Delivery of c-myb Antisense Oligodeoxynucleotides to Human Neuroblastoma Cells Via Disialoganglioside GD_2-Targeted Immunoliposomes: Antitumor Effects

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Background: **Advanced-stage neuroblastoma resists conventional treatment; hence, novel therapeutic approaches are required. We evaluated the use of c-myb antisense oligodeoxynucleotides (asODNs) delivered to cells via targeted immunoliposomes to inhibit c-Myb protein expression and neuroblastoma cell proliferation *in vitro*. *Methods:* Phosphorothioate asODNs and control sequences were encapsulated in cationic lipid, and the resulting particles were coated with neutral lipids to produce coated cationic liposomes (CCLs). Monoclonal antibodies directed against the disialoganglioside GD_2 were covalently coupled to the CCLs. ^{3}H-labeled liposomes were used to measure cellular binding, and cellular uptake of asODNs was evaluated by dot-blot analysis. Growth inhibition was quantified by counting trypan blue dye-stained cells. Expression of c-Myb protein was examined by western blot analysis. *Results:* Our methods produced GD_2-targeted liposomes that stably entrapped 80%–90% of added c-myb asODNs. These liposomes showed concentration-dependent binding to GD_2-positive neuroblastoma cells that could be blocked by soluble anti-GD_2 monoclonal antibodies. GD_2-targeted liposomes increased the uptake of asODNs by neuroblastoma cells by a factor of four-fold to 10-fold over that obtained with free asODNs. Neuroblastoma cell proliferation was inhibited to a greater extent by GD_2-targeted liposomes containing c-myb asODNs than by nontargeted liposomes or free asODNs. GD_2-targeted liposomes containing c-myb asODNs specifically reduced expression of c-Myb protein by neuroblastoma cells. Enhanced liposome binding and asODN uptake, as well as the antiproliferative effect, were not evident in GD_2-negative cells. *Conclusions:* Encapsulation of asODNs into immunoliposomes appears to enhance their toxicity toward targeted cells while shielding nontargeted cells from antisense effects and may be efficacious for the delivery of drugs with broad therapeutic applications to tumor cells. [J Natl Cancer Inst 2000;92:253–61]**

Advanced-stage neuroblastoma is refractory to conventional treatments such as radiation therapy and chemotherapy *(1,2)*. Thus, novel therapeutic approaches are required. The identification of activated oncogenes and inactivated tumor suppressor genes as fundamental genetic differences between malignant cells and normal cells has made it possible to consider such genes as targets for antitumor therapy. The c-Myb proto-oncogene is the best characterized member of a family of transcription factor genes. Its protein product plays a fundamental role in the proliferation of normal and leukemic cells *(3–5)*, and c-Myb gene expression has been reported in several solid tumors of different embryonic origins *(6,7)*, including neuroblastoma, where it is linked to cell proliferation and differentiation *(8,9)*.

In recent years, antisense oligodeoxynucleotides (asODNs) have shown efficacy in the selective inhibition of gene expression *(10–13)*, but therapeutic applications of asODNs are limited by their low physiologic stability, unfavorable pharmacokinetics, low cellular uptake, and lack of tissue specificity. Instability has been largely overcome by employing backbone-modified oligodeoxynucleotides, such as phosphorothioate oligodeoxynucleotides *(14)*. These analogues are more resistant to nucleases *(10,14,15)*, but their animal pharmacokinetics and cytotoxicity appear relatively non-sequence specific. For example, the same morphologic and functional abnormalities (mainly involving the spleen, liver, and kidneys) were observed in animals treated with several oligodeoxynucleotides of different sequences, likely as a result of their accumulation in these organs *(16–18)*.

The problem of low cellular uptake of asODNs has also been difficult to overcome. Endogenous uptake pathways have insufficient capacity to deliver the quantities of asODNs required to suppress gene expression *(14,19,20)*. Forming complexes between asODNs and cationic liposomes *(21,22)* or polylysine *(23)* has enhanced intracellular delivery, but such complexes have disadvantages for *in vivo* applications. Cationic lipid complexes, for example, are rapidly cleared by the reticuloendothelial system and can be nonspecifically cytotoxic *(24)*. Liposomes sterically stabilized with polyethylene glycol derivatives have circulation half-lives of approximately 12 hours and good stability in the presence of plasma, and they have recently been shown to facilitate delivery of asODNs *in vivo (25,26)*. Sterically stabilized immunoliposomes, which have cell surface-directed antibodies on their exteriors, have been recognized as efficient tools for delivery of drugs and diagnostic agents to specific kinds of cells *(27)*.

Among the antigens found on malignant cells, the disialoganglioside GD_2 is an attractive target for immunoliposomal therapy of tumors of neuroectodermal origin *(28,29)*, since it is extensively expressed in these tumors *(30,31)* but is less common in nonmalignant tissue. We have previously used GD_2-targeted, sterically stabilized immunoliposomes to deliver the apoptosis-inducing drug fenretinide to melanoma cells *in vitro (32)*. In this study, we investigated whether GD_2-targeted, sterically stabilized liposomes containing c-myb asODNs selectively delivered to neuroblastoma cells sufficient asODNs to reduce c-Myb protein expression and to inhibit cell growth.

Materials and Methods

Chemicals and Monoclonal Antibodies

Hydrogenated soy phosphatidylcholine (HSPC), cholesterol, 1,2-distearoylglycero-3-phosphatidylethanolamine-*N*-polyethylene glycol-2000 (DSPE-PEG), and 1,2-dioleoyl-3-trimethylammonium pro-

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See "Notes" following "References."

pane (DOTAP) were from Avanti Polar Lipids, Inc. (Alabaster, AL). A derivative of DSPE-PEG with a maleimide group at the distal terminus of the polyethylene glycol chain (DSPE-PEG-MAL) was synthesized by Shearwater Polymers (Huntsville, AL). [^{3}H]Cholesterol hexadecylether ([^{3}H]CHE) was from Du Pont NEN (Boston, MA). All the other reagents of biochemical and molecular biology grade were obtained from Sigma Chemical Co. (St. Louis, MO).

Two different monoclonal antibodies (MAbs) directed against disialoganglioside GD_2 (anti-GD_2s) were employed. In the first set of experiments, we used 14.G2a, a murine MAb of immunoglobulin (Ig) G2a isotype subclass, specific for the GD_2 antigen, provided by R. A. Reisfeld (The Scripps Institute, La Jolla, CA) *(33).* To reduce the possibility of adverse reactions to murine antibodies, we used, in a second set of experiments, the chimeric human–mouse MAb ch14.18. This antibody was constructed by combining constant regions of human IgG molecules with variable regions of a GD_2-specific murine MAb *(29)* and was provided by R. Handgretinger (University of Tubingen, Tubingen, Germany).

Oligodeoxynucleotides

Three 24-mer phosphorothioate oligodeoxynucleotides were prepared, purified, and dried under vacuum by Lynx Therapeutics, Inc. (Hayward, CA). Our asODN, which is referred to hereafter as myb-as, is complementary to codons 2–9 of human c-Myb messenger RNA (mRNA) and has the sequence 5′-TATGCTGTGCCCGGGTCTTCGGGC-3′. It is currently being used in phase II clinical studies, where it is known as LR-3001 *(34)* . A sense oligodeoxynucleotide (myb-s) (5′-GCCCGAAGACCCGGGCACAGCATA-3′) and a scrambled sequence (5′-TCGCGGATGTCCGGGTCTGTCGCT-3′) oligodeoxynucleotide were used as controls.

Liposome Preparation and Oligodeoxynucleotide Encapsulation

A 700-μg portion of a given oligodeoxynucleotide with a trace of ^{125}I-labeled oligodeoxynucleotide (to allow us to estimate the percentage of oligodeoxynucleotide present at various stages of the procedure) was dissolved in 0.25 mL distilled deionized water. Next, 0.51 mL methanol and 0.25 mL $CHCl_3$ containing 2.17 μmol DOTAP were added, and the mixture was gently vortex mixed to form a monophase. After a 30-minute incubation at room temperature, 0.25 mL distilled deionized water and 0.25 mL $CHCl_3$ were added. After mixing and centrifugation (800*g* for 7 minutes at room temperature), the aqueous methanol layer was removed. Under these conditions, which were developed in pilot studies using 10 μg oligodeoxynucleotide and various amounts of DOTAP, 90%–95% of the oligodeoxynucleotide was recovered in the organic phase. The resulting oligodeoxynucleotide-to-lipid mole ratio is 1 : 24, and a positive-to-negative charge ratio of 1 : 1 is obtained.

Following the extraction, 4.3 μmol cholesterol and appropriate amounts of HSPC, DSPE-PEG, or DSPE-PEG-MAL to give cholesterol-to-phospholipid mole ratios of 1 : 2 were added to the organic phase. Next, 0.2 mL distilled deionized water was added, and the mixture was vortex mixed and then emulsified by sonication for 2 minutes. The organic phase was evaporated with the use of a rotary evaporator (Buchi, Flawil, Switzerland). Liposomes, which form as the organic phase evaporates, were freeze-thawed in liquid nitrogen 10 times and then reduced in size to approximately 100 nm by extrusion six times through 200-nm and five times through 100-nm polycarbonate membranes (Avestin, Inc., Ottawa, ON, Canada) in a Liposofast extruder (Avestin, Inc.), as previously described *(32,35).* Liposomes prepared in this manner are called coated cationic liposomes (CCLs) *(26,27).*

Coupling of Anti-GD_2 MAbs to Liposomes

A previously described method *(36),* slightly modified by us *(32),* was used to covalently link MAbs to the maleimide terminus of DSPE-PEG-MAL. To activate the anti-GD_2s for reactivity toward the maleimide, we utilized 2-iminothiolane (Traut's reagent) to convert exposed amino groups on the antibody into free sulfhydryl groups. A 20 : 1 mole ratio of 2-iminothiolane to MAb and 1 hour of incubation at room temperature with occasional mixing gave optimal MAb activation. After separation of thiolated MAb from iminothiolane, with the use of Sephadex G-25 column chromatography, the MAb was slowly added to a 5-mL test tube containing the liposomes (with encapsulated oligodeoxynucleotides) and a small magnetic stirring bar. Optimal coupling was obtained with the use of a phospholipid-to-MAb mole ratio of 1500–2000 : 1. Oxygen was displaced by running a slow stream of nitrogen over the reaction mixture. The tube was capped and sealed with Teflon tape, and the reaction mixture was incubated overnight at room temperature with continuous slow stirring. The resulting immunoliposomes were separated from unreacted MAb by chromatography with the use of Sepharose CL-4B, sterilized by filtration through 0.2-μm pore cellulose membranes (Millipore Corp., Bedford, MA), and stored at 4 °C. Coupling of MAbs to liposomes was quantified by adding trace amounts of ^{125}I-labeled MAb to the coupling reaction with liposomes, followed by γ-counting (Cobra 5002; Canberra Packard, Meriden, CT).

Coated cationic liposomes with covalently attached anti-GD_2 MAb and encapsulated myb-as are referred to as GD_2-targeted liposomes and abbreviated aGD2-CCL-myb-as throughout this report. Those that lack the antibody are referred to as nontargeted liposomes, and the "aGD2" prefix is omitted from the abbreviation. Liposomes with encapsulated myb-s have "myb-s" as their suffix.

Cell Lines and Culture Conditions

To broadly cover the phenotypes exhibited by neuroblastoma cells *in vitro,* we used four GD_2-positive human neuroblastoma cell lines: GI-LI-N, ACN, HTLA-230, and LAN-5 *(32,37).* Four GD_2-negative cell lines, adherent cell lines A431 (human epidermoid carcinoma) and HeLa (human cervical carcinoma) and suspension cell lines Jurkat (human T cell) and HL-60 (human leukemia), were used in some experiments. All cell lines were maintained in the logarithmic phase of growth at 37 °C in 75-cm^2 plastic culture flasks (Corning Inc., Corning, NY) in a 5% CO_2–95% air humidified incubator. They were split, washed, counted, and replated in fresh complete medium (RPMI-1640 medium [Biochrom, Berlin, Germany], supplemented with 10% heat-inactivated fetal calf serum [Biochrom], 50 IU/mL sodium penicillin G, 50 μg/mL streptomycin sulfate, and 2 m*M* L-glutamine), as previously described *(32,38).* A 1 m*M* EDTA solution in Hanks' balanced salt solution (Flow Laboratories, Milan, Italy) was used to release adherent cell lines from the surface of the culture flasks.

Binding and Uptake of Liposomes

Neuroblastoma cells or control GD_2-negative cells were incubated in complete medium for 2 hours at 37 °C with various concentrations of [^{3}H]CHE-labeled liposomes with encapsulated myb-as. Cells were washed extensively, treated with trypsin, and lysed with 1 N NaOH prior to measurement of radioactivity. In competition experiments, a 50-fold excess of free MAb was added 30 minutes before addition of the liposomes. In some experiments, the chimeric human–mouse variant ch14.18 anti-GD_2 MAb or the nonspecific isotype-matched antibody, code X 0943 (Dako Corp., Glostrup, Denmark), was coupled to myb-as-entrapping liposomes, as described above.

Oligodeoxynucleotide Release

Release of oligodeoxynucleotides from liposomes was measured by dialyzing the oligodeoxynucleotide-containing liposomes in 25% human plasma from healthy donors or in complete medium against a large volume of the same solvent at 37 °C, using dialysis tubing with a molecular mass cutoff of 100 000 daltons. The dialysis bag was sampled at intervals, and the radioactivity was measured.

Uptake of asODNs by Neuroblastoma Cells

For the uptake studies, 50 μg/mL free or liposome-encapsulated myb-as was added to each well of six-well culture plates (Corning, Bibby Sterilin, Ltd., Staffordshire, U.K.) containing 5×10^5 cells/well. After incubation at 37 °C for 2 hours, the medium was removed, and cells were washed twice with ice-cold 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), pH 7.4. Cells were resuspended in Versene (Life Technologies, Inc. [GIBCO BRL], Gaithersburg, MD), washed again with BSA in PBS, and centrifuged at 300*g* for 5 minutes at 4 °C. The cell pellet was suspended in 0.5 *M* NaCl in 0.2 *M* acetic acid (pH 2.5) and held at 4 °C for 10 minutes to elute surface-bound oligodeoxynucleotides. Samples were centrifuged at 300*g* for 5 minutes at 4 °C. The pellet was resuspended at a volume of 100 μL and then frozen at −20 °C. For dot-blot analyses, 400 μL 12.5 m*M* EDTA in 0.5 *M* NaOH was added to each frozen sample, and the samples were boiled for 10 minutes. They were then blotted onto a nylon membrane (Amersham Life Science, Inc., Arlington Heights, IL) by use of a dot-blot or a slot-blot apparatus (Bio-Rad Laboratories, Richmond, CA). The membrane was washed with 0.4 *M* NaOH and with 2× standard saline citrate (i.e., 300 m*M* NaCl and 30 m*M* sodium citrate), dried in an oven at 80 °C for 1 hour, and then hybridized with a ^{32}P-labeled probe complementary to myb-as, as outlined in the ExpressHyb protocol (Clontech Laboratories, Inc., Palo Alto, CA). Time-

dependence studies were carried out in the same way, except that the time of incubation was varied. Competition experiments were carried out by addition of a 50-fold excess of free anti-GD_2 MAb 30 minutes before the addition of aGD2-CCL-myb-as.

Inhibition of Cell Growth

GI-LI-N or HeLa cells were plated in T-25 culture flasks (Corning) and treated with free myb-as, nontargeted liposomes (CCL-myb-as), GD_2-targeted liposomes (aGD2-CCL-myb-as), or control oligodeoxynucleotide sequences (free or encapsulated in targeted or nontargeted liposomes) at an initial concentration of 80 μg/mL. After 18, 36, and 72 hours, additional oligodeoxynucleotides (same formulations as were added initially) were added at a concentration of 40 μg/mL.

In some experiments, 100 μg/mL of the various oligodeoxynucleotide formulations was added to cells at the beginning of the experiment and every 2 days thereafter. Two hours after each addition, the cells were washed to remove unbound oligodeoxynucleotides and transferred to fresh complete medium.

All experiments were continued for 8 days; at 2-day intervals, the cells were detached with EDTA, stained with trypan blue dye, and counted microscopically.

Western Blot Analysis of c-Myb in Neuroblastoma Cells

GI-LI-N cells (5×10^5) were treated with free myb-as, CCL-myb-as, or aGD2-CCL-myb-as at an initial concentration of 100 μg/mL. After 18 and 36 hours, additional myb-as (same concentration and formulations as were used initially) was added. Two hours after each addition, the cells were washed and transferred to fresh complete myb-as-free medium. Control cells were untreated with any myb-as formulation, washed, and transferred in the same way as the experimental cells. At 12-hour intervals from the beginning of the experiment, cells were harvested and stored frozen at −80 °C for later analysis. Frozen cell samples were solubilized in lysis buffer (i.e., 0.01 *M* Tris–HCI [pH 7.5], 0.144 *M* NaCl, 0.5% Nonidet P-40, 0.5% sodium dodecyl sulfate [SDS], 0.1% aprotinin, 10 μg/mL leupeptin, and 2 m*M* phenylmethylsulfonyl fluoride) and sonicated. The protein content of the samples was quantified with the use of the BCA protein assay (Pierce Chemical Co., Rockford, IL). Forty-microgram samples of protein were subjected to 10% SDS–polyacrylamide gel electrophoresis, with the use of prestained molecular weight markers (Amersham International, Little Chalfont, Buckinghamshire, U.K.) run in parallel to aid in the localization of the 75-kd c-Myb or 64- to 67-kd c-Myc protein. (The latter protein was used to test for equivalence of protein gel loading and transfer to the nitrocellulose membranes and the specificity of the myb-as.) The resolved proteins were blotted onto nitrocellulose membranes, and an anti-human c-Myb MAb (clone UBI 05–175; Upstate Biotechnology, Lake Placid, NY) or an anti-c-Myc MAb (clone 9E10; Cambridge Research Biochemicals, London, U.K.) was used to localize the c-Myb or c-Myc polypeptides on the blots. Peroxidase-conjugated goat anti-mouse antibodies (Bio-Rad Laboratories) were used as secondary antibodies. Immune complexes (i.e., proteins of interest) were visualized with the use of an enhanced chemiluminescence system (Pierce Chemical Co.). The relative amount of transferable c-Myb protein in a given sample was quantified by densitometry of x-ray films and normalization by staining the blotted nitrocellulose membranes with Ponceau red to estimate the amount of protein loaded.

Statistics

Results are expressed as means ± 95% confidence intervals (CIs). All data derive from at least four independent experiments.

RESULTS

Characterization of GD_2-Targeted Immunoliposomes

In experiments for which data are not shown, we noted the following: 1) Liposomes prepared by our procedure are typically 70–120 nm in diameter, as observed by electron microscopy *(35);* 2) an average of 65% (95% CI = 50%–80%) of the anti-GD_2 antibody was associated with liposomes (presumably covalently attached through thioether bonds), with an antibody density of 60–80 μg MAb/μmol phospholipid; 3) the efficiency of asODN trapping in liposomes was estimated to be 80%–90%; 4) during 2–4 hours of dialysis at 37 °C in 25% human plasma, 10%–20% of asODN was released, and these asODNs were probably associated with the liposomal exterior; 5) continued dialysis for up to 1 week produced no more free asODN; and 6) our dialyzed liposomes retained their ability to bind to GD_2-expressing cells over these same time periods.

Binding of GD_2-Targeted Immunoliposomes to GD_2-Positive and GD_2-Negative Cells

We studied the concentration dependence of aGD2-CCL-myb-as-binding to GD_2-positive (GI-LI-N and ACN) and GD_2-negative (HeLa and HL-60) cells by measuring uptake of phospholipid from liposomes. Phospholipid uptake by GD_2-positive neuroblastoma cell lines increased with phospholipid (i.e., liposome) concentration, showing subsaturation at 400 nmol phospholipid/mL (Fig. 1). Similar results were obtained with the use of both mouse 14.G2a or its chimeric human–mouse variant ch14.18 anti-GD_2 MAbs (data not shown). The same liposome preparation without anti-GD_2 MAb (CCL-myb-as) showed very low phospholipid uptake with no evidence of saturation. Preincubation of cells for 30 minutes with soluble anti-GD_2 MAb, but not with an unrelated MAb, almost completely blocked phospholipid uptake by cells, suggesting that aGD2-CCL-myb-as binding occurred through specific antigen–antibody recognition. Moreover, coupling to liposomes of nonspecific isotype-matched antibody did not increase phospholipid uptake by neuroblastoma cells (data not shown). Both GD_2-negative cell lines showed very low cell phospholipid uptake in all cases examined (Fig. 1).

Uptake of Free and Encapsulated Oligodeoxynucleotides by Neuroblastoma Cells

Four GD_2-positive neuroblastoma cell lines and four GD_2-negative unrelated tumor cell lines were incubated with free or encapsulated myb-as, and uptake of the oligodeoxynucleotide was measured by dot-blot analysis. Since there were no obvious differences in the degree of uptake of liposome-encapsulated myb-as by the four GD_2-positive cell lines, only the data obtained with two GD_2-positive cell lines (GI-LI-N and ACN) and one GD_2-negative cell line (HeLa) are shown (Fig. 2). Fig. 2, A, is an example of the dot-blot analysis of myb-as uptake. The amount of myb-as taken up by GD_2-positive cells treated with aGD2-CCL-myb-as (dots 7 and 8) was substantially greater than that taken up by cells treated with free myb-as (dots 3 and 4) or CCL-myb-as (dots 5 and 6). By using densitometry, we estimated that the uptake of myb-as into GI-LI-N and ACN cells was fourfold to fivefold higher when they were incubated with aGD2-CCL-myb-as than when they were incubated with an equal amount of free myb-as or CCL-myb-as (Fig. 2, B). Longer incubation led to an approximately 10-fold greater uptake of myb-as from aGD2-CCL-myb-as, relative to uptake of free myb-as. After 24 hours' incubation, uptake from aGD2-CCL-myb-as relative to uptake from CCL-myb-as decreased to only twofold (Fig. 2, C). In a competition experiment, the uptake of aGD2-CCL-myb-as was inhibited by a 50-fold excess of free anti-GD_2 (Fig. 2, B).

Effect of Liposome-Encapsulated myb-as on Neuroblastoma Cell Proliferation

To determine the effects of myb-as on neuroblastoma cell proliferation, we treated GI-LI-N cells with free or liposome-encapsulated myb-as, using a pro-

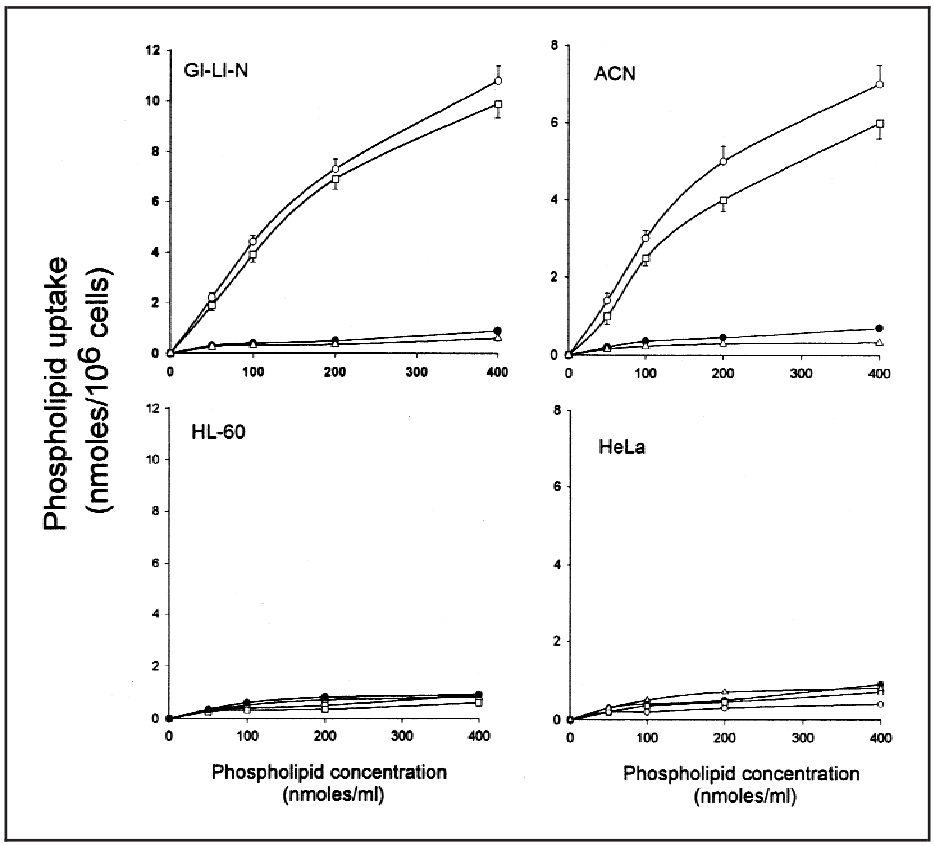

Fig. 1. Concentration dependence of liposome binding to disialoganglioside GD_2-positive (GI-LI-N and ACN) and GD_2-negative (HL-60 and HeLa) tumor cells. Cells were incubated for 2 hours with the indicated concentrations of ^{3}H-labeled liposomes with encapsulated c-myb antisense oligodeoxynucleotides (myb-as) and with (**open circles**) or without (**open triangles**) coupled anti-GD_2 monoclonal antibodies (14.G2a MAb). In competition experiments, cells were preincubated for 30 minutes with a 50-fold excess of either 14.G2a MAb (**closed circles**) or an isotype-matched nonspecific antibody (code X 0943; **open squares**) before addition of ^{3}H-labeled liposomes with coupled anti-GD_2 MAb and encapsulated myb-as. Cells were washed, treated with trypsin, and lysed with 1 N NaOH prior to measurement of radioactivity. **Error bars in the top two panels** are 95% confidence intervals. Only the upper portions of the error bars are shown for the upper curve, and only the lower portions are shown for the lower curves.

tocol previously shown to inhibit neuroblastoma cell growth by asODNs *(9)*. As expected, free myb-as inhibited GI-LI-N cell proliferation up to 70%. Treatment of cells with CCL-myb-as or aGD2-CCL-myb-as induced a more rapid and stronger inhibition of neuroblastoma cell growth (Fig. 3, A). Conversely, in GD_2-negative HeLa cells, myb-as, in all formulations, affected cell proliferation to a smaller extent (Fig. 3, B). To demonstrate the sequence specificity of these effects, we also delivered myb-s into GI-LI-N and HeLa cells. With the use of formulations of the same types as those used with myb-as, myb-s showed little or no growth inhibition (Fig. 3, A and B). Scrambled-sequence oligodeoxynucleotides also were ineffective (data not shown).

To assess the efficiency of GD_2-targeted liposomes in delivering myb-as to target cells, we used a protocol that allows high levels of antibody-mediated binding while minimizing nonspecific adsorption of liposomes to cells *(32,37)*. In these experiments, tumor cells were exposed to oligodeoxynucleotides for 2 hours at the beginning of the experiment and every 2 days thereafter (for 8 days) and then washed and transferred to oligodeoxynucleotide-free complete medium. Under these conditions, free myb-as, CCL-myb-as, free myb-s, CCL-myb-s, and aGD2-CCL-myb-s had no substantial cytotoxic effect on GI-LI-N cells (Fig. 3, C). In contrast, aGD2-CCL-myb-as induced a marked decrease in neuroblastoma cell proliferation over the same 8-day period. Under the same conditions, none of our oligodeoxynucleotide formulations inhibited proliferation of the GD_2-negative cell lines HeLa (Fig. 3, D) or HL-60, a cell line that has been shown to be strictly dependent on c-Myb protein for cell proliferation *(5,39)* (data not shown). Hence, antiproliferative activity under these conditions appears to require expression of the GD_2 antigen on the cell surface to allow binding followed by internalization of the aGD2-CCL-myb-as.

To investigate whether the enhanced cytotoxicity of aGD2-CCL-myb-as depends on specific recognition of cells by antibodies, competition studies were performed. Addition of a 50-fold excess of free anti-GD_2 MAb to a culture of GI-LI-N cells that were growing in the presence of aGD2-CCL-myb-as caused a substantial reduction in the antiproliferative effect of aGD2-CCL-myb-as, while addition of an unrelated MAb had little effect **(**data not shown).

Effect of myb-as on c-Myb Expression in Neuroblastoma Cells

The expression of c-Myb protein in GI-LI-N cells treated with free myb-as, CCL-myb-as, or aGD2-CCL-myb-as was analyzed by western blot analysis (Fig. 4, A). Cells treated with free myb-as or CCL-myb-as (by addition of 100 μg/mL inhibitor at 0, 18, and 36 hours followed, 2 hours later, by transfer to fresh inhibitor-free complete medium) showed levels of protein expression similar to those in the control (untreated) cells. In contrast, GI-LI-N cells treated with the aGD2-CCL-myb-as showed a reduction in c-Myb protein levels of about 70% in comparison with control cells. Free or liposome-entrapped sense or scrambled analogues of myb-as did not affect c-Myb protein expression (data not shown). The time dependence of this effect is shown in Fig. 4, B. To examine the specificity of protein repression by myb-as, the effect of aGD2-CCL-myb-as on the expression of c-Myc protein was determined. As expected, there was no repression of c-Myc expression by aGD2-CCL-myb-as (Fig. 4, C).

DISCUSSION

We have shown that an asODN complementary to eight contiguous codons of c-Myb mRNA inhibited the growth of neuroblastoma cells *in vitro* and that its inhibitory effect was greatest when it was delivered to the cells in GD_2-targeted, sterically stabilized liposomes. Our immunoliposomal asODN preparation also inhibits expression of c-Myb protein by these cells. Our results demonstrate the feasibility of the selective delivery of myb-as to GD_2-positive neuroblastoma

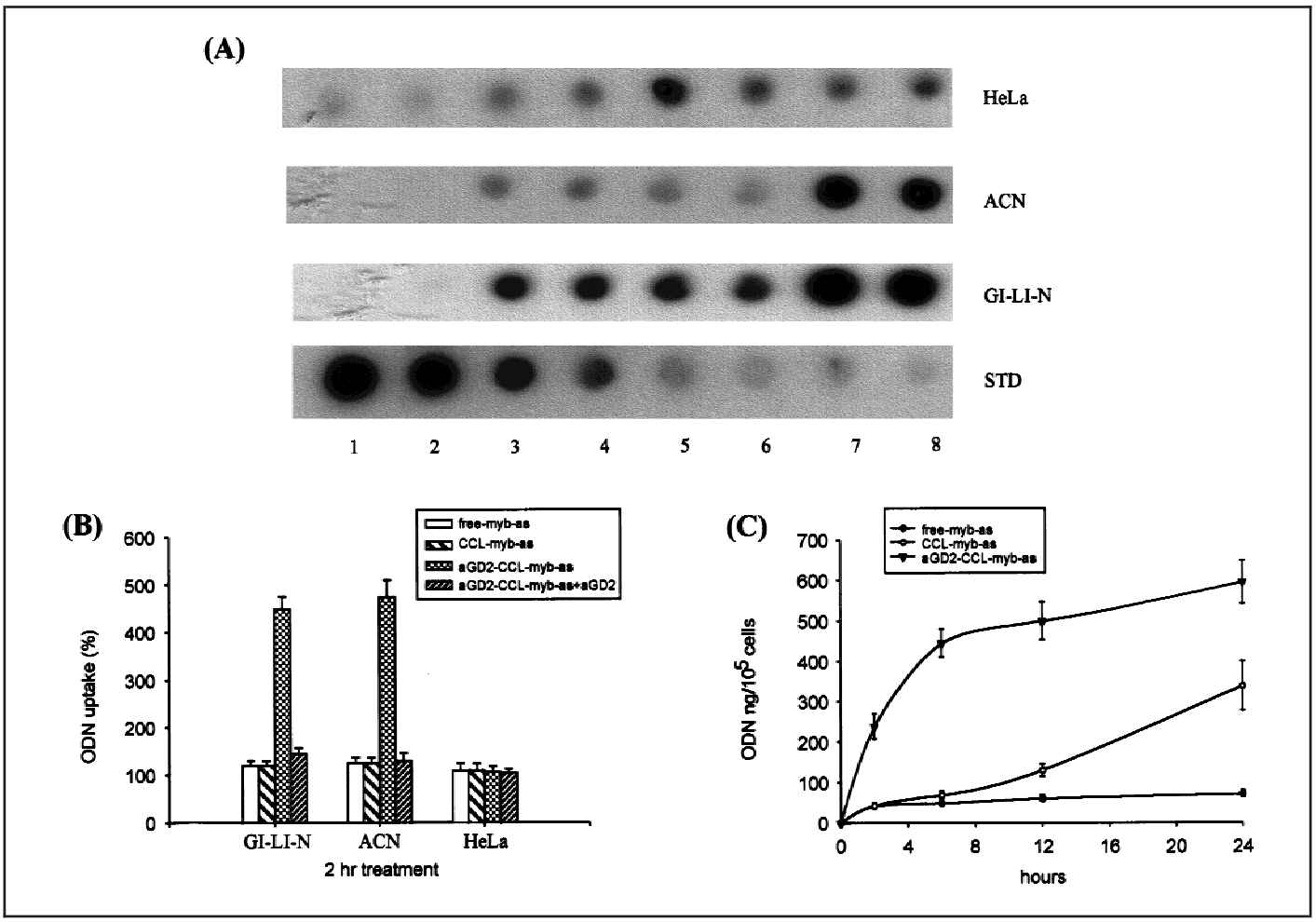

Fig. 2. Cellular uptake of c-myb antisense oligodeoxynucleotides (myb-as). **Panel A:** dot-blot evaluation of intracellular myb-as. For the standards lane (STD), various amounts of free myb-as (200, 100, 50, 25, 12.5, 6.25, 3.12, 1.55 ng in **dots 1–8,** respectively) were added to frozen untreated cell samples. **Dots in the other lanes** show myb-as released from the indicated types of cells incubated at 37 °C for 2 hours with the following additions: none (**dots 1 and 2**), free myb-as (**dots 3 and 4**), myb-as encapsulated in liposomes without anti-disialoganglioside GD_2 monoclonal antibody (MAb) (**dots 5 and 6**), and myb-as encapsulated in liposomes with anti-GD_2 MAb (**dots 7 and 8**). After addition of free myb-as (**STD lane**) or incubation (**other lanes**), 400 μL 12.5 m*M* EDTA in 0.5 *M* NaOH was added to the mixtures, and the samples were boiled for 10 minutes. They were then blotted onto a nylon membrane by use of a dot-blot apparatus. The membrane was washed with 0.4 *M* NaOH and with 2× standard saline citrate (i.e., 300 m*M* NaCl and 30 m*M* sodium citrate), dried in an oven at 80 °C for 1 hour, and then hybridized with a ^{32}P-labeled probe complementary to myb-as. **Panel B:** densitometric analysis, after hybridization and autoradiography, of released intracellular myb-as. The uptake of myb-as mediated by liposomes was expressed as percentage of the uptake obtained with free myb-as in these independent experiments taken as 100%. CCL-myb-as = cells incubated with myb-as encapsulated in liposomes without anti-GD_2 MAb. aGD2-CCL-myb-as = cells incubated with myb-as encapsulated in liposomes with anti-GD_2 MAb. aGD2-CCL-myb-as + aGD2 = cells incubated with myb-as encapsulated in liposomes with anti-GD_2 MAb following preincubation with 50-fold excess free anti-GD_2 MAb for 30 minutes. **Error bars** are 95% confidence intervals. **Panel C:** time-dependent intracellular uptake into GI-LI-N cells of myb-as from free myb-as, CCL-myb-as, or aGD2-CCL-myb-as (abbreviations as in panel B). Scanning densitometry was used to quantify myb-as, using data from the STD lane in panel A to generate a standard curve. **Error bars** are 95% confidence intervals. ODN = oligodeoxynucleotide.

cell lines by targeted immunoliposomes. The myb-as oligodeoxynucleotide, LR-3001, used in this study is being tested in an ongoing phase II clinical trial for the treatment of chronic myelogenous leukemia *(34).*

Neuroblastoma is a pediatric neuroectoderm-derived tumor that often presents at an advanced stage and is characterized by a very poor prognosis. Despite development of very aggressive multimodal therapeutic approaches, the long-term survival of patients with neuroblastomas has not improved substantially over the last two decades *(1,2).* Additional therapeutic strategies are continuously being explored for their clinical potential as alternative or adjuvant treatment.

asODNs are being investigated as possible therapeutic agents that take direct advantage of molecular complementarity *(10,17,18)* to inhibit gene expression in a specific manner. The antisense approach uses a short oligodeoxynucleotide designed to bind to a target mRNA transcript through Watson-Crick base-pairing to form an asODN–RNA heteroduplex that inhibits gene expression.

While this conceptually simple, straightforward, and rational approach to novel drug design is attractive for the treatment of cancers and other human diseases *(10,40),* using it therapeutically to reduce or to eliminate expression of gene products important for the growth of neuroblastoma cells is much more complex for several reasons. These include the need to deliver asODNs selectively to specific areas of the body in order to maximize their action and to minimize their side effects *(41),* instability, and impermeability. A previously developed therapeutic strategy that addresses many of these potential problems uses liposomes as carriers for asODNs *(42).*

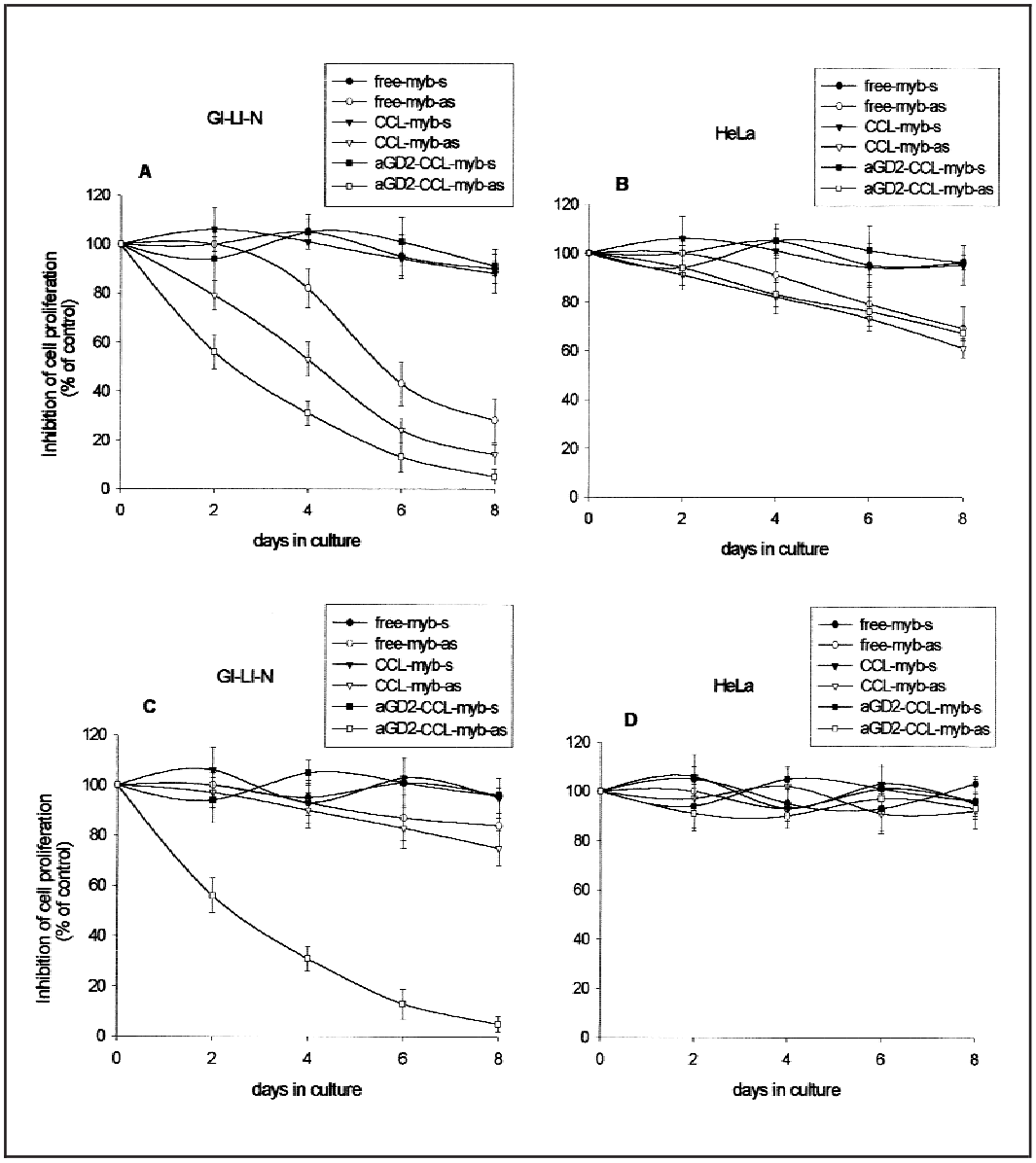

Fig. 3. Growth inhibition of neuroblastoma cells by c-myb antisense oligodeoxynucleotides (myb-as) encapsulated in anti-disialoganglioside GD_2 liposomes. **Panels A** and **B** represent GI-LI-N and HeLa cells, respectively, treated with free sense oligodeoxynucleotide (free-myb-s), free-myb-as, sense oligodeoxynucleotide encapsulated in liposomes without anti-GD_2 monoclonal antibody (CCL-myb-s), antisense oligodeoxynucleotides encapsulated in liposomes without anti-GD_2 monoclonal antibody (CCL-myb-as), sense oligodeoxynucleotide encapsulated in liposomes with anti-GD_2 monoclonal antibody (aGD2-CCL-myb-s), or myb-as encapsulated in liposomes with anti-GD_2 monoclonal antibody (aGD2-CCL-myb-as). The initial oligodeoxynucleotide concentration was 80 μg/mL. Oligodeoxynucleotides were added at 40 μg/mL after 18, 36, and 72 hours. At the beginning of each experiment and at 2-day intervals thereafter, the cells were detached with EDTA, stained with trypan blue, and counted microscopically. **Panels C** and **D** represent the same cell lines treated by adding the same oligodeoxynucleotide preparations to the growing cells at a concentration of 100 μg/mL at the beginning of the experiment and every 2nd day thereafter. Two hours after each of these additions, the cells were washed to remove unbound oligodeoxynucleotides and transferred to fresh complete medium. Cells were detached, stained, and counted as in the experiments shown in panels A and B. Data are expressed as percentage of control, and **error bars** are 95% confidence intervals.

Here, we have described a system in which asODNs are encapsulated within small (about 100 nm) liposomes that have high trapping efficiencies (up to 90% of added oligodeoxynucleotide is stably associated with lipids) and bear covalently attached MAbs specific for the GD_2 antigen on their exterior. The rationale for this approach is the extensive expression of GD_2 on neuroblastoma cells in comparison to its limited presence on normal tissues, such as the peripheral nervous system and the cerebellum *(28–31)*.

We have compared the cellular uptakes of free asODN, asODN encapsulated in GD_2-targeted liposomes, and asODN encapsulated in nontargeted liposomes. Encapsulation of asODN in GD_2-targeted liposomes was found to promote a fourfold to 10-fold increase in cellular uptake of asODNs when compared with the free oligodeoxynucleotide. The increase in uptake relative to CCL-myb-as approaches 10-fold at 6 hours' incubation time, but the relative size of the increase diminishes with time, to only twofold at 24 hours. Uptake was cell type specific: Of the cell types we examined, only those that express GD_2 antigens took up more asODN from receptor-targeted liposomes than from nontargeted liposomes. That the uptake of asODN by neuroblastoma cells could be competitively inhibited by excess free anti-GD_2 MAb is consistent with the hypothesis that intracellular delivery of asODN from GD_2-targeted liposomes is mediated by GD_2 molecules in the plasma membrane. Similar findings have been reported for the folate receptor *(13,43)*.

Although we did not perform fractionation assays to assess the subcellular localization of immunoliposome-delivered asODN, we can conclude that it was not bound to the plasma membrane, since acetic acid was used to elute surface-bound asODN in our studies of asODN uptake. Because aGD2-CCL-myb-as was able to inhibit cell growth and to suppress c-Myb expression, we conclude that myb-as was delivered intracellularly in functionally active form. Since it has been shown that 14.G2a MAb was internalized by melanoma cells, even when conjugated to ricin A-chain *(44)*, these findings suggest that, in our experimental model system, liposomes bind to the cell surface and are then internalized via a receptor-dependent endocytic pathway. This conclusion agrees with previous reports

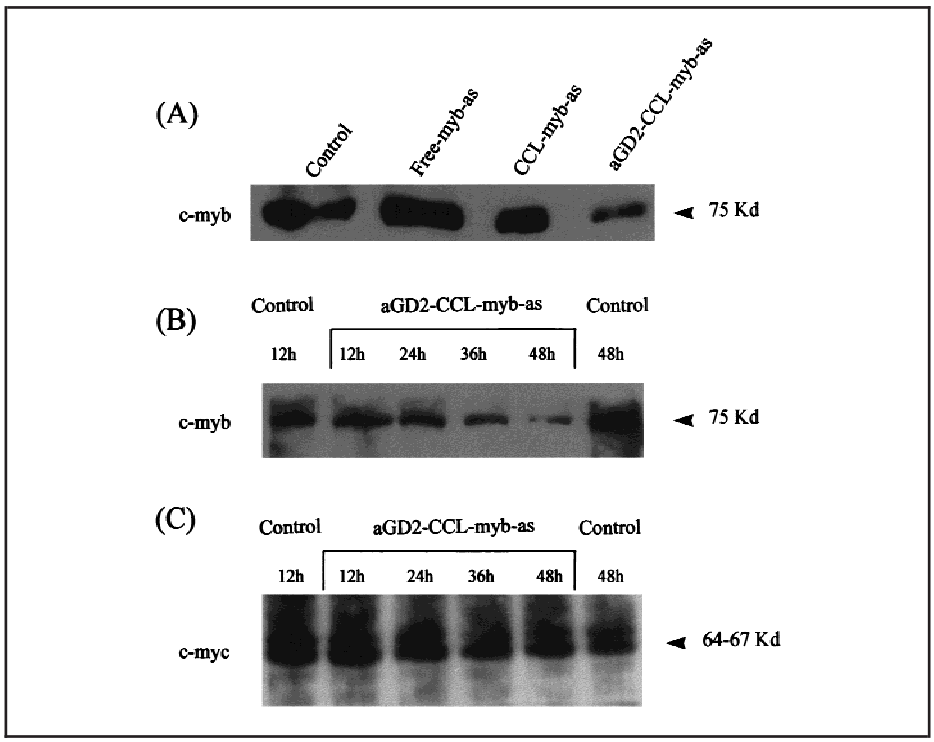

Fig. 4. Selective inhibition of c-Myb protein expression in GI-LI-N cells. Except in control experiments, free c-myb antisense oligodeoxynucleotide (free-myb-as), myb-as encapsulated in liposomes without anti-disialoganglioside GD_2 monoclonal antibodies (CCL-myb-as), or myb-as encapsulated in liposomes with anti-GD_2 monoclonal antibody (aGD2-CCL-myb-as) was added to GI-LI-N cells at a concentration of 100 μg/mL at the beginning of the experiment and 18 and 36 hours later. Two hours after each addition, the cells were washed and transferred to fresh myb-as-free complete medium. The cells were harvested at 48 hours (**panel A**) or at the indicated time points (**panels B** and **C**) and frozen for later analysis. Analysis for protein expression (c-Myb for panels A and B and c-Myc for panel C) was by immunoblotting. (*See* "Materials and Methods" section for more details.) Kd = kilodalton.

(13,45) that showed that endocytosis of immunoliposomes leads to nondestructive release of much of the internalized material into the cytosol, and it confirms the importance of internalizing receptors *(27,36)*.

One of the most attractive features of antisense-based inhibitors is the potential for great specificity of their inhibitory effects *(40)*. Demonstrating such specificity is usually considered to be the most important criterion for concluding that a true antisense mechanism explains the biologic effects of a particular oligodeoxynucleotide. For this reason, we have examined the specificity of inhibition of c-Myb expression and tumor growth by LR-3001, an oligodeoxynucleotide complementary to codons 2–9 of human c-Myb mRNA, and have demonstrated that the effects of this oligodeoxynucleotide are specific, as measured by a number of parameters. Scrambled and sense analogues of LR-3001 had no effect on c-Myb protein expression or on neuroblastoma growth in cell culture. LR-3001 inhibits c-Myb protein expression but does not affect the expression of c-Myc, a related transcription factor with a similar half-life *(46)*. These findings suggest that the inhibitory effects of LR-3001 on c-Myb expression and its biologic consequences occur through an antisense mechanism.

Our results illustrate that encapsulation of asODN in GD_2-targeted liposomes can protect nontargeted cells from a potentially toxic molecule while simultaneously enhancing the molecule's toxicity toward a targeted cell population. One cannot, however, expect that even a carefully targeted liposome will end up only at the intended tumor site; a portion of the dose could accumulate at other bodily locations with potentially toxic consequences. In future *in vivo* studies, the toxicity of aGD2-CCL-myb-as at nontargeted sites deserves attention.

The great advantages of liposome-encapsulated cytotoxic agents over free molecules have been unquestionably demonstrated in several animal models of cancer *(47–50)*, mainly against small primary and micrometastatic solid tumors *(36,51)*. Thus, the use of myb-as, encapsulated into GD_2-targeted liposomes, may hold promise for the treatment of neuroblastoma in the minimal residual disease setting. Moreover, the great selectivity of aGD2-CCL-myb-as for GD_2-positive cells suggests exploring using it to purge contaminating neuroblastoma cells from bone marrow before reinfusion into patients undergoing autologous bone marrow transplantation *(52)*, possibly in combination with $CD34^+$ cell selection.

Finally, the fact that many melanoma specimens have altered expression of c-Myb, coupled with the previously demonstrated inhibition of melanoma growth by c-myb asODNs, suggests that there may be a potential role for c-myb antisense therapy in the treatment of this tumor *(53)*. Thus, immunotargeting of liposomes to neuroectoderm-derived tumor cells may provide an effective strategy for tumor site-selective delivery of drugs with broad therapeutic applications.

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