analytical chemistry

Article

pubs.acs.org/ac

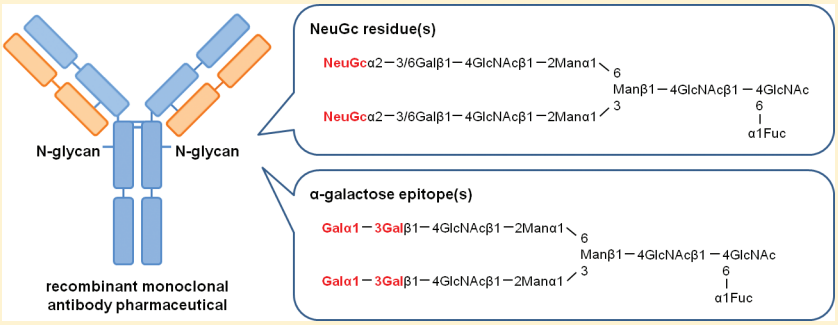
Analysis of Nonhuman *N*-Glycans as the Minor Constituents in Recombinant Monoclonal Antibody Pharmaceuticals

Eiki Maeda,[†] Soichiro Kita,[‡] Mitsuhiro Kinoshita,[‡] Koji Urakami,[†] Takao Hayakawa,[§] and Kazuaki Kakehi*[,‡]

[†]Analytical Development Laboratories, CMC Center, Takeda Pharmaceutical Company Limited, Jusohonmachi 2-17-85, Yodogawa-ku, Osaka 532-8686, Japan

[‡]Faculty of Pharmaceutical Sciences, and [§]Pharmaceutical Research and Technology Institute, Kinki University, Kowakae 3-4-1, Higashi-Osaka 577-8502, Japan

Supporting Information



ABSTRACT: Minor N-linked glycans containing *N*-glycolylneuraminic acid residues and/or α-Gal epitopes (i.e., galactose-α1,3-galactose residues) have been reported to be present in recombinant monoclonal antibody (mAb) therapeutics. These contaminations are due to their production processes using nonhuman mammalian cell lines in culture media containing animal-derived materials. In case of the treatment of tumors, we inevitably use such mAbs by careful risk−benefit considerations to prolong patients' lives. However, expanding their clinical applications such as for rheumatism, asthma, and analgesia demands more careful evaluation of the product characteristics. The present work for detailed evaluations of *N*-glycans demonstrates the methods using capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) and a combination of high-performance liquid chromatography and electrospray ionization time-of-flight mass spectrometry. The CE-LIF method provides excellent separation of both major and minor *N*-glycans from six commercial mAb pharmaceuticals within 30 min and clearly indicates that a possible trigger of immunogenicity in humans due to the presence of nonhuman *N*-glycans is present. We strongly believe that the proposed method will be a powerful tool for the analysis of *N*-glycans of recombinant mAb products in various development stages, such as clone selection, process control, and routine release testing to ensure safety and efficacy of the products.

Therapeutic recombinant monoclonal antibodies (mAbs) normally contain several N-linked glycans at asparagine residues on heavy chains.[1] The attached *N*-glycans play important biological and physicochemical roles such as resistance against protease, elongation of circulatory half-life in vivo, and antibody-dependent cellular cytotoxicity.[2−6] Although the attached *N*-glycans are mostly biantennary and core-fucosylated complex-type, their composition usually varies with the changes in manufacturing process conditions even if the same cell line is used.[7−10] In order to ensure quality of the mAb pharmaceuticals, all the *N*-glycans have to be analyzed as a critical parameter for product characterization and lot-to-lot consistency assessment.

Determination of nonhuman *N*-glycans that are not present in humans is especially important, because this will be a big problem in relation to immunogenicity and possible factor for incidence of some diseases, and possible masking of existing antigenic sites on the peptide backbone causes a crucial side effect or insufficient efficacy.[11] Recently, some important research works on the presence of nonhuman *N*-glycans in mAb products were reported. The one is galactose-α1,3-galactose (known as α-Gal epitope) attached to the non-reducing terminal of *N*-glycans. Chung et al. reported that cetuximab, a chimeric mouse−human IgG_1 mAb against the epidermal growth factor receptor produced in murine myeloma SP2/0 cell line, induced hypersensitivity reactions to subjects having IgE antibodies specific for α-Gal epitope on the Fab portion of the cetuximab heavy chain.[12] This epitope is not biosynthesized in old world monkeys, apes, humans, and CHO

Received: November 24, 2011
Accepted: February 13, 2012
Published: February 13, 2012

 © 2012 American Chemical Society dx.doi.org/10.1021/ac300234a | *Anal. Chem.* 2012, 84, 2373−2379

cells because of inactivation of the gene coding for α-1,3-galactosyltransferase (EC 2.4.1.151).[12−15] Other isotypes against the same epitope are also reported, such as IgG antibodies in serum (approximately 20−100 μg/mL) and also IgA antibodies in body secretions from saliva, milk, and colostrum.[16−18] It is now recognized that the epitope is one of the major barriers in the transplantation of organs from other mammals to humans because of inducing complement-mediated destruction and antibody-dependent cell-mediated destruction.[19,20] The other is the presence of *N*-glycolylneuraminic acid (NeuGc) residues attached to the nonreducing terminal of *N*-glycans. NeuGc cannot be synthesized in humans either, because of an irreversible mutation of the human gene *CMAH*, encoding CMP-*N*-acetylneuraminic acid hydroxylase, which is responsible for producing CMP-*N*-glycolylneuraminic acid from CMP-*N*-acetylneuraminic acid.[21,22] NeuGc exists in almost all animal cells but is not found in human tissues.[23] In several controversial reports published previously, small amounts of NeuGc have been claimed to exist in fetal human tissue, certain human tumors, and human tumor cell lines.[24,25] Normal humans have variable amounts of circulating IgA, IgM, and IgG antibodies against Neu5Gc.[26,27] Recently, Ghaderi et al. demonstrated the presence of covalently bound NeuGc in a therapeutic mAb, cetuximab, but not in panitumumab. Anti-NeuGc antibodies from healthy humans interacted with cetuximab in a NeuGc-specific manner and generated immune complexes in vitro. In addition, mice having a human-like defect in NeuGc synthesis generated antibodies to NeuGc after injection of cetuximab, and circulating anti-NeuGc antibodies can promote drug clearance.[28] These findings relevant to nonhuman *N*-glycans containing α-Gal epitopes and NeuGc residues are considered as warnings against pharmaceutical companies which have been developing therapeutic mAb pharmaceuticals.

The combination of high-performance liquid chromatography (HPLC) coupled with mass spectrometry and endoglycosidase digestion provides sufficient resolution and sensitivity for *N*-glycans.[29−34] In addition, capillary electrophoresis with laser-induced fluorescence detection (CE-LIF) has also been used for profiling of the fluorescently labeled *N*-glycans because of its throughput and high-resolution separation capability.[7,8,33−38] Chen et al. reported structural analysis of *N*-glycans of ribonuclease B, fetuin, and erythropoietin by CE-LIF after fluorescent labeling with 8-aminopyren-1,3,6-trisulfonate (APTS).[33] APTS-labeled *N*-glycans of mAbs, namely, asialo 0, 1, or 2 galactosylated biantennary complex-type *N*-glycans, were also reported.[34,36] Not only the separation methodologies described above, but also the labeling strategies of glycans for introducing a chromophore or fluorophore to the glycans with recent developments and advances of the glycan analysis, have also been published.[39−41] In the previous study, we developed an *N*-glycan profiling method for therapeutic mAb products using CE-LIF with 2-aminobenzoic acid (2-AA) as a fluorescent tag.[8] By comparison of migration times with those of 2-AA-derivatized *N*-glycans of which structures had been determined by a combination of HPLC and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS), all the *N*-glycans including minor species from rituximab were identified.

In the present study, we determined nonhuman *N*-glycans, the possible antigenic carbohydrate epitopes like α-Gal and NeuGc, in commercially available mAb pharmaceuticals in a detailed structural and quantitative manner using CE-LIF and a combination of HPLC and high-resolution time-of-flight mass spectrometry equipped with an electrospray ionization source and hybrid ion trap (LC/ESI-IT-TOF MS). To determine the interproduct variety of their minor *N*-glycans, chimeric Fab−human IgG_1 (cetuximab and infliximab), mouse CDR−human IgG_1 and IgG_4 (tocilizumab, bevacizumab, and gemtuzumab ozogamicin), and human IgG_1 (adalimumab) were selected and investigated. Furthermore, a lot-to-lot repetitive analysis was performed using three different lots of gemtuzumab ozogamicin in order to evaluate intraproduct variation. From the results obtained in this study, we strongly suggest that careful considerations are essentially required for assuring safety and efficacy of the therapeutic recombinant mAb products even if they are fully humanized proteins.

EXPERIMENTAL SECTION

Materials. Therapeutic mAb pharmaceuticals, cetuximab, infliximab, gemtuzumab ozogamicin, tocilizumab, bevacizumab, and adalimumab were donated from Kinki University Nara Hospital. γ-Globulins (human: from Cohn fraction II, III) as a human IgG antibody were purchased from Sigma (St. Louis, MO, U.S.A.). Each mAb preparation was dialyzed against distilled water for 3 days, with changing water several times at 4 °C using cellulose membrane tubing (Sanko Junyaku, Chiyoda-ku, Tokyo, Japan), and then freeze-dried. Other reagents and solvents are described in the Supporting Information.

Preparation of 2-AA-Labeled *N*-Glycans. The released *N*-glycans mixture was labeled with 2-AA according to the method reported previously.[8] After the purification of 2-AA-labeled *N*-glycans by a Sephadex LH-20 column, the collected fluorescent fraction was dried and redissolved in water (100 μL), and a portion were used for further analyses (procedures are described in the Supporting Information).

Structural Determination of the Fractionated *N*-Glycans by LC/ESI-IT-TOF MS. Structures of the *N*-glycans were analyzed by LC/ESI-IT-TOF MS (Shimadzu, Kyoto, Japan). Liquid chromatography was conducted with a CBM-20A system controller, two LC-20AD pumps, an SIL-20AC autosampler, and a CTO-20A column oven (Shimadzu). Chromatographic separation was done with a $5C_{18}$-PAQ (2.0 mm i.d. × 150 mm length, Nacalai Tesque, Kyoto, Japan) at 45 °C using a linear gradient formed by 5% acetonitrile containing 0.1% formic acid (solvent A) and 95% acetonitrile containing 0.1% formic acid (solvent B) at 0.2 mL/min. The column was initially equilibrated and eluted with 97% solvent A for 2 min, from which point solvent B was increased to 25% over 20 min and kept at this composition for additional 5 min. LC/ESI-IT-TOF MS was operated in data-dependent tandem mass spectrometry (MS/MS) mode. The curved desolvation line temperature, heat block temperature, detection voltage, and nebulizer gas flow rate were set at 200 °C, 200 °C, 1.7 kV, and 1.5 L/min, respectively. Data were collected for 80 ms for MS mode and 150 ms for MS/MS mode.

Tandem Mass Spectrometry Analysis for α-Gal-Containing *N*-Glycans by MALDI-QIT-TOF MS. For confirmation of the *N*-glycan structure containing α-Gal epitope, tandem mass analyses were conducted for several *N*-glycans by MALDI-quadrupole ion trap (QIT)-TOF mass spectrometry. MALDI-QIT-TOF mass spectra were acquired on an AXIMA-QIT-TOF mass spectrometer (Shimadzu). A nitrogen laser was used to irradiate samples, and an average shot of 50 times was taken. Argon was used for collision-

dx.doi.org/10.1021/ac300234a | *Anal. Chem.* 2012, 84, 2373−2379

induced dissociation. The instrument was operated in positive and reflectron mode. An aqueous sample solution (2 μL) was mixed with a matrix solution (2 μL) of 1% DHB in ethanol/water (1:1), and the mixture was applied to a polished stainless-steel target and dried in atmosphere for a few hours.

CE-LIF of 2-AA-Labeled *N*-Glycans. CE-LIF was performed on a P/ACE MDQ system (Beckman Coulter, Fullerton, CA, U.S.A.) equipped with a He−Cd laser-induced fluorescence detector (ex 325 nm, em 405 nm) using a DB-1 capillary (100 μm i.d., 30 cm effective length, 40 cm total length, Agilent/J&D Scientific, Palo Alto, CA, U.S.A.) in 100 mM Tris−borate buffer (pH 8.3) containing 5% poly(ethylene glycol) as a running buffer. Poly(ethylene glycol) was added to diminish electroosmotic flow and improve the resolution. For pressure injection, sample solutions were introduced to the capillary at 1 psi for 10 s. Separation was performed at 25 kV of applied voltage at 25 °C in reverse polarity.

RESULTS AND DISCUSSION

Structural Analysis of 2-AA-Labeled *N*-Glycans by LC/ESI-IT-TOF MS and MALDI-QIT-TOF MS. The 2-AA-labeled *N*-glycans from six recombinant mAb products and a human IgG antibody were analyzed by HPLC (Figure 1), and the assigned structures of the *N*-glycans from each peak by LC/ESI-IT-TOF MS are listed on each chromatogram and summarized in Table S-1 in the Supporting Information. In total, 46 *N*-glycans were determined from the tested products. Biantennary *N*-glycans **3**, **4**, and **6** were commonly detected as major *N*-glycans in all the products, and their singly charged deprotonated molecules $[M - H]^-$ in gemtuzumab ozogamicin were observed at *m/z* 1582.6, 1744.6, and 1906.7, respectively (Table S-1 in the Supporting Information). Although agalactosylated biantennary *N*-glycan **3** was commonly observed abundantly in all products, mono- and digalactosylated biantennary *N*-glycans **4** and **6** were significantly low in bevacizumab (Figure 1c) and adalimumab (Figure 1f). In addition, the amount of digalactosylated biantennary *N*-glycan **6** in tocilizumab (Figure 1b) and cetuximab (Figure 1d) was much smaller than other mAb products, although they contained a similar amount of monogalactosylated biantennary *N*-glycan **4**. These results indicate that the variety of major *N*-glycans is remotely related to the humanization levels of mAb products.

For minor *N*-glycans (see also the enlarged chromatograms in Figure 1), tocilizumab (Figure 1b) and adalimumab (Figure 1f) contained relatively larger amounts of high-mannose-type *N*-glycans **13**−**17**, and cetuximab (Figure 1d) and infliximab (Figure 1e) contained larger amounts of hybrid-type *N*-glycans **18**−**23**, respectively. Sialylated *N*-glycans **24**−**45** were commonly observed in all mAb products. Interestingly, it was revealed that the major sialylated *N*-glycans of three mAb pharmaceuticals [gemtuzumab ozogamicin (Figure 1a), cetuximab (Figure 1d), and infliximab (Figure 1e)] were occupied with NeuGc residue **33**−**41**, **45** as identified from the results of LC/ESI-IT-TOF MS (Figure 2). Two doubly charged deprotonated molecules $[M - 2H]^{2-}$ at *m/z* 1098.3 (Figure 2a) and *m/z* 1106.3 (Figure 2b) due to *N*-glycans **26** and **36** were observed in gemtuzumab ozogamicin. Both molecules provided the same singly charged deprotonated molecule $[M - H]^-$ corresponding to digalactosylated biantennary *N*-glycan **6** in their MS/MS spectra (Figure S-1, parts a and b, in the Supporting Information). The calculated mass difference between *N*-glycans **26** and **36** was 16.0, and this clearly

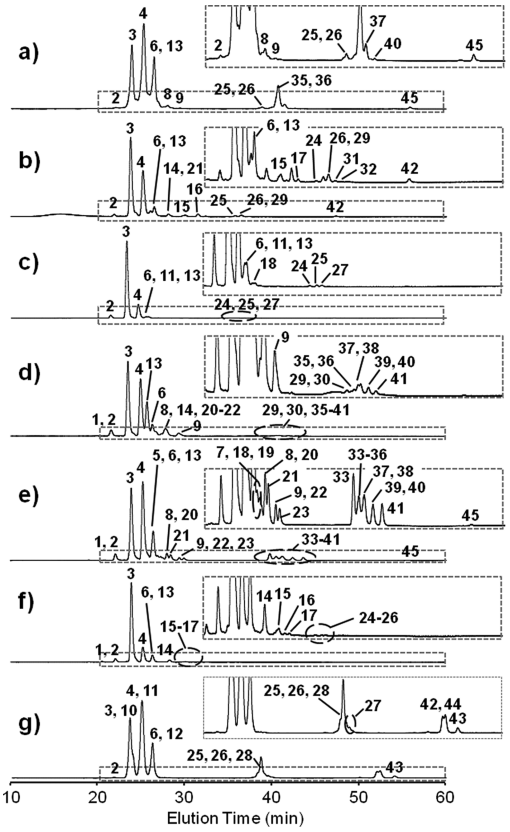


Figure 1. Analysis of 2-AA-labeled *N*-glycans by HPLC: (a) gemtuzumab ozogamicin, (b) tocilizumab, (c) bevacizumab, (d) cetuximab, (e) infliximab, (f) adalimumab, and (g) human IgG antibody. The numbers represent the *N*-glycans observed in each peak, and their structures are summarized in Table S-1 in the Supporting Information. In the box, the region between 20 and 60 min is enlarged. Analytical conditions: column, Asahi Shodex NH2P-50 4E (4.6 mm i.d. × 250 mm length); eluent, solvent A, 2% acetic acid in acetonitrile, solvent B, 5% acetic acid containing 3% triethylamine in water; gradient condition, a linear gradient (30−95% solvent B) from 2 to 82 min, keep for 15 min (95% solvent B); detection, ex 350 nm, em 425 nm.

indicated the presence of NeuGc in gemtuzumab ozogamicin. Not only gemtuzumab ozogamicin manufactured in NS0 murine myeloma cell line, but chimeric antibodies, infliximab and cetuximab, produced in murine myeloma SP2/0 cell lines, also contained NeuGc residues instead of NeuAc. The results are consistent with both the results of sialic acid analysis (see the Supporting Information) and the previous report that cetuximab has NeuGc residues at the nonreducing terminal of *N*-glycans on its Fab region.[28] In contrast, human IgG antibody and other three therapeutic mAbs manufactured in CHO cell lines, tocilizumab, bevacizumab, and adalimumab, did not contain NeuGc-containing *N*-glycans.

Two peaks (**8** and **9**) observed at 28.0 and 29.4 min in gemtuzumab ozogamicin (Figure 1a) provided doubly charged deprotonated molecules $[M - 2H]^{2-}$ at *m/z* 1033.9 (Figure 2c) and *m/z* 1115.3 (Figure 2d), respectively. The structures of these peaks were easily assigned as follows. The mass differences (Δ162.2 and Δ324.1) from digalactosylated biantennary *N*-glycan **6** indicated the presence of additional hexose molecule(s) compared to the *N*-glycan **6**. This means that one or two molecules of galactose attach to the nonreducing ends of *N*-glycan **6** to form one or two α-Gal

dx.doi.org/10.1021/ac300234a | *Anal. Chem.* 2012, 84, 2373−2379

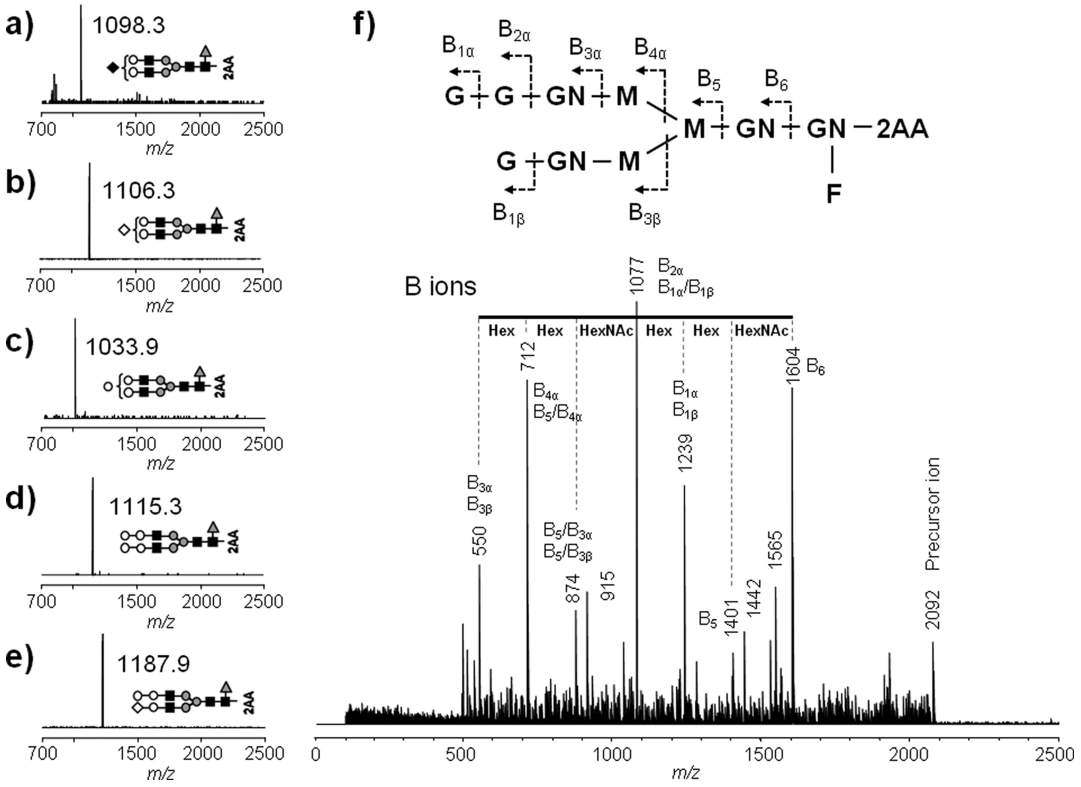


Figure 2. Negative MS spectra and positive MS/MS spectrum of 2-AA-labeled *N*-glycans from gemtuzumab ozogamicin: (a) *N*-glycan **26** containing one NeuAc residue, (b) *N*-glycan **36** containing one NeuGc residue, (c) *N*-glycan **8** containing one α-Gal epitope, (d) *N*-glycan **9** containing two α-Gal epitopes, (e) *N*-glycan **37** containing one NeuGc residue and one α-Gal epitope, and (f) *N*-glycan **8**. The dotted lines on the MS/MS spectrum indicate the type of cleavage according to Domon and Costello's nomenclature (ref 45).

epitopes. In addition, the peak detected at 41.4 min (Figure 1a) showed doubly charged deprotonated molecules $[M - 2H]^{2-}$ at *m/z* 1187.9 (Figure 2e) due to *N*-glycan **37**, and the ion provided singly charged ion $[M - H]^-$ at *m/z* 2069.8 (Figure S-1c in the Supporting Information) corresponding to *N*-glycan **8** by MS/MS analysis. These galactose residues at the nonreducing ends of *N*-glycans were specifically released by enzymatic digestion with α-galactosidase (data not shown). In addition, MS/MS analysis by MALDI-QIT-TOF MS demonstrated the presence of α-Gal epitope in its structure. Figure 2f shows the positive MS/MS spectrum of *N*-glycan **8** corresponding to the peak observed at 28.0 min in Figure 1a. The positive MS/MS spectrum derived from precursor ion $[M + Na]^+$ (*m/z* 2092.0) was mainly consisted by B-type fragment ions. The characteristic B ions at *m/z* 1401.0 (B5) and 1604.0 (B6) corresponding to a loss of 691 and 488 from $[M + Na]^+$ were observed. These mass differences corresponded to a loss of two *N*-acetylhexosamine (HexNAc) residues having a fucose residue with 2-AA and a HexNAc residue having a fucose residue with 2-AA, respectively. This result indicates that the B5 ion at 1401.0 contains three mannose and two HexNAc residues with additional three residues of hexose at the nonreducing end of *N*-glycan. The same fragment ions were observed in other mAb products as well (data not shown). These results obtained by MALDI-QIT-TOF MS obviously show the presence of α-Gal epitope in *N*-glycan **8**.

From the results described above, gemtuzumab ozogamicin contains nonhuman *N*-glycan having α-Gal epitope in its structure. Cetuximab and infliximab also contained these *N*-glycans as well (see Table S-1 in the Supporting Information), and both mAb products induced anaphylaxis mediated by IgE antibody which binds to α-Gal epitope specifically.[12,42] For infliximab, the mechanism of the anaphylaxis is under discussion, but the obtained results indicate that the presence of α-Gal epitope seems to be one of the root causes of the anaphylactic response.

The combination of HPLC and MS provides lots of structural information concerning attached *N*-glycans having NeuGc residues and α-Gal epitopes. However, due to incomplete resolution of the peaks as shown in Figure 1, detailed quantitative analysis of the *N*-glycans is a hard work. For example, two *N*-glycans, digalactosylated biantennary *N*-glycan **6** and high-mannose-type *N*-glycan **13**, which were supposed to be present in almost all mAb products, were observed at the same elution time.

CE-LIF Analysis of 2-AA-Labeled *N*-Glycans. 2-AA-labeled *N*-glycans from mAb products were analyzed by CE-LIF according to the method reported previously (Figure 3).[8] All peaks observed in each electropherogram were assigned using HPLC eluents by comparing migration times. CE-LIF provides much higher resolution than HPLC, and the *N*-glycans were observed in the order of disialo-, monosialo-, high-mannose-type, hybrid-type, and biantennary complex-type asialo-*N*-glycans. It should be noted that the *N*-glycans having the same molecular weight such as monogalactosylated biantennary complex-type *N*-glycans **4** were resolved into two peaks as indicated by asterisks in Figure 3a (at 22.3 and 22.6 min, respectively) depending on the linkage position of galactose residues at the nonreducing end. In addition, as shown in Figure 3b, digalactosylated biantennary complex-type *N*-glycan **6** and high-mannose-type *N*-glycan **13**, which could not be separated by HPLC, were completely resolved, and

dx.doi.org/10.1021/ac300234a | *Anal. Chem.* 2012, 84, 2373−2379

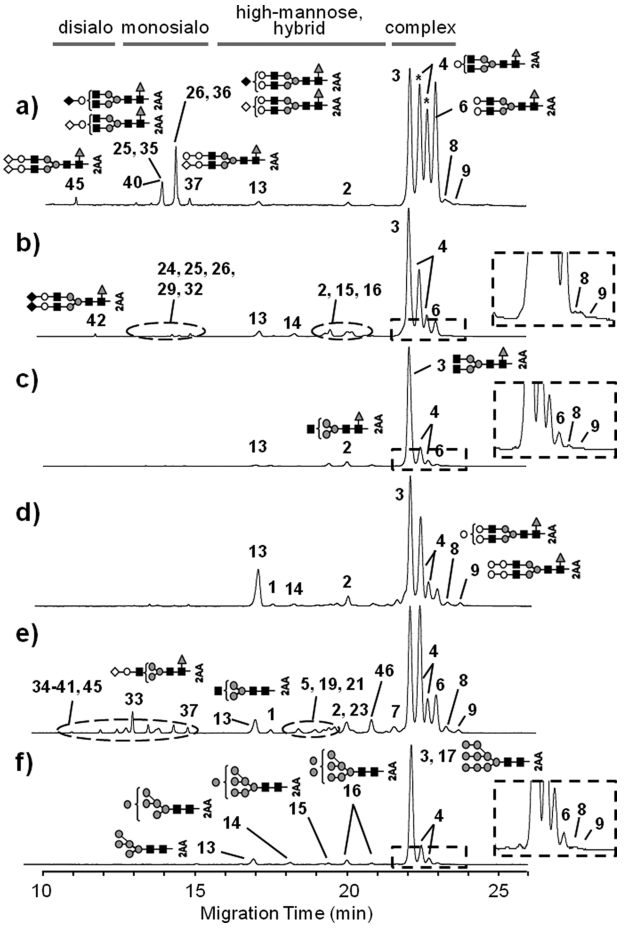


Figure 3. CE-LIF analysis of 2-AA-labeled *N*-glycans: (a) gemtuzumab ozogamicin, (b) tocilizumab, (c) bevacizumab, (d) cetuximab, (e) infliximab, and (f) adalimumab. The numbers represent the *N*-glycans observed in each peak, and their structures are summarized in Table S-1 in the Supporting Information. In the box, the region between 21.5 and 24.0 min is enlarged. Analytical conditions: capillary, DB-1 capillary (100 μm i.d., 30 cm effective length, 40 cm total length); running buffer, 100 mM Tris−borate buffer (pH 8.3) containing 5% PEG70000; injection, 1 psi for 10 s; applied voltage, −25 kV; temperature, 25 °C; detection, ex 325 nm, em 405 nm.

observed at quite different migration times. These results definitely demonstrate that CE-LIF is much more suitable for *N*-glycan profiling than HPLC because of its speed, throughput, and resolution.

From the electropherogram of gemtuzumab ozogamicin, two characteristic peaks were observed at 23.1 and 23.5 min (**8** and **9**, respectively) after four major peaks around 22 min (Figure 3a). These two peaks were observed in all the tested products but not observed in the previous study.[8] By peak assignment using fractionated HPLC eluents of gemtuzumab ozogamicin, these two peaks were identified as *N*-glycans having one and two α-Gal epitopes, respectively (Figure S-3 in the Supporting Information). In the same manner, biantennary complex-type *N*-glycans having one or two NeuGc or NeuAc residues at the nonreducing end (*N*-glycans **25**, **26**, **35**, **36**, and **45**) and hybrid-type *N*-glycan **40** containing a NeuGc residue at the nonreducing ends were also identified (Figure S-3 in the Supporting Information). However, complete separation between *N*-glycans **25** and **35** was not achieved even if using CE-LIF, because their structural difference is only the attached sialic acid types at the nonreducing end, namely, NeuGc or NeuAc. For the same reason, *N*-glycans **26** and **36** were not resolved from each other. For distinguishing terminal sialic acid species, both commonly used separation techniques in the pharmaceutical industry,[43] i.e., HPLC and CE, could not provide a complete separation because *N*-glycans usually contain six or more monosaccharides and the structural difference between NeuGc and NeuAc is much smaller (Δ16.0) than those of total masses. Terminal sialic acid modification could be alternatively accessed by sialic acid analysis, and the dominant types were over 93% as described in the Supporting Information. Therefore, we believe that current CE-LIF method is quite useful for rapid and extensive evaluation of nonhuman *N*-glycans of mAb pharmaceuticals.

The peak identification of nonhuman *N*-glycans containing NeuGc (**33**−**41** and **45**) at the nonreducing end from infliximab was also conducted by using fractionated eluents by HPLC (Figure S-4 in the Supporting Information). All the *N*-glycans having NeuGc (summarized in Table S-1 in the Supporting Information) were detected from infliximab, although one of them (*N*-glycan **39**) could not be observed by LC/ESI-IT-TOF MS. Separation between hybrid-type *N*-glycans **40** and monogalactosylated biantennary complex-type *N*-glycans **35** was achieved. The results obtained by CE-LIF indicate that the CE-LIF method affords good separation of over 40 *N*-glycans within 30 min, and the method can be used as a rapid *N*-glycan profiling in several development stages of therapeutic recombinant mAbs for monitoring and controlling product consistency as well as assuring safety and efficacy of the products.

Quantitative CE-LIF Analysis of 2-AA-Labeled *N*-Glycans. The percent compositions of detected *N*-glycans in each mAb product are summarized in Table 1. Three major *N*-glycans, **3**, **4**, and **6**, in gemtuzumab ozogamicin, tocilizumab,

Table 1. Quantitative Analysis of Attached *N*-Glycans in Recombinant mAb Products

	sialo- (%)			complex-type (%)					
product[a]	type	di-	mono-	**3**	**4**	**4**	**6**	**8, 9**	others (%)[b]
gem	NeuGc	0.4	8.9	23.9	23.1	17.5	22.1	2.3	1.8
toc	NeuAc	0.3	1.7	47.8	24.2	7.6	5.9	0.8	11.6
bev	NeuAc	0.0	0.3	75.5	12.7	3.7	1.3	1.7	6.1
cet	NeuGc	0.1	1.0	37.5	26.1	7.0	5.5	2.3	20.6
inf	NeuGc	0.1	7.4	27.6	28.7	7.9	9.0	2.4	17.1
ada	NeuAc	0.0	0.4	71.2	11.9	4.0	1.1	0.3	10.9
hIgG	NeuAc	0.6	11.1	37.8	22.7	10.9	14.0	0.0	2.9

[a]Symbols: gem, gemtuzumab ozogamicin; toc, tocilizumab; bev, bevacizumab; cet, cetuximab; inf, infliximab; ada, adalimumab; hIgG, human IgG antibody. [b]High-mannose-type and hybrid-type *N*-glycans.

dx.doi.org/10.1021/ac300234a | *Anal. Chem.* 2012, 84, 2373−2379

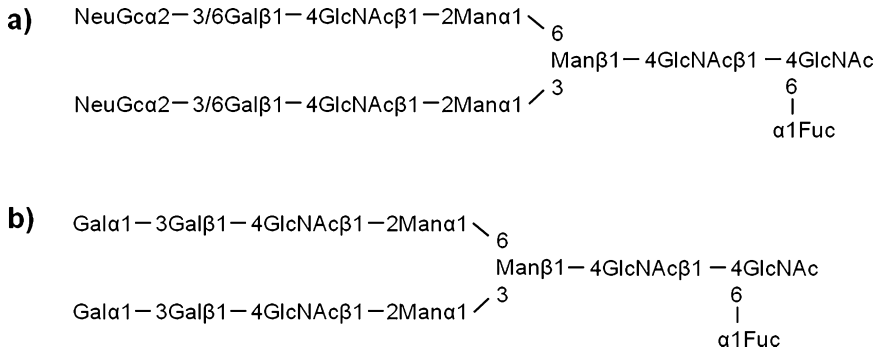


Figure 4. Representative scheme of nonhuman *N*-glycans containing NeuGc residues (a) and α-Gal epitopes (b).

bevacizumab, cetuximab, infliximab, adalimumab, and human IgG antibody (as the standard) occupied 86.6%, 85.5%, 93.2%, 76.1%, 73.2%, 88.2%, and 85.4%, respectively. For agalactosylated biantennary *N*-glycan **3**, the range of the compositions was varied from 23.9% to 75.5%. For the two peaks due to monogalactosylated biantennary complex-type *N*-glycans **4** which could be separated into two peaks, the earlier detected molecule showed a higher percent composition than the latter one in all the products. For example, these were 23.1% and 17.5% in gemtuzumab ozogamicin, respectively.

The percent compositions of minor *N*-glycans showed higher diversity than those observed for major peaks. For example, tocilizumab, cetuximab, infliximab, and adalimumab contained over 10% of high-mannose-type and hybrid-type *N*-glycans. Gemtuzumab ozogamicin and infliximab contained over 7% of monosialylated *N*-glycans of which sialic acids were exclusively NeuGc. It should be noted that all the mAb products examined in the present study except for human IgG antibody have nonhuman *N*-glycans containing α-Gal epitope(s) (**8** and **9**) as examined by CE-LIF. Again, we would like to emphasize that careful considerations are strongly needed not only for types of *N*-glycans but also for the amounts of each *N*-glycan, especially having nonhuman constituents like NeuGc residue and α-Gal epitope in their structure. It should be also emphasized that this is conveniently achieved by CE-LIF method.

Lot-to-Lot Variation of *N*-Glycan Distribution. In order to address the intraproduct variations of attached *N*-glycans, lot-to-lot analysis was performed using three different lots of gemtuzumab ozogamicin as a model mAb product. Relative standard deviations (RSDs) of percent compositions for major *N*-glycans **3**, **4**, and **6** were not higher than 2.3%, and it showed that good consistency was kept between each lot (summarized in Table S-2 in the Supporting Information). Moreover, for minor *N*-glycans having disialo-, monosialo-, and one or two α-Gal epitope(s) showed reproducible results (RSDs ≤ 5.5%). However, the sum of high-mannose-type and hybrid-type *N*-glycans showed over 10% in RSD values due to their small abundances. From the results described here, the attached *N*-glycans kept constant distributions among the production lots, although they showed wide diversity among the products due to the differences in their manufacturing process.

CONCLUSION

Detailed structural and quantitative analyses of N-linked glycans from six therapeutic recombinant mAb products by CE-LIF and LC/ESI-IT-TOF MS demonstrate their variety due to differences in the manufacturing process, especially cell types. Forty-six *N*-glycans were successfully assigned from the tested mAbs, and they include nonhuman *N*-glycans containing NeuGc residues instead of NeuAc (*N*-glycans **33**−**41** and **45**) and α-Gal epitope(s) (*N*-glycans **8**, **9**, and **37**). The former structure (representative structure is shown in Figure 4a) was observed in three mAb pharmaceuticals produced in nonhuman mammalian cell lines such as murine myeloma SP2/0 and NS0 cell lines, whereas it was absent in other mAbs produced in CHO cell lines. However, even if a CHO cell line is used for the production, NeuGc can be taken up and metabolically incorporated into secreted glycoprotein when animal-derived materials are used as a culture medium during the manufacturing process. Therefore, pharmaceutical companies should have their own strategy to reduce its uptake by adding a human sialic acid (i.e., NeuAc) to the culture medium.[28,44] The latter structure (Figure 4b) was observed in all the tested mAb products by CE-LIF analysis.

It should be noticed that fully humanized mAb, adalimumab, still contains α-Gal epitope. Since the detailed mechanism of this contamination is not clear, further investigation must be necessary in order to reduce or eliminate the risk of crucial side effect or insufficient efficacy in humans. Unfortunately, the detailed correlation between the risks led by these contaminations and the amount of the glycans is not still clear. Furthermore, since the peptide portion to which these nonhuman *N*-glycans are attached is not accessed in this study, it should be verified in a future work on peptide analysis of the mAb pharmaceuticals.

Hyphenated techniques of HPLC/CE with MS are powerful approaches for getting structural information, even if minor glycans are present at low levels. Actually, in tocilizumab, bevacizumab, and adalimumab, the *N*-glycans containing α-Gal epitope are present at very low levels compared to the whole amounts of *N*-glycans. When analyzed by LC/MS, as shown in Figure 1, parts b, c, and f, it was hard to detect these *N*-glycans accurately due to their very low amounts and interference by other *N*-glycans which coeluted in HPLC. In contrast, in CE-LIF, these *N*-glycans, especially *N*-glycans **8** and **9**, could be obviously separated and observed at the end of the run, around 23−24 min, as described in Figure 3, parts b, c, and f, respectively. These results clearly indicate that the CE-LIF method provides notable information regarding the presence of α-Gal epitope in mAb products rather than the HPLC method, from the aspect of glycan analysis. In the glycan analysis of mAb pharmaceuticals, both identity and quantity of nonhuman *N*-glycans are quite important information. For further investigation in order to find out the threshold of the glycan contaminations, the proposed CE-LIF method is quite useful for rapid, quantitative, and extensive evaluations of *N*-glycans of

dx.doi.org/10.1021/ac300234a | *Anal. Chem.* 2012, 84, 2373−2379

mAb pharmaceuticals in various development stages such as, for example, clone selection, bioprocess control, detailed characterization for approval application, and lot release testing.

Although the CE/MS method for glycan analysis sounds to be a really appropriate strategy, CE cannot provide sufficient separation of *N*-glycans without the sieving effect by neutral polymers in the separation buffers. This problem leads to less quantitative information in CE/MS analysis. Therefore, when applying CE/MS to a detailed glycan analysis, this discrepancy between "resolutions of glycans" and "obtaining quantitative data" needs to be improved.

The indications of recombinant mAb products have been remarkably expanding into solid tumor, leukemia, rheumatism, asthma, and analgesia. Some of them need long-term drug exposure for obtaining sufficient results. In order to improve safety and efficacy of the products, pharmaceutical companies have to monitor and control the variations of *N*-glycans in their products especially when possible antigenic carbohydrate constituents, NeuGc residue and/or α-Gal epitope, are present, through all the development and clinical stages, even if they are fully humanized products. We believe that the present report will be a useful guide for evaluating attached *N*-glycans of mAb products.

■ ASSOCIATED CONTENT

Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +81-6-6721-2332. Fax: +81-6-6721-2353. E-mail: k_kakehi@phar.kindai.ac.jp.

Notes

The authors declare no competing financial interest.

■ REFERENCES

(1) Sutton, B. J.; Phillips, D. C. *Biochem. Soc. Trans.* **1983**, *11*, 130−132.
(2) Tao, M. H.; Morrison, S. L. *J. Immunol.* **1989**, *143*, 2595−2601.
(3) Flintegaard, T. V.; Thygesen, P.; Rahbek-Nielsen, H.; Levery, S. B.; Kristensen, C.; Clausen, H.; Bolt, G. *Endocrinology* **2010**, *151*, 5326−5336.
(4) Umaña, P.; Jean-Mairet, J.; Moudry, R.; Amstutz, H.; Bailey, J. E. *Nat. Biotechnol.* **1999**, *17*, 176−180.
(5) Shields, R. L.; Lai, J.; Keck, R.; O'Connell, L. Y.; Hong, K.; Meng, Y. G.; Weikert, S. H. A.; Presta, L. G. *J. Biol. Chem.* **2002**, *277*, 26733−26740.
(6) Shinkawa, T.; Nakamura, K.; Yamane, N.; Shoji-Hosaka, E.; Kanda, Y.; Sakurada, M.; Uchida, K.; Anazawa, H.; Satoh, M.; Yamasaki, M.; Hanai, N.; Shitara, K. *J. Biol. Chem.* **2003**, *278*, 3466−3473.
(7) Kamoda, S.; Nomura, C.; Kinoshita, M.; Nishiura, S.; Ishikawa, R.; Kakehi, K.; Kawasaki, N.; Hayakawa, T. *J. Chromatogr., A* **2004**, *1050*, 211−216.
(8) Kamoda, S.; Ishikawa, R.; Kakehi, K. *J. Chromatogr., A* **2006**, *1133*, 332−339.
(9) Mizuochi, T.; Taniguchi, T.; Shimizu, A.; Kobata, A. *J. Immunol.* **1982**, *129*, 2016−2020.
(10) Fujii, S.; Nishiura, T.; Nishikawa, A.; Miura, R.; Taniguchi, N. *J. Biol. Chem.* **1990**, *265*, 6009−6018.
(11) Feizi, T.; Childs, R. A. *Biochem. J.* **1987**, *245*, 1−11.
(12) Chung, C. H.; Mirakhur, B.; Chan, E.; Le, Q.-T.; Berlin, J.; Morse, M.; Murphy, B. A.; Satinover, S. M.; Hosen, J.; Mauro, D.; Slebos, R. J.; Zhou, Q.; Gold, D.; Hatley, T.; Hicklin, D. J.; Platts-Mills, T. A. E. *N. Engl. J. Med.* **2008**, *358*, 1109−1117.
(13) Joziasse, D. H.; Shaper, J. H.; Jabs, E. W.; Shaper, N. L. *J. Biol. Chem.* **1991**, *266*, 6991−6998.
(14) Smith, D. F.; Larsen, R. D.; Mattox, S.; Lowe, J. B.; Cummings, R. D. *J. Biol. Chem.* **1990**, *265*, 6225−6234.
(15) Lantéri, M.; Giordanengo, V.; Vidal, F.; Gaudray, P.; Lefebvre, J.-C. *Glycobiology* **2002**, *12*, 785−792.
(16) Galili, U.; Rachmilewitz, E. A.; Peleg, A.; Flechner, I. *J. Exp. Med.* **1984**, *160*, 1519−1531.
(17) Galili, U. *Springer Semin. Immunopathol.* **1993**, *15*, 155−171.
(18) Galili, U. *Transplantation* **2004**, *78*, 1093−1098.
(19) Macher, B. A.; Galili, U. *Biochim. Biophys. Acta* **2008**, *1780*, 75−88.
(20) Kobayashi, T.; Cooper, D. K. *Subcell. Biochem.* **1999**, *32*, 229−257.
(21) Varki, A. *Nature* **2007**, *446*, 1023−1029.
(22) Hayashi, N.; Kawasaki, N.; Nakajima, Y.; Toyoda, M.; Katagiri, Y.; Itoh, S.; Harazono, A.; Umezawa, A.; Yamaguchi, T. *J. Chromatogr., A* **2007**, *1160*, 263−269.
(23) Nakamura, K.; Ariga, T.; Yahagi, T.; Miyatake, T.; Suzuki, A.; Yamakawa, T. *J. Biochem.* **1983**, *94*, 1359−1365.
(24) Varki, A. *Glycobiology* **1992**, *2*, 25−40.
(25) Marquina, G.; Waki, H.; Fernandez, L. E.; Kon, K.; Carr, A.; Valiente, O.; Perez, R.; Ando, S. *Cancer Res.* **1996**, *56*, 5165−5171.
(26) Tangvoranuntakul, P.; Gagneux, P.; Diaz, S.; Bardor, M.; Varki, N.; Varki, A.; Muchmore, E. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 12045−12050.
(27) Padler-Karavani, V.; Yu, H.; Cao, H.; Chokhawala, H.; Karp, F.; Varki, N.; Chen, X.; Varki, A. *Glycobiology* **2008**, *18*, 818−830.
(28) Ghaderi, D.; Taylor, R. E.; Padler-Karavani, V.; Diaz, S.; Varki, A. *Nat. Biotechnol.* **2010**, *28*, 863−867.
(29) Anumula, K. R.; Dhume, S. T. *Glycobiology* **1998**, *8*, 685−694.
(30) Nakano, M.; Kakehi, K.; Tsai, M.-H.; Lee, Y. C. *Glycobiology* **2004**, *14*, 431−441.
(31) Kamoda, S.; Nakano, M.; Ishikawa, R.; Suzuki, S.; Kakehi, K. *J. Proteome Res.* **2005**, *4*, 146−152.
(32) Naka, R.; Kamoda, S.; Ishizuka, A.; Kinoshita, M.; Kakehi, K. *J. Proteome Res.* **2006**, *5*, 88−97.
(33) Chen, F. T.; Evangelista, R. A. *Electrophoresis* **1998**, *19*, 2639−2644.
(34) Ma, S.; Nashabeh, W. *Anal. Chem.* **1999**, *71*, 5185−5192.
(35) Kakehi, K.; Funakubo, T.; Suzuki, S.; Oda, Y.; Kitada, Y. *J. Chromatogr., A* **1999**, *863*, 205−218.
(36) Raju, T. S. *Anal. Biochem.* **2000**, *283*, 125−132.
(37) Nakajima, K.; Oda, Y.; Kinoshita, M.; Kakehi, K. *J. Proteome Res.* **2003**, *2*, 81−88.
(38) Ma, S.; Lau, W.; Keck, R. G.; Briggs, J. B.; Jones, A. J. S.; Moorhouse, K.; Nashabeh, W. *Methods Mol. Biol.* **2005**, *308*, 397−409.
(39) Ruhaak, L. R.; Zauner, G.; Huhn, C.; Bruggink, C.; Deelder, A. M.; Wuhrer, M. *Anal. Bioanal. Chem.* **2010**, *397*, 3457−3481.
(40) Huhn, C.; Selman, M. H. J.; Ruhaak, L. R.; Deelder, A. M.; Wuhrer, M. *Proteomics* **2009**, *9*, 882−913.
(41) Pabst, M.; Kolarich, D.; Pöltl, G.; Dalik, T.; Lubec, G.; Hofinger, A.; Altmann, F. *Anal. Biochem.* **2009**, *384*, 263−273.
(42) Vultaggio, A.; Matucci, A.; Nencini, F.; Pratesi, S.; Parronchi, P.; Rossi, O.; Romagnani, S.; Maggi, E. *Allergy* **2010**, *65*, 657−661.
(43) Read, E. K.; Park, J. T.; Brorson, K. A. *Biotechnol. Appl. Biochem.* **2011**, *58*, 213−219.
(44) Bardor, M.; Nguyen, D. H.; Diaz, S.; Varki, A. *J. Biol. Chem.* **2005**, *280*, 4228−4237.
(45) Domon, B.; Costello, C. E. *Biochemistry* **1988**, *27*, 1534−1543.