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Tunicamycin blocks neuritogenesis and glucosamine labeling of gangliosides in developing cerebral neuron cultures

(nerve cells/regeneration/development)

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ABSTRACT **Fetal cerebral neurons at the initiation of active neurite outgrowth in culture incorporate 4-fold more [^{3}H]glucosamine into glycoproteins than into the cellular lipid fraction. After 8 days or longer, when a well-developed fiber network is apparent, lipids are labeled more extensively than the glycoproteins. Labeling of the latter is inhibited 95% and 89% by 0.5 μg of tunicamycin per ml added to 1-day-old and 8-day-old cultures, respectively. Labeling of glycolipids is inhibited 30% in 1-day-old and 86% in 8-day-old cultures. Tunicamycin blocks incorporation of glucosamine label into practically all ganglioside species except for a resorcinol-positive, sialidase-sensitive band tentatively identified as G_{Q1b} tetrasialoganglioside (Svennerholm ganglioside nomenclature). It also substantially reduces binding of ^{125}I-labeled tetanus toxin to intact cells. There is 14% and 27% reduction in the total ganglioside sialic acid content in 1-day-old and 8-day-old cells treated for 24 hr with 0.5 μg of tunicamycin per ml, but no substantial compositional changes are encountered. Tunicamycin blocks neurite outgrowth when added to cells soon after plating but causes no retraction or losses of fibers once the fiber network is established. Therefore, inhibition of neurite outgrowth by tunicamycin is not due to an effect on cellular gangliosides but can be correlated to an inhibition of protein glycosylation.**

Gangliosides are acidic glycosphingolipids present at the cell surface and believed to participate, like other glycoconjugates, in events such as cell–cell recognition and signal transduction (1, 2). The nervous system is especially rich in gangliosides containing several sialosyl residues compared to other tissues and is also remarkably rich in lipid-bound sialic acid (3). Changes in ganglioside metabolism and composition have been correlated with distinct sites (4) and events of neuronal differentiation, particularly with progressive axodendritic proliferation (5, 6) and, in tissue culture with neuronal cells maturation (7, 8). Despite that, the actual functions of gangliosides and also of glycoproteins in neuritogenesis are unknown (9).

Tunicamycin, an amphipatic analog of UDP-GlcNAc, is one of the few agents now widely used as a selective inhibitor of protein glycosylation (10). An inhibitory effect of tunicamycin on *in vitro* glycosylation of gangliosides by Golgi preparations, presumably by sugar nucleotide-transport inhibition, also was reported (11). Tunicamycin blocked glucosamine labeling of cellular gangliosides and, as expected, of cellular glycoproteins when added to somatic neurohybrid NG108-C15 cells (12). It also affected cell adhesion and neurite outgrowth in N115 neuroblastoma cells (13). We now have extended these observations to examine the effect of tunicamycin on the initiation of neurite outgrowth and its possible correlation to glycoconjugate metabolism in normal rat cerebral neuron cultures.

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MATERIALS AND METHODS

Culture System. Cerebral hemispheres of 16-day-old rat embryos were pooled and dissociated mechanically in 10 ml of basal metabolic Eagle's medium (BME medium) supplemented with 20% fetal calf serum and 10% whole egg ultrafiltrate. Cells were collected and seeded on poly(L-lysine)-coated 24-well Costar culture plates; after 2 hr, the serum-containing medium was replaced with BME medium supplemented with insulin (10 μg/ml), hydrocortisone (10 nM), transferrin (5 μg/ml), glycyl-L-histidyl-L-lysine (10 ng/ml), and somatostatin (10 ng/ml) and was maintained as reported elsewhere (14). More than 92% of the cells after 1 day and up to 3 weeks in culture were tetanus toxin-positive by indirect immunofluorescence technique.

Metabolic Studies. The tunicamycin used in these studies was purchased from Calbiochem and used as a mixture of all homologues present. Several experiments were performed with a purified homologue of tunicamycin, A2-tunicamycin, prepared as described by Mahoney and Duksin (15). The antibiotic was dissolved in 0.01 M NaOH, lyophilized, stored at −70°C, and reconstituted with the growth medium prior to use. Labeling of cells was performed in the absence or presence of tunicamycin in the BME medium supplemented with the above noted growth factors. 1-D-[^{14}C]Glucosamine (56 μCi/μmol; 1 Ci = 37 GBq) or D-[1,6-^{3}H(N)]glucosamine (32 Ci/mmol) from New England Nuclear was added 30–60 min after addition of tunicamycin.

Ganglioside Analysis. Cells were harvested, and total lipids were extracted and prepared for TLC as described (16). Alternatively, the upper methanol/water layer after organic solvent partition was reduced to a volume of 50–100 μl by evaporation and was dialyzed in an Eppendorf tube for 18 hr against two changes of water to remove water-soluble dialyzable components. The residue was adsorbed onto a reversed-phase Sep-Pak, C_{18} column (Waters Associates) and was eluted as described by Williams and McCluer (17) prior

Abbreviations: G_{M1}, galactosyl-β1→3-*N*-acetylgalactosaminyl-β1→4-(*N*-acetylneuraminyl-α2→3)-galactosyl-β1→4-glucosyl-β1→1-ceramide; G_{M2}, *N*-acetylgalactosaminyl-β1→4-(*N*-acetylneuraminyl-α2→3)-galactosyl-β1→4-glucosyl-β1→1-ceramide; G_{D1a}, (*N*-acetylneuraminyl-α2→3)-galactosyl-β1→3-*N*-acetylgalactosaminyl-β1→4-(*N*-acetylneuraminyl-α2→3)-galactosyl-β1→4-glucosyl-β1→1-ceramide; G_{D1b}, galactosyl-β1→3-*N*-acetylgalactosaminyl-β1→4-(*N*-acetylneuraminyl-α2→3-*N*-acetylneuraminyl-α2→3)-galactosyl-β1→4-glucosyl-β1→1-ceramide; G_{T1b}, (*N*-acetylneuraminyl-α2→3)-galactosyl-β1→3-*N*-acetylgalactosaminyl-β1→4-(*N*-acetylneuraminyl-α2→3-*N*-acetylneuraminyl-α2→3)-galactosyl-β1→4-glucosyl-β1→1-ceramide; G_{Q1b}, (*N*-acetylneuraminyl-α2→3-*N*-acetylneuraminyl-α2→3)-galactosyl-β1→3-*N*-acetylgalactosaminyl-β1→4-(*N*-acetylneuraminyl-α2→3-*N*-acetylneuraminyl-α2→3)-galactosyl-β1→4-glucosyl-β1→1-ceramide. The ganglioside nomenclature is that of Svennerholm (see ref. 26) where G is for ganglioside; M, monosialo; D, disialo; T, trisialo; Q, tetrasialo.

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to TLC analysis. Aliquots were spotted on 10 × 10 cm silica gel G plates (Merck Darmstadt no. 5760) and developed for 40 min in a solvent system composed of methylacetate/*n*-propanol/chloroform/methanol/0.25% KCl, 50:40:40:40:34 (vol/vol), or by a two-directional solvent system using the above solvent system for the first direction and a solvent mixture consisting of chloroform/methanol/acetone/acetic acid/water, 100:20:40:30:10 (vol/vol), for the second direction as described elsewhere (17). Labeled gangliosides were detected by exposure to an x-ray film (Kodak); whenever appropriate individual gangliosides were visualized by spraying with resorcinol, the percentage distribution of the individual ganglioside bands was determined by the direct densitometric scanning method of Ando *et al.* (18). The radioactive, resorcinol-positive bands were quantified by scraping off and assaying the silica gel by use of Aquasol scintillation mixture (New England Nuclear). Alternatively, bands on the x-ray films were monitored by direct densitometry. The nomenclature convention used for individual gangliosides is described in the abbreviations footnote.

Miscellaneous Methods. *Ganglioside sialic acids.* These were determined after acid hydrolysis by a modified thiobarbituric acid procedure (16).

DNA. DNA was determined on the delipidated cell pellet by the diphenylamine method as described elsewhere (16).

Tetanus toxin binding. Binding of ^{125}I-labeled tetanus toxin was performed essentially as described (14). Each one of the experiments was done at least twice, and statistical analysis was performed on 4–6 cultures.

RESULTS

Effect of Tunicamycin on Cell Morphology and General Metabolism. Dissociated fetal rat cerebral cells seeded on culture plates precoated with poly(L-lysine) attached and extended small slender neuritic processes by 2 hr after seeding. Within 24 hr, neurite outgrowth was remarkably enhanced as shown in Fig. 1*B*. Addition of tunicamycin (0.2 μg/ml) 6–24 hr after seeding prevented neurite outgrowth (Fig. 1*A*), and 2–3 days later losses of cells were encountered. In contrast, there were no morphologically identifiable toxic effects on cells bearing neurites in 8-day-old cultures treated with tunicamycin at 1 μg/ml (Fig. 1*C*). In contrast to the effect of tunicamycin on neuroblastoma cells (13), the 8-day-old cerebral cells did not retract their fibers.

The degree of cytotoxicity was tested by using a number of labeled precursors for synthesis of cellular constituents. Uptake of labeled serine or tyrosine into cellular proteins was partially (15–20%) inhibited by tunicamycin at 0.5 μg/ml. There were no effects on serine labeling of phosphatidylserine nor any changes in the rates of ^{32}P incorporation into phospholipids (data not shown), suggesting that at low doses the antibiotic is only slightly toxic.

Effect of Tunicamycin on Incorporation of [^{3}H]Glucosamine. Addition of A_2-tunicamycin at 0.5 μg/ml for 24 hr to the culture medium of cells maintained for 1 or 8 days *in vitro* caused 81.3% and 87.5% inhibition of D-[^{3}H]glucosamine uptake, respectively, in comparison to control values. This is in line with other studies using cells in culture, which show selective inhibition of glycosylation after administration of tunicamycin (19). About 78% of the cellular label from glucosamine in 1-day-old inhibited cultures resided in the delipidated cell fraction and consisted mainly of labeled proteins. In contrast, <45% of radioactivity was present in the protein fraction by day 8 in culture. In 8-day-old cultures, the majority of the radioactive label incorporated in the methanolic water layer was found in glycolipids. This suggests that 8-day-old cerebral cells incorporate glucosamine into glycolipids more effectively than do the immature cells and also that the inhibitory effect of tunicamycin on ganglioside labeling

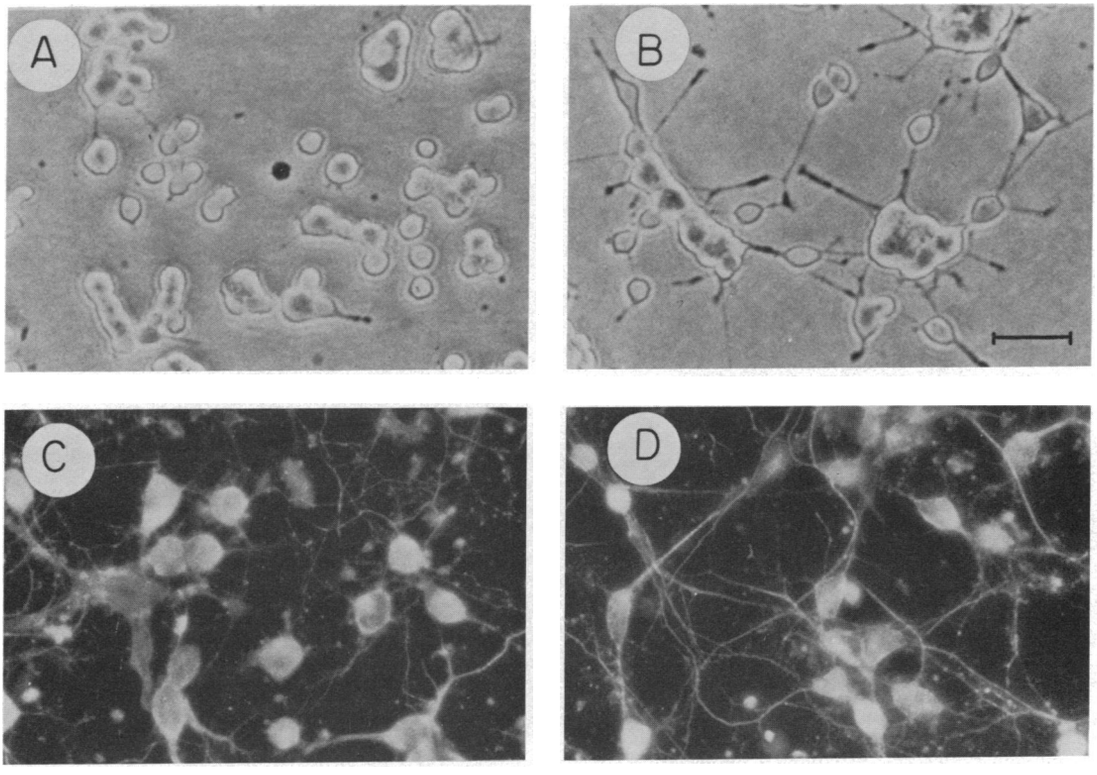

FIG. 1. Effect of tunicamycin on morphology of cerebral neurons after 1 and 8 days in culture. Phase-contrast micrographs of 1-day-old cells treated with tunicamycin at 0.2 μg/ml for 24 hr (*A*) and the corresponding control cells (*B*) and of 8-day-old cells treated with tunicamycin at 1 μg/ml (*C*) and the corresponding controls of the same age (*D*). Both *C* and *D* are stained with a monoclonal anti-synaptosomal antibody (unpublished data). (Bar = 25 μm.)

Table 1. Inhibitory effect of tunicamycin on D-[^{3}H]glucosamine uptake and ganglioside sialic acid content

Culture exposure	Ganglioside sialic acid, nmol/μg of DNA	D-[^{3}H]glucosamine uptake, cpm $\times 10^{-3}/\mu$g of DNA		
		Total	Delipidated protein pellet	Lipids
Day 1				
Control	0.28	2.57 ± 0.18	2.01 ± 0.25	0.56
Tunicamycin	0.24	0.48 ± 0.20	0.09 ± 0.05	0.39
% inhibition	14	81.3	95.5	30.4
Day 8				
Control	0.52	4.88 ± 0.32	2.16 ± 0.14	2.72
Tunicamycin	0.38	0.61 ± 0.04	0.23 ± 0.04	0.38
% inhibition	27	87.5	89.4	86.0

Cells were pulse-labeled for 24 hr with D-[^{3}H]glucosamine (5 μCi/0.5 ml per well) in the presence or absence of A_2-tunicamycin at 0.5 μg/ml. Excess isotope was removed by rinsing twice with phosphate-buffered saline, and cells were transferred into Eppendorf conical tubes and precipitated with 5% trichloroacetic acid. The soluble radioactivity was discarded by centrifugation, and the insoluble pellet was extracted with chloroform/methanol, 1:2 (vol/vol), as described. The pellet was hydrolyzed with 0.5 M NaOH, and aliquots were taken for DNA and radioactivity assays. Values represent averages of ± SEM of four culture wells. Organic solvent extracts from four culture wells were combined, and aliquots were taken for sialic acid determination (16) and radioactivity assay.

in 1-day-old cells is less pronounced than in 8-day-old cultures (Table 1). The ganglioside sialic acid content, an index for ganglioside ontogenesis (20), was reduced by 14% and 27% in 1-day-old and 8-day-old cultures, respectively, after exposure to tunicamycin.

The composition of gangliosides in older cultures did not change substantially between controls and tunicamycin-treated cells (Fig. 2). The percentage distribution of G_{Q1b}, G_{T1b}, and G_{D1a} slightly increased from 9% to 13%, from 30% to 33%, and from 17% to 21%, respectively, after treatment with tunicamycin for 24 hr. G_{M3}, G_{D3}, and G_{D1b}, which accounted in control cells for 10%, 21%, and 7% of the total ganglioside sialic acid, were reduced to 6%, 14%, and 4%, respectively.

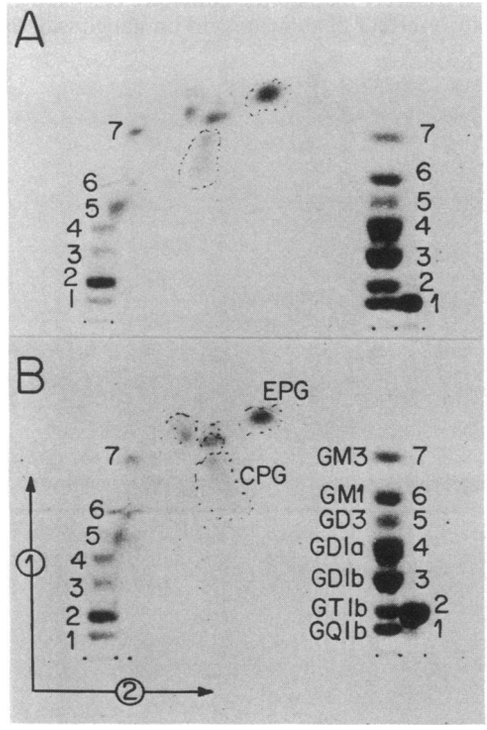

FIG. 2. Thin-layer chromatogram of cerebral neuron gangliosides in 9-day-old control (*A*) and tunicamycin (0.5 μg/ml)-treated (*B*) cells. Total lipid extract was separated in two directions and stained with resorcinol as described. Bands: 1, tetrasialo G_{Q1b}; 2, trisialo G_{T1b}; 3, disialo G_{D1b}; 4, disialo G_{D1a}; 5, monosialo G_{M1}; 6, monosialo G_{M2}; and 7, monosialo G_{M3}. EPG and CPG are the locations of ethanolamine and choline phosphoglycerides, respectively, after visualization with iodine vapors.

The patterns of [^{14}C]glucosamine labeling in cellular gangliosides in control and tunicamycin (0.5 μg/ml)-treated cultures is illustrated in Fig. 3. [^{14}C]Glucosamine was effectively incorporated into all major cellular gangliosides when added to cells in the absence of tunicamycin (Fig. 3*A*). Clearly the labeling pattern does not match the steady-state concentration of the various ganglioside species (compare to Fig. 2) presumably because of differences in the turnover of each of the sugar or sialic acid constituents. Label was highest in G_{D1a} (33.3%) followed by G_{T1b} (26.9%), G_{D1b} (14.8%), and G_{M1} (11.5%), as determined by radioactivity assay of the silica gel. Addition of vibrio cholera neuraminidase to cells for a period of 6 hr prior to termination of the experiment caused a 6-fold increase of monosialo G_{M1} (peak 5) and also G_{M3} (peak 7) species at the expense of trisialo G_{T1b} (peak 2) and disialo G_{D1b} (peak 3) and G_{D1a} (peak 4) species (Fig. 3*B*). A different result emerged in the glycolipid extract from cells that were subjected to 0.5 μg of tunicamycin and subsequently were separated on TLC. Of the total radioactivity incorporated into the lipids of tunicamycin-treated cells (one-fifth of the normal values), nearly no radioactivity could be detected in the G_{T1b} and G_{D1b} ganglioside species (Fig. 3*C*). The major radioactive peak comigrated with tetrasialoganglioside G_{Q1b} (67.3%), the remaining counts being distributed between G_{D1a} (10.9%), G_{M1} (3.7%), and G_{M2} (5.4%) species. The major radioactive peak was nondialyzable and Pronase insensitive. It was partially sensitive to sialidase treatment (Fig. 3*D*); the loss of radioactivity of this peak was concomitant to an increase in the G_{M1} ganglioside.

The effect of tunicamycin concentration on labeling of gangliosides with [^{14}C]glucosamine in 3-week-old cultures is shown in Fig. 4. At 0.2 μg of tunicamycin per ml, labeling of disialoganglioside species G_{D1a} and G_{D1b} and trisialo species G_{T1b} was reduced by approximately 86%, 54%, and 82%, respectively, as determined by scanning the radioactive tracing. In contrast, there were no substantial changes in the radioactivity associated with the tetrasialoganglioside species G_{Q1b}, which remained the major cellular labeled ganglioside in cultures treated with tunicamycin at 1 μg/ml. Other radioactively labeled ganglioside species (Fig. 4 *Inset*) and an unidentified radioactive band [lane 1 between the origin (bottom) and G_{Q1b} in Fig. 4 *Inset*] were also markedly inhibited by tunicamycin.

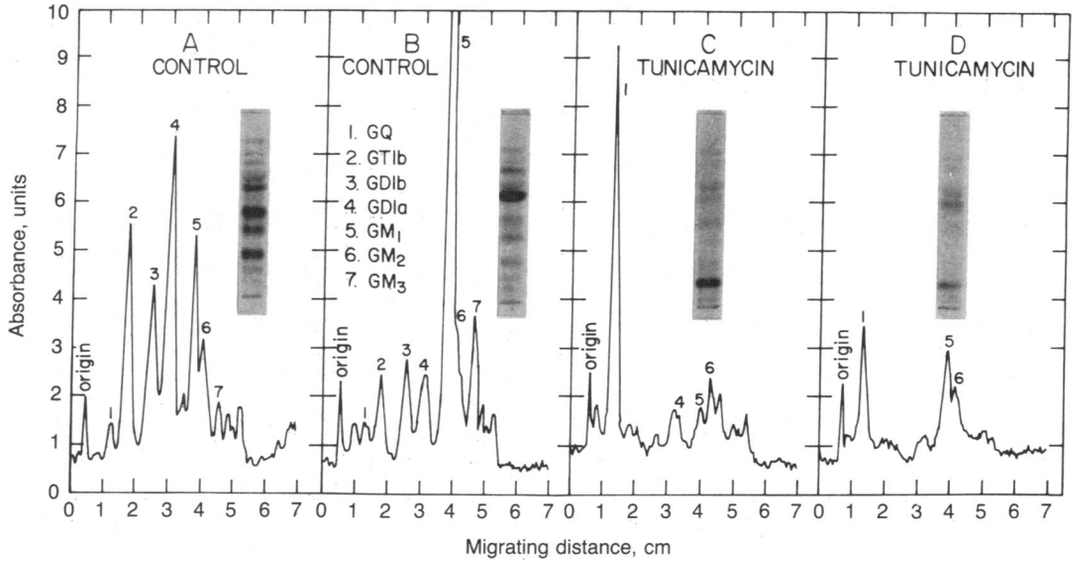

FIG. 3. Autoradiograph tracing of D-[^{14}C]glucosamine-labeled cellular gangliosides. Cerebral neurons, grown for 10 days in culture, were pulse labeled for 36 hr with 1 μCi of D-[^{14}C]glucosamine per 0.5 ml per well in the absence (*A* and *B*) or in the presence (*C* and *D*) of 0.5 μg of tunicamycin per ml. Cells were also treated for 6 hr prior to termination with vibrio cholera neuraminidase (5 milliunits; Behringswerke) (*B* and *D*). The radioactive medium was discarded, cells were washed twice with phosphate-buffered saline, collected in Eppendorf Microfuge tubes, and subjected to chloroform/methanol extraction. The combined lipid extracts from six wells were isolated and separated on TLC as described.

Effect of Tunicamycin on Binding Activity of ^{125}I-Labeled Tetanus Toxin to Nerve Cells. Addition of tunicamycin at 1 μg/ml to 8-day-old nerve cells resulted in a 50% decrease in toxin binding by 6 hr (Fig. 5*A*). Treatment with tunicamycin for up to 24 hr resulted in a loss of 63.5% of binding, followed by an apparent plateau. Tunicamycin at 0.1 μg/ml, added for up to 40 hr, caused a 17% loss of toxin binding activity, whereas tunicamycin at 0.5 μg/ml added to cells for the same time period caused a 62% drop in toxin binding activity (Fig. 5*B*). The age-in culture-dependent inhibition of ^{125}I-tetanus toxin binding by tunicamycin is shown in Fig. 5*C*. Evidently, the 2-day-old fetal cerebral cells encountered lesser losses of tetanus toxin binding sites compared to 9-day-old cultures (about 30% inhibition) after a 3-hr exposure to tunicamycin at either 1 or 2 μg/ml.

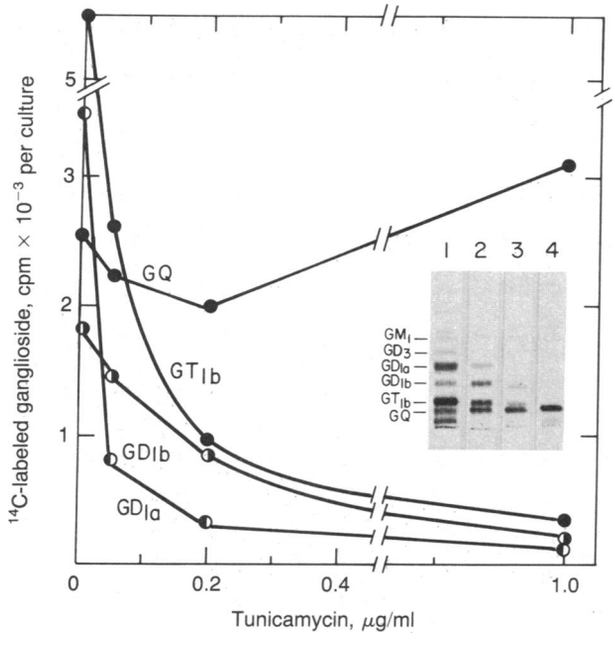

FIG. 4. Effect of tunicamycin on [^{14}C]glucosamine distribution into major cellular ganglioside species. Cerebral cells grown for 3 weeks in culture were incubated with D-[^{14}C]glucosamine, and cellular gangliosides were extracted and isolated by using experimental conditions similar to those described in the legend to Fig. 3. The radioactive tracings (*Inset*) correspond to control cultures (lane 1) and to cultures treated with 0.05 μg (lane 2), 0.2 μg (lane 3), and 1 μg (lane 4) of tunicamycin per ml. Values expressed as cpm per culture represent pooled lipid extracts from four wells.

These observations raise an intriguing question as to the ontogenesis of cell-surface gangliosides, which are putative receptors for tetanus toxin (16, 21, 22). Although tunicamycin virtually abolishes labeling of gangliosides by still unclear mechanisms (11, 12), it does not cause substantial bulk loss of these compounds. This result could be understood if it is postulated that only a limited fraction of the bulk gangliosides is subject to labeling. Because tunicamycin also drastically inhibits tetanus toxin binding and also reduces some of the presumed putative receptors (i.e., G_{D1b} but not G_{T1b}), it is possible that the limited fraction of ganglioside labeled by glucosamine is also the fraction associated with the toxin binding.

DISCUSSION

The aim of this study has been to investigate glycoconjugate metabolism and neurite outgrowth and their mutual relationship in normal rat cerebral cortex cultures. These virtually pure, nondividing neuronal cell preparations adapted to grow in serum-free, hormone-supplemented medium incorporate labeled glucosamine into cellular glycoproteins 4-fold more effectively than into cellular glycolipids during the first 2 days in culture. Higher labeling of the glycoprotein pool could reflect a stimulation of synthesis related to disruption of tissue integrity, followed by resynthesis of cellular constituents necessary for neurite outgrowth. Compared to glycoproteins, labeling of glycolipids is substantially higher in cells that already have established a well-developed fiber network in culture. Most of the label in the glycolipid species resides in the di- and trisialoganglioside species. This is compatible with the net increase in the total ganglioside sialic acid and enhanced complexity of species with maturation in culture noted by several laboratories (7, 8).

In this report, we have demonstrated that total incorporation of labeled glucosamine into the cellular glycoprotein

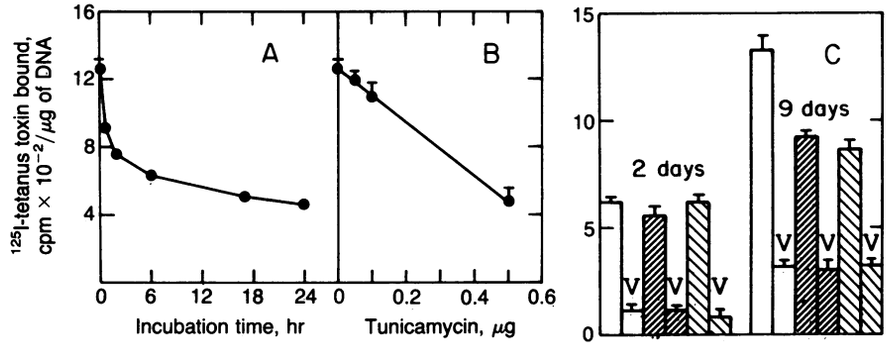

FIG. 5. Time course and concentration dependence of inhibition of ^{125}I-labeled tetanus toxin (^{125}I-tetanus toxin) binding to cerebral cells by tunicamycin. Cerebral neurons grown for 8 days in culture were incubated with tunicamycin at 1 μg/ml for the times designated (*A*) or for 46 hr with various concentrations of tunicamycin (*B*). ^{125}I-tetanus toxin (62,500 cpm per culture; 7050 cpm/ng) in 20 mM Tris/0.25% sucrose/5% fetal calf serum was added for 90 min at 37°C, and cell-associated radioactivity after three rinses with phosphate-buffered saline was determined. (*C*) Cells maintained for 2 or 9 days *in vitro* were incubated for 3 hr with tunicamycin at either 1 (▨) or 2 (▧) μg/ml in the presence (V) or absence of vibrio neuraminidase (5 milliunits per culture), followed by incubation with ^{125}I-tetanus toxin as detailed above. □, Control. Values are expressed as cpm/μg of DNA and represent the average of duplicate cultures (*A*) or 3–4 cultures (±SEM) as detailed for *B* and *C*.

fraction is markedly inhibited in both 1- and 8-day-old cultures with nontoxic concentrations of tunicamycin. This is in agreement with the well-established effect of this antibiotic on glycosylation of glycoproteins (10).

Second, we showed that incorporation of label into cellular glycolipids is blocked in a concentration-dependent fashion by tunicamycin in these neuronal cultures, which already contain an extensive fiber network. In contrast, glycolipid labeling is less affected in 1- to 2-day-old cells. Lack of inhibition correlates with the lower rates of ganglioside biosynthesis in the nerve cells at this particular stage of development. This period is also the most critical for neurite outgrowth. In other words, the addition of tunicamycin at this stage inhibits the formation of neuritic fibers. Once the fiber network has been established, such as in 8-day-old cultures, the addition of tunicamycin does not cause them to retract, although glycolipid synthesis is most affected at this later stage. Therefore, these results indicate that *de novo* synthesis of glycolipids is not a critical process for the rapidly growing neurite. The observation that tunicamycin affects neurite outgrowth at an early stage, when glycoprotein synthesis is dominant over glycolipids, indicates rather a correlation between glycoprotein synthesis and neurite outgrowth.

The biochemical characterization of tunicamycin inhibition of glycolipid biosynthesis also has revealed a novel observation with regards to synthesis of complex gangliosides. Unlike NG108-C15 neurohybrid cells (12) or other transformed cells of neural origin (23), normal neuronal cells also synthesize G_{D3} and its higher ganglioside homologues (7, 8). This report demonstrates that the *de novo* synthesis of G_{D3} and its higher homologues, with the possible exception of G_{Q1b}, is also blocked by tunicamycin. G_{Q1b} species, tentatively identified by adsorptive, hydrophobic, and thin-layer chromatographic procedures, is still extensively labeled in the presence of tunicamycin. It is also present as a normal constituent of cellular gangliosides and highly labeled in 3-week-old cultures. Its specific labeling in the presence of tunicamycin can be understood if a pool of its immediate precursor (i.e., G_{T1b}) can serve as an acceptor for CMP-sialic acid intermediate. The existence of such a pathway has been documented (24). Further, accumulation of CMP-sialic acid in tunicamycin-treated neurohybrid cells was shown (12). Whether labeling of G_{Q1b} in tunicamycin-treated cells reflects the existence of a transient metabolic pool as suggested by Maccioni *et al.* (25) remains to be seen.

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