An innovative approach for the characterization of the isoforms of a monoclonal antibody product

Shanmuuga Sundaram, Alice Matathia, Jun Qian, Jingming Zhang, Ming-Ching Hsieh, Tun Liu, Richard Crowley, Babita Parekh* and Qinwei Zhou*

Bioanalytical Science; ImClone Systems, a wholly owned subsidiary of Eli Lilly & Co.; Branchburg, NJ USA

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Protein biopharmaceuticals, such as monoclonal antibodies (mAbs) are widely used for the prevention and treatment of various diseases. The complex and lengthy upstream and downstream production methods of the antibodies make them susceptible to physical and chemical modifications. Several IgG1 immunoglobulins are used as medical agents for the treatment of colon, breast and head and neck cancers, and at least four to eight isoforms exist in the products. The regulatory agencies understand the complex nature of the antibody molecules and allow the manufacturers to set their own specifications for lot release, provided the safety and efficacy of the products are established in animal models prior to clinical trials. During the manufacture of a mAb product, we observed lot-to-lot variability in the isoform content and, although the variability is within the set specifications for lot release, made attempts to gain mechanistic insight by isolating and characterizing the individual isoforms. Matrix-assisted laser desorption/ionization (MALDI) and liquid chromatography (LC)/mass spectrometry (MS)/MS analyses of the isolated isoforms indicate that this variability is caused by sialic acid content, as well as truncation of C-terminal lysine of the individual isoforms. Sialidase and carboxypeptidase treatment of the product confirm the observations made by MALDI and LC/MS/MS.

Variability of isoforms of IgG1 product due to process change. The IEF pattern of different lots of the product produced using two different processes and different locations are shown in Figures 1 and 2. Irrespective of the process or the manufacturing location, six to eight isoforms are present in most of the lots with pl’s between 7.9–8.9; however, the relative abundance of each isoform showed some variability. Hence, we made attempts to isolate and characterize each isoform of the product to determine
migration of the protein in IEF gel. Band 1 contained neutral oligosaccharide, mainly G2F-2α-gal; Band 7 and 8 contained mostly sialylated oligosaccharides with low amount of α-gal species. The relative abundances of oligosaccharides residues with terminal α-gal and NGNA vs. G0F, which was set as the internal reference for each band calculation (% relative abundance = oligosaccharide MS area/G0F MS area), are shown in Table 1. The species with different glycans are enriched differently in each band compared with the total pool of oligosaccharides. From the MALDI analysis of the released glycans from each isoform, it is evident that isoforms 2–7 contain sialylated oligosaccharides and isoform 7 contains fully sialylated oligosaccharides and less α-galactosylated oligosaccharides.

Sialidase treatment of isoform 7. To better understand the affect of the variation observed in IEF, characterization studies were performed to determine the species present in IEF isoform 7. To determine if isoform 7 contained sialylated species, the mAb product was treated with sialidase and analyzed by IEF. As shown in Figure 5, isoform 7 was not present in the sample of the product treated sialidase, demonstrating presence of terminal sialic acid.

C-terminal lysine. The C-terminal lysine may also contribute to charge heterogeneity. To determine the presence of C-terminal lysine in isoform 7, the mAb product was treated with carboxypeptidase and analyzed by IEF. The carboxypeptidase-treated sample showed the presence of isoform 7 (data not shown); however, isoforms 1 and 2 were no longer observed. This result indicates that isoforms 1 and 2 contain species with C-terminal lysine.

Peptide map of the isoforms of the IgG1 product. The individual isoforms of the IgG1 product were reduced and alkylated, subjected to trypsin digestion and the peptides extracted from the membrane using 1% Zwittergent 3–16 at 37°C for 2 h. These samples were subjected to liquid chromatography (LC)/mass spectrometry (MS)/MS analysis; the peptide maps of each isoform are presented in Figure 6. The C-terminal lysine containing peptide and truncated C-terminal form were identified by MS/MS data. From the analysis, the percent of lysine of isoforms 1–8 were determined to be 80.2, 54.3, 21.0, 14.3, 8.5, 5.4, 5.1 and 5.7, respectively, indicating lysine truncation in isoforms 5–8. This also confirmed the result from Figure 5 that both isoforms 1 and 2 have significant amounts of C-terminal lysine.

Biological activity of the IgG1 product. The biological activities of all the lots used in this investigation are presented in Table 2. Although variation in the number of isoforms was observed, no significant difference in the biological activity was observed between these lots. This result indicates that the isoform variability did not affect the potency of the molecule.

Discussion

Due to the complexity of the upstream and downstream processing conditions used during mAb manufacturing, maintenance of the structural integrity of the molecule is a challenge. C-terminal
Additional factors such as three dimensional structure and exposure to solvent may also influence the rate of deamidation. It is known that the succinimide intermediate spontaneously hydrolyzes to isoAsp or Asp at an approximate ratio of 3:1.\textsuperscript{23-25} The isoAsp can distort the confirmation of the protein,\textsuperscript{17} which may result in charge heterogeneity.\textsuperscript{26} Charge variation can also occur due to the enzymatic removal of a C-terminal lysine from either one or both heavy chains. It is frequently observed in mAbs with up to three charge variants resulting from the removal of terminal lysine.\textsuperscript{27} The extent of C-terminal lysine removal depends of the endogenous carboxypeptidase activity of the host cell line, and that peptidase level may vary from batch-to-batch fermentation conditions. Another common form of IgG heterogeneity involves cysteine residues, such as oxidation status, disulfide linkage or decomposition.\textsuperscript{28,29} Pristatsky et al.\textsuperscript{30} provided evidence for the presence of trisulfide bonds in a human IgG2 mAb and speculated that dissolved hydrogen sulfide generated during the fermentation process might have contributed to the formation of trisulfide bonds.

The carbohydrate moiety of IgGs also exhibit heterogeneity in sugar content and structure. A negatively charged sialic acid molecule typically caps the end of the carbohydrate chain, and
as a consequence of the variable nature of sialic acid content, isoforms with differences in charge can be formed. Catlin et al. reported that the number of sialic acid molecules influenced the pI of rHuEPO.

Antibody products produced in murine cell lines have several oligosaccharide structures. The IEF of the product used in this investigation displayed banding patterns with a narrow pI range (<0.1 pH units) with different densities, which indicated protein heterogeneity. It is necessary for pharmaceutical manufacturers to define and characterize the pattern of protein heterogeneity and assure lot-to-lot consistency for the review by regulatory agencies. Hence, we attempted to isolate and characterize the isoforms to identify the difference between the various subsets. Separation of the IgG1 isoforms poses a major challenge because the differences in pI are <0.1 pH units. We took a novel but simple approach to isolate the isoforms by performing the IEF on the flatbed surface, followed by blotting the isoforms on PVDF membrane. Using this approach, we generated enough material to perform peptide mapping and characterize the oligosaccharides. Our study revealed that the sialic acid content of the isoforms varies with each form, and the peptide map analysis of the isoforms indicated that the C-terminal lysine content also contributed to the differences in the migration pattern. Sialidase and carboxypeptidase treatments of the IgG1 product demonstrated the involvement of C-terminal lysine and sialic acid in charge heterogeneity of this molecule. Based on the results presented in this investigation, we propose that variation in the sialic acid content, as well as the differences in the C-terminal lysine content, contributed to variation in the IEF pattern in the product. The biological activity of all lots used

Figure 4. Relative abundance of selected oligosaccharides in each band (isoform).
in the investigation indicates no significant differences, which suggests that the variability in the isoform content of the IgG1 product may not result in drastic modification of the molecule.

There are numerous reports implicating the involvement of deamidation as one of the origins of charge heterogeneity; in most cases, loss of biological activity and shift in the pl’s of the isoforms to the acidic region were reported. In this investigation, we did not observe loss of biological activity or a drastic shift in the pl’s of the isoforms to the acidic region. However, it should be pointed out that after sialidase and carboxy peptidase treatments, three charged variants were still present in this molecule (Fig. 5). We isolated these variants and performed further characterization using LC/MS/MS. Our studies indicated that the isoforms remaining after sialidase and carboxy peptidase digestion had different degrees of deamidation on the heavy chain T34 (PENNY peptide) with a similar glycosylation pattern.

Apart from the inhibition of tumor cell proliferation, some IgG1 molecules are also capable of lysing cells through antibody-mediated cellular cytotoxicity (ADCC), which is triggered upon binding of lymphocyte receptors to the Fc region of the antibody. It is well-documented that fucosylation of the IgG1 affects ADCC activity; however, the role of sialic acid in effector function is under debate. Antibody galactosylation has been observed to affect complement activation, but it has no impact on ADCC. The affect of glycosylation on the ADCC activity of the mAb product discussed here was not within the scope of this report, but may be investigated in the future.

It is interesting to note that variability of the isoform pattern was observed irrespective of the process and location changes. During the production of antibodies, ranges for all processing conditions are generally used. For example, the fermentation conditions may not have an absolute limit for pH, stripping method, dissolved oxygen content, harvest time or the raw materials. It is likely that the minor changes (within the set acceptance range) in the upstream processing and other production conditions might have contributed to modification of glycosylation pattern and truncation of C-terminal lysine of the IgG1 product, thereby resulting in charge heterogeneity. Regulatory agencies understand the complex nature of antibodies and allow manufacturers to set their own specifications for lot quality control. However, the safety and efficacy of the released lots must be established in animal models prior to the initiation of clinical trials. Apart from this, it is also expected that the mechanism of action and the stability of the molecule will be understood. As required by the regulatory guidelines, the lots used in this investigation were tested in animal models and showed no significant differences in safety and efficacy.

### Materials and Methods

The IgG1 mAb product used in this investigation was manufactured at ImClone Systems (Branchburg, NJ). Peptide
Figure 6. LC/MS/MS peptide map of IgG1 isoforms. The percent of Lysine of the isoforms 1–8 were determined to be 80.2, 54.3, 21.0, 14.3, 8.5, 5.4, 5.1 and 5.7, respectively indicating lysine truncation in isoforms 5–8.
N-glycosidase was purchased from Prozyme. Sodium cyanoborohydride, antracitic acid (2-AA), 2,5-dihydroxybenzoic acid, 5-methoxysalicylic acid were obtained from Sigma-Aldrich.

Isolation of isoforms using isoelectric focusing. IEF of the IgG1 product was performed using Multiphor™ Electrophoresis (GE Healthcare, Piscataway, NJ). Briefly, 10 μL (20 μg) of the desalted samples were applied on the IEF agarose pH 3–10 gels (Lonza, Wakersville, MD) and focused for 1 h 15 min at 1,000 V and 15 W. The anode and cathode solutions were 0.5 M acetic acid and 0.5 M NaOH, respectively. The separated isoforms were then transferred to a polyvinylidene fluoride (PVDF) membrane by press blotting for 15 min, stained with Coomassie blue and the bands were excised using a razor blade. Each band was cut from the PVDF membrane as closely as possible to minimize overlap between the bands, divided into a number of smaller pieces washed and destained in 500 μL 50% methanol overnight. The membrane pieces were then purified for further analysis.

N-glycan release and anthranilic acid labeling. N-linked oligosaccharides for each band were released by treatment with PNGase F for approximately 18 h at 37°C. The released N-glycans were labeled with 2-AA and sodium cyanoborohydride in methanol/acetic acid for 1.5 h at 80°C. The labeled glycans were purified by SPE cartridge.

MALDI analysis. Small aliquots of the reductively-aminated N-glycans were dialyzed for 45 min using an MF-Millipore membrane filter (0.25 μm pore, 47 mm dia., floating on water). The dialyzed sample was dried in a Speed-Vac, redisolved in a small amount of water, and mixed with a solution of 2,5-dihydroxybenzoic acid (9 g/L) and 5-methoxysalicylic acid (1 g/L) dissolved in water/acetonitrile (50:50). The mixture was dried onto a MALDI target and analyzed using an Applied Biosystems DE-Pro mass spectrometer operated in the linear/negative-ion mode. Oligosaccharides were assigned based on the observed mass-to-charge ratio.

Biological activity of the IgG1 product. The biological activity of the IgG1 product was monitored by plating target cells expressing specific receptor for the IgG1 in 96-well plate followed by the addition of IgG1 at concentrations ranging from 7.5 nM to 0.01 nM. The cells treated with the antibody were incubated for 72 h at 37°C/7% CO2. The cell growth was measured by the addition of MTS (3-(4,5-dimethyl-2-y)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) to purple formazan crystals. The biological activity was expressed as the ratio of percent growth inhibition based on the ED50 values of the test sample and the reference standard.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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| Table 2. Biological activity of the IgG1 product |
|-----------------|---------|---------|-----------------|
| Sample Lot #   | Process | Location| Percent biological activity* |
| 1              | 1       | A       | 100              |
| 2              | 1       | A       | 100              |
| 1              | 2       | A       | 100              |
| 2              | 2       | A       | 100              |
| 1              | 3       | B       | 100              |
| 2              | 3       | B       | 100              |
| 3              | 3       | B       | 100              |
| 4              | 3       | C       | 100              |

*Potency values are reported as the ratio of percent growth inhibition based on the ED50 values of the test sample and the reference standard.

Trypsin PVDF membrane peptide mapping. The bands were cut from PVDF membrane and wet by 100% MeOH before de-stained by water/acetonitrile (50:50). The bands were reduced with 0.02 M DTT in 0.05 M Tris pH 7.5 at 37°C for an hour followed by alkylation with 0.1 M iodoacetamide at room temperature in dark for 30 min. The digestion was performed using trypsin (5 ng/ml) in 0.05 M Tris buffer pH 7.5 with 1% (w/v) Triton X-100 (Calbiochem, San Diego, CA) at 37°C for 2 h. The digested peptides were extracted using water/acetonitrile (60:40) with 0.1% trifluoroacetic acid, and the resulting solution was dried down before analysis by LC-MS/MS. The resultant peptides were resolved using a C18 reverse phase column (Zorbax C18 300SB, 300 A, 5 μm, 4.6 x 150 mm) using an Agilent HPLC 1100 series (Agilent, Wilmington, DE) interfaced to a LCQ Deca ion trap mass spectrometer (Thermo Scientific). The digested sample was eluted with a gradient from 98% solvent A (0.1% TFA) to 40% solvent B (100% acetonitrile, 0.085% TFA) in 95 min at a flow rate of 0.5 ml/min. The scale of X axis of all the chromatograms is tailored to maximize the intensity of the C-terminal peptides.
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