Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 1551–1555, March 1983
Biochemistry

Tunicamycin inhibits ganglioside biosynthesis in neuronal cells

(glycolipids/glycoproteins/nucleotide sugars/neuroblastoma–glioma hybrid cells)

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Communicated by Saul Roseman, December 13, 1982

ABSTRACT **The antibiotic tunicamycin blocks the transfer of GlcNAc-1-*P* from UDP-GlcNAc to dolichol phosphate, thereby blocking the synthesis of *N*-linked oligosaccharide chains on glycoproteins. Its effect on the biosynthesis of gangliosides has not been reported. We report that tunicamycin caused a 70–80% reduction in incorporation of [^{3}H]GlcN into gangliosides and neutral glycosphingolipids of the neuroblastoma–glioma hybrid cell line NG 108-15 at antibiotic concentrations that caused a 90% reduction of the radiolabel incorporation into glycoproteins. The effect of tunicamycin on ganglioside biosynthesis was apparent after only 4 hr of incubation, and maximum inhibition was seen within 6 hr. When control or tunicamycin-treated (5 μg/ml) cells were collected and fractionated to separate glycoproteins, neutral glycosphingolipids, gangliosides, and nucleotide sugar-precursor pools, the following results were obtained: (*i*) UDP-GlcNAc and UDP-GalNAc pool sizes increased >3-fold, and specific activities decreased 50% upon treatment with tunicamycin; (*ii*) when corrected for this value, the percentage inhibition of GlcN incorporation into various glycoconjugates by tunicamycin in these cells was 82% for glycoproteins, 54% for neutral glycosphingolipids, and 50% for gangliosides; and (*iii*) the different gangliosides were affected differentially, with the most striking inhibition apparent in GM_3 biosynthesis, which was decreased 78% in the presence of tunicamycin. These data suggest that the effects of tunicamycin on glycosphingolipids as well as on glycoproteins must be considered when interpreting its effects on intact cells and organisms.**

Tunicamycin, a streptomycete antibiotic (1), has been reported to specifically inhibit the glycosylation of proteins *N*-glycosylated at asparagine residues (2–4) by blocking the enzymatic transfer of GlcNAc-1-*P* from UDP-GlcNAc to dolichol phosphate (5, 6), thereby inhibiting dolichol-linked saccharide synthesis. Tunicamycin's effects on intact cells (7–17) and developing organisms (18–20), where it has been shown to alter cell morphology and function, have been attributed specifically to the loss of *N*-asparagine-linked oligosaccharides on glycoproteins. Potential actions of tunicamycin on gangliosides and neutral glycosphingolipids (GSL) in these systems have not been investigated.

We tested the effect of tunicamycin on ganglioside, GSL, and glycoprotein biosynthesis in the neuroblastoma–glioma hybrid cell line NG 108-15, a cell line with a high rate of ganglioside biosynthesis (21). Addition of tunicamycin caused a marked inhibition of ganglioside and GSL biosynthesis as well as protein glycosylation. While the mechanism of the inhibition is not known, the results suggest that the effects of tunicamycin on intact cells and organisms may not be exclusively due to the inhibition of protein glycosylation.

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MATERIALS AND METHODS

Materials. Tissue culture media and calf serum were obtained from GIBCO; Sephadex G-25 and DEAE-Sepharose CL-6B, from Pharmacia; silica gel 60 TLC plates, from Merck, Darmstadt, Federal Republic of Germany; D-[6-^{3}H]glucosamine hydrochloride, from New England Nuclear (22 Ci/mmol; 1 Ci = 3.7×10^{10} Bq) or ICN (8 Ci/mmol); and [4,5-^{3}H]leucine from New England Nuclear (5 Ci/mmol). Tunicamycin was the gift of J. Douros (National Cancer Institute, Bethesda, MD). GlcN·HCl and GalN·HCl, obtained from Sigma, were recrystallized before use. Ganglioside standards were purified from bovine brain (22), and GSL standards were the gift of S. Roseman (Dept. of Biology, The Johns Hopkins University, Baltimore, MD).

Cell Culture and Metabolic Radiolabeling. NG 108-15 hybrid cells were grown as described (23). Confluent flasks or plates were incubated with [^{3}H]GlcN·HCl or [^{3}H]leucine by using the following protocol. Growth medium was removed and replaced with low-glucose medium (GIBCO 430-1600) supplemented (final concentration, 0.2–5 μg/ml) with small volumes of concentrated tunicamycin (5 mg/ml in 25 mM NaOH) or (in control flasks) with small volumes of carrier (25 mM NaOH). After the preincubation times given in *Results*, [^{3}H]GlcN dissolved in the appropriate medium was added to each flask, and the flasks were returned to the incubator for the indicated times. After the incubations, the cells were collected and washed as described (23). The resulting cell pellets were frozen on dry ice and stored at −20°C for up to 10 days before analysis.

Glycoconjugate Separation and Analysis. Routinely, gangliosides were isolated as described (23) with the following modifications. After extraction and partitioning, the gangliosides were dissolved in 2 ml of solvent I (chloroform/methanol/water, 120:60:9, vol/vol), applied to a 5-ml column of Sephadex G-25 superfine, and eluted with 2.5 ml of the same solvent and 1.25 ml of solvent II (chloroform/methanol, 2:1, vol/vol). Column effluents were combined, the solvents were evaporated, the residue was redissolved in 2 ml of water, and the solution was dialyzed versus water for 18 hr. The Sephadex adsorption chromatography removed nucleotide sugars and free sugars from the ganglioside fraction (24), which was then analyzed by TLC.

To isolate nucleotide sugars for determination of specific activity of the precursor pools, cells were collected from 50 flasks of metabolically radiolabeled control or tunicamycin-treated cells. The pellets (3–4 × 10^8 cells) were suspended in water to a volume of 22.5 ml and homogenized (20 strokes) in a Dounce homogenizer. Methanol (60 ml) and chloroform (30 ml) were added, and the mixture was agitated vigorously and centrifuged at 3,000

Abbreviation: GSL, neutral glycosphingolipids. Ganglioside nomenclature is that of Svennerholm (see ref. 31).

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× g for 30 min. The supernatant was decanted, 75 ml of ethanol/water, 3:2 (vol/vol), were added, and the pellets were rehomogenized as above. The ethanol mixture was heated to boiling on a steam bath, chilled, and recentrifuged as above. The supernatants were combined and evaporated to dryness at 30°C. The pellet was retained for protein determination (see below). The residue from the organic phase was redissolved in 112.5 ml of chloroform/methanol/water, 4:8:3 (vol/vol). Upon addition of water (19.5 ml) and vigorous agitation, two phases appeared and were separated by brief centrifugation. The lower phase was removed, and the upper phase was reextracted with prepared lower phase. The lower phases were combined for neutral glycolipid analysis (see below). The solvent was evaporated from the upper phases, and the residue was redissolved in 45 ml of solvent I, applied to an 11.8-ml column of Sephadex G-25 superfine, and eluted with 30 ml of the same solvent followed by 30 ml of solvent II. The effluents were combined and stored for ganglioside analysis (see below). More polar compounds, including nucleotide sugars, were then eluted from the column with 60 ml of solvent III (methanol/water, 1:1, vol/vol), solvents were removed by evaporation, and the residue was redissolved in a small volume (275 μl) of water.

Nucleotide sugars, UDP-GlcNAc and UDP-GalNAc, were purified by paper chromatography (25) in ethanol/1.0 M ammonium acetate, pH 7.5, 7:3 (vol/vol), and by paper electrophoresis in 0.1 M sodium borate (pH 8.5) at 7 V/cm for 80 min. Sodium was removed by treatment with cation exchange resin (Bio-Rex AG 50 X-8, 100–200 mesh, H^+ form), the resulting liquid was evaporated, and borate was removed by sequential addition and evaporation of methanol. A portion of the purified nucleotide sugars was treated with 6 M HCl at 105°C for 4 hr in a sealed ampule. After hydrolysis, the acid was evaporated, the residue was resuspended in sodium citrate buffer, and a portion was subjected to HPLC (see Table 3).

CMP-sialic acid in the nucleotide sugar pool was analyzed in the following manner. A portion of the mixed nucleotide sugar pool was further subjected to hydrolysis in 1 M formic acid at 100°C for 45 min. After hydrolysis, the solvents and acid were evaporated, and the residue was redissolved in water and applied to a small column of anion exchange resin (Bio-Rad AG1 X-8, formate form). The column was washed with water, and then sialic acid was eluted with 0.2 M formic acid. The eluate was evaporated to dryness, redissolved in a small volume of 50% aqueous ethanol, and subjected to TLC in solvent IV (ethyl acetate/acetic acid/water, 4:2:2, vol/vol). The sialic acid was detected by using resorcinol reagent and quantitated by using a Kontes fiber optic scanner. This analysis revealed a 2.4-fold increase of sialic acid in this pool after tunicamycin treatment (compared to controls). Radiolabeled species other than sialic acid on the chromatograph precluded accurate determination of the CMP-sialic acid specific activity.

The gangliosides that were eluted from the Sephadex G-25 column were further purified by evaporating the solvents, redissolving in 5 ml of H_2O, and dialyzing versus water for 18 hr. The samples were evaporated to dryness, and monosialo- and disialogangliosides were separated by DEAE-Sepharose chromatography as described (22, 23). The two fractions contained 83.8–90.7% of the applied radiolabel. The column effluents containing gangliosides were evaporated to dryness, resuspended in 2 ml of water, and dialyzed versus water for 18 hr.

Gangliosides were analyzed by TLC and by enzymatic and acid degradation. Ganglioside TLC was performed on precoated silica gel plates (Merck) with routine use of chloroform/methanol/0.25% aqueous KCl, 60:35:8 (vol/vol), as the developing solvent. Gangliosides were detected by iodine vapor, the appropriate areas were scraped from the plate into vials containing 1 ml of water, scintillation fluor was added, and the radiolabel was determined in a scintillation counter. All radiolabel determinations were corrected for quenching by internal standardization. Parallel lanes to those scraped for radiolabel determination were stained for sialic acid with a resorcinol reagent (26). Degradation analysis (with neuraminidase or formic acid) was performed as described (23) with the following addition. Released polar radiolabel was eluted from the Sephadex G-25 column with solvent III, the solvent was evaporated, the residue was resuspended in a small volume of water, and a portion was subjected to silicic acid TLC with solvent IV. The radiolabel comigrated with standard sialic (*N*-acetylneuraminic) acid. Radiolabel in the GSL core, GalN, was determined by TLC after acid hydrolysis as described (23).

Protein pellets were dissolved in 0.1 M NaOH (2 ml per flask of cells). Aliquots were removed for determination of protein concentration by the method of Lowry *et al.* (27). Radiolabel in the crude protein fraction was determined by diluting a portion of the dissolved pellet in a 10-fold volume of ice-cold 10% trichloroacetic acid. After 15 min at 0°C, the precipitated protein was collected by centrifugation (27,000 × g for 30 min) and resuspended in 1 M NH_4OH; radiolabel was determined by scintillation counting.

RESULTS

Identity of Glycoconjugates Isolated from NG 108-15 Cells. Gangliosides were identified by their characteristic partitioning into the upper phase in chloroform/methanol/water, 4:8:5.6 (vol/vol); elution from DEAE-Sepharose chromatography; migration with bovine brain gangliosides standards (GM_3, GM_2, GM_1, and GD_{1a}) on silicic acid TLC in three solvent systems; staining with the sialic acid-specific resorcinol reagent on TLCs; stability to treatment with base (0.2 M NaOH at 37°C for 90 min); and conversion to the expected products after treatment with neuraminidase or formic acid (23).

GSLs partitioned into the lower phase in chloroform/methanol/water, 4:8:5.6, were stable to alkali (0.2 M NaOH at 37°C for 90 min) and to acid (0.1 M HCl in tetrahydrofuran) and migrated with the mobility of tri- or tetraglycosylceramides on silicic acid TLC.¶

Nucleotide sugars (UDP-GlcNAc and UDP-GalNAc) comigrated with standard UPD-GlcNAc upon paper chromatography and paper electrophoresis. After acid hydrolysis, all of the radioactivity comigrated with GlcN and GalN upon HPLC analysis.

The glycoprotein pool was defined as that material which was insoluble in chloroform/methanol/water, 4:8:3 (vol/vol), and which could be precipitated by trichloroacetic acid after resolubilization in mild alkali.

Tunicamycin Inhibition of Radiolabel Incorporation into Gangliosides and GSLs. When tunicamycin was added to cells for 20 hr, followed by a 2-hr pulse with radiolabeled GlcN, near-maximal inhibition of radiolabel incorporation into glycoproteins was accomplished with 0.5 μg of the antibiotic per ml. A sharp decrease in incorporation of radiolabel into both gangliosides and GSL paralleled the inhibition of incorporation into glycoproteins, with maximal inhibition accomplished by using tunicamycin at 0.5 μg/ml and half-maximal inhibition accom-

¶ Dolichol-linked saccharides are not considered to be significant contaminants of these GSL fractions because the dolichol-linked saccharides would be labile to the acid or alkali treatments described in the text. In addition, experiments that included a 3-hr radiolabeled pulse followed by a 3-hr cold chase resulted in no significant decrease of radiolabel in the GSL pools (see Table 1) Radiolabel in dolichol-linked saccharides would be expected to be markedly reduced after such a chase.

plished by using ≈0.2 μg/ml (Fig. 1).

When briefer incubations were used, higher concentrations of tunicamycin were needed to inhibit radiolabel incorporation into glycoconjugates. For example, inhibition (>70%) of radiolabel incorporation into glycoproteins during a 4-hr pulse required 5 μg of tunicamycin per ml (data not shown), a concentration used in all subsequent experiments reported.

In the presence of tunicamycin at 5 μg/ml, kinetic studies revealed rapid inhibition of GSL biosynthesis (Table 1). In three experiments, near maximal inhibition was found after a 1-hr preincubation with tunicamycin, followed by a 3-hr pulse of radiolabeled GlcN in the presence of tunicamycin. Longer preincubation times (4 or 7 hr) resulted in higher incorporation into control cells [perhaps because of the longer incubation in low-glucose medium (28)] and in maximal inhibition by tunicamycin of radiolabeled GlcN incorporation into proteins, gangliosides, and GSL. Protein synthesis was not significantly inhibited under these conditions (4-hr preincubation with tunicamycin and 3-hr pulse with [^{3}H]leucine in the presence of tunicamycin), remaining at 94% of control levels (data not shown).

A 3-hr chase in unlabeled medium (Table 1) resulted in a significant increase (70%) in radiolabel incorporation into control cell glycoproteins, no significant change in radiolabel incorporation into control cell gangliosides or GSL, and no significant change in the percentage inhibition of radiolabel incorporation by tunicamycin.

Further purification of gangliosides by DEAE-Sepharose chromatography, followed by silicic acid TLC, revealed differential tunicamycin inhibition of radiolabel incorporation into different gangliosides (Table 2). The antibiotic caused the most marked decrease of radiolabel incorporation into ganglioside GM_3 (89%), comparable to inhibition of protein glycosylation. Lower levels of inhibition (70–74%) of radiolabel incorporation into more complex gangliosides were found. The radiolabel in the purified gangliosides was characterized in two ways. (*i*) A portion of the radioactivity was released by treatment with neuraminidase or formic acid, or both, and cochromatographed with standard *N*-acetylneuraminic acid on TLC. (*ii*) After complete acid hydrolysis, the remaining radioactivity cochromatographed with standard GalN on TLC.

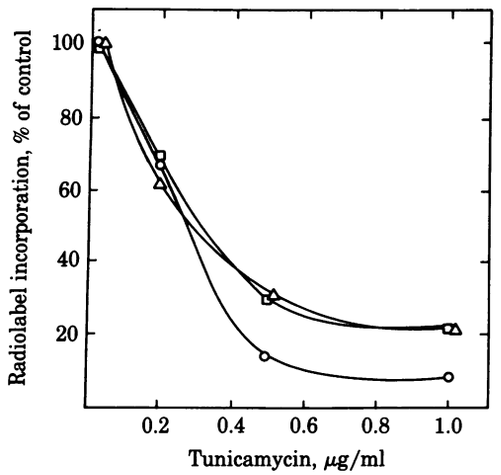

FIG. 1. Tunicamycin inhibition of radiolabel incorporation from [^{3}H]GlcN into glycoconjugates of NG 108-15 cells. ○, Glycoproteins; □, gangliosides; and △, GSLs. The medium was removed from confluent 75-cm^2 flasks of NG 108-15 cells (one flask per data point; 7–9 × 10^6 cells per flask) and replaced with complete medium containing the indicated amounts of tunicamycin. After 20 hr of incubation, the medium was removed and replaced with 10 ml of labeling medium (low glucose) containing 50 μCi of [^{3}H]GlcN. After 2 hr of incubation in the presence of the radiolabel, the cells were collected and analyzed. Control flasks had the following levels of incorporation: glycoproteins, 140,973 dpm/mg of protein; gangliosides, 89,416 dpm/mg of protein; and GSLs, 29,050 dpm/mg of protein.

Table 1. Kinetics of tunicamycin inhibition

Preincubation time, hr	Tunicamycin	[^{3}H]GlcN incorporation, dpm/mg of protein × 10^{-3}					
		Protein		Gangliosides		GSL	
		dpm	%I*	dpm	%I*	dpm	%I*
1	−	775	—	380	—	113	—
	+	138	82	182	52	26	77
4	−	2,181	—	752	—	425	—
	+	156	93	289	62	78	82
4 (+ chase)	−	3,687	—	819	—	363	—
	+	266	93	264	68	72	80
7	−	2,392	—	847	—	345	—
	+	126	95	256	70	81	77

The medium was removed from confluent plates (60 mm) of NG 108-15 cells and replaced with 2 ml of low-glucose control medium or the same medium containing tunicamycin at 5 μg/ml. After the indicated preincubation times, an additional 0.5 ml of the appropriate medium containing 25 μCi of [^{3}H]GlcN was added. After 3 hr in the presence of radiolabel, cells were collected from most of the plates for glycoconjugate analysis. One set of plates was subjected to a cold chase before collection of the cells; the radiolabeled medium was removed and the cell layer was gently washed with fresh medium and incubated for an additional 3 hr in 2 ml of the appropriate medium supplemented with 4 μM unlabeled GlcN.

*%I, % inhibition by tunicamycin.

Nucleotide Sugar Pool Sizes and Specific Activities in Control and Tunicamycin-Treated NG 108-15 Cells. Nucleotide sugars were extracted from control and tunicamycin-treated NG

Table 2. Distribution of radiolabel from [^{3}H]GlcN into glycoconjugates of control and tunicamycin-treated NG 108-15 cells

Glycoconjugate	[^{3}H]GlcN incorporation in cells, dpm/mg of protein		Tunicamycin inhibition, %
	Control	Tunicamycin-treated	
Glycoproteins	12,289	1,101	91
Neutral glycolipids	4,824	1,199	75
Gangliosides			
Total (crude)	12,472	3,719	70
Partially purified			
Monosialogangliosides	3,581	950	73
Disialogangliosides	6,254	1,689	73
Purified*			
GM_3	239	27	89
GM_2	1,989	576	71
GM_1	2,103	637	70
GD_{1a}	5,289	1,359	74

The growth medium was removed from each of 100 flasks (75 cm^2) and replaced with 6.5 ml of either control (low glucose) medium or the same medium containing 5 μg of tunicamycin per ml. After 3 hr of incubation, 1 ml of the appropriate medium containing 10 μCi of [^{3}H]GlcN was added to each flask. After an additional 3 hr in the presence of the radiolabel, the cells were collected and analyzed.

*Gangliosides were analyzed by TLC. At least 10,000 cpm (control) or 4,000 cpm (tunicamycin) of mono- or disialoganglioside was applied for each chromatographic analysis. Each value is the average of two analyses. All gangliosides were clearly separated from each other, and the least abundant ganglioside (GM_3) comigrated with more than 600 cpm of radiolabel (control), with adjacent TLC regions having less than 50 cpm.

Table 3. Nucleotide sugar pool sizes and specific activities from control and tunicamycin-treated NG 108-15 cells

Sugar nucleotide	Pool size, pmol/mg of protein: Control cells	T-treated cells	Ratio*	Specific activity, Ci/mol: Control cells	T-treated cells	Ratio*
UDP-GlcNAc	152	496	3.3	35.2	17.2	0.49
UDP-GalNAc	63	200	3.2	54.2	30.7	0.57

Control and tunicamycin-treated (T-treated) NG 108-15 cells were radiolabeled with [^{3}H]GlcN as described in Table 2. Nucleotide sugars were extracted, purified, and hydrolyzed. A portion of the released hexosamines was dissolved in sodium citrate buffer (pH 2.2; 0.2 M Na^+), and GlcN and GalN were separated and quantitated on a Durrum D-500 automatic amino acid analyzer equipped with a programmable fraction collector that permitted the collection of the individual amino sugars for subsequent radioactivity determination.
*Tunicamycin treatment value/control value.

108-15 cells after a 3-hr incubation with radiolabeled GlcN and analyzed as described. The results (Table 3) demonstrate a marked increase in nucleotide sugar pool sizes upon treatment of the cells with tunicamycin. Both UDP-GlcNAc and UDP-GalNAc pools were increased >3-fold in the presence of the antibiotic. Radiolabel incorporation increased <2-fold, resulting in diminished specific activities after tunicamycin treatment. Although we were unsuccessful in measuring the specific activity of the small and labile CMP-sialic acid pool, quantitative TLC revealed a 2.4-fold increase in the relative pool size after tunicamycin treatment, similar to the increases for UDP-GlcNAc and UDP-GalNAc. The decreases in the specific activities of the nucleotide sugars in tunicamycin-treated cells were not due to differential use of radiolabel in the medium during the incubation because samples of the medium from cell incubations revealed comparable reductions of free [^{3}H]GlcN in the medium of control and tunicamycin-treated cells, with 65% of the radiolabel remaining in the medium as [^{3}H]GlcN after 3 hr.

Tunicamycin Inhibition of Ganglioside Biosynthesis. Purified mono- and disialoganglioside pools were treated with neuraminidase and formic acid as described (23). The data are consistent with the presence of radiolabel in both the sialic acids and core GalNAc of the gangliosides. The above treatments revealed the distribution of radiolabel in these residues. Estimation of the specific activities of the precursor pools (Table 3) allowed determination of the effects of tunicamycin on ganglioside biosynthesis. For these calculations, the specific activity of CMP-sialic acid was estimated to be equal to that of its precursor, UDP-GlcNAc, because we were unsuccessful in determining the specific activity of the small and labile CMP-sialic acid pool in these cells.

Table 4. Effect of tunicamycin on the incorporation of sialic acid and GalNAc residues into gangliosides of NG 108-15 cells

Ganglioside	Radiolabeled residue	Incorporation, pmol/mg of protein: Control cells	T-treated cells	Tunicamycin inhibition, %
GM_3	Sialic acid	3.1	0.7	78
GM_2/GM_1	Sialic acid	11.8	2.6	78
	GalNAc	26.1	16.4	37
GD_{1a}	Sialic acid (terminal)	17.1	3.7	79
	Sialic acid (internal)	20.7	8.9	57
	GalNAc	21.2	13.4	37

Gangliosides were purified from control and tunicamycin-treated (T-treated) NG 108-15 cells as described in Table 2 and the text. Subsequent degradation with neuraminidase and formic acid (23) allowed the determination of radioactivity in sialic acid and GalNAc residues. The resulting values were corrected for the specific activities of the nucleotide sugar precursor pools (Table 3).

The results of this analysis are given in Table 4. Tunicamycin inhibition of the incorporation of sialic acid into gangliosides GM_3, GM_2, and GM_1 and the terminal sialic acid of ganglioside GD_{1a} was most marked—nearly 80%. Incorporation of other residues into these ganglioside pools was less affected, with GalNAc incorporation being blocked 34–37%.

DISCUSSION

Tunicamycin has been shown to block the first step in dolichol-linked saccharide synthesis—the transfer of GlcNAc-1-*P* from UDP-GlcNAc to dolichol phosphate (5, 6)—thereby selectively blocking glycosylation of proteins at asparagine residues (2–5). Tunicamycin also has been reported to cause marked changes in the morphology of the cells cultured *in vitro* (7–9), alterations in cell recognition and adhesion (10–12), inhibition of cell differentiation (13, 14), and decreases in cell-surface receptor binding (15–17). In addition, it arrests the development of sea urchin embryos at the gastrula stage (18, 19) and blocks normal development in the mouse embryo (20). Tunicamycin-induced changes in intact cells and organisms have been attributed to changes in protein glycosylation.

Our studies on the neuroblastoma–glioma hybrid cell line NG 108-15 demonstrate that tunicamycin inhibits the biosynthesis of gangliosides and GSLs in addition to blocking protein glycosylation. We report that (*i*) tunicamycin caused a marked inhibition of glucosamine incorporation into gangliosides and GSL; (*ii*) the tunicamycin concentration necessary to cause inhibition was the same as for inhibition of protein glycosylation (Fig. 1); and (*iii*) inhibition was rapid—near maximum inhibition was measured after a 1-hr preincubation with tunicamycin (Table 1).

Purification of the individual gangliosides from NG 108-15 cells (GM_3, GM_2, GM_1, and GD_{1a}) revealed differential inhibition of radiolabeled saccharide incorporation by tunicamycin (Table 2). The most markedly affected ganglioside (89% inhibition) was GM_3. Although this ganglioside comprised less than 3% of the total radioactivity incorporated into gangliosides and less than 15% of the steady-state ganglioside concentration (23), it is thought to be an obligatory intermediate in the biosynthesis of the other gangliosides (29). Tunicamycin inhibition of radiolabel incorporation into the other gangliosides was somewhat less (70–75% inhibition). However, enzymatic and acid treatments revealed differences in GlcN incorporation into the different carbohydrate residues of these gangliosides.

The tunicamycin-induced decrease in [^{3}H]GlcN incorporation could be due to either a decrease in the biosynthetic rate or a decrease in the precursor-pool specific activity. Because it has been shown that nucleotide sugar pools can increase markedly in size (28), these pools were isolated and their specific activities were determined in order to distinguish between the above possibilities. The results demonstrate a marked increase in the size of the nucleotide sugar pools in the presence of tunicamycin (>3-fold) and a decrease in the precursor-pool specific activity by ≈50% (Table 3). However, this decrease does not account for the marked decrease in radiolabel incorporation into gangliosides and GSL in the presence of tunicamycin.

The radiolabel from [^{3}H]GlcN was incorporated into the gangliosides as two different carbohydrate residues—sialic acid and GalNAc. Sequential enzymatic and acid treatments allowed the determination of the distribution of the radioactivity between these residues. In conjunction with the determination of the

nucleotide sugar precursor-pool specific activity, the relative rates of GlcN incorporation into these residues in the different gangliosides was calculated (Table 4), confirming a marked inhibition by tunicamycin. If one assumes that the CMP-sialic acid precursor pool was affected similarly to the UDP-GlcNAc and UDP-GalNAc pools, ganglioside GM_3 biosynthesis was decreased nearly 80%. Sialic acid incorporation in the GM_2/GM_1 pool also was decreased nearly 80%; however, GalNAc incorporation was decreased only 37%, perhaps because of addition of labeled GalNAc to preexisting unlabeled GM_3 [pool size, 400 pmol/mg of cell protein (23)].

The different residues in the ganglioside GD_{1a} also were affected differentially. Incorporation of GlcN into the terminal sialic acid residue was inhibited nearly 80%; into the internal sialic acid, ≈60%; and into the core GalNAc, only 37%. While confirming the inhibition of ganglioside biosynthesis by tunicamycin, these data present an enigma. Because GD_{1a} is synthesized by the action of sialyltransferase on GM_1 (29), one would expect radioactivity incorporation into the terminal sialic acid of GD_{1a} to be equal to or greater than incorporation into the internal sialic acid of GD_{1a}. The results (Table 4) show the opposite. Two possible explanations for these data are: (*i*) a temporal increase in tunicamycin inhibition or a decrease in CMP-sialic acid specific activity, or both, occur over the 3-hr period of the labeling; or (*ii*) two separate pools of CMP-sialic acid exist with markedly different specific activities. Our data cannot distinguish between these or other possibilities.

The data reported here demonstrate that a marked decrease in the synthesis of gangliosides and GSL is caused by tunicamycin, even though the pool size of their immediate precursors, the nucleotides sugars, increases. Although the mechanism of this inhibition is not known, two possibilities include (*i*) direct inhibition of one or more of the glycosyltransferases involved in ganglioside and GSL biosynthesis or (*ii*) inhibition of glycosylation of the glycosyltransferases (which may themselves be glycoproteins), thereby decreasing their stability (30) or specific activity.

Whether direct or indirect, the effects of tunicamycin on ganglioside and GSL biosynthesis, as well as on glycoprotein biosynthesis, must be considered when interpreting effects of this antibiotic on intact cells and organisms.

We are grateful to Patti Hill for assistance in cell culture and Nancy Standish for manuscript preparation. This work was supported by National Institutes of Health Grants HD 14010 and CA 30471 and was aided by Basil O'Connor Starter Research Grant 5-302 from The March of Dimes–Birth Defects Foundation. R.L.S. is a recipient of an American Cancer Society Junior Faculty Research Award.

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