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ARTICLES

Role of Nucleotide Sugar Pools in the Inhibition of NCAM Polysialylation by Ammonia

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Ammonia in animal cell cultures has been shown to specifically inhibit terminal sialylation of N- and O-linked oligosaccharides of glycoproteins. For example, we have previously shown that as little as 2.5 mM NH_4Cl can decrease neural cell adhesion molecule (NCAM) polysialylation in both small cell lung cancer (SCLC) and Chinese hamster ovary (CHO) cells. Besides its potential involvement in SCLC metastasis, polysialic acid (PolySia) is a sensitive marker for measuring changes in sialylation. The role of UDP-*N*-acetylglucosamine (UDP-GlcNAc) in ammonia's inhibition of NCAM polysialylation was examined by adding glucosamine (GlcN) and uridine (Urd) to the cultures. This bypassed feedback inhibition of GlcN-6-P synthase and increased UDP-GlcNAc content by 25-fold in SCLC cells. After 3 days, PolySia levels were reduced to 10% of control with little effect on NCAM protein content. The extensive decrease in PolySia was confirmed in CHO cells. The effects of GlcN or Urd alone were less extensive, lending support to a specific role for UDP-GlcNAc in inhibition by ammonia. By comparison, 20 mM NH_4Cl decreased PolySia content by 45% and increased UDP-GlcNAc in SCLC cells by 2-fold. The discrepancy between the {GlcN+Urd} and NH_4Cl effects on UDP-GlcNAc and PolySia suggests that accumulation of UDP-GlcNAc is only partially responsible for decreased polysialylation in response to NH_4Cl. In an attempt to increase NCAM polysialylation, *N*-acetylmannosamine and cytidine were added to cultures in order to circumvent the feedback inhibition of CMP-sialic acid synthesis. However, this only slightly increased PolySia levels and failed to counter ammonia's inhibition of NCAM polysialylation.

Introduction

The neural cell adhesion molecule (NCAM) is a cell membrane glycoprotein that exhibits a unique polysialic acid polymer (PolySia) attached to the terminal α(2,3)-linked sialic acid on several complex N-linked oligosaccharides (Figure 1). NCAM polysialylation is a key determinant of the metastatic potential of small cell lung cancer (SCLC; Scheidegger et al., 1994a) and a sensitive marker for changes in glycoprotein sialylation on live cells (Zanghi et al., 1998).

Increasing the concentration of ammonia[1] in the culture medium results in a decrease in NCAM polysialylation (Zanghi et al., 1998). Ammonia rapidly accumulates in acidic cell compartments, including the trans-Golgi where polysialylation occurs, until the pH gradients collapse (de Duve et al., 1974; Anderson and Pathak, 1985; Knepper et al., 1989; Scheidegger et al., 1994b). The increase in intraorganelle pH may inhibit the activity of pH-sensitive sialyltransferases, including polysialyltransferase, leading to decreased polysialylation (Andersen and Goochee, 1995; Oka et al., 1995). Andersen and Goochee (1995) developed a model to show that an observed 2-fold decrease in sialyltransferase activity over the same pH change as that induced by ammonia in the trans-Golgi could explain the observed decrease in O-linked sialylation in recombinant G-CSF secreted by CHO cells. However, Thorens and Vassalli (1986) observed decreased sialylation of immunoglobulins secreted by plasma cells that could not be explained by decreased enzyme activity alone. They showed that sialyltransferase activity in plasma cell extracts was unaffected over a broad range of pH values and concluded their report with no clear explanation for ammonia's effect on sialylation.

Ryll et al. (1994) presented a completely different hypothesis for the mechanism of growth inhibition by

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[1] "Ammonia" is used here to represent collectively the base NH_3 in equilibrium with its acid conjugate NH_4^+.

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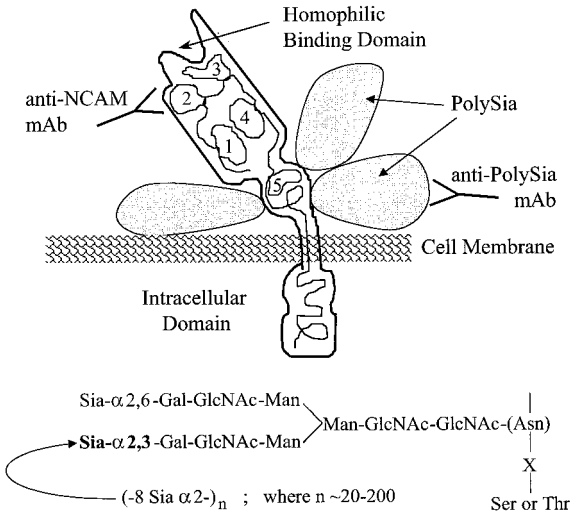

Figure 1. Structure of the neural cell adhesion molecule (NCAM; Goridis and Brunet, 1992; Troy, 1992; Scheidegger et al., 1994a; picture adapted from the Scripps Institute Web page). NCAM mediates cell−cell adhesion in a variety of cells and tissues. The extracellular domain consists of five immunoglobulin-like domains. The polysialic acid polymer (PolySia) consists of α(2,8)-linked sialic acid (NeuAc) residues attached to the terminal α(2,3)-linked sialic acids in up to three N-linked complex oligosaccharides on NCAM's fifth immunoglobulin-like domain. Three other N-linked oligosaccharides are attached to the third and fourth immunoglobulin-like domains. The adhesion properties of NCAM and other cell surface molecules are regulated by the extent of NCAM polysialylation. Two distinct monoclonal antibodies recognize NCAM—one that binds exclusively to PolySia chains greater than three residues, and another that binds to the peptide backbone. With these antibodies, flow cytometry can be used to quantitate both PolySia and NCAM content on single, viable cells under various culture conditions.

ammonia. They suggested that ammonia toxicity is mediated by the intracellular concentration of UDP-*N*-acetylglucosamine (UDP-GlcNAc) and UDP-*N*-acetylgalactosamine (UDP-GalNAc), collectively referred to here as UDP-GNAc. They showed that ammonia accumulation in batch and perfusion cultures is accompanied by an increase in UDP-GNAc levels for various cell lines (Ryll and Wagner, 1992) and that exogenous addition of NH_4Cl also results in a rapid increase in the UDP-GNAc pool, accompanied by a depletion of UTP (Ryll et al., 1994). Depletion of UTP and the subsequent depletion of ATP may result in growth inhibition, but numerous studies have shown that the UDP-GNAc precursor glucosamine (GlcN) can be extremely toxic to cells, even when the UTP and ATP pools are maintained by exogenous addition of uridine (Urd) (e.g., Krug et al., 1984).

Since nucleotide sugars, including UDP-GlcNAc, serve as the sugar donors for protein glycosylation, the intraluminal concentrations of these substrates may be one level of regulation in the biosynthesis of glycoproteins (Schachter, 1986). Pels Rijcken et al. (1995) examined the effects of increased nucleotide sugar pools on the incorporation of sugars into glycoconjugates by rat hepatocytes. By adding 0.5 mM Urd to the medium, they expanded UDP-GNAc intracellular pools 5-fold after 40 h. The treatment also increased incorporation of GlcNAc into cell-associated and secreted glycoconjugates, accompanied by reduced sialylation of both fractions. This implies that ammonia may also inhibit NCAM polysialylation by increasing UDP-GNAc pools. In this regard, Gawlitzek et al. (1995) reported that addition of ammonia or GlcN to BHK-21 cell cultures resulted in decreased sialylation and fucosylation and increased antennarity in the complex N-linked oligosaccharides on a recombinant human mutant interleukin-2 (IL-Mu6). We examined this hypothesis by analyzing SCLC N417 and CHO MT2-1-8 cells cultured in medium containing combinations of GlcN, Urd, and NH_4Cl. Both wild-type CHO K1 and most SCLC cell lines naturally express abundant levels of polysialylated NCAM (Aletsee-Ufrecht et al., 1990; Bloch, 1992). We have previously shown that {GlcN+Urd} causes a rapid increase in the UDP-GNAc pools in SCLC N417 cells by circumventing the negative feedback regulation of GlcN-6-P synthase (see Figure 2) (Pederson et al., 1992).

Pels Rijcken et al. (1995) also showed that, while 0.5 mM Urd or 0.5 mM cytidine (Cyt) caused an increase in hepatocyte UTP and CTP pools, this treatment had no effect on the level of CMP-*N*-acetylneuraminic acid (CMP-NeuAc). This is due to feedback inhibition of UDP-GlcNAc 2-epimerase by CMP-NeuAc (Figure 2). Analogous to the {GlcN+Urd} treatment, we examined the effects of increased CMP-NeuAc pools on NCAM polysialylation by incubating SCLC N417 and CHO MT2-1-8 cells with *N*-acetylmannosamine (ManNAc) and Cyt, thus bypassing the feedback inhibition and potentially increasing NCAM polysialylation. We also examined the possibility that treatment with {ManNAc+Cyt} would offset ammonia's inhibition of polysialylation.

Materials and Methods

Materials. Fetal bovine serum (FBS; Lot No. 31N6560) and MEM nonessential amino acid (NEAA) solution were obtained from Gibco (Grand Island, NY). The CHO MT2-1-8 cell line was obtained from SmithKline Beecham Pharmaceuticals (King of Prussia, PA) and the SCLC NCI-N417 (SCLC N417) cell line (passage no. 40) from the National Cancer Institute (Bethesda, MD). NCAM14.2 monoclonal antibody (mAb; mouse IgG1) was obtained from Becton Dickinson (Research Triangle Park, NC), H28 mAb (rat IgG2a) from Immunotech (Westbrook, ME), and the 5a5 mAb (mouse IgM) hybridoma (Dodd et al., 1988) from the Developmental Studies Hybridoma Bank (Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, and Department of Biological Sciences, University of Iowa). FITC-labeled secondary antibodies included sheep anti-mouse IgG (heavy and light chains) (Sigma, St. Louis, MO; F-2883; used at 1:40 dilution in PBS), goat anti-mouse IgM (μ chain) (Sigma F-9529; 1:40 dilution), and goat anti-rat IgG (heavy and light chains) (Caltag, Burlingame, CA; R40101; 1:30 dilution). All other chemical reagents were purchased from Sigma.

Cell Culture. CHO MT2-1-8 cells were cultured as adherent monolayers and were passaged or harvested using a nonenzymatic dissociating solution (Sigma C-5914). Culture medium consisted of DME/F12 (pH 7.4) supplemented with 29 mM sodium bicarbonate, 25 mM HEPES, 6 mM (final concentration) glutamine, 1% NEAA, 200 nM methotrexate, and 3.5% (v/v) FBS. The SCLC N417 cell line was maintained in culture as loose, floating aggregates. SCLC culture medium consisted of RPMI 1640 (pH 7.4−7.6) supplemented with 24 mM sodium bicarbonate, 25 mM HEPES, 6 mM (final concentration) glutamine, 17.5 mM (final concentration) glucose, 1 mM pyruvate, 1% NEAA, SIT (30 nM sodium selenite, 5 μg/mL bovine insulin and 10 μg/mL human holo-transferrin), and 2.5% (v/v) FBS. Both cell lines were grown without antibiotics at 37 °C, 98% relative humidity, and 5% CO_2 atmosphere. Cell concentration and viability were measured using a hemacytometer and the trypan blue dye exclusion method. Glucose and lactate concentrations were measured simultaneously

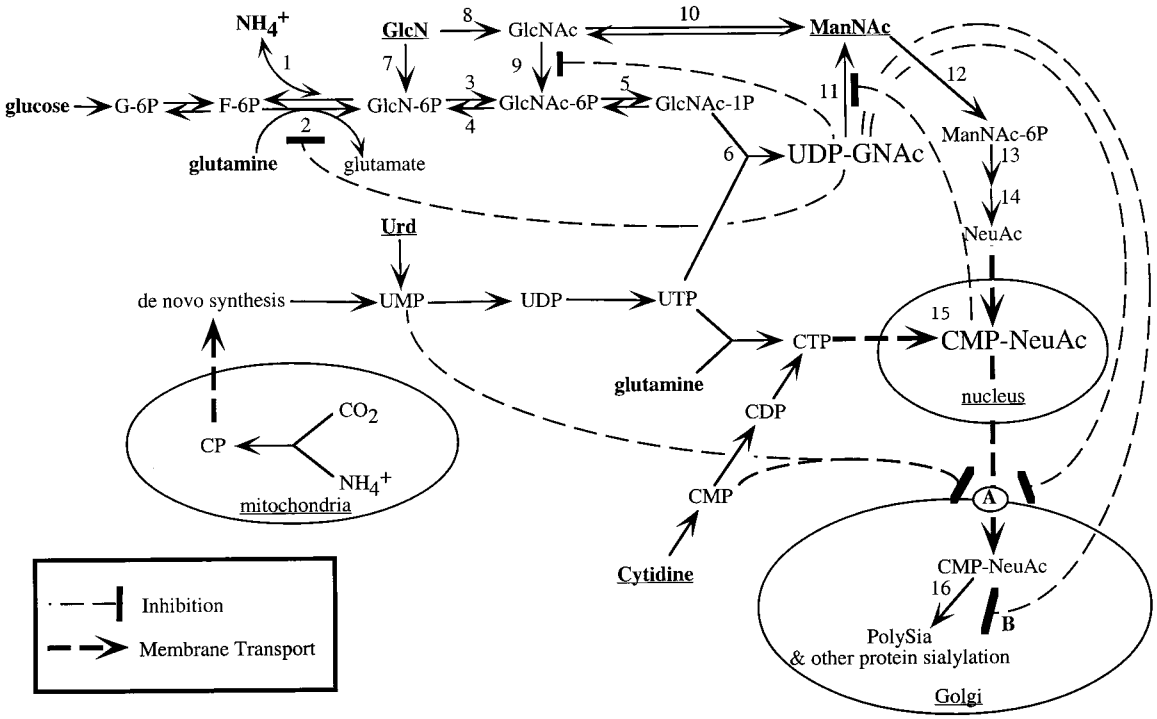

Figure 2. Synthesis and regulation of nucleotide sugars UDP-GNAc and CMP-NeuAc and their role in glycosylation (Corfield and Schauer, 1982; Ryll et al., 1994). Important enzymes are numbered as follows: 1, GlcN-6-P deaminase; 2, GlcN-6-P synthase; 3, GlcN-6-P *N*-acetyltransferase; 4, GlcNAc-6-P deacetylase; 5, GlcNAc-6-P mutase; 6, UDP-GlcNAc-1-P pyrophosphorylase (the product of this reaction is UDP-GlcNAc, which is in equilibrium with UDP−GalNAc via the UDP-GlcNAc 4-epimerase; the collective pool of UDP-GlcNAc and UDP-GalNAc is referred to here as UDP-GNAc); 7, hexokinase/glucokinase; 8, GlcN *N*-acetyltransferase; 9, GlcNAc kinase; 10, GlcNAc 2-epimerase; 11, UDP-GlcNAc-2-epimerase; 12, ManNAc kinase; 13, NeuAc-9-P synthase; 14, NeuAc-9-P phosphatase; 15, CMP-NeuAc synthase; 16, sialyltransferase or polysialyltransferase. Additional notes: UDP-GlcNAc, UMP, and CMP can inhibit the translocation of CMP-NeuAc across the Golgi membrane (Carey et al., 1980); elevated levels of UDP-GlcNAc may inhibit sialylation by increasing the antennarity of the protein oligosaccharide. It has been shown that exogenous GlcN and ammonia increase the antennarity in the complex N-linked oligosaccharides on a recombinant human mutant interleukin-2 (IL-Mu6) (Gawlitzek et al., 1995), and an altered oligosaccharide may be a less favorable substrate for the terminal sialylation reaction (Mostafapour and Goldstein, 1993; Oka et al., 1995).

using a YSI 2700 Biochemistry Analyzer (YSI, Yellow Springs, OH).

Cell growth experiments were conducted in 24-well (SCLC) or 6-well (CHO) plates (Corning, NY) as described previously (Zanghi et al., 1998). SCLC N417 cells were seeded at a density of 0.025×10^6 cells/mL and a volume of 1.25 mL, with the eight inner wells containing cells and the remaining outer wells filled with water. The cells were added at time zero to test medium previously equilibrated to the culture conditions. CHO MT2-1-8 cells were seeded at a density of 0.020×10^6 cells/mL and a volume of 4 mL. Seeding time was 12−18 h prior to the beginning of an experiment (time $t = 0$) to allow sufficient time for the cells to adhere to the plates. At $t = 0$, seeding medium was replaced with test medium preequilibrated to the culture conditions. A single plate was sacrificed for each treatment per time point ($n = 4$, remaining wells filled with water). Cultures for flow cytometry experiments were set up in a similar manner, except that T-flasks were used in order to obtain sufficient cell numbers for analysis. One flask was analyzed per condition per time point, and three flow cytometry samples were prepared per antibody per condition as described below.

Flow Cytometry Sample Preparation. Cells were washed once with PBS FACS (PBS, 5% v/v FBS, and 0.02% NaN_3 at 4 °C) and placed in polystyrene test tubes (Falcon No. 2008, Becton Dickinson Labware, Lincoln Park, NJ) with 0.50×10^6 total cells per sample for CHO MT2-1-8 cells and 0.75×10^6 total cells per sample for SCLC cells. Twenty microliters of primary antibody diluted in PBS FACS was added per sample. SCLC cell surface NCAM content was measured with the NCAM14.2 mAb prepared from mouse ascites fluid diluted 1:40, and CHO cell NCAM content was measured with the H28 mAb diluted 1:8. Cell surface polysialic acid content was measured with 5a5 mAb hybridoma supernatant diluted 1:5. We have previously demonstrated the specificities of these mAbs in our culture system (Zanghi et al., 1998). Background samples were prepared by adding PBS FACS without antibody. Following the addition of primary antibody, cells were incubated on ice for 30−60 min, washed twice with PBS FACS, and resuspended in 20 μL of FITC-labeled secondary antibody (see also Materials section) or 20 μL of PBS FACS (background). Isotype controls were not used because previous studies showed that the cell fluorescence for isotype controls was the same as background cell fluorescence (Zanghi et al., 1998). Cells were incubated on ice and in the dark for 30−60 min, washed once, and resuspended in 500 μL of PBS FACS. Live and dead cells were discriminated by the addition of 10 μg/mL of propidium iodide (PI) 5−10 min prior to flow cytometric analysis. SCLC cells were occasionally filtered through a 41-μm polyester mesh (Spectrum Medical Industries, Houston, TX) to remove large clumps.

Flow Cytometry Acquisition and Analysis. Data were acquired on a FACScan (Becton Dickinson, Franklin Lakes, NJ) flow cytometer and analyzed with CELLQuest software (Becton Dickinson). The PMT voltage for FL1 (FITC fluorescence) and FL2 (PI fluorescence) was adjusted such that the mean background fluorescence for

the control sample was 4−6 relative fluorescence units (RFI) on a 4-decade log scale (1−10 000). A total of 10 000 or 20 000 total events were acquired per sample, and data were limited to single, viable cells by gating on forward scatter (FSC), side scatter (SSC), and FL2 fluorescence. All flow cytometry data are presented normalized to control using the following formula:

$$\text{normalized MFI} = \frac{\overline{\text{MFI}}_{\text{mAb (test)}} - \overline{\text{MFI}}_{\text{bk (test)}}}{\overline{\text{MFI}}_{\text{mAb (control)}} - \overline{\text{MFI}}_{\text{bk (control)}}} \quad (1)$$

where $\overline{\text{MFI}}_{\text{mAb (test or control)}}$ is the average MFI on replicate samples for the test or control culture stained with anti-NCAM or anti-PolySia mAb, and $\overline{\text{MFI}}_{\text{bk (test or control)}}$ is the average MFI on replicate background samples for the test or control culture. Error bars for the normalized MFI values were calculated from the following approximation:

$$\text{SD} = (\text{normalized MFI})\left[\left(\frac{\overline{\text{MFI}}_{\text{mAb (test) SD}}}{\overline{\text{MFI}}_{\text{mAb (test)}}}\right)^2 + \left(\frac{\overline{\text{MFI}}_{\text{mAb (control) SD}}}{\overline{\text{MFI}}_{\text{mAb (control)}}}\right)^2\right]^{1/2} \quad (2)$$

where $\overline{\text{MFI}}_{\text{mAb (test or control) SD}}$ is the standard deviation on $\overline{\text{MFI}}_{\text{mAb (test or control)}}$. Normalization was necessary to compensate for variations between different days of analysis.

Nuclear Magnetic Resonance (NMR) Spectroscopy. A total of 10^8 viable cells were suspended in 50 mL of medium containing the desired test agent(s) and incubated for 4−5 h. Longer incubation times were not possible due to the depletion of medium nutrients. The cells were pelleted and immediately extracted with 5 mL of a 2:1 mixture (−20 °C) of chloroform and methanol, followed by 2.5 mL of water. The extract was centrifuged for 10 min at 450*g*, and the top aqueous layer was carefully removed and concentrated to about 50 μL without drying. Samples were stored at −80 °C until NMR analysis. Prior to analysis, each sample was reconstituted in 0.45 mL of D_2O containing 10−20 mM EDTA (pH 8.2). Data were obtained at 9.4 T using a GE Omega 400 spectrometer (General Electric, Fremont, CA) with a transmitter frequency of 169.4 MHz, a 60° tip angle, a 2-s repetition time, 800 acquisitions, 32K block size, 20 000-Hz sweep width, and 0.5-Hz line broadening prior to Fourier transformation. The samples were proton-decoupled (Waltz-16) during acquisition. Using these parameters, the spectral intensity changes accurately reflect changes in the intracellular concentrations (Pederson et al., 1992). Relative changes in UDP-GNAc content were determined by comparing the ratio of UDP-GNAc:glycerophosphocholine peak heights between samples and normalizing to control.

Calculation of Metabolic Parameters. Specific glucose consumption (q_{glc}) and lactate production (q_{lac}) rates were calculated from the following formula:

$$C_s = q_s \int_0^t X_v \, d\tau + C_s(0) \quad (3)$$

where C_s (mmol/mL) is the concentration of substrate (glucose or lactate), q_s (mmol cell^{-1} h^{-1}) represents $-q_{glc}$ or q_{lac}, and X_v is the viable cell concentration (cells/mL). The integral represents the cumulative viable cell-hours and was calculated at each time point t from the viable cell concentration vs time data. q_s was obtained by plotting C_s vs cumulative cell-hours and calculating the slope using regression analysis, assuming constant q_s. The curve fit was weighted by the standard errors of the individual data points.

Sialidase Activity. Cell-free cell culture supernatants (stored at −20 °C) were centrifuged at 16000*g* for 15 min in a microcentrifuge (Eppendorf, Westbury, NY) at room temperature prior to use. For the determination of cell lysate sialidase activity, cells from high-viability cultures were lysed at a density of 1 million total pelleted cells per 100 μL of lysis buffer (Sigma). The cells were vortexed and pipetted to ensure complete cell lysis. Sialidase activity was determined fluorometrically with (4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (4MU-NeuAc) using a modification of the protocol developed by Gramer and Goochee (1993) and Munzert et al. (1996). To a new 1.5-mL microcentrifuge tube, the following reagents were added sequentially: 25 μL of 4 mM 4MU-NeuAc, 25 μL of 1 M KH_2PO_4 buffer (adjusted to pH 7.0 with saturated NaOH), and 250 μL of cell-free supernatant or cell lysate (diluted with water). This mixture was allowed to incubate in a 37 °C water bath for 1 h for cell lysate samples or 3 h for supernatant samples. The reaction was then quenched with 700 μL of 0.375 M glycine buffer (adjusted to pH 10.4 with saturated KOH) and centrifuged for 5 min at 16000*g*. Samples were diluted to a total of 3 mL with the glycine/KOH buffer and then loaded into UV-transparent cuvettes (1 cm path length, VWR); 250 μL of DMEM/F12 was added to cell lysate samples prior to the final dilution. Negative controls consisted of samples containing only DMEM/F12 or lysis buffer with DMEM/F12, with or without 4MU-NeuAc. A positive control was prepared containing 4 munits/mL neuraminidase V. Fluorescence measurements were carried out at 362 (excitation) and 448 nm (emission) on a SPEX Fluorolog (SPEX, Edison, NJ) fluorescence spectrometer with a SPEX 1681 0.22-m excitation spectrometer and SPEX 1680 0.22-m emission double spectrometer, employing 0.5-mm slits in the light path. Data were collected on a Packard Bell 486 computer running DM-3000 software (SPEX). 4MU in DME/F12 was used as a calibration reference. Assay sensitivity was determined to be 0.5 nmol of 4MU per milliliter of sample per hour using serial dilutions of the 4 munits/mL neuraminidase V positive control.

Results

Glucosamine and Uridine vs Ammonia. *SCLC Cell Growth and Metabolism.* We used the SCLC N417 cell line because the effects of GlcN and Urd had been previously characterized in our laboratory (Pederson et al., 1992). In that study, it was shown that the molar ratio of GlcN to glucose (GlcN:Glc) is important in determining the effects of GlcN on the nucleotide pools, presumably due to competition by GlcN for the glucose transporter. At a ratio of 1:1, UDP-GNAc levels increased and reached a steady value after 6 h. This was accompanied by accumulation of GlcNAc-6-P and depletion of UTP. Subsequent addition of Urd caused an additional increase in UDP-GNAc and a decrease in GlcNAc-6-P, consistent with UTP limitation for GlcN alone. In contrast, a 1:10 GlcN:Glc ratio resulted in conversion of GlcN to UDP-GNAc without depletion of UTP or phosphocreatine (Pederson et al., 1992). We wished to examine the effects of {GlcN+Urd} treatment on NCAM polysialylation over longer culture periods comparable to those in our previous experiments with

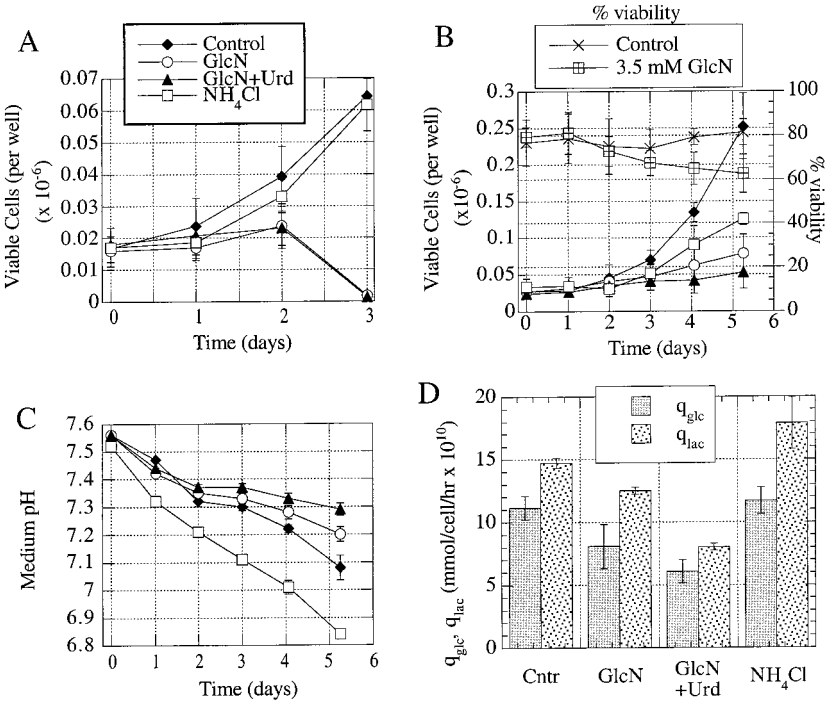

Figure 3. Effect of glucosamine, uridine, and ammonia on SCLC N417 cell growth and metabolism. In the first experiment (A), cells were cultured in well plates containing medium supplemented with 17.5 mM GlcN, 17.5 mM GlcN plus 1 mM Urd, or 10 mM NH_4Cl. In the second experiment (B−D), cells were cultured with 3.5 mM GlcN, 3.5 mM GlcN plus 1 mM Urd, or 20 mM NH_4Cl. The legend in plot A also applies to the appropriate symbols in plots B and C. Error bars in plots A−C represent one standard deviation ($n = 4$). Error bars in plot D represent the standard errors on the linear curve fits (see Materials and Methods).

ammonia (Zanghi et al., 1998). In those experiments, PolySia decreased rapidly and reached a plateau 1−3 days after NH_4Cl addition. We first examined a 1:1 ratio of GlcN:Glc, with or without Urd. At this ratio, GlcN was toxic to the cells, regardless of whether Urd was present (Figure 3A). There was a delay in the response as cell numbers increased slightly after 2 days in culture and then dramatically decreased 1 day later. On the other hand, 10 mM NH_4Cl had little effect on the cells, consistent with previous results for this cell line (Zanghi et al., 1998).

The growth experiment was repeated using a 1:5 GlcN:Glc ratio and 20 mM NH_4Cl to better match the cells' response to the two conditions. The cells did grow in the presence of 3.5 mM GlcN, although very slowly (Figure 3B). Growth was further reduced by the addition of Urd. Several qualitative differences in the cells' responses to NH_4Cl vs GlcN or {GlcN+Urd} are apparent in Figure 3. First, a decrease in cell viability was never observed for cells cultured in 20 mM NH_4Cl (not shown, similar to control culture in Figure 3B), but the viability decreased even with 3.5 mM GlcN (Figure 3B, {GlcN+Urd} treatment gave similar results). A second qualitative difference is shown in Figure 3C. The decrease in medium pH over the course of a batch culture was considerably greater in the presence of 20 mM NH_4Cl, with pH approaching 6.8 after 5 days. This difference in pH cannot be explained by differences in lactic acid production, as the measured lactate concentration for these cells was equal to or less than that for the control culture. A similar pH decrease was observed in other SCLC cell cultures supplemented with NH_4Cl (Zanghi et al., 1998). The slightly higher pH values for GlcN and {GlcN+Urd} cultures can be attributed to lower lactate accumulation. Finally, GlcN and ammonia had opposite effects on glycolysis. q_{glc} and q_{lac} were lower for cells treated with GlcN or {GlcN+Urd} (Figure 3D). In contrast, q_{glc} and q_{lac} remained the same or increased following NH_4Cl addition, consistent with previous studies (Miller et al., 1988; Zanghi et al., 1998).

SCLC Cell NCAM and Polysialic Acid Content. N417 cells were cultured in T-flasks for 3 days in the presence of 3.5 mM GlcN, 3.5 mM GlcN plus 1 mM Urd, or 20 mM NH_4Cl. The combination of GlcN and Urd had a dramatic effect on PolySia, with levels decreasing to 10% of control (Figure 4A). There was a much smaller effect on PolySia content with GlcN alone, perhaps due to UTP limitation. In the presence of NH_4Cl, PolySia content decreased to roughly 50% of control, in agreement with previous results (Zanghi et al., 1998). NCAM content increased when cells were exposed to GlcN, although to a lesser extent when Urd was also present. The large increase in NCAM following GlcN treatment may explain the smaller decrease in PolySia compared to that for {GlcN+Urd}. However, even if PolySia is normalized to NCAM, the decrease with GlcN alone is no greater than that for NH_4Cl and much less than that for {GlcN+Urd}.

UDP-GNAc Pools. To investigate the role of UDP-GNAc in the observed changes in PolySia, the intracellular UDP-GNAc concentration was measured in N417 cells after a 4-h incubation at the same conditions as those in the flow cytometry experiment discussed above. Based on ^{31}P NMR analysis of cell extracts, UDP-GNAc levels increased by 6-, 25-, and 2-fold compared to control for cells cultured with GlcN, {GlcN+Urd}, and NH_4Cl, respectively (Figure 4B). The elevated UDP-GNAc levels that are associated with decreased PolySia content in cells treated with either {GlcN+Urd} or NH_4Cl suggest that ammonia inhibits NCAM polysialylation at least in part via elevated UDP-GNAc levels. In this regard, the treatment with the greatest increase in UDP-GNAc levels produced the greatest decrease in PolySia. However, it should be noted that the relative increase in UDP-GNAc

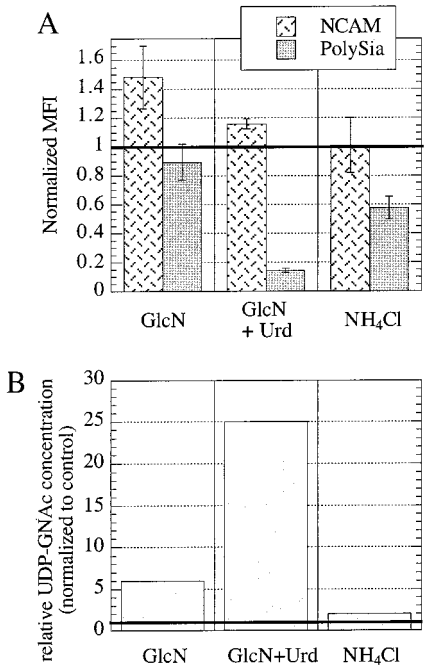

Figure 4. Effects of glucosamine, glucosamine plus uridine, and NH_4Cl on SCLC N417 cell (A) NCAM and PolySia content after 3 days and (B) UDP-GNAc intracellular concentration after 4 h. For each experiment, cells were cultured in T-flasks with medium containing 3.5 mM GlcN, 3.5 mM GlcN plus 1 mM Urd, or 20 mM NH_4Cl and then analyzed at the indicated times. Data in plot A were obtained by normalizing the mean fluorescence intensity (MFI, see Materials Methods; $n = 3$ for each parameter) and averaging the normalized MFI from duplicate experiments. The error bars represent the standard deviation between the two experiments. The data in plot B were obtained from the ^{31}P NMR spectra of sample extracts prepared from a separate cell culture experiment.

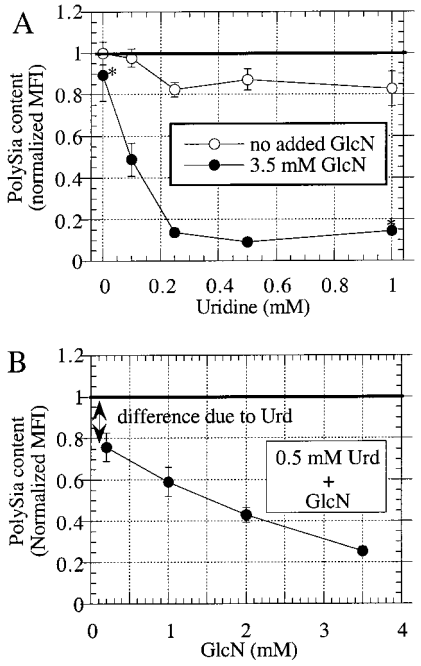

Figure 5. Effects of the uridine and glucosamine dose on SCLC N417 cell PolySia content. Cells were cultured for 3 days in medium containing (A) different concentrations of Urd, with or without 3.5 mM GlcN, or (B) different concentrations of GlcN with 0.5 mM Urd, and then analyzed by flow cytometry. PolySia content is expressed as the MFI normalized to control (no GlcN or Urd added). The data points with an asterisk in plot A were reproduced from Figure 4. Error bars represent the standard deviation after normalization ($n = 3$).

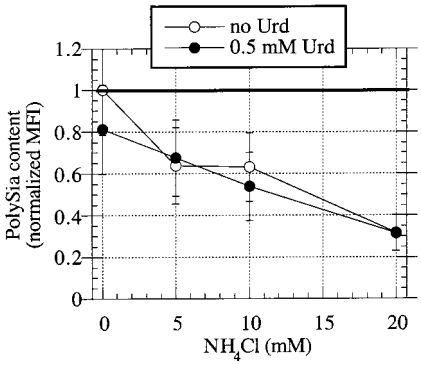

Figure 6. Effects of uridine and ammonia on SCLC N417 cell PolySia content. Cells were cultured for 3 days in medium containing different concentrations of NH_4Cl, with or without 0.5 mM Urd and then analyzed by flow cytometry. PolySia content is expressed as the MFI normalized to control (no Urd or NH_4Cl added). Error bars represent the standard deviation after normalization ($n = 3$).

does not strictly correlate with the decrease in PolySia. While 3.5 mM GlcN alone results in UDP-GNAc levels after 4 h that are about 3 times higher than those produced by 20 mM NH_4Cl, the decrease in PolySia after 3 days is less extensive for the GlcN treatment. Even adjusting for the increase in NCAM content with GlcN by normalizing PolySia content with respect to NCAM, the relative decrease in PolySia is then similar for the two treatments, despite the difference in UDP-GNAc levels.

Different Doses and Combinations of Uridine, Glucosamine, and Ammonia. Alone or with 3.5 mM GlcN, there was a dose-dependent decrease in PolySia up to 0.25 mM Urd with no additional change in PolySia at higher Urd concentrations (Figure 5A). In contrast, GlcN caused a dose-dependent decrease in NCAM polysialylation under conditions of excess Urd, with a 50% decrease in PolySia at 1.5 mM GlcN (Figure 5B). The role of UTP limitation in the inhibition of PolySia under elevated levels of ammonia was examined by culturing N417 cells with increasing concentrations of NH_4Cl, with and without Urd. There was a 20% decrease in PolySia when 0.5 mM Urd was added alone (Figure 6), similar to that discussed above (compare to Figure 5A). However, there was no additional decrease in PolySia content when Urd was added in the presence of NH_4Cl.

CHO MT2-1-8 Cell NCAM and Polysialic Acid Content. The effects of a 2-day exposure to 3.5 mM GlcN, 1 mM Urd, or a combination of the two additives were examined in CHO MT2-1-8 cells to determine if inhibition of NCAM polysialylation by UDP-GNAc is a general phenomenon. Urd had no effect on cell growth or viability, GlcN decreased the final cell concentration by 10% with no decrease in viability, and {GlcN+Urd} decreased the cell concentration by 20% and the viability to 87% (from 95%; not shown). PolySia decreased to 45% and 25% of control when the cells were exposed to GlcN or {GlcN+Urd}, respectively (Figure 7). The decrease in PolySia with GlcN alone was much greater than that observed for N417 cells, while the decrease for {GlcN+Urd} was less dramatic. With GlcN alone, the difference may be explained in part by increased NCAM levels in the N417 cells. However, even after normalizing to NCAM, the change in PolySia in response to GlcN is about 50% greater (60% vs 40% decrease relative to control) for the CHO cells. Thus, inhibition of CHO MT2-1-8 cell PolySia by GlcN is less dependent on the presence of Urd than is inhibition of SCLC N417 cell PolySia. This suggests that

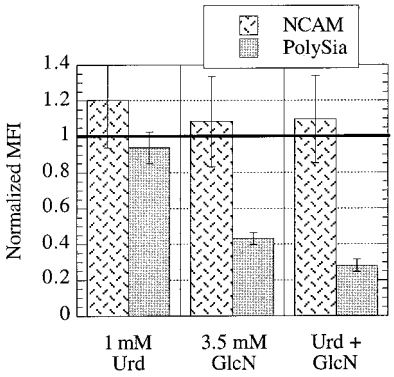

Figure 7. Effects of glucosamine and uridine on CHO MT2-1-8 cell NCAM polysialylation. Cells were cultured for 2 days in medium containing 3.5 mM GlcN, 1 mM Urd, or 3.5 mM GlcN plus 1 mM Urd and then analyzed by flow cytometry. The error bars represent the standard deviation between duplicate experiments ($n = 3$ for each experiment).

UTP was not as limiting for UDP-GNAc formation in CHO MT2-1-8 cells under these conditions and is consistent with the small change in CHO cell PolySia in response to 1 mM Urd (Figure 7). Similar to the results found for N417 cells, there was no effect of Urd on the response by CHO cells to 10 mM NH_4Cl and no difference between the effects of 0.5 or 1 mM Urd (not shown).

Modulation of Intracellular CMP-NeuAc Pools. *N*-Acetylmannosamine (ManNAc) and a combination of ManNAc and cytidine (Cyt) were added to N417 and MT2-1-8 cell cultures in an effort to increase NCAM polysialylation and to further examine the role of nucleotide sugars in the inhibition of PolySia by ammonia. Since CMP-NeuAc is the precursor for sialylation reactions (including NCAM polysialylation) in the trans-Golgi, it is possible that elevated levels of CMP-NeuAc in the Golgi lumen will increase the PolySia content. Similarly, if observed decreases in PolySia under elevated levels of NH_4Cl are due to competitive inhibition by UDP-GNAc of the CMP-NeuAc transporter on the Golgi membrane (see point A in Figure 2), then elevated concentrations of CMP-NeuAc may prevent this from happening, provided that levels of CMP-NeuAc in the cytosol are increased sufficiently to out-compete elevated levels of UDP-GNAc. The CMP-NeuAc concentration was not measured in this study, but evidence in the literature suggests that the treatments used here will increase the intracellular CMP-NeuAc concentration by as much as 30-fold (see Discussion). Combinations of {ManNAc+Cyt} and NH_4Cl (10 mM for MT2-1-8 cells, 20 mM for N417 cells) were also examined, and Cyt alone was added as a control.

Based on estimates from cell samples collected prior to flow cytometry, Cyt alone had little effect on cell growth, viability, q_{glc}, or q_{lac} in either cell line. ManNAc and {ManNAc+Cyt} resulted in a slight decrease in growth and an increase in q_{glc} and q_{lac} for both cell lines. The change was more apparent for the N417 cells, in which a decrease in viability was also noted (from ~70% to ~50%). ManNAc and {ManNAc+Cyt} caused a slight increase in PolySia (about 110% of control) in both cell lines (Figure 8). The presence of ManNAc appears to offset the inhibitory effects of Cyt alone on PolySia content, especially for CHO cells. The Cyt concentration (0.1, 0.2, or 0.5 mM) did not affect the responses of N417 cells, with or without ManNAc (not shown). Finally, inhibition of PolySia by NH_4Cl was not significantly affected by {ManNAc+Cyt} (Figure 8) or (for CHO cells) by ManNAc alone (not shown).

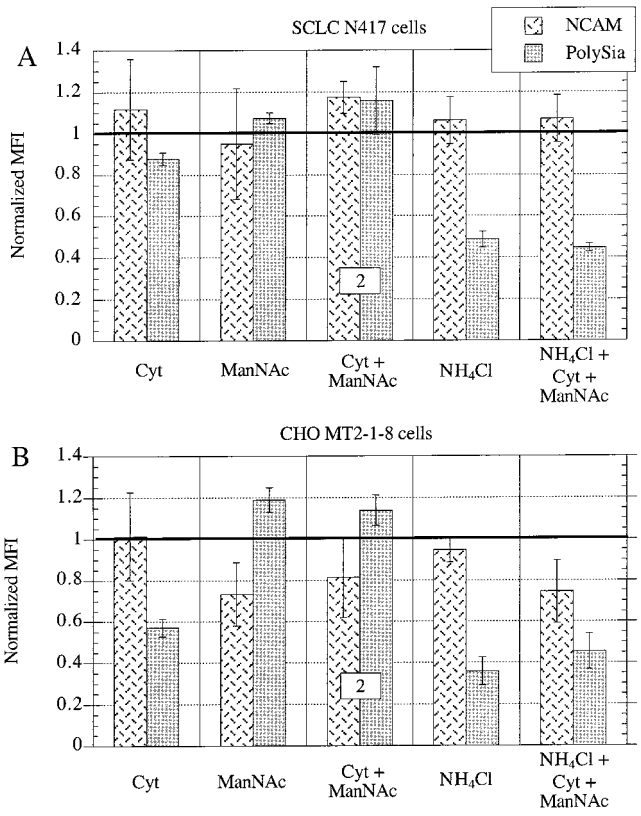

Figure 8. Effect of *N*-acetylmannosamine, cytidine, and ammonia on (A) SCLC N417 and (B) CHO MT2-1-8 cell NCAM and PolySia content. Cells were cultured for 2 days (MT2-1-8) or 3 days (N417) in medium containing combinations of 0.5 mM Cyt, 20 mM ManNAc, and 10 mM (MT2-1-8) or 20 mM (N417) NH_4Cl and then analyzed by flow cytometry. The boxed number indicates that two experiments were performed for the indicated treatment; the other histograms represent one experiment. Error bars on unnumbered histograms represent the standard deviation on triplicate samples, and error bars on numbered histograms represent the standard deviation between experiments ($n = 3$ for each experiment).

Sialidase Activity. Cell culture supernatant samples were analyzed to ensure that observed decreases in PolySia content were not due to extracellular degradation by sialidase released from nonviable cells (Gramer and Goochee, 1993). There was little sialidase activity (<0.5 nmol 4MU h^{-1} mL^{-1} vs ~65 nmol h^{-1} mL^{-1} for 4 munits/mL neuraminidase V) in any of the CHO cell culture supernatants and no difference in sialidase activity for any of the culture conditions (not shown). Similar results were obtained for supernatant samples from N417 cells exposed to ammonia. Samples were not analyzed for N417 cells exposed to GlcN, Urd, ManNAc, and/or Cyt. However, our previous results indicate that N417 cells do not have detectable intracellular sialidase activity (Zanghi et al., 1998).

Discussion

Ryll et al. (1994) proposed that NH_4^+ serves as a precursor in UDP-GNAc formation by reacting with fructose-6-phosphate (F-6P) to form GlcN-6P (Figure 2, reaction 1) (Ghosh et al., 1960; Ryll et al., 1994). This reaction normally proceeds in the opposite direction (Gryder and Pogell, 1959), but when GlcN-6P deaminase is coupled with GlcN-6P-acetylase in the presence of acetyl CoA, GlcN-6P may be formed from NH_4^+ and F-6P. Decreased PolySia content and increased levels of UDP-GNAc in cells exposed to ammonia or {GlcN+Urd}

suggest that ammonia may inhibit NCAM polysialylation via increased intracellular UDP-GNAc levels (Figure 4). This is consistent with a previous report that ammonia and GlcN can inhibit sialylation by this pathway (Gawlitzek et al., 1995). However, in comparison to the other treatments, the relative decrease in PolySia content for ammonia was much greater than the relative increase in UDP-GNAc content (Figure 4). This discrepancy between the decrease in PolySia and the increase in UDP-GNAc may be due in part to the difference in exposure time prior to PolySia (3 days) vs UDP-GNAc (4 h) analysis. The kinetics of the response to ammonia or GlcN, whether measuring PolySia or UDP-GNAc, are cell line-dependent and difficult to predict (Pederson et al., 1992; Ryll et al., 1994; Zanghi et al., 1998). However, other evidence also suggests that ammonia affects sialylation via mechanisms other than an increase in UDP-GNAc. Unlike GlcN, the effects of ammonia are not potentiated by Urd (Figure 6). This indicates that UTP is not limiting and that a bottleneck at the final reaction in the synthesis of UDP-GNAc does not occur during exposure to NH_4Cl. While it is possible that accumulation of NH_4^+ in the mitochondria may cause elevated levels of carbamoyl phosphate (CP) to be produced and diffuse into the cytoplasm where the de novo UTP synthesis reactions occur (Figure 2), this phenomenon has not been substantiated in the type of cells examined here (Schneider et al., 1996).

Together, our results suggest that ammonia also decreases sialylation via other mechanisms, such as an increase in the trans-Golgi pH. This explanation is consistent with the results of Andersen and Goochee (1995) (see Introduction) and with recent reports of decreased sialylation of recombinant proteins by CHO and BHK-21 cells exposed to NH_4Cl. Gawlitzek et al. (1998a) showed that 5 mM GlcN, which yields an increase in UDP-GNAc similar to 13 mM NH_4Cl, had no effect on the galactose or sialic acid content of a recombinant immunoadhesin produced in CHO cells. In contrast, 165 μM of the weak base chloroquine, which caused an increase in the pH of acidic compartment similar to 13 mM NH_4Cl, decreased galactosylation and sialylation similar to the effect of 13 mM NH_4Cl. Similarly, Grammatikos et al. (1998) showed that addition of {12.5 mM GlcN + 2 mM Urd} to glutamine-free medium did not decrease the fractional sialylation of IL-Mu6 by BHK-21 cells, even though intracellular UDP-GNAc levels increased by 15-fold. In contrast, when adenosine was added to prevent an associated increase in UDP-GNAc levels, 12 mM NH_4Cl decreased the fractional sialylation of IL-Mu6 by 12%.

Regardless of whether UDP-GNAc production is responsible for inhibition of PolySia by ammonia, it is clear that GlcN and/or Urd decreases PolySia content (Figures 4A, 5 and 7). As shown in Figure 4B, GlcN and {GlcN+Urd} significantly increase UDP-GNAc levels in N417 cells. Pederson et al. showed that 0.5−10 mM Urd alone increased UDP-GNAc levels by 2-fold in N417 cells (Pederson et al., 1992; Pederson, 1997). The data are consistent with the hypothesis that UDP-GNAc inhibits NCAM polysialylation, and it appears that UTP availability limits UDP-GNAc formation in N417 cells at Urd concentrations below 0.25 mM (Figure 5A). When Urd or GlcN alone is added to N417 cell cultures, there is an accumulation of intermediate metabolites in the UDP-GNAc synthesis pathway (Pederson et al., 1992). With Urd alone, the metabolites include UTP and presumably UMP and UDP. With GlcN alone, the intermediates are primarily GlcNAc-6P and GlcNAc-1P; GlcN-6P is probably present as well (Figure 2). Subsequent addition of GlcN or Urd, respectively, results in depletion of the intermediate metabolites and increased levels of UDP-GNAc (Pederson et al., 1992). Since the combination is much more effective in decreasing N417 cell PolySia content, the data in Figures 4 and 5A provide compelling evidence that it is UDP-GNAc and not the intermediate metabolites that inhibits NCAM polysialylation. Although we have not measured UDP-GNAc levels in CHO MT2-1-8 cells, the data in Figure 7 suggest that UTP production is not as limiting in these cells. Our results are consistent with previous reports that GlcN or Urd can inhibit sialylation via UDP-GNAc (Gawlitzek et al., 1995; Pels Rijcken et al., 1995). However, the effects are likely to be cell-type- or protein-specific because an increase in UDP-GNAc in response to 5 mM GlcN did not affect the sialylation of a recombinant immunoadhesin by another CHO cell line (Gawlitzek et al., 1998a). The results obtained may also be affected by the presence of uridine. For example, sialylation of IL-Mu6 has been reported to decrease in BHK-21 cells exposed to 10 mM GlcN (Gawlitzek et al., 1998b), but not in cells exposed to {12.5 mM GlcN + 2 mM Urd} (Grammatikos et al., 1998). Interestingly, in our hands, adding Urd to GlcN further enhanced the decrease in PolySia content.

UDP-GNAc may affect sialylation via several mechanisms. Previous studies with hepatocytes have shown that elevated levels of UDP-GNAc increase incorporation of GlcNAc into cell-associated and secreted glycoconjugates (Pels Rijcken et al., 1995). This would change the oligosaccharide structure to which sialic acids, including PolySia (Figure 1), are added. Indeed, Pels Rijcken et al. (1995) did observe decreased sialylation of both cell-associated and secreted glycoconjugages. Gawlitzek et al. (1995, 1998b) showed that GlcN and/or ammonia increased the antennarity on the N-linked oligosaccharide of IL-Mu6 produced by BHK-21 cells. However, they could not explain how this would result in decreased sialylation, since the increased antennarity created more available sites for addition of sialic acid to the oligosaccharide. Grammatikos et al. (1998) showed that {GlcN+Urd} increased UDP-GNAc levels and increased the oligosaccharide antennarity of IL-Mu6 with no change in the fraction of available galactose sites that were sialylated. Incorporation of sialic acid into glycoconjugates is catalyzed by sialyltransferases that recognize the terminal and subterminal sugars, their sequence, and other specific linkages on the oligosaccharide (Mostafapour and Goldstein, 1993). Thus, increased antennarity may decrease sialyltransferase recognition, resulting in a decrease in the number of α(2,3)-linked sialic acid sites available for polysialylation. Polysialyltransferase (PST) activity is also highly dependent on the oligosaccharide and peptide structure of NCAM (Oka et al., 1995). Reduced PST activity would result in fewer and/or shorter PolySia chains.

Alternatively, increased levels of UDP-GNAc may inhibit the transport of CMP-NeuAc into the Golgi lumen (point A in Figure 2) where sialylation and polysialylation occur. Using mouse liver microsomes in a cell-free system, it was shown that UDP-GlcNAc specifically inhibits the transport of CMP-NeuAc by 30% (Carey et al., 1980). If ammonia acts via UDP-GlcNAc by decreasing the availability of CMP-NeuAc in the Golgi lumen, we hypothesized that an increase in the CMP-NeuAc pool would offset ammonia inhibition of PolySia. However, although ManNAc may have slightly increased PolySia

levels, especially in CHO cells, {ManNAc+Cyt} had no effect on the decrease in PolySia in response to NH_4Cl (Figure 8).

We did not measure the CMP-NeuAc concentration, so it is possible that the ManNAc and {ManNAc+Cyt} treatments had little effect on the CMP-NeuAc pool. However, this is unlikely based on several reports in the literature. For example, addition of 10 and 20 mM ManNAc, which bypasses feedback control of NeuAc synthesis via inhibition of UDP-GlcNAc 2-epimerase by CMP-NeuAc (reaction 11 in Figure 2), resulted in 14- and 40-fold increases, respectively, in the free NeuAc concentration in normal human fibroblasts (Thomas et al., 1985a,b). Addition of GlcN alone to these cells had no effect on free sialic acid levels, demonstrating that feedback control was operating in these cells and that the synthesis of ManNAc directly from GlcN (by way of reactions 8 and 10, Figure 2) is not significant (Thomas et al., 1985a). ManNAc alone did not increase the CMP-NeuAc content in normal human fibroblasts (Thomas et al., 1985b), perhaps because of CTP limitation. CTP limitation can be overcome by adding Cyt. When Cyt was added to sialuria fibroblasts, which contain high levels of free NeuAc under control conditions, CMP-NeuAc levels increased 4-fold (Krasnewich et al., 1993). A recent report with CHO cells indicates that addition of ManNAc alone can dramatically increase intracellular levels of CMP-NeuAc, with 2.3- and 30-fold increases in response to addition of 2 and 20 mM ManNAc, respectively (Gu and Wang, 1998). There was an associated increase in the fully sialylated fraction of the Asn^{97} oligosaccharide of IFN-γ from the control level of 65% to 74% and 80% for 2 and 20 mM ManNAc, respectively. Similar NCAM PolySia levels in cultures with ManNAc or {ManNAc+Cyt} (Figure 8) suggest that CTP did not limit CMP-NeuAc production after addition of ManNAc to the CHO or SCLC cells used in our experiments.

The experiments with Cyt alone and in combination with ManNAc point to another possible mechanism of decreased PolySia in CHO cells: inhibition of the Golgi CMP-NeuAc transporter by CMP. Addition of Urd or Cyt to cell cultures results in accumulation of nucleotide di- and triphosphates (Pederson et al., 1992; Ryll and Wagner, 1992; Ryll et al., 1994; Pederson, 1997). Without measuring the nucleotide monophosphates (NMPs), it is difficult to predict how these concentrations are affected by the various treatments. However, it is clear that NMPs inhibit sialylation. CMP is a strong inhibitor of CMP-NeuAc transport into prepared mouse thymocyte or liver vesicles (Carey et al., 1980; Cacan et al., 1984). This may be responsible for inhibition of PolySia with Cyt alone (Figure 8). With ManNAc also present, the presumed increase in CMP-NeuAc may have decreased CMP levels by increasing the flux of cytidine into CMP-NeuAc, and/or the increase in CMP-NeuAc may have been sufficiently high that transport into the Golgi was not inhibited by the presence of CMP. Monophosphates of the antiviral nucleoside analogues BVdU ((*E*)-5-(2-bromovinyl)-2′-deoxyuridine) and PdU (5-propyl-2-deoxyuridine) inhibit incorporation of galactose and sialic acid into N-linked oligosaccharides of HSV-infected cells and block the transport of pyrimidine sugar nucleotides across Golgi membranes in a cell-free system (Olofsson et al., 1988, 1993). The action of these compounds is specific, as they do not inhibit galactosyltransferase activity, viral glycoprotein synthesis or transport, or UDP-hexose formation. Inhibition of the nucleotide sugar transporters may be a general property of all NMPs, including UMP, but more work will be necessary to resolve this issue. The Golgi CMP-NeuAc transporter gene was recently cloned (Eckhardt et al., 1996) and may prove to be a suitable target for the genetic or biochemical modulation of sialylation or polysialylation.

Besides studying the mechanisms of PolySia inhibition by ammonia, our intention was to examine the potential for biochemically modulating polysialic acid levels by way of nucleotide sugars—upregulation by {ManNAc+Cyt} and downregulation by {GlcN+Urd}. This may have applications in cancer research, where it has been reported that the progression of PolySia-bearing tumors may be regulated by changes in the levels of polysialylation (Figarella-Branger et al., 1990; Roth and Zuber, 1990; Scheidegger et al., 1994a). The data presented here show that downregulation of polysialylation by {GlcN+Urd} is a distinct possibility. Since the correlation between polysialylation and metastasis is positive, our findings suggest that glucosamine's role as a chemotherapeutic agent should be extended beyond cytotoxicity to the inhibition of SCLC tumor metastasis.

Notation

4MU-NeuAc	(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid
BVdU	(*E*)-5-(2-bromovinyl)-2′-deoxyuridine
CHO	Chinese hamster ovary
CMP	cytidine monophosphate
CMP-NeuAc	CMP-*N*-acetylneuraminic acid
CP	carbamoyl phosphate
CTP	cytidine triphosphate
Cyt	cytidine
F-6P	fructose-6-phosphate
FBS	fetal bovine serum
FSC	forward scatter
FITC	fluorescein isothiocyanate
G-CSF	granulocyte colony stimulating factor
GlcN	glucosamine
{GlcN+Urd}	combination of GlcN and Urd added to the culture medium
GlcNAc	*N*-acetylglucosamine
mAb	monoclonal antibody
ManNAc	*N*-acetylmannosamine
{ManNAc+Cyt}	combination of ManNAc and Cyt added to the culture medium
IL-Mu6	recombinant human mutant interleukin-2
MFI	mean fluorescence intensity
NCAM	neural cell adhesion molecule
NEAA	MEM nonessential amino acids
NeuAc	*N*-acetylneuraminic acid
NMP	nucleotide monophosphate
NMR	nuclear magnetic resonance
PBS	phosphate-buffered saline
PI	propidium iodide
PolySia	polysialic acid
PdU	5-propyl-2-deoxyuridine
q_{glc}	specific glucose consumption rate
q_{lac}	specific lactate production rate
RFI	relative fluorescence intensity
SCLC	small cell lung cancer
SSC	side scatter
UDP	uridine diphosphate
UDP-GalNAc	UDP-*N*-acetylgalactosamine
UDP-GlcNAc	UDP-*N*-acetylglucosamine

UDP-GNAc	collectively UDP-GlcNAc and UDP-GalNAc
Urd	uridine
UTP	uridine triphosphate

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