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Membrane-associated CD19–LYN complex is an endogenous p53-independent and Bcl-2-independent regulator of apoptosis in human B-lineage lymphoma cells

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ABSTRACT **CD19 receptor is expressed at high levels on human B-lineage lymphoid cells and is physically associated with the Src protooncogene family protein-tyrosine kinase Lyn. Recent studies indicate that the membrane-associated CD19–Lyn receptor–enzyme complex plays a pivotal role for survival and clonogenicity of immature B-cell precursors from acute lymphoblastic leukemia patients, but its significance for mature B-lineage lymphoid cells (e.g., B-lineage lymphoma cells) is unknown. CD19-associated Lyn kinase can be selectively targeted and inhibited with B43–Gen, a CD19 receptor-specific immunoconjugate containing the naturally occurring protein-tyrosine kinase inhibitor genistein (Gen). We now present experimental evidence that targeting the membrane-associated CD19–Lyn complex *in vitro* with B43–Gen triggers rapid apoptotic cell death in highly radiation-resistant p53$^-$Bax$^-$ Ramos-BT B-lineage lymphoma cells expressing high levels of Bcl-2 protein without affecting the Bcl-2 expression level. The therapeutic potential of this membrane-directed apoptosis induction strategy was examined in a *scid* mouse xenograft model of radiation-resistant high-grade human B-lineage lymphoma. Remarkably, *in vivo* treatment of *scid* mice challenged with an invariably fatal number of Ramos-BT cells with B43–Gen at a dose level <1/10 the maximum tolerated dose resulted in 70% long-term event-free survival. Taken together, these results provide unprecedented evidence that the membrane-associated anti-apoptotic CD19–Lyn complex may be at least as important as Bcl-2/Bax ratio for survival of lymphoma cells.**

CD19 antigen is a B-lineage-specific surface receptor that is expressed on B-lineage lymphoma cells from 85% of patients but it is absent on the parenchymal cells of life-maintaining nonhematopoietic organs, circulating blood myeloid and erythroid cells, T cells, and bone marrow stem cells (1–6). CD19 is physically and functionally associated with Src protooncogene family protein-tyrosine kinases (PTKs) to form transmembrane receptor tyrosine kinases with ancillary signal transducing functions (7). Src family PTKs in these CD19 receptor–PTK complexes act as signal transducers and couple CD19 to downstream cytoplasmic signaling pathways (7–14). Src family PTKs are abundantly expressed in B-lineage lymphoid cells and are thought to regulate their vital functions (7, 15). Recent studies indicate that the membrane-associated CD19–Lyn receptor–enzyme complex plays a pivotal role for survival and clonogenicity of immature B-cell precursors from acute lymphoblastic leukemia patients (16), but its significance for mature B-lineage lymphoid cells (e.g., B-lineage lymphoma cells) is unknown.

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CD19-associated Lyn kinase can be selectively targeted and inhibited with B43–Gen (16), a CD19 receptor-specific immunoconjugate containing the naturally occurring PTK inhibitor genistein (Gen), an isoflavone (5,7,4′-trihydroxyisoflavone) isolated from the fermentation broth of *Pseudomonas* spp., which is also present in soybeans (17). We now present experimental evidence that targeting the membrane-associated CD19–Lyn complex *in vitro* with B43–Gen triggers rapid apoptotic cell death in highly radiation-resistant p53$^-$Bax$^-$ Ramos-BT B-lineage lymphoma cells expressing high levels of Bcl-2 protein without affecting Bcl-2 expression level. The therapeutic potential of this membrane-directed apoptosis induction strategy was examined in a *scid* mouse xenograft model of radiation-resistant high-grade human B-lineage lymphoma. Remarkably, *in vivo* treatment of *scid* mice challenged with an invariably fatal number of Ramos-BT cells with B43–Gen at a dose level <1/10 the maximum tolerated dose resulted in 70% long-term event-free survival. By comparison, neither unconjugated B43 monoclonal antibody nor B43 pokeweed antiviral protein immunotoxin improved the outcome of *scid* mice in this human lymphoma xenograft model. These results (*i*) establish the importance of the membrane-associated CD19–Lyn complex for survival of B-lineage lymphoma cells and (*ii*) demonstrate that targeting of this antiapoptotic complex with B43–Gen immunoconjugate is a viable biotherapeutic strategy against high-grade B-lineage lymphoma.

MATERIALS AND METHODS

Cells. Ramos-BT is a highly radiation-resistant subclone of the CD19$^+$ Ramos Burkitt lymphoma cell line. Ramos-BT cells lack p53 as well as Bax and express high levels of the antiapoptotic oncoprotein Bcl-2. Ramos-BT cells are resistant to anti-CD19 immunotoxins and anti-CD19 × CD19 monoclonal antibody homoconjugates. When injected into *scid* mice, Ramos-BT cells cause fatal human B-lineage lymphoma.

Preparation of the B43(Anti-CD19)–Gen Immunoconjugate. We used a two-step procedure for conjugation of Gen to the CD19-specific monoclonal antibody B43, as recently described in detail (16). This procedure involves attachment of the heterobifunctional cross-linking agent sulfo-succinimidyl 6-(4′-azido-2′-nitrophenylamino)hexanoate (SANPAH) to free amino groups on B43 to form a succinimidyl linkage, followed by photolytic generation of a reactive singlet nitrene on the other terminus of the crosslinker in the presence of a 25-fold molar excess of Gen. The chemical composition, CD19

Abbreviations: PTK, protein-tyrosine kinase; Gen, genistein; PKC, protein kinase C; PAP, pokeweed antiviral protein.

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Table 1. Selective binding of B43(anti-CD19)–Gen immunoconjugate to CD19$^+$ lymphoma cells

		B43(anti-CD19)–^{125}I-Gen binding				
		% inhibitable binding				
Cell line	Immunophenotype	Excess α-CD19	Excess α-CD7	Specific binding, cpm per 10^8 cells	fmol per 10^8 cells	Molecules per cell $\times 10^{-4}$
Ramos	CD19$^+$	28.6	0	620 (±20)	3444 (±111)	2.1 (±0.1)
Nalm-6	CD19$^+$	33.6	0	900 (±28)	5000 (±155)	3.1 (±0.1)
Nalm-16	CD19$^+$	34.2	0	780 (±32)	4333 (±178)	2.7 (±0.1)
Molt-3	CD19$^-$ (T-ALL)	<0.1	ND	<10	NE	NE
HL-60	CD19$^-$ (AML)	<0.1	ND	<10	NE	NE

Binding of B43–^{125}I-GEN (1.8×10^5 cpm/nmol) to CD19$^+$ vs. CD19$^-$ leukemia cells was determined in the presence and absence of 10-fold molar excess of nonradioactive B43(anti-CD19) monoclonal antibody or TXU(anti-CD7) monoclonal antibody as described (14). Each determination was performed in triplicate. Background cpm in blank tubes with no added B43–Gen did not exceed 35 cpm. Binding data are expressed as percentage inhibitable binding (100% binding was the value obtained in the absence of excess unlabeled antibodies), cell-associated specific cpm inhibitable by excess unlabeled anti-CD19 monoclonal antibody B43, estimated fmol of B43–Gen bound to 10^8 cells, and number of radioiodinated B43–Gen molecules bound per cell. ND, not determined; NE, not examined.

specificity, affinity, internalization, and *in vitro* as well as *in vivo* PTK inhibitory activity of the B43–Gen immunoconjugate against CD19-associated Src family PTKs have been reported elsewhere (16). The final preparation was contaminated with <5% unconjugated B43 antibody or Gen and was found, in four independent conjugations, to contain on average 1 (range, 0.9–1.3) molecule of Gen per B43 antibody molecule, as determined by the specific activity of immunoconjugates prepared using ^{125}I-Gen (16). Gen (in 65% ethanol/35% PBS, pH 7.5) was radioiodinated at room temperature in Reacti-Vials containing Iodo-Beads (Pierce) and ^{125}I (Na, carrier-free; 17.4 Ci/mg; 1 Ci = 37 GBq; NEN) according to the manufacturer's instructions (16). The specific activity of ^{125}I-Gen was 2.6×10^5 cpm/nmol. The purity of the B43–^{125}I-Gen immunoconjugate was assessed by SDS/PAGE (5% separating gels, nonreducing conditions) and autoradiography using intensifying screens and Kodak XAR-5 film. Ligand binding assays with B43–^{125}I-Gen (1.8×10^5 cpm/nmol) were performed as described (17–20).

Immune Complex Kinase Assays and Kinase Renaturation Assays. Ramos-BT lymphoma cells were treated for 4 hr at 37°C with the indicated concentrations of B43–Gen, pelleted, and lysed in Nonidet P-40 lysis buffer; Lyn or Syk immune complex kinase assays were performed as described (7). Control reagents included (*i*) unconjugated B43 monoclonal antibody mixed with unconjugated Gen and (*ii*) TXU–Gen immunoconjugate that does not react with CD19$^+$ B-lineage lymphoma cells. The effect of B43–Gen on the enzymatic activity of protein kinase C (PKC) and PKC-dependent serine/threonine kinases was examined by a kinase renaturation assay as described (7).

Apoptosis Assays. After various incubation times with the B43–Gen immunoconjugate, Ramos-BT cells were analyzed for apoptotic changes by DNA flow cytometry as described (21). In addition, cells were harvested 30 min to 24 hr after exposure to the B43–Gen immunoconjugate and DNA was prepared for analysis of fragmentation. DNA was then electrophoresed through a 1% agarose gel and visualized by UV light after staining with ethidium bromide, as reported (22).

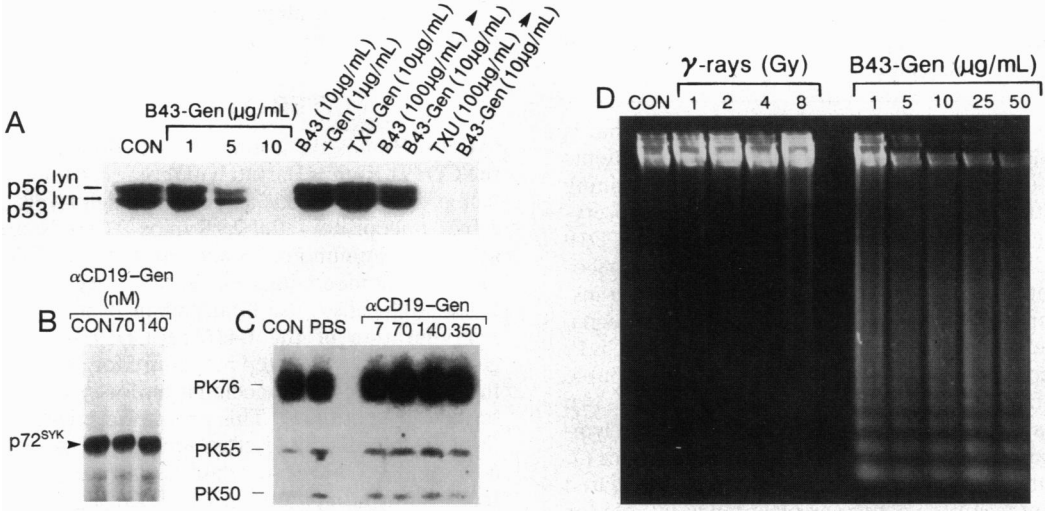

FIG. 1. Biologic effects of B43–Gen on human B-lineage lymphoma cells. (*A*) B43–Gen inhibits CD19-associated Lyn kinase. Ramos cells were treated for 4 hr at 37°C with the indicated concentrations of B43–Gen, pelleted, and lysed in Nonidet P-40 lysis buffer; and Lyn immune complex kinase assays were performed as described (7, 16). Control (CON) reagents included unconjugated B43 monoclonal antibody mixed with unconjugated Gen and TXU–Gen immunoconjugate, which does not react with CD19$^+$ B-lymphoma cells. To demonstrate the CD19 specificity of Lyn inhibition, excess B43(anti-CD19) antibody versus TXU(anti-CD7) antibody were used for blocking. (*B*) B43–Gen does not inhibit Syk kinase. Syk immune complex kinase assays were performed as described (18). (*C*) B43–Gen does not inhibit renaturable serine kinases. Serine kinase renaturation assays were performed as described (7). (*D*) B43–Gen causes apoptosis in radiation-resistant B-lymphoma cells. Ramos cells were harvested 24 hr after exposure to the B43–Gen immunoconjugate or γ-rays and DNA was prepared for analysis of fragmentation as described (16, 22).

Bcl-2 Western Blot Analysis. The expression of Bcl-2 protein in Ramos-BT cells was studied by immunoblotting with an anti-human Bcl-2 antibody (Dako) as described (23).

***scid* Mouse Model of Human B-Lineage Lymphoma.** *scid* mice challenged i.p. with 5×10^6 Ramos-BT cells received three consecutive daily i.p. injections of B43–Gen (25 μg per mouse, which is at least 10-fold lower than the maximum tolerated dose) starting 24 hr after lymphoma cell inoculation. Control mice were treated with unconjugated Gen (10 μg per mouse), unconjugated B43 monoclonal antibody (50 μg per mouse), TXU(anti-CD7)–Gen (50 μg per mouse) control immunoconjugate, or PBS. For comparison, some mice were treated with B43 pokeweed antiviral protein (PAP) immunotoxin, which is an active antileukemia agent (24, 25). Survival of mice was monitored by daily observation and event times were measured from the day of inoculation of lymphoma cells to the day of death. The probability of event-free survival was determined and event-free interval curves were generated by the Kaplan–Meier product limit method as reported (24, 26).

RESULTS AND DISCUSSION

Effects of B43–Gen Immunoconjugate on PTK Activity in B-Lineage Lymphoma Cells. We first investigated the immunoreactivity of radioiodinated B43–Gen with CD19 antigen-positive target Ramos-BT, Nalm-6, and Nalm-16 cells and CD19 antigen-negative Molt-3 (T-leukemia/lymphoma) and HL-60 (acute myeloid leukemia) nontarget cells in the presence and absence of a 10-fold molar excess of unlabeled B43(anti-CD19) antibody by standard ligand binding assays (Table 1). Anti-CD19 monoclonal antibody B43, but not the control anti-CD7 monoclonal antibody TXU, blocked binding of B43–^{125}I-Gen to CD19$^+$ target lymphoma/leukemia cells; 3444 ± 111 fmol of B43–^{125}I-Gen was specifically bound to 10^8 Ramos-BT lymphoma cells, which corresponds to 2.1 ± 0.1 × 10^4 B43–^{125}I-Gen molecules per cell. By comparison, we observed no detectable binding of B43–^{125}I-Gen to Molt-3 T-lineage leukemia cells or HL-60 acute myelocytic leukemia cells (Table 1).

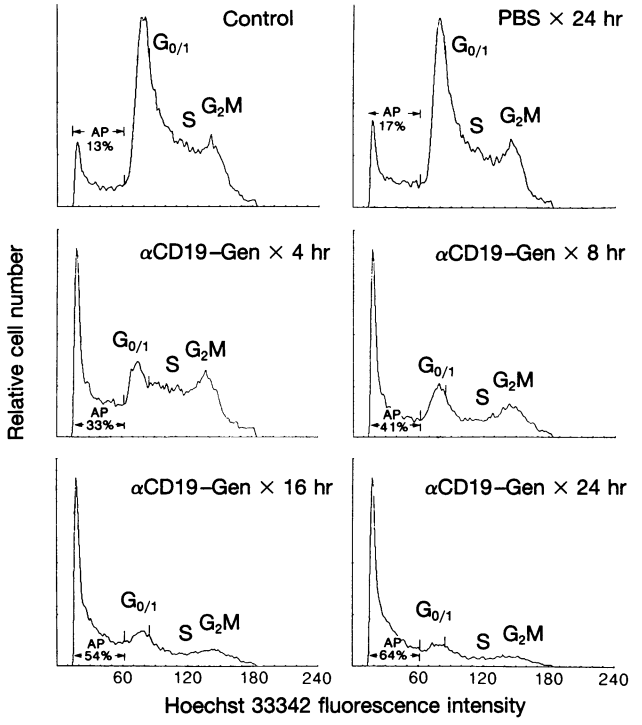

FIG. 2. DNA flow cytometric analysis of B43(anti-CD19)–Gen-treated B-lymphoma cells. After treatment with 70 nM B43–Gen for 30 min to 24 hr, DNA flow cytometry was used to identify and quantify the fraction of apoptotic (AP) Ramos cells based on their decreased stainability with the DNA-specific fluorochrome Hoechst 33342 as described (21).

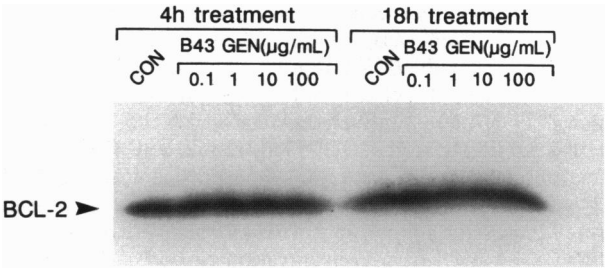

FIG. 3. B43–Gen does not decrease Bcl-2 protein levels in B-lymphoma cells. Proteins from whole cell lysates of untreated and B43–Gen-treated Ramos cells were separated on 10.5% polyacrylamide gels, transferred to a 0.45-μm Immobilon poly(vinylidene difluoride) membrane, and immunoblotted with anti-human Bcl-2 antibody. CON, control.

We previously reported that CD19 is physically and functionally associated with Lyn kinase, which is the predominant Src family member PTK in B-lineage lymphoma and leukemia cells (7). To examine whether, upon binding to the CD19 receptor, B43–Gen might inhibit the CD19-associated Lyn kinase, Ramos-BT lymphoma cells were treated with nanomolar concentrations of the immunoconjugate for 4 hr, and the PTK activity of Lyn was estimated by immune complex protein kinase assays. B43–Gen treatment of Ramos-BT lymphoma cells resulted in inhibition of Lyn kinase, as reflected by decreased autophosphorylation, which is reminiscent of our findings with leukemic B-cell precursors (Fig. 1*A*). No inhibition of Lyn kinase was observed in control Ramos-BT cells that were treated with unconjugated B43 mixed with unconjugated Gen or with the TXU–Gen immunoconjugate directed against the CD7/Tp41 T-cell surface antigen. These results indicate that both the tyrosine kinase inhibitory Gen moiety as well as the CD19-specific targeting B43 monoclonal antibody moiety are required for B43–Gen induced inhibition of Lyn kinase in

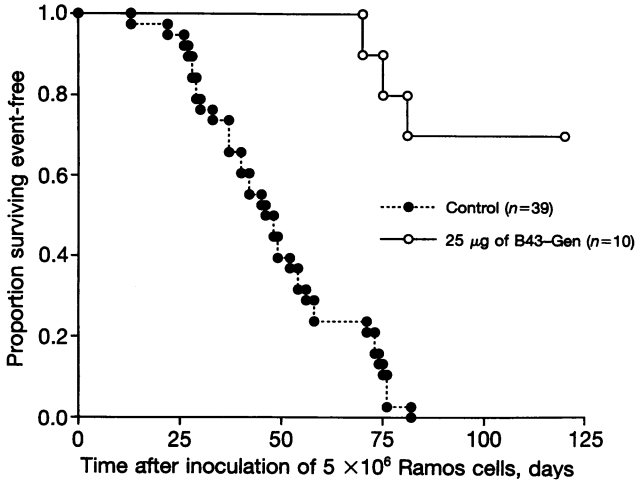

FIG. 4. Anti-lymphoma activity of the B43–Gen immunoconjugate against human B-lineage lymphoma in *scid* mice. *scid* mice challenged i.p. with 5×10^6 Ramos cells received three consecutive daily i.p. injections of B43–Gen (25 μg per mouse) starting 24 hr after lymphoma cell inoculation. Control mice were treated with PBS, unconjugated Gen (10 μg per mouse), conjugated B43 monoclonal antibody (50 μg per mouse), TXU(anti-CD7)–Gen (50 μg per mouse) control immunoconjugate, or B43–PAP immunotoxin (30 μg per mouse). The probability of event-free survival was determined and event-free interval curves were generated by the Kaplan–Meier product limit method.

B-lineage lymphoma cells. The cellular inhibition of Lyn kinase by B43–Gen was CD19 receptor specific because excess anti-CD19 antibody B43 but not excess anti-CD7 antibody TXU could block this effect. Unlike the Lyn kinase, Syk kinase, which does not constitutively associate with the CD19 receptor, was not inactivated in B43–Gen-treated cells (Fig. 1*B*). We also examined the effects of B43–Gen treatment of cells on the enzymatic activity of PKC and PKC-dependent renaturable serine kinases and found that serine kinases were not inhibited by B43–Gen even at a concentration of 350 nM (Fig. 1*C*). Taken together, these experiments demonstrated that the B43(anti-CD19)–Gen immunoconjugate is a potent and cell type-specific PTK inhibitor, which selectively inhibits the CD19-associated Lyn kinase.

B43–Gen Induces Apoptotic Death in Radiation-Resistant B-Lineage Lymphoma Cells Without Decreasing Their Bcl-2 Oncoprotein Levels. A frequent mechanism that causes cell death is known as apoptosis or programmed cell death. Apoptosis is identified by distinct ultrastructural features and a ladder-like DNA fragmentation pattern resulting from endonuclease-mediated cleavage of DNA into oligonucleosome-length fragments (27, 28). Human cells experience apoptosis when exposed to ionizing radiation or one of several chemotherapeutic drugs that affect diverse molecular targets (22, 27–29). B43–Gen has been shown to kill immature leukemic B-cell precursors by inducing apoptosis (17). Therefore, we were interested in whether B43–Gen-induced Lyn kinase inhibition could trigger apoptosis in mature B-lineage lymphoma cells as well. B43–Gen caused apoptosis of Ramos-BT cells as evidenced by agarose gel electrophoresis of DNA (Fig. 1*D*) or by DNA flow cytometry (Fig. 2). At 24 hr after initiation of B43–Gen treatment, 64% of Ramos-BT cells were apoptotic as determined by DNA flow cytometry (Fig. 2).

Bcl-2 protein has been shown to repress most types of apoptotic cell death (30). Bcl-2 has been shown to heterodimerize with Bax protein that accelerates apoptosis (31).

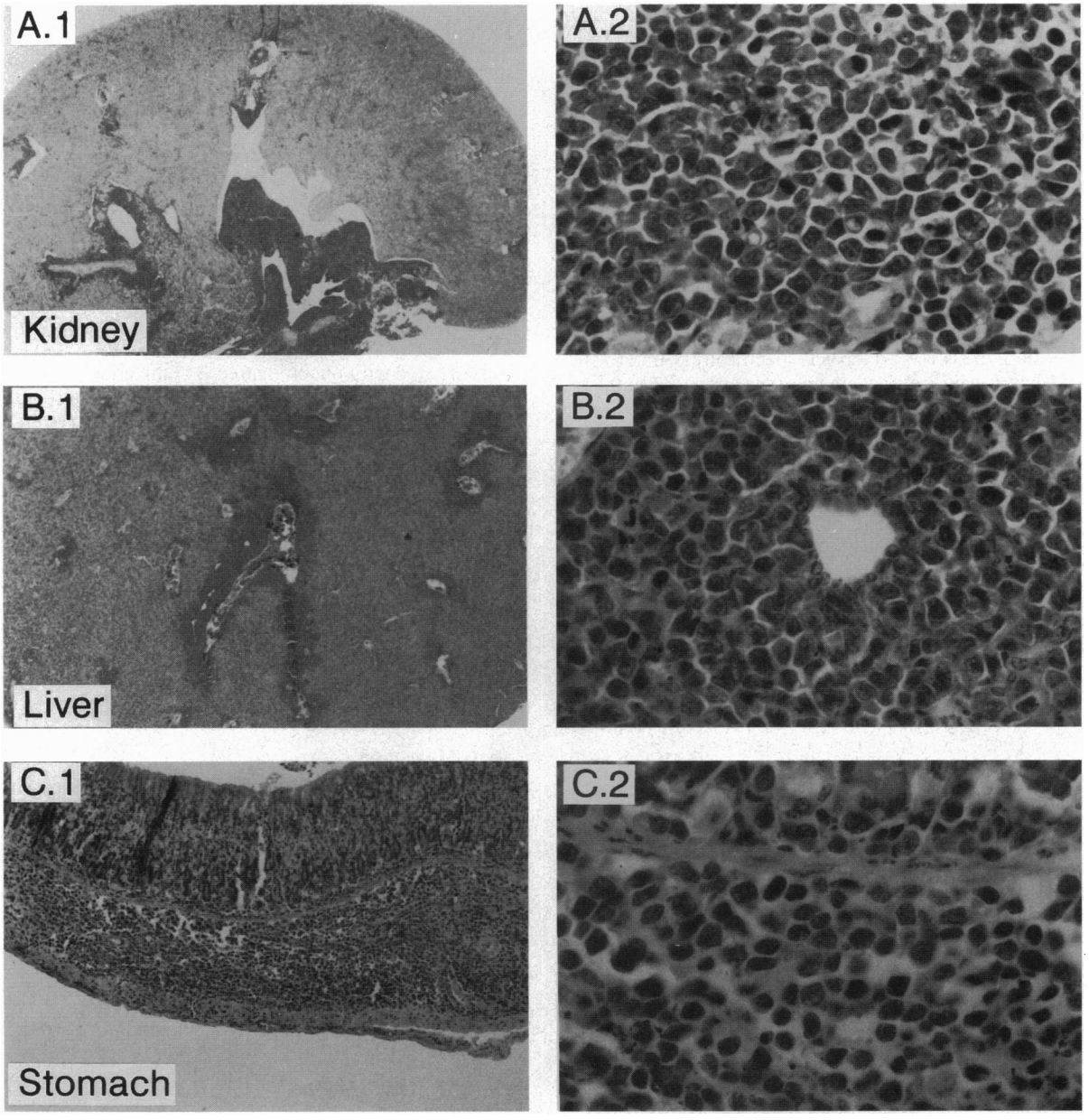

FIG. 5. Disseminated lymphoma in *scid* mice inoculated with Ramos cells. (*A*) Kidney. A dense pelvic infiltrate as well as several perivascular cortical infiltrates of highly pleiomorphic lymphoid cells were apparent. (*B*) Liver. Generalized infiltration of portal areas by pleiomorphic lymphoid cells with a high mitotic rate was observed. (*C*) Stomach. A mass of highly pleiomorphic lymphoid cells is attached to the serosal surface. Lymphoma cells invaded the wall of the organ and distended the submucosal region.

Recent studies indicate that the ratio of Bcl-2 to Bax determines survival or death after exposure to an apoptotic agent (31). Ramos-BT, the target human B-lineage lymphoma clone used in this study, expresses high levels of Bcl-2 protein, but it does not express detectable levels of Bax protein. The ability of B43–Gen to cause apoptosis in Ramos-BT cells provides unprecedented evidence that the membrane-associated anti-apoptotic CD19–Lyn complex may be at least as important as Bcl-2/Bax ratio for survival of lymphoma cells. B43–Gen-induced apoptosis of Ramos-BT cells was not triggered by a decrease in the expression level of Bcl-2 protein (Fig. 3). Induction of apoptosis by some anticancer agents has been shown to be p53 dependent (27–29). Since Ramos-BT cells are p53 deficient, these results further demonstrate that B43–Gen-induced apoptosis does not depend on p53 expression.

B43–Gen Confers Long-Term Event-Free Survival in *scid* Mice Xenografted with Fatal Human B-Lineage Lymphoma. B43–Gen is not toxic to *scid* mice at doses ranging from 10 to 250 μg (16). Thus, the maximum tolerated dose of B43–Gen is >250 μg per mouse. We examined the effect of 25 μg of B43–Gen, which is <1/10 the maximum tolerated dose, on the survival of *scid* mice challenged with an otherwise invariable fatal inoculum of Ramos lymphoma cells. All control mice that were treated with PBS, unconjugated Gen (10 μg per mouse), unconjugated B43 antibody (50 μg per mouse), TXU(anti-CD7)–Gen (50 μg per mouse) control immunoconjugate, or B43–PAP immunotoxin (30 μg per mouse) died of disseminated human Burkitt lymphoma after inoculation of 5×10^6 Ramos cells (median event-free survival, 48.0 days) (Fig. 4). These mice had large abdominal masses with extensions to the abdominal organs and histologic evidence of organ infiltration by human lymphoma cells (Fig. 5). Sheets of neoplastic cells obliterated the normal tissue elements of bone marrow, spleen, and abdominal nodes. Colonization of the brain was apparent by the presence of thin rafts of lymphoma cells in the leptomeninges, and in some cases extensive invasion by lymphoma cells was seen in gray matter of the cerebral cortex and brain stem. The heart showed thin, short rafts in the epicardium, and the alveolar septa of the lung contained a light infiltrate of Ramos cells. Kidneys had large accumulations in perirenal fat, as well as light to extensive interstitial accumulations and small numbers of neoplastic perivascular cuffs in the cortex (Fig. 5). Large accumulations were visible in the portal areas of the liver, along with numerous small nests in the sinusoidal and subcapsular spaces (Fig. 5). The mitotic rate was very high, averaging 15–20 mitotic figures per high-power field in most tissues and up to 40–50 per high-power field in some areas. In contrast, B43–Gen prevented disseminated human lymphoma in the majority of *scid* mice. Seven of 10 mice treated with the B43–Gen immunoconjugate (25 μg per mouse = 168 pmol per mouse) remained alive without clinical evidence of lymphoma for >4 months (probability of event-free survival at 100 days, 70% ± 15%; median event-free survival, >125 days) (Fig. 4).

This study expands previous reports (7, 15) on the role of Src family PTK in vital cellular functions of human lymphoid cells, in particular by exploring the induction of apoptosis after inhibition of CD19-associated Lyn kinase in B-lineage lymphoma cells. Our results indicate that the membrane-associated CD19–Lyn complex is an important regulator of apoptosis not only in B-cell precursors (15) but in mature B-lineage lymphoid cells as well. B43–Gen may provide the basis for an effective strategy for B-lineage lymphoma patients who have failed standard therapy.

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