Ammonium Ion and Glucosamine Dependent Increases of Oligosaccharide Complexity in Recombinant Glycoproteins Secreted from Cultivated BHK-21 Cells

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Abstract: The effect of different ammonium concentrations and glucosamine on baby hamster kidney (BHK)-21 cell cultures grown in continuously perfused double membrane bioreactors was investigated with respect to the final carbohydrate structures of a secretory recombinant glycoprotein. The human interleukin-2 (IL-2) mutant glycoprotein variant IL-Mu6, which bears a novel *N*-glycosylation site (created by a single amino acid exchange of Gln_{100} to Asn), was produced under different defined protein-free culture conditions in the presence or absence of either glutamine, NH_4Cl, or glucosamine. Recombinant glycoprotein products were purified and characterized by amino acid sequencing and carbohydrate structural analysis using matrix-assisted laser desorption ionization time of flight mass spectrometry, high-pH anion-exchange chromatography with pulsed amperometric detection, and methylation analysis. In the absence of glutamine, cells secreted glycoprotein forms with preponderantly biantennary, proximal fucosylated carbohydrate chains (85%) with a higher NeuAc content (58%). Under standard conditions in the presence of 7.5 m*M* glutamine, complex-type *N*-glycans were found to be mainly biantennary (68%) and triantennary structures (33%) with about 50% containing proximal α1-6-linked fucose; 37% of the antenna were found to be substituted with terminal α2-3-linked *N*-acetylneuraminic acid. In the presence of 15 m*M* exogenously added NH_4Cl, a significant and reproducible increase in tri- and tetraantennary oligosaccharides (45% of total) was detected in the secretion product. In glutamin-free cultures supplemented with glucosamine, an intermediate amount of high antennary glycans was detected. The increase in complexity of N-linked oligosaccharides is considered to be brought about by the increased levels of intracellular uridine diphosphate-GlcNAc/GalNAc. These nucleotide sugar pools were found to be significantly elevated in the presence of high NH_3/NH_4^+ and glucosamine concentrations. © 1998 John Wiley & Sons, Inc. *Biotechnol Bioeng* **57:** 518–528, 1998.

Keywords: ammonium; UDP-GlcNAc; *N*-glycosylation; BHK-21 cells

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INTRODUCTION

The majority of recombinant human proteins produced for therapeutical use are glycoproteins: erythropoietin, tissue-plasminogen activator, factor VIII, interleukin 2 (IL-2), interferon-β, and immunoglobulins, etc. Carbohydrate chains of polypeptides play an important role in protein folding and tertiary structure maintenance, secretion, proteolytic stability, and in vivo clearance rate. Furthermore, protein-bound glycans are involved in cellular targeting and receptor-binding properties of glycoprotein hormones and are known to contribute to the antigenic properties of molecules (reviewed by Varki, 1993). Therefore, it is necessary to fully characterize recombinant glycoprotein products that are obtained from controlled biotechnological processes with respect to their carbohydrate structures. Several bioprocess parameters have been shown to significantly affect glycosylation of recombinant polypeptides produced from mammalian host cells: serum or protein content of the medium, glucose levels, culture pH, and growth of cells in suspension or on carriers (Curling et al., 1990; Gawlitzek et al., 1995a,b; Hooker et al., 1995; Patel et al., 1992; Watson et al., 1994).

Ammonia in mammalian cell cultures is mainly a by-product of glutamine metabolism and the thermal degradation of glutamine to D-pyroglutamic acid. Depending on the glutamine content of the medium, ammonia can accumulate in batch or fed batch cultures to concentrations up to 10 m*M* or more (Ozturk et al., 1992). Different effects of ammonia present in animal cell cultures have been reported (reviewed by Schneider et al., 1996). Increased concentrations of NH_3/

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NH_4^+ have been shown to inhibit cell growth and productivity (Doyle and Butler, 1990; Martinelle and Häggström, 1993; McQueen and Bailey, 1991). In previous experiments we demonstrated a dose dependent increase of intracellular uridine diphosphate (UDP)-*N*-acetylglucosamine and UDP-*N*-acetylgalactosamine in four different cell lines [baby hamster kidney (BHK), Chinese hamster ovary (CHO), Ltk^- 929, and hybridoma] as a quick response to exogenously applied ammonium chloride (Ryll et al., 1994). These nucleotide sugars are biosynthetic precursor molecules in biosynthetic pathways of polypeptide glycosylation located in the cytoplasm and the Golgi apparatus (Abeijon and Hirschberg, 1992; Kornfeld and Kornfeld, 1985). Moreover, Pedersen et al. (1992) reported a rapid increase in UDP-hexosamine levels accompanied by uridine triphosphate limitations after adding glucosamine to the medium. Different effects of ammonia on product quality have been described. A decrease in sialylation of glycoproteins synthesized in the presence of elevated ammonia concentrations has been observed by several researchers (Andersen and Goochee, 1995; Maiorella, 1992; Thorens and Vassalli, 1986). The N-glycosylation of mouse placental lactogen-I expressed by CHO cells was inhibited by increasing levels of ammonium chloride in the medium (Borys et al., 1994).

In the present study we examined the effects of different glutamine and ammonium concentrations, as well as glucosamine, in controlled perfused BHK-21 cell cultures on the N-linked oligosaccharide structures of a recombinant human IL-2 N-glycosylation mutant protein (IL-Mu6). This model glycoprotein was generated by site directed mutagenesis of a human IL-2 cDNA (resulting in a single amino acid exchange of Gln_{100} to Asn; Dittmar et al., 1988). Carbohydrate structural analysis of glycoprotein products obtained under different culture conditions was performed by matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI/TOF-MS), high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD mapping), and methylation analysis of the enzymatically liberated *N*-glycan chains.

MATERIALS AND METHODS

Cell Lines

The BHK-21 (C13) cell line was transfected with the pBEH-IL-Mu6 plasmid (Artelt et al., 1988; Dittmar et al., 1988) harboring a cDNA encoding a genetically engineered IL-2 variant glycoprotein (IL-Mu6). This polypeptide contains a single amino acid substitution (Glu_{100} to Asn), thus creating the potential N-glycosylation recognition site Asn_{100}-Thr-Thr (Gawlitzek et al., 1995b).

Culture Medium Composition

The standard production medium consisted of a 1:1 mixture of Iscove's modified Dulbecco medium (IMDM) and Ham's F-12 (Gibco–BRL, Eggenstein, Germany) and the following supplements: $NaHCO_3$ (3.61 g L^{-1}), D(+)-glucose (0.8 g L^{-1}), Na-pyruvate (0.22 g L^{-1}), ethanolamine (1.2 μL L^{-1}), aspartic acid (0.377 g L^{-1}), asparagine (0.02 g L^{-1}), glutamate (0.055 g L^{-1}, 0.441 g L^{-1} in glutamine-free medium), glutamine (0.73 g L^{-1}), glycine (0.03 g L^{-1}), histidine (0.08 g L^{-1}), leucine (0.06 g L^{-1}), lysine (0.07 g L^{-1}), methionine (0.08 g L^{-1}), phenylalanine (0.03 g L^{-1}), serine (0.08 g L^{-1}), and tryptophane (0.02 g L^{-1}); all supplements were purchased from Sigma (Deisenhofen, Germany). Under serum-containing conditions, 2% (v/v) FCS (Gibco–BRL) was added.

Bioreactor System

Long-term cultivations were carried out in continuously perfused 2.5-L double membrane stirrer bioreactor systems as described previously (Ryll et al., 1990).

Cell Culture Conditions

Three different processes were used to produce IL-Mu6 from recombinant BHK-21 cells under different culture conditions. In all systems cells were grown in suspension. The pH was maintained at 7.20 ± 0.1, and dissolved oxygen was maintained at $40 \pm 5\%$ of air saturation. In all processes (cultures I–III, see Results Section) 0.5–2 L day^{-1} of microfiltrated culture supernatants were harvested continuously for subsequent purification and biochemical characterization of the product.

Analysis of Daily Culture Samples

The total cell number was determined by nuclei fixing and staining with a solution containing 0.1 mol L^{-1} citric acid, 0.1 mg mL^{-1} crystal violet, and 1% (v/v) of Triton-X 100 and the subsequent counting by means of a hemocytometer (Lin et al., 1991). Viable cells were counted by using the trypan-blue exclusion method. Glucose and lactate concentrations were determined using a YSI glucose/lactate analyzer (model 2000, Yellow Springs Instruments, Yellow Springs, OH). Free amino acids were quantified by reverse phase-HPLC (RP-HPLC) after precolumn derivatization with *o*-phthaldialdehyde (OPA, Serva Feinbiochemica, Heidelberg, Germany) as depicted earlier (Ryll et al., 1990). The ammonium concentration in the culture samples was determined using a gas-sensitive ammonia electrode (Ingold Messtechnik AG, Urdorf, Switzerland). IL-Mu6 bioactivity was determined by using a cloned murine IL-2 dependent cytotoxic T-lymphocyte cell line as described by Conradt et al. (1988). Quantification of intracellular nucleotides was performed by ion-pair HPLC after perchloric acid extraction of the cells according to the method described in detail by Ryll and Wagner (1991).

SDS-PAGE and Western Blotting

DS-PAGE was performed according to Laemmli (as described by Conradt et al., 1989) using 15% polyacrylamide gels with 3% stacking gels. Separated proteins were stained by Coomassie brilliant blue R250 (Serva Heidelberg, Germany). Protein transfer onto nitrocellulose membranes (0.45 μm, Millipore, Eschborn, Germany) and the following immunodetection was performed as described by Conradt et al. (1988).

Purification of IL-Mu6

IL-Mu6 glycoprotein was purified from the microfiltrated supernatant of different cell cultures using a three step procedure. The product was quantitatively adsorbed onto a controlled pore glass column (CPG 10, 11.5 nm, 200–400 mesh, Serva). Elution was performed using phosphate buffered saline (PBS, pH 7.0) containing 50% ethylene glycol and 0.8*M* NaCl (flow rate 8 mL h^{-1}). IL-Mu6 containing fractions were further purified by gel permeation chromatography using an Ultrogel AcA54 column (2.6 × 90 cm, IBF Biotechnics, Villeneuve-la-Garenne, France) with PBS (flow rate 30 mL h^{-1}). Final purification was achieved by RP-HPLC on a C_{18}-stationary phase (Nucleosil 100, Macherey & Nagel, Düren, Germany) as described by Grabenhorst et al. (1993).

Amino Acid Sequencing

Purified IL-Mu6 polypeptide preparations were subjected to automated Edman degradation using an Applied Biosystems model 475A sequencer. About 0.1–0.5 nmol of protein was applied onto glass fiber disks of the cartridge after precycling with polybrene. Phenylthiohydantoin derivatives were analyzed in the on-line mode. The programs used were those provided by the manufacturer (Grabenhorst et al., 1993).

Liberation and Isolation of N-Linked Oligosaccharides from IL-Mu6 Polypeptides

In order to remove organic solvent, biologically active fractions from the RP-HPLC step were pooled and dialyzed against PNGase buffer of 50 m*M* sodium phosphate (pH 7.5) containing 10 m*M* EDTA and 0.02% (w/v) NaN_3. After dialysis the protein was evaporated using a Speedvac concentrator (Savant Instruments Inc., Farmingdale, NY). *N*-Glycans were liberated using recombinant peptide-*N*-glycosidase F (5 U according to the manufacturer's specifications) from *F. memingosepticum* (PNGase F, expressed in *Escherichia coli,* Boehringer–Mannheim, Mannheim, Germany). Incubations were performed for 16 h at 37°C. Released oligosaccharides were separated from the protein by RP-HPLC on a C_{18}-phase column (4.6 × 250 mm, Vydac, Hesperia, CA) using the same solvents as described above and were recovered in the flow through. Complete removal of N-linked oligosaccharides by PNGase F was checked by SDS-PAGE. Oligosaccharide fractions were pooled, neutralized with 25% NH_4OH, and evaporated to dryness.

Desalting of *N*-Glycans

For analytical HPAEC-PAD and further *N*-glycan characterization, oligosaccharides were desalted by fast PLC (FPLC) on a Sephadex G-25 Superfine Fast Desalting HR 10/10 column at a flow rate of 1 mL min^{-1} (Pharmacia, Upsalla, Sweden). Oligosaccharides were detected by absorption at 206 nm.

Enzymatic Removal of Terminal *N*-Acetylneuraminic Acid

Neutral oligosaccharides were obtained by incubating native *N*-glycans in 35 μL 0.05*M* sodium acetate buffer (pH 5.0) containing 5 m*M* $CaCl_2$, 0.02% (w/v) NaN_3, and 10 mU neuraminidase (*Vibrio cholerae* sialidase, Calbiochem, Bad Soden, Germany) for at least 2 h at 37°C.

Analytical HPAEC-PAD

For analytical HPAEC-PAD of neutral oligosaccharides, a Dionex BioLC System (Dionex, Sunnyvale, CA) equipped with a CarboPac PA1 column (4 × 250 mm) was used in combination with a pulsed amperometric detector (detector potentials and pulse durations: $E_1 = +50$ mV, $t_1 = 480$ ms; $E_2 = +500$ mV, $t_2 = 120$ ms; $E_3 = -500$ mV; $t_3 = 60$ ms) with an output range of 300 nA. Elution of desialylated oligosaccharides was performed by applying a 0–20% linear gradient of solvent B for 40 min, followed by a 10-min linear run to 100% solvent B (solvent A, 0.1*M* NaOH; solvent B, 0.1*M* NaOH containing 0.6*M* sodium acetate). The flow rate was 1 mL min^{-1}. Native *N*-glycans were eluted using a 0–35% linear gradient of solvent B for 50 min, followed by a 10-min linear run to 100% solvent B (solvent A, 0.1*M* NaOH; solvent B, 1*M* NaOH containing 0.6*M* sodium acetate) at a flow rate of 1 mL min^{-1}. Neutral and sialylated oligosaccharides were identified based on the comparison of retention times of reference oligosaccharides of known structure (Nimtz et al., 1993).

Methylation Analysis of PNGase F Liberated *N*-Glycans

For methylation analysis, *N*-glycans were reduced, permethylated, and purified on a Sephadex LH20 column (Pharmacia). After hydrolysis, reduction, and peracetylation, methylated alditol acetates were separated and identified on a Carlo Erba Mega series gas chromatograph equipped with a 39-m DB1 capillary column connected to a Kratos MS-50 fast-scan mass spectrometer (Nimtz et al., 1993). Values were corrected using response factors obtained from standard oligosaccharides (Nimtz et al., 1990, 1993).

MALDI/TOF-MS

2,5-Dihydroxbenzoic acid (DHB) was used as the UV-absorbing matrix. DHB (10 mg mL^{-1}) was dissolved in 10% ethanol in water. For analysis by MALDI/TOF-MS, reduced and permethylated oligosaccharides were mixed with the same volume of matrix. The sample (1 μL) was applied onto a stainless steel probe tip and dried at room temperature. The concentration of the analyte was approximately 10 pmol μL^{-1}. Measurements were performed on a Bruker REFLEx™ MALDI/TOF mass spectrometer using an N_2 laser (337 nm) with 3-ns pulse width and 10^7–10^8 W cm^{-2} irradiance at the surface (0.2-mm^2 spot). Both positive and negative ion spectra were recorded at an acceleration voltage of 20 kV using the reflectron for enhanced resolution.

RESULTS

Cell Cultures

Three different cultivation processes for the production of IL-Mu6 from transfected BHK-21 cells were analyzed (Fig. 1A,B) and were controlled by daily analyses as described in the Materials and Methods Section. In culture I, the effect of long-term growth of cells under glutamine-free medium conditions was investigated. BHK cells were seeded at a concentration of 9.5×10^4 mL^{-1} in medium containing 2% FCS. After a lag phase of 5 days, cells entered an exponential growth phase that persisted until the end of the process. At day 9, serum was removed by perfusion with protein-free medium at a rate of 2 L day^{-1}. After reaching protein-free conditions at day 11 (protein concentration between 0.1 and 0.2 g L^{-1} culture medium), perfusion was stopped for 2 days and was restarted again after this adaption phase with an initial rate of 0.5 L day^{-1} and increased up to 2.1 L day^{-1} until the end of the process when cells reached a concentration of 1.06×10^7 mL^{-1} (see Fig. 1). During the entire process the ammonium concentration in the medium was below 0.5 m*M*. Products harvested at days 15 and 16, 17 and 18, and 19 and 20 were separately pooled.

In culture II the sequential change in medium conditions depicted in Fig. 1B led to different NH_4^+ concentrations. Under standard medium conditions with 7.5 m*M* glutamine, an ammonium concentration of 2–4 m*M* was measured that increased to 19 m*M* upon perfusion with medium supplemented with 15 m*M* NH_4Cl (compare Fig. 1B). Products were harvested on days 4–7 (standard conditions), 10–12, 13, and 14–15 for +15 m*M* NH_4Cl, and days 18–22, 23 and 24, and 25 and 26 for Gln free.

In culture III (not shown), cells were initially seeded into the bioreactor at a concentration of 1×10^5 mL^{-1} in the presence of glutamine-free medium supplemented with 2% FCS. A cell concentration of 1×10^6 mL^{-1} was reached after 11 days in the same medium. Thereafter, culture conditions were changed by perfusion with protein-free and glutamine-free medium (4 L day^{-1}). Perfusion with the same medium containing 10 m*M* glucosamine was started at

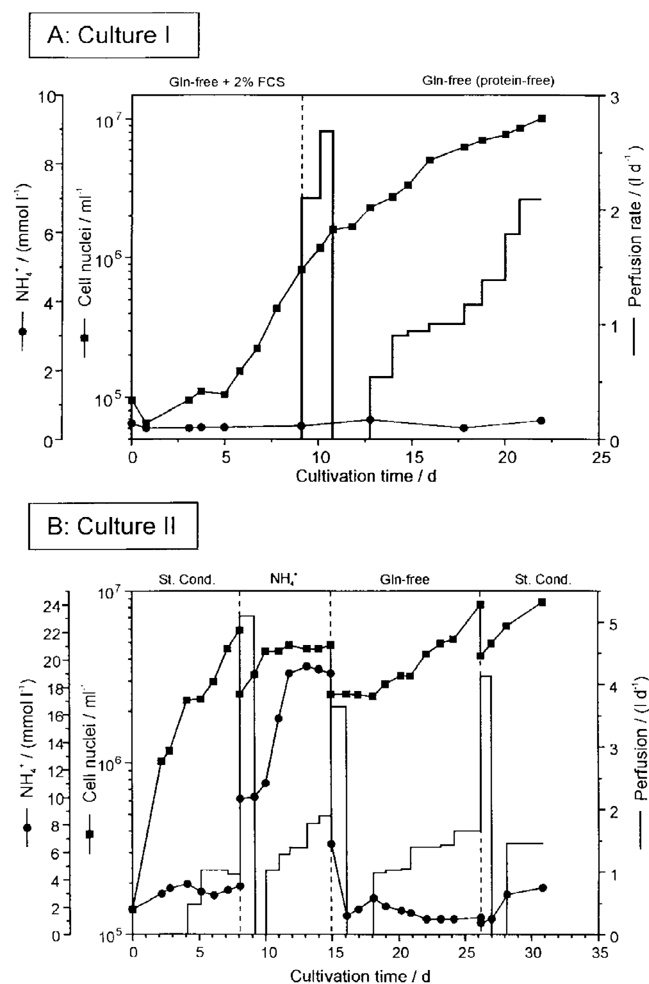

Figure 1. Continuous cultivation of recombinant BHK-21 cells secreting IL-Mu6 protein. Several product harvests were made from cultures I and II as described in the text. Daily analyses (including ammonia determinations) were performed as detailed in the Materials and Methods Section.

day 21. NH_4^+ concentrations remained constantly below 0.5 m*M* over the entire process. Products were harvested separately on days 26–28 and 29–30 (glucosamine conditions) for subsequent protein purification.

The cell specific productivity in all cultures studied was about 0.2 μg (day $10^6)^{-1}$. Cell viability during culture processes was >95% as judged from trypan-blue exclusion, except for 15 m*M* NH_4Cl conditions where at day 14 an increase of dye-positive cells was observed (80% trypan-blue negative).

Purification of Products

The secreted IL-Mu6 protein from individual harvests was characterized by Western-blot analysis (not shown). Separate purification of each product was achieved by a three-step purification procedure (described in the Materials and Methods Section) resulting in 80–90% final yields. Western-blot analysis of samples from purified IL-Mu6 preparations revealed protein patterns identical to those obtained

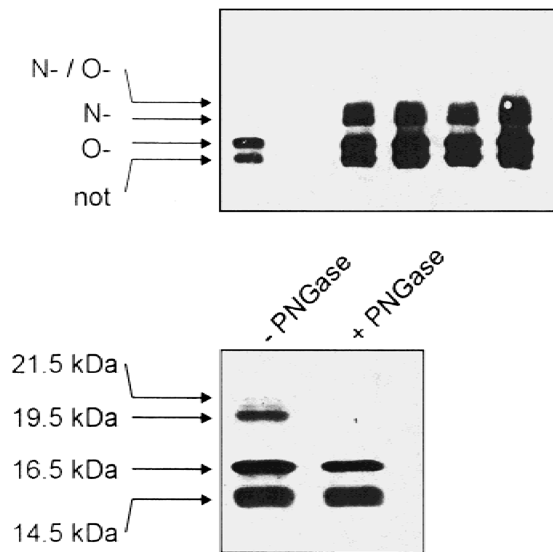

Figure 2. SDS-PAGE and Western-blot analysis of purified IL-Mu6 protein produced under different culture conditions. Each lane was loaded with 0.2 µg of protein. Upper panel: Left: wild-type IL-2 produced from BHK-21 cells (Conradt et al., 1989); lanes 1–4 represent IL-Mu6 preparations obtained from culture II under standard conditions (1), 15 m*M* NH_4Cl conditions (2), Gln-free (3), and standard conditions (4). Arrows indicate the migration positions of the nonglycosylated, O-glycosylated, N-glycosylated, and N + O-glycosylated glycoprotein forms. Lower panel: arrows indicate the apparent molecular weight of the above glycoforms before and after digestion with PNGase F.

for polypeptide forms contained in the culture supernatant. As exemplified in Fig. 2 (upper panel), four major protein forms could be distinguished. The low molecular weight form was nonglycosylated, followed by the O-glycosylated, N-glycosylated, and the N- plus O-glycosylated form of the IL-Mu6 variant. Both N-glycosylated polypeptide forms could be separated in the final HPLC purification step (not shown) and were used for N-linked carbohydrate structural characterization. NH_2-terminal sequencing revealed the expected amino acid sequence (A_1-P-?-S-S-S-T-K-K-T-Q-L-Q-L_{14}, no amino acid derivative was detected in cycle 3 due to O-glycosylation of Thr_3; Dittmar et al., 1988) for the O-glycosylated polypeptide forms; additionally, truncated species lacking the first two or five amino acids were observed when O-glycosylation at Thr-3 was absent (Gawlitzek et al., 1995b; Grabenhorst et al., 1993).

N-Linked Oligosaccharide Analysis

Quantitative release of N-linked oligosaccharides from IL-Mu6 protein by PNGase F was verified by SDS-PAGE analysis (Fig. 2, lower panel).

MALDI/TOF-MS

Desialylated *N*-glycans were reduced and permethylated and subjected to MALDI/TOF-MS analysis. As shown in Figure 3A, *N*-glycans from IL-Mu6 produced in culture I under glutamine-free conditions yielded a major molecular ion signal at $m/z = 2262$ Da and two minor signals at 2711 and 3161 Da, which can be explained by the methylated diantennary, triantennary, and tetraantennary lactosamine-type structures, respectively, containing proximal fucose (see also inserted schemes in Fig. 3A).

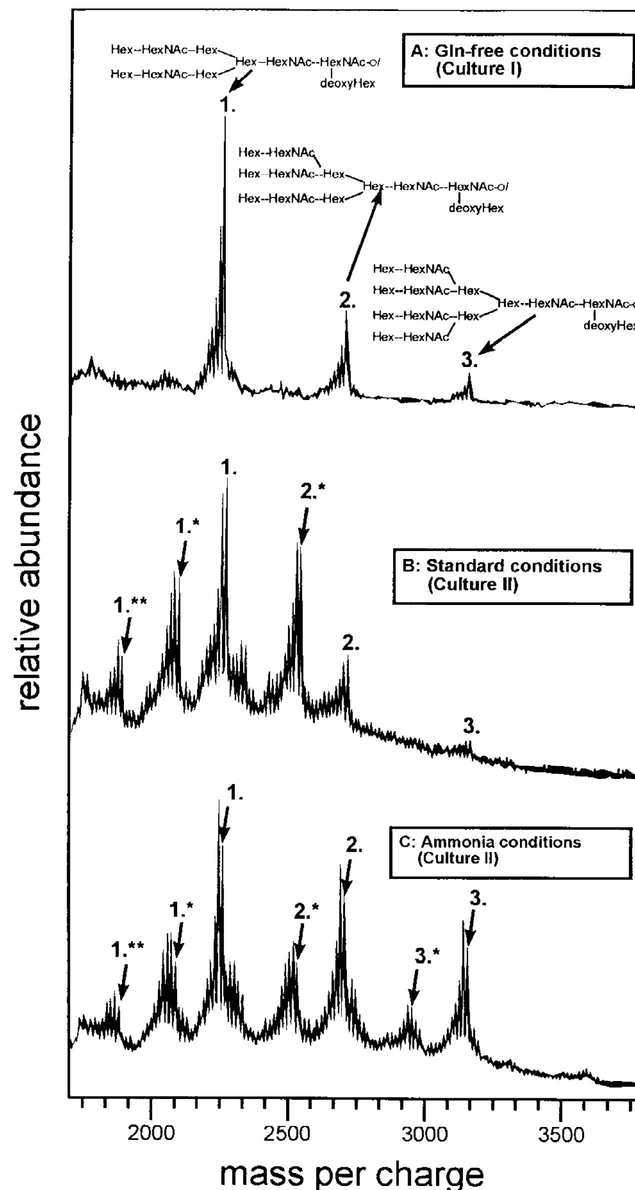

Figure 3. MALDI/TOF-MS of reduced and permethylated desialylated oligosaccharides isolated from IL-Mu6 produced under different culture conditions. (A) The molecular ion pattern of IL-Mu6 oligosaccharides produced under glutamine-free conditions in culture I. The proposed primary sequence of glycan structures as depicted by the inserts is compatible with molecular ion signals ($M + Na^+$) detected: (1) $m/z = 2262$ Da; (2) $m/z = 2711$ Da; and (3) $m/z = 3161$ Da for bi-, tri-, and tetraantennary carbohydrates with proximal fucose, respectively. In culture II, (B) standard conditions and (C) ammonia conditions, a significant increase in high antennary oligosaccharides is observed. Additionally, signals corresponding to afuco- and afuco-agalacto biantennary chains (1* and 1**, $m/z =$ 2088 and 1884 Da) and afuco tri-/tetraantennary chains (2* and 3*, $m/z =$ 2537 and 2986 Da) were detected. Signals for partially undermethylated structures are also present (M minus 14).

By contrast, *N*-glycans from two glycoprotein preparations produced in culture II (Fig. 3B,C) yielded a more complex molecular ion signal pattern. For oligosaccharides from IL-Mu6 obtained under standard culture conditions, the prominent ion at 2262 Da again corresponds to a diantennary and fucosylated structure (signals at 1884 and 2088 being produced by an afuco derivative and a structure lacking fucose and one Gal; Fig. 3B), and 2711 and 2537 Da correspond to the triantennary and afuco-triantennary oligosaccharides, respectively. Desialylated oligosaccharides from IL-Mu6 produced in the presence of 15 m*M* NH_4Cl afforded a more complex pattern of molecular ion signals with relative intensities shifted toward the higher molecular mass region (Fig. 3C). Apart from the biantennary structures, intense ions at 2711 and 3161 Da, representing tri- and tetraantennary structures with proximal fucose, were observed. (For detailed structural assignments of signals, see the legend to Fig. 3 and results discussed below.)

These observations indicated that in the presence of glutamine, resulting in 2–4 m*M* NH_4^+ concentrations in the medium, or exogenously added 15 m*M* NH_4Cl as well as glucosamine, BHK cells synthesized higher antennary oligosaccharide chains compared to culture conditions with low ammonia content (glutamine free). Because the relative abundance of ion signals that are observed in MALDI/TOF-MS cannot be quantified confidently, further analysis by HPAEC-PAD mapping of native and enzymatically desialylated *N*-glycans, as well as methylation analysis, were performed.

HPAEC-PAD Mapping of Oligosaccharides

Aliquots from sialidase incubation mixtures of individual *N*-glycan preparations were subjected to HPAEC (see Figs. 4, 5). Carbohydrate peaks obtained were compared to elution times of authentic oligosaccharide reference structures (Conradt et al., 1987; Nimtz et al., 1990, 1993) and were quantitated using experimentally determined response factors for NeuAc and neutral carbohydrate standards of known structure. Significant differences in the mapping profiles were apparent in each sample corroborating the above MALDI/TOF-MS data. For glutamine-free culture conditions, biantennary, proximal fucosylated oligosaccharides predominated (85% of chains) and were accompanied by only small amounts of tri- and tetraantennary *N*-glycans. Under standard culture conditions, the relative proportion of triantennary chains were increased (the fucosylated Man-6 branched structure being superimposed by the afuco derivative of the Man-3 branched isomer as shown by enzymatic defucosylation of glycans; data not shown) and about half of the structures lacked proximal fucose. [Compare also MALDI/TOF-MS data in Fig. 3 (signals 1*, 1**, and 2*) and methylation analysis in Table I (detection of the 1,3,5,6-tetra-*O*-methyl-GlcNAc derivative)]. A large increase in highly branched structures was detected in the oligosaccharide profile from IL-Mu6 produced in culture II in the presence of NH_4Cl (up to 45% of total glycans; see Fig. 4B, peaks 4, 4b, 4c). The proximal fucose content was significantly higher compared to standard conditions. These characteristics were also observed, but were less pronounced, in the profile of N-linked oligosaccharides synthesized in glutamine-free medium supplemented with 10 m*M* glucos-

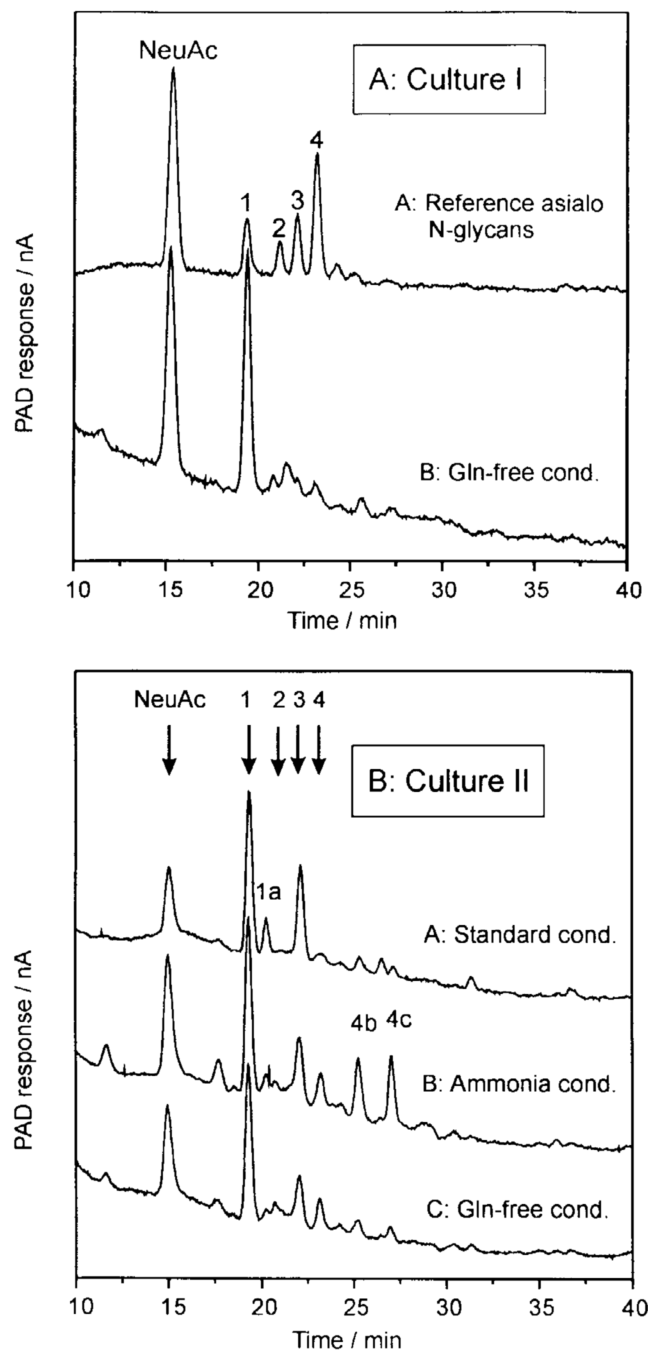

Figure 4. High pH anion-exchange mapping of enzymatically desialylated N-linked oligosaccharides of IL-Mu6 produced under various culture conditions. (A) Gln-free conditions: the upper panel shows the elution of a reference standard glycan mixture (NeuAc, 1 = bi-, 2 + 3 = tri-(two isomeric forms), and 4 = tetraantennary lactosamine-type oligosaccharides with α1–6 linked proximal fucose. (B) Oligosaccharides from IL-Mu6 produced in culture II under standard, ammonia, and glutamine-free conditions. Arrows indicate the elution positions of reference oligosaccharides; peak 1a is the biantennary afuco glycan; 4b and 4c indicates the position of tetraantennary chains with one or two Galβ1-3GlcNAc branches.

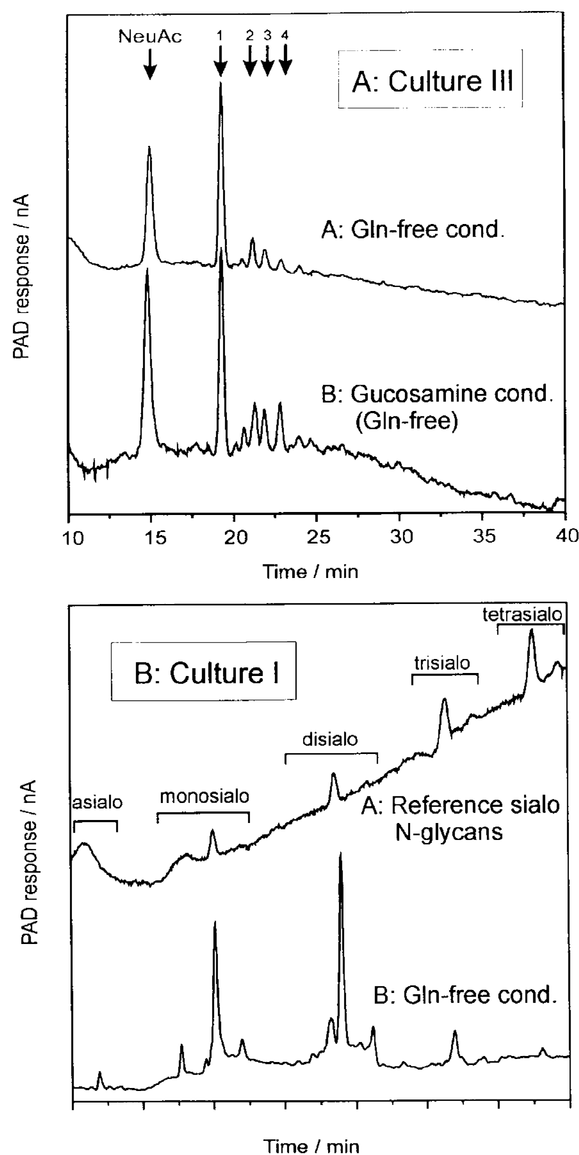

Figure 5. High pH anion-exchange mapping of enzymatically desialylated N-linked oligosaccharides of IL-Mu6. (A) *N*-Glycan maps from the glycoprotein produced under Gln-free and glucosamine conditions in culture III. (B) Native oligosaccharides of IL-Mu6 produced in culture I under Gln-free conditions. The upper panel indicates the elution positions of glycan standards containing no and 1–4 NeuAc residues.

amine (compare Fig. 5A). When culture conditions were shifted back from high ammonia conditions to glutamine-free conditions, the proportion of high antennary chains was significantly reduced (see panel C in Fig. 4B).

Methylation Analysis

The partially methylated alditol acetate derivatives obtained for neutral oligosaccharides supported the structural assignments proposed from data obtained by MALDI/TOF-MS and HPAEC-PAD analysis. Terminal GlcNAc was detected (3,4,6-tri-*O*-methyl derivative) in about 5% of oligosaccharides from the product obtained under standard and high ammonia conditions, respectively. (Compare also the corresponding signals in MALDI/TOF spectra: e.g., agalacto diantennary structure at 1884 Da.) Significant amounts of 3-substituted GlcNAc (4,6-di-*O*-methyl derivative) were observed, indicating partially modified antennae with Galβ1-3GlcNAc in place of the common Galβ1-4GlcNAc motif in oligosaccharides synthesized under standard conditions. They were detected in higher amounts in the product secreted from cells grown in the presence of NH_4Cl (see also tetraantennary peaks 4b and 4c in Fig. 4). In MALDI/TOF-MS, these isomeric forms yielded the same molecular ion signal (3161 Da). The 3-substituted galactose derivative (2,4,6-tri-*O*-methyl-galacitol) was detected in very small amounts after desialylation, indicating the possibility of *N*-glycan structures modified with lactosamine repeats. Methylation analysis from the sialylated oligosaccharides yielded the 2,4,6-tri-*O*-methyl-galactose as the only substituted derivative of this monosaccharide (data not shown), thus confirming that NeuAc is exclusively α2-3-linked to Gal-GlcNAc branches in *N*-glycans of recombinant glycoproteins produced from BHK-21 host cells (Conradt et al., 1989).

Sialylation of Oligosaccharides

The NeuAc content of each oligosaccharide sample was calculated from the corresponding peak areas obtained in HPAEC-PAD profiles of the enzymatically desialylated samples. Based on the known number of Galβ1-4/3GlcNAc branches of individual neutral oligosaccharides, 37, 42, and 58% of the outer antennae can be assumed to be α2-3-sialylated in carbohydrate preparations from IL-Mu6 produced under standard and high ammonia, and glutamine-free conditions in cultures II and I, respectively. As exemplified in Fig. 5B, a ratio of 4.5:5.5 of monosialylated and disialylated glycan structures was detected under glutamine-free conditions but in the presence of glucosamine (data not shown) a ratio of 7:3 was observed; neutral, tri-, and tetrasialylated carbohydrates were present in low amounts.

UDP-GlcNAc/GalNAc Content of Cells

Nucleoside-diphosphate amino sugar concentrations of cells cultivated under defined culture conditions were determined according to the method described by Ryll and Wagner (1991). Average values ($n = 2$) for intracellular UDP-GlcNAc/GalNAc and ammonia during different culture conditions are depicted in Table II. Increased NH_4^+ levels in cultures were paralleled by a concomitant increase in UDP-GlcNAc/GalNAc pools of cells and, furthermore, gave rise to secretion of a recombinant glycoprotein product with N-linked oligosaccharides of significantly higher antennarity (Table II).

O-Linked Carbohydrates

No detailed structural analysis of polypeptide *O*-glycans was performed in this study. However, it is known that recombinant human IL-2 when expressed from different host cell lines, contains invariantly the classical α2-3-

Table I. Methylation analysis of desialylated oligosaccharides from IL-Mu6 produced in culture I (Gln-free) and culture II (standard and ammonia conditions).

Peracetylated derivative of	Molar ratio			Substitution position
	Gln-free condition	Standard condition	Ammonia condition	
Fucitol				
2,3,4-Tri-*O*-methyl-	1.0	0.4	0.7	6
Galacitol				
2,3,4,6-tetra-*O*-methyl-	2.5	1.5	2.0	Terminal
2,4,6-tri-*O*-methyl-	0.1	0.1	0.1	3
2,3,4-tri-*O*-methyl-	ND	(+)	(+)	
2,4-di-*O*-methyl-	ND	ND	ND	
Mannitol				
2,3,4,6-tetra-*O*-methyl-	(+)	0.1	0.2	Terminal
3,4,6-tri-*O*-methyl-	1.6	1.7	1.5	2
2,4-di-*O*-methyl-	1.0	1.0	1.0	3,6
3,4-di-*O*-methyl-	0.2	0.3	0.5	2,6
3,6-di-*O*-methyl-	0.1	0.2	0.3	2,4
2-*O*-methyl-	ND	ND	ND	
2-*N*-Methylacetamido-2-deoxyglucitol				
1,3,5,6-tetra-*O*-methyl-	(+)	0.6	0.3	4
1,3,5-tri-*O*-methyl-	0.9	0.4	0.7	4,6
3,4,6-tri-*O*-methyl-	ND	0.4	0.4	Terminal
3,6-di-*O*-methyl-	3.3	2.8	3.0	4
4,6-di-*O*-methyl-	ND	0.2	0.4	3
6-*O*-methyl-	(+)	ND	ND	
3-*O*-methyl-	(+)	0.1	0.1	3,6
2-*N*-Methylacetamido-2-deoxygalcitol				
3,4,6-tri-*O*-methyl-	(+)	0.1	0.1	Terminal
4,6-di-*O*-methyl-	(+)	(+)	(+)	3

ND, not detected; (+) trace.

monosialylated or α2-3- and α2-6-disialylated core Galβ1-3GalNAc mucin-type forms (Conradt et al., 1989). The sialylated forms can be distinguished from the neutral form and the nonglycosylated polypeptide in SDS-PAGE (Conradt et al., 1988, 1989). Apparently, under all culture conditions a very similar ratio of nonglycosylated and O-glycosylated forms with one or two NeuAc is secreted by BHK cells (Fig. 2, upper panel), the Galβ1-3GalNAc derivative being detectable as a faint minor band migrating between the two major low molecular weight polypeptides (compare Fig. 2, lower panel).

Table II. Comparison of ammonia concentration, UDP-GlcNAc/GalNAc pools, and oligosaccharide antennarity under different culture conditions.

Culture conditions[a]	NH_4^+ (mmol L^{-1})	Cell spec. UDP-GlcNAc/GalNAc[b] (fmol)	*N*-Glycan antennarity (%)		
			Bi-	Tri-	Tetra-
Gln free	< 0.5	0.25	85	12	3
Standard	2–4	0.5	67.5	32.5	2.5
NH_4^+	10–19	1.5	52.4	14	33.6
GlcN (Gln free)	< 0.5	1.5	65	21	14

[a]Gln free, culture I; GlcN, culture III.
[b]Nucleotide-sugar pools were determined as described by Ryll and Wagner (1991); average values of duplicate determinations.

DISCUSSION

Recombinant human glycoproteins destined for clinical applications can be obtained from biotechnological processes by cultivating mammalian host cells harboring the desired foreign gene in a stably integrated form (see Goochee et al., 1991 for review). Several human glycoproteins of therapeutic importance have been expressed in different mammalian hosts, and the carbohydrate structures of a few purified products have been analyzed structurally in remarkable detail by using sophisticated analytical techniques (Hokke et al., 1990; Nimtz et al., 1993; Parekh et al., 1989a,b; Sasaki et al., 1987; Spellman et al., 1989; Tsuda et al., 1988). The published data indicate that in general, each recombinant glycoprotein bears its own typical set of oligosaccharide chains with respect to antennarity, even at individual glycosylation domains, when expressed from different host cell lines (reviewed in Conradt et al., 1990). However, considerable outer chain microheterogeneity is encountered that is characteristic for a given producer cell (Conradt et al., 1990; Takeuchi and Kobata, 1991). This microheterogeneity reflects the different expressions of terminal glycosyltransferase activities in each host.

Unfortunately, in most of the carbohydrate structural studies on recombinant glycoprotein preparations that have been reported so far, no detailed information is presented on the production conditions or on the final yield of the puri-

fied proteins. The IL-Mu6 model glycoprotein used here was purified reproducibly with 80–90% final yield, such that any contribution from the purification procedure to the oligosaccharide pattern of the product obtained under different conditions can be excluded. Furthermore, the cell specific productivity was roughly the same under all culture conditions [0.2 μg (day $10^6)^{-1}$].

It is conceivable that environmental factors in bioprocesses contribute to the final oligosaccharide heterogeneity of glycoproteins. This has been well documented in several publications (reviewed in Goochee et al., 1991; and Jenkins et al., 1996). Different possible mechanisms have been suggested to explain environmental effects on protein glycosylation (e.g., Gawlitzek et al., 1995b; Hooker et al., 1995; Rijcken et al., 1995; Thorens and Vassalli, 1986). In two recent studies, different detrimental ammonia effects on protein glycosylation have been discussed. Borys et al. (1993) described a pH dependent decrease in glycosylation caused by supplemented NH_4Cl (0–9 m*M*). Glycosylation was significantly inhibited at pH 8 but hardly affected at pH 7.2, suggesting that the unprotonated NH_3 was responsible for altered glycosylation. Andersen and Goochee (1995) reported an inhibitory and dose dependent effect of ammonia on sialylation of O-linked glycans from granulocyte colony-stimulating factor produced by CHO cells. Interestingly enough, the largest decrease in sialic acid content was observed between 0 and 2 m*M* NH_4^+; thereafter (2–10 m*M* NH_4^+) sialylation decreased only slightly.

The results presented here strongly suggest that an increase of the intracellular UDP-GlcNAc/GalNAc pool, caused by elevated concentrations of NH_4^+ or glucosamine in the culture medium, contributes to the formation of more complex oligosaccharide structures (increased branching). *N*-Glycans synthesized under low ammonia conditions (Gln free) revealed the most homogenous oligosaccharide pattern with the highest content of terminal sialic acid and proximal fucose. *N*-Glycan heterogeneity increased with ammonium (and glucosamine). In contrast, sialylation decreased only under standard (and glucosamine) conditions but was not further influenced in the presence of 15 m*M* NH_4Cl. Proximal fucosylation was significantly decreased under normal conditions but was moderately improved in medium supplemented with 15 m*M* NH_4Cl.

Our results are supported by the recent data from Pels Rijcken et al. (1995). The authors describe an increased incorporation of *N*-acetylhexosamines into cell associated and secreted glycoproteins due to elevated levels of UDP-GlcNAc/GalNAc caused by supplementing the medium with glucosamine and the pyrimidine nucleoside uridine. Furthermore, high UDP-hexosamine concentrations were found to decrease sialylation. The latter observation was partly confirmed in our experiments where sialylation decreased under standard and glucosamine conditions (elevated UDP-hexosamine conditions compared to Gln-free conditions). However, a further increase of UDP-GlcNAc/GalNAc caused by 15 m*M* NH_4Cl did not result in even lower sialylation.

Because glucosamine did not increase the carbohydrate complexity of IL-Mu6 to the same extent as ammonia, it can be assumed that the elevated UDP-GlcNAc/GalNAc concentration is not the only physiological factor involved. There are a variety of potential mechanisms by which ammonia could interfere with protein glycosylation (e.g., branching, sialylation, and fucosylation). Modulation of glycosylation patterns of polypeptides may be controlled at the transcriptional or translational level of the pertinent transferases. High UDP-GlcNAc/GalNAc concentrations have been discussed as impairing the transport of CMP-NeuAc into the *trans* Golgi compartment, resulting in decreased sialylation (Pels Rijken et al., 1995). Furthermore, weak bases such as ammonia or chloroquine are known to raise the pH of acidic intracellular compartments, e.g., the *trans* Golgi (Thorens and Vassalli, 1986 reviewed in Schneider et al., 1996). An intracellular pH change could affect the glycosylation machinery by several mechanisms. Waldman and Rudnik (1989) suggest that the nucleotide sugar antiport system in the *trans* Golgi membrane may be regulated by acidification of this compartment by the resident ATPase H^+ pump. A Golgi pH change might also affect (decrease or increase; Barasch et al., 1991) the activity of different glycosyltransferases (e.g., the branching enzymes GlcNAc-transferases III and IV; Schachter, 1986). Andersen and Goochee (1995) described a sharp pH optimum of 6.5–6.75 for the O-linked α2,6-sialyltransferase in CHO and calculated a twofold decrease in enzyme activity in the presence of 10 m*M* NH_4Cl.

Glycosidases are considered to be potential factors that can contribute to oligosaccharide heterogeneity (Gramer and Goochee, 1993; Gramer et al., 1995). In our experiments, α-fucosidase and β-galactosidase activities were determined in cell culture supernatants (data not shown). Levels did not change with culture conditions but correlated with cell number during the processes (Gawlitzek, 1995), suggesting that differences in fucosylation or galactosylation were not caused by respective glycosidase action. Moreover, both lysosomal enzymes released from BHK cells have acidic pH optima (pH 4–5.5; Gawlitzek, 1995) and show only a little activity at culture pH (7.2). Sialidase has been shown to decrease sialylation of recombinant glycoproteins in CHO batch cultures (Gramer et al., 1995). Nonetheless, it can be assumed that sialidase activities, although not measured in our experiments, are very low in perfusion cultures with high cell viability (>95%).

It is noteworthy that apart from the alterations in antennarity, sialylation, and proximal fucosylation described above, oligosaccharides synthesized in the presence of ammonia, either derived from glutamine or supplemented exogeneously, reproducibly bear a significant proportion of outer chains with Galβ1-3GlcNAc branches that were not detected under glutamine-free conditions or were present in only negligible amounts. In a previous report on the structural analysis of recombinant erythropoietin from BHK-21 cells, no structures containing Galβ1-3GlcNAc branches were detected (Nimtz et al., 1993). However, Tsuda et al.

(1988) described such oligosaccharide chains in their recombinant EPO preparations from the same host cell line. Different cell culture conditions (e.g., ammonia concentration), although not described in this article, could explain the discrepancy in results.

Discrepancies in the observed effects of ammonia might be explained by the different cell lines (e.g., BHK, CHO, and fibroblasts) and glycoproteins used and by various environmental factors (e.g., FCS, media composition, T flasks vs. bioreactors) involved in these experiments. It is important to mention that carbohydrate moieties of glycoproteins can only be modified or manipulated within the range given by the cell line (e.g., glycosyltransferase activities present) and the 3-dimensional structure of the protein, which determines the accessibility for glycosyltransferases (and also glycosidases). Finally, further studies on the glycosylation characteristics of recombinant glycoproteins produced under different carefully controlled long-term culture conditions may allow future design of bioprocesses with mammalian cell lines by employing media formulations that yield glycosylated products with predictable posttranslational modifications of the desired polypeptides.

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