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In vivo treatment of human leukemia in a *scid* mouse model with c-myb antisense oligodeoxynucleotides

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ABSTRACT **The c-*myb* protooncogene encodes proteins that are critical for hematopoietic cell proliferation and development. Disrupting c-*myb* function might, therefore, prove an effective therapeutic strategy for controlling leukemic cell growth. Antisense oligodeoxynucleotides have been utilized for this purpose *in vitro*, but their *in vivo* efficacy has not been reported. We therefore established human leukemia–*scid* mouse chimeras with K562 cells and treated diseased animals with phosphorothioate-modified antisense oligodeoxynucleotides. K562 cells express the c-*myb* protooncogene, which served as the target for the antisense DNA. They also express the tumor-specific *bcr–abl* oncogene that was utilized to track the human cells in the mouse host. Once circulating leukemic blast cells had been detected, the survival of untreated control mice was 6 ± 3 days (mean ± SD). The survival of animals treated for 7 or 14 days with either sense or scrambled-sequence c-myb oligodeoxynucleotides was not statistically different from the control animals. In distinct contrast, animals treated for similar lengths of time with antisense c-myb oligodeoxynucleotides survived at least 3.5 times longer than the various control animals. In addition, animals receiving antisense c-myb DNA had significantly less disease at the two sites most frequently manifesting leukemic cell infiltration, the central nervous system and the ovary. These results suggest that phosphorothioate-modified antisense DNA may be efficacious for the treatment of human leukemia *in vivo*, and by analogy, for the treatment of other human neoplasias.**

Protooncogenes encode proteins critical for cell growth and development. Their activation may also be important in the pathogenesis of many human malignancies including those of the hematopoietic system (1–4). Disrupting protooncogene function might, therefore, form the basis of a strategy for controlling the growth of leukemic blood cells. Antisense oligodeoxynucleotides have been employed for this purpose *in vitro* (5–9). We have demonstrated that antisense c-myb (10) and c-kit (11) DNAs can markedly inhibit leukemic cell growth and in some cases are cytocidal. We have also shown (10) that leukemic colony-forming units are more sensitive to the effects of antisense c-myb oligodeoxynucleotides than are their normal counterparts (10). Accordingly, it has been hypothesized that antisense DNA might prove useful in the treatment of human leukemia (5, 6, 10). Problems concerning oligodeoxynucleotide delivery and stability *in vivo* prompted us to suggest that, at least in the near term, the therapeutic use of these compounds might be restricted to *ex vivo* applications such as bone marrow purging (10).

Recent technological advances in the development of more nuclease-resistant oligodeoxynucleotides, such as those with phosphorothioate-modified internucleoside phosphate backbones ([S]oligodeoxynucleotides) (12), the availability of miniature implantable infusion pumps for drug delivery in small animals (13), and the establishment of normal and leukemic human hematopoiesis in severe combined immunodeficiency (*scid*) mice (14–17), allowed us to evaluate the efficacy of this material for *in vivo* leukemia treatment. We report herein experiments that demonstrate that phosphorothioate-modified antisense DNA may prove efficacious for the treatment of human leukemia *in vivo*, and by analogy, of other human neoplasias.

MATERIALS AND METHODS

Establishment of *scid* Mouse–Human K562 Cell Leukemia Chimera. Six- to 7-week-old female mice (CB-17/ACRTAC/*scid*/SDS; Taconic Laboratories, Taconic, NY) were injected intraperitoneally with cyclophosphamide [150 mg/kg (body weight); total dose, ≈3 mg] on each of two successive days. Twenty-four hours after the second injection, animals were transplanted by tail vein injection with 1×10^7 K562 human leukemia cells (American Type Culture Collection) that had been washed and seeded into fresh medium [RPMI 1640 medium/10% (vol/vol) fetal bovine serum] 12 h prior to use. After transplantation of the K562 cells, animals were monitored for the presence of circulating blast cells by blood sampling from the retroorbital plexus.

Oligodeoxynucleotides. Phosphorothioate oligodeoxynucleotides corresponding to c-*myb* codons 2–9 (18) were prepared on an Applied Biosystems model 380B or 390Z automated synthesis instrument using published methodologies (12). The sense and antisense c-myb sequences were 5′-GCCCGAAGACCCCGGCACAGCATA-3′ and 5′-TATGCTGTGCCGGGGTCTTCGGGC-3′, respectively. Phosphorothioate oligodeoxynucleotides with base content identical to the antisense sequence but in "scrambled" order (5′-GCACGCAGCTGAAGCACAAGCACC-3′) and compounds corresponding to c-kit sequences (11) were also prepared.

Oligodeoxynucleotide Administration. Beginning 4 weeks after leukemic cell transplantation, animals were monitored for the development of overt leukemia by retroorbital plexus bleeding thrice weekly. When the peripheral-blood blast-cell content was at least 1–5% of the total leukocyte count, as determined by a 200-cell differential count in a standard hemocytometer chamber, treatment was initiated. Animals were anesthetized and prefilled constant infusion miniosmotic pumps (Alzet, Palo Alto, CA) were inserted subcutaneously into a paraspinal pocket that was entered through a small interscapular incision. Implanted pumps released their contents at a rate of 1 μl/h. Oligomers were diluted in sterile

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Abbreviations: RT, reverse transcriptase; nt, nucleotide(s); CML, chronic myelogenous leukemia; CNS, central nervous system.

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distilled water so that each animal received a total dose of 100 μg/day [5 mg/kg (body weight); 1 μM].

Reverse Transcription–Polymerase Chain Reaction (RT–PCR). RT–PCR for detection of bcr–abl mRNA transcripts was carried out as described (18). The following primers were utilized: 3′ *abl* exon 2 [22 nucleotides (nt)], GCTTCACACCATTCCCCATTGT; 5′ *bcr* exon 2 (22 nt), CACAGCATTCCGGTGACCATCA. After 60 cycles, 10 μl of amplified product was electrophoresed on a 4% agarose gel and then transferred to a nylon filter. Filters were prehybridized and then probed with a ^{32}P-end-labeled oligonucleotide probe corresponding to a 39-nt abl sequence (CCAGTGAAAATGACCCCAACCTTTTCGTTGCACTGTATG) contained within the amplified region. Hybridization was detected by autoradiography.

Statistical Analysis. Statistical significance of survival differences among animals in the various oligomer treatment groups was determined using the Student *t* test for unpaired samples. Survival was determined from the time that the infusion pumps were implanted. *P* values of <0.05 were judged to be of statistical significance.

RESULTS

Establishment and Growth Characteristics of K562 Cells in *scid* Mice. K562 cells were derived from a patient with chronic myelogenous leukemia (CML) (19). They carry the tumor-specific Philadelphia chromosome translocation t(9;22)(q34;q11) (20), which creates the *bcr–abl* hybrid gene thought to be required for the development of CML (21, 22). K562 cells were used for these studies because they express the c-*myb* protooncogene (23, 24) and this laboratory has demonstrated (5, 6, 11) that growth of c-myb-expressing CML cells can be inhibited with antisense c-myb DNA. In addition, the *bcr–abl* marker allows for the sensitive specific RT–PCR-based detection of tumor cells (10) in the *scid* mice.

scid mice have proven excellent hosts for xenogenic tissue grafts, including human hematopoietic cells (16, 17), because defective T- and B-lymphocyte development (25) renders these animals severely immunocompromised. Nevertheless, the presence of residual immune cells usually requires that such animals be irradiated prior to attempts to engraft human hematopoietic tissue (14–17, 26). This was not possible in our facility, so that animals were prepared for transplant by conditioning with cyclophosphamide as described above.

After K562 cell transplantation, animals were monitored for circulating blast cells by retroorbital plexus blood sampling. Blast cells were demonstrable in peripheral blood within 5–6 weeks. They were morphologically distinguished from mouse leukocytes by their large size. Blast cell measurements ($n = 50$) revealed a diameter of ≈28 μm, in comparison to ≈8 μm for the mouse leukocytes. Blasts were also distinguished by their high nuclear/cytoplasmic ratio, open nuclear chromatin, and prominent nucleoli (Fig. 1*A*).

Shortly after manifesting peripheral leukemia, the animals became weak and sickly in appearance. Neurologic manifestations were a prominent part of the clinical picture. Limb paresis, circling gait, and other manifestations of central nervous system (CNS) disease including marked bulging of the cranium from hydrocephalus developed in 100% of the control animals. Abdominal enlargement secondary to the formation of large granulocytic sarcomas was also noted on occasion. Mean (±SD) survival of the animals once K562 cells became detectable in peripheral blood was 6 ± 3 days ($n = 20$) with none living longer than 12 days.

At the time of their demise, the animals were autopsied. As predicted by the clinical manifestations of the disease, examination of the cranial contents revealed involvement in 100% of the cases. At the microscopic level, meningeal infiltration and packing of the subarachnoid space with sparing of the brain substance was the most prominent feature (Fig. 1 *C* and *D*). Though tumor nodules and studding of serosal surfaces were frequently observed at various locations throughout the abdominal cavity, the only other organ besides the brain that was consistently involved by leukemic-cell infiltration in this model was the ovary (Fig. 1*B*). These organs were often massively enlarged and were easily palpable on abdominal examination. Of interest, though the bone marrows of such animals demonstrated a marked granulocytic hyperplasia and the spleens were occasionally enlarged, gross leukemic infiltration with large blasts was not usually evident. Nevertheless, RT–PCR analysis of mouse bone marrow cells revealed the presence of the tumor-specific bcr–abl mRNA (10), thereby establishing the presence of human leukemic cells in the animals' bone marrow. Accordingly, although the disease developed by these animals did not resemble human CML in blast crisis in every detail, the hyperplastic marrow, presence of granulocytic sarcomas, and presence of meningeal leukemia suggested that this was a relevant model with which to test the efficacy of the antisense c-myb oligodeoxynucleotides.

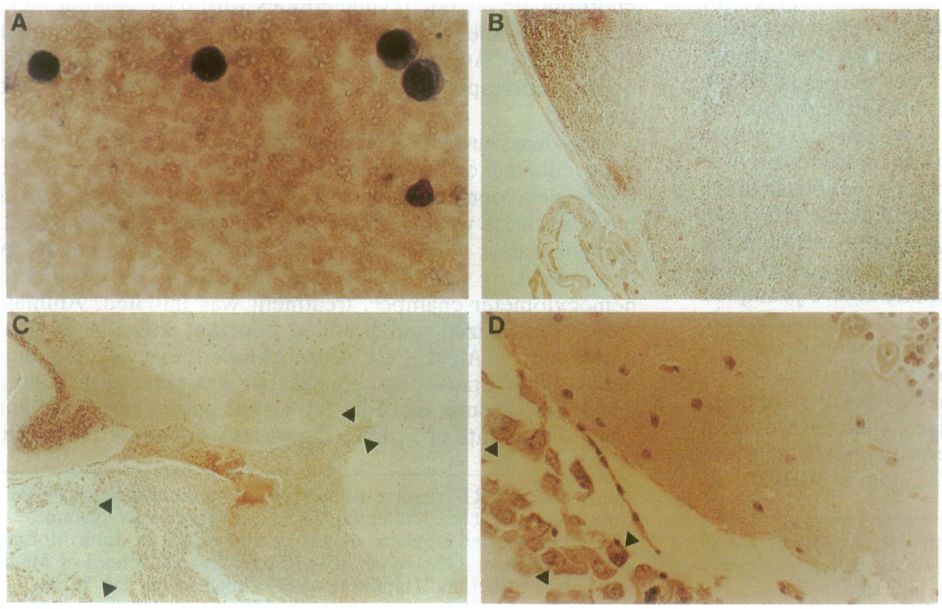

FIG. 1. Composite photomicrographs of peripheral blood (*A*) (Wright's stain), ovary (*B*), and brain (*C* and *D*) (hematoxylin/eosin stain) obtained from autopsied chimeric *scid*–human K562 cell leukemia-bearing mice. Circulating blast cells are shown in *A*. Measurement of 50 such cells revealed a diameter of ≈28 μm, in comparison to 8 μm for the mouse leukocytes. Total effacement of normal ovarian architecture by K562 leukemia cells is shown in *B*. Heavy meningeal and subarachnoid infiltration with apparent sparing of the brain parenchyma is shown at low (×75) and high (×300) powers in *C* and *D*, respectively. Arrowheads indicate leukemic blast cells filling the subarachnoid space in *C* and a high-power view of the blast cells in *D*.

Effect of c-myb Oligodeoxynucleotide Treatment of K562 Cell Growth *in Vivo*. Establishment of the *scid*-human leukemia animal model allowed the effect of the phosphorothioate c-myb oligodeoxynucleotides on human leukemic cell growth *in vivo* to be determined. Accordingly, mice were transplanted with K562 cells and then infused with the oligodeoxynucleotides when clinical leukemia was detected. Treatment was not initiated until the onset of measurable disease to obtain an accurate assessment of the oligomers' antitumor activity in a clinically relevant model. No other antileukemic therapy was administered to the animals.

Three experiments were carried out in which mice received continuous infusion of oligomers for 3, 7, and 14 days. Mice were arbitrarily assigned to equally sized experimental groups. The treatment group received antisense oligomer infusions. The control groups received sense oligomers (experiments 1–3), scrambled-sequence oligomers (experiments 1 and 3), or no treatment (experiments 2 and 3).

In the first experiment, animals received a 3-day constant infusion of sense, antisense, or scrambled-sequence phosphorothioate c-myb oligodeoxynucleotides. Fifteen similarly aged healthy female mice were arbitrarily assigned to one of three experimental groups. Implanted pumps contained a total of 300 μg of 24-nt c-myb [S]oligodeoxynucleotide in 72 μl of sterile distilled water; each pump delivered 100 μg/day for 3 days. The mice in the antisense-treated group appeared to have a longer disease-free interval than those in the control groups. By the 8th treatment day, only 1 of 5 animals in the antisense treatment group had died compared to 3 of 5 in the sense and scrambled-sequence groups. However, this apparent survival advantage did not persist and by the 11th day after pump implantation, all animals except 2 in the sense-oligomer-treated group had died. Both of these latter animals were paralyzed and gravely ill and thus were sacrificed to prevent unwarranted suffering.

A second experiment was then carried out to test the hypothesis that a longer antisense oligomer exposure would translate into an enhanced survival for animals receiving this treatment (Fig. 2). Larger pumps that released their contents over 7 days were employed. Three groups were again established, but mouse availability restricted the number of animals to four mice per group instead of the five mice previously employed. One group of animals consisted of an untreated control. Animals in the other groups received sense or antisense compounds delivered by the implantable infusion pumps. Pumps were loaded with 700 μg of oligomers delivered so that the total daily dose to each animal was the same as in the first experiment. As shown in Fig. 2*A*, the four untreated control animals and three of four animals receiving infusions of the sense oligomers were dead by the 5th day after pump implantation. In contrast to these groups, three of the four antisense-treated animals were alive and apparently well. The sole surviving animal from the sense group expired on the 11th day but the two remaining antisense animals survived considerably longer. These animals died on the 24th and 34th days after pump implantation. The mean survival (±SD) of the control and sense oligomer-treated animals in this experiment was 2 ± 2 (mean ± SD) and 3 ± 4 days, respectively. Mean survival of animals in the antisense group was 17 ± 12 days, an 8.5- and 5.7-fold increase in survival when compared to the untreated and sense controls, respectively.

Antisense-treated animals also appeared to have a lower total body disease burden. For example, all control and sense-treated animals were paralyzed at the time of death secondary to CNS disease. Clinical indications of CNS involvement were noted at death in only two of the four antisense-treated animals. Microscopic analysis of brain tissue from the animals that had no clinical brain involvement revealed that they were indeed free of disease (Fig. 3).

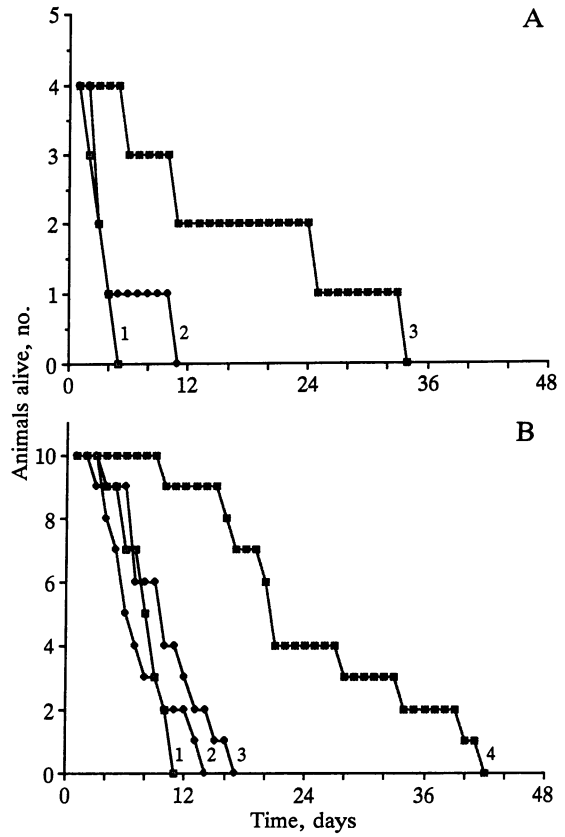

FIG. 2. Survival curves of *scid*-human chimeric animals transplanted with K562 chronic myelogenous leukemia cells. (*A*) Animals received a 7-day infusion of oligomers at 100 μg/day. Curves: 1, control; 2, sense; 3, antisense. (*B*) Animals received a 14-day infusion of oligomers at 100 μg/day. Curves: 1, control; 2, scrambled; 3, sense; 4, antisense.

Similarly, ovarian replacement by tumor cells was much less marked in the antisense group. The ovaries were grossly involved with disease in three of the four animals in the control group (the fourth animal was cannibalized by its cagemates and could not be autopsied) and in all four animals receiving sense oligomers. In contrast, ovarian involvement was grossly evident in only two of the antisense-treated animals and the ovaries of these animals were less extensively involved as reflected by their weight. Weight of the involved ovaries in autopsied untreated control animals was 2.0 ± 0.8 g (mean ± SD), 1.7 ± 0.7 g in the sense-treated animals, and 1.0 ± 0.9 g in the antisense group.

To confirm that infusion of the antisense DNA was responsible for increased survival and impaired leukemic cell growth in the treated animals, a third experiment was carried out with a total of 40 animals (Fig. 2*B*). As in the previous experiments, the mice were implanted with infusion pumps that delivered oligomers at a dose of 100 μg/day, but the infusions were continued for a total of 14 days. Survival of animals in the untreated control group and groups receiving sense or scrambled-sequence oligomers was quite similar. Fifty percent of the animals in these groups were dead by the 8th, 6th, and 9th days after pump implantation, respectively. In marked contrast, 9 of 10 animals in the antisense-treated group were alive and well on the 10th day. By the 17th day, all animals in the various control groups were dead whereas 7 of 10 animals in the antisense-treated group remained alive. Autopsy of the dead animals in all groups revealed substantial CNS involvement that was the presumed cause of death. Between the 17th and 21st days after treatment, 3 additional animals in the antisense group died (Fig. 2*B*), but 4 animals

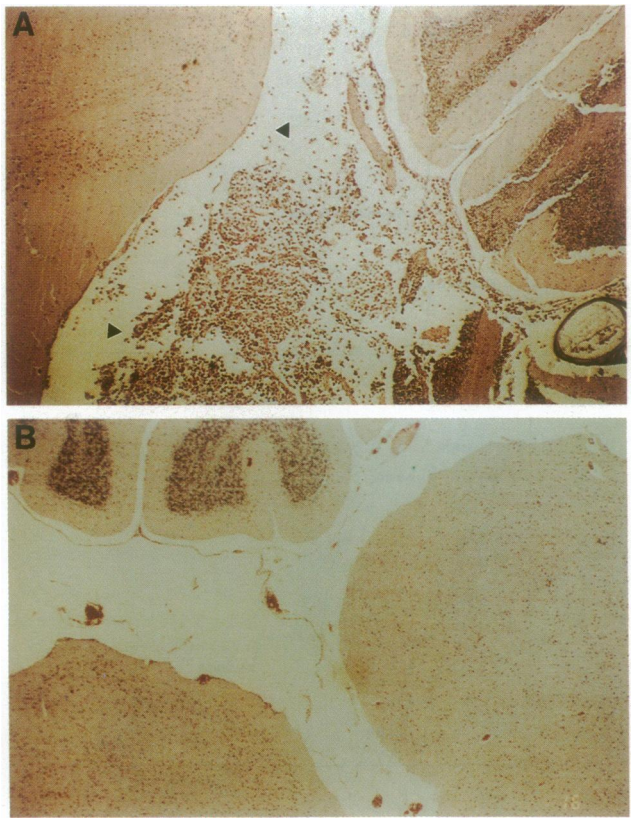

FIG. 3. Composite photomicrographs (×400) of brain stained with hematoxylin/eosin and obtained from autopsied chimeric *scid*–human K562 cell leukemia-bearing mice treated with sense (*A*) or antisense (*B*) phosphorothioate c-myb oligodeoxynucleotides. Note extensive meningeal and subarachnoid infiltration of the meninges (arrows) in *A* and the lack of involvement in *B*.

remained alive and were apparently well. Peripheral blood of these animals was sampled to determine if *bcr–abl*-expressing cells could be detected by RT–PCR. As shown in Fig. 4, all animals had detectable *bcr–abl* expression at variable levels. Although these determinations are admittedly semiquantitative, we noted that the 2 animals with apparently highest disease burden (lanes 1 and 2) died on days 28 and 33, and animals with intermediate (lane 4) and low level *bcr–abl* expression (lane 3) died on days 39 and 41, respectively. Though all of these animals were clinically without CNS disease, scattered infiltration of the meninges was noted on microscopic examination in 2 animals. The immediate cause of death of these 4 animals was uncertain but presumably was leukemia-related. All had scattered foci of disease but as noted in the previous experiment, total body disease burden was substantially less than in the control animals.

Effect of c-kit Oligodeoxynucleotide Treatment on K562 Cell Growth *in Vivo*. As an additional sequence-specificity control, we also studied the effect of phosphorothioate oligodeoxynucleotides targeted to the c-*kit* protoonocogene on growth of K562 cells *in vivo*. We have previously shown that antisense c-kit oligomers can inhibit the growth of benign and malignant hematopoietic cells that express c-*kit* (11). Since K562 cells do not express this protooncogene (27), one would predict that the growth of these cells *in vivo* would not be affected by these oligomers if growth inhibition was truly sequence-specific. Alternatively, if growth inhibition was a nonspecific toxic effect of the oligodeoxynucleotides, one might see a prolongation of the animals' survival. Accordingly, animals were transplanted with K562 cells and exposed to phosphorothioate c-kit oligomers corresponding to codons 1–6 (11) as outlined above except that twice the daily dose was employed (200 μg/day) for a total of 14 days. As shown in Table 1, c-kit oligomers had no effect on animal survival or the extent of disease.

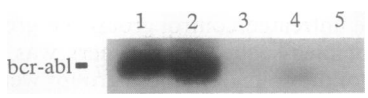

FIG. 4. Detection of bcr–abl mRNA transcripts in peripheral blood cells derived from long-term-surviving *scid*–human K562 cell leukemia chimeric mice. Total RNA was extracted from light-density nucleated blood cells and reverse-transcribed with a 3′ primer specific for the second c-*abl* exon, and the resulting cDNA was amplified by PCR with a primer pair specific for the *bcr–abl* junction. Amplified DNA was detected by Southern blot analysis with a 40-nt c-abl probe (16). Lanes: 1–4, representative of *bcr–abl* content of blood of each individual mouse; 5, water control lane.

Table 1. Effect of phosphorothioate c-kit and c-myb oligodeoxynucleotides on survival of *scid*–human leukemia (K562) chimeric mice

Gene target	Survival time, days			
	Untreated control	Sense oligomers	Scrambled oligomers	Antisense oligomers
c-*kit*	6 ± 3	NT	5 ± 4	5 ± 2
c-*myb*	7 ± 2	9 ± 4	7 ± 4	24 ± 11*

Chimeric animals were established and treated with oligodeoxynucleotides for 14 days as described in the text. The dose of c-kit oligomers employed (200 μg/day) was twice the dose of c-myb administered. Survival is expressed in days (mean ± SD). NT, not tested.
*$P < 0.001$ in comparison to survival in control groups.

DISCUSSION

The results of these experiments provide clear evidence that phosphorothioate-modified c-myb antisense oligodeoxynucleotides have significant single-agent antileukemic activity in an *in vivo* model system. The simplest and perhaps most compelling evidence in support of this statement is that we were able to significantly prolong the life of animals with established leukemia with sequence-dependent oligodeoxynucleotides. The leukemia in this model behaved in a very aggressive manner. Once circulating leukemia cells were detectable in the peripheral blood, mean (±SD) survival of all the animals in the various control groups ($n = 60$ animals) was remarkably similar and was between 6 and 7 days (± 3 to 5 days). In contrast, mice treated exclusively with antisense c-myb DNA had a statistically significant 3- to 8-fold longer survival compared to untreated control animals. The clinicopathologic manifestations of the disease were also highly reproducible with an extremely prominent CNS component and a propensity for the leukemic cells to grow in the form of granulocytic sarcomas. Accordingly, another important antileukemic effect of the antisense compounds was the dramatic protection of the animal's CNS and a >50% inhibition of granulocytic sarcoma growth in the animal's ovaries. No survival advantage was observed in mice that received nonhybridizing c-myb sequences or c-kit sequences, a gene that the K562 leukemia cells do not express. Accordingly, fortuitous effects of oligodeoxynucleotides on disruption of transcription (28, 29) or nonspecific effects such as inhibition of polymerases (30), interferon induction (31), or protein binding (32), are unlikely to have contributed to the therapeutic effects observed.

Responses were also dose-dependent. Seven- and 14-day infusions (100 μg/day) of antisense oligodeoxynucleotides were effective in prolonging survival, whereas a 3-day infusion of the same daily dose was not. Of additional interest was

the observation that constant infusion delivery of antisense oligodeoxynucleotides, even at relatively low doses, had significant therapeutic utility. This finding suggests that intravenous bolus injections of large amounts of material, which are probably less efficient because of the rapid renal clearance of molecules delivered in this fashion (33), may prove unnecessary for treatment. Accordingly, cost-effective tumor inhibition at clinically achievable oligomer concentrations may prove quite feasible.

We presume that the cause of death in mice treated with the antisense c-myb DNA was due to direct or indirect effects of the animals' leukemic cell burden. It is highly unlikely that toxic effects of the oligomers contributed to the demise of these animals since it has been reported (33, 34) that mice may be given much higher doses of phosphorothioate oligodeoxynucleotides than we employed without apparent ill effect. In addition, since we were targeting the human c-*myb* gene, not the mouse c-*myb* gene, direct effects on mouse hematopoiesis were not observed nor would they be expected. The human and mouse c-*myb* sequences differ by 5 nt (18, 35) in the region targeted, and Anfossi *et al.* (5) have shown that as little as a 2-base-pair mismatch is enough to result in loss of antisense-DNA-mediated inhibition of c-*myb* gene function (5). These c-*myb* gene sequence differences also preclude us from accurately predicting potential toxicity of the antisense treatment in a human patient. Differential sensitivity of normal and leukemic cells has been reported *in vitro* (10), but only *in vivo* clinical trials will allow determination of the therapeutic index of this molecule.

Although none of the antisense-treated animals were cured, it is useful to emphasize that actual treatment duration was short in these pilot studies. One could fairly hypothesize that longer or repeated infusions of even higher doses of the antisense DNA might effect longer disease-free intervals. One could also hypothesize that survival might be improved if antisense DNA were delivered after disease reduction with more conventional therapy. Regardless, the optimal use of antisense oligodeoxynucleotides and their ultimate therapeutic utility in the treatment of human leukemia, either as single agents or in combination with other specifically targeted oligomers or drugs, can only be determined in human disease trials. We believe that the results reported herein support the institution of such trials. It is an unfortunate fact that the vast majority of human leukemias ultimately develop chemotherapy resistance. Alternative treatment strategies are, therefore, sorely needed and targeted gene disruption with antisense oligodeoxynucleotides may prove useful in this regard. Finally, since most leukemias are not known to express a single disease-specific gene of pathogenetic importance, such as *bcr–abl*, the ability to disrupt other critical but more widely expressed genes may prove an important underpinning in the therapeutic strategy upon which the use of these molecules will be built.

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