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Suppression of Philadelphia[1] leukemia cell growth in mice by *BCR–ABL* antisense oligodeoxynucleotide

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ABSTRACT **When injected into SCID mice, the Philadelphia chromosome-positive chronic myeloid leukemia-blast crisis cell line BV173 induces a disease process closely resembling that seen in leukemia patients. At 1 and 3 weeks after injection of 10^6 BV173 cells, $CD10^+$ cells were detected in the bone marrow of the mice, leukemic colonies grew from bone marrow and spleen cell suspensions, and *BCR–ABL* transcripts were detectable in bone marrow, spleen, peripheral blood, liver, and lungs. Systemic treatment of the leukemic mice with a 26-mer *BCR–ABL* antisense oligodeoxynucleotide (1 mg/day for 9 days) induced disappearance of $CD10^+$ and clonogenic leukemic cells and a marked decrease in *BCR–ABL* mRNA in mouse tissues. Untreated mice or mice treated with a *BCR–ABL* sense oligodeoxynucleotide or a 6-base-mismatched antisense oligodeoxynucleotide were dead 8–13 weeks after leukemia cell injection; in marked contrast, mice treated with *BCR–ABL* antisense oligodeoxynucleotide died of leukemia 18–23 weeks after injection of leukemic cells. These findings provide evidence for the *in vivo* effectiveness of an anticancer therapy based on antisense oligodeoxynucleotides targeting a tumor-specific gene.**

The Philadelphia chromosome (Ph^1) translocation found in most chronic myelogenous leukemia (CML) patients and in a cohort of acute lymphocytic leukemia (Ph^1 ALL) patients (1) results in the juxtaposition of the *ABL* gene, normally residing on chromosome 9 (2), and the *BCR* gene on chromosome 22. Distinct *BCR–ABL* mRNAs are produced from the splicing of either the first, second, or third exon of the *BCR* gene to the second exon of *ABL* (3, 4). The *BCR–ABL* genes contribute to the pathogenesis of CML, as CML-like syndromes develop in mice injected with constructs encoding the BCR–ABL fusion products (5, 6).

Synthetic oligodeoxynucleotides (ODNs) complementary to either the b2/a2 or the b3/a2 breakpoint junction simultaneously suppress Ph^1 cell proliferation *in vitro* and spare the growth of normal progenitors (7).

Using an *in vivo* model of leukemia in which immunodeficient (SCID) mice injected with human leukemic cells develop a disease process closely resembling that in humans (8), we now show that *BCR–ABL* antisense phosphorothioate ODNs ([S]ODNs) effectively suppress leukemia growth *in vivo*.

METHODS

Cell Lines. The Ph^1 BV173 cell line (9) and the promyelocytic leukemia HL-60 cell line (10) were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum.

Mice and Leukemia Cell Inoculation. SCID mice obtained from Taconic Farms were maintained under sterile conditions by the Laboratory Animal Services at Thomas Jefferson University. Male SCID mice (6–8 weeks old) were injected intravenously with 10^6 BV173 or HL-60 cells in 0.2 ml of RPMI 1640 medium.

Single-Cell Suspensions. Single-cell suspensions from peripheral blood, bone marrow, and spleen were obtained as described (11).

Flow Cytometry. Cells (10^5 in 20 μl) were stained with fluorescein isothiocyanate-conjugated mouse anti-CD10 monoclonal antibody (Becton Dickinson) to human CD10 (also known as common ALL antigen, CALLA) and analyzed by flow cytometry with an EPICS Profile analyzer (Coulter). The assay detected 10^3 BV173 cells mixed with 10^5 murine marrow cells (sensitivity = 10^{-2}).

Colony Assay. Marrow cells from healthy SCID mice did not form colonies in methylcellulose (HCC 4230, Terry Fox Laboratory, Vancouver) in the absence of growth factors. By contrast, BV173 cells formed numerous, rapidly growing colonies. As few as 10^2 BV173 cells among 10^5 murine bone marrow cells were detectable in clonogenic assays (sensitivity = 10^{-3}). Reverse transcription–polymerase chain reaction (RT–PCR) analysis for detection of *BCR–ABL* (b2/a2) transcripts in individual colonies confirmed that the colonies analyzed were leukemic.

Detection of 210-kDa BCR–ABL Protein. For detection of BCR–ABL protein, BV173 cells (10^5 per ml in 25 ml) were untreated or exposed to *BCR–ABL* ODNs (15 μM) for 60 hr. Lysates (100 μl) of 10^6 cells from each culture were centrifuged at 11,000 × *g* at 4°C for 15 min and postnuclear supernatants were collected. Sample separation by SDS/PAGE, electroblotting to nitrocellulose, probing with the 8E9 mouse anti-ABL antibody (gift of R. Arlinghaus, M.D. Anderson Cancer Center, University of Texas, Houston), and protein detection were as described (12).

RT–PCR Analysis. RNA extraction, RT, PCR amplification, and hybridization with specific ^{32}P-end-labeled probes were as described (13). Use of RT–PCR enabled detection (7-day exposure to x-ray film with intensifying screen at −70°C) of 10^2 BV173 cells in a mixture of 10^6 murine cells and BV173 cells (assay sensitivity = 10^{-4}).

[S]ODN Treatment. [S]ODNs were prepared on an Applied Biosystems model 390Z automated synthesizer (14). For *in vitro* studies, BV173 or HL-60 cells were cultured in the presence of [S]ODNs as described (13). For *in vivo* studies, mice were injected intravenously with 26-mer (sense, 6-base-mismatched or antisense) [S]ODNs at 1 mg/day for 9 days.

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Abbreviations: ALL, acute lymphocytic leukemia; CML, chronic myelogenous leukemia; ODN, oligodeoxynucleotide; [S]ODN, phosphorothioate ODN; RT, reverse transcription.

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Tissue Distribution of Intact [S]ODNs. Mice were injected intravenously with b2/a2 antisense [S]ODN (1 mg/day, containing 1 μg of uniformly ^{35}S-labeled [S]ODN) for 9 days. At various times after the last injection, DNA was isolated from single-cell suspensions of each organ. Equal amounts of DNA (5 μg) were electrophoresed in a 15% polyacrylamide/7 M urea gel and electroblotted onto 0.1-μm Nytran membranes (Schleicher & Schuell). Filters were hybridized with a 26-mer ^{32}P-end-labeled sense ODN at 37°C for 18 hr in 5× standard saline citrate (SSC)/0.1% SDS containing salmon sperm DNA (100 μg/ml), washed twice in 2× SSC/0.1% SDS at 37°C, and autoradiographed.

Tissue Distribution of ^{35}S-Labeled [S]ODNs. Tissues of injected mice were solubilized in Soluene-350 (Amersham; 1 ml/0.5 g) and mixed with 4 ml of scintillation fluid (Hydrocount LSD, Baker), and radioactivity was measured in a Beckman LS7500 liquid scintillation counter. [S]ODN concentrations (μmol/g of tissue) were derived by dividing cpm/g of tissue by the specific activity of the injected [S]ODN (cpm/μmol). Since tissues have a density near 1 g/ml, data are presented as molarities.

RESULTS

Inhibition of BV173 Cell Proliferation by *BCR–ABL* Antisense [S]ODNs. To assess their *in vitro* sensitivity to b2/a2 antisense [S]ODNs, BV173 cells were cultured in the presence of the b2/a2 antisense [S]ODN (5′-CGC-TGA-AGG-GCT-TCT-TCC-TTA-TTG-AT-3′), a mismatched [S]ODN (5′-CGG-TGT-ACG-GCA-TCT-TCG-TTA-TTC-AT-3′; six mismatches underlined), or the sense [S]ODN (5′-ATC-AAT-AAG-GAA-GAA-GCC-CTT-CAG-CG-3′). Only the b2/a2 antisense [S]ODN inhibited the proliferation of BV173 cells (Fig. 1*A*). After 9 days in culture, inhibition was complete at concentrations as low as 3 μM, >50% at 1.5 μM, but no longer detectable at lower concentrations (Fig. 1*A*). Specificity of the inhibition was confirmed by the lack of any effect of b2/a2 antisense [S]ODN on proliferation of HL-60 cells, in which the *ABL* locus is not translocated (Fig. 1*B*).

Inhibition of BV173 cell proliferation by b2/a2 antisense [S]ODN was accompanied by a specific decrease in p210 BCR–ABL protein (Fig. 1*A*).

Disease Process in SCID Mice Injected with BV173 Cells. We injected 10^6 BV173 cells into the tail vein of SCID mice and monitored the disease process at various times after injection by (*i*) immunofluorescence analysis of cells positive for CD10, expressed by BV173 cells but not HL-60 cells; (*ii*) clonogenic assay in which BV173 cells, but not murine cells, form colonies in the absence of hematopoietic growth factors; and (*iii*) RT–PCR for detection of b2/a2 transcripts in total RNA isolated from murine tissues.

At 7 days after injection, RT–PCR revealed leukemic cells only in bone marrow cell, spleen cell, and peripheral blood lymphocyte suspensions. A few leukemic colonies (6.0 ± 4.2) formed only from bone marrow cell suspensions, and immunofluorescence studies did not detect leukemic cells. At 21 days, RT–PCR revealed the presence of leukemic cells in bone marrow, spleen, peripheral blood, liver, and lungs, but not in brain (Fig. 2). Numerous leukemic colonies (284 ± 52) formed from bone marrow and few (4.0 ± 1.4) from spleen. At least 6 ± 4% of marrow cells were CD10$^+$. Thus, SCID mice injected with 10^6 leukemic cells have a lower leukemic load at 7 days than at 21 days.

***In Vivo* Effects of *BCR–ABL* [S]ODNs on Leukemia Progression in SCID Mice.** Male SCID mice (8–10 weeks old, 20–22 g) were injected intravenously with 1 mg of sense or antisense b2/a2 BCR–ABL [S]ODN each day for 9 days beginning either 7 days (group +7) or 21 days (group +21) after injection of 10^6 BV173 cells. Clonogenic assays and flow cytometry analysis of bone marrow cell suspensions from an untreated mouse or a mouse treated with either sense or antisense [S]ODN beginning 7 days after injection of 10^6 BV173 cells and sacrificed 12 days after the last treatment (28 days of leukemia growth) indicated leukemic cells in the untreated and sense [S]ODN-treated mice but not after injection of *BCR–ABL* antisense [S]ODN (Table 1, group +7, day 28). Amplification of *BCR–ABL* transcripts in RNA isolated from peripheral blood, spleen, bone marrow, liver, lung, and brain

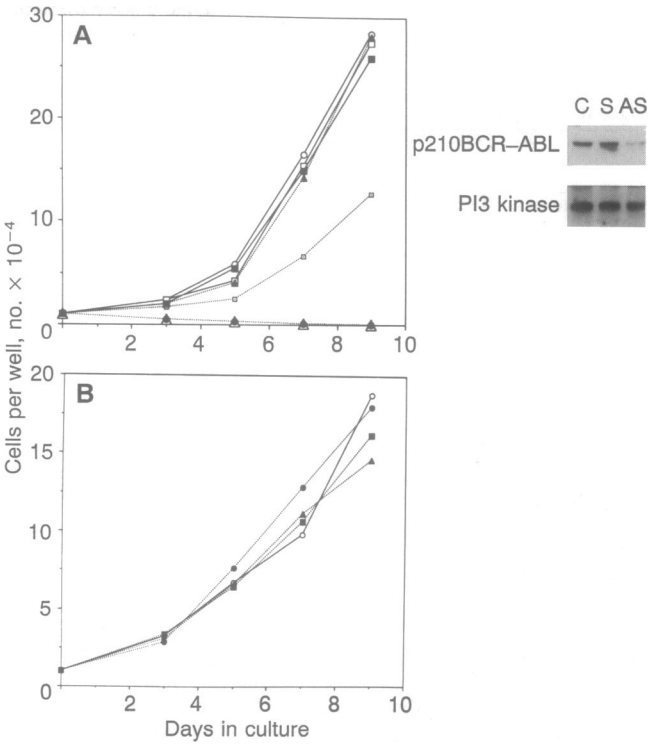

FIG. 1. Effects of b2/a2 antisense [S]ODN on BV173 proliferation and *BCR–ABL* expression. BV173 cells (10^5 per ml) were untreated (○) or exposed to b2/a2 sense [S]ODN (□; final concentration, 15 μM), b2/a2 antisense [S]ODN (●, △, ⊡, and ▲; final concentrations, 15, 3, 1.5, and 0.75 μM, respectively), or a 6-base-mismatched b2/a2 antisense [S]ODN (■; final concentration, 15 μM). For detection of BCR–ABL protein (*Right*), BV173 cells (10^5 per ml in 25 ml) were untreated (control, C) or exposed to *BCR–ABL* sense (S) or antisense (AS) [S]ODN (15 μM) for 60 hr. Levels of the p85 subunit of phosphatidylinositol 3-kinase (PI3 kinase) were measured as a control for amount of protein loaded. (*B*) HL-60 cells were untreated (○) or exposed to b2/a2 antisense [S]ODN (■, ●, and ▲; 10, 20, and 30 μM, respectively).

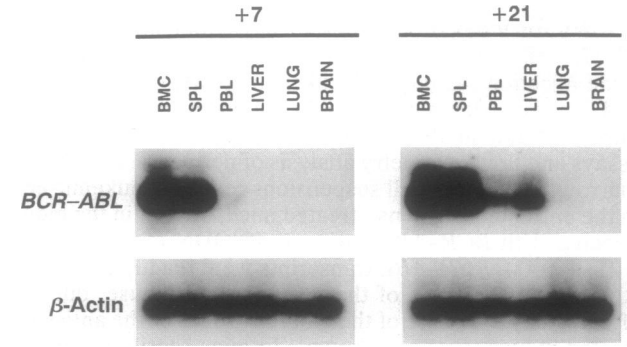

FIG. 2. *BCR–ABL* b2/a2 transcripts in SCID mouse tissues 7 days (+7) and 21 days (+21) after intravenous injection of 10^6 BV173 cells. *BCR–ABL* and β-actin transcripts were separately detected by RT–PCR and hybridization of the amplified products to specific probes. BMC, bone marrow cells; SPL, spleen cells; PBL, peripheral blood leukocytes.

Table 1. Flow cytometry analysis and leukemic colony formation in SCID mice injected with BV173 cells and treated with *BCR–ABL* antisense (AS) or sense (S) [S]ODN or not treated (C, control)

Group	[S]ODN	Day tested	% CD10$^+$ cells			Leukemic colonies per 10^5 cells		
			PBL	SPL	BMC	PBL	SPL	BMC
+7	C	28	0	0	5.2 ± 2.3	0	3.8 ± 1.0	353.0 ± 52.3
		42	NT	NT	NT	NT	NT	NT
	S	28	0	0	5.2 ± 0.7	0	2.0 ± 1.4	380.0 ± 25.5
		42	1.9 ± 0.2	4.1 ± 0.2	16.8 ± 1.8	7.5 ± 1.6	87.5 ± 7.8	681.5 ± 71.4
	AS	28	0	0	0	0	0	0
		42	0	0	0	0	0	0
+21	C	42	1.0 ± 0.1	1.9 ± 0.1	17.7 ± 1.8	11.0 ± 8.5	80.5 ± 12.0	575.5 ± 83.8
		56	NT	NT	NT	NT	NT	NT
	S	42	0.9 ± 0.1	1.9 ± 0.8	20.0 ± 0.5	14.5 ± 4.9	116.0 ± 29.7	668.5 ± 59.1
		56	2.0 ± 0.3	9.9 ± 1.8	23.9 ± 5.0	51.0 ± 14.1	548.5 ± 128.0	839.5 ± 41.7
	AS	42	0	0	0	0	0	0
		56	0	0	0	0	0	0

On day 0 mice were injected intravenously with 10^6 BV173 cells. [S]ODNs (1 mg per mouse per day) were injected intravenously for 9 days starting on day +7 or +21. Control mice received diluent only. Leukemia growth in the mice was analyzed on days 28 and 42 (group +7) or on days 42 and 56 (group +21) by assessing peripheral blood leukocytes (PBL), spleen cells (SPL), and bone marrow cells (BMC) for CD10 by flow cytometry and for clonogenic growth in methylcellulose. Numbers are mean ± SD from two or four determinations (flow cytometry and clonogenic assays, respectively). NT, not tested.

revealed *BCR–ABL* transcripts in each of these tissues, except brain, in the untreated or sense-treated mouse but not in the antisense-treated mouse (data not shown). In mice sacrificed 26 days after the last [S]ODN injection (42 days of leukemia growth), spleens were enlarged, and distinct leukemic nodules were visible in liver and kidneys of the untreated or sense-treated mouse but not in those of the antisense-treated mouse. Clonogenic assays and flow cytometry analysis of peripheral blood, bone marrow, and spleen cell suspensions demonstrated the presence of leukemic cells in the samples of untreated and sense-treated mice, but not in those from the *BCR–ABL* antisense-treated mouse (Table 1, group +7, day 42).

BCR–ABL (b2/a2) transcripts were detected in all tissues of the sense [S]ODN-treated mouse, but such levels were substantially reduced in bone marrow, spleen, and liver of the antisense [S]ODN-treated mouse (Fig. 3, group +7, day 42). To exclude the possibility that residual *BCR–ABL* antisense [S]ODN interfered with reverse transcriptase, preventing detection of *BCR–ABL* transcript, we performed RT–PCRs with human-specific primers corresponding to the 3′ *ABL* portion of *BCR–ABL* mRNA. *BCR–ABL* transcripts were readily detected only in the various tissues of the sense-treated mouse (data not shown), indicating that the markedly reduced levels of *BCR–ABL* transcripts from tissues of antisense-treated mice reflected a striking reduction in the number of infiltrating leukemic cells.

In mice treated with *BCR–ABL* [S]ODN (1 mg per mouse per day for 9 days) beginning 21 days after injection of 10^6 BV173 cells and analyzed 12 days after the last oligonucleotide injection (42 days of leukemia growth), splenomegaly and liver metastases were visible in control and sense-treated mice, but not in the antisense-treated mouse. Clonogenic assays and flow cytometry analysis of peripheral blood, bone marrow, and spleen cell suspensions revealed leukemic cells in the untreated and sense-treated mice, but not in the mouse injected with *BCR–ABL* antisense [S]ODN (Table 1, group +21, day 42). *BCR–ABL* transcripts were readily detected in RNA from all tissues of the sense-treated mouse, but only after a 7-day exposure of the blot for tissues of the antisense-treated mouse (data not shown). In mice sacrificed 26 days after the last [S]ODN injection (56 days of leukemia growth), leukemic colonies and CD10$^+$ cells were detected in bone marrow, spleen cell, and peripheral blood lymphocyte suspensions of the mouse treated with sense [S]ODN but not in similar preparations from the antisense-treated mouse (Table 1, group +21, day 56). The mouse treated with sense [S]ODN had numerous metastases in the liver, a few metastases in the kidneys, and splenomegaly (Fig. 4 *Left*), whereas the corresponding organs of the antisense-treated mouse appeared normal (Fig. 4 *Right*). Low levels of *BCR–ABL* transcripts were detected in each tissue except brain of the antisense-treated mouse, but only after a 7-day exposure of the blot (Fig. 3, group +21, day 56).

These results (and additional RT–PCR data from two other mice) indicate that SCID mice injected with 10^6 BV173 cells

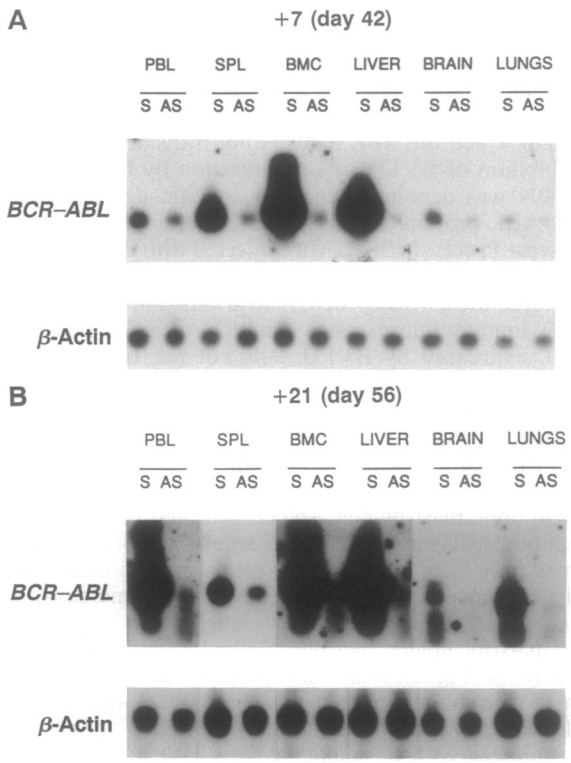

FIG. 3. *BCR–ABL* b2/a2 transcripts in RNA from tissues of sense-treated (S) or antisense-treated (AS) leukemic SCID mice. *BCR–ABL* and β-actin transcripts were separately detected by RT–PCR of RNA isolated from 5×10^5 cells for each tissue sample (abbreviations as in Fig. 2) except peripheral blood (2.5×10^5 cells). Blots were exposed 7 days (*BCR–ABL*) and 2 hr (β-actin), except for the spleen sample of group +21, day 56 (24 hr and 2 hr of exposure, respectively).

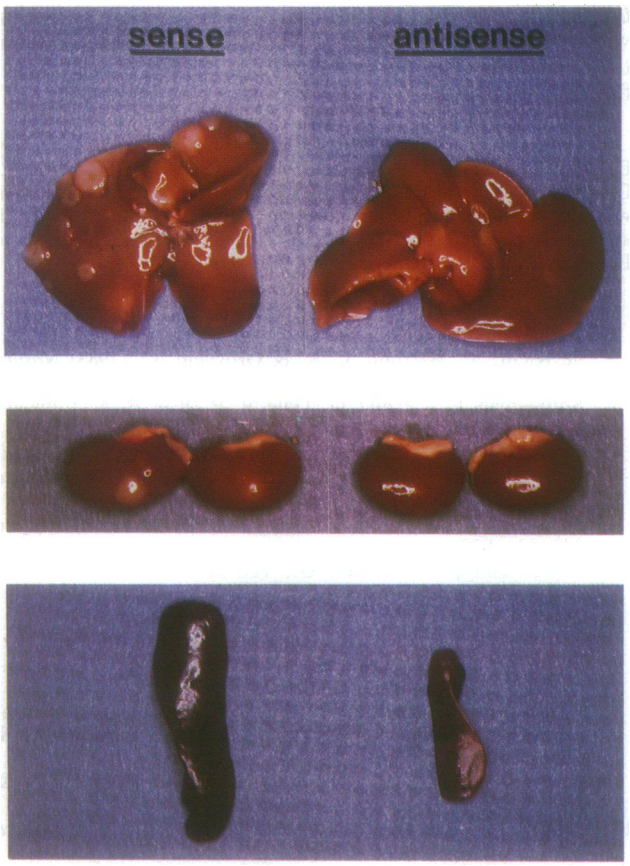

FIG. 4. Leukemic infiltration in livers, kidneys, and spleens of leukemic SCID mice after treatment with *BCR–ABL* sense (*Left*) or antisense (*Right*) [S]ODN.

and then treated intravenously with *BCR–ABL* antisense [S]ODN had only molecular evidence of "minimal residual disease" at 42 and 56 days after injection of leukemic cells. By contrast, untreated and sense [S]ODN-treated mice had macroscopic, microscopic, and molecular evidence of diffuse leukemia.

These differences among the groups of mice were reflected in their mortality rates; all 9 untreated, 10 *BCR–ABL* sense-treated, and 10 *BCR–ABL*-mismatched-[S]ODN-treated leukemic mice died with diffuse leukemia, as confirmed by necropsy, 8–13 weeks after intravenous injection of 10^6 BV173 leukemia cells (median survival time, 9.7 ± 0.9 weeks). In marked contrast, the 10 *BCR–ABL* antisense-treated mice died of leukemia 18–23 weeks after injection of 10^6 leukemic cells (Fig. 5*A*) (median survival time, 19.4 ± 1.4 weeks; $P < 0.001$ compared with control groups). Thus, the 9-day schedule of treatment with *BCR–ABL* [S]ODN did not eradicate leukemia but did prolong survival. Similar experiments using SCID mice injected with HL-60 cells demonstrated the specificity of the *in vivo* effects of *BCR–ABL* antisense [S]ODN, all 10 untreated and 10 antisense-treated mice (starting from day +7 or +21; 5 mice in each group) died 7–9 weeks after intravenous injection of 10^6 HL-60 cells (Fig. 5*B*).

Distribution of *BCR–ABL* Antisense [S]ODN in Mouse Tissues. *BCR–ABL* antisense [S]ODN molecules injected into mice were distributed, in intact form, throughout the body but became concentrated in the liver as shown by blot hybridization of tissue DNA isolated 24 and 72 hr after the last injection (Fig. 6). Lower levels were detected in all the other organs, except brain (Fig. 6). Accumulation of *BCR–ABL* [S]ODN in various organs was also assessed by measuring the amount of ^{35}S-labeled material in weighed organ

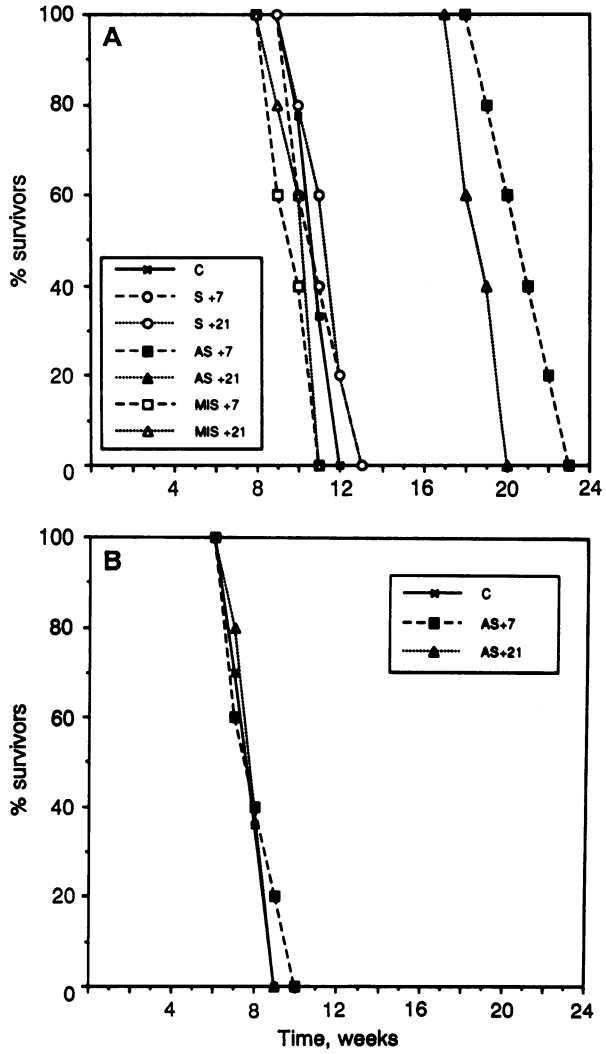

FIG. 5. Survival of *BCR–ABL* [S]ODN-treated leukemic SCID mice. (*A*) Mice injected with 10^6 BV173 cells were treated 7 or 21 days later (+7 or +21) with *BCR–ABL* sense (S), mismatched (MIS), or antisense (AS) [S]ODN. Control mice (C) received diluent only. (*B*) Irradiated mice (350 cGy) were injected 24 hr later with 10^6 HL-60 leukemic cells and treated 7 or 21 days later with *BCR–ABL* antisense (AS) [S]ODN. Control mice (C) received diluent only.

samples; tissue concentrations correlated with the relative levels of intact [S]ODN detected in the same tissues and ranged from 3 to 26 μM (Fig. 6).

DISCUSSION

The sequence specificity of the effects of *BCR–ABL* antisense [S]ODN described here strongly supports an antisense mechanism of action as opposed to other processes reported for phosphorothioate-modified nucleic acids such as inhibition of polymerases (15), induction of interferon (16), or protein binding (17), although adventitious interference with transcription (18) cannot be ruled out. The *in vivo* antileukemic effects correlated well with our estimates of *BCR–ABL* [S]ODN concentrations reached in mouse tissues, indicating that in all tissues, except brain, the concentration of ^{35}S-labeled material was close to or higher than 3 μM. The absence of detectable amounts of hybridizable [S]ODN in the brain is consistent with the reduced levels of *BCR–ABL* transcripts in some of the antisense-treated mice as compared with the sense-treated mice (Fig. 3), since leukemic cells were most likely affected by the [S]ODN before reaching the

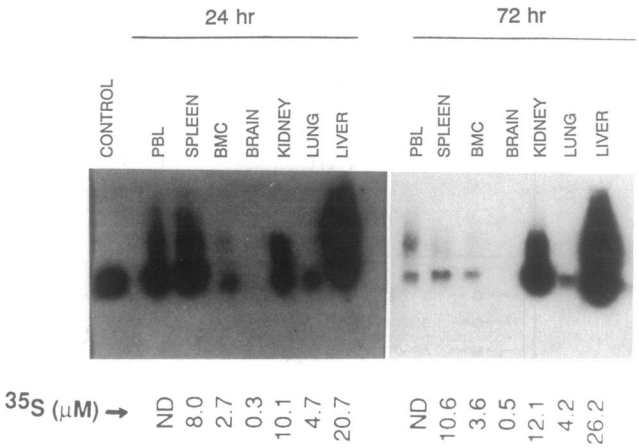

FIG. 6. Tissue distribution of *BCR–ABL* b2/a2 antisense [S]ODN in SCID mice. The control lane contained ≈10 ng of stock [S]ODN used for the intravenous injection. ^{35}S concentrations are given at the bottom. ND, not done. Tissues are abbreviated as in Fig. 2.

brain. Even though a portion of the ^{35}S-labeled material presumably represents some degraded [S]ODNs, our own findings of intact *BCR–ABL* antisense [S]ODN in various tissues up to 2 weeks after termination of the treatment (data not shown) suggest that a significant amount of the injected [S]ODN remains intact. Since our *in vitro* experiments indicate a highly significant antileukemic effect of *BCR–ABL* antisense [S]ODN at concentrations as low as 1.5 μM (Fig. 1), it is not surprising that a significant antileukemic effect was also observed *in vivo*.

The leukemic process was only temporarily suppressed in SCID mice, even though BV173 cells are exquisitely sensitive to BCR–ABL deprivation, perhaps reflecting the absence of a normal *ABL* gene product in the cells (unpublished observations), and undergo apoptosis following down-regulation of *BCR–ABL* expression (19).

Mice treated with *BCR–ABL* antisense [S]ODN revealed only a temporary weight loss after completion of [S]ODN injection but showed no significant morphological alterations in major organs, as analyzed by histopathology, and no inflammatory response. These observations suggest the favorable pharmacological properties of antisense [S]ODNs.

The strategy of suppressing leukemic growth by *BCR–ABL* antisense ODNs is based on the specific inhibition of the expression of genes that confer a growth advantage to neoplastic cells. By contrast, most anticancer treatments exploit differences in metabolic processes (e.g., growth rate) between normal and tumor cells rather than specific effects of a drug on genetically defined characteristics of the neoplastic cells. Owing to their selective effect, ODNs complementary to the unique breakpoint sequences of the *BCR–ABL* transcripts of Ph^1 leukemia, either alone or in combination with other oncogene-targeted ODNs or with more conventional antileukemic therapy (20), appear to hold promise for future clinical use.

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