Immune Cell–Mediated Antitumor Activities of GD\textsubscript{2}-Targeted Liposomal c-myb Antisense Oligonucleotides Containing CpG Motifs

Chiara Brignole, Fabio Pastorino, Danilo Marimpietri, Gabriella Pagnan, Angela Pistorio, Theresa M. Allen, Vito Pistoia, Mirco Ponzoni

**Background:** Expression of the c-myb proto-oncogene in neuroblastoma, the most common extracranial solid tumor of infancy, is linked with cell proliferation and differentiation. Neuroblastoma can be selectively targeted via monoclonal antibodies against the disialoganglioside (GD\textsubscript{2}) tumor-associated antigen. Liposomes coated with anti-GD\textsubscript{2} antibodies (targeted liposomes) and entrapping a c-myb antisense oligonucleotide have antitumor activity. Because antisense oligonucleotides containing CpG motifs can stimulate immune responses, we evaluated the effect of CpG-containing c-myb antisense oligonucleotides encapsulated within targeted liposomes. Methods: Antisense (myb-as) and scrambled (myb-scr) control oligonucleotides with CpG motifs were encapsulated within GD\textsubscript{2}-targeted and nontargeted liposomes. Two murine (nude and SCID-bg) xenograft models of neuroblastoma were established. Mice (groups of 10) were injected intravenously with various oligonucleotide and liposome formulations, and life span, long-term survival, immune cell activation, and cytokine release were measured over time. Results: Tumor-bearing mice injected with targeted liposome-CpG-myb-as or targeted liposome-CpG-myb-scr lived longer than mice in any other group, although long-term survival (i.e., more than 120 days) was obtained only in mice injected with targeted liposome-CpG-myb-as. Splenocytes isolated from mice injected with targeted liposome-CpG-myb-as contained activated macrophages, B cells, and natural killer (NK) cells, but only activated NK cells were associated with antitumor cytotoxic activity. In vivo immune cell activation was accompanied by the time-dependent increases in plasma levels of the cytokines interleukin 12 (IL-12; maximum level reached by 2 hours) and interferon gamma (IFN-\gamma; maximum level reached by 18 hours) and was observed on the oligonucleotide CpG motif. Ablation of macrophages or NK cells resulted in a loss of in vivo antitumor activity. Conclusion: Immune cell activation, involving the time-dependent activation of macrophages and NK cells, contributes to the antitumor activity of targeted liposome-CpG-myb-as against neuroblastoma and could improve the effectiveness of antitumor targeted liposomes. [J Natl Cancer Inst 2004;96:1171–80]

Neuroblastoma, a neoplasm derived from the sympathetic nervous system, is the most common extracranial solid tumor of childhood (1,2). Despite advances in treatment strategies, long-term survival for patients with advanced-stage disease is poor (3,4).

The identification of cancer-associated molecules and/or genes holds promise for the development of novel therapeutic strategies that selectively target tumor cells. Among these strategies, antisense oligonucleotides can be used to decrease tumor-associated gene expression, resulting in specific anticancer effects and minimal damage to normal tissues. In addition to their direct effects on gene expression, antisense oligonucleotides can have antitumor effects via indirect, immune-stimulatory mechanisms, if they are designed to contain unmethylated cytosine–guanine (CpG) motifs. These motifs can induce cytokine secretion and stimulate innate immune responses (5–7). These features make CpG-containing antisense oligonucleotides potentially useful as immune adjuvants.

Although antisense oligonucleotides, with or without CpG motifs, show promise as therapeutic agents for direct or indirect tumor cell killing, there are a number of problems associated with their clinical use. First, antisense oligonucleotides are sensitive to cleavage by ubiquitous nuclease enzymes in vivo—a problem that can be circumvented by the synthesis of chemically modified antisense oligonucleotides. However, structural modifications to antisense oligonucleotides, e.g., the addition of phosphoroethioate groups to increase their resistance to nuclease degradation, may compromise their activities or lead to their binding to nonspecific DNA sequences (9,10). Second, delivery of free antisense oligonucleotides is often limited by their low blood stability, cellular uptake, and specificity. These problems can be reduced by incorporating the antisense oligonucleotides into lipid vesicles or liposomes. Indeed, liposome encapsulation of antisense oligonucleotides specific for the raf oncogene led to increased levels of antisense oligonucleotides in plasma and tissues and improved antitumor effects relative to the free oligonucleotides (11). Liposomes have been found to facilitate the delivery of antisense oligonucleotides targeting the Bcl-2, MDR1, or c-myc genes in vivo (12–15).

Sterically stabilized (i.e., containing polyethylene glycol [PEG] grafted onto the liposome exterior) immunoliposomes that have cell surface–directed antibodies coupled to the PEG terminus can selectively deliver drugs and diagnostic agents to target cells [reviewed in (16)]. Principles similar to those used in the therapeutic applications of sterically stabilized immunoliposomal drugs can be applied to selectively deliver antisense oligonucleotides to target cells in vivo.

**Affiliations of authors:** Laboratory of Oncology (CB, FP, DM, GP, VP, MP), and Epidemiology and Biostatistics Service (AP), G. Gaslini Children’s Hospital, Genoa, Italy; Department of Pharmacology, University of Alberta, Edmonton, AB, Canada (TMA).

**Correspondence to:** Mirco Ponzoni, PhD, Differentiation Therapy Unit, Laboratory of Oncology, G. Gaslini Children’s Hospital, Largo G. Gaslini 5, 16147 Genoa, Italy (e-mail: mirco.ponzoni@ospedale-gaslini.ge.it).

**See “Notes” following “References.”**

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The disialoganglioside GD$_2$ is a tumor-associated antigen that has appeal for use in targeting applications involving liposome-encapsulated drugs or liposome-encapsulated antisense oligonucleotides because it is widely expressed on cancer cells of neuronal origin. Moreover, ligand or antibody binding to GD$_2$ leads to the internalization of the receptor. In normal tissues, GD$_2$ is expressed at low levels and only in the cerebellum and peripheral nerves.

The c-myb proto-oncogene is the best characterized member of the myb family of transcription factor genes. Expression of c-myb has been detected in several solid tumors of different origin, including neuroblastoma, and is linked to cell proliferation and/or differentiation (17,18). Recently, we demonstrated that c-myb antisense oligonucleotide entrapped in GD$_2$-targeted liposomes inhibited neuroblastoma growth in vitro by specifically reducing c-myb protein expression (19,20). Here, we investigated the direct (i.e., anti-c-myb-mediated) and the indirect (i.e., CpG-mediated) antitumor effects of GD$_2$-targeted c-myb-antisense–containing liposomes in xenograft mouse models of human neuroblastoma that mimic the natural clinical course of this disease (21,22).

**MATERIALS AND METHODS**

**Chemicals and Monoclonal Antibodies**

Hydrogenated soy phosphatidylcholine, cholesterol, 1,2-distearoyl-3-phosphatidylethanolamine-N-polyethylene glycol-2000 (DSPE-PEG), and 1,2-dioleoyl-3-trimethylammonium propane were purchased from Avanti Polar Lipids (Alabaster, AL). A derivative of DSPE-PEG with a maleimide group (N,N-diethyl-N-[2-[2-(2-maleimidyl)ethoxy]]aminomethyl]glycol-2000 (DSPE-PEG), and 1,2-dioleoyl-3-trimethylammonium propane were purchased from Avanti Polar Lipids (Alabaster, AL). A derivative of DSPE-PEG with a maleimide group at the distal terminus of the polyethylene glycol chain was synthesized by Shearwater Polymers (Huntsville, AL). [3 H]cholesteryl hexadecyl ether was purchased from DuPont NEN (Boston, MA). The hybridoma cell line 14.G2a, which secretes a murine immunoglobulin G2a (IgG2a) specific for the GD$_2$ antigen, was a generous gift of R. A. Reisfeld (The Scripps Institute, La Jolla, CA). All of the biochemical- and molecular biology–grade reagents were obtained from Sigma Chemical (St. Louis, MO).

**Oligonucleotides**

We used a 24-base phosphorothioate c-myb antisense oligonucleotide that contains CpG sequences, which is referred to hereafter as CpG-myb-as. The antisense sequence is complementary to codons 2–9 of the human c-myb mRNA and has the following sequence: 5'-TATGCTGTCCCAGCTTTCCCG GC-3'. We also used a scrambled sequence, 5'-TTTGCACT CCTGGGGTGCTGGGCC-3', which contains a CpG motif (CpG-myb-scr), as a control oligonucleotide. Both CpG-myb-as and CpG-myb-scr were provided by Inex Pharmaceuticals (Burnaby, BC, Canada). A second scrambled sequence, 5'-TGTACCGTTGGCGCTGTCGACC-3', lacking the CpG motifs (myb-scr) was purchased from TIB Molbiol (Genoa, Italy) and was used as an additional control sequence.

**Liposome Preparation**

Oligonucleotides were encapsulated within coated cationic liposomes by using the method of Stuart et al. (13), with our slight modification (19). Fab' fragments of the anti-GD$_2$ monoclonal antibody and of an isotype-matched control monoclonal antibody (isotype-matched Fab) were coupled to the terminus of maleimide-containing DSPE-PEG on the external surface of the liposome (targeted liposome) by a procedure that we recently described (21,23).

**In Vivo Therapeutic Studies**

HTLA-230 cells were a gift from E. Bogenmann (Children’s Hospital Los Angeles, Los Angeles, CA). HTLA-230 cells were isolated from a patient with metastatic stage IV neuroblastoma. These cells exhibit high levels of N-myc gene amplification and of GD$_2$ expression and grow in athymic nude mice (21,24). This cell line was grown in complete medium, which consisted of Dulbecco’s Modified Eagle Medium, supplemented with 10% fetal bovine serum (Sigma), 50 IU/mL sodium penicillin G, 50 μg/mL streptomycin sulphate, and 2 mM L-glutamine, as previously described (19). Five-week-old female athymic (nude) mice and SCID-bg (C.B-17/IcrHsd-scid-bg) mice were purchased from Harlan Laboratories (Harlan Italy-S. Pietro al Natisone, Udine, Italy) and housed under specific pathogen-free conditions. Mice received intravenous injections via the tail vein of 3.5 × 10$^6$ HTLA-230 tumor cells in HEPES-buffered saline (21). After 4 hours, mice were randomly assigned to groups of 10, and each mouse received the first intravenous oligonucleotide or control treatment. Treatments were administered once a day, 4 days per week (a single course), with a 3-day interval between courses, for a total of 2 weeks. Each treatment consisted of 50 μg of oligonucleotide per mouse. Oligonucleotides were administered in various formulations: CpG-myb-as, liposome-CpG-myb-as, liposome-CpG-myb-scr, liposome-myb-scr, targeted liposome-CpG-myb-as, and targeted liposome-CpG-myb-scr. Mice assigned to the control group received HEPES-buffered saline, CpG-myb-as plus anti-GD$_2$ Fab' fragments, empty targeted liposome, and isotype-matched Fab’–targeted liposome-CpG-myb-scr.

Therapeutic studies were also performed in mice depleted of macrophages, which was accomplished via administration of liposomes containing dichloromethylene bisphosphonate (clodronate; Sigma) (25), as previously described (26). To deplete the macrophages, mice were given intravenous injections of clodronate-containing liposomes 2 days before receiving the HTLA-230 tumor cells and beginning treatment (25,26).

The body weight and general physical status of the mice were recorded daily, and mice were killed when signs of poor health became evident. All experiments involving animals have been reviewed and approved by the licensing and ethical committee of the National Cancer Research Institute and by the Italian Ministry of Health. All in vivo experiments were repeated at least twice, with each set of experiments yielding similar results.

**Studies on Immune Stimulation**

Stimulation of immunocompetent cells was investigated both in vitro and ex vivo. Briefly, for the in vitro studies, spleens from nude mice that had received neither tumor cells nor oligonucleotide treatments were collected and reduced mechanically into single-cell suspensions. Splenocytes were then cultured in complete medium in the presence of CpG-myb-as or targeted liposome-CpG-myb-as at an oligonucleo-
tide concentration of 20 μg/mL. Splenocytes harvested at various time points after incubation with the oligonucleotides were processed for flow cytometry to determine the phenotype of the cells. To detect specific cell populations, we used anti-CD11b (M1/70) antibodies for monocytes, anti-CD19 (1D3) antibodies for B lymphocytes, anti-NK 1.1 (PK136) antibodies for NK cells, anti-GR-1 (RB6-8C5) antibodies for granulocytes, and anti-CD11c (HL3) antibodies for myeloid dendritic cells. All antibodies were conjugated with phycoerythrin and were purchased from BD Biosciences (San Diego, CA). Cell activation was assessed by double-staining B lymphocytes, monocytes, NK cells, granulocytes, and myeloid dendritic cells with a fluorescein–isothiocyanate-conjugated anti-CD69 monoclonal antibody (BD Biosciences), which detects an early activation antigen, in conjunction with the cell-specific monoclonal antibodies. In all experiments, splenocytes were incubated first with FcBlock (BD Biosciences) to block Fc receptors and subsequently with test monoclonal antibodies or isotype-matched monoclonal antibodies of irrelevant specificity, both conjugated with the same fluorochromes. Cells were analyzed on a FACScan flow cytometer using Cell Quest software (Becton Dickinson, Mountain View, CA). A total of 1 × 10^6 events were collected for each sample.

The threshold for positive expression was based on the maximum staining obtained with the isotype-matched monoclonal antibody (control antibodies) used at the same concentration as the test antibody. If fewer than 1% of the cells stained positive with control antibodies, then the cell population was considered negative for the marker. If cells labeled with test antibody were brighter than those stained with isotype-matched control antibody, then the cell population was considered positive for the marker.

For the ex vivo studies, 1 day after receiving the tumor cell inoculation, nude mice received a single intravenous injection of either liposome-CpG-myb-as, targeted liposome-CpG-myb-as, or CpG-myb-as. Each injection delivered 80 μg of oligonucleotide per mouse. HEPES-buffered saline, in a volume equivalent to that used for the injection of the oligonucleotide, was injected into control mice. At different times after injection, mice (three per group) were given injections of HEPES-buffered saline. Mice were killed at different times after injection, and splenocytes and peritoneal macrophages were collected from each mouse and pooled within the same treatment group.

The peritoneal macrophages (approximately 1 × 10^5 cells/mL to 2 × 10^5 cells/mL) were cultured overnight in complete medium. Splenocytes were cultured overnight in Dulbecco’s Modified Eagle Medium in the absence or presence of recombinant IL-2 (1000 IU/mL; Proleukin Chiron, Emeryville, CA). The HTLA-230 neuroblastoma target cells were labeled for 2 hours with Na_2^{51}CrO_4 (100 μCi/1 × 10^6 cells) (ICN Biomedicals, Asse, Belgium). Target cells were then incubated for 6 hours at 37 °C with splenocytes or peritoneal macrophages as effector cells. The effector:target cell ratio was 100:1 for splenocytes and 10:1 for macrophages. Supernatants were collected, and the amount of radioactivity released into the supernatants was counted with a gamma counter (Cobra 5002; Canberra Packard, Meriden, CT). The percentage of specific target cell lysis was calculated using the formula [(Exp – S)/(M – S)] × 100%, in which Exp is the observed released ^51Cr value, S is the spontaneously released ^51Cr value, and M is the maximum released ^51Cr value.

**Statistical Methods**

Results are expressed as mean ± 95% confidence intervals (CIs). All in vitro data are from at least three independent experiments. All in vivo experiments were performed at least twice. Survival curves were constructed by using the Kaplan–Meier method, and the log-rank test was used to compare the curves. Bonferroni’s adjustment was applied as a correction for multiple comparisons to explore post hoc differences between pairs of groups. A P value of less than .05 was considered statistically significant. Hazard ratios of death were calculated with 95% confidence intervals. Statistical analyses were performed using Statistica (StatSoft, Tulsa, OK) and Stata, release 7.0 software (Stata, College Station, TX).

**RESULTS**

**In Vivo Effects of Targeted Liposomal myb Antisense Oligonucleotides**

To evaluate the antitumor efficacy of different liposome formulations of myb antisense oligonucleotides in a murine model of human neuroblastoma, initial experiments were performed in a biologically and clinically relevant xenograft model already established in our laboratory (21,22). Mice injected with the targeted liposome-CpG-myb-as lived longer (mean = 4 months) than control mice injected with buffer (mean = 1.5 months) (hazard ratio [HR] of death = 0.31, 95% CI = 0.12 to 0.80; P < .001) (Fig. 1) or than control mice injected with the targeted liposome-CpG-myb-scr (HR = 0.55, 95% CI = 0.22 to 1.39; P = .004). However, a statistically significant, although smaller, increase in life span was also seen in mice injected with the targeted liposome-CpG-myb-scr compared with mice in-
In Vitro and In Vivo Administration of Targeted Liposome-CpG-myb-as and Immune Activation

To assess the potential involvement of CpG motifs in the induction of an innate immune response by our liposome formulations, we evaluated the expression of the CD69 antigen, an early marker of cellular activation, both \textit{ex vivo} and \textit{in vitro}, on various immunologically relevant cell types (28,29). The \textit{ex vivo} studies were performed on splenocytes and peripheral blood cells collected from tumor-bearing nude mice that had received targeted or non-targeted liposome-CpG-myb-as or CpG-myb-as. Flow cytometric analysis of splenocytes demonstrated that injection of targeted liposome-CpG-myb-as resulted in a rapid (within 2 hours) increase of CD69 antigen expression on monocytes, B cells, and NK cells (Fig. 2, A). Liposome-CpG-myb-as did not increase CD69 expression on any splenocyte population (data not shown). However, injection of liposome-CpG-myb-as activated monocytes from peripheral blood, although to a lesser extent and on a different time scale than injection of targeted liposome-CpG-myb-as (Fig. 2, B). Compared with splenocytes or blood cells from mice injected with HEPES buffer, splenocytes or blood cells from mice injected with CpG-myb-as or CpG-myb-as plus the Fab' fragment of anti-GD_{2} antibodies were only marginally activated, if at all (data not shown).

We next evaluated whether the \textit{in vitro} stimulation of spleen cells could mimic the \textit{ex vivo} stimulation. Splenocytes were collected from tumor-free nude mice, cultured in a complete medium, and treated \textit{in vitro} with CpG-myb-as, liposome-CpG-myb-as, targeted liposome-CpG-myb-as, or NS-targeted liposome-CpG-myb-scr at an oligonucleotide concentration of 20 \mu{g}/mL. After splenocytes were treated with the targeted liposome-CpG-myb-as, CD69 expression increased in a time-dependent manner for each of the different cell populations, i.e., B cells, monocytes, NK cells, granulocytes, and dendritic cells. Maximum CD69 expression was detected after 18 hours (Fig. 3). CD69 expression was only marginally increased in splenocytes treated with liposome-CpG-myb-as or CpG-myb-as (data not shown). Thus, intravenous injection of targeted liposome-CpG-myb-as induced a marked immune response in neuroblastoma-bearing mice.

Intravenous Injection of Targeted Liposome-myb-as and Cytokine Secretion

Because cytokine secretion is another marker of immune-cell activation, we next assessed the effect of intravenous injections of liposome-CpG-myb-as on cytokine release over time in tumor-bearing mice. Levels of IL-12, IFN-\gamma, IL-18, and TNF-\alpha cytokines were higher in mice injected with targeted liposome-CpG-myb-as than in mice injected with liposome-CpG-myb-as (Fig. 4). Cytokine levels in mice injected with CpG-myb-as were comparable to cytokine levels in control mice injected with HEPES buffer. After mice were injected with targeted liposome-CpG-myb-as, the maximum IL-12 level was achieved within 2 hours and was approximately eightfold (4425 pg/mL, 95\% CI = 4312 to 4538 pg/mL) higher than the level in control mice (570 pg/mL, 95\% CI = 485 to 655 pg/mL) injected with buffer or in mice injected with CpG-myb-as. The levels of IL-12 in mice injected with non-targeted liposomes were lower than the levels in mice injected with targeted liposomes, but the kinetics was similar, reaching a peak within 2 hours and declining rapidly thereafter (Fig. 4, A).

\begin{figure}
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\includegraphics[width=\textwidth]{figure1.png}
\caption{Survival of neuroblastoma-bearing nude mice after injection with liposomes containing oligonucleotides. Nude mice were injected intravenously with 3.5 \times 10^{6} HTLA-230 neuroblastoma cells and randomly assigned to groups of 10 mice. After 4 hours, each mouse received 50 \mu{g} of oligonucleotide, either free or encapsulated in either targeted or non-targeted liposomes. Control mice received HEPES-buffered saline. Survival of mice was monitored daily. For targeted liposome-CpG-myb-as versus control, hazard ratio of death = 0.31 [95\% confidence interval = 0.12 to 0.80; \textit{P} < 0.001].}
\end{figure}

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\caption{Maximum CD69 expression was detected after 18 hours (Fig. 3). CD69 expression was only marginally increased in splenocytes treated with liposome-CpG-myb-as or CpG-myb-as (data not shown). Thus, intravenous injection of targeted liposome-CpG-myb-as induced a marked immune response in neuroblastoma-bearing mice.

Intra- and \textit{In Vivo Administration of Targeted Liposome-CpG-myb-as and Immune Activation

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\begin{figure}
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\includegraphics[width=\textwidth]{figure3.png}
\caption{Survival of neuroblastoma-bearing nude mice after injection with liposomes containing oligonucleotides. Nude mice were injected intravenously with 3.5 \times 10^{6} HTLA-230 neuroblastoma cells and randomly assigned to groups of 10 mice. After 4 hours, each mouse received 50 \mu{g} of oligonucleotide, either free or encapsulated in either targeted or non-targeted liposomes. Control mice received HEPES-buffered saline. Survival of mice was monitored daily. For targeted liposome-CpG-myb-as versus control, hazard ratio of death = 0.31 [95\% confidence interval = 0.12 to 0.80; \textit{P} < 0.001].}
\end{figure}
Levels of IFN-γ in mice injected with targeted liposome-CpG-myb-as began to increase after 8 hours and were approximately sevenfold (256 pg/mL, 95% CI = 233 to 279 pg/mL) higher than levels in control mice (34 pg/mL, 95% CI = 31 to 37 pg/mL) after 18 hours (Fig. 4, B). The levels of IFN-γ were lower in mice injected with liposome-CpG-myb-as than in mice injected with targeted liposome-CpG-myb-as. These results are consistent with the hypothesis that formulations containing CpG sequences initially stimulate production of IL-12 by macrophages, which may, in turn, activate NK cells to secrete IFN-γ. Mice injected with targeted liposome-CpG-myb-as sustained high levels of IL-1β and TNF-α compared with mice injected with HEPES-buffer or with CpG-myb-as. Mice injected with liposome-CpG-myb-as had lower levels of IL-1β and TNF-α than mice injected with targeted liposome-CpG-myb-as (Fig. 4, C and D). Thus, intravenous injection of targeted liposome-CpG-myb-as elicited a strong systemic secretion of immunostimulatory cytokines in neuroblastoma tumor-bearing mice.

**Intravenous Injection of Targeted Liposome-CpG-myb-as and NK Cell Cytotoxic Activity**

Because oligonucleotide-containing liposomes appear to work, in part, by stimulating the innate immune system, we designed experiments to identify the immunologic effectors of tumor cell killing. In an *in vitro* ⁵¹Cr-release assay, peritoneal macrophages collected at different times after tumor-bearing nude mice were injected with either targeted or non-targeted liposome-CpG-myb-as did not lyse the HTLA-230 neuroblastoma target cell line (data not shown). By contrast, peritoneal macrophages isolated from tumor-bearing mice and incubated with the anti-GD₂ monoclonal antibody mediated antibody-dependent cellular cytotoxicity activity against the target cells (data not shown), as expected (27).

We repeated the *in vitro* ⁵¹Cr-release assay with splenocytes from tumor-bearing mice injected with either targeted or non-targeted liposome-CpG-myb-as as effector cells. Splenocytes from mice injected with targeted liposome-CpG-myb-as specifically lysed the neuroblastoma HTLA-230 target cells in a time-dependent manner (Fig. 5). Cytotoxic activity was increased when the splenocytes were incubated with IL-2 to promote NK cell activation (30,31). Splenocytes from mice injected with targeted liposome-CpG-myb-as were more effective than splenocytes from mice injected with liposome-CpG-myb-as at...
mediating the lysis of the neuroblastoma HTLA-230 target cells (Fig. 5), confirming the in vivo results (Fig. 1). Thus, intravenous administration of targeted liposome-CpG-myb-as elicited a strong functional activation of NK cells. Because the experiments were done with cells from nude mice, which lack T cells, the functional activation of the NK cells is T-cell-independent.

**Effects of Depletion of Macrophages or NK Cells on Antitumor Efficacy of Oligonucleotide-Containing Liposomes**

To gain additional insights into the in vivo relevance of our observations, therapeutic experiments were conducted in mice chemically depleted of macrophages with liposomes containing clodronate (26). Macrophage depletion led to a complete loss of antitumor efficacy in neuroblastoma-bearing mice injected with liposome-CpG-myb-as (Fig. 6, A). As a further control, we also used a liposome formulation in which the scrambled oligonucleotide sequence lacked the CpG motifs. Survival curves showed a complete loss of antitumor effects in macrophage-depleted mice treated with liposome-myb-scr (data not shown).

With the final aim of distinguishing the specific antisense activity of our oligonucleotide-containing liposomes from the nonspecific CpG-mediated immune responses, we conducted therapeutic experiments using SCID-bg mice, which lack NK cells. These mice were chosen because NK cells were the only effector cells identified in the in vitro tumor cytotoxicity assay (Fig. 5). Neuroblastoma-bearing SCID-bg mice injected with targeted liposome-CpG-myb-as lived statistically significantly longer than control mice (HR of death = 0.63, 95% CI = 0.25 to 1.59; \( P = .002 \)) or mice injected with targeted liposome-CpG-myb-scr (Fig. 6, B), although not as long as nude mice injected with targeted liposome-CpG-myb-as. In this experiment, 120 days after receiving the tumor cells, 20% of the mice injected with targeted liposome-CpG-myb-as were still alive compared with none of the mice in the other treatment groups. These data demonstrate that the therapeutic results with targeted liposome-CpG-myb-as obtained in our neuroblastoma tumor nude mouse model were likely attributable to both the decreased expression of the c-myb proto-oncogene (19) and to the nonspecific stimulation of the host immune system mediated by the CpG motifs.

**DISCUSSION**

This study demonstrated that the systemic administration of CpG-containing antisense oligonucleotides, directed against the c-myb oncogene, to mice bearing human neuroblastoma induced antitumor effects leading to long-term survival only when the antisense oligonucleotides were encapsulated in coated cationic liposomes specifically targeted to the neuroblastoma surface antigen GD3 that internalizes after binding its ligand. Increased life span, but not long-term survival, was also obtained in mice injected with targeted liposomes of CpG-containing scrambled c-myb oligonucleotides and, to a lesser degree, in mice injected with non-targeted liposomes of CpG-containing antisense or scrambled oligonucleotides. These findings support a dual mechanism of action of the antisense oligonucleotide liposomes: direct inhibition of cell growth (resulting from decreased c-myb...
proto-oncogene expression) and indirect CpG-dependent immune stimulation (resulting from NK cell-mediated lysis of tumor cells).

Therapeutic effects of antisense oligonucleotides were thought to arise primarily from molecular complementarity (10,32), which leads to inhibition of gene expression in a sequence specific manner. Recently, activities unrelated to antisense effects, such as immune stimulation, have been reported (7,33). This immunostimulatory activity is strongly dependent on the presence of unmethylated CpG motifs (5). In contrast to bacterial DNA, which contains a large number of unmethylated CpG motifs, mammalian DNA contains mostly methylated CpG motifs (34). Immune effector cells seem to have evolved pattern recognition receptors that, by binding the CpG sequences, evoke protective immune responses (35). Specific recognition of CpG motifs occurs via binding to the TLR9 receptor, which belongs to the superfamily of Toll-like receptors (35). It has recently been demonstrated that the intravenous injection of lipid–DNA complexes into tumor-bearing mice induces antitumor activity (28,36) and that the encapsulation of CpG-containing oligonucleotides in lipicid particles greatly increases their immunostimulatory effects (37). Immune stimulation mediated by CpG sequences may therefore have a role in anticancer therapy.

Targeted lipid-based carriers, such as coated cationic liposomes, may solve many of the problems associated with the in vivo use of antisense oligonucleotides (13,14,19,38,39). Our previous studies (19,21) showed that the encapsulation of either myb-as or myc-as oligonucleotides within GD2-targeted coated cationic liposomes improved their efficacy against neuroectodermived tumor cells. In fact, treatment of neuroblastoma cells in vitro, and of melanoma cells in vitro and in vivo, with GD2-targeted coated cationic liposomes containing myb-as or myc-as oligonucleotides, respectively, led to an inhibition of tumor cell proliferation via the specific loss of the expression of proliferative proteins (19,21,40).

In this study, we investigated the antitumor effects of GD2-targeted liposomes containing CpG-myb-as oligonucleotides in a biologically and clinically relevant experimental mouse model of human neuroblastoma (21,22). We found that the systemic administration of GD2-targeted liposomes containing CpG-myb-as to neuroblastoma-bearing mice led to long-term survival. Some of the observed effects were the result of CpG-mediated immune stimulation, because we demonstrated that the antitumor activity was associated with both targeted and non-targeted liposomes containing oligonucleotides with CpG mo-
tifs, in agreement with previous findings (41). Further evidence derives from the observation that no increase in life span was found for neuroblastoma-bearing mice treated with a non-targeted liposome containing oligonucleotides without CpG motifs.

We speculate that long-term survival in neuroblastoma-bearing mice was related to several factors working in cooperation: long circulation times of the liposomes (required for localization to the neuroblastoma), selective targeting of the liposomes to the neuroblastoma tumor cells via GD2 (required to reduce nonspecific uptake of the liposomes), and CpG-mediated immune stimulation enhancing the specific antisense activity of myb-as oligonucleotides (required for loss of c-myb expression). Thus, on the basis of our in vivo results and previous studies (28,41), we hypothesize that macrophages initiate a cascade that ends with activated NK cells killing neuroblastoma cells. This hypothesis is supported by the observations that abrogation of macrophages by liposomes containing clodronate (26) abolished the antitumor activity associated with liposome-CpG-myb-as. The critical involvement of NK cells was demonstrated by the total loss of antitumor activity in experiments in which targeted liposomes entrapping a scrambled CpG-containing oligonucleotide were administered to neuroblastoma-bearing NK-cell–depleted SCID-bg mice. However, in the SCID-bg mouse model, treatment with targeted liposomes containing CpG-myb antisense oligonucleotide still resulted in long-term survival for 20% of the mice, suggesting that the specific inhibition of c-myb plays a pivotal, but partial, role in its antitumor efficacy. These experiments allowed us to separate the antitumor activity associated with the delivery of the CpG sequence to macrophages from that associated with the specific loss of c-myb expression.

The mechanisms underlying the immunogenicity of our liposome formulations were investigated both in vitro and ex vivo. Targeted and non-targeted liposomes increased CD69 expression on macrophages from peripheral blood. However, only the targeted liposomes rapidly induced activation of immune cells from splenocytes. Compared with targeted liposomes, non-targeted liposomes have longer circulation times and lower splenic uptake, which may explain the difference in effects between the two formulations (13). Targeted liposomes showed some accumulation in the spleen (15,20), which may give rise to rapid activation of splenocytes. The intravenous injection of oligonucleotide-containing liposomes led to the release of a number of immunostimulatory cytokines into plasma. The kinetics of secretion are consistent with the idea that macrophages are the first mediators of the immune response with an early release of IL-12 (peak at 2 hours), which triggers, in turn, the release of IFN-γ by NK cells (peak at 18 hours). This finding is in agreement with previous results (42). However, in our murine model, the contribution of other cell types to the release of IFN-γ and IL-12 cannot be ruled out. Indeed, IL-12–activated macrophages and B cells are induced to secrete IFN-γ (43,44). The increased, sustained levels of TNF-α could cooperate with IL-12 to induce lytic activity of NK cells, confirming previous results, which demonstrated that both IL-12 and TNF-α are required for the induction of NK cell lytic activity by CpG-containing oligonucleotides (42,45,46). Likewise, IL-1β can be involved early in the generation of lymphokine-activated killer cells (47). Finally, the hypothesis that NK cells play a pivotal role in killing neuroblastoma cells was supported by the observation that splenocytes collected from mice injected with our oligonucleotide-containing liposomes were able to lyse neuroblastoma cells.

In conclusion, the encapsulation of antisense oligonucleotides in targeted liposomes substantially increased their therapeutic activity against neuroblastoma cells by a direct mechanism involving the loss of expression of the c-myb proto-oncogene and by an indirect mechanism involving antisense oligonucleotides that contain immune-stimulatory CpG motifs. Because many melanomas express high levels of GD2, have altered expression of c-myb, and are growth inhibited by c-myb antisense oligonucleotides, there may be a potential role for targeted c-myb antisense therapy in the treatment of melanoma. However, as a note of caution, targeted liposomes appear to lose their efficacy when they are used to treat established advanced solid tumors (48,49), likely because the binding site barrier restricts penetration of liposomes into the tumor (50,51). We contend that targeted liposome-CpG-myb-as deserves clinical evaluation as an adjuvant therapy for advanced-stage neuroblastoma disease or for disease resulting from incomplete surgery or early micrometastatic lesions.

A final comment addresses the potential relevance of the experimental therapeutic approach described herein in a tumor-bearing host with an intact immune system. It may be envisaged that the availability of a large amount of IFN-γ, consequent to CpG stimulation of the innate immune system, has a strong impact on the tumor cells themselves. In human neuroblastoma, IFN-γ can sensitize tumor cells to apoptosis (52) or induce their differentiation, especially in conjunction with TNF-α (53); IFN-γ alternatively could increase expression of the class I major histocompatibility complex antigen on the cell surface (54), thus allowing killing of neuroblastoma cells by tumor-specific cytotoxic T lymphocytes. In this scenario, it is apparent that administration of CpG-containing oligonucleotides to a tumor-bearing immunocompetent host would give rise to multiple opportunities for the host immune system to destroy cancer cells. The contribution of each mechanism to tumor cell killing in this setting remains to be established.

References


NOTES

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