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Short Communication

Rapid profiling of carbohydrate glycoforms in monoclonal antibodies using MALDI/TOF mass spectrometry

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1. Introduction

Mass spectrometry is now making significant contributions to the characterization of biologically important molecules. The development of two ionization methods, electrospray and matrix-assisted laser desorption [1], has extended the utility of mass spectrometry to the characterization of both protein and carbohydrate structure. As more peptides and proteins are carried through the process of regulatory registration for use as therapeutic agents, mass spectrometry will serve as an indispensable tool for the certification of product identity and consistency.

There is at least one monoclonal antibody currently in therapeutic use (ORTHOCLONE OKT®3 [2], Ortho Biotech, Raritan, NJ) and many others are in various stages of pharmaceutical development. While the first monoclonal antibodies were of murine origin, future antibodies will be produced on a large scale by cell cultures using recombinant technology. Although the carbohydrate portion is an integral part of antibody structure and function, relatively little information is available concerning these non-peptide portions of antibodies produced by artificial means and under different cell culture conditions [3]. Therefore, it is important to determine the presence and nature of post-translational modifications when characterizing recombinant proteins, since these modifications could affect the heterogeneity or biological activity of the product [4]. Classical methods of protein structure determination (e.g. amino acid analysis and Edman sequencing) do not detect many post-translational modifications. However, mass spectrometry can provide useful data about both amino acid and non-amino acid constituents of proteins [5].

We have been involved in confirming the protein structure of monoclonal antibodies synthesized by genetic engineering using a peptide mapping procedure to fragment the protein into smaller components amenable to amino acid sequence analysis. Mass spectrometry has been used as an adjunct to automated Edman sequencing, particularly in confirming the carboxy terminus of peptide fragments and in diagnosing modification of amino acids that occured as degradation products. Specifically, MALDI/TOF instrumentation has made this a routine procedure in our laboratory. In the process of characterizing monoclonal antibodies using peptide mapping techniques, we isolated the glycopeptide tryptic fragment containing the conserved, heavy-chain Asn-linked carbohydrate, and found MALDI/TOF mass spectral analysis of the peptide to be a rapid method for obtaining an indication of the structural heterogeneity of the carbohydrate. This report describes the application of

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MALDI/TOF mass spectrometry to the profiling of the carbospectrometry to the profiling of the carbohydrate in the F_c region of several monoclonal antibodies, both of murine origin and humanized versions.

2. Experimental

The monoclonal antibodies used in this study were from various research and production batches of material. In general, the murine monoclonal antibodies were produced from hydridoma cell lines grown either as ascites tumors or in cell culture. Humanized antibodies were obtained from transfected murine myeloma cell lines, using either a COS or NS_0 parent cell line. The cells were grown in a proprietary, defined serum free medium. Antibodies were purified from diafiltered and concentrated media by protein A affinity chromatography for research materials, and additional chromatography and finishing steps for production batches.

Peptide mapping of the antibodies was performed as described in detail previously [6]. Briefly, the antibody was Cys-sulfonated and the heavy and light chain separated. The isolated chains were digested with trypsin and the digests analyzed by reversed-phase LC using a C_{18} Dynamax column (250 × 4.6 mm i.d., Rainin Instrument Co. Inc.) eluted with a gradient of acetonitrile in 0.1% trifluoroacetic acid. The fraction containing the glycopeptide of interest (identity confirmed by amino acid sequencing) was collected and dried on a Speedvac rotary evaporator. The peptide was redissolved in 0.1% trifluoroacetic acid for analysis by mass spectrometry.

A solution of the peptide (0.2 μl) containing 1–10 pmol of material was applied to the sample target simultaneously with 0.5 μl of the matrix solution consisting of α-cyano-4 hydroxycinnamic acid at 10 μg μl^{-1} in acetonitrile–0.1% trifluoroacetic acid (7:3 v/v). The sample was allowed to dry before introduction into the mass spectrometer. Mass spectra were recorded on Finnigan MAT (San Jose, CA) LaserMAT UV MALDI/TOF mass spectrometer equipped with a 337 nm pulsed nitrogen laser and operated in the positive ion mode with 20 kV acceleration potential. The mass spectra shown are summations of multiple laser shot acquisitions (typically 5–10 laser shots per spectrum). Mass assignments are based on an external calibation using the commercially available standard peptide melittin (Sigma; St. Louis, MO). All data analysis were performed using the standard software supplied by the manufacturer.

3. Results and discussion

The peptide maps of the heavy chain of the antibodies were generated by reversed-phase LC of the tryptic digest as described previously. The peptide fragment containing the conserved site of glycosylation, Asn^{297} (numbering according to the E_u index of Kabat et al. [7]), eluted as a broad, multiple peak about one-third of the way through the gradient. Although the amino acid sequence of this peptide was not the same in all of the antibodies studied, the glycopeptide could readily be located by the peak shape and confirmed by amino acid sequencing. The total glycopeptide peak was collected and the dried sample was subjected to MALDI/TOF mass spectral analysis. Although several techniques exist for the carbohydrate characterization of glycoproteins [8], MALDI/TOF mass spectrometry provides a simple, sensitive method that gives a basic profile of the carbohydrate features which could be used for comparison purposes (e.g. in batch release of a therapeutic protein) [9–12].

Initially, sinapinic acid was used as matrix to obtain desorption/ionization, but after substitution with α-cyano-4-hydroxy-cinnamic acid we found significant improvement in the mass spectra of glycopeptides in the 1500–4000 u mass range. The glycopeptides ionized very efficiently in this matrix, almost exclusively giving the singly charged ion. The resulting signals were sufficiently resolved from sodium adduct peaks (observed as distinctly separate signals of varying intensity 22 u higher in mass than the protonated molecular ion signals of interest) to obtain molecular weights accurate to within 2 Daltons with the use of an external standard. Assuming that the structure of any one of the glycoforms is correctly assigned, one can than utilize such an ion as an internal standard for improved accuracy in the mass assignments of other ions in the spectrum. An example of a mass spectrum of an antibody glycopeptide is given in Fig. 1.

Fig. 1 shows the MALDI/TOF mass spectrum resulting from the glycoforms of the tryptic glycopeptide of a humanized IgG_4

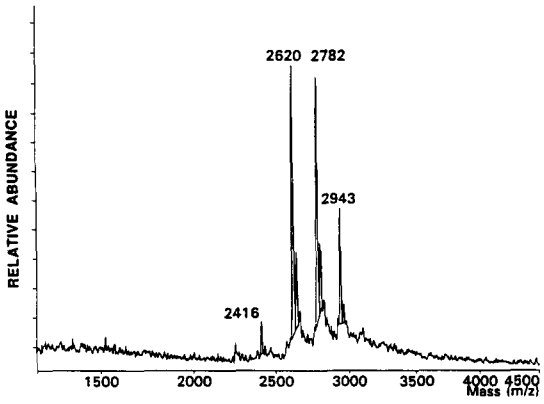

Fig. 1. Positive-ion MALDI mass spectrum of a glycopeptide generated from the tryptic digest of a humanized IgG_4 monoclongal antibody. The labeled masses are the protonated molecular ions of the four major glycoforms. The signals observed immediately adjacent (higher mass) to the major peaks are sodium adduct ions.

monoclonal antibody. The relative abundances of the individual ions were quite consistent in different samples from the same batch of antibody, and high quality spectra are generally obtainable providing care is taken in the preparation of samples. Different batches of this antibody produced via the same methodology showed a very similar glycoform mass spectrometry profile. In general, quantification of peptides in mixtures using MALD ionization with TOF mass analysis has proved difficult owing to factors including differential detection due to the mass and charge state of the ions, as well as variations in desorption efficiency attributable to the structural or chemical nature of the various peptides in the mixture. However, for glycopeptides of closely related structure (differing only in their carbohydrate component), we proceeded under the assumption that the relative abundances of the ions observed for the various species were proportional to the relative concentrations of these glycoforms present at the spot sampled by the laser pulses. A similar assumption used in the analysis of carbohydrates released from glycoproteins by enzymatic digestion has been affirmed through the use of conventional carbohydrate analysis techniques [13].

Immunoglobulins of the G class (IgG) are known to have a conserved *N*-glycosylation site at asparagine-297 of the heavy chain (in the F_c region of the antibody). Since the core structure of the carbohydrate at this site is also conserved [14], structures of the glycoforms can be deduced from the masses of the individual ions observed in the mass spectra. For example, the calculated mass of the tryptic fragment of human IgG_4 containing Asn^{297} is 1173 u based on the amino acid sequence. Subtracting this weight from mass of the major MH^+ ions in Fig. 1 gives the following masses as being contributed by the carbohydrate: 1242, 1446, 1608 and 1769 u. The 1769 u mass is consistent with one of the known antibody carbohydrate structures, a fucosylated biantennary complex type, structure A in Fig. 2. The 1608 u and 1446 u masses, derived from other signals in the spectrum shown in Fig. 1 can be accounted for by removal of the terminal galactose from one or both arms of structure A to give structures B and C (also shown in Fig. 2). Positional isomerism of the glycan linkages, which differentiates between the two arms (i.e. there are two forms of structures B, D and E that differ in whether the longer arm is 1,6 or 1,3-linked) cannot be determined by the mass spectral data. Further loss of one *N*-acetylglucosamine produces structure D with carbohydrate mass equal to 1242 u. Smaller amounts of additional glycoforms are evident in the mass spectra of other antibody samples (see below). These carbohydrate structures have been identified in other monoclonal antibodies by comparing chromatographic retention times of released oligosaccharides to those obtained for authentic standards. Such carbohydrates result from incomplete processing of the protein by various glycotransferases and glycosidases in the endoplasmic reticulum, and Golgi apparatus during protein synthesis in eukaryotic cells. Although quantification of mixture components can be an uncertain enterprise (see above), the relative amounts of the glycoforms present as determined from the relative abundances of their representative ions (approximately 5:100:98:40 for the relevant ions in Fig. 1) were in agreement with those found for the other monoclonal antibodies analyzed using traditional techniques [3,15].

Fig. 3(top) shows the mass spectrum of the glycopeptide from a murine $IgG2_a$ monoclonal antibody derived from a hybridoma cell line grown in cell culture, and Fig. 3(bottom) shows the mass spectrum of the glycopepide from a different murine $IgG2_a$ antibody derived from ascites. The peptide portion of this glycopeptide has a mass of 997 u. From the additional mass of the ions observed in these spectra, it can be concluded that the major glycoforms have the same structures (A through D) as in

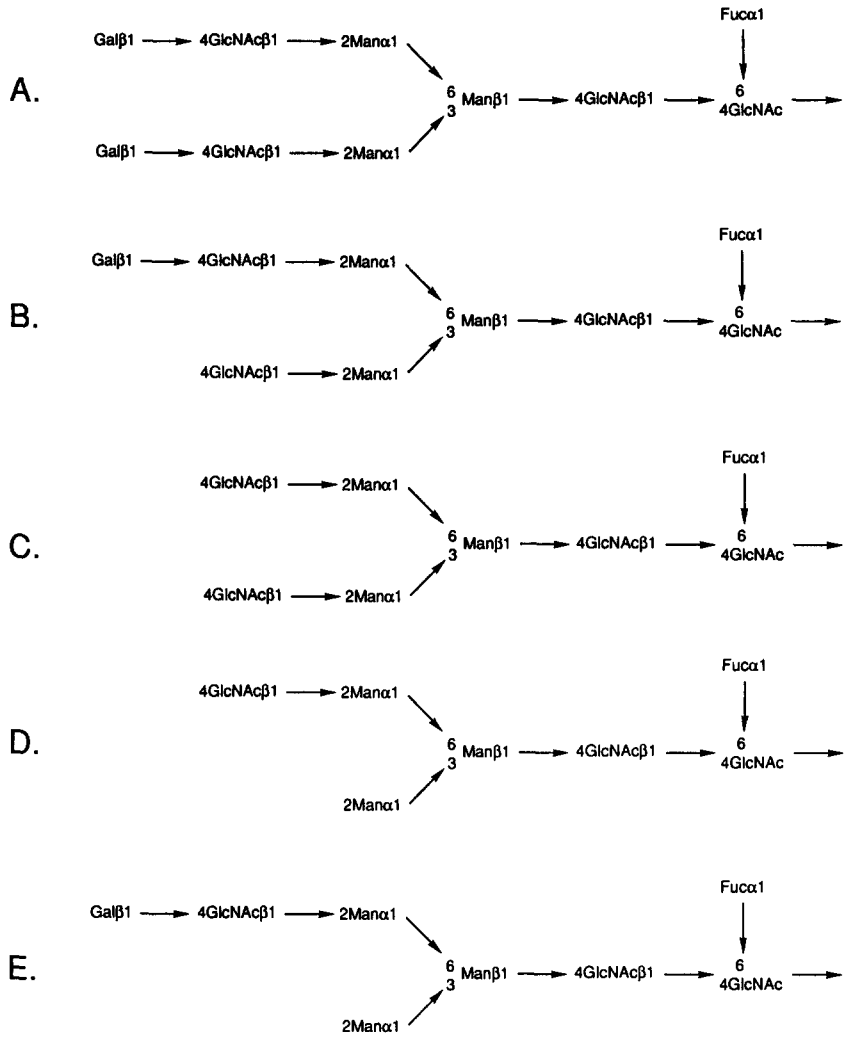

Fig. 2. Five representative carbohydrate structures known to occur in IgG antibodies (for details, see Refs. [15] and [16]).

the antibody analyzed in Fig. 1. The glycoform profiles of these murine antibodies show relatively less of structure A and more of structure B than any of the humanized antibodies (all derived from the same cell line) that we have examined to date. Other minor glycoforms were observed in the ascite-derived antibody with carbohydrate masses of 1405 u and 1080 u (deduced from the appearance of ions at masses of 2403 u and 2078 u, respectively). The 1405 u structure can reasonably be assigned the structure E in Fig. 2, as this glycoform has been identified in other IgG antibodies [15]. The 1080 u carbohydrate is consistent with the loss of a mannose residue from structure E.

Another murine monoclonal antibody, an IgG_1 subtype derived from a different parent cell line to the $IgG2_a$ antibodies above, showed a considerably different mass spectral carbohydrate profile (Fig. 4). The contribution of the peptide portion to the mass of this glycopeptide is 1157 u, so the most prominent glycoform at m/z 2604 has carbohydrate structure C. The structures of two additional glycoforms were deduced from the ions in this mass spectrum. The ion at m/z 3076 can be accounted for by the addition of sialic acid to one of the arms of carbohydrate structure A with concomitant loss of the fucose moiety. Antibodies derived from murine hybridomas are generally known to have both small amounts of sialated carbohydrate structure as well as structures without fucose [16]. In a detailed analysis of one such murine monoclonal antibody, it was observed and that the carbohydrate structures present with sialic acid did not contain the

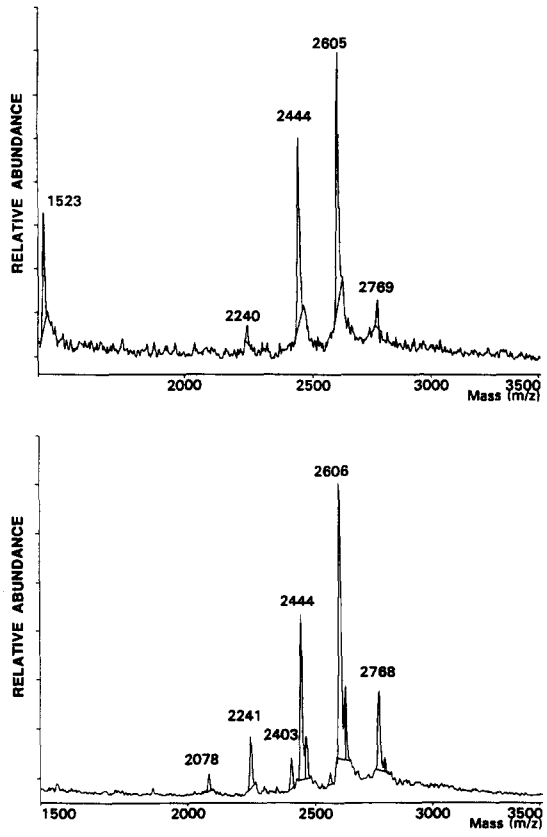

Fig. 3. Positive-ion MALDI mass spectra glycopeptides generated from the tryptic digest of a murine $IgG2_a$ monoclonal antibody derived from (top) a cultured cell line, and (bottom) an ascites-derived murine antibody. The signal at m/z 1523 is attributed to a coeluting (and unrelated) peptide in the tryptic map.

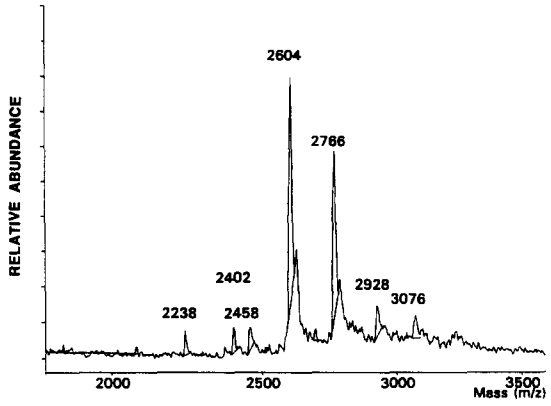

Fig. 4. Positive-ion MALDI mass spectrum of a glycopeptide from an IgG_1 murine monoclonal antibody derived from a cell culture. The parent cell line of the hybridoma was different from that used for the antibodies that generated the glycopeptides yielding the mass spectrum in Fig. 3. The contribution of the peptide portion to the mass of the glycopeptide is 1157 u.

fucose substituent [17]. In the present study, small amounts of this higher molecular weight glycoform were also found in some samples of humanized antibodies. An additional non-fucosylated structure was observed as the ion at m/z 2458 in Fig. 4. This glycoform is most probably structure C without fucose. The mono-galactosyl structure B without the fucose may also be present, but the ion indicative of this species would coincide with the sodium adduct of structure C, and therefore could not be identified unequivocally.

4. Conclusion

MALDI/TOF mass spectrometry has proved to be a useful, sensitive analytical technique for the characterization of recombinant biotechnology products. In the peptide mapping of proteins, MALDI/TOF mass spectrometry can be used to rapidly scan peptide fragments and match the masses with those expected if a putative sequence of the protein is known. Glycopeptides can usually be readily identified by the appearance of multiple ions in the mass spectrum that differ by 162 u (a hexose unit), 203 u (an *N*-acetylhexosamine unit) or 291 u (a sialic acid unit). Structures of the glycoforms can often be deduced from the molecular weight data in proteins that possess relatively conserved carbohydrate structures, such as antibodies. MALDI/TOF mass spectrometry is a convenient way to profile carbohydrate glycoforms for batch comparisons of pharmaceutical proteins. Our results confirm literature reports that different cell lines and production conditions can affect the distribution of glycoforms of monoclonal antibodies. The potential biological effects of these differences remains to be determined. The availability of relatively inexpensive, easy to operate instruments for the application of this technique makes MALDI/TOF mass spectrometry a highly desirable component of the protein/carbohydrate chemistry laboratory.

References

[1] F. Hillenkamp, M. Karas, R.C. Beavis and B.T. Chait, Anal. Chem., 63 (1991) 1193A–1202A.
[2] J.R. Thistlewaite, A.O. Gaber, B.W. Haag, A.J. Aronson, C.E. Broelsch, J.K. Stuart and F.P. Stuart, Transplantation, 43 (1987) 176–184.
[3] J. Lund, N. Takahashi, H. Nakagawa, M. Goodall, T.

Bentley, S.A. Hindley, R. Tyler and R. Jefferis, Mol. Immunol., 30 (1993) 741–748.
[4] J.C. Paulson, TIBS, 14 (1989) 272–276.
[5] K. Biemann, Annu. Rev. Biochem., 61 (1992) 977–1010.
[6] D.J. Kroon, A. Baldwin-Ferro and P. Lalan, Pharm. Res., 9 (1992) 1386–1393.
[7] E.A. Kabat, T.T. Wu, M. Reid-Miller, H.M. Perry and K.S. Gottesman, Sequences of Proteins of Immunological Interest, 4th edn, US Department of Health and Human Services, Bethesda, MD, 1987 (NIH Publication No. 91-3242).
[8] M.W. Spellman, Anal. Chem., 62 (1990) 1714–1722.
[9] D.J. Harvey, Glycoconjugate J., 9 (1992) 1–12.
[10] M.J. Treuheit, C.E. Costello and B.H. Halsall, Biochem. J., 283 (1992) 105–112.
[11] T.M. Billeci and J.T. Stults, Anal. Chem., 65 (1993) 1709–1716.
[12] M.C. Huberty, J.E. Vath, E. James, W. Yu and S.A. Martin, Anal. Chem., 65 (1993) 2791–2800.
[13] C.W. Sutton, J.A. O'Neill and J.S. Cottrell, Anal. Biochem., 218 (1994) 34–36.
[14] T. Mizuochi, T. Taniguchi, A. Shimizu and A. Kobata, J. Immunol., 129 (1982) 2016–2020.
[15] R. Jefferis, J. Lund, H. Mizutani, H. Nakagawa, Y. Kawazoe, Y. Arata and N. Takahashi, Biochem. J., 268 (1990) 529–537.
[16] R.J. Rothman, L. Warren, J.F.G. Vliegenthart and K.J. Hard, Biochemistry, 28 (1989) 1377–1384.
[17] H. Krotkiewski, G. Gronberg, B. Krotkiewska, B. Nilsson and S. Svensson, J. Biol. Chem., 265 (1990) 2019–2020.