Site- and Branch-Specific Sialylation of Recombinant Human Interferon-γ in Chinese Hamster Ovary Cell Culture

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Abstract: Since sialic acid content is known to be a critical determinant of the biological properties of glycoproteins, it is essential to characterize and monitor sialylation patterns of recombinant glycoproteins intended for therapeutic use. This study reports site- and branchspecific differences in sialylation of human interferon-y (IFN-γ) derived from Chinese hamster ovary (CHO) cell culture. Sialylation profiles were quantitated by reversed-phase HPLC separations of the site-specific pools of tryptic glycopeptides representing IFN- γ 's two potential N-linked glycosylation sites (i.e., Asn^{25} and Asn^{97}). Although sialylation at each glycosylation site was found to be incomplete, glycans of Asn²⁵ were more heavily sialylated than those of Asn⁹⁷. Furthermore, Man(α1-3) arms of the predominant complex biantennary structures were more favorably sialylated than $Man(\alpha 1-6)$ branches at each glycosylation site. When the sialylation profile was analyzed throughout a suspension batch culture, sialic acid content at each site and branch was found to be relatively constant until a steady decrease in sialylation was observed coincident with loss of cell viability. The introduction of a competitive inhibitor of sialidase into the culture supernatant prevented the loss of sialic acid after the onset of cell death but did not affect sialylation prior to cell death. This finding indicated that incomplete sialylation prior to loss of cell viability could be attributed to incomplete intracellular sialylation while the reduction in sialylation following loss of cell viability was due to extracellular sialidase activity resulting from cell lysis. Thus, both intracellular and extracellular processes defined the sialic acid content of the final product. © 1997 John Wiley & Sons, Inc. Biotechnol Bioeng 55: 390-398, 1997.

Keywords: γ -IFN; sialylation; glycosylation; sialidase, CHO cell culture

INTRODUCTION

Glycoproteins are often highly dependent upon sialic acid content for their biological function (Jenkins and Curling, 1994). Among the properties of a glycoprotein which can be influenced by sialylation are solubility (Lawson et al.,

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1983), thermal stability (Tsuda et al., 1990), resistance to protease attack (Aquino et al., 1980), antigenicity (Schauer, 1988), and specific activity (Smith et al., 1990). Most generally, sialylation can shield a glycoprotein from recognition by hepatocytic asialoglycoprotein receptors (Weiss and Ashwell, 1989) thereby dramatically increasing circulatory lifetime. Due to this recognized biological significance, sialylation is often a desirable feature of recombinant glycoproteins intended for therapeutic use (e.g., Fukuda et al. (1989) and Tsuda et al. (1990) have found that desialylation of erythropoietin abolishes its in vivo biological activity). Consequently, the characterization and monitoring of sialylation patterns of recombinant therapeutic glycoproteins is essential to ensure product efficacy, homogeneity, and consistency. Several reports have suggested that culture time can play a critical role in defining product sialylation. Sliwkowski et al. (1992) have reported the qualitative loss of sialic acid from CHO-derived human deoxyribonuclease I over the course of a batch culture, and extracellular sialidase activity arising from cell lysis (Gramer and Goochee, 1993) has been reported to be capable of desialylating exogenously-added glycoproteins (Gramer et al., 1993; Warner et al., 1993) in CHO cell culture. However, quantitative analysis of the sialylation profile of a recombinant glycoprotein product throughout the course of a CHO culture process has not been previously reported.

This paper presents the site- and branch-specific quantitative monitoring of sialylation of recombinant human interferon-y (IFN-y) throughout batch CHO cell culture and explores possible factors leading to incomplete sialylation in the final product. IFN- γ is a cytokine exhibiting antiviral, antiproliferative, and immunomodulatory activities (Farrar and Schreiber, 1993). This 143-amino acid polypeptide contains two potential N-linked glycosylation sites (i.e., Asn²⁵ and Asn⁹⁷). The glycans of IFN-γ derived from CHO cell cultures are predominantly complex biantennary structures with lesser proportions of complex tri- and tetraantennary structures (James et al., 1995; Harmon et al., 1996). With respect to sialic acid content, Mutsaers et al. (1986) observed by ${}^{1}H$ -NMR that the Man($\alpha 1$ -3) branches of the predominant biantennary structures are more heavily sialylated than the Man(α 1-6) arms. However, quantitative

site-specific differences in sialylation of CHO-derived IFN- γ have not been previously reported.

MATERIALS AND METHODS

CHO Cell Culture

Recombinant human IFN-γ was produced by a CHO cell line cotransfected with genes for dihydrofolate reductase and human IFN-γ originally provided by Dr. Walter Fiers (University of Ghent, Belgium). The cell line was selected for growth in the presence of $2.5 \times 10^{-7} M$ methotrexate (Sigma, St. Louis, MO) and adapted to serum-free SFM-CHO-II (Gibco, Gaithersburg, MD) medium supplemented with $2.5 \times 10^{-7} M$ methotrexate, 5 units ml⁻¹ penicillin (Sigma), 5 µg ml⁻¹ streptomycin (Sigma), and 10 µg ml⁻¹ neomycin (Sigma). A 100-ml suspension batch culture was performed in spinner flasks (50 rpm) in a 37°C incubator (10% CO₂). A culture was inoculated at a density of 3×10^5 cells ml $^{-1}$, and a maximum viable cell density of 1.8×10^6 cells ml⁻¹ was achieved in 72 h. Two additional cultures were performed in which 1 mM 2,3-dehydro-2-deoxy-Nacetylneuraminic acid (Sigma) was introduced into the culture supernatant at the beginning and at 72 h of cultivation, respectively. Each culture lasted seven days, and supernatant samples were collected for analysis at 24-h intervals. Cell density and viability were determined with a Neubauer Hemacytometer (Reichert, Buffalo, NY). Prior to cell counting, samples were diluted with an equal volume of 0.4% trypan blue solution (Sigma).

Site- and Branch-Specific Quantitation of Sialylation

All chromatographic separations were performed using an INTEGRAL Micro-Analytical Workstation (PerSeptive Biosystems, Framingham, MA) equipped with a highpressure microbore flow cell (Model 9550-0150, Linear Instruments, Fremont, CA). For purification of IFN-y from CHO cell culture supernatant, samples were filtered (0.22μm Millex-GS, Millipore, Bedford, MA); 1- to 1.5-ml aliquots were loaded at a flow rate of 200 μ l min⁻¹ onto a 0.76 × 150 mm column packed with immunoaffinity resin (Reselute-γ, Celltech Ltd., Slough, UK). This resin previously had been equilibrated with a loading buffer (10 mM pH 7.2) sodium phosphate (Mallinckrodt, Paris, KY) and with 150 mM NaCl (Mallinckrodt)). The immunoaffinity column was then washed with a loading buffer and step-eluted by an elution buffer (10 mM HCl (Mallinckrodt) with 150 mM NaCl). Eluted IFN-y was monitored at 220 nm, manually collected, and immediately neutralized with an equal volume of trypsin digestion buffer (200 mM pH 8.5 Tris (Mallinckrodt) with 200 mM urea (EM Science, Gibbstown, NJ)). IFN-γ concentrations were calculated based upon integrated peak areas of immunoaffinity elution peaks.

Tryptic digestion of IFN-γ was performed by adding

modified sequencing-grade trypsin (Boehringer Mannheim, Indianapolis, IN), which was reconstituted in 1 mM HCl to IFN- γ fraction in an approximately 1:5 (enzyme:substrate) mass ratio, and by allowing proteolysis to proceed at 37°C for 24 hours. Reversed-phase fractionation of tryptic peptides was performed by loading the digestion mixture on a 1 × 250 mm Vydac C18 analytical reversed-phase HPLC column (The Separations Group, Hesperia, CA); washing with 95% HPLC-grade water (EM Science), 5% acetonitrile (EM Science), containing 0.1% trifluoroacetic acid (TFA; American Bioanalytical, Natick, MA); and eluting with a 60-min linear gradient to 5% HPLC-grade water, and 95% acetonitrile, with 0.085% TFA at a flow rate of 50 µl min⁻¹. Eluted peptides were monitored at 220 nm, and fractions were manually collected and identified by matrix-assisted laser-desorption ionization/time-of-flight (MALDI/TOF) mass spectrometry.

MALDI/TOF was performed using a Voyager BioSpectrometry Workstation (PerSeptive Biosystems). Samples were prepared by mixing a 2-μl aliquot with 2 μl of matrix solution (7 mg ml⁻¹ solution of 2,5-dihydroxybenzoic acid (Aldrich, Milwaukee, WI) in 50% water:50% acetonitrile). A 1-μl sample of the mixture was spotted onto a well of the MALDI sample plate and allowed to air-dry prior to introduction into the mass spectrometer. Data for 64 3-ns pulses of the 337-nm nitrogen laser were averaged for each spectrum, and linear, positive-ion TOF detection was performed using an accelerating voltage of 28,125 V. External calibration was performed using a mixture of bradykinin (MW 1060.2; Sigma) and bovine insulin (MW 5733.5; Calbiochem, San Diego, CA).

For sialic acid-based separations of glycopeptides, sitespecific glycopeptide fractions were diluted in water, loaded onto a 1 × 250 mm Vydac C18 analytical reversed-phase HPLC column, and washed at 50 μl min⁻¹ with buffer A of pH 7 20 mM triethylamine (EM Science) and 300 mM boric acid (Mallinckrodt) in 90% HPLC-grade water:10% acetonitrile. Glycopeptides of Asn²⁵ were eluted by a 60-min linear gradient from a mixture of 80% buffer A:20% buffer B (pH 7 20 mM triethylamine and 300 mM boric acid in 50% HPLC-grade water:50% acetonitrile) to 100% buffer B. Glycopeptides of Asn⁹⁷ were eluted by a 60-min linear gradient from 100% buffer A to a mixture of 30% buffer A:70% buffer C (pH 7 20 mM triethylamine and 300 mM boric acid in 70% HPLC-grade water: 30% acetonitrile). Eluted glycopeptides were monitored at 220 nm, and fractions were manually collected and identified by MALDI/ TOF as described above.

Identification of Monosialylated Biantennary Glycopeptide Fractions

Digestion by *Streptococcus pneumoniae* β -galactosidase (Oxford Glycosystems, Abingdon, UK) and *Streptococcus pneumoniae* β -N-acetylhexosaminidase (Oxford Glycosystems) was performed by mixing 1.7 μ l of glycopeptide fraction with 0.7 μ l of digestion buffer (pH 5 50 mM sodium

citrate (Mallinckrodt), 50 mM sodium phosphate, and 25 mM ZnCl₂ (Mallinckrodt)), containing 0.24 milliunits of each enzyme and allowing reaction to proceed at 37°C for 24 h. Reversed-phase separation of digested glycopeptides from enzymes was performed by loading a digestion mixture onto a 1 × 250 mm Vydac C18 analytical reversedphase HPLC column, washing with a mixture of 95% HPLC-grade water:5% acetonitrile containing 0.1% TFA, and eluting by a 60-min linear gradient to 5% HPLC-grade water:95% acetonitrile with 0.085% TFA at a flow rate of 50 μ l min⁻¹. Digestion by jack bean α -mannosidase (Oxford Glycosystems) was performed by mixing 0.7 µl of digestion buffer containing 14 milliunits of enzyme with 1.7 ul of glycopeptide fraction and by allowing reaction to proceed at 37°C for 24 h. MALDI/TOF of digested glycopeptides was performed as described above.

Site-Specific Quantitation of Asialoantennarity

Desialylation was achieved by mixing 1 µl of site-specific glycopeptide fraction and 1 µl of 20 mM pH 5.0 sodium acetate (Fisher, Fair Lawn, NJ), which contained 4 milliunits of *Arthrobacter ureafaciens* sialidase (Oxford Glycosystems), in a well of the MALDI sample plate. The reaction was allowed to proceed at 37°C, until the mixture was completely dried due to evaporation. A 1-µl aliquot of matrix solution was then added, and the mixture was allowed to air-dry prior to MALDI/TOF analysis as described above.

RESULTS AND DISCUSSION

Analytical Methodology

Several analytical methods have been employed to quantitate sialylation of glycoproteins. To estimate total sialic acid content, charge-based separations of intact glycoproteins, such as isoelectric focusing (Righetti, 1983) or capillary electrophoresis (Watson et al., 1993), as well as colorimetric or chromatographic analysis of sialic acids chemically or enzymatically released from glycoproteins (Reuter and Schauer, 1994) can be utilized. However, site- or branchspecific information is not obtainable by such techniques. Furthermore, these analyses cannot detect differences in the number of available sialylation sites, due to variability in the site occupancy or variability in the antennarity of glycans. Quantitation of sialic acid content by high-pH anionexchange chromatography (Townsend et al., 1989) of free glycans released by chemical or enzymatic methods can reveal antennarity and branch-specificity. However, if the glycans are cleaved from the intact glycoprotein, sitespecific information is lost. Site-specific sialylation is best obtained by the analysis of glycopeptides that is obtained by proteolysis of the glycoprotein, so that the potential glycosylation sites are isolated on separate fragments. In our previous publication (Harmon et al., 1996), we described the determination of the site-specific glycosylation microheterogeneity of CHO-derived IFN-γ using a combination of immunoaffinity chromatography to purify the product from culture supernatant, the tryptic digestion to obtain the proteolytic fragments, the low-pH reversed-phase HPLC fractionation of the resultant peptides, and the matrix-assisted laser-desorption ionization/time-of-flight (MALDI/TOF) mass spectrometry of the two glycopeptide fractions representing the respective glycosylation sites to identify their oligosaccharide structures. Although asialoglycan structures could be quantitated by this methodology if the glycopeptides were desialylated by sialidase treatment prior to MALDI/TOF analysis (Sutton et al., 1994), MALDI/TOF did not quantitate adequately the sialylated glycopeptides of IFN-γ, due to the lability of the glycosidic linkage of sialic acid that led to source fragmentation (Huberty et al., 1993), and because of the variable presence of negatively-charged sialic acid residues that affected the relative ionization efficiencies of the glycopeptides (Sutton et al., 1994). Furthermore, MALDI/TOF could not distinguish between mass degenerate structures, e.g., the two isomers of the monosialo complex biantennary structure differing in the branch of sialylation, $Man(\alpha 1-3)$ or $Man(\alpha 1-6)$, which are based on the masses of the intact glycopeptides. The determination of branching by MALDI/TOF requires the interpretation of post-source decay fragmentation patterns obtained with reflectron TOF detection and is highly qualitative.

In this study, site- and branch-specific quantitation of sialylation of CHO-derived IFN- γ was obtained by introducing an additional analytical step, sialic acid-based separations of the site-specific pools of glycopeptides using a neutral pH/borate complexation reversed-phase HPLC method, previously reported by Rice et al. (1990). To illustrate the sialic acid-based separations obtained by this methodology, Figure 1 shows the separation of the Asn⁹⁷-linked tryptic glycopeptides of IFN- γ after 168 h of suspension

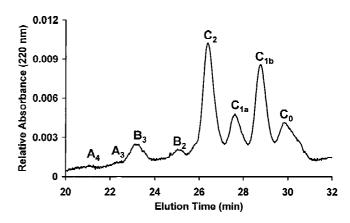


Figure 1. Neutral pH/borate complexation reversed-phase HPLC separation of Asn⁹⁷-linked IFN- γ tryptic glycopeptides, following the addition of 168 h of suspension batch CHO cell culture. A₃ and A₄ represent nonfucosylated complex tetraantennary glycans with 3 and 4 sialic acids, respectively; B₂ and B₃ indicate nonfucosylated complex triantennary glycans with 2 and 3 sialic acids, respectively; and C₀, C_{1a}, C_{1b}, and C₂ correspond to nonfucosylated complex biantennary glycans with 0, 1 (on α1-6 branch), 1 (on α1-3 branch), and 2 sialic acids, respectively.

batch CHO cell culture. Following MALDI/TOF analysis of each fraction, oligosaccharide structures were assigned, based upon the observed masses of the glycopeptides relative to the known masses of their respective amino acid sequences (i.e., 1523 and 2253 Da for the glycopeptides of

 Asn^{97} and Asn^{25} , respectively). As listed in Table 1, the observed masses of fractions C_0 , C_{1a} , C_{1b} , and C_2 in Figure 1 corresponded to glycopeptides containing complex biantennary oligosaccharide structures with zero, one, one, and two sialic acids, respectively. Fractions B_2 and B_3 were

Table I. Oligosaccharide structures for Asn⁹⁷-linked glycopeptide fractions (Figure 1) identified by MALDI-TOF mass spectrometry.

Symbol				$(M+H)^+$	mass (Da)
			Glycan structure	Observed	Expected
A_3	-GlcNAc-GlcNAc-Man	<	Man \(\text{GlcNAc-Gal} \\ \text{GlcNAc-Gal} \\ \text{GlcNAc-Gal} \\ \text{GlcNAc-Gal} \\ \text{GlcNAc-Gal} \\ \text{Man} \(\text{GlcNAc-Gal} \)	4754	4752
${ m A_4}$	–GlcNAc–GlcNAc–Man	<	Man GlcNAc-Gal-NeuAc GlcNAc-Gal-NeuAc GlcNAc-Gal-NeuAc GlcNAc-Gal-NeuAc Man GlcNAc-Gal-NeuAc	5045	5043
B_2	-GlcNAc-GlcNAc-Man	<	Man GlcNAc-Gal GlcNAc-Gal GlcNAc-Gal Aman-GlcNAc-Gal 2 NeuAc	4097	4095
\mathbf{B}_3	-GlcNAc-GlcNAc-Man	<	GlcNAc-Gal-NeuAc Man GlcNAc-Gal-NeuAc Man-GlcNAc-Gal-NeuAc	4388	4386
C_0	-GlcNAc-GlcNAc-Man	<	Man-GlcNAc-Gal Man-GlcNAc-Gal	3148	3147
C_{1a}	–GlcNAc–GlcNAc–Man	3 6	Man-GlcNAc-Gal 1 Man-GlcNAc-Gal-NeuAc	3439	3438
С _{1ь}	–GlcNAc–GlcNAc–Man	3 (Man-GlcNAc-Gal-NeuAc 1 Man-GlcNAc-Gal	3439	3438
C_2	-GlcNAc-GlcNAc-Man	<	Man-GlcNAc-Gal-NeuAc Man-GlcNAc-Gal-NeuAc	3730	3730

identified as complex triantennary glycans containing two and three sialic acids, respectively, and fractions A_3 and A_4 were classified as complex tetraantennary glycans with three and four sialic acids, respectively.

The mass-degeneracy of fractions C_{1a} and C_{1b} suggested that the separation of two monosialo biantennary structures was based upon the branch of sialylation, i.e., sialic acid on a Man(α 1-3) or Man(α 1-6) arm. Because insufficient material was available for definitive ¹H-NMR analysis, the specificity of exoglycosidase digestion and sensitivity of MALDI/TOF were employed to identify C_{1a} and C_{1b}. Since the presence of terminal sialic acid would block the digestion of sialylated branches, the two monosialo biantennary fractions exhibited similar masses, which corresponded to the losses of single Gal and GlcNAc residues following incubation with β-galactosidase and β-N-acetylhexosaminidase. However, upon treatment with jack bean α-mannosidase, only fraction C_{1a} demonstrated a mass shift of 162 Da corresponding to the loss of Man. Since jack bean α -mannosidase will cleave a single Man residue α 1-3linked to the core β -Man but will not cleave a single α 1-6-linked Man at relatively low concentrations (Yamashita et al., 1980), fractions C_{1a} and C_{1b} were identified as monosialo complex biantennary structures sialylated on the Man $(\alpha 1-6)$ and Man $(\alpha 1-3)$ branches, respectively, as depicted in Table 1. Thus, by combining tryptic digestion and neutral pH/borate complexation reversed-phase HPLC separations of the site-specific pools of tryptic glycopeptides, both sitespecific and, for the predominant complex biantennary glycan, branch-specific quantitation of sialylation was obtained. Similar separations were obtained for the glycopeptides of Asn²⁵. However, in agreement with the site-specific fucosylation of CHO-derived IFN-γ previously reported by James et al. (1995), the glycopeptides of Asn⁹⁷ each demonstrated mass shifts indicative of nonfucosylated glycans, whereas the masses of Asn²⁵-linked glycopeptides corresponded to structures containing a fucose.

A limitation of this methodology was the inability to distinguish the sialic acid linkages. Two distinct sialic acid linkages, NeuAc (α 2-3) Gal and NeuAc (α 2-6), Gal, catalyzed by α 2-3 sialyltransferase and α 2-6 sialyltransferase, respectively, have been identified in N-linked glycoproteins purified from cultured human cell lines (Takeuchi et al., 1988). However, in N-linked oligosaccharides of glycoproteins derived from CHO culture, only NeuAc (α 2-3) Gal linkages have been observed (Takeuchi et al., 1988; Parekh et al., 1989). As a result, this limitation was not of significance for the analysis of CHO-derived IFN- γ .

Site- and Branch-Specific Sialylation of IFN-γ

Since complex biantennary structures represented the majority of oligosaccharides at each glycosylation site, the sialylation percentage calculated for each glycosylation site was based upon the occupancy of the available sialylation sites of biantennary glycans:

sialylation percentage =

$$\frac{2 A_{C2} + A_{C1a} + A_{C1b}}{2(A_{C2} + A_{C1a} + A_{C1b} + A_{C0})} \times 100\%$$

where A_n is the integrated peak area of fraction n from the neutral pH/borate complexation HPLC chromatogram. Using this definition, the complex biantennary glycans of Asn²⁵ were calculated to be 80% sialylated, whereas those of Asn⁹⁷ were 70% sialylated following 168 h of cultivation. Since each glycosylation site would be expected to have similar availability of nucleotide sugar precursors, this site-specific difference in sialylation was most likely the result of differences in sialyltransferase activity due to local environmental effects. This finding is consistent with other previously-reported site-specific differences in the glycosylation of CHO-derived IFN-y, including greater site occupancy of the Asn²⁵ site (Rinderknecht et al., 1984), higher average antennarity of Asn²⁵-linked oligosaccharides than those of Asn⁹⁷ (James et al., 1995; Harmon et al., 1996), and site-specific fucosylation of the glycans of Asn²⁵ (James et al., 1995). Each of these observations suggest that the Asn²⁵ site offers a greater accessibility to the various enzymatic processes involved in glycosylation. This greater accessibility of the Asn²⁵ site could be explained by the NMR data of Grzesiek and coworkers (1992) for E. coli-derived human IFN-γ, which indicated that Asn²⁵ is contained within a loop region of high flexibility whereas Asn⁹⁷ is the final residue of an α -helical region. Such site-specific differences in sialylation have been observed for other glycoproteins. For example, Sasaki et al. (1988) have reported that the Asn²⁴-linked oligosaccharides of recombinant human erythropoietin are much less sialylated than those at Asn³⁸, despite their proximity.

The ability to separate the isomeric monosialo biantennary structures allowed the sialylation percentage of each branch to be calculated:

sialylation percentage of Man($\alpha 1$ –3) branch =

$$\frac{A_{C2} + A_{C1b}}{A_{C2} + A_{C1a} + A_{C1b} + A_{C0}} \times 100\%$$

sialylation percentage of Man(α 1–6) branch =

$$\frac{A_{C2} + A_{C1a}}{A_{C2} + A_{C1a} + A_{C1b} + A_{C0}} \times 100\%$$

At each glycosylation site, the Man(α 1-3) branch was more favorably sialylated than the Man(α 1-6) arm. Approximately 85% and 78% of Man(α 1-3) branches were sialylated at the Asn²⁵ and Asn⁹⁷ sites, respectively, while 74% and 61% of Man(α 1-6) arms contained sialic acid at the Asn²⁵ and Asn⁹⁷ sites, respectively, at 168 h of cultivation. This finding was in agreement with the ¹H-NMR data of Mutsaers et al. (1986), which indicated that the Man(α 1-3) branch of CHO-derived IFN- γ was more heavily sialylated than the Man(α 1-6) arm. Although α 2-3-sialyltransferase from CHO cells has not been studied extensively, preferential sialylation of the Man(α 1-3) arm has been previously reported for α 2-6-sialyltransferases (Gra-

benhorst et al., 1995; Joziasse et al., 1985; van den Eijnden et al., 1980). Since the Man(α 1-3) branch exhibits a rigid exposed orientation with respect to the Man(β 1-4)-GlcNAc(β 1-4) GlcNAc portion of the core, whereas the Man(α 1-6) arm possesses greater flexibility with a most stable conformation in which it folds back to the core, Joziasse et al. (1985) have suggested that this branch specificity could be related to a lower accessibility of Gal on the Man (α 1-6) branch sterically hindering sialyltransferase activity.

Due to the approximately 1 h exposure of the acid-labile glycosidic linkages of sialic acid to low pH during the initial reversed-phase HPLC fractionations of the tryptic peptides, the potential loss of sialic acid during this step was examined. The glycopeptides were incubated in 0.1% TFA for an additional 4 hours prior to neutralization and analysis by the neutral pH/borate complexation reversed-phase HPLC method. Less than 2% losses in sialylation for each site and branch were observed relative to glycopeptide fractions that did not undergo extended acidic exposure. This finding was similar to the report by Rohrer et al. (1993) of less than 3% loss of sialic acid from fetuin tryptic digests during 1 h exposures to 0.1% TFA. Thus, the incomplete sialylation and site- and branch-specific sialylation differences observed for IFN-γ were not artifacts of the analytical method.

Site- and Branch-Specific Sialylation During Batch CHO Cell Culture

An advantage of the analytical methodology presented was its sensitivity. Site- and branch-specific quantitation of sialic acid content was obtained from as little as 0.5 μg of IFN- γ . Due to these small sample requirements, sialylation could be monitored throughout a single batch culture without introducing the additional uncertainty inherent in the use of parallel cultures for single time points. Figure 2 shows IFN- γ concentrations and viable cell densities for a 100-ml suspension batch CHO cell culture performed in a spinner flask using a serum-free medium. Maximum viable cell density of 1.8×10^6 cells ml⁻¹ was obtained at 72 h and declined rapidly thereafter, whereas IFN- γ concentration reached its maximum titer at about day five and subse-

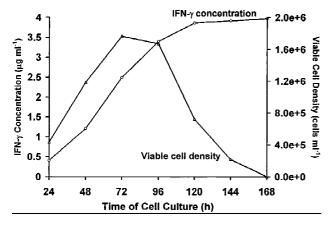


Figure 2. Viable cell density and IFN- γ concentration in a suspension batch CHO cell culture.

quently remained relatively constant. As shown in Figure 3, the relative proportions of bi-, tri-, and tetraantennary oligosaccharide structures determined by MALDI/TOF analysis of the site-specific pools of glycopeptides following desialylation did not change significantly at either site during the course of the culture. No significant accumulations of other oligosaccharide structures were detected. This finding was in contrast to the report of Hooker and coworkers (1995), who, using similar analytical techniques, observed the accumulation of oligomannose and truncated glycans for IFN-γ during batch CHO cell cultures.

In Figure 4, the percentage sialylation of the complex biantennary glycans at each glycosylation site throughout the culture is presented. Sialylation was incomplete but relatively constant during the early stages of batch culture (i.e., approximately 92% and 79% at Asn²⁵ and Asn⁹⁷, respectively). However, after 96 h, a steady decrease in sialylation was observed at each glycosylation site with sialylation percentages of 80% and 70% at Asn²⁵ and Asn⁹⁷, respectively, observed at 168 h. The loss of sialic acid content coincided with the observed decrease in viable cell density. Warner et al. (1993) have previously reported the isolation of an extracellular sialidase from CHO culture capable of desialylating exogenously-added glycoproteins, and Gramer et al. (1995) have observed a substantial increase in extracellular sialidase activity in CHO cell culture following loss of cell viability. These reports suggested that the observed decline in sialylation of IFN-γ may have been caused by extracellular sialidase released from lysed cells following cell death. Furthermore, since there was minimal biosynthesis of IFN- γ from 120 to 168 h, the observed decline in sialylation could not be attributed to the synthesis of poorly sialylated IFN-γ.

In order to verify that the observed decrease in sialic acid content following cell death was due to extracellular sialidase activity, a similar suspension batch CHO cell culture was performed in which 1 mM 2,3-dehydro-2-deoxy-N-acetylneuraminic acid (2,3-D) was introduced into the cul-

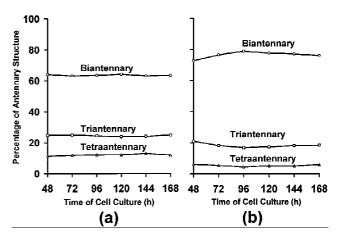


Figure 3. Effect of culture time on relative proportions of (a) Asn^{25} - and (b) Asn^{97} -linked asialoantennary glycan structures of IFN- γ in a suspension batch CHO cell culture.

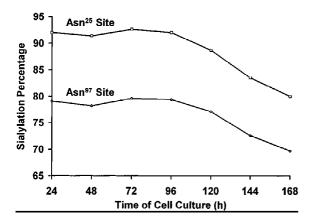


Figure 4. Effect of culture time on site-specific sialylation of complex biantennary glycans of IFN- γ in a suspension batch CHO cell culture.

ture supernatant on day 4. An analogue of sialic acid, 2,3-D has been shown to inhibit competitively (K_i of 0.025 mM) the activity of sialidase derived from CHO culture supernatant Gramer et al. (1995). Figure 5 depicts the site-specific sialylation percentages during days 4 to 7 for the culture performed with 2,3-D. The introduction of sialidase inhibitor prevented loss of sialic acid following cell death, as sialylation percentage remained essentially unchanged during the remainder of the culture. This finding confirmed that the decline in sialic acid content for the culture without 2,3-D addition was due to extracellular sialidase, presumably released during cell lysis. An approximately 12% decrease in sialylation of both Asn²⁵- and Asn⁹⁷-linked biantennary glycans was observed between days 4 and 7 for the culture lacking 2,3-D, thus indicating that, although there was preferential sialylation of the glycans of Asn²⁵, extracellular desialylation did not discriminate significantly between the glycosylation sites. A similar 12% loss of sialic acid has been previously reported for a 6-day incubation of gp120 glycoprotein exogenously added to a batch CHO cell culture Gramer et al. (1995). The lack of significant accumulation of truncated oligosaccharide structures during the

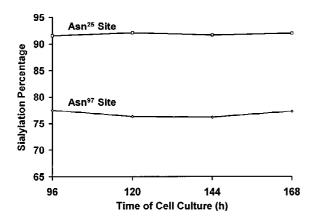


Figure 5. Effect of sialidase inhibitor 2,3-dehydro-2-deoxy-N-acetylneuraminic acid on site-specific sialylation of complex biantennary glycans of IFN- γ in a suspension batch CHO cell culture.

culture indicated that there was no significant activity of glycosidases other than sialidase.

While the loss in sialylation following cell death could be attributed to extracellular sialidase activity, incomplete sialylation was observed at each glycosylation prior to cell lysis. Work by Gramer et al. (1995) has indicated that secretion of lysosomal sialidase does not contribute significantly to the accumulation of extracellular sialidase activity in CHO culture. As a result, the release of cytosolic sialidase resulting from damage to the cellular membrane is likely to be the predominant mechanism for extracellular sialidase activity, and the lack of sialylation prior to cell lysis is expected to result from intracellular processes. To verify that incomplete sialylation observed for CHOderived IFN- γ in the early stages of the cultivation was not due to extracellular desialylation, a culture was performed in which 2,3-D was added to the supernatant at cell inoculation. The sialylation percentages at day 4 for this culture (i.e., 92% and 80% for Asn²⁵- and Asn⁹⁷-linked glycans, respectively) were nearly identical to those obtained without sialidase inhibitor. This result confirmed that the incomplete sialylation observed prior to loss of cell viability was a result of incomplete intracellular sialylation rather than extracellular desialylation. Based upon this experiment, it cannot be stated whether the observed incomplete intracellular sialylation was due to incomplete biosynthetic sialylation or to intracellular degradation of more fully sialylated product. However, since intracellular IFN- γ is expected to be either in the Golgi complex or within secretory vesicles with minimal exposure to cytosolic sialidase, incomplete synthetic sialylation is suspected to be predominantly responsible for incomplete intracellular sialylation.

Figure 6 shows the branch-specific sialylation of the complex biantennary glycans of IFN- γ at each glycosylation site during the suspension batch CHO cell culture. Prior to loss of cell viability, the sialylation of each branch was relatively constant, i.e., approximately 98% and 91% sialylation of Man(α 1-3) and 86% and 67% sialylation of

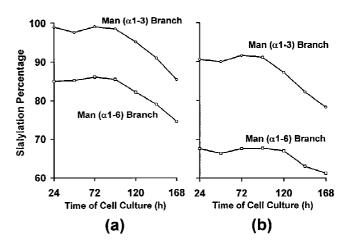


Figure 6. Effect of culture time on branch-specific sialylation of (a) Asn^{25} - and (b) Asn^{97} -linked complex biantennary glycans of IFN- γ in a suspension batch CHO cell culture.

 $Man(\alpha 1\text{-}6)$ branches at Asn^{25} and Asn^{97} , respectively. Following cell lysis, sialylation of each arm declined steadily at each glycosylation site, thus indicating that both branches were vulnerable to extracellular sialidase activity. In observing the various glycosylation sites and branches, it was clear that the wide range of values for intracellular sialylation and the similar susceptibilities to extracellular desialylation suggest either that 1) the predominant cause for incomplete intracellular sialylation was incomplete biosynthetic sialylation arising from variances in steric accessibility for sialyltransferase, or 2) intracellular desialylation proceeded by a different mechanism than extracellular desialylation.

CONCLUSIONS

Based upon data obtained prior to loss of cell viability, both site- and branch-specific differences in intracellular sialylation of CHO-derived human IFN-γ have been observed. The glycans of Asn²⁵ were found to be more heavily sialylated than those of Asn^{97} , and $Man(\alpha 1-3)$ branches of the predominant complex biantennary glycans were more favorably sialylated than $Man(\alpha 1-6)$ arms at each site. The wide variability in sialylation of the sites and branches indicated that intracellular sialylation was highly dependent upon local environments. As a result, in attempting to optimize sialic acid content of recombinant glycoprotein products (e.g., by increasing the availability of nucleotide sugar precursors or sialyltransferase activities), such physical constraints inherent in the conformations of specific glycoproteins may limit the maximal sialylation which can be achieved. During the cultivation of a suspension batch CHO cell culture, extracellular desialylation of IFN-y was observed following cell lysis. The oligosaccharides at each glycosylation site as well as each branch of the predominant complex biantennary glycans were found to be susceptible to extracellular desialylation. As a result, both intracellular and extracellular processes defined the sialic acid content of the final product. Although most batch cultures performed in industry are terminated prior to massive cell death and its desialylating effects, significant accumulation of extracellular sialidase during the course of extended cultures (e.g., fed-batch cultures) may also yield undesired desialylation of glycoprotein products.

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