Antisense c-myc and Immunostimulatory Oligonucleotide Inhibition of Tumorigenesis in a Murine B-Cell Lymphoma Transplant Model

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Background: Because the development of drug-resistant cells can lead to relapses in patients with lymphoma treated with chemotherapy, new approaches are needed for effective disease management, such as those targeting the c-MYC proto-oncogene with antisense oligonucleotides. Our goal was to investigate whether antisense c-myc oligonucleotides could prevent tumorigenesis in a B-cell lymphoma model. Methods: Immunocompetent mice received subcutaneous injections of tumor cells from a transgenic mouse model of Burkitt’s lymphoma. For 7 consecutive days, beginning 1 day after tumor cell transplantation, the mice were given either a DNA phosphorothioate oligonucleotide complementary to c-myc codons 1–5 (myc6) or other c-myc-related oligonucleotides at a dose of 0.76 mg per day subcutaneously. Myc protein expression, normalized to β-actin expression, was measured by western blotting of tumor and splenic proteins. To determine whether tumor inhibition by myc6 could be a result of B-cell activation, we compared the activity of myc6 with that of an immunostimulatory oligonucleotide, mcg. Results: In comparison with control treatments (saline vehicle, scrambled-sequence oligonucleotide, or double-mismatch oligonucleotide), treatment with myc6 delayed tumor onset by 3 days, decreased total tumor mass at sacrifice (i.e., 17 days after tumor cell transplantation) by 40% ± 16% (mean ± standard error), and decreased the splenic Myc-to-actin ratio. Inhibition of tumors by myc6 and mcg (both of which share a dACGTT motif) was comparable. Administration of an oligonucleotide sequence complementary to c-myc codons 384–388 (myc55) delayed tumor onset by 5–6 days, decreased total tumor mass at sacrifice by 65% ± 6%, and reduced the splenic Myc-to-actin ratio to below that produced by myc6. A 14-day treatment regimen of myc55 alternating with mcg completely inhibited tumor formation during the therapeutic schedule. Conclusions: A combined oligonucleotide regimen, based on antisense c-MYC and immunostimulatory oligonucleotides, should be investigated to increase the number and duration of complete remissions obtained after standard chemotherapy for B-cell lymphoma.

Activation of the c-MYC proto-oncogene, whose expression is required for cell proliferation, has been implicated in a broad spectrum of cancers, which generally result from a multistep process of oncogene activation and suppressor gene inactivation (1). For example, Burkitt’s lymphoma is a frequent hematologic cancer of childhood in which virtually all patients display a translocation involving chromosomes 8 and 14 that places c-MYC from chromosome 8 under the transcriptional control of the immunoglobulin M heavy-chain enhancer (Eμ) from chromosome 14 (2).

The initial control of lymphoma currently relies on intensive chemotherapy using four to six agents, achieving complete remission in 60%–85% of the patients, depending on the treatment strategy (3). Analysis of the time course of treatment failure revealed that relapse is due to the development of resistant cells, rather than to the escape of sensitive cells. Therefore, new approaches are needed for effective management of disease.

Cloning and sequencing of pathogenic genes during the last decade have made possible a direct genetic approach to the treatment of disease by use of synthetic oligonucleotides or recombinant DNA vectors (4–6). Antisense oligonucleotides provide one new approach for adjuvant, or consolidation, therapy to prevent relapse. These oligonucleotides target specific genes implicated in tumorigenesis, thereby inhibiting cell proliferation (7). Clinical trials with antisense DNA (6) have demonstrated their safety and efficacy against several diseases, including follicular lymphoma (8). Consequently, complementary oligonucleotides have the potential to eliminate drug-resistant cells, with a subsequent decrease in the recurrence of lymphoma.

The c-MYC proto-oncogene is a frequent target of antisense DNA for the purpose of inhibiting cellular proliferation (9–17). In vitro studies using antisense c-MYC oligonucleotides targeted against various sites in human c-MYC messenger RNA (mRNA) have reported the inhibition of proliferation of HL-60 promyelocytic leukemia cells (10,12,14). The antiproliferative effect is associated with a 50%–93% reduction in the Myc protein level (10,12,14).

Translating this approach to a murine model in which animals carry a murine immunoglobulin enhancer/c-myc fusion transgene (Eμ-myc), a single administration of antisense c-myc DNA was observed to decrease Myc expression in splenic and peripheral lymphocytes (15). For the determination of the effects of continuous oligonucleotide therapy over an extended period, an antisense-c-myc DNA phosphorothioate (myc6 in Table 1) was administered prophylactically to Eμ-myc-carrying mice for 6 weeks, beginning at the time of weaning (16). This regimen prevented tumor onset in 75% of the antisense cohort.

The ability of the myc6 antisense c-myc oligonucleotide to
protect Eμ-myc-carrying transgenic mice from tumor development over a 6-month period suggested that this sequence might provide reliable consolidation therapy for lymphoma patients. In this context, lymphoma cells transplanted into an immunocompatible recipient would provide a better model for residual disease. Therefore, the parental (C57BL/6J × SJL/J)F1 hybrids were used as the recipients of tumor transplants; these mice contain all possible antigens present in the donors, thereby eliminating the potential for tumor rejection independent of the oligonucleotide treatments.

For the determination of the general sequence specificity of antisense c-myc oligonucleotides, a series of antisense c-myc oligonucleotides (Table 1) was analyzed for the ability to inhibit tumorigenesis. In addition to the antisense c-myc sequence controls, an oligonucleotide with known immunostimulatory activity (mcg, Table 1) (18) was also included. Remarkably, purified bacterial DNA is sufficient to induce B-cell proliferation, immunoglobulin production, and secretion of cytokines (19). On the other hand, vertebrate DNA does not normally stimulate an immune response because of 5-methylation of cytosines in dCg dimers (20). Indeed, methylation of cytosine in immunostimulatory oligonucleotides eliminates immunostimulation (18), whereas substitution of phosphorothioate linkages for phosphodiester amplifies the response (21).

Optimal immunostimulation requires the presence of the DNA motif dPuPuCGPyPy: oligonucleotides containing one or more copies of this motif can induce more than 95% of all spleen B cells to begin proliferating (18). Variations from the consensus motif dPuPuCGPyPy result in a significant loss of immune stimulation (22). The location of this motif in the oligonucleotide is also important. Oligonucleotides with dPuPuCGPyPy motifs located at or near the 5' or 3' termini show little or no immunostimulatory activity. In addition, multiple dPuPuCGPyPy motifs result in greater stimulatory capacity. The presence of a dPuPuCGPyPy-like motif in both myc6 and myc55 implied that additional controls were necessary to distinguish antisense effects from immunostimulatory effects.

We compared the abilities of myc6, myc55, and a known immunostimulatory sequence, mcg, to inhibit Myc expression and used our results to select oligonucleotides for testing in a 14-day trial of a potential antitumor regimen.

**Materials and Methods**

Tumor cell preparation and recipient mice. Tumors were aseptically removed from Eμ-myc-carrying transgenic male mice (23) after euthanasia by CO2 inhalation. The tumors were minced, homogenized in sterile phosphate-buffered saline (PBS), filtered, pelleted, and washed two times in PBS. The cell suspension was adjusted to 2 × 10^6 trypan blue-dye-excluding cells/mL, so that 0.1 mL contained 2 × 10^6 tumor cells per injection. The recipient mice were four to 6-week-old (C57BL/6J × SJL/J)F1 males (The Jackson Laboratory, Bar Harbor, ME). Animal facilities at Thomas Jefferson University are accredited by the American Association for Accreditation of Laboratory Animal Care, and all animal experiments followed the National Institutes of Health guidelines. Tumors were detected by palpation, and tumor volumes were estimated from two perpendicular measurements (length × width/2 ± standard error of the means [SEM]).

Tumor cell transplantation. Tumors (2 × 10^6) were injected subcutaneously on the rear leg of recipient mice. One day after the tumor cell transplantation, phosphorothioate oligodeoxynucleotides were administered subcutaneously on the opposite hind leg at a dose of 0.76 mg (150 nmol or 30 mg/kg) either every 2nd day for 14 days—equivalent to the continuous dose rate previously reported to prevent tumorigenesis (16)—or every day for 7 or 14 days. Controls included a scrambled myc6 sequence (scr6, Table 1) and the PBS vehicle.

**Oligodeoxynucleotide synthesis.** The phosphorothioate oligonucleotides in Table 1 were synthesized as previously described (16), then analyzed by denaturing gel electrophoresis, and visualized with Stains-All (Bio-Rad Laboratories, Richmond, CA). Greater than 90% of each sequence was full length. For additional quality control, random batches of oligonucleotide were tested by mass spectrometry (24) to show full-length oligomers or by nuclear magnetic resonance (25) to show complete sulfuration. Oligomers were sterilized by centrifugation at 14600 rpm for 10 minutes at room temperature through sterile 0.22-μm pore filter units (Nalgene Scientific, South Plainfield, NJ).

**Western blot analysis.** Forty micrograms (when available) of total protein lysate isolated from each sample (16) was subjected to electrophoresis, blotted, and probed with monoclonal mouse anti-Myc antibody C-33 (Santa Cruz Bio-technology, CA). Myc was then visualized by chemiluminescence (16). To normalize protein loadings, we stripped the membranes and reprobed them with a monoclonal anti-β-actin antibody (clone AC-74; Sigma Chemical Co., St. Louis, MO). Band intensities were measured by densitometry by use of a Bio-Imager (Bio-Image, Ann Arbor, MI). Densitometric values for Myc were normalized to the respective actin intensity for each sample ± SEM.

**Measurements of cytokines.** Retro-orbital blood samples were collected from mice under isoflurane anesthesia 1 hour after intraperitoneal injections of 0.76 mg of phosphorothioate oligodeoxynucleotide or PBS. Plasma was separated from blood cells by centrifugation at 230g for 10 minutes at room temperature and stored at −20 °C. Serial dilutions of the appropriate cytokine standards (Pharmingen, San Diego, CA), from 2000 pg/mL to 15 pg/mL, were used to determine murine plasma cytokine levels of interleukin 12 (IL-12), tumor necrosis factor-α (TNF-α) and interferon gamma (IFN γ) by enzyme-linked immunosorbent assays.

**Statistical analyses.** Differences in the median values of tumor volumes and western blot band densities among treatment groups were analyzed by Kruskal–Wallis one-way analysis of variance on ranks (26), with the use of SigmaStat (Jandel Scientific, San Rafael, CA). To isolate the group or groups that differed from the others, we used the Mann–Whitney rank sum test and the Student–Newman–Keuls, Dunn’s, and Dunnett’s pairwise multiple comparison procedures (26). All P values are two-sided.

**Results**

**Repression of Tumorigenesis by myc6 Antisense c-myc Oligonucleotide**

We first compared a regimen of 0.76 mg per day of myc6 given every other day for 14 days (i.e., seven doses) in our tumor transplant model with a comparable regimen of scr6 or of PBS alone in mice given injections of 2 × 10^6 tumor cells. Tumors were detected 9 days after tumor cell transplantation in all three treatment groups; the tumors were measured 10, 14, and 17 days after tumor cell transplantation, but they displayed indistinguishable growth rates.

The lack of tumor inhibition under this regimen led us to carry out a second trial at twice the average daily dose rate (i.e.,
by daily administration of 0.76 mg of either myc6 or scr6 or PBS vehicle) for 7 days. When the number of cells transplanted was increased to $2 \times 10^6$, the tumors grew more rapidly. Tumor development was assessed daily, and tumor volumes were estimated 11 and 14 days after tumor cell transplantation.

Both the scr6-treated and the PBS-treated control mice developed tumors 9 days after tumor cell transplantation, whereas the mice treated with myc6 did not begin to develop tumors until 12 days after transplantation (Fig. 1). The differences among the groups were greater than would be expected by chance ($P = .010$ at 11 days; $P = .001$ at 14 days). Multiple pairwise comparisons revealed significant differences between the myc6-treated group and both the PBS-treated and the scr6-treated groups (all $P < .05$) at 11 and 14 days, whereas the PBS-treated and the scr6-treated groups did not differ significantly from each other (all $P > .05$).

Therapy with myc6 resulted in slower tumor growth and a 40% ± 16% reduction in total tumor mass at sacrifice, 17 days after tumor cell transplantation. Treatment with myc6 reduced the Myc-to-actin ratios in spleens to 0.30 ± 0.13, whereas the Myc-to-actin ratios in the tumors were reduced to only 0.76 ± 0.28 (Fig. 2).

**Sequence Dependence of Tumor Inhibition**

A derivative of myc6 with two central mismatches, myc61 (Table 1), was designed to maintain the integrity of the dCAC-GTT and dGGGG motifs, while eliminating the potential for antisense activity. Tumor growth was not significantly inhibited by myc61 (Fig. 3). On the other hand, treatment with the immunostimulatory sequence mcg strongly inhibited tumorigenesis under the same conditions. The differences among the groups were greater than would be expected by chance ($P = .012$ at 14 days; $P = .001$ at 16 days). Multiple pairwise comparisons revealed significant differences between the mcg-treated group and both the myc61-treated group and the untreated mice (all $P < .05$) at 14 and 16 days, whereas the myc61-treated and the untreated groups did not differ significantly from each other (all $P > .05$).

Treatment with myc61 failed to reduce the Myc-to-actin ratios in tumors or spleens (Fig. 2; Fig. 4, A and B), as hypothesized for an antisense mechanism, because of the two central mismatches. In contrast, mcg treatment reduced the Myc-to-actin ratios in the tumors to 0.20 ± 0.10 but reduced the splenic Myc-to-actin ratios to only 0.39 ± 0.19 (Fig. 2; Fig. 4, A

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**Fig. 1.** Inhibition of tumorigenesis in mice treated with myc6. Lymphoma cells ($2 \times 10^6$) were implanted subcutaneously into six mice per treatment group, followed by daily subcutaneous administration of 0.76 mg of DNA phosphorothioate in 0.1 mL of sterile phosphate-buffered saline (PBS), or vehicle only, from day 1 to day 7 after tumor cell transplantation. Tumor volumes (length × width²/2 ± standard error of the means) were estimated at 6, 7, 9, 11, 12, and 14 days after tumor cell transplantation. PBS vehicle only; ▲, scr6 scrambled control; ▓, myc6, complementary to codons 1–5.

**Fig. 2.** Western blot analysis of Myc expression in mice treated with mcg, myc61, myc6, or myc55 for 7 days. The spleens (S) and tumors (T) of mice in each treatment group were removed at the time of sacrifice, 17 days after tumor cell transplantation, and processed for western blotting (see ‘‘Materials and Methods’’ section). Ratios of Myc-to-actin band densities ± standard error are shown on the y-axis for each treatment group (four to six mice per group). DNA sequence administered is shown at the bottom of each column. Oligonucleotides administered are shown on the x-axis below each column.

**Fig. 3.** Inhibition of tumorigenesis in mice treated with mcg (immunostimulatory sequence). Lymphoma cells ($2 \times 10^6$) were implanted subcutaneously into six mice per treatment group, followed by daily subcutaneous administration of 0.76 mg of DNA phosphorothioate in 0.1 mL of sterile phosphate-buffered saline from day 1 to day 7 after tumor cell transplantation. Tumor volumes (length × width²/2 ± standard error of the means) were estimated at 6, 7, 9, 11, 12, and 14 days after tumor cell transplantation. ●, untreated mice; ■, myc61 mismatch-treated mice; ◆, mcg-treated mice.
Treatment with myc55 (Fig. 5) delayed tumor onset by 5–6 days and resulted in negligible tumor growth and 65% ± 6% inhibition in total tumor mass at sacrifice. This treatment reduced the Myc-to-actin ratios in the tumors to 0.27 ± 0.23 and the Myc-to-actin ratios in the spleens to 0.08 ± 0.08 (Fig. 2; Fig. 4, C and D).

Effect of Treatment for 14 Days

To determine whether extended therapy would be more effective, we treated groups of mice with oligodeoxynucleotide phosphorothioates for 14 days. In addition to mcg and myc55, a scrambled myc55 sequence (scr55 in Table 1), a derivative of myc55 containing three mismatches (myc56 in Table 1), and PBS were included as controls. Mice were also administered myc55 or mcg in the absence of tumor cells in order to determine the effects that these sequences might have on the immune system response.

Extending the therapy from 7 to 14 days resulted in 100% mortality in the mcg treatment group. Two of three mice died before the end of the therapeutic schedule, confirming an unacceptable level of toxicity during prolonged exposure to a known immunostimulatory sequence. Ascites accumulation was detected in the single mouse that survived 2 days beyond the termination of mcg injections (16 days after tumor cell transplantation).

The fatal toxicity to the other mice in this treatment group occurred 10 and 13 days after tumor cell transplantation, significantly earlier than ascites accumulation would have been detected. One mouse receiving mcg in the absence of a tumor cell transplant accumulated a small amount of ascites. Treatment with myc56, a control sequence with the dGTCGTT motif of myc55 disrupted (Table 1), also proved to be fatally toxic: All of the mice receiving tumor cells and myc56 died during therapy. One mouse that received myc55 in addition to tumor cells and one that received myc55 without tumor cells died shortly after the therapeutic regimen was completed. One mouse from the scr55 treatment group and one from the PBS treatment group

![Fig. 4](https://academic.oup.com/jnci/article-abstract/90/15/1146/2519719)

Fig. 4. Western blot analysis of mice treated with oligodeoxynucleotide phosphorothioates. Spleens and tumors were removed at the time of sacrifice and were processed for western blotting (see “Materials and Methods” section). The Myc levels in spleens of normal mice were below detection limits. myc61 and mcg (A and B) or myc55 (C and D) was administered intraperitoneally at 0.76 mg per day for 7 days into groups of three to six mice each. Myc (A) and actin (B) levels in spleen (S) or tumor (T) extracts from two mice treated with myc61 and one mouse treated with mcg. Myc (C) and actin (D) levels in spleen (S) or tumor (T) extracts from four mice treated with myc55. In the two tumor lanes on the right of C and D, much less than 40 μg of total protein was applied because the tumors were too small to extract sufficient protein. E: mcg or myc55 was administered intraperitoneally at 0.76 mg per day for 14 days to groups of three mice each. Left three lanes in E: spleen (S) extracts from mice treated with mcg in the absence of tumor cell transplants; next four lanes: spleen (S) or tumor (T) extracts from mice treated with myc55 after tumor cell transplants; right two lanes: spleen extracts (S) from mice treated with mcg in the absence of tumor cell transplants.

and B). However, the mice treated with mcg displayed notable ascites accumulation, which implies blockage of the lymphatic system (27,28). No ascites accumulation was observed during treatment with any of the other sequences (Table 1) administered under this dosage regimen.

Although myc6 significantly inhibited tumor progression (Fig. 1), complete tumor prevention was not observed. Therefore, we analyzed the inhibitory capacity of three antisense DNAs targeted against additional c-myc mRNA sites (myc51, myc53, and myc55, Table 1) that have been found to be susceptible to complementary DNA inhibition in HL-60 cells (10,12,14). Treatment with myc51 and myc53 slowed tumor growth, resulting in tumors that were about half as large as those in the untreated mice (Fig. 5). The differences among the groups were greater than would be expected by chance (P = .022 at 7 days; P = .020 at 9 days; P = .009 at 11 days). Multiple pairwise comparisons revealed significant differences between the myc55-treated group and the untreated mice (all P<.05) at 7, 9, and 11 days, whereas the myc51-treated, myc53-treated, and control, untreated groups did not differ significantly from each other (all P>.05). Incomplete tumor inhibition in both of these treatment groups correlated with the high levels of Myc detected in both the spleens and the tumors from these mice (data not shown).
died on the day that they were to be sacrificed. Tumor onset in both the PBS and scr55 control treatment groups began 8 days after tumor cell transplantation (data not shown), similar to the time of onset detected in mice treated for 7 days with control oligonucleotides (Fig. 1).

The myc55 treatment group developed tumors 11–13 days after tumor cell transplantation, as observed in the 7-day regimen. These results indicate that 14 days of treatment with 0.76 mg (150 nmol) per day of myc55 was no more effective than 7 days of treatment in preventing or further inhibiting tumorigenesis. However, extending the myc55 therapy from 7 to 14 days fully ablated Myc in both the spleens and the tumors of mice analyzed in this treatment group (Fig. 4, E). A fourfold increase in the exposure time of this western blot revealed the presence of low levels of Myc in tumors from only one of the myc55-treated mice (data not shown). The quantity of Myc detected in this mouse, when normalized to that of actin, was similar to that detected in the normal control group that did not receive tumor transplants, but only PBS vehicle.

The toxicity of mcg resulted in 100% mortality between 10 and 16 days after tumor cell transplantation. These premature deaths resulted in either the absence of detectable tumors or tumors too small to process. Therefore, the levels of Myc in this group remained undetermined. Spleen extracts from the three mice receiving mcg in the absence of tumor cells were also analyzed for Myc expression (Fig. 4, E). Myc was detected in only one of the mice, yielding an average Myc-to-actin ratio of 0.03 ± 0.03 when the western blot was overexposed fourfold (data not shown). The myc56 treatment group developed tumors 8 days after tumor cell transplantation, similar to the findings in the PBS-treated and the scr55-treated control groups. However, treatment with myc56 was also toxic, resulting in 100% mortality between 10 days and 16 days after tumor cell transplantation. The postmortem instability of Myc prevented analysis of Myc levels in spleens and tumors from these mice.

**Effect of Alternating myc55 With mcg for 14 Days**

The significant, and similar, tumor inhibition resulting from treatment with myc55, an antisense oligonucleotide, or with mcg, an immunostimulatory oligonucleotide, led us to analyze the efficacy of treatment with alternating doses of these two sequences. Since the results above cannot rule out the possibility that the dGTCGTT motif in myc55 may, in part, be responsible for the tumor inhibitory activity of this oligonucleotide, an additional control oligonucleotide (myc57 in Table 1) was synthesized. This sequence maintains the integrity of the dGTCGTT hexamer of myc55, while creating a pair of two nucleotide mismatches to disrupt the myc55 antisense sequence. Initially, 0.76 mg of mcg was administered per day, but this dose regimen proved to be fatally toxic (see above). Therefore, we administered 0.76 mg of myc55 every other day or the control sequence myc57, alternating with 0.65 mg of mcg given every other day. Treatment was begun 1 day after tumor cell transplantsations, as previously described, and was continued for 14 consecutive days, with alternating oligonucleotide injections.

Fig. 6 shows that treatment with myc57 alone or treatment with PBS vehicle alone resulted in tumor development between 8 and 10 days after tumor cell transplantation. Addition of mcg treatment to either the myc57 or the PBS treatment groups postponed tumor development by an additional 3–4 days. Treatment with myc55 alone given every other day inhibited tumor development until 10–14 days after tumor cell transplantation, as did treatment with mcg alone given every other day. However, when 0.76 mg of myc55 was alternated every other day with 0.65 mg of mcg, no palpable tumors were detected until 14 days after tumor cell transplantation, the end of the therapeutic regimen. The differences among the groups were greater than would be expected by chance up to 11 days (P = .02 at 9 days; P = .017 at 10 days; P = .017 at 11 days). After 11 days, tumors in both the PBS and myc57 treatment groups grew much more rapidly than those in the other treatment groups. By 14 days after tumor cell transplantation, these mice were sacrificed to prevent further suffering. The average tumor volume for these two groups was 4.4 mL ± 1.0 mL. The average tumor volume observed in mice from the myc55-only, myc55-plus-mcg, myc57-plus-mcg, or PBS-plus-mcg treatment groups was 0.82 mL ± 0.31 mL. However, statistical analysis at 14 days revealed that the differences in median values among all treatment groups were not great enough to exclude the possibility that the differences were due to random sampling variability (P = .078).

These results suggest that treatment with an antisense oligonucleotide, myc55, in addition to an immunostimulatory oligonucleotide, mcg, was more effective than treatment with either oligonucleotide alone. Injection of 0.65 mg of mcg was less toxic than the earlier injection of the 0.76-mg dose. One myc55-plus-mcg-treated mouse died at the end of therapy (14 days after tumor cell transplantation), and two of the myc57-plus-mcg-treated mice died 11–14 days after tumor cell transplantation, near the end of therapy. The fatal toxicity seen with mcg treatment suggests that minimal amounts of this oligonucleotide should be used in future experiments.
Analysis of Circulating Cytokines

Weekly measurements of plasma TNF-α, IL-12, and IFN γ levels were performed 1 hour after intraperitoneal oligonucleotide injections. Both the TNF-α and the IL-12 concentrations remained below detectable levels throughout therapy. Sporadic increases in the amounts of IFN γ were detected, with no correlation to the therapeutic regimen.

Effects of Oligonucleotides on Spleen Mass

The mass of the spleen is also an indicator of spleen cell proliferation. The spleens of tumor-bearing mice receiving only PBS were threefold to 3.5-fold larger than those of untreated mice (Fig. 7). Spleen enlargement in mice treated with all myc-related sequences in Table 1 was threefold to fivefold larger than normal, slightly greater than in the PBS-treated group. This enhanced splenomegaly is most likely due to the effect of the phosphorothioate backbone (29). However, treatment with an immunostimulatory oligonucleotide, mcg, resulted in a sixfold to sevenfold increase in the spleen mass. This increase in spleen mass was greater than would be expected by chance when compared with either the PBS-treated control group or the normal, untreated control mice (all P<.05). However, the splenic enlargement observed in each of the other treatment groups was not statistically significant (all P>.05). Relative to normal, untreated mice (normal, Fig. 7), only the mcg and myc61 treatment groups were statistically different (all P<.05), whereas those from all other treatment groups were not (all P>.05).

Discussion

We analyzed the efficacy of c-myc complementary oligonucleotides involved in the inhibition of tumorigenesis to gain insight into the sequence specificity and mechanisms involved. It would be desirable to identify a safe and efficacious oligonucleotide for consolidation therapy in lymphoma patients, to reduce or eliminate residual drug-resistant cells, and thereby to increase the number of patients who undergo a complete remission (3). In this study, myc6 (16) inhibited the growth of B-cell lymphoma transplants from Eμ-myc-carrying transgenic mice into immunocompetent hosts (Fig. 1). This oligonucleotide has been reported to show some stimulation of spleen cell proliferation in tumor-free mice, but several-fold less than that for mcg (30). The ability of dGGGG to inhibit proliferation (31–33) has been attributed to tetraplex formation. However, myc6 cannot form a tetraplex structure in physiologic salts at 37 °C (34). Both the dCACGTT and the dGGGG motifs were intentionally retained in myc61, which represents myc6 with a central 2-base-pair mismatch (Table 1). Nevertheless, treatment with myc61 did not significantly inhibit tumor growth (Fig. 3); moreover, myc61 did not decrease Myc levels in tumors or spleens (Fig. 2; Fig. 4, A and B).

The Myc-to-actin ratios at sacrifice (17 days) in the spleens of mice treated with myc6 were reduced relative to those in the spleens of mice treated with myc61 (Fig. 2; Fig. 4, A and B). This phenomenon probably reflects unimpeded uptake of myc6 by circulating lymphocytes and tumor lymphoblasts, both of which can become sequestered in the spleen, the body’s largest reservoir of lymphocytes. This would result in the observed inhibition of Myc expression in the spleen. The lack of reduction of Myc levels in tumors at sacrifice probably reflects the resistance of established tumors to penetration by circulating drugs (35). This in turn correlates with the growth of these tumors in treated mice.

Tumor development was delayed and tumor growth was reduced in the mice treated with myc6. The Myc protein levels detected in the spleens of these mice were reduced twofold relative to those of the tumors. In contrast, myc61 treatment did not inhibit tumorigenesis, and the levels of Myc detected in the spleens and the tumors of these mice were not significantly different from each other (P = .608). The effect of the mismatches suggests that myc6 treatment inhibited Myc expression and subsequent tumor growth in a sequence-dependent manner, independent of either dGGGG or dCACGTT.

Inhibition of tumorigenesis by mcg (Fig. 3) was accompanied by the accumulation of ascites, in part a result of blockage of the lymphatic channels following stimulation of B-cell proliferation by the dCAGTT motif of mcg. This blockage compromised drainage of the peritoneal cavity (27,28), thus inhibiting the critical flow of lymph fluid (excess interstitial fluid) into the circulation; disruption of this flow can be fatal (28).

In mcg, dCAGTT occurs in the middle of the sequence. However, oligonucleotides containing a complete dPuPuCG-PyPy consensus motif at either end of the sequence were found to be ineffective in their immunostimulatory activity (22). In myc6 and myc61, the dCACGTT motif is located at the 5′ terminus and has only one purine, rather than two purines, 5′ to the CG. Both the 5′ terminal location and the purine-to-pyrimidine transversion should make immunostimulatory activity by myc6 or myc61 unlikely (22).

The incomplete inhibition of tumors by myc6 led us to analyze additional target sites on the mRNA to achieve more efficient inhibition. Tumorigenesis was inhibited similarly by treatment with either mcg (Fig. 3) or myc55 (Fig. 5) but not by treatment with myc57 (Fig. 6). These data, along with the decreased ratios of Myc to actin observed with myc55 treatment,
imply that the efficacy of myc55 therapy depends on the entire sequence. In mice treated with myc55, tumors were detected only at the injection site; no lymph node metastases were observed. However, we observed metastases to cervical and inguinal lymph nodes, in addition to minor lymph nodes throughout the body, in all other treatment groups, including mice receiving mcg. In addition, the accumulation of ascites in mice treated with mcg indicates a level of toxicity that requires cautious administration.

The injection of tumor cells (antigen) 1 day before administration of mcg, which is a known immunostimulant (18,22,35), could prime the immune system to the tumor. This pretreatment may thus induce an immune response that might subsequently inhibit tumor development. The observation that Myc levels in the spleens of mice treated with mcg were twofold greater than those measured in their tumors (Figs. 2; Fig. 4, A and B) further supports the hypothesis that the oligonucleotide activates B cells within the spleens of this treatment group. The lower Myc antigen levels detected in tumors of mice treated with mcg may reflect an immune response against the tumor cells. Although no Myc antigen could be detected in either circulating lymphocytes or spleens of normal, nontransgenic, nontransplanted mice, B cells from transgenic Eμ-myc-carrying mice display very high constitutive Myc levels in circulating lymphocytes, spleens, and tumors (15,16).

However, administration of the immunostimulatory sequence mcg to normal mice in the absence of Eμ-myc lymphoma cells (Fig. 4, E; data not shown) yielded a splenic Myc-to-actin ratio of 0.03 ± 0.03, detectable only when the western blot was overexposed fourfold to allow detection. This boost in Myc levels (resulting from the stimulation of normal spleen cells by mcg) is modest relative to the high levels expressed in Eμ-myc lymphoma cells. This modest stimulation of Myc expression by mcg would be difficult to measure above the high levels observed constitutively in Eμ-myc lymphoma cells. Scrambled and mismatched sequence controls did not affect the high levels of Myc antigen expressed in tumors or spleens.

Secretion of cytokines should follow immune system activation, yet no increase in TNF-α, IL-12, or IFN-γ levels was detected in the plasma 1 hour after the injection of mcg. The increase in cytokine levels induced by dPuPuCGPyPy motifs depends on both the route of administration of dPuPuCGPyPy-containing DNA and the time after administration at which the mice are bled for cytokine analysis. Yi et al. (36) reported that intravenous injection of Escherichia coli DNA resulted in an increase in the serum IL-6 concentration by 2 hours after injection, followed by a rapid decline to below detectable levels at 8 hours. Cowdery et al. (37) reported that the serum IFN-γ levels remained below detectable levels until 5 hours after the intravenous injection of E. coli DNA. In addition, mice receiving E. coli DNA alone exhibited an increase in TNF-α and IL-6 levels that was only 10% of the levels achieved following administration of a combination of DNA and lipopolysaccharide. The significant increases in cytokines detected in these reports may also be due to the use of E. coli DNA, which contains numerous unmethylated dPuPuCGPyPy motifs. In vitro analysis of cytokine stimulation by dPuPuCGPyPy-containing oligonucleotides revealed that the greater the number of dPuPuCGPyPy motifs in an oligonucleotide, the greater the secretion of cytokines (22). Yi et al. (36) analyzed 11 different dPuPuCGPyPy-containing oligonucleotides to determine the optimal nucleotide sequence for induction of cytokines. However, their results indicated that the presence of unmethylated dPuPuCGPyPy motifs in oligonucleotides does not necessarily result in immune stimulation. In addition, both the time course of cytokine release and the serum levels of cytokines depend on the route of administration of the DNA. The cytokines measured in this study (TNF-α, IL-12, and IFN-γ) may have remained below detectable levels because of the route of administration, the time at which the mice were bled for samples, and/or the inability of these oligonucleotides to stimulate an immune response.

When mice were treated with myc55, the level of Myc expression in their tumors was similar to that in the tumors of mice treated with mcg. However, the Myc levels in the spleens of mice treated with myc55 were only one fourth of the levels detected in their tumors (Fig. 2; Fig. 4, A and B). Lymphocyte proliferation occurs primarily in the lymph nodes, the spleen being the largest such lymphatic organ (28). Expression of Myc is a prerequisite for cell cycle progression (9); therefore, the low levels of Myc detected in the spleens of the myc55 treatment group imply an inhibition of lymphocyte proliferation. This inhibition correlates with the elimination of detectable metastases, a decreased rate of tumorogenesis, and a reduction in the total tumor mass. The excretion half-life of DNA phosphorothioates is 40–60 hours, and these compounds accumulate in highly perfused organs such as the spleen (29). This observation suggests that treatment with myc55 inhibits spleen cell (primarily lymphocyte) proliferation and subsequently Myc expression.

The dGTCGTT motif in myc55 differs from the dPuPuCGPyPy consensus (18,22) by having a pyrimidine (T) rather than a purine on the 5’ side of the dPuPuCGPyPy, which correlates with the report that immune stimulation is significantly reduced when a purine was substituted for a pyrimidine, or vice versa (22). The myc57 control includes a pair of two nucleotide mismatches that disrupt complementarity to the myc55 target mRNA, but myc57 still retains the dGTCGTT motif. Nevertheless, treatment with myc57 failed to inhibit tumorogenesis. This result further supports the model that the effect of the myc55 antisense oligonucleotide is dependent on its entire sequence, irrespective of the presence of the dGTCGTT motif.

Extending myc55 treatment to 14 days, while maintaining a dose of 0.76 mg per day, proved ineffective. Although prolonging the therapy to 14 days did not further inhibit tumorogenesis, this result may be due to selection for a tumor cell subpopulation that does not require Myc expression for proliferation (23,38,39). The absence of detectable Myc in the spleens and tumors of the myc55 treatment group (Fig. 4, E) supports this hypothesis. The similar times of tumor onset and similar total tumor masses detected in the 7-day and 14-day regimens, in addition to the existence of metastasis in the 14-day treatment group, further support the potential development of Myc-independent tumors in the 14-day treatment group.

The similar, yet incomplete, tumor inhibition resulting from treatment with myc55 alone or with mcg alone suggested that a therapeutic regimen in which myc55, an antisense oligonucleotide, is alternated with mcg, an immunostimulatory oli-
gonucleotide, might prove to be more effective in preventing or limiting tumor onset. Although either myc55 or mcg alone delayed tumor onset by 3–7 days, alternating the daily therapy between 0.76 mg of myc55 and 0.65 mg of mcg was found to be much more effective than either sequence alone. This alternating regimen postponed tumor onset by 7–9 days, which was beyond the end of the therapeutic schedule (Fig. 6).

Thus, in addition to being used for consolidation therapy following standard chemotherapy, antisense therapy might serve to sensitize tumor cells for subsequent chemotherapy (8). Furthermore, current chemotherapeutic regimens for cancer therapy often result in development of drug-resistant tumors (3). To increase the number of patients who are able to maintain complete remission, antisense therapeutics could be added to the standard chemotherapeutic regimen.

Having created a mouse model for residual disease by transplanting Eμ-myc tumor cells into immunocompatible mice, we found that alternating injections of the antisense oligonucleotide myc55 with the immunostimulatory oligonucleotide mcg allowed complete prevention of tumorigenesis during the therapeutic schedule. These results suggest that patients treated with a similar oligonucleotide therapeutic regimen, in addition to standard chemotherapeutic agents, might experience a more favorable outcome.

References

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Notes

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