Monitoring Recombinant Human Interferon-Gamma N-Glycosylation During Perfused Fluidized-Bed and Stirred-Tank Batch Culture of CHO Cells

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Abstract: Chinese hamster ovary cells producing recombinant human interferon-γ were cultivated for 500 h attached to macroporous microcarriers in a perfused, fluidized-bed bioreactor, reaching a maximum cell density in excess of 3×10^7 cells (mL microcarrier)$^{-1}$ at a specific growth rate (μ) of 0.010 h^{-1}. During establishment of the culture, the N-glycosylation of secreted recombinant IFN-γ was monitored by capillary electrophoresis of intact IFN-γ proteins and by HPLC analysis of released N-glycans. Rapid analysis of IFN-γ by micellar electrokinetic capillary chromatography resolved the three glycosylation site occupancy variants of recombinant IFN-γ (two Asn sites occupied, one Asn site occupied and nonglycosylated) in under 10 min per sample; the relative proportions of these variants remained constant during culture. Analysis of IFN-γ by capillary isoelectric focusing resolved at least 11 differently sialylated glycoforms over a pI range of 3.4 to 6.4, enabling rapid quantitation of this important source of microheterogeneity. During perfusion culture the relative proportion of acidic IFN-γ proteins increased after 210 h of culture, indicative of an increase in N-glycan sialylation. This was confirmed by cation-exchange HPLC analysis of released, fluorophore-labeled N-glycans, which showed an increase in the proportion of tri- and tetrasialylated N-glycans associated with IFN-γ during culture, with a concomitant decrease in the proportion of monosialylated and neutral N-glycans. Comparative analyses of IFN-γ produced by CHO cells in stirred-tank culture showed that N-glycan sialylation was stable until late in culture, when a decline in sialylation coincided with the onset of cell death and lysis. This study demonstrates that different modes of capillary electrophoresis can be employed to rapidly and quantitatively monitor the main sources of glycoprotein variation, and that the culture system and operation may influence the glycosylation of a recombinant glycoprotein. *Biotechnol Bioeng* **60**: 596–607, 1998.

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INTRODUCTION

To date, the vast majority of industrial bioprocesses for the production of recombinant proteins by animal cells employ homogeneous, low cell density (<10^7 cells mL^{-1}) suspension culture systems, which are relatively simple to operate and scale-up (Lubiniecki, 1990). By contrast, more complex high cell density (>10^7 cells mL^{-1}) culture systems, where cells are physically retained in the reactor by a variety of means, are an increasingly attractive alternative for continuous production. These compact systems offer the potential advantages of high volumetric production rate at low cell growth rate, but may be considered difficult to operate reliably. While the major engineering challenges (e.g., reactor design, aeration, mixing, media formulation) associated with these systems have been addressed (Ozturk, 1996), such that large-scale industrial operation is possible (Bhat et al., 1995; Gray et al., 1996), few reports describe the effect of long-term continuous culture on the processing and modification of complex recombinant proteins produced in this way.

In the case of batch and fed-batch cultures, it is now clear that complex modifications such has glycosylation, which may affect the immunogenicity, half-life, bioactivity and stability of a potential therapeutic product (Jenkins and Curling, 1994), and which lead to a structurally heterogeneous protein product, are not constant during culture. In this laboratory, the N-glycosylation microheterogeneity (diversity of N-glycan structures at a glycosylation site) of recombinant IFN-γ produced by CHO cells was demonstrated to alter markedly during batch culture, with an increase in the proportion of high-mannose and truncated oligosaccharides (Hooker et al., 1995). Similar data have pre-

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viously been obtained for the glycosylation of a monoclonal antibody (Robinson et al., 1994). In fact, multiple bioprocess factors are known to affect recombinant protein heterogeneity, and control of recombinant protein glycosylation may be achieved at a number of levels: choice of host cell, genetic engineering of glycan processing, or control of bioprocess parameters such as culture environment, method of cell culture, and culture time (Jenkins et al., 1996).

The above considerations emphasize the necessity to monitor glycosylation of a recombinant protein or antibody accurately during all stages of a bioprocess, during both production and downstream processing (Paliwal et al., 1993), as a means of ensuring reproducible product quality, batch-to-batch consistency, and stability. Indeed, regulatory authorities such as the US Food and Drug Administration are requiring increasingly sophisticated carbohydrate analyses as part of the ''well-characterized product'' or process validation (Liu, 1992). Technical advances in a variety of analytical techniques now permit detailed assessment of recombinant protein primary structure and glycosylation offline [e.g., mass spectrometry (James, 1996)], however, rapid monitoring of recombinant protein glycosylation would enable (1) control of fermentation operations for enhancement of product quality (e.g., prevention of protein degradation), (2) control of downstream chromatographic operations for recovery of specific product variants, and (3) rapid assessment of the effect of changes in bioprocess operations on product heterogeneity. Recent reports highlight the use of mass spectrometry to ''rapidly'' monitor the site-specific glycosylation of recombinant human interferon-γ (Harmon et al., 1996). However, the procedures reported are not amenable to automation, do not permit quantitation of key sources of variability such as N-glycan sialylation, and extensive interpretation of mass spectral data is necessary for each assay.

Capillary electrophoresis (CE) may be employed to rapidly assay the concentration of recombinant proteins in crude process streams (Freitag et al., 1996; Strege and Lagu, 1995), and is proving itself to be a versatile analytical tool for recombinant (glyco)protein analysis both by itself (Novotny, 1996; Teshima and Wu, 1996), and in combination with other analytical techniques such as mass spectrometry (Figeys et al., 1997; Kelly et al., 1996). There are now several examples of glycoprotein analysis by CE, from analysis of intact glycoproteins (Hoffstetterkuhn et al., 1996; James et al., 1994; Moorhouse et al., 1996; Morbeck et al., 1994), glycopeptides (Rush et al., 1993), released oligosaccharides (Kakehi et al., 1996; Okafo et al., 1996; Weber and Lunte, 1996) to individual monosaccharides (Guttman, 1997). These examples employ a variety of separation mechanisms (e.g., capillary zone electrophoresis, micellar electrokinetic capillary chromatography, capillary isoelectric focusing) designed to enhance the resolution of the specific analyte(s) in question. It is this flexibility of approach that promotes the utility of capillary electrophoresis as a tool for recombinant protein monitoring and analysis.

Recombinant IFN-γ produced by chinese hamster ovary (CHO) cells has two N-linked glycosylation sites, Asn_{25} and Asn_{97}, which are both variably occupied and predominantly associated with complex-type oligosaccharides (James et al., 1995; James et al., 1996). In this article we demonstrate that different capillary electrophoretic separations of intact IFN-γ proteins can be employed to both rapidly and quantitatively monitor changes in N-glycosylation and sialylation of recombinant IFN-γ produced by mammalian cells in culture. In combination with complementary HPLC analyses of variably sialylated oligosaccharides, we show that these sources of heterogeneity vary with respect to method of cell culture (growth of cells in fluidized-bed perfusion or stirred-tank bioreactors) and time in culture.

MATERIALS AND METHODS

Chemicals

Analytical grade reagents were purchased from Sigma Chemical Company (Poole, UK) unless otherwise stated. Monoclonal antibody 20B8 coupled to Sepharose was provided by Lonza Biologics (Slough, UK). Neuraminidase from *Arthrobacter ureafaciens* was purchased from Oxford Glycosciences Ltd. (Abingdon, UK).

Cell Line and Culture

Recombinant human IFN-γ was produced by the Duk cell line derived from mutant CHO-K1 cells lacking dihydrofolate reductase ($DHFR^-$) co-transfected with the genes for IFN-γ and DHFR at Wellcome Research Laboratories (Beckenham, UK). The IFN-g gene was inserted using a plasmid vector derived from pSV2-dhfr, which contains the SV40 early promoter. This cell line (clone 320) was adapted for growth in serum-free medium in this laboratory (RPMI 1640 supplemented with 5 mg mL^{-1} bovine serum albumin (Miles Biochemicals, Slough, UK), 5 μg mL^{-1} bovine insulin, 5 μg mL^{-1} human transferrin, 1 m*M* sodium pyruvate, 0.1 m*M* alanine, 2 m*M* glutamine, 1 μ*M* putrescine, 3 μ*M* $ZnSO_4$, 10 n*M* Na_2SeO_3, 10 n*M* $CuSO_4$ (Sigma, Poole, UK) (Hayter et al., 1991). For batch culture, initial glucose concentration was 9 m*M*, glucose concentrations employed in perfusion culture media are described in the text. Expression of IFN-γ and DHFR genes was coamplified by methotrexate selection (1 μ*M*).

Perfusion culture employed a 2 L Cytopilot®-Mini fluidized bed reactor (Pharmacia Biotech, St. Albans, UK) with a 200 mL Cytoline 1 (Pharmacia Biotech) microcarrier bed volume. Conditions of constant dissolved oxygen tension (40% of air saturation by sparging), pH 7.2 (maintained by CO_2 and 0.1 *M* NaOH addition), and a temperature of 37°C were maintained by two independent controllers (Anglicon, Brighton, UK). An inoculum density of 2.0×10^6 cells mL $microcarrier^{-1}$ was employed. The agitation rate was maintained at 150 rpm in packed-bed mode for 3 h to encourage

cell attachment and 450 rpm during bed fluidization. Aliquots of perfusate (1 L) were collected throughout the culture and stored at −80°C. Cell density on microcarriers was determined by addition of 1 mL of 0.1*M* citric acid, 0.1% (w/v) crystal violet, 1.0% (v/v) Triton X-100 to a 1 mL sample of microcarriers with constant rotation for 2 h at 37°C to remove and stain cell nuclei, followed by counting in a Neubauer chamber. The released and fixed cell nuclei were stained with propidium iodide for subsequent flow cytometric analysis of cell cycle stage with a Coulter Epics II instrument as previously described (Jenkins and Hovey, 1993). Routine glucose, lactate, and ammonia determinations were made using a Biolyzer (Kodak IBI Ltd., Cambridge, UK).

Batch suspension culture of CHO cells was performed with a 15 L bioreactor (InCeltech, Reading, UK), using an operating culture volume of 12 L, as described previously (Hooker et al., 1995). Bioreactor cultures were seeded at 0.15×10^6 cells mL^{-1} using cells washed and resuspended in fresh medium to avoid spent medium carryover. Conditions of constant dissolved oxygen tension (40% air saturation by sparging with air), pH 7.2 (adjusted by CO_2 addition), and a temperature of 37°C were maintained. After each removal of a culture sample for IFN-γ analysis, the stirrer speed was stepped down with decreasing culture volume to maintain a constant power-to-volume ratio of 5×10^3 $W \cdot L^{-1}$.

Scanning Electron Microscopy of Macroporous Microcarriers

A 1 mL sample of Cytoline 1 microcarriers was removed from the reactor and transferred to a 15 mL centrifuge tube. The microcarriers were allowed to settle, and the supernatant removed by aspiration. The microcarriers were then washed in 1 mL of PBS followed by the addition of 1 mL of 2% (v/v) glutaraldehyde in PBS. After incubation overnight at 4°C the microcarriers were washsed twice with PBS and dehydrated in ethanol. Ethanol was subsequently substituted by acetone in increasing proportion: 25%, 50%, 75%, and 100% (v/v). The microcarriers were treated by critical point drying with liquefied carbon dioxide, and then coated with gold in an argon atmosphere. Cell adhesion was observed with a 525*M* scanning electron microscope (Phillips, Eindhoven, The Netherlands).

Immunoaffinity Purification of IFN-γ

Cell-free supernatant from cell culture was adjusted to pH 6.5 with 50 m*M* 2-[N-morpholino]ethanesulfonic acid (MES) and 0.02% (w/v) NaN_3 (buffer 1). Samples were loaded onto a 1 × 10 cm immunoaffinity column (monoclonal antibody anti-IFN-γ 20B8, covalently coupled to cyanogen bromide activated Sepharose beads) at a flow rate of 1.0 mL min^{-1}. Unbound protein was washed off the matrix with 100 mL of 250 m*M* MES, 1*M* NaCl, 0.1% Tween-20 pH 6.5, followed by 100 mL of buffer 1. Bound IFN-γ was eluted in 13.5 mL of 0.1*M* glycine-HCl, pH 2.5, and immediately neutralized with 1.5 mL of 1*M* Tris-HCl (pH 8.0). Purified IFN-γ was concentrated and desalted by ultrafiltration with Centriprep® concentrators (Amicon Ltd., Stonehouse, UK), and then buffer-exchanged with 10 m*M* ammonium bicarbonate buffer, containing 0.02% (w/v) NaN_3 (pH 8.0), and stored at 4°C. Monoclonal antibody 20B8 has previously been shown to be specific for the IFN-γ peptide by epitope mapping (Hooker et al., 1997), thus, the immunoaffinity purification is independent of N-glycans associated with IFN-γ. Recombinant IFN-γ prepared by this method was >98% pure, as determined by analysis of silver-stained SDS polyacrylamide gels (data not shown), and yields were typically >95%, as determined by ELISA (data not shown).

ELISA

Sandwich ELISA of IFN-γ was performed using 96-well assay plates (Dynatech, Billinghurst, UK) coated with a rabbit polyclonal antibody raised against a mixed population of CHO-derived recombinant human IFN-γ. Captured recombinant IFN-γ was subsequently detected using monoclonal antibody 20B8 directed against human IFN-γ (Lonza Biologics, Slough, UK) and anti-mouse polyvalent immunoglobulins-horseradish peroxidase conjugate. Plates were read at 490 nm with a Dynatech MRX plate reader (Dynatech). Purified recombinant human IFN-μγ derived from CHO cells had a specific activity of 0.5×10^7 IU mg^{-1}.

Protein Assay

Protein assays were performed with an assay kit supplied by Bio-Rad Laboratories Ltd., Hemel Hempstead, UK. BSA was used as standard.

Capillary Electrophoresis

All separations were performed with a PACE 2100 capillary electrophoresis system supplied by Beckman Instruments (United Kingdom) Ltd., High Wycombe, UK. Data were analyzed with ''System Gold'' software supplied by Beckman Instruments. Separation of protein glycoforms by micellar electrokinetic capillary chromatography (MECC) occurred in uncoated fused silica capillaries (50 cm × 50 μm i.d.) and was performed using electrophoresis buffers containing borate and SDS, as described previously (James et al., 1994). Rapid analyses typically employed a 50 m*M* borate, 50 m*M* SDS, pH 8.5 buffer system. Separations by capillary isoelectric focusing were performed using an eCAP cIEF 3-10 kit (Beckman Instruments) using protocols, reagents, and neutral coated capillaries supplied by the manufacturer. Final sample concentration in sample solution containing ampholytes was adjusted to 250 μg mL^{-1} and cIEF was performed at a constant voltage of 13.5 kV, at a temperature of 20°C and detection at 280 nm (0.04 AU,

negative offset: 20%). Separations were calibrated using synthetic pI standards supplied.

Release of Sialylated N-Glycans from Recombinant IFN-γ

Prior to digestion with PNGase F (recombinant *Flavobacterium meningosepticum* produced in *E. coli;* Oxford Glycosystems Ltd.), approximately 500 μg IFN-γ protein was denatured by addition of 8 μL 10% (w/v) SDS and boiling for 2 min. Proteins were then digested at 30°C for 18 h in a final volume of 200 μL, contaiing 0.5% (v/v) Nonidet P-40 (BDH, Poole, UK), 0.1% (w/v) SDS, 20 units of PNGase F (desalted by repeated ultrafiltration with Microcon™ centrifugal concentrators) and 50 m*M* NH_4HCO_3, pH 8.1.

Released sialylated N-glycans were separated from deglycosylated IFN-γ polypeptide and detergents by RP-HPLC with a Waters 616 system (Waters Ltd., Watford, UK). The entire digestion mixture was loaded onto a Waters Delta-Pak™ C_{18} column (300 Å, 5 μg, 2 × 150 mm) at a flow rate of 0.2 mL min^{-1}. N-glycans were collected in the first 8 mL of column flow-through in solvent A (0.05% TFA in H_2O), then concentrated and desalted by lyophilization.

Analysis of 2-AB Labeled Sialylated N-Glycans by HPLC

Sialylated N-glycans released from recombinant IFN-γ proteins were reductively aminated at the reducing terminus with the fluorophore 2-aminobenzamide (2-AB; excitation 330 nm, emission 420 nm) using a kit supplied by Oxford Glycosystems Ltd., Abingdon, UK. All procedures were carried out according to manufacturer's instructions. 2-AB labeled N-glycans were separated into neutral, mono-, di-, tri-, or tetra-sialylated structures by HPLC with a Glycosep™ C cation-exchange column (4.6 × 100 mm; Oxford Glycosystems Ltd.). By this procedure, neutral oligosaccharides elute in the void volume while charged N-glycans are progressively eluted using a linear gradient of 100% solvent A (20% acetonitrile, 80% H_2O) to 100% solvent B (20% acetonitrile, 80% 250 m*M* ammonium formate, pH 4.5) at a flow rate of 0.3 mL min^{-1} over 35 min. Oligosaccharide elution was monitored with a Waters 474 scanning fluorescent detector.

RESULTS AND DISCUSSION

Growth and Productivity of CHO Cells Secreting Recombinant Human IFN-γ in Fluidized-Bed Perfusion Culture

The experiment described employed a modular fluidized-bed perfusion bioreactor that has previously been shown to be suitable for culture of CHO cells at high cell density (Reiter et al., 1991). A schematic diagram of medium flow within the bioreactor, and the macroporous nature of the Cytoline 1 microcarriers is illustrated in Figure 1. Cells

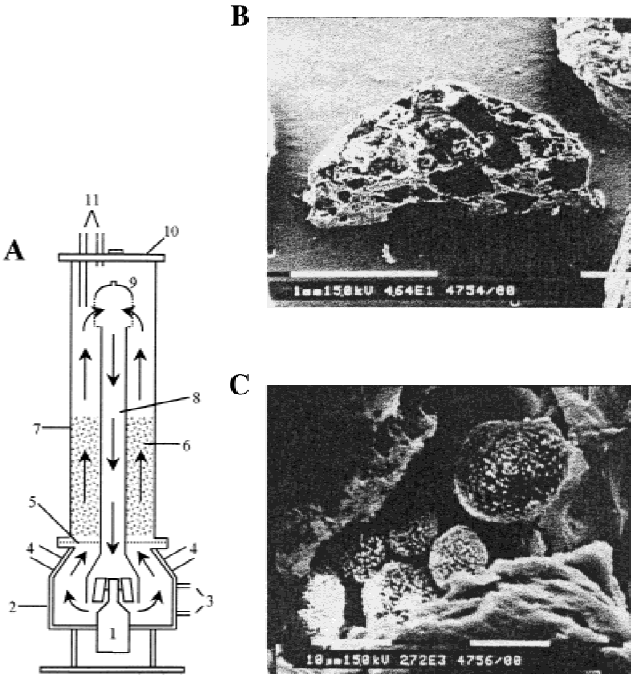

Figure 1. The modular fluidized-bed bioreactor and macroporous microcarriers employed in this study. **(A)** Schematic diagram of the principal components and flow within the reactor during fluidized-bed mode: 1 = magnetic stirrer; 2 = stainless-steel double jacketed housing; 3 = inlet and outlet for heating circuit; 4 = probe nozzles; 5 = porous gas/liquid distribution plate; 6 = macroporous microcarriers; 7 = glass cylinder; 8 = downflow section of draft tube; 9 = retention sieve; 10 = headplate; 11 = medium inlet and outlet for continuous-mode operation. **(B)** Scanning electron micrograph of a sectioned Cytoline 1 macroporous microcarrier (bar = 1 mm). **(C)** Scanning electron micrograph of CHO320 cells attached to a microcarrier (bar = 10 μm).

were visualized within the pores by scanning electron microscopy.

During establishment of the perfusion culture, glucose consumption rate was calculated daily and was used as a guide to determine the required perfusion rate to maintain a glucose concentration of approximately 2 m*M* (Fig. 2). The initial decrease in glucose concentration was matched by a characteristic sharp increase in lactate concentration, which reached a peak of 14 m*M* after 140 h, a typical value for CHO320 suspension cultures at the end of batch growth. The concentration of ammonia increased streadily during the first 250 h, reaching a maximum of 1.6 m*M*. Glucose concentration has previously been shown to be appropriate for direct feedback control of medium perfusion rate through long-term cultures of CHO cells (Konstantinov et al., 1996). We assume that cell growth was limited primarily by glucose concentration, as shown previously for glucose-limited chemostat culture of this cell line in this laboratory (Hayter et al., 1992), where glucose feed concentrations of 2.75 m*M* and 4.25 m*M* were employed. Cell density on the macroporous microcarriers, monitored by nuclei counts, was observed to increase steadily, reaching a maximum of about 3.8×10^7 cells mL microcarriers^{-1} after approximately 400 h. This correlated with a decline in glucose

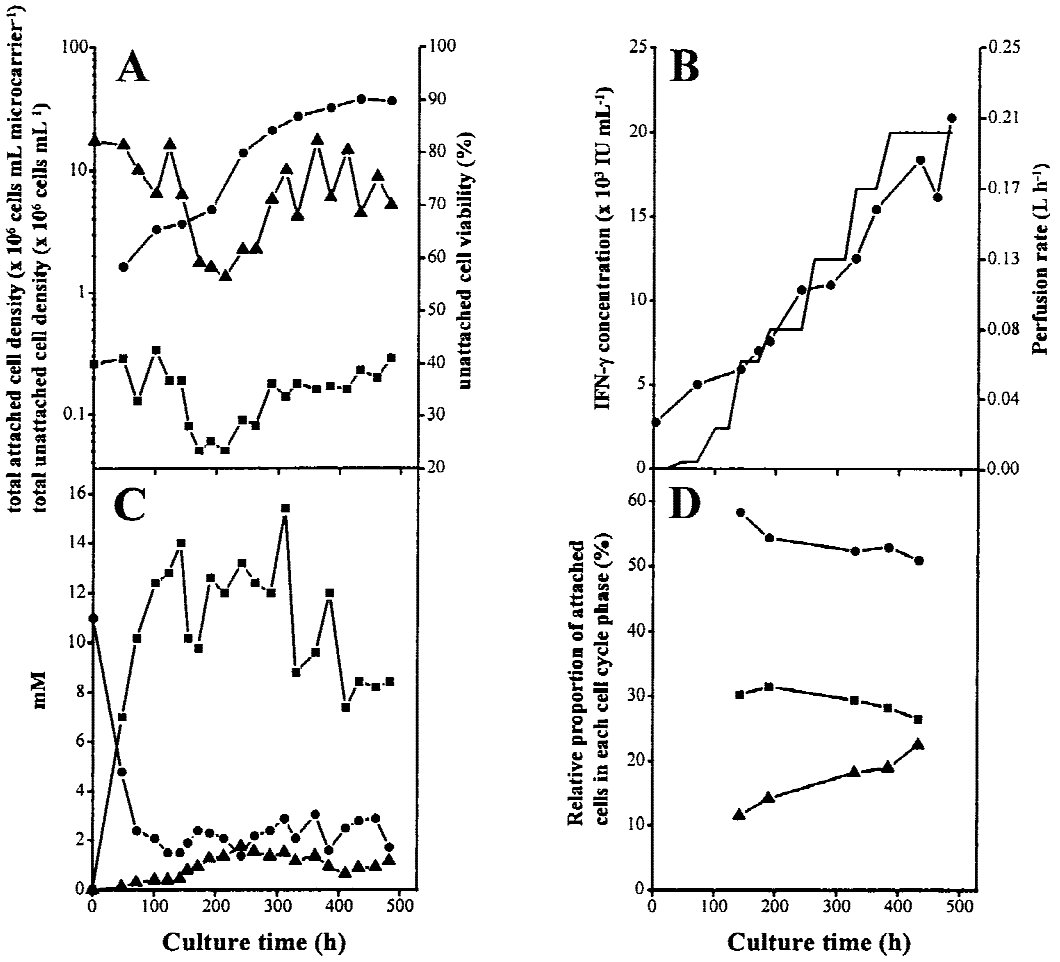

Figure 2. Growth and productivity of CHO320 cells during fluidized-bed perfusion culture. **(A)** Total density of attached (●) and unattached (■) cells; viability of unattached cells (▲). **(B)** Recombinant IFN-γ concentration in perfusate (●) and perfusion rate (−). **(C)** Glucose (●), lactate (■), and ammonia (▲) concentrations in perfusate. **(D)** Relative proportion of cells in different phases of the cell cycle; G_1-phase (●), S-phase (■), G_2/M-phase (▲).

consumption rate (data not shown), at a maximum perfusion rate of 0.20 L h^{-1} (or 25-fold bed volumes per day) by the end of the culture, thus indicating that a maximum population density had been reached under the conditions employed. The specific growth rate (μ) in perfusion culture was 0.010 h^{-1}, which was lower than that observed during stirred-tank culture of this cell line [0.028 h^{-1} in this study (Fig. 6) and typically 0.020–0.030 h^{-1}].

The maximum viable cell density obtained during stirred-tank culture of the CHO320 cell line in the same medium was 0.54×10^6 cells mL^{-1} (Fig. 6; and typically $0.6–1.0 \times 10^6$ cells mL^{-1}; Hooker et al., 1995). Thus, we observed an approximate 4–5 fold increase in cell density if the culture systems are compared on the basis of reactor volume (the 200 mL microcarrier bed occupied 10% of the total reactor volume). This perfusion system may be operated with a maximum microcarrier bed volume of 500 mL, so there is the potential for an overall 10-fold increase in cell density over stirred-tank culture.

The viability of unattached cells was monitored during the perfusion culture by trypan blue exclusion. During the majority of the culture lifetime, cell viability remained above 70% although after 120 h viability dropped to 57% before recovering after 210 h. This drop in viability may be related to the rapid reduction in medium glucose concentration from above 11 m*M* to below 2 m*M*. After an adaptation period to this lower glucose concentration cell viability recovered. It has previously been determined by confocal microscopy of microcarriers stained with fluorescein diacetate and ethidium bromide that the viability of unattached cells is an accurate indicator of attached cell viability within microcarriers (G. Blüml, Institute of Applied Microbiology, Vienna, Austria; personal communication). We infer that cells may detach from microcarriers regardless of their viability.

During the course of perfusion culture the cell cycle of cells attached to microcarriers was monitored by flow cytometry. The proportion of cells in G_1 and S phases declined very slowly while the proportion of cells in G_2/M phase increased, suggesting a gradual arrest of the cell cycle in this phase. Comparison of these data with similar analyses of unattached cells showed that cells attached to microcarriers generally had a lower proportion of cells in G_2/M (−5–10%), a higher proportion in S (+5–10%), and similar pro-

portion in G_1. This observation suggests that cells in G_2/M-phase, and possibly G_1-phase may detach more easily than those in S-phase. It is known that cells ''round-up'' during preparation for cell division during G_2/M, and this change from flatter, attached morphology may be significant; newly formed cells in G_1 would share this morphology before they begin to spread across the microcarrier surface.

The role of cell cycle in determining cellular productivity is more difficult to establish. Previous studies have shown that protein expression varies with CHO cell line and the gene expressed. Recombinant gene expression in CHO cells has been reported as being aphasic (Feder et al., 1989), occurring maximally in G_1-phase (Kubbies and Stockinger, 1990), S-phase (Gu et al., 1993; Kubbies and Stockinger, 1990), or G_2/M (Aggeler et al., 1982). Recent work by M. Al-Rubeai and colleagues using CHO320 cells producing IFN-γ has shown that cell specific productivity is only partly related to cell cycle, although maximum specific productivity in batch culture was always associated with a high proportion of S-phase cells (personal communication). However, recent reports highlight the possibility of maintaining cells in G_1 phase as a method for extending the productivity of viable cells. This may be achieved by lowering culture temperature (Chuppa et al., 1997; Moore et al., 1997) or by genetic engineering of the cell cycle (Fussenegger et al., 1997).

Recombinant IFN-γ concentration, measured by ELISA, increased in perfusate to a maximum of 21×10^3 IU mL^{-1} (4.2 μg mL^{-1}), approximately double that observed at the end of stirred-tank culture (~9–10×10^3 IU mL^{-1}; Fig. 6). During perfusion culture, average cell specific productivity, qIFN, was 397 IU 10^6 $cells^{-1}$ h^{-1}, which also exceeded maximum specific productivity in stirred-tank culture [303 IU 10^6 $cells^{-1}$ h^{-1} in this study (Fig. 6), and typically 270–300 IU 10^6 $cells^{-1}$ h^{-1}; Hooker et al., 1995]. While the apparent increase in recombinant IFN-γ concentration in perfusate appeared to correlate well to increases in attached cell density, this factor alone does not define the productivity of the system. In fact, the dynamics of cell growth and production of recombinant proteins in perfused and fixed-bed systems, as compared to more conventional batch systems has only recently been examined. Production of monoclonal antibodies by BHK cells in a fluidized bed reactor has been shown to be influenced by a combination of process parameters—high productivity is not primarily the result of greater cell numbers within the system, but more the physicochemical definition of the system (Griffiths and Racher, 1994).

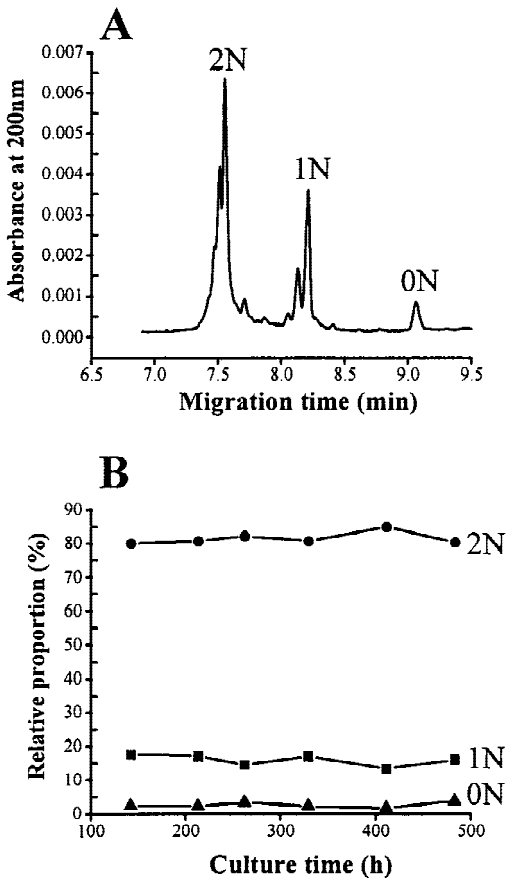

Figure 3. Monitoring recombinant human IFN-γ glycosylation site occupancy by micellar electrokinetic capillary chromatography. **(A)** Typical separation of immunoaffinity purified recombinant IFN-γ glycosylation site occupancy variants by MECC in 50 m*M* sodium borate, 50 m*M* SDS, pH 8.5; 5 s hydrodynamic injection of IFN-γ at 200 μg mL^{-1}. 2N, glycosylated at Asn_{25} and Asn_{97}; 1N, glycosylated at Asn_{25}; 0N, non-glycosylated. **(B)** Quantitative analysis of glycosylation site occupancy during culture.

Monitoring Variable N-Glycosylation Site-Occupancy of Recombinant IFN-γ by Micellar Electrokinetic Capillary Chromatography

Time-course samples from the perfusion culture were harvested and the immunoaffinity-purified recombinant IFN-γ analyzed by MECC (Fig. 3A). High-resolution separations of recombinant human IFN-γ glycoforms by MECC have been developed in this laboratory previously (James et al., 1994). This technique, which is reliant on anionic borate-sugar diol complexation and variable electrostatic repulsion with slowly migrating SDS micelles, provides quantitative information on the relative proportions of the three site-occupancy variants of IFN-γ: two Asn sites may be occupied (2N; Asn_{25} and Asn_{97}), one site occupied (1N; Asn_{25}) or no sites are occupied (0N). SDS-gel capillary electrophoresis (PAGCE; Beckman Instruments (United Kingdom) Ltd., High Wycombe, UK) was evaluated as an alternative to MECC as a strategy for monitoring (data not shown). While the three major variants were resolved by this technique, PAGCE was inferior in a number of respects—lower component resolution, reduced sensitivity, and longer migration times (~20 min). Molecular weight determinations by PAGCE were also markedly inflated by glycosylation. The PAGCE gel-sieving method required IFN-γ protein concentrations in excess of 1 mg mL^{-1} for quantitative analyses of site occupancy, whereas the MECC method

quantitated IFN-γ glycoforms reliably with sample protein concentrations below 25 μg mL^{-1}. Therefore, rapid MECC was far more suitable for routine quantitation of site occupancy.

The quantitative analysis of site-occupany variation during perfusion culture is shown in Fig. 3B. The electropherograms demonstrated similar profiles for each of the time-course samples (not shown), which indicated no major changes in the proportion of site-occupancy variants; no more than 5% of IFN-γ molecules were nonglycosylated at any stage, and the 2N variant was predominant throughout. Although there was some variation in the individual proportions of each glycoform, there was no apparent overall change during the 500 h culture lifetime. Integration of peak areas permitted calculation of the mean number of N-glycans per IFN-γ polypeptide. This was found to be consistent and within the range 1.77 to 1.83 N-glycans per IFN-γ monomer.

Monitoring IFN-γ N-Glycosylation Microheterogeneity by Capillary Isoelectricfocusing

N-Glycan sialylation is a key determinant of the circulatory half-life of serum glycoproteins (Morell et al., 1971) and therefore may have direct influence on the pharmacokinetic behavior of a therapeutic protein product. Cellular sialylation is inherently variable and has been shown to respond to culture environment (Andersen and Goochee, 1995). Therefore, rapid monitoring of this source of heterogeneity is extremely desirable. As employed in this laboratory, MS and some HPLC approaches do not easily lend themselves to rapid monitoring strategies and consequently, we evaluated CE as a technique to rapidly quantitate sialylation of IFN-γ during perfusion culture. It was possible to increase the concentration of borate and SDS in the MECC electrophoresis buffers to provide a high-resolution separation of IFN-γ glycoforms (James et al., 1994). However, this method will only provide separations based on the overall amount of carbohydrate associated with the IFN-γ polypeptide and would not adequately resolve IFN-γ molecules with different contents of negatively-charged sialic acids per se. Slab-gel isoelectric-focusing (IEF) has proved a versatile tool for the analysis of charge-variants of proteins and this technique has recently been adapted for operation in polymer gel-filled capillaries using standard CE equipment. A number of workers now report their use of cIEF for analysis of recombinant glycoproteins (Chen et al., 1996; Moorhouse et al., 1996; Thorne et al., 1996; Yowell et al., 1993).

Purified IFN-γ samples were analyzed by cIEF, and typical electropherograms of sialylated and enzymatically desialylated proteins are presented in Figure 4. For the sialylated IFN-γ, eleven major isoelectric point variants were repeatedly obtained, with pI values ranging between 6.4 and 3.4 (Fig. 4B). Desialylation of the protein with a non-specific neuraminidase from *A. ureafaciens* resulted in a major shift

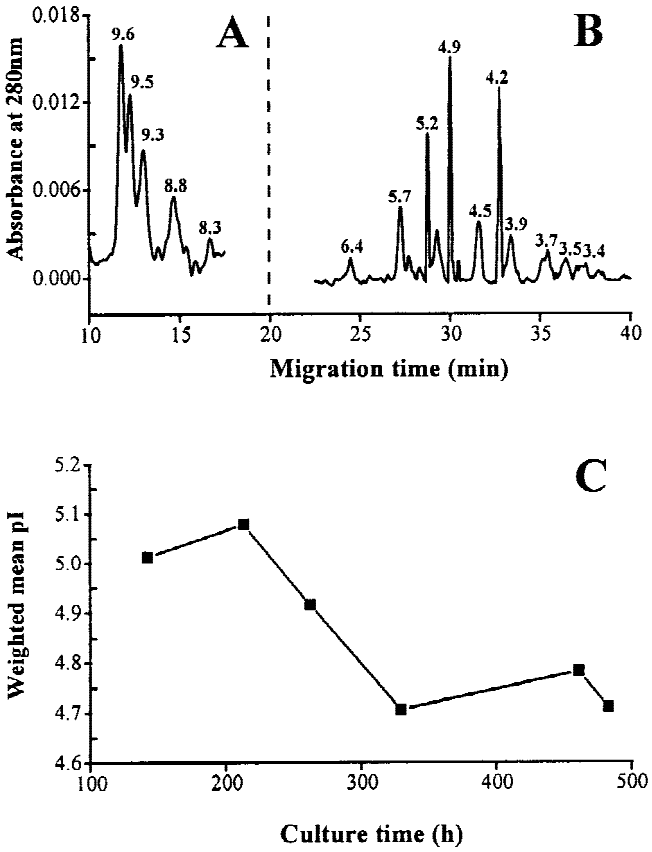

Figure 4. Monitoring recombinant human IFN-γ microheterogeneity by capillary isoelectric focusing. **(A)** IFN-γ desialylated with neuraminidase from *A. ureafaciens.* **(B)** Typical analysis of immunoaffinity purified recombinant IFN-γ from perfusion culture of CHO cells; IFN-γ concentration at 250 μg mL^{-1}. **(C)** The weighted mean pI of recombinant IFN-γ variants secreted by CHO cells during perfusion culture.

in the molecular pI to a narrow peak grouping with pI values between 9.6 and 8.3 (Fig. 4A). The continued presence of heterogeneity in pI after desialylation is likely to be the result of variable C-terminal cleavage of CHO cell derived IFN-γ, as previously determined by MS in this laboratory (James et al., 1996). The observed pI's are consistent with theoretical values (calculated using the Swiss-Prot database ''compute pI/MW'' sequence analysis tool) for the most prevalent C-terminally truncated recombinant human IFN-γ polypeptides produced by CHO cells terminating at Lys_{128} (pI 8.99), Arg_{129} (pI 9.18), Lys_{130} (pI 9.3), and Arg_{131} (pI 9.42). It is clear that the degree of sialylation has a marked effect on the apparent pI of recombinant IFN-γ, a lower pI indicating increased sialylation of N-glycans. Peak groups did not fall into two distinct groupings, representing 2N and 1N glycoforms, reflecting the extensive variability of N-glycan sialylation. No non-sialylated molecules were evident.

For quantitative analysis of these data, each cIEF peak was integrated using System Gold software (Beckman) to determine the area under each peak and the weighted (based on peak area) mean pI of IFN-γ molecules calculated. The weighted mean pI of the IFN-γ population demonstrated a

clear decline after 210 h of perfusion culture (Fig. 4C). This corresponds to about half the time required to colonize the bed of the bioreactor. This decrease in net pI could be the result of modifications to the IFN-γ polypeptide, increased sialylation of IFN-γ glycans, or a combination of both. We have shown previously that C-terminal clipping of the IFN-γ polypeptide occurs during stirred-tank culture CHO cells (Goldman et al., 1997). However, as indicated above, it is unlikely that these minor alterations in amino acid content would result in the large changes in pI observed. We infer that IFN-γ sialylation is increased during establishment of the perfusion culture and thereafter becomes relatively constant. The MECC monitoring data described previously showed a constant level of glycosylation site occupancy, therefore this phenomenon would potentially be due to increased sialylation of individual N-glycans.

Analysis of Sialylated N-Glycans by HPLC

To test the above hypothesis, we analyzed sialylated N-glycans released from purified IFN-γ by cation-exchange HPLC after derivatization at the reducing terminus with the fluorophore 2-aminobenzamide (Bigge et al., 1995). This analysis separates N-glycans substituted with 0, 1, 2, 3, or 4 sialic acids (Fig. 5A) and calculation of mean sialic acid content of N-glycans is possible after computerized integration of peak areas. These data showed that the mean sialic acid content of IFN-γ N-glycans increased during the perfusion culture from 1.74 to 1.91 sialic acids per N-glycan (i.e., an increase of 9%; Fig. 5B). The latter value is typical of that obtained on analysis of IFN-γ derived from early to mid-exponential phase CHO320 batch culture samples (Fig. 6). Taking the mean N-glycan content of IFN-γ polypeptides into account, as determined by MECC, it is possible to calculate the molar sialylation of IFN-γ. This increases from 3.1 mol sialic acid/mol IFN-γ at the start of culture to 3.4 mol sialic acid/mol IFN-γ at the end. This increase in sialylation was due mainly to an increase in the proportion of tri- and tetrasialylated N-glycans associated with IFN-γ, with a concomitant reduction in mono- and non-sialylated oligosaccharides (Fig. 5C). The most obvious increase in IFN-γ sialylation was evident after 210 h of culture, which correlates with a rapid increase in cell growth. There were no major alterations in the relative proportions of core, desialylated N-glycans as determined by MALDI-MS of desialylated IFN-γ glycopeptides during culture (as detailed in James et al., 1995; data not shown).

Comparative Analysis of Recombinant IFN-γ Glycosylation During Stirred-Tank Batch Culture

To compare the glycosylation of IFN-γ produced by fluidized-bed perfusion culture with that of IFN-γ produced by more conventional stirred-tank batch culture, IFN-γ produced during the course of a typical batch fermentation was analyzed by MECC and cation-exchange HPLC of released

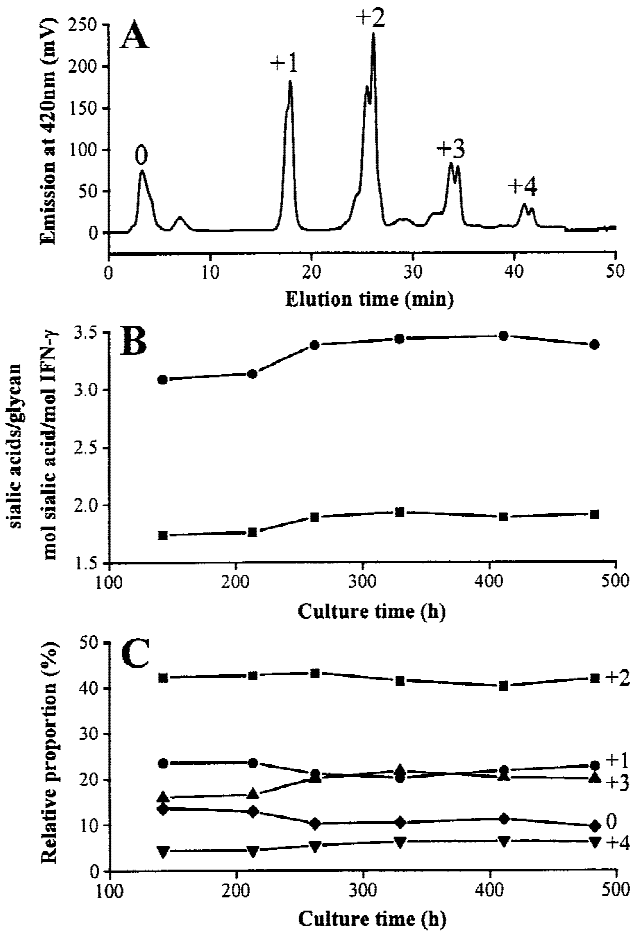

Figure 5. Quantitative analysis of the sialylation of N-glycans associated with recombinant human IFN-γ during fluidized-bed perfusion culture. **(A)** Typical analysis of 2-AB derivatized N-glycans derived from IFN-γ by cation-exchange HPLC: 0, non-sialylated; +1, mono-sialylated; +2, di-sialylated; +3, tri-sialylated; +4, tetra-sialylated. **(B)** Calculated mean sialic acid content of individual N-glycans (■) and molar sialylation of IFN-γ monomer (●) produced during the course of perfusion culture. **(C)** Changes in the proportion of differently sialylated N-glycans during perfusion culture (0, +1, +2, +3, +4; as for **A**).

N-glycans (Fig. 6). As described above, combination of these data permitted calculation of IFN-γ molar sialylation during culture for direct quantitative comparison.

The 12 L batch culture exhibited typical growth characteristics, reaching a maximum viable cell density of 0.54×10^6 cells mL^{-1} after 90 h of culture (Fig. 6A). Viable cell density then dropped sharply, with 9% cell viability after 200 h culture. Glucose, lactate, and ammonia concentrations were monitored through culture (data not shown). Lactate reached a maximum concentration of 16 m*M* after approximately 75 h and thereafter remained constant, the glucose concentration declined steadily from an initial 9 m*M*, and was exhausted after 96 h, and the ammonia concentration gradually increased to a maximum of 5 m*M* after 200 h culture. Maximum specific growth rate (μ) was 0.028 h^{-1}, typical for this cell line. As noted above, this was approximately 3 times that observed in fluidized-bed culture. Recombinant IFN-γ productivity (Fig. 6B) was lower than that

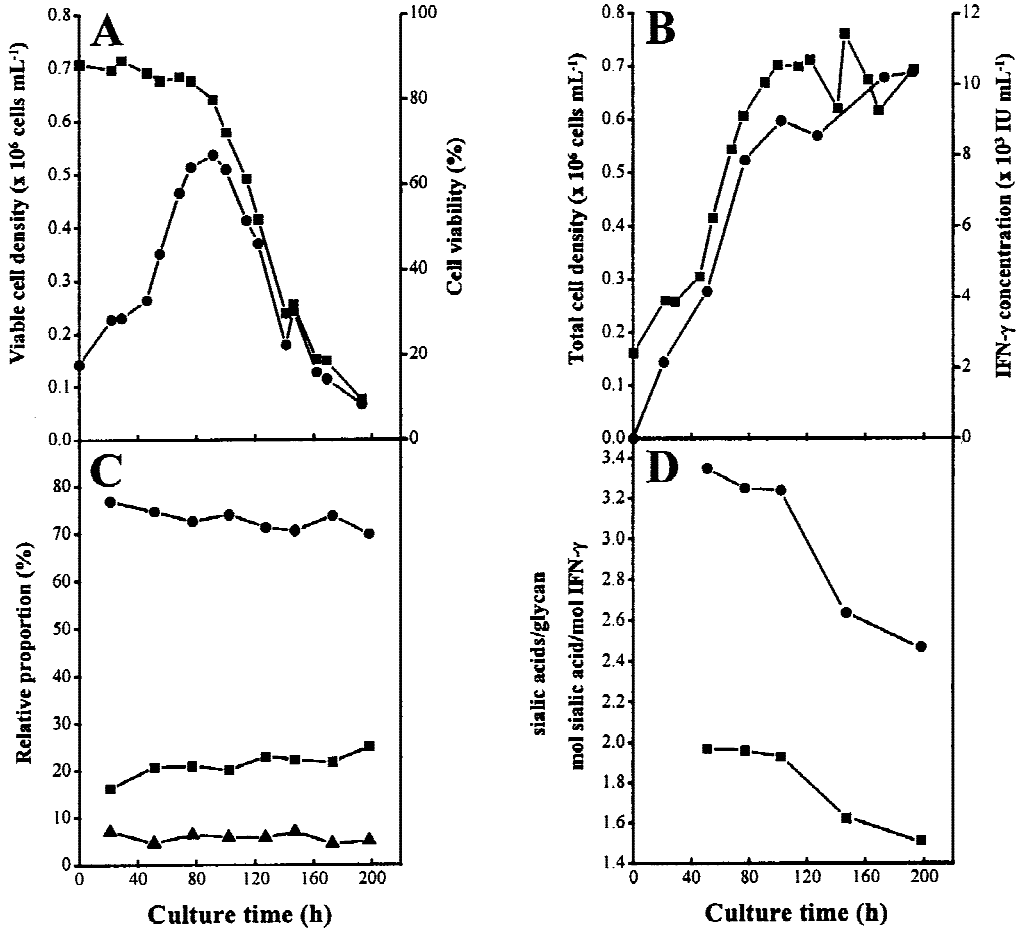

Figure 6. Analysis of recombinant human IFN-γ glycosylation produced during stirred-tank batch culture of CHO cells. **(A)** Viable cell density (●) and cell viability (■); **(B)** total cell density (■) and IFN-γ concentration in supernatant (●); **(C)** monoitoring IFN-γ glycosylation site occupancy by MECC (as for Fig. 3); 2N (●), glycosylated at Asn_{25} and Asn_{97}; 1N (■), glycosylated at Asn_{25}; 0N (▲), non-glycosylated; **(D)** analysis of N-glycan sialylation (as for Fig. 5); calculated mean sialic acid content of individual N-glycans (■) and molar sialylation of IFN-γ monomer (●).

observed in perfusion culture, with a maximum specific production rate, (qIFN) during exponential growth of 303 IU 10^6 cells^{-1} h^{-1}, which compares to a qIFN of 397 IU 10^6 cells^{-1} h^{-1} during perfusion culture. The rate of IFN-γ production declined markedly after approximately 96 h, concomitant with a cessation in cell growth.

Glycosylation site occupancy was monitored by MECC (Fig. 6C). These data indicate that site occupancy was not constant throughout culture, as observed during the perfusion culture (Fig. 3), but delined gradually and slightly throughout the batch fermentation. The proportion of doubly glycosylated molecules diminished while that of the singly glycosylated variant increased; the non-glycosylated variant remained constant. Taking into account that cell growth and maximum IFN-γ production had ceased by 100 h culture, these minor changes in site occupancy observed may be considered insignificant. In contrast, perfusion culture produced stable site occupancy, reminiscent of that previously reported during chemostat culture of CHO320 cells (Hayter et al., 1992). However, there were generally higher proportions of the doubly glycosylated (2N) IFN-γ glycoform and lower proportions of the non-glycosylated (0N) variant compared to stirred-tank culture, i.e, the IFN-γ protein was more heavily glycosylated during perfusion culture.

At the cellular level, N-glycosylation site occupancy may be related to several factors including oligosaccharyldolichol substrate availability, oligosaccharyl transferase activity, rate of protein synthesis, competition with protein folding and other co-translational events (Shelikoff et al., 1996). Despite a higher cell specific productivity (qIFN) in perfusion culture (397 IU 10^6 cells^{-1} h^{-1}) than routinely obtained for this cell line in stirred-tank culture (303 IU 10^6 cells^{-1} h^{-1} (Fig. 6), typically 270–300 IU 10^6 IU 10^6 cells^{-1} h^{-1}), N-glycan transfer was consistently, but only marginally, more efficient in the perfused cells. The continuously changing environment and physiological state of cells in stirred-tank culture may negatively affect their capability for glycosylation. The combination of attached growth, constant nutrient/growth factor availability, low shear stress conditions, and toxic metabolite removal in perfusion culture function to promote a more favorable cellular environment for glycosylation. In this laboratory we have previously shown that the proportion of fully glycosylated re-

combinant IFN-γ molecules increases during transient periods of glucose excess in glucose-limited chemostat culture of CHO cells, also suggesting that the culture environment influences N-glycan transfer (Hayter et al., 1992). Increases in ammonia concentration in culture have been shown to decrease N-glycosylation of a recombinant protein produced by CHO cells (Borys et al., 1994), however, this effect was pH dependent and required ammonia concentrations significantly in excess (9 m*M*) of those reached in the perfusion culture reported here (1.6 m*M*). Gawlitzek et al. (1995) have shown that short-term changes in cell culture conditions (glucose, amino acids, and pO_2 limitation) may affect glycosylation of an interleukin-2 variant produced by BHK cells.

The mean sialic acid content of N-glycans associated with IFN-γ produced in stirred tank culture was relatively stable up to 100 h culture (1.93–1.97 sialic acids per N-glycan; Fig. 6D), and very similar to that observed after 210 h of perfusion culture (1.91 sialic acids per N-glycan). Thus, for both cell culture systems, attainment of maximal IFN-γ sialylation was associated with periods of rapid cell growth. However, N-glycan sialylation subsequently declined markedly to 1.5 sialic acids per glycan after 200 h batch culture. Molar sialylation exhibited a concomitant reduction from approximately 3.3 mol sialic acid/mol IFN-γ protein to 2.5 mol/mol during this death phase.

These data indicate strongly that between 100 h and 200 h of batch culture, a significant proportion of IFN-γ already produced is desialylated extracellularly. During the death phase, while there is a further 12.6% increase in the total IFN-γ content of the batch culture supernatant, there is a 22% reduction in N-glycan sialylation (i.e., even if all IFN-γ proteins produced between 100 and 200 h of culture were non-sialylated then this would not wholly account for the level of desialylation observed).

Consistent glycosylation of IFN-γ does not occur during stirred-tank culture of CHO cells. Our data is similar to that reported recently by Gu et al. (1997) who also demonstrated that sialylation of IFN-γ produced by CHO320 cells decreased in parallel with loss in cell viability in batch culture. In this laboratory we have shown previously by MS that the microheterogeneity of N-glycans associated with IFN-γ also alters during stirred-tank culture of CHO320 cells (Hooker et al., 1995); during culture the proportion of high mannose and truncated forms increases relative to the proportion of complex type N-glycans. Similarly, N-glycosylation of a chimeric MAb has also been shown to deteriorate during fed-batch culture of NS0 cells (Robinson et al., 1994). Differences in the sialylation of recombinant human tissue kallikrein produced by CHO cells from microcarrier culture and from a serum-free suspension cell recycle process have been reported (Watson et al., 1994), however, the authors did not report on cell viability or density.

A decline in N-glycan sialylation during the death phase of stirred-tank culture is most likely to result from the action of extracellular sialidases released from lysed cells (Gramer et al., 1995; Munzert et al., 1997), or possibly be due to compromised cellular sialylation. The latter could be related to the build up of toxic metabolites in the batch culture environment (which does not occur in continuous culture). Metabolites include ammonia, which may directly affect cell growth (Ryll et al., 1994), and thereby also specifically inhibit sialylation by diffusing across cellular membranes and increasing the pH in the *trans*-Golgi network contributing to a sub-optimal pH for sialyltransferase activity. Ammonia has also recently been implicated as a factor contributing to increased complexity (antennarity) of N-glycans, brought about by an elevation of intracellular uridine diphosphate-GlcNAc/GalNAc pools (Gawlitzek et al., 1998). However, there is likely to be a multiplicity of factors involved in control of sialylation in cells, independent of any effects of ammonia, (which only accumulates to low concentration in perfusion culture). In fact, the N-glycans of all recombinant glycoproteins studied to date are incompletely sialylated. In vivo, this may be due to (1) deficient Golgi α2,3 sialyltransferase activity, (2) rate of transport of glycoprotein substrate through the Golgi, (3) competition between glycoprotein substrates in the Golgi (4) steric inaccessibility of glycan acceptor, and/or (5) control of nucleotide-sugar substrate (CMP-NeuAc) transport into the Golgi lumen. In the case of the latter, recent research on rat hepatocytes has suggested that elevated cytosolic levels of UDP-N-acetylhexosamine impairs transport of CMP-NeuAc into the Golgi lumen, resulting in reduced glycoconjugate sialylation; there was evidence for feedback inhibition of CMP-sialic acid synthesis in response to increased cytosolic concentrations of CMP-sialic acid (Pels-Rijcken et al., 1995).

CONCLUSIONS

We have shown quantitatively that both long-term perfusion and batch cultures of CHO cells produce recombinant human IFN-γ with a consistent and highly comparable degree of glycosylation, monitored here as glycosylation site occupancy and level of N-glycan sialylation. However, our data suggest that the sialylation of recombinant glycoproteins produced in stirred-tank batch culture may decline significantly during the stationary and death phase of batch culture. Such deleterious changes to recombinant glycoprotein product consistency may be averted by implementation of appropriate monitoring technologies capable of rapid quantitative analyses of intact glycoprotein populations, enabling control of bioreactor operations, e.g., to avoid late harvest of under-sialylated product. As demonstrated in our study, this may be achieved by fast capillary electrophoretic separations, capable of providing a variety of useful analytical information.

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idized-bed bioreactor, and Dr. Gerald Blüml (Institute of Applied Microbiology, Vienna) for helpful advice and discussions.

NOMENCLATURE

CHO	Chinese hamster ovary
cIEF	capillary isoelectricfocusing
IFN-γ	interferon-γ
MECC	micellar electrokinetic capillary chromatography
PNGase F	peptide-n-glycosidase F
rp-HPLC	reverse-phase high performance liquid chromatography
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

References

Aggeler, J., Kapp, L. N., Tseng, S. C. G., Werb, Z. 1982. Regulation of protein secretion in chinese-hamster ovary cells by cell-cycle position and cell-density—plasminogen activator, procollagen and fibronectin. Exp. Cell Res. **139**: 275–283.

Andersen, D. C., Goochee, C. F. 1995. The effect of ammonia on the O-linked glycosylation of granulocyte-colony-stimulating factor produced by chinese-hamster ovary cells. Biotechnol. Bioeng. **47**: 96–105.

Bhat, R. U., Freeberg, J., McKown, M. C., Mock, K., Stambolija, L., Helgeson, E. A. 1995. Structural-analysis of recombinant factor-VIII produced by continuous perfusion cell culture. Thromb. Haemost. **73**: 1213.

Bigge, J. C., Patel, T. P., Bruce, J. A., Goulding, P. N., Charles, S. M., Parekh, R. B. 1995. Nonselective and efficient fluorescent labeling of glycans using 2-aminobenzamide and anthranilic acid. Anal. Biochem. **230**: 229–238.

Borys, M. C., Linzer, D. I. H., Papoutsakis, E. T. 1994. Ammonia affects the glycosylation patterns of recombinant mouse placental lactogen-1 produced by chinese-hamster ovary cells in a pH-dependent manner. Biotechnol. Bioeng. **43**: 505–514.

Chen, A. B., Rickel, C. A., Flanigan, A., Hunt, G., Moorhouse, K. G. 1996. Comparison of ampholytes used for slab gel and capillary isoelectricfocusing of recombinant tissue-type plasminogen-activator glycoforms. J. Chromatogr. A **744**: 279–284.

Chuppa, S., Tsai, Y. S., Yoon, S., Shackleford, S., Rozales, C., Bhat, R., Tsay, G., Matanguihan, C., Konstantinov, K., Naveh, D. 1997. Fermentor temperature as a pool for control of high-density perfusion cultures of mammalian cells. Biotechnol. Bioeng. **55**: 328–338.

Feder, J. N., Assaraf, Y. G., Seamer, L. C., Schimke, R. T. 1989. The pattern of dihydrofolate-reductase expression through the cell-cycle in rodent and human cultured-cells. J. Biol. Chem. **264**: 20583–20590.

Figeys, D., Ducret, A., Aebersold, R. 1997. Identification of proteins by capillary electrophoresis tandem mass spectrometry—evaluation of an on-line solid-phase extraction device. J. Chromatogr. A **763**: 295–306.

Freitag, R., Reif, O. W., Weidemann, R., Kretzmer, G. 1996. Production of recombinant h-ATIII with mammalian cell cultures using capillary electrophoresis for product monitoring. Cytotechnol. **21**: 205–215.

Fussenegger, M., Mazur, X., Bailey, J. E. 1997. A novel cytostatic process enhances the productivity of chinese hamster ovary cells. Biotechnol. Bioeng. **55**: 927–939.

Gawlitzek, M., Conradt, H. S., Wagner, R. 1995. Effect of different cell culture conditions on the polypeptide integrity and N-glycosylation of a recombinant model glycoprotein. Biotechnol. Bioeng. **46**: 536–544.

Gawlitzek, M., Valley, U., Wagner, R. 1998. Ammonium ion and glucosamine dependent increases of oligosaccharide complexity in recombinant glycoproteins secreted from cultivated BHK-21 cells. Biotechnol. Bioeng. **57**: 518–528.

Goldman, M. H., James, D. C., Ison, A. P., Bull, A. T. 1997. Monitoring proteolysis of recombinant human interferon-gamma during batch culture of chinese hamster ovary cells. Cytotechnol. **23**: 103–111.

Gramer, M. J., Goochee, C. F., Chock, V. Y., Brousseau, D. T., Sliwkowski, M. B. 1995. Removal of sialic-acid from a glycoprotein in CHO cell culture supernatant by the action of an extracellular CHO cell sialidase. Bio/Technol. **13**: 692–698.

Gray, D. R., Chen, S., Howarth, W., Inlow, D., Maiorella, B. L. 1996. CO_2 in large-scale and high-density CHO cell perfusion culture. Cytotechnol. **22:** 65–78.

Griffiths, J. B., Racher, A. J. 1994. Cultural and physiological factors affecting expression of recombinant proteins. Cytotechnol. **15**: 3–9.

Gu, M. B., Todd, P., Kompala, D. S., 1993. Foreign gene-expression (beta-galactosidase) during the cell-cycle phases in recombinant CHO cells. Biotechnol. Bioeng. **42**: 1113–1123.

Gu, X. J., Harmon, B. J., Wang, D. I. C. 1997. Site- and branch-specific sialylation of recombinant human interferon-gamma in chinese hamster ovary cell culture. Biotechnol. Bioeng. **55**: 390–398.

Guttman, A. 1997. Analysis of monosaccharide composition by capillary composition by capillary electrophoresis. J. Chromatogr. A **763**: 271–277.

Harmon, B. J., Gu, X. J., Wang, D. I. C. 1996. Rapid monitoring of site-specific glycosylation microheterogeneity of recombinant human interferon-gamma. Anal. Chem. **68**: 1465–1473.

Hayter, P. M., Curling, E. M. A., Baines, A. J., Jenkins, N., Salmon, I., Strange, P. G., Bull, A. T. 1991. Chinese hamster ovary cell growth and interferon production kinetics in stirred-batch culture. Appl. Microbiol. Biotechnol. **34**: 559–564.

Hayter, P. M., Curling, E. M. A., Baines, A. J., Jenkins, N., Salmon, I., Strange, P. G., Tong, J. M., Bull, A. T. 1992. Glucose-limited chemostat culture of chinese-hamster ovary cells producing recombinant human interferon-gamma. Biotechnol. Bioeng. **39**: 327–335.

Hoffstetterkuhn, S., Alt, G., Kuhn, R. 1996. Profiling of oligosaccharide-mediated microheterogeneity of a monoclonal-antibody by capillary electrophoresis. Electrophoresis **17**: 418–422.

Hooker, A. D., Goldman, M. H., Markham, N. H., James, D. C., Ison, A. P., Bull, A. T., Strange, P. G., Salmon, I., Baines, A. J., Jenkins, N. 1995. N-glycans of recombinant human interferon-gamma change during batch culture of chinese hamster ovary cells. Biotechnol. Bioeng. **48**: 639–648.

Hooker, A. D., Green, N. H., James, D. C., Strange, P. G., Baines, A. J., Bull, A. T., Jenkins, N. 1997. Epitope determination for antibodies raised against recombinant human interferon-gamma, pp. 277–282. In: M. J. T. Carrondo et al. (eds.), Animal cell technology: From vaccines to genetic medicine. Kluwer Academic Publishers, Dordrecht.

James, D. C. 1996. Analysis of recombinant glycoproteins by mass spectrometry. Cytotechnol. **22**: 17–24.

James, D. C., Freedman, R. B., Hoare, M., Jenkins, N. 1994. High-resolution separation of recombinant human interferon-gamma glycoforms by micellar electrokinetic capillary chromatography. Anal. Biochem. **222**: 315–322.

James, D. C., Freedman, R. B., Hoare, M., Ogonah, O. W., Rooney, B. C., Larionov, O. A., Dobrovolsky, V. N., Lagutin, O. V., Jenkins, N. 1995. N-glycosylation of recombinant human interferon-gamma produced in different animal expression systems. Bio/Technol. **13**: 592–596.

James, D. C., Goldman, M. H., Hoare, M., Jenkins, N., Oliver, R. W. A., Green, B. N., Freedman, R. B. 1996. Post-translational processing of recombinant human interferon-gamma in animal expression systems. Prot. Sci. **5**: 331–340.

Jenkins, N., Curling, E. M. A. 1994. Glycosylation of recombinant proteins: Problems and prospects. Enz. Mic. Technol. **16**: 354–364.

Jenkins, N., Hovey, A. 1993. Temperature control of growth and productivity in mutant chinese hamster ovary cells synthesizing a recombinant protein. Biotechnol. Bioeng. **42**: 1029–1036.

Jenkins, N., Parekh, R. B., James, D. C. 1996. Getting the glycosylation right—implications for the biotechnology industry. Nat. Biotechnol. **14**: 975–981.

Kakehi, K., Hirose, A., Tamai, T., Taga, A., Honda, S. 1996. Analysis of N-acetylneuraminic acid oligomers by high-performance capillary electrophoresis. Anal. Sci. **12**: 171–176.

Kelly, J. F., Locke, S. J., Ramaley, L., Thibault, P. 1996. Development of electrophoretic conditions for the characterization of protein glycoforms by capillary electrophoresis electrospray mass-spectrometry. J. Chromatogr. A **720**: 409–427.

Konstantinov, K. B., Tsai, Y. S., Moles, D., Matanguihan, R. 1996. Control of long-term perfusion chinese hamster ovary cell culture by glucose auxostat. Biotechnol. Prog. **12**: 100–109.

Kubbies, M., Stockinger, H. 1990. Cell cycle dependent DHFR and tPA production in cotransfected, mtx- amplified CHO cells revealed by dual-laser flow cytometry. Exp. Cell Res. **188**: 267–271.

Liu, D. T. Y. 1992. Glycoprotein pharmaceuticals—scientific and regulatory considerations, and the United States Orphan Drug Act. Trends Biotechnol. **10**: 114–120.

Lubiniecki, A. S. 1990. Large-scale mammalian cell culture technology. Marcel Dekker, New York.

Moore, A., Mercer, J., Dutina, G., Donahue, C. J., Bauer, K. D., Mather, J. P., Etcheverry, T., Ryll, T. 1997. Effects of temperature shift on cell cycle, apoptosis and nucleotide pools in CHO cell batch cultures. Cytotechnol. **23**: 47–54.

Moorhouse, K. G., Rickel, C. A., Chen, A. B. 1996. Electrophoretic separation of recombinant tissue-type plasminogen-activator glycoforms—validation issues for capillary isoelectric-focusing methods. Electrophoresis **17**: 423–430.

Morbeck, D. E., Madden, B. J., McCormick, D. J. 1994. Analysis of the microheterogeneity of the glycoprotein chorionic gonadotropin with high-performance capillary electrophoresis. J. Chromatogr. A **680**: 217–224.

Morell, A. G., Gregoriadis, G., Scheinberg, I. H., Hickman, J., Ashwell, G. 1971. The role of sialic acid in determining the survival of glycoproteins in the circulation. J. Biol. Chem. **246**: 1461–1467.

Munzert, E., Heidemann, R., Buntemeyer, H., Lehmann, J., Muthing, J. 1997. Production of recombinant human antithrombin III on 20-L bioreactor scale: Correlation of supernatant neuraminidase activity, desialylation, and decrease of biological activity of recombinant glycoprotein. Biotechnol. Bioeng. **56**: 441–448.

Novotny, M. V. 1996. Glycoconjugate analysis by capillary electrophoresis. Meth. Enzymol. **271**: 319–347.

Okafo, G., Burrow, L., Carr, S. A., Roberts, G. D., Johnson, W., Camilleri, P. 1996. A coordinated high-performance liquid chromatographic, capillary electrophoretic, and mass-spectrometric approach for the analysis of oligosaccharide mixtures derivatized with 2-aminoacridone. Anal. Chem. **68**: 4424–4430.

Ozturk, S. S. 1996. Engineering challenges in high-density cell culture systems. Cytotechnol. **22**: 3–16.

Paliwal, S. K., Nadler, T. K., Regnier, F. E. 1993. Rapid process monitoring in biotechnology. Trends Biotechnol. **11**: 95–101.

Pels-Rijcken, W. R. P., Overdijk, B., Vandeneijnden, D. H., Ferwerda, W. 1995. The effect of increasing nucleotide sugar concentrations on the incorporation of sugars into glycoconjugates in rat hepatocytes. Biochem. J. **305**: 865–870.

Reiter, M., Bluml, G., Gaida, T., Zach, N., Unterluggauer, F., Doblhoffdier, O., Noe, M., Plail, R., Huss, S., Katinger, H. 1991. Modular integrated fluidized-bed bioreactor technology. Bio/Technol. **9**: 1100–1102.

Robinson, D. K., Chan, C. P., Ip, C. Y., Tsai, P. K., Tung, J., Seamans, T. C., Lenny, A. B., Lee, D. K., Irwin, J., Silberklang, M. 1994. Characterization of a recombinant antibody produced in the course of a high yield fed-batch process. Biotechnol. Bioeng. **44**: 727–735.

Rush, R. S., Derby, P. L., Strickland, T. W., Rohde, M. F. 1993. Peptide-mapping and evaluation of glycopeptide microheterogeneity derived from endoproteinase digestion of erythropoietin by affinity high-performance capillary electrophoresis. Anal. Chem. **65:** 1834–1842.

Ryll, T., Valley, U., Wagner, R. 1994. Biochemistry of growth inhibition by ammonium ions in mammalian cells. Biotechnol. Bioeng. **44**: 184–193.

Shelikoff, M., Sinskey, A. J., Stephanopoulos, G. 1996. A modeling framework for the study of protein glycosylation. Biotechnol. Bioeng. **50**: 73–90.

Strege, M. A., Lagu, A. L. 1995. Capillary electrophoretic separations of biotechnology-derived proteins in *Escherichia coli* fermentation broth. Electrophoresis **16**: 642–646.

Teshima, G., Wu, S. L. 1996. Capillary electrophoresis analysis of recombinant proteins. Meth. Enzymol. **271**: 264–293.

Thorne, J. M., Goetzinger, W. K., Chen, A. B., Moorhouse, K. G., Karger, B. L. 1996. Examination of capillary zone electrophoresis, capillary isoelectric-focusing and sodium dodecyl sulfate capillary electrophoresis for the analysis of recombinant tissue plasminogen activator. J. Chromatogr. A **744**: 155–165.

Watson, E., Shah, B., Leiderman, L., Hsu, Y. R., Karkare, S., Lu, H. S., Lin, F. K. 1994. Comparison of N-linked oligosaccharides of recombinant human tissue kallikrein produced by chinese hamster ovary cells on microcarrier beads and in serum-free suspension culture. Biotechnol. Prog. **10**: 39–44.

Weber, P. L., Lunte, S. M. 1996. Capillary electrophoresis with pulsed amperometric detection of carbohydrates and glycopeptides. Electrophoresis **17**: 302–309.

Yowell, G. G., Fazio, S. D., Vivilecchia, R. V. 1993. Analysis of a recombinant granulocyte-macrophage colony-stimulating factor dosage form by capillary electrophoresis, capillary isoelectric-focusing, and high-performance liquid chromatography. J. Chromatogr. A **652**: 215–224.