labs-15 and 16), and the results were a little different each other with respect to the levels of monosialo and triantennary species. In most measurements employing MALDI MS or LC/ESI MS, the fucosylated species was more abundant than the triantennary branching species, consistent with the chromatographic data. In summary, the results from three different methods, which were carried out in different laboratories, were similar each other as shown in Figure 1b.

IgG

The results for sample B IgG are presented in Figure 2a (labs 5-8, 11-16) with attention to the galactosylation levels as well as the minor component, monogalactosylated biantennary oligosaccharide bearing bisecting GlcNAc. Galactosylation in this sample was evaluated according to the relative abundance of three major species as a percentage of the total. Repeated measurements were performed in labs-12 and 16. The CVs in lab-12 with permethylation and MALDI MS were quite small. The standard deviations of the

measurement by lab-16 were exceptionally large for this specific sample, but the CVs for these species were less than 10% for samples A and C in the same laboratory. With either MALDI or LC/ESI MS, the most abundant *N*-linked oligosaccharide from sample B IgG was the monogalactosylated species by both MALDI and LC/ESI MS. The galactosylation levels calculated from the MALDI MS measurements ranged from 0.80 to 1.10 (0.93 ± 0.10) mol per fucosylated biantennary oligosaccharide chain, but those calculated from LC/ESI MS measurements (1.10 and 1.29 mol) were slightly higher (Table 3). The relative levels of the monogalactosylated and fucosylated glycan bearing bisecting GlcNAc calculated by mass spectrometric measurements were over the range of 2.2 - 14.0 % of total oligosaccharides, which was comparable to that obtained by chromatography. Similarly to the transferrin study described above, the results on IgG oligosaccharides from three different methods were comparable each other (Figure 2b). Typical mass spectra of permethylated oligosaccharides are shown in Figure 4.

Reproducibility of MALDI MS quantitation of permethylated oligosaccharides

Reproducibility of MALDI MS quantitation was evaluated by a supplementary experiment carried out in one laboratory (lab-17), where sample B (0.3 mg) was divided into three portions and each sample was separately subjected to permethylation followed by MS.

The coefficients of variation (CVs) of the five repeated measurements, or intra-assay CV, were less than 10% (1.3 – 8.8% for three major oligosaccharide species and 12 - 34% for the minor one (Table 3). The inter-assay CV was 0.1 - 4.2% for major species and 13% for the minor one. In this experiment, the sensitivity for small components was approximately 0.4% of their proportion among total oligosaccharides, when defined by the detection of ions with a signal-to-noise ratio of more than 2.

MS of glycopeptides

Nine laboratories performed MS of glycopeptides, and seven presented qualitative data or quantitative results for a single sample. Two laboratories presented sufficient, as well as remarkable, data with LC/ESI MS/MS (lab-15) or MALDI TOF MS (lab-17).

First, the difference between the oligosaccharide profiles at the two glycosylation sites of transferrin was revealed as shown in Figure 5a, where the levels of fucosylation and triantennary branching of transferrin were higher for the oligosaccharide attached to Asn-630 than for that attached to Asn-432, and this site-specific oligosaccharide profile difference was quite consistent between the results from these two laboratories. As shown by the MALDI TOF mass spectra in Figures 5b and 5c, the heterogeneity of oligosaccharide structures was more obvious at Asn-630, while a very low level of fucosylation could be identified in the Asn-432 oligosaccharides by tandem MS of the ion observed at *m/z* 3830 (data not shown). In addition, the level of fucosylated biantennary oligosaccharide exceeded that of the triantennary oligosaccharide, consistent with the chromatographic or mass spectrometric

analyses of the released oligosaccharides. On the other hand, sialic acid loss was observed in the glycopeptide ions generated by MALDI. Good reproducibility was reported by lab-17 (Figure 5a).

Serum IgG is polyclonal and is thus a mixture composed of different primary protein structures. The amino acid sequence of the tryptic peptide involving the *N*-glycosylation site Asn-297 is heterogeneous; and EEQYNSTYR and EEQFNSTFT representing two subclass molecules, IgG1 and IgG2, respectively, are abundant. In this study, three IgG samples (A, B and C) from different individuals were analyzed. The relative abundances of subclass molecules were estimated as 1/3, 1/4 and 2/1 (IgG1/IgG2), for samples A, B and C, respectively, based on the total intensities of the corresponding groups of ions in the MALDI linear TOF mass spectra of enriched glycopeptides (Supplementary Figure 1). The amino acid sequences of these glycopeptide ions were verified by tandem MS in a separate experiment (data not shown). The galactosylation levels of IgG1 or IgG2 for each sample were calculated from the corresponding signals observed in the LC/ESI or MALDI mass spectrum (Supplementary Figure 2a) and summarized in Table 4. The results from these independent analyses were consistent; *i.e.* the agalacto species was more abundant than the digalactosylated species in the IgG2 from samples A and B, but not in the IgG1 from sample C. The oligosaccharides on the major subclass molecules, IgG2 for samples A or B, and IgG1 for sample C, should contribute more to the global glycan profiles of total IgG in each sample. The results from the MS of IgG glycopeptides were quite consistent with the chromatographic or mass spectrometric measurements of the corresponding released oligosaccharides (Supplementary Figure 2b). Hypogalactosylation of IgG2 relative to IgG1 is not a common finding among different individuals (unpublished observation by YW).

The levels of minor glycans with bisecting GlcNAc in the major IgG subclass molecules were comparable between LC/ESI MS and MALDI MS (data not shown). The numbers of oligosaccharide structures identified as constituting more than 1% of the total oligosaccharides from sample B IgG were compared by different methods. Fifteen structures were seen by lab-1 using chromatography, nine and fourteen by lab-7 and lab-8, respectively, analyzing permethylated oligosaccharides with MALDI MS, fifteen by LC/ESI MS of glycopeptides and ten by MALDI TOF MS of glycopeptides (Supplementary Figure 3), indicating glycopeptide analysis to be sufficiently sensitive to detect minor glycans on IgG. No significant amounts of glycopeptides derived from other regions of IgG were found in these samples.

DISCUSSION

Oligosaccharides

The chromatographic quantitation of the reductively aminated oligosaccharides showed a significant variance among laboratories, despite the fact that the method has been accepted as established. Whether or not labeling efficiency is uniform for different glycan structures

and/or different fluorescent aromatic amines remains unclear, but the variance seems to be attributable to the use of different reaction protocols, which can result in incomplete derivatization. In addition, the insufficient separation of specific glycans, *e.g.* those with or without bisecting GlcNAc, resulted in a considerable variation in the quantitation (Figure 2b), while the chromatographic method discriminates isomers, *e.g.* monogalacto species bearing a galactose at either antenna in IgG.

It is generally accepted that MS does not allow real quantitation for oligosaccharides unless stable isotope-labeled analogues are incorporated as internal standards. In this multi-institutional study analyzing the same transferrin and IgG glycoprotein samples, however, MS has yielded quite comparable results with chromatography in the (relative) quantitation of oligosaccharides (Figures 1b and 2b). For MALDI, especially, the in-source and post-source decay of glycans are so well-known that one suspects it would be too difficult to detect their intact structures. Parameters such as laser wavelength and power, extraction voltages, and "hot" or "cool" matrices directly influence the internal energy of generated ions and the resulting fragmentation (Harvey, 1999). Furthermore, the distribution of molecular species in complex glycan mixtures within the matrix spot on the target depending on sample preparation is also a critical point and sufficient "averaging" of laser shots is essential. However, there are publications describing good quantitation of oligosaccharides based on the signal intensities observed in the MALDI mass spectrum (Harvey, 1993; Garozzo *et al.*, 1994). Indeed, a comparative study of the signal intensities generated from a mixture of equimolar amounts of various *N*-linked glycans did not show any significant difference for compounds with molecular masses over about 1 kDa (Naven and Harvey, 1996). In the present study, although different MALDI TOF instruments were used, the analytical parameters, such as the type of laser (nitrogen that emits at 337 nm), the choice of matrix (2,5-dihydroxybenzoic acid, DHB), the type of ion species for permethylated oligosaccharide detection (sodium-adduct ions $[M+Na]^+$) and the mode of TOF analysis (reflectron), were the same among the laboratories and yielded consistent results. In MALDI MS, the ions generated by hundreds of laser shots, each of which produces one mass spectrum, are accumulated from different points of laser irradiation. The procedure makes the reproducibility excellent in the repeated measurements by lab-12 and in the supplemental experiment, and the inter-assay reproducibility in the same laboratory was also quite good (Table 2).

Regarding the determination of sialylation levels, the glycosidic linkage of sialic acid is susceptible to prompt (in-source) decay in the MALDI process, and the sialic acid-substituted oligosaccharides are likely to decompose in the flight tube in the TOF instrument as well. In addition, the number of sialic acids affects the relative ionization efficiencies (Sutton et al., 1994). Consequently, the signal intensity of underivatized sialylated oligosaccharides in the MALDI mass spectrum cannot be directly related to concentration as shown by the results obtained by lab-13 for oligosaccharides only derivatized at the reducing end by aminopyridine (Figure 1). Permethylation, which has been used for more than 40 years in order to modify

hydroxyl groups of sugars (Hakomori, 1964; Ciucanu and Kerek, 1984; Ciucanu and Cotello, 2003), methylates the carboxylic acid group of the sialic acid to produce a neutral sugar, whereupon esterified sialic acid loss is avoided and the ionization efficiency becomes equivalent to that of natively neutral oligosaccharides. In addition, this type of derivatization prevents salt formation which complicates the mass spectrum and impairs the signal-to-noise ratio for the individual molecular ion species. Furthermore, the lack of hydroxyl groups also prevents the cleavage of other glycosidic bonds (Lemoine *et al.*, 1996), making the permethylated oligosaccharides resistant to in-source fragmentation. In fact, the sialylation levels determined by MALDI MS after permethylation were acceptable, although a variance exists among laboratories as was the case for chromatography. A special setup of derivatization required a smaller amount of samples (5 μg glycoprotein) to meet the high sensitivity of MALDI MS for permethylated oligosaccharides (Kang *et al.*, 2005), while routine analysis used ~100 μg in this study.

The levels of triantennary oligosaccharides determined by MALDI MS were lower than those from chromatography, though not markedly. It is not possible to decide which value is the correct one, but the decreased triantennary structures seen by MS may be due to the increased mass of 810.4 Da of an additional antenna, making the detector response for the triantennary oligosaccharide ions at *m/z* 3602.8 weaker than that for the biantennary ions at *m/z* 2792.4, or there may be increased collision-induced dissociation (CID) of an enlarged cross-section of structure. The peak broadening effect due to isotopes is only 17% for this mass increase and thus does not produce a substantial error even when the quantitation is based on the peak height in the MALDI mass spectrum. On the other hand, core fucosylation, which occurs at the 6-position of the reducing terminal GlcNAc in the transferrin oligosaccharides, does not affect quantitation by MALDI MS (Naven and Harvey, 1996). It is worth noticing that the level of the triantennary oligosaccharide was lower than that of the fucosylated species in measurements with either MALDI MS or chromatography, indicating that MALDI MS is similar in its ability to detect minor glycans. Similarly, good relative quantitation by MALDI MS was confirmed by IgG analysis, as the monogalactosylated species was the most abundant and the calculated level of galactosylation was comparable to that obtained by chromatography.

ESI MS was employed by two laboratories, where oligosaccharide alditols were separated by graphitized carbon chromatography and introduced on-line to an ESI mass spectrometer operated in the negative ion mode (Karlsson *et al.*, 2004). The reproducibility of LC/ESI MS was acceptable from the data of lab-16. Regarding sialylated oligosaccharides, ESI is soft enough to detect the intact oligosaccharides with sialylation unless high nozzle-skimmer potential is applied, even when sialic acids are not derivatized. However, the charge of sialic acids affects the efficiency of negative ion formation, and thus the distribution of multiply-charged ions in the mass spectrum is different from that for neutral oligosaccharides, disturbing straightforward quantitation of sialic acid-substituted

oligosaccharides in the mass spectrum or ion chromatogram at the same time as neutral glycans. Nevertheless, LC/ESI MS results were comparable to those from MALDI MS or chromatography as shown in Figures 1b and 2b, except that an increased level of monosialylated oligosaccharides from the transferrin sample was reported by one laboratory, possibly due to sialic acid loss during sample preparation. A brief summary of the methods for oligosaccharide analysis including capillary electrophoresis-MS (Zamfir *et al.*, 2000) is presented in Table 5.

In this study, some laboratories also presented MS/MS spectra of permethylated or reductively aminated oligosaccharides. For example, the product ion mass spectra provided branching and linkage information (Supplementary Figure 4). This method is used for structure verification, qualitatively, and is not primarily directed toward quantitation. However, along with the establishment of the strategies for *de novo* carbohydrate sequencing by an algorithm incorporating the fragmentation database and informatics (Ashline *et al.*, 2005; Tang *et al.*, 2005), quantitation of oligosaccharide isomers will be realized in the near future.

Glycopeptides

Glycopeptide analysis allows identification of site-specific oligosaccharide structures which are essential to understanding the role of local oligosaccharide structures in protein folding and functions. However, it is conceivable that the analysis of glycopeptides is difficult as compared to that of unglycosylated peptides or oligosaccharides, since chemical properties differ between the glycan and peptide components. Moreover, each glycopeptide is often a minor constituent in the peptide mixtures after enzymatic digestion of glycoproteins because of the large number of glycoforms expressed by many proteins at each site. These problems may be overcome by employing LC/MS and selecting the glycopeptides in the chromatogram by detection of the CID-generated glycan-specific oxonium ions (Huddleston *et al.*, 1993). Alternatively, glycopeptide fraction can be enriched by lectin or other extraction tools, and the fraction can be directly analyzed by MS, especially when the glycoprotein has a small number of glycosylation sites, as is the case for transferrin and IgG (Wada *et al.*, 2004). The glycan and peptide structures of glycoproteins can then be elucidated by MS/MS of glycopeptide ions (Liu *et al.*, 1993; Krokhin *et al.*, 2004).

The MS of glycopeptides is usually performed in the positive ion mode to detect $[M+H]^+$ ions. In this case, protonation occurs on the peptide portion, and thus the signal intensity of the glycopeptide is largely dependent on the proton affinity of the peptide component of the molecule, thus rationalizing the quantitation of different glycoforms of a peptide according to their signal intensities. However, there are still a few concerns. Firstly, glycopeptides are larger than oligosaccharides and thus are accompanied by an increased risk of CID. Since the glycan moiety is more labile than the peptide backbone, the resulting cleavage of the glycosidic bonds may result in overestimation of the glycoforms with a smaller number of

building saccharides. This effect will be evident in the MALDI produced ions in which internal energies are higher than those of ESI ions. In previous reports, however, neutral oligosaccharides had minimal affect upon the ionization efficiencies of glycopeptides, and consequently, the integration of MALDI TOF signals of several desialylated glycopeptides yielded excellent quantitative correlations with published data obtained by established HPLC techniques (Sutton *et al.*, 1994; Harmon *et al.*, 1996). Secondly, the sialic acids of glycopeptides cannot be appropriately derivatized without deleterious modifications of the peptide component. In MALDI MS, the sialic acid loss occurs both in-source and post-source as described above, and occurs over a significant time frame in which a hydrogen transfers between suitably positioned functional groups (Harvey, 1999). Consequently, the sialic acid loss increases with the longer delay time before extraction (Naven *et al.*, 1997); and is prominent in the MALDI ion-trap type of mass separators. Most studies of sialylated glycopeptides with MALDI TOF MS are conducted in the linear mode so that the postsource decay ions are not separated. On the other hand, the sialic acid loss is minimal in ESI, but there are charge-dependent effects on the ionization efficiency and on the distribution of multiply-charged ions of the oligosaccharides.

Despite these problems, the site-specific analysis of glycoprotein oligosaccharides by MS is becoming popular. In the present study, the results from two laboratories on glycopeptides, one with MALDI MS and another with LC/MS, were consistent with each other, and were informative in terms of the oligosaccharide profiles specific to the protein sequence. The galactosylation levels at different polymorphic sequences of IgG explained well the global glycan profiles determined by the analyses of released oligosaccharides, and the oligosaccharide profiles of two glycosylation sites of transferrin were clearly shown to be different from each other. Although these findings could be obtained by isolation of each glycopeptide and subsequent analysis of released oligosaccharides, the direct analysis of glycopeptides performed herein is by far easier and more rapid as well as being sufficiently sensitive for minor glycans. The glycopeptide analysis requires a fairly small amount of samples (less than 10 μg glycoprotein in this study), and thus will contribute a great deal to the emerging field of glycoproteomics.

In conclusion, MALDI MS of permethylated oligosaccharides is as reliable as chromatographic methods for elucidating glycan profiles based on mass mapping of the compositional analysis. The publicly available murine and human MALDI MS glycomics data, which is being acquired by the Consortium for Functional Glycomics (www.functionalglycomics.org), for systems biology research are based on this approach. Good relative quantitation data can be achieved without neutralization of sialic acid residues by LC/ESI MS utilizing porous graphitized carbon as a separation medium for oligosaccharide alditols. Quantitative and relative quantitative datasets can be obtained by peptide sequencing and determination of glycosylation sites by LC-MS/MS of native

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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