

Bcl-2 Antisense Oligodeoxynucleotide Therapy of Epstein-Barr Virus-associated Lymphoproliferative Disease in Severe Combined Immunodeficient Mice¹

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Abstract

Bcl-2 is upregulated by Epstein-Barr virus (EBV) in immortalized lymphoblastoid (LCL) B cells and is expressed in the majority of EBV-associated posttransplant lymphoproliferative disorders (PTLDs). Given the antiapoptotic function and chemoprotective effects of Bcl-2, it represents a rational target for modulation using antisense oligodeoxynucleotides in Bcl-2-expressing, EBV-associated lymphoproliferative disorders. Using a fully phosphorothioated oligodeoxynucleotide targeted to the first six codons of Bcl-2, we examined the effects of Bcl-2 antisense both *in vitro* in LCLs and *in vivo* in the human/severe combined immunodeficient chimeric model of EBV-associated lymphoproliferative disorders. *In vitro* treatment of LCLs with Bcl-2 antisense in the presence of cationic lipid was associated with decreased expression of Bcl-2 protein, inhibition of proliferation, and stimulation of apoptotic cell death; these effects were sequence-dependent. Furthermore, treatment of LCL-bearing severe combined immunodeficient mice with Bcl-2 antisense but not control oligodeoxynucleotides completely prevented or significantly delayed the development of fatal EBV-positive lymphoproliferative disease *in vivo*. These studies demonstrate that Bcl-2 antisense oligodeoxynucleotides mediate sequence-dependent antitumor effects in EBV-associated B-cell lymphoproliferations both *in vitro* and *in vivo*. These findings suggest that Bcl-2 antisense therapy may represent a novel antitumor treatment strategy for EBV-associated PTLDs and other Bcl-2-expressing, EBV-positive malignancies.

Introduction

EBV³ is causally associated with PTLDs and is believed to play a central role in the pathogenesis of these malignancies (1). The biological activity of EBV that links it to lymphomagenesis is its capacity to transform resting B cells to immortalized lymphoblastoid cells that proliferate indefinitely and harbor the virus in a latent state (2, 3). Latent EBV infection is characterized by restricted expression of viral gene products, including six nuclear antigens (EBNA-1, -2, -3a, -3b, -3c, and -LP) and two transmembrane proteins (LMP-1 and -2) that function cooperatively to initiate and maintain transformation (4, 5). Although the precise role of each of these latent gene products in transformation is not fully understood, they mediate their transforming functions by constitutively activating cellular genes that are involved in physiological B-cell activation, proliferation, and survival.

Although transformation of B cells by EBV requires the expression of at least five latent viral genes (*EBNA-1*, *EBNA-2*, *EBNA-3a*, *EBNA-3c*, and *LMP-1*) and cannot be mediated by a single viral gene (3, 6–9), *LMP-1* most closely mimics a classical oncogene. *LMP-1* is

capable of transforming immortalized rodent fibroblasts, rendering them tumorigenic *in vivo* (10, 11), and in B cells, *LMP-1* confers a phenotype resembling activated lymphocytes (12, 13). An important oncogenic function of *LMP-1* is to protect cells from apoptotic cell death (14–16). The antiapoptotic function of *LMP-1* is mediated by up-regulation of cellular antiapoptotic genes (15, 16). Gene transfer studies in EBV-negative Burkitt cells have demonstrated that *LMP-1* induces Bcl-2 expression, in the absence of other latent viral genes, and that this effect confers resistance to apoptosis (15, 16). Thus, *LMP-1*-mediated induction of Bcl-2 expression plays a key role in EBV transformation by promoting cell survival.

The majority of PTLDs are EBV-positive and express both *LMP-1* and abundant quantities of Bcl-2 (17, 18). Although Bcl-2 expression in follicular lymphomas bearing the t(14;18) translocation is recognized as an important pathogenetic feature, the pathogenetic significance of Bcl-2 expression in PTLD is less clear. Nonetheless, given the role of Bcl-2 in conferring resistance to apoptotic cell death and protecting cells from the cytotoxic effects of an array of chemotherapeutic agents, it represents a logical target for modulation using antisense strategies in *LMP-1*- and Bcl-2-expressing human PTLDs as well as in other Bcl-2-expressing EBV-associated malignancies.

Recent preclinical and clinical studies using a fully phosphorothioated antisense oligodeoxynucleotide targeted to the first six codons of the Bcl-2 open reading frame have demonstrated its efficacy as a chemosensitizing agent in a mouse melanoma model and its lack of toxicity in animals and humans (19, 20). Using this oligodeoxynucleotide, we have investigated the antitumor effects of Bcl-2 antisense in EBV-associated lymphoproliferative disease using the SCID/human chimeric model of PTLD. In this animal model, injection of established LCLs intraperitoneally in SCID mice gives rise to fatal EBV-positive lymphoid tumors that exhibit characteristics of EBV-positive PTLD in allograft recipients (21, 22). These EBV-positive tumors are human B cell in origin, usually involve the abdominal cavity and viscera, and histopathologically resemble diffuse large-cell or immunoblastic lymphomas. They may be monoclonal, oligoclonal, or polyclonal, and express both lytic and latent viral gene products, including *LMP-1*. Using this animal model of PTLD, we have shown that Bcl-2 antisense treatment of LCL-bearing SCID mice had a dramatic antitumor effect *in vivo* by preventing or delaying the development of fatal lymphoproliferative disease in these animals. Our *in vitro* studies demonstrated that Bcl-2 antisense treatment was associated with protein-specific declines in Bcl-2 protein and stimulation of apoptosis in LCLs, which suggested an antisense-mediated mechanism of action. These findings suggest that Bcl-2 antisense oligodeoxynucleotide therapy may represent a novel and potentially nontoxic treatment strategy for EBV-associated PTLD.

Materials and Methods

Cell Lines. 11-23 and Sweig are EBV-immortalized LCLs. 11-23 was derived in this laboratory by infecting umbilical cord lymphocytes with the FF41 strain; Sweig was derived by infecting adult B cells with the B958 strain and was a generous gift of Tyler Curiel (Baylor Institute for Immunology

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³ The abbreviations used are: EBV, Epstein-Barr virus; LCL, lymphoblastoid cell line; LMP-1, latent membrane protein; NK, natural killer; PTLD, posttransplant lymphoproliferative disorder; SCID, severe combined immunodeficient/immunodeficiency; MM, mismatch; RV, reverse.

Research, Houston, TX). BJAB is an EBV-negative Burkitt-like line and was provided by William Summers (Yale University, New Haven, CT). All of the cells were maintained in RPMI-1640 plus 10% FCS.

Incubation of Cells with Oligodeoxynucleotides. Eighteen-mer fully phosphorothioated oligodeoxynucleotides corresponding to the first six codons of the human Bcl-2 open reading frame were provided by Genta, Inc. (San Diego, CA). The sequences of the antisense and control oligodeoxynucleotides are as follows: antisense (G3139), 5'-TCTCCCAGCGTGCGCCAT-3'; RV control (G3622), 5'-TACCGCGTGCACCCTCT-3'; and two-base MM control (G4126), 5'-TCTCCCAGCATGTGCCAT-3'. The lyophilized oligodeoxynucleotides were resuspended in water immediately prior to use.

For the *in vitro* studies, cells in log phase of growth (2×10^5 /ml) were cultured with or without oligodeoxynucleotide in RPMI-1640 plus 10% FCS for 3 or 4 days. Cells were exposed to oligodeoxynucleotide in the presence of cationic lipid to enhance uptake for 8 h daily. Each day, the medium was removed, and cells were cultured with oligodeoxynucleotide (0.2, 1.0, or 10 μ M) in the presence of commercially available cationic lipid (Eu-Fectin 8; JBL, San Luis Obispo, CA) in serum-free artificial medium (Opti-MEM I; Life Technologies, Inc., Gaithersburg, MD). After 8 h of exposure to oligodeoxynucleotide and cationic lipid, the serum-free medium was removed, and the cells were placed in RPMI-1640 with 10% FCS. For untreated controls, cells were cultured with lipid in serum-free medium in the absence of oligodeoxynucleotides for 8 h daily.

Cellular Proliferation Assays. Cells (2×10^5 /ml) were plated in triplicate in 200 μ l of medium in microtiter wells and cultured for 72 h with and without oligodeoxynucleotides, as described above. During the last 16 h of culture, each well was pulsed with 1 μ Ci of [3 H]thymidine. Cells were harvested with a multiple automated sample harvester (Cambridge Technology, Cambridge, MA), and incorporation of [3 H]thymidine was measured by standard scintillation counting and was expressed as the mean \pm the SD of triplicate assays. The two-tailed unpaired *t* test was used to determine the significance of differences in [3 H]thymidine incorporation between antisense oligodeoxynucleotide- and control oligodeoxynucleotide-treated cells.

Immunoblotting. The preparation of cell extracts, electrophoresis, and transfer were carried out as described previously (23). In brief, exactly 2×10^5 viable cells were used for each condition; cell viability was determined by trypan blue exclusion after 72 h of culture with or without oligodeoxynucleotide, as described previously (23). Total protein from whole cell lysates was resolved by SDS-PAGE and transferred electrophoretically to nitrocellulose paper. The immunoblotting procedure was performed according to the manufacturer's protocol for the chemiluminescent detection of proteins using the Phototope-HRP Western Blot Detection kit (New England Biolabs, Beverly, MA). The following primary antibodies were used: for Bcl-2 detection, mouse monoclonal antibody was purchased from Alexis (San Diego, CA); and for actin detection, goat polyclonal antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Relative amounts of Bcl-2 protein were quantitated by densitometric analysis of the protein bands and normalized to actin (Molecular Dynamics, Sunnyvale, CA).

Apoptosis Assays. To detect and quantitate apoptosis, a flow cytometric assay based on quantitating DNA breaks was used to measure apoptosis (24). This method utilizes terminal deoxynucleotide transferase and bromo-dUTP to label exposed 3'-OH DNA ends in fixed cells; bromodeoxyuridine-tagged DNA is then quantitated by flow cytometry using fluorescein-conjugated anti-bromodeoxyuridine antibody. Nonapoptotic cells do not incorporate significant amounts of bromo-dUTP because of the lack of exposed 3'-OH DNA ends and, consequently, have relatively little fluorescence compared with apoptotic cells, which have an abundance of 3'-OH ends. The assay was performed with the APO-BRDU kit (Pharmingen, San Diego, CA) according to the manufacturer's protocol using 1×10^6 cells per sample after culture with or without oligodeoxynucleotide for 72 or 96 h. The percentage of cells stimulated to undergo apoptosis by antisense and/or drug treatment was calculated as follows: (percentage of apoptotic treated cells - percentage of apoptotic control cells)/(100 - percentage of apoptotic control cells) \times 100.

Evaluation of Oligodeoxynucleotide Effects *In Vivo*. Female SCID/NCR mice, 5 to 7 weeks old, were obtained from the National Cancer Institute breeding colony (Bethesda, MD) and housed in a pathogen-free environment. Food supplies and instruments were autoclaved, and all manipulations were performed in a laminar-flow hood. Animals were randomly assigned to experimental groups ($n = 6-7$). Each mouse was injected i.p. with 20,000,000

Sweig cells in 0.5 ml of sterile PBS. Animals were monitored daily and killed when they developed clinical signs of disease. All of the animals were subjected to necropsy to determine the gross pattern of tumor development; tumor and organ specimens from selected animals from each treatment group were processed for histology. Animals that did not develop clinical signs of disease were killed at 185 days (experiment 1) and 168 days (experiment 2) after cell injection and were examined for gross tumor. The mice were randomized to receive Bcl-2 antisense G3139, RV control oligodeoxynucleotide, MM control oligodeoxynucleotide, or placebo (sterile saline). The oligodeoxynucleotides were administered i.p. at a dose of 10 mg/kg/day for 12 days (total dose, 125 mg/kg) in five divided doses at 72-h intervals beginning on day 1 (or day 10 in experiment 2) after the injection of LCLs. Each dose of oligodeoxynucleotide was administered in 0.5 ml of sterile saline.

Statistical Analysis. The end point for the therapeutic trial was survival, based on the day of sacrifice for each animal. Survival of each group was described by a Kaplan-Meier plot. Experimental groups were statistically compared with the log-rank (Mantel-Cox) test for analysis of mortality data.

Results

Effect of Bcl-2 Antisense on Proliferation and Apoptosis of LCLs *In Vitro*. Previous investigators have shown that Bcl-2 antisense treatment of some Bcl-2-expressing cell lines is associated with nearly complete loss of Bcl-2 mRNA within 24 h and reduced expression Bcl-2 protein by 48 h (25, 26). To determine whether Bcl-2 antisense treatment of EBV-positive LCLs is associated with decreased Bcl-2 expression, two different LCLs were treated with Bcl-2 antisense in the presence of cationic lipids to enhance uptake for 72 h. Antisense treatment (1.0 μ M or 0.2 μ M) was associated with a marked decline in Bcl-2 protein compared with untreated cells (>90%), MM control oligodeoxynucleotide-treated cells (>80%), and RV control oligodeoxynucleotide-treated cells (>90%; Fig. 1). The antisense effect was sequence-dependent, inasmuch as the control oligodeoxynucleotides had no effect (RV) or minimal effect (MM; <25%) on Bcl-2 levels, and protein-specific, inasmuch as there was no effect on actin (Fig. 1) or other nonspecific proteins seen on long exposure of the autoradiograph (not shown).

Expression of Bcl-2 has been shown to protect EBV-transformed B cells from apoptotic cell death (14-16). To determine whether the marked decline in Bcl-2 protein levels in antisense-treated LCLs

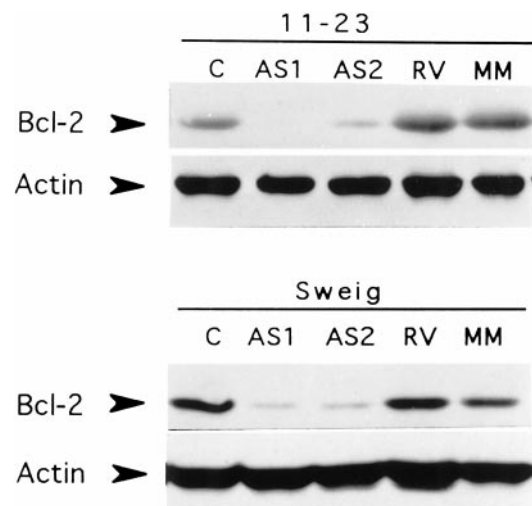


Fig. 1. Effect of Bcl-2 antisense oligodeoxynucleotide treatment on Bcl-2 protein levels in EBV-positive LCLs. Western blot analysis of Bcl-2 expression in two EBV-positive LCLs, 11-23 and Sweig, was performed after exposure to oligodeoxynucleotide for 72 h. C, untreated, cultured in cationic lipid without oligodeoxynucleotide; AS1, Bcl-2 antisense-treated (1.0 μ M); AS2, Bcl-2 antisense-treated (0.2 μ M); RV, RV oligodeoxynucleotide-treated (0.2 μ M); MM, MM oligodeoxynucleotide-treated (0.2 μ M). Blots were sequentially exposed to anti-Bcl-2 antibody and anti-actin antibody.

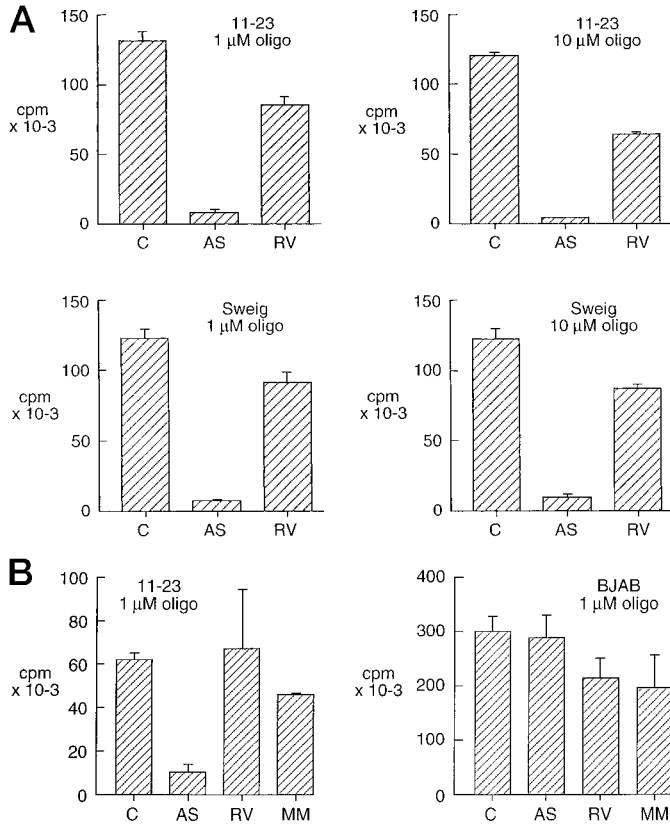


Fig. 2. Effect of Bcl-2 antisense oligodeoxynucleotide treatment on proliferation of EBV-positive LCLs and an EBV-negative, Bcl-2 negative Burkitt line. Proliferation was assayed by [³H]thymidine incorporation in LCLs 11-23 and Sweig (A) and in an EBV-negative Burkitt line (BJAB) and LCL 11-23 (B) after exposure to oligodeoxynucleotide at a concentration of 1 or 10 μM for 72 h. Values represent the mean ± SD of triplicate assays. C, untreated, cultured in cationic lipid without oligodeoxynucleotide; AS, Bcl-2 antisense-treated; RV, reverse oligodeoxynucleotide-treated; MM, MM oligodeoxynucleotide-treated. *P*s for the mean difference between AS-treated and untreated control, MM-treated, or RV-treated in 11-23 and Sweig in all assays were <0.004. *P*s for the mean difference between AS-treated and untreated control, MM-treated, or RV-treated in BJAB were >0.06.

affected cell growth or survival, we performed proliferation assays on two different LCLs and on an EBV-negative, Bcl-2-negative Burkitt-like lymphoid line (BJAB). As shown in one of three representative experiments, Bcl-2 antisense treatment (1.0 or 10 μM) of both the 11-23 and Sweig LCLs for 72 h in the presence of cationic lipid significantly inhibited proliferation as measured by thymidine uptake compared with untreated or RV control oligodeoxynucleotide-treated cells (Fig. 2A); similar results were obtained using the MM control oligodeoxynucleotide (Fig. 2B). In contrast, Bcl-2 antisense treatment (1.0 μM) had no significant effect on the proliferation of Bcl-2-negative BJAB cells compared with MM or RV oligodeoxynucleotide-treated cells (Fig. 2B).

To determine whether the antiproliferative effect of Bcl-2 antisense in LCLs was attributable, in part, to apoptotic cell death, oligodeoxynucleotide-treated and -untreated Sweig cells were assayed for apoptosis by terminal deoxynucleotidyl transferase-mediated nick end labeling assay after 72 or 96 h of treatment. Bcl-2 antisense treatment for 72 h stimulated apoptosis relative to untreated cells (15%) or MM control oligodeoxynucleotide-treated cells (18%; Table 1). More prolonged treatment with Bcl-2 antisense (96 h) further increased apoptosis relative to both untreated cells (51–66%) or MM control oligodeoxynucleotide-treated cells (50–54%). In contrast, treatment with control oligodeoxynucleotide had no discernable effect on apoptosis compared with untreated cells (Table 1). Similar results were obtained using RV oligodeoxynucleotide as the control (not shown).

In Vivo Antitumor Effects of Bcl-2 Antisense in the SCID Model of EBV-associated Lymphoproliferative Disease. The antiproliferative and proapoptotic effects of Bcl-2 antisense in LCLs *in vitro* suggested that Bcl-2 antisense oligodeoxynucleotides might have antitumor effects in EBV-positive, Bcl-2-expressing lymphoproliferative diseases. We tested the feasibility of this concept *in vivo* using the human/SCID chimeric model of EBV-associated lymphoproliferative disease in two separate experiments. In pilot studies, we demonstrated that injection of 20,000,000 Sweig cells resulted in the reproducible development of fatal lymphoproliferative disease involving the abdominal cavity and viscera 30–50 days postinjection. These tumors were shown to be EBV-positive by *in situ* hybridization for EBV RNA and expressed both LMP-1 and Bcl-2 by Western blotting (not shown). Morphologically, they resembled diffuse large-cell lymphomas with immunoblastic features.

In the first experiment, animals were injected with Sweig cells and were randomly assigned to the mock-treated (untreated) group (*n* = 7), Bcl-2 antisense treatment (*n* = 7), or control (RV) oligodeoxynucleotide treatment (*n* = 7). Six animals did not receive cell injections and were treated with Bcl-2 antisense. Treatment was started on the day of injection for all of the three groups. The dosage of oligodeoxynucleotide used for these studies (10 mg/kg/day for 12 days) has been shown by other investigators to be well tolerated and unassociated with any adverse effects in SCID or nude mice (19, 27). As expected, we observed no clinical signs of toxicity in the six antisense-treated animals that did not receive cells, and there was no evidence of organ damage at necropsy. All of the animals in the mock-treated (untreated) and RV oligodeoxynucleotide treatment groups died with gross tumor by day 46 after cell injection (Fig. 3). In contrast, five of seven animals in the Bcl-2 antisense treatment group remained alive and without clinical signs of tumor for 185 days after cell injection (Fig. 3). There was no significant difference in survival between the untreated and RV oligodeoxynucleotide treatment arms, with a median survival of 46 days in both of the groups. In contrast, the survival of the Bcl-2 antisense-treatment group was significantly prolonged compared with either the untreated (*P* < 0.001) or control oligodeoxynucleotide-treated (*P* < 0.001) arms. Only two animals in the Bcl-2 antisense group developed tumor (days 87 and 126 postinjection). The remaining five animals were free of disease at the time of sacrifice (day 185).

In the second experiment, animals were injected with Sweig cells and were randomly assigned to one of four groups: mock-treated (untreated) group (*n* = 6); control (RV) oligodeoxynucleotide treatment (*n* = 6); immediate Bcl-2 antisense treatment (*n* = 6); and delayed Bcl-2 antisense treatment (*n* = 6). Treatment was started on the day of cell injection except for the delayed Bcl-2 antisense treatment; the delayed antisense treatment was started on day 10 after cell injection. As in the first experiment, there was no significant difference in survival between the untreated and RV oligodeoxynucleotide-treated animals (median survival, 43 days in both of the groups), whereas the survival of the immediate Bcl-2 antisense treatment group was significantly prolonged compared with the untreated control group (*P* < 0.001) and the RV oligodeoxynucleotide-treated group

Table 1 Percentage of EBV + LCLs induced to undergo apoptosis by Bcl-2 antisense (AS) treatment

Experiment (duration)	% apoptotic cells (untreated)	% apoptotic cells (MM-treated)	% apoptotic cells (AS-treated)	% AS-induced apoptosis ^a
1 (72 h)	20	18	32	15 (18)
2 (96 h)	32	34	62	51 (50)
3 (96 h)	19	29	73	66 (54)

^a The percentage of antisense (AS)-induced apoptosis is calculated relative to untreated controls and MM oligodeoxynucleotide-treated cells (value shown in parentheses).

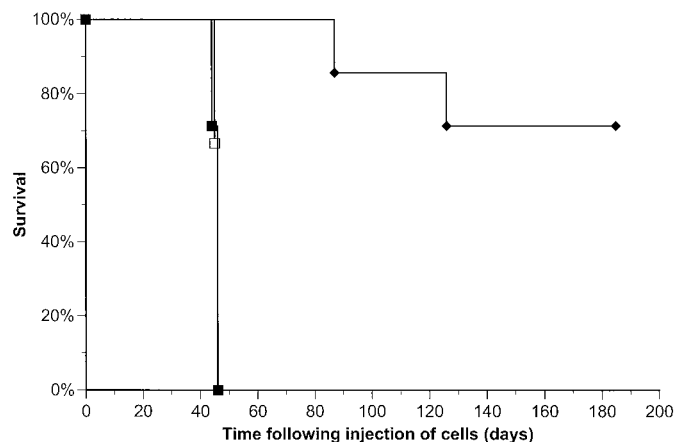


Fig. 3. Effect of Bcl-2 antisense treatment *in vivo* on the survival of LCL (Sweig)-bearing scid mice. Animals were injected with 20,000,000 Sweig cells i.p. on day 1 and treated with Bcl-2 antisense oligodeoxynucleotide ($n = 7$; ◆), RV control oligodeoxynucleotide ($n = 7$; ■), or saline without oligodeoxynucleotide ($n = 7$; □) on days 1, 4, 7, 10, and 13. Animals were killed when they developed clinical signs of tumor. On day 185, all of the surviving animals were killed. All of the 14 animals in the RV oligodeoxynucleotide or saline arms died with gross tumor. In the antisense arm, two of seven animals died with gross tumor, and five of seven remained tumor-free.

($P < 0.03$) (Fig. 4). Two animals in the immediate Bcl-2 antisense treatment group developed tumor (days 118 and 125 postinjection), and four animals were free of disease at the time of sacrifice (day 168). Delaying the onset of Bcl-2 antisense treatment by 10 days resulted in significant prolongation of survival compared with the untreated control group ($P < 0.04$; median survival, 76 *versus* 43 days). However, only one of six animals in the delayed Bcl-2 antisense treatment group remained free of disease at the time of sacrifice.

Discussion

PTLD remains a significant cause of morbidity and mortality in immunosuppressed allograft recipients (28, 29). These tumors are highly associated with EBV and have been shown to express EBV latent viral proteins, including the oncogenic EBV-encoded latent membrane protein, LMP-1 (17, 18). A critical transforming function of LMP-1 is up-regulation of the antiapoptotic cellular protein, Bcl-2 (15). Not surprisingly, strong expression of Bcl-2 has been shown to be a consistent feature of EBV-associated PTL (17, 18). Given the known function of Bcl-2 in promoting cell survival and protecting cells from apoptotic stimuli, including DNA-damaging chemotherapeutic agents, overexpression of Bcl-2 likely plays a role in the pathogenesis and chemoresistance of PTL.

Given the potential role of Bcl-2 in the pathogenesis of PTL, we have tested the hypothesis that antisense-mediated reductions in Bcl-2 expression in PTL will promote cell death and mediate antitumor effects. We have demonstrated that exposure of LCLs to Bcl-2 antisense oligodeoxynucleotide *in vitro* caused a sequence-dependent decline in Bcl-2 protein. Furthermore, this effect was associated with the inhibition of proliferation and stimulation of apoptosis under conditions of serum deprivation. The functional effects of Bcl-2 antisense in LCLs were also sequence-dependent. The antiproliferative effect of Bcl-2 antisense was significantly greater than the antiproliferative effect of either of the control oligodeoxynucleotides, and the control oligodeoxynucleotides had no appreciable effect on apoptosis. Furthermore, Bcl-2 antisense had no effect on proliferation of a Bcl-2-negative B-cell line, BJAB, compared with the control oligodeoxynucleotides. Moreover, we have demonstrated a profound antitumor effect of Bcl-2 antisense *in vivo* using the human/SCID chimeric model of EBV-associated PTL. Bcl-2 antisense oligodeoxynucle-

otide treatment of LCL-bearing SCID animals for 12 days after injection of cells completely prevented the development of fatal tumors in the majority of animals in two separate studies. In contrast, the control oligodeoxynucleotides had virtually no effect on tumor engraftment and the survival of LCL-bearing animals. Delaying the onset of antisense treatment also had a significant antitumor effect. Delayed treatment significantly prolonged survival of LCL-bearing animals compared with untreated animals, but the majority of these animals ultimately did succumb to overwhelming tumor.

The sequence-dependency of effects of the Bcl-2 antisense oligodeoxynucleotide in LCLs both *in vitro* and *in vivo* is consistent with an antisense mechanism of action of G3139. However, we can not definitively exclude sequence-independent or alternative sequence-dependent mechanisms of action that may contribute to or mediate beneficial antitumor effects *in vivo*. A nonspecific sequence-independent mechanism of action seems unlikely, inasmuch as our control oligodeoxynucleotides were therapeutically ineffective. We have not observed antitumor effects in this animal model using the RV control oligodeoxynucleotide G3622, the two-base MM control oligodeoxynucleotide G4126 (not shown), or two other unrelated phosphorothioated oligodeoxynucleotides (not shown). An important nonspecific yet sequence-dependent effect of oligodeoxynucleotides is immunomodulation. The immunomodulatory effects of oligodeoxynucleotides are dependent on the presence of unmethylated 5'-CpG-3' sequence elements in the context of specific flanking sequences (reviewed in Ref. #30). Specific CpG-containing oligodeoxynucleotides have been shown to activate murine NK cells and macrophages, stimulate B-cell activation, and promote the T helper 1 (Th1) immune response (30–33). The Bcl-2 antisense sequence used in these studies does contain two CpG motifs, and this sequence has been shown to activate mouse NK cells *in vitro* (34). Thus, it is possible that NK stimulation may have contributed the antitumor effect that we observed *in vivo*. However, the inactive RV control oligodeoxynucleotide sequence also contains two CpG motifs, and, furthermore, two additional unrelated CpG-containing oligodeoxynucleotides that we have tested in this animal model have failed to demonstrate antitumor

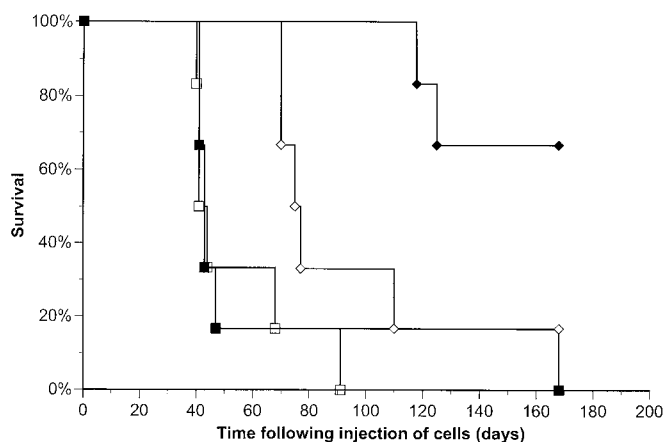


Fig. 4. Effect of Bcl-2 immediate and delayed antisense treatment *in vivo* on survival of LCL (Sweig)-bearing SCID mice. Animals were injected with 20,000,000 Sweig cells i.p. on day 1 and treated with Bcl-2 antisense oligodeoxynucleotide ($n = 6$; ◆), RV control oligodeoxynucleotide ($n = 6$; ■), or saline without oligodeoxynucleotide ($n = 6$; □) on days 1, 4, 7, 10, and 13. For delayed antisense treatment, animals ($n = 6$; ◇) were injected with 20,000,000 Sweig cells i.p. on day 1 and were treated with Bcl-2 antisense oligodeoxynucleotide on days 10, 13, 16, 19, and 22. Animals were killed when they developed clinical signs of tumor. On day 168, all of the surviving animals were killed. Eleven of 12 animals in the saline and RV oligodeoxynucleotide arms died with gross tumor. In the immediate Bcl-2 antisense arm, two of six animals died with tumor, and four remained tumor-free. In the delayed Bcl-2 antisense arm, five of six animals died with tumor, and one remained tumor free.

activity. Thus, the mere presence of CpG motifs does not confer antitumor activity.

These studies support the concept that the Bcl-2 antisense oligodeoxynucleotide G3139 may represent a new treatment option for PTLD. PTLD remains a significant cause of morbidity and mortality in immunosuppressed allograft recipients (35, 36). A variety of therapeutic modalities have been used in these patients, including reducing immunosuppression, IFN chemotherapy, and donor leukocyte infusions (37). Despite the array of treatment options, the mortality remains high for patients with poor prognostic features. Failure is attributable to both unresponsive disease and treatment-related morbidity in this patient population. The Bcl-2 antisense oligodeoxynucleotide G3139 has been evaluated in clinical trials and is remarkably well tolerated in humans (20). The only significant adverse effect reported with G3139 by Webb *et al.* (20) in a Phase I dose-escalation trial was an inflammatory reaction at the infusion site; treatment-related end-organ damage was not observed with G3139. Furthermore, given the excellent toxicity profile of phosphorothioated oligodeoxynucleotides, it may be feasible to combine G3139 treatment with cytotoxic chemotherapeutic regimens. *In vitro* and *in vivo* studies using Bcl-2 antisense oligodeoxynucleotides have demonstrated that depletion of Bcl-2 protein levels is associated with reversal of chemoresistance *in vitro* (26) and chemosensitization *in vivo* (19). The use of G3139 for PTLD may not only enhance the antitumor efficacy of standard chemotherapeutic regimens but may also permit the use of lower and less toxic doses of chemotherapeutic drugs without compromising efficacy. In conclusion, G3139 may represent a potentially nontoxic yet effective biologically targeted treatment strategy for PTLD, as well as for other LMP-1- and Bcl-2-expressing EBV-associated malignancies.

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