

# The Anti-CD30 Monoclonal Antibody SGN-30 Promotes Growth Arrest and DNA Fragmentation *in Vitro* and Affects Antitumor Activity in Models of Hodgkin's Disease

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## ABSTRACT

The leukocyte activation marker CD30 is highly expressed on the Reed Sternberg cells of Hodgkin's disease (HD). On normal tissues, CD30 has a restricted expression profile limited to activated T cells, activated B cells, and activated natural killer cells. This expression profile makes CD30 an ideal target for monoclonal antibody (mAb)-based therapies of Hodgkin's disease. CD30 mAbs have been shown to be effective in *in vitro* and *in vivo* models of hematologic malignancies such as anaplastic large cell lymphoma, yet these mAb have not been efficacious in HD models. We have found that a mAb against CD30, AC10, was able to inhibit the growth of HD cell lines *in vitro*. To generate a more clinically relevant molecule, the variable regions from AC10 were cloned into an expression construct containing the human  $\gamma 1$  heavy chain and  $\kappa$  light chain constant regions. The resulting chimeric antibody, designated SGN-30, retained the binding and *in vitro* growth-inhibitory activities of the parental antibody. Treatment of HD cell lines with SGN-30 *in vitro* resulted in growth arrest in the G<sub>1</sub> phase of the cell cycle and DNA fragmentation consistent with apoptosis in the HD line L540cy. Severe combined immunodeficient mouse xenograft models of disseminated HD treated with SGN-30 produced significant increases in survival. Similarly, xenograft models of localized HD demonstrated dose-dependent reduction in tumor mass in response to SGN-30 therapy. SGN-30 is being developed for the treatment of patients who have HD that is refractory to initial treatment or who have relapsed and have limited therapeutic options.

## INTRODUCTION

The leukocyte activation marker CD30 is a  $M_r$  105,000–120,000 integral membrane glycoprotein and a member of the TNF-R<sup>3</sup> superfamily. This family of key immunoregulatory molecules includes CD27, CD40, CD95, OX40, TNF-R1, and TNF-R2. Originally identified on Reed-Sternberg cells in HD using the Ki-1 mAb (1), CD30 has subsequently been found on ALCL and subsets of non-Hodgkin's lymphomas (NHLs), as well as in rare solid tumors such as embryonal carcinomas and seminomas (2). The expression of CD30 in normal cells is restricted to activated T and B cells and is absent from resting lymphocytes and resting monocytes, and from normal cells outside of the immune system. CD30 is expressed at high levels by activated cells in autoimmune disease and is detectable in the circulation of patients with rheumatoid arthritis (3), multiple sclerosis, and systemic sclerosis (4, 5). Histological examination for CD30 expression identifies rare, large lymphoid cells in sections of lymph node, tonsil,

thymus, and decidual endometrial cells at the placental interface (6). CD30 is transiently expressed on T cells in culture after mitogen activation or antigen receptor cross-linking, and is constitutively expressed after viral infection (7).

Whereas the function of CD30 is not clearly defined, ligation of CD30 has been implicated in the activation-induced cell death of thymocytes, a negative selection process for deletion of autoreactive T cells (2). Negative selection is severely diminished in CD30<sup>-/-</sup> mice, giving rise to increased thymocyte numbers (8). Conversely, overexpression of CD30 has been shown to enhance negative selection of autoreactive T cells in the thymus, which suggests an important coregulatory role for CD30 (9).

Approximately 20% of HD cases are refractory to initial treatment or will relapse with limited options for secondary therapies. The utility of CD30 as a diagnostic marker for HD is well established, and its highly restricted expression profile in normal settings has led to the investigation of this antigen as a target for immunotherapy (10). *In vitro*, several antibodies to CD30 have been shown to inhibit the growth of ALCL cells but not CD30-positive HD cells, and initial observations by Gruss *et al.* (11) demonstrated that the growth of HD cells was actually stimulated by anti-CD30 mAbs.

The differential effects of anti-CD30 mAb on ALCL compared with HD cell lines have recently been attributed to the constitutive expression of NF $\kappa$ B by HD cell lines (12). Signaling through anti-CD30 mAbs without the ability to activate the pro-survival factor NF $\kappa$ B resulted in subsequent apoptosis of ALCL cells. In contrast, it was proposed that constitutive NF $\kappa$ B activation in HD lines resulted in insensitivity to mAb-induced CD30 activation. The basis of this differential sensitivity in HD lines remains controversial (13, 14).

The anti-CD30 mAb BerH2 was tested clinically in HD, and, despite its ability to target malignant cells, patients experienced no therapeutic benefit from this treatment (15). In a subsequent clinical trial using mAb BerH2 conjugated to saporin toxin, the treatment of four patients with refractory HD demonstrated rapid and substantial reductions in tumor mass, yet the effects were transient and nondurable (16). Investigators continue to refine the approaches for treating CD30-expressing neoplastic cells using anti-CD30-conjugates, including toxins linked to single-chain Fab fragments and bispecific antibodies (17, 18); yet taken together, these data did not suggest that efficacy could be achieved using an unconjugated CD30 mAb.

Genetically engineered mAbs such as Rituxan, a chimeric mAb targeted to CD20, have become part of standard chemotherapy regimens for certain B-cell malignancies (19). To date, no unmodified anti-CD30 mAb has been shown to have antitumor activity on HD cells either *in vitro* or *in vivo*. In this report, we describe the antiproliferative activities of the mouse antihuman CD30 mAb AC10 (20) on HD cells and the antitumor activity of SGN-30, a chimeric antibody derived from AC10. To use human mAb effector functions and to minimize immunogenicity, SGN-30 contains the V<sub>H</sub>s and V<sub>L</sub>s from AC10 genetically fused to the human  $\gamma 1$  and  $\kappa$  constant regions, respectively. AC10 and SGN-30 can both suppress HD cell growth *in*

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<sup>3</sup> The abbreviations used are: TNF, tumor necrosis factor; TNF-R, TNF receptor; BrdUrd, bromodeoxyuridine; SCID, severe combined immunodeficient; HD, Hodgkin's disease; mAb, monoclonal antibody; ALCL, anaplastic large cell lymphoma; NF $\kappa$ B, nuclear factor  $\kappa$ B; CHO, Chinese hamster ovary; FBS, fetal bovine serum; PI, propidium iodide; V<sub>H</sub>, heavy-chain variable region; V<sub>L</sub>, light-chain variable region.

*in vitro* and, importantly, SGN-30 has potent antitumor efficacy in xenograft models of disseminated and localized human HD.

## MATERIALS AND METHODS

**Cells and Reagents.** Murine hybridoma line AC10 producing IgG2b was obtained from Dr. E. Podack (University of Miami, Miami, FL). The hybridoma was grown in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% FBS. Antibody was purified from culture supernatants by protein A chromatography. CD30-positive HD lines L540, KM-H2, HDLM-2, and L428, as well as the ALCL line Karpas-299, were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany); L540cy was provided by Dr. Phil Thorpe (University of Texas, Southwestern Medical School, Dallas, TX); HL-60 and Daudi were obtained from American Type Culture Collection (Manassas, VA). DG44 CHO cells were obtained from Lawrence Chasin (Columbia University, New York, NY). Goat-antimouse-FITC or goat-antihuman-FITC were from Jackson Immunoresearch, (West Grove, PA). AntiCD30 mAb Ki-1 was from Accurate Chemicals (Westbury, NY).

**Fluorescence-activated Cell Sorting Analysis.** To evaluate CD30 expression on cell lines,  $3 \times 10^5$  cells were combined with saturating levels (4  $\mu\text{g}/\text{ml}$ ) of either AC10 or SGN-30 in ice-cold 2% FBS/PBS (staining medium) for 20 min on ice and washed twice with ice-cold staining medium to remove unbound mAbs. Cells were then stained with secondary mAbs diluted 1:50 in ice-cold staining medium, goat-antimouse-FITC for AC10 or goat-antihuman-FITC for SGN-30, incubated for 20 min on ice, washed as described above, and resuspended in 5  $\mu\text{g}/\text{ml}$  PI. Labeled cells were examined by flow cytometry on a Becton Dickinson FACScan flow cytometer and were gated to exclude the nonviable cells. Data were analyzed using Becton Dickinson CellQuest software version 3.3, and the background-corrected mean fluorescence intensity was determined for each cell type.

For antibody saturation binding,  $3 \times 10^5$  Karpas-299 cells were combined with increasing concentrations of AC10 or SGN-30 diluted in ice-cold staining medium for 20 min on ice, washed twice with ice-cold staining medium to remove free mAbs, and incubated with 1:50 goat-antimouse-FITC or goat-antihuman-FITC, respectively. The labeled cells were washed, resuspended in PI, and analyzed as described above. The resultant mean fluorescence intensities were plotted *versus* mAb concentration.

For analysis of cell cycle position, cells were cultured in complete medium and at the indicated times were labeled with BrdUrd (10  $\mu\text{M}$  final; Sigma, St. Louis, MO) for 20 min to detect nascent DNA synthesis, and with PI to detect total DNA content as described previously (21). Labeled cells were analyzed for cell cycle position and apoptosis by flow cytometry using the Becton-Dickinson CellQuest program as described previously (21).

**In Vitro Growth Inhibition.** Evaluation of growth inhibition by murine mAbs was carried out by immobilizing the mAbs at 10  $\mu\text{g}/\text{ml}$  in 50 mM Tris-HCl (pH 8.5), to plastic 96-well tissue culture plates overnight at 4°C. Plates were washed twice with PBS to remove unbound mAbs followed by the addition of cells in 100  $\mu\text{l}$  complete medium at 5000 cells/well. After a 48-h incubation at 37°C, 5% CO<sub>2</sub>, cells were labeled with [<sup>3</sup>H]thymidine by the addition of 50  $\mu\text{l}$  complete medium containing 0.5  $\mu\text{Ci}$  of [<sup>3</sup>H]thymidine for 2 h, and the level of DNA synthesis was determined relative to cells in untreated control wells. Evaluation of growth inhibition by SGN-30 was carried out using soluble mAbs and secondary cross-linker. Cells were plated at 5000 cells/well in 180  $\mu\text{l}$  of complete medium in a 96-well format. SGN-30 in complete medium containing a corresponding 10-fold excess of goat-antihuman IgG was added at the concentrations noted, in 20  $\mu\text{l}$ . At 96-h postincubation, cells were labeled with [<sup>3</sup>H]thymidine for 4 h followed by cell harvest and scintillation counting to quantify the level of nascent DNA synthesis. The percentage of inhibition relative to untreated control wells was plotted *versus* SGN-30 concentration.

**Xenograft Models of Human HD.** For the disseminated HD model,  $1 \times 10^7$  L540cy cells were injected via the tail vein into C.B-17 SCID mice. Treatment with SGN-30 was initiated at the indicated times and administered via i.p. injection every 4 days for a total of five injections. Animals were evaluated daily for signs of disseminated disease, in particular hind-limb paralysis. Mice that developed these or other signs of disease were then killed. For the localized model of HD,  $2 \times 10^7$  L540cy cells were implanted into the

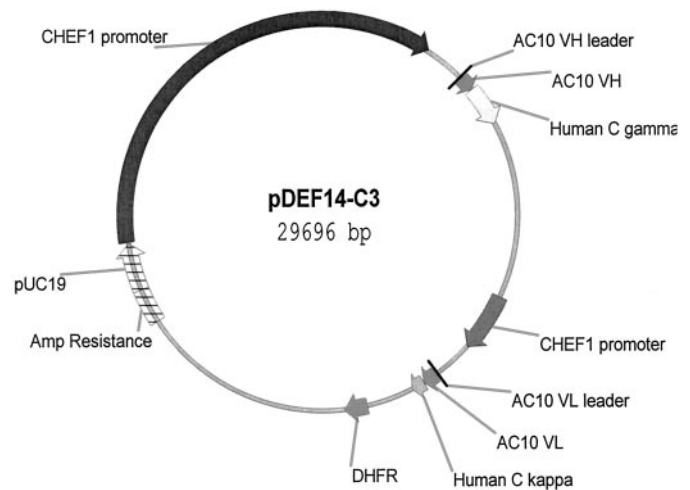


Fig. 1. SGN-30 expression vector. DNA encoding the V<sub>H</sub> of mAb AC10 was joined to the sequence encoding the human  $\gamma$ 1 constant region (*Human C gamma*), and the AC10 V<sub>L</sub> was similarly joined to the human  $\kappa$  constant region (*Human C kappa*) in separate cloning vectors. The heavy- and light-chain chimeric sequences were cloned into plasmid pDEF14 for expression of intact chimeric mAb in CHO cells. pDEF14 uses the Chinese hamster elongation factor 1 $\alpha$  gene promoter, which drives transcription of heterologous genes.<sup>4</sup>

right flank of SCID mice. Therapy with SGN-30 was initiated when the tumor size in each group of five animals averaged  $\sim 50 \text{ mm}^3$ . Treatment consisted of i.p. injections of SGN-30 every 4 days for five injections. Tumor size was determined using the formula  $(L \times W^2)/2$ , where  $L$  = length and  $W$  = width.

## RESULTS

**Construction and Expression of SGN-30.** For construction of SGN-30, the V<sub>H</sub>s and V<sub>L</sub>s were cloned from the AC10 hybridoma, essentially as described previously (22). Total RNA was isolated from the AC10 hybridoma and cDNA of the variable regions was generated using mouse  $\kappa$  and IgG2b gene-specific primers. DNA encoding the AC10 V<sub>H</sub> was joined to sequence encoding the human  $\gamma$ 1 constant region (huC $\gamma$ 1, SwissProt accession no. P01857) in a cloning vector and the AC10 V<sub>L</sub> was similarly joined to the human  $\kappa$  constant region (huC $\kappa$ ; PID G185945) in a separate cloning vector. Both the heavy- and light-chain chimeric sequences were cloned into pDEF14 for expression of intact chimeric mAb in CHO cells. The plasmid pDEF14 uses the Chinese hamster elongation factor 1 $\alpha$  gene promoter that drives transcription of heterologous genes<sup>4</sup> leading to high levels of expression of recombinant proteins without the need for gene amplification. The resulting plasmid was designated pDEF14-C3 (Fig. 1).

For the generation of the SGN-30-expressing cell line, pDEF14-C3 was linearized and transfected into DG44 CHO cells by electroporation. After electroporation, the cells were allowed to recover for 2 days in complete DMEM/F12 medium containing 10% FBS, after which the medium was replaced with selective medium without hypoxanthine and thymidine. Only those cells that incorporated the plasmid DNA, which includes the dihydrofolate reductase gene, were able to grow in the absence of hypoxanthine and thymidine. High titer clones were selected and cultured in bioreactors. SGN-30 antibody was purified by protein A, ion exchange, and hydrophobic interaction chromatographies. The final product was homogeneous as determined by size exclusion high-performance liquid chromatography with >99% of the protein eluting as a single peak of  $M_r \sim 150,000$ .

<sup>4</sup> D. A. Allison, Hamster EF-1 $\alpha$  transcriptional regulatory DNA. United States Patent 5888809, 1999.

Table 1 Binding of AC10 and SGN-30 to cell lines

| Cell Line | Lineage <sup>a</sup>       | MFI <sup>b</sup> |        | Binding ratios <sup>c</sup> |        |
|-----------|----------------------------|------------------|--------|-----------------------------|--------|
|           |                            | AC10             | SGN-30 | AC10                        | SGN-30 |
| HDLM2     | HD (T-cell-like)           | 507.2            | 591.8  | 156                         | 176    |
| L540      | HD (T-cell-like)           | 435.8            | 582.5  | 183                         | 251    |
| L540cy    | HD (T-cell-like)           | 363.3            | 495.9  | 120                         | 156    |
| Karpas    | ALCL                       | 399.9            | 579.2  | 158                         | 176    |
| KM-H2     | HD (B-cell-like)           | 102.0            | 105.8  | 33                          | 41     |
| L428      | HD (B-cell-like)           | 174.4            | 186.0  | 67                          | 67     |
| HL60      | Acute myelogenous leukemia | 1.0              | 3.8    | 1                           | 2      |
| Daudi     | Burkitt's lymphoma B-cell  | -0.6             | 0.9    | 1                           | 1      |

<sup>a</sup> Gruss *et al.*, 1994 (11).

<sup>b</sup> MFI, mean fluorescence intensity.

<sup>c</sup> Binding ratios were determined by dividing the geometric MFI of cells stained with primary (AC10 or SGN-30 at 4  $\mu$ g/ml) and appropriate secondary (goat antimouse or goat antihuman IgG, respectively) -FITC conjugate, by the geometric MFI of cells stained with respective secondary antibody alone.

**Binding of Murine AC10 and SGN-30 to HD Cell Lines.** AC10 was originally produced by immunizing mice with the CD30-positive large granular lymphoma cell line YT and was shown to be specific for CD30 (20). Before evaluating the effects of AC10 and SGN-30 on the growth of HD cells, we compared the level of CD30 expression on several cultured cell lines. As expected, all four HD lines tested were CD30-positive based on flow cytometry fluorescence ratios (Table 1). The T-cell-like HD cell lines, HDLM-2 and L540, as well as the ALCL line Karpas-299 expressed qualitatively similar, high levels of CD30, whereas expression on two B-cell-like HD lines KM-H2 and L428 were somewhat lower. L540cy, a subclone of L540, displayed an intermediate level of CD30 expression. Although the binding of SGN-30 and AC10 to these cell lines was detected using different secondary antibodies, FITC-conjugated goat antihuman or goat antimouse, respectively, these data indicated that the chimerization process did not diminish SGN-30-specific binding to cell surface CD30. The promyelocytic leukemia line HL-60 and the Burkitt's lymphoma line Daudi were both CD30-negative and served as controls in subsequent studies.

To further compare the binding activity of the murine and chimeric antibodies, Karpas-299 cells were incubated with titrations of AC10 or SGN-30 followed by labeling with goat-antimouse-FITC or goat-antihuman-FITC (Jackson ImmunoResearch, West Grove, PA), respectively, to determine levels required for saturation. Labeled cells were examined by flow cytometry, and the mean fluorescence intensity was plotted against mAb concentration. Binding saturation for both forms of the mAb occurred at  $\sim$ 0.5  $\mu$ g/ml (Fig. 2). Saturation was consistent for all of the CD30-positive cell lines examined (data not shown), further demonstrating that SGN-30 retained the binding activity of the parental murine antibody.

Freshly isolated peripheral blood mononuclear cells did not react with SGN-30 and showed no signal above background in this assay. Similarly, isolated human primary B cells and T cells did not bind SGN-30. Primary human peripheral T cells, activated with anti-CD3 and anti-CD28, and B cells, activated by pokeweed mitogen, both showed transient, low level binding of SGN-30 at 72 h postactivation, which diminished thereafter (data not shown).

**In Vitro Activities of AC10 and SGN-30 in Vitro.** Anti-CD30 antibodies such as M44 and M67 have been shown to have antiproliferative effects on ALCL lines, whereas they either have no effect on the growth of HD lines or else stimulate the growth (11, 23). To initially evaluate the effect of the mAb AC10 on HD cell proliferation, AC-10 was compared with mAb Ki-1 under the previously reported solid phase conditions (11). For these studies, mAbs were immobilized onto plastic tissue culture plates before the addition of HD cells as described in "Materials and Methods." After incubation for 48 h at 37°C, cells were labeled with [<sup>3</sup>H]thymidine, and the level of DNA

synthesis was determined relative to cells in untreated control wells. Fig. 3A shows that the presence of immobilized mAb Ki-1 had nominal effect on the growth of the HD lines. In contrast, the presence of immobilized AC10 resulted in significant growth inhibition of all of the HD lines tested. The CD30-negative B-cell line Ramos showed no sensitivity to either Ki-1 or AC10.

*In vivo*, a therapeutic mAb would be expected to function either monomerically or to be aggregated with antigen via Fc receptor-mediated clustering. After chimerization, the growth-inhibitory effects of AC10 and SGN-30 were compared in a solution assay in the presence of respective secondary cross-linking antibody, goat antimouse, or mouse antihuman IgG, on CD30-positive or -negative cells. Fig. 3B shows that the murine and chimeric mAbs have growth-inhibitory activities that are comparable with each other on the CD30-positive lines Karpas, L540 and L428, and show no growth inhibition on the CD30-negative line Ramos. The secondary antibodies alone showed little inhibitory effect or were slightly stimulatory to cell proliferation (Fig. 3B). To examine the potency of SGN-30, a titration was performed on the HD cell lines L540, L540cy, and L428, the ALCL line Karpas-299, and the CD30-negative line HL-60 (Fig. 3C). SGN-30 was added in solution at the concentrations noted in the presence of excess goat-antihuman IgG. Cross-linking antibody was added to potentiate the effects of SGN-30 and to approximate the effects of Fc receptor-mediated cross-linking that could occur *in vivo*. The CD30-positive ALCL line was highly sensitive to SGN-30, with an IC<sub>50</sub> (concentration of mAb that inhibited 50% of cell growth) of 2 ng/ml. Moreover, the HD lines L428, L540, and L540cy showed IC<sub>50</sub> sensitivities to SGN-30 of 100 ng/ml, 80 ng/ml, and 15 ng/ml, respectively. In parallel studies, these cells, treated with a nonbinding control mAb and cross-linker, showed no significant decrease in DNA synthesis over the concentration range tested; the percentage inhibition values for Karpas, L428, L540, L540cy, and HL-60 were each <5% at 400 ng/ml of control IgG. The CD30-negative line HL-60 showed a maximum inhibition of 14% by SGN-30 at the highest level tested (2  $\mu$ g/ml; Fig. 3C).

**Cell Cycle Effects of SGN-30.** Hubinger *et al.* (24) have recently shown that anti-CD30 mAbs can inhibit the growth of ALCL cells, including Karpas-299, through induction of cell cycle arrest and without the induction of apoptosis. However, these mAbs did not have an inhibitory effect on HD cells, and, in some cases, they stimulated proliferation. To more closely examine the cell cycle effects of

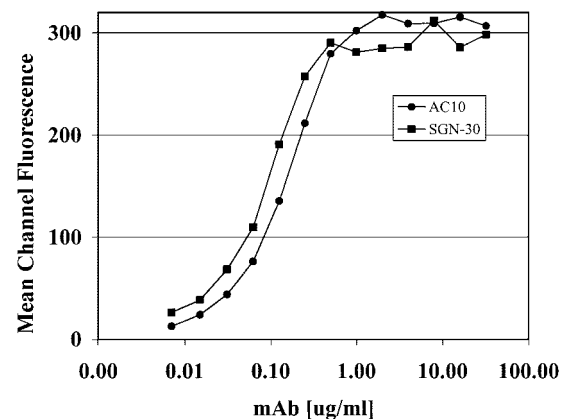


Fig. 2. Antibody saturation. Binding saturation of AC-10 and SGN-30 to CD30-positive Karpas-299. Cells were combined with increasing concentrations of AC10 or SGN-30 for 20 min, washed with 2% FBS/PBS (staining medium) to remove free mAbs, and incubated with goat-antimouse-FITC or goat-antihuman-FITC, respectively. The labeled cells were washed again with staining medium and examined by flow cytometry. The resultant mean fluorescence intensities were plotted *versus* mAb concentration as described in "Materials and Methods." Results are representative of three determinations.



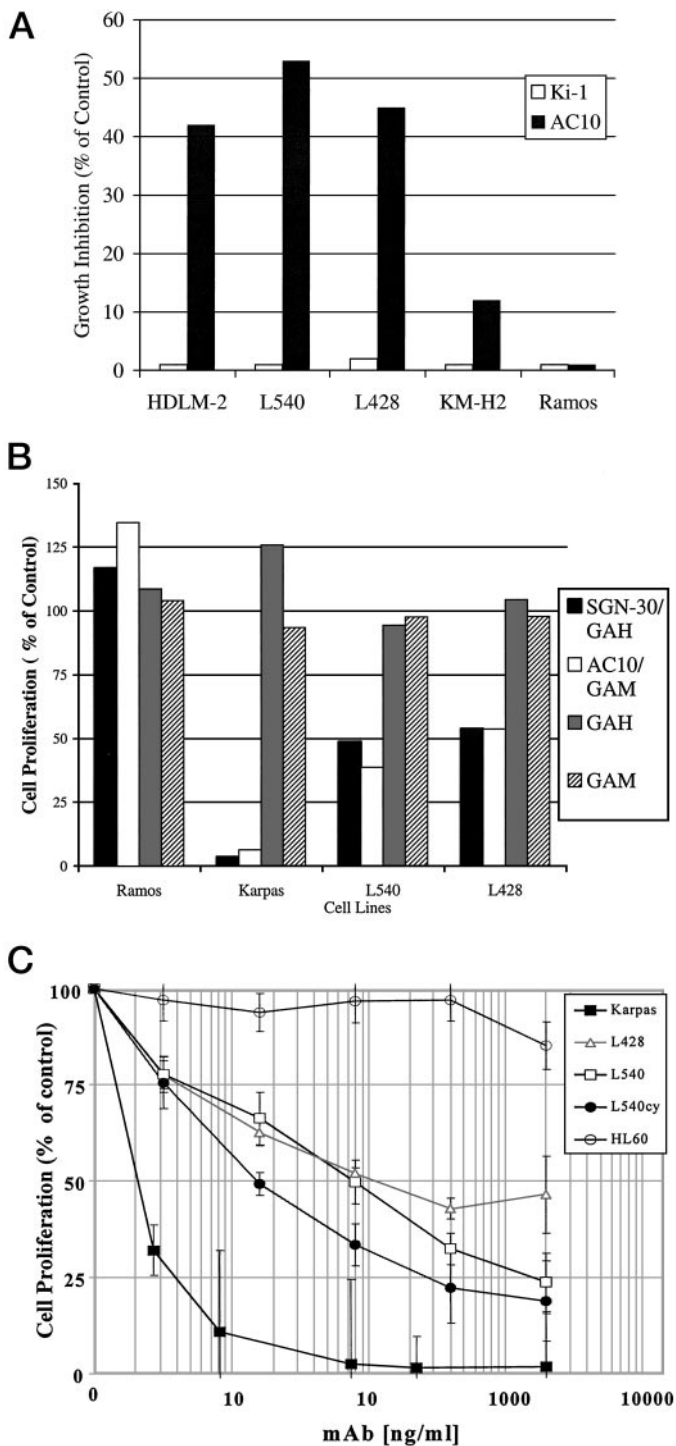


Fig. 3. *In vitro* growth inhibition by AC10 and SGN-30. A, AC10 or the anti-CD30 mAb, Ki-1, were immobilized at 10  $\mu\text{g/ml}$  to plastic 96-well tissue culture plates, and four different CD30-positive HD cell lines, HDLM-2, L540, L428, and KM-H2, and the CD30-negative line Ramos were plated in complete medium at 5000 cells/well. DNA synthesis was determined at 48 h relative to cells in untreated control wells. B, CD30-positive lines and the CD30-negative line Ramos were plated at 5000 cells/well. AC-10 or SGN-30 was added at 2  $\mu\text{g/ml}$  in the presence of a corresponding 4-fold excess of goat-antimouse IgG (GAM) for AC-10 or goat-antihuman IgG (GAH) for SGN-30, or the secondary IgG alone, and cell proliferation was determined relative to untreated cells. C, CD30-positive lines and the CD30-negative line HL-60 were plated at 5000 cells/well. SGN-30 was added at the concentrations noted in the presence of a corresponding 10-fold excess of goat-antihuman IgG. The percentage of inhibition relative to untreated control wells was plotted *versus* SGN-30 concentration. Results for each study are the average of four determinations.

SGN-30 *in vitro*, the HD cell line L540cy was cultured in complete medium containing 1.0  $\mu\text{g/ml}$  of SGN-30 complexed with goat-antihuman IgG at 10  $\mu\text{g/ml}$ . At the indicated times, cells were labeled with BrdUrd for 20 min to detect nascent DNA synthesis, and with PI to detect total DNA content. Labeled cells were analyzed for cell cycle position by flow cytometry using the Becton-Dickinson Cellfit program as described previously (21).

Fig. 4 shows a representative shift in DNA content and DNA synthesis in L540cy HD cells after exposure to SGN-30. The percentage of the population in each region was quantified as described in "Materials and Methods" and is shown in Table 2. Exposure of L540cy to SGN-30 results in time-dependent loss of the S-phase cells from 40% in the untreated population to 13% at 2 days postexposure. Coordinately, the  $G_1$  content of this population increased from 40% in untreated cells to 65% at 3 days postexposure. The region of less than  $G_1$  content gives an accurate indication of apoptotic cells undergoing DNA fragmentation (21), and this population increased from 6% in the untreated population to 29% at 48 h post-SGN-30 exposure. These flow cytometric studies were corroborated by a parallel dye-exclusion assay using a hemocytometer. As measured by dye exclusion, untreated L540cy cells were 93% viable; this decreased to 72% at 48 h post-SGN-30 exposure. Karpas cells treated with SGN-30 showed a similar decrease in S phase from 40 to 11%, and increase in apoptosis from 2 to 12% at 48 h post-SGN-30 with soluble SGN-30 and the cross-linking secondary antibody (Table 2). Previous studies with immobilized mAbs to CD30 induced apoptosis in ALCL cells but not in HD cells (12). These data are representative of three independent experiments. Data for untreated populations are those of cells at the end of the time course. No significant changes in cell cycle distribution or apoptotic cells were seen in untreated populations at the different time points (data not shown).

In control studies, the CD30-negative B-cell line Daudi showed only nominal modulation of cell cycle and little increase in apoptosis after treatment with SGN-30 (Table 2). Taken together, these data indicate that SGN-30 induced the growth arrest in, and accumulation of, the  $G_1$  population and a diminution of S phase in CD30-positive lines, and induced apoptosis in L540cy HD and Karpas ALCL cells *in vitro*.

***In Vivo* Efficacy of SGN-30 against HD Xenografts.** The *in vivo* activity of SGN-30 was evaluated in SCID mice using L540cy cells. The establishment of human HD models in mice has proven to be difficult. Unlike other HD-derived cell lines that give very poor engraftment in immunodeficient mice, L540cy HD tumor cell models can be successfully established in SCID mice (25). Two separate disease models using L540cy cells, a disseminated model and a localized s.c. tumor model were used to evaluate the *in vivo* efficacy of SGN-30.

Previous studies have shown that L540cy cells injected i.v. into SCID mice spread in a manner comparable with the dissemination of human HD and show preferential localization to the lymph nodes (25). To evaluate SGN-30 in this disseminated HD model,  $1 \times 10^7$  L540cy cells were injected via the tail vein into C.B-17 SCID mice. Untreated mice, or those that were treated with an equivalent dose of nonbinding control mAbs (data not shown), developed signs of disseminated disease, in particular, hind-limb paralysis, within 30–40 days of tumor cell injection (Fig. 5A). Mice that developed these or other signs of disease were then sacrificed in accordance with Institutional Animal Care and Use Committee guidelines. Therapy with SGN-30 was initiated 1 day after tumor cell injection and administered via i.p. injection every 4 days for a total of 5 injections. All of the animals (five of five) that received the 4-mg/kg/injection dose regimen, and four of five that received either the 1-mg/kg/injection or the 2-mg/

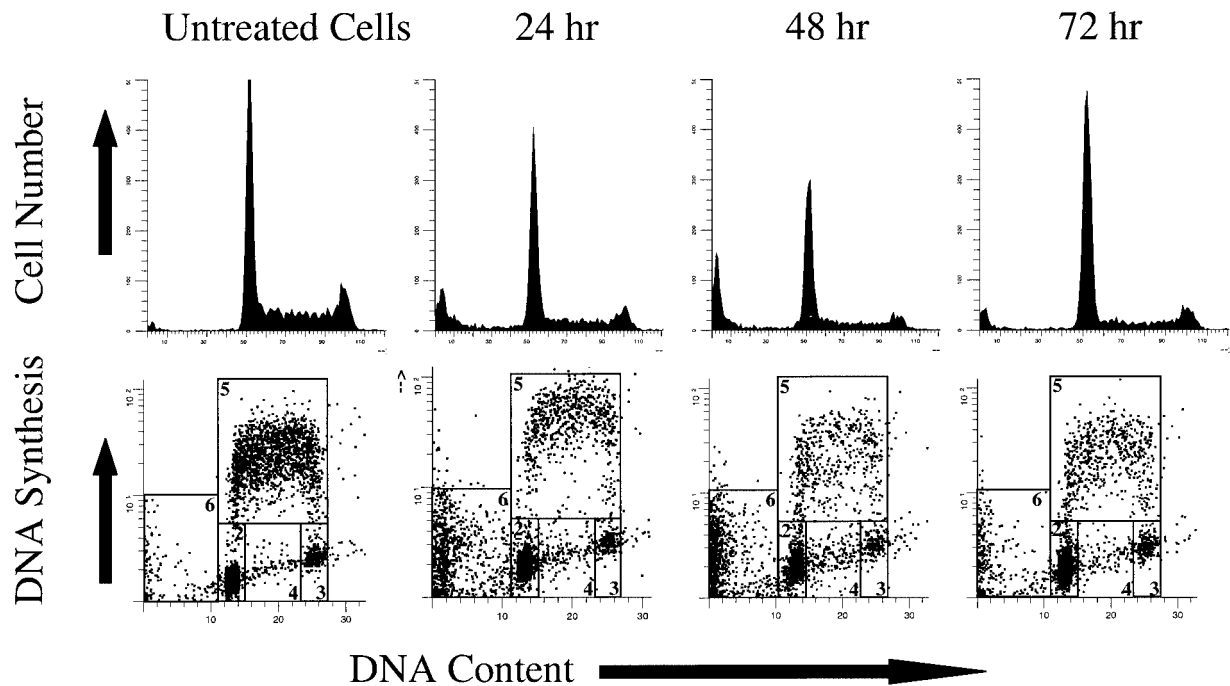


Fig. 4. Cell cycle effects of SGN-30 on L540cy HD cells. Cells were treated with 1  $\mu\text{g/ml}$  SGN-30 and 10  $\mu\text{g/ml}$  of goat antihuman secondary antibody. At the times indicated, cells were labeled with BrdUrd, permeabilized and stained with anti-BrdUrd to detect nascent DNA synthesis (*bottom panels*), and stained with PI to detect total DNA content (*top panels*). *Top panels* profile  $G_1$ , S-phase, and  $G_2$  content via PI staining; the *bottom panels* show content and DNA synthesis as detected by BrdUrd incorporation. *Region 2*,  $G_1$ ; *region 5*, S phase; *region 3*,  $G_2$ ; *region 4*, containing DNA of sub- $G_2$  content not undergoing DNA synthesis; *region 6*, DNA of sub- $G_1$  content indicate cells with apoptotic DNA fragmentation (21). Results are representative of three independent studies.

kg/injection, survived for more than 120 days (the length of the study) with no signs of disease.

In a subsequent study, the efficacy of SGN-30 was further evaluated by varying the day on which therapy was initiated. For this study, L540cy cells were injected into SCID mice via the tail vein on day 0, and therapy was initiated on day 1, day 5, or day 9 (Fig. 5B). In all of the treated groups, SGN-30 was administered at 4 mg/kg using a schedule of every 4 days for 5 injections. Consistent with the previous study, SGN-30 significantly impacted the survival of animals that received therapy starting on day 1, with four of five animals disease-free after 140 days. When the initiation of therapy was delayed, SGN-30 still demonstrated significant efficacy; three of five animals that received therapy starting on day 5, and two of five starting on day 9, remained disease-free for the length of the study.

SGN-30 also demonstrated efficacy in s.c. L540cy HD tumor models. Two  $\times 10^7$  cells were implanted in the flank of SCID mice. Therapy with SGN-30 was initiated when the tumor size in each group of five animals averaged  $\sim 50 \text{ mm}^3$ . Treatment consisted of i.p. injections of SGN-30 every 4 days for 5 injections using the same doses as in the disseminated model, i.e., 1, 2, and 4 mg/kg/injection. Tumors in the untreated animals grew rapidly and reached an average of  $>800 \text{ mm}^3$  by day 34. Tumor progression in animals treated with an equivalent dose of isotype-matched irrelevant control mAb was not significantly different from those of untreated controls (data not shown). SGN-30 produced a significant delay in tumor growth at all of the concentrations tested in a dose-dependent manner (Fig. 5C).

## DISCUSSION

Advances in chemotherapy and radiotherapy regimens for treating HD represent a significant breakthrough in clinical oncology and have increased the remission rate from  $<5\%$  in the 1960s to greater than 80% today (1999; Ref. 26). Nonetheless, these treatments are associated with significant morbidity and mortality. One-third of HD pa-

tients relapse and one-fifth are nonresponsive to initial therapy with little chance of long-term survival even with salvage therapy (27). One significant aspect of HD relapse is the development of tumor cell clones that are refractive to salvage therapy. Targeted mAb therapy represents an alternative strategy that could potentially bypass this resistance or work in concert with standard therapies to selectively sensitize antigen-positive cells for killing by chemotherapy.

CD30 is a potentially significant target for immunotherapy of HD. It is highly expressed on malignant Reed-Sternberg cells, whereas its expression in normal cells is restricted to activated T cells, activated B cells, and activated natural killer cells. Indeed, mAbs to CD30 have been investigated for the treatment of CD30-expressing malignancies, and several studies have shown that anti-CD30 mAbs possessing signaling properties inhibit the growth of ALCL cells but not of HD cells *in vitro* nor in xenografted SCID mice (11, 23). The mechanism by which activating antibodies inhibit ALCL cells is unclear and has variously been attributed to the induction of apoptosis (12, 14) or to

Table 2. Cell cycle effects of SGN-30

|              | Untreated | Treated |      |      |
|--------------|-----------|---------|------|------|
|              |           | 24 h    | 48 h | 72 h |
| L540cy       |           |         |      |      |
| % $G_1$      | 40        | 52      | 51   | 65   |
| % S phase    | 40        | 21      | 13   | 17   |
| % $G_2$ -M   | 13        | 5       | 5    | 7    |
| % Apoptosis  | 6         | 20      | 29   | 10   |
| Karpas = 299 |           |         |      |      |
| % $G_1$      | 41        | 71      | 64   | 59   |
| % S phase    | 40        | 7       | 11   | 17   |
| % $G_2$ -M   | 15        | 17      | 10   | 11   |
| % Apoptosis  | 2         | 5       | 12   | 10   |
| Daudi        |           |         |      |      |
| % $G_1$      | 25        | 26      | 24   | 25   |
| % S phase    | 53        | 49      | 53   | 53   |
| % $G_2$ -M   | 13        | 16      | 12   | 13   |
| % Apoptosis  | 7         | 8       | 8    | 7    |

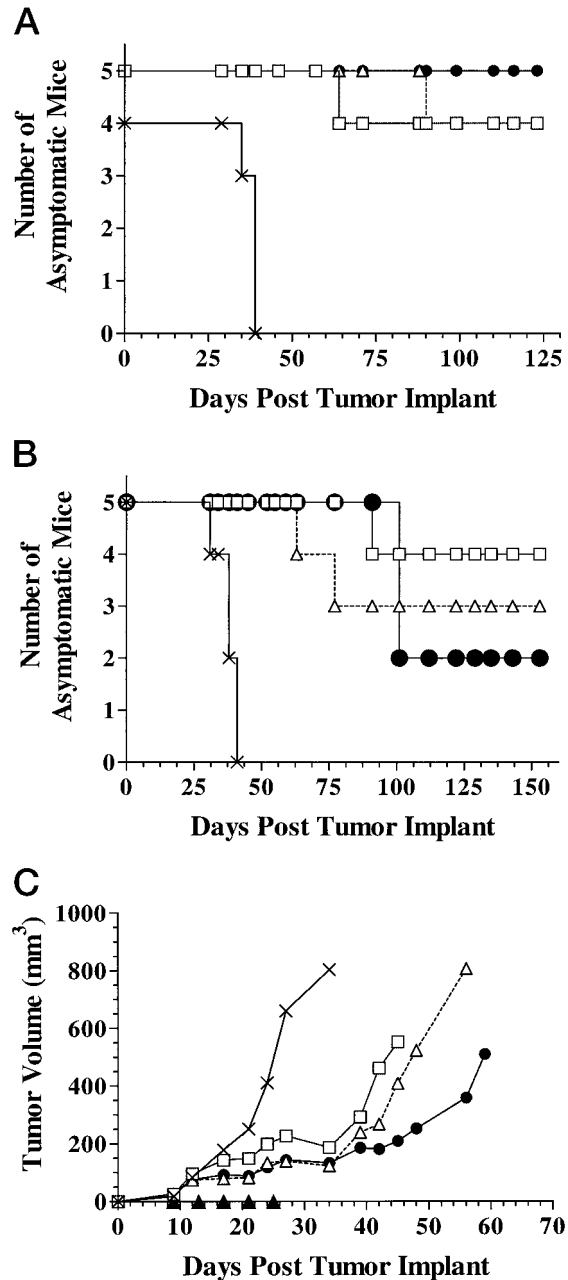


Fig. 5. Efficacy of SGN-30 in HD models. A, antitumor activity of SGN-30 on disseminated L540cy HD in SCID mice. Groups of mice (five/group) were either left untreated (×) or received 1 (□), 2 (△), or 4 (●) mg/kg SGN-30 (every 4 days for five injections) starting on day 1 after tumor inoculation. B, disseminated L540cy HD in SCID mice in which groups of mice (five/group) were either left untreated (×) or received therapy initiated on day 1 (□), day 5 (△), or day 9 (●) by SGN-30 administered at 4 mg/kg using a schedule of every 4 days for five injections. C, s.c. L540cy HD tumor model in SCID mice. Two  $\times 10^7$  L540cy HD cells were implanted into the right flank of the mice. Groups of mice (five/group) were either left untreated (×) or received 1 (□), 2 (△), or 4 (●) mg/kg SGN-30 (every 4 days for five injections; ▲) starting when the tumor size in each group of five animals averaged  $\sim 50$  mm<sup>3</sup>.

cell cycle arrest in the absence of apoptosis (13). In either case, the reported effects of anti-CD30 mAbs on HD cell lines have been in sharp contrast to that on ALCL cell lines. No effect and a stimulatory effect on the proliferation of HD cells *in vitro* have both been reported (11). One possible explanation for the differing effects on ALCL and HD cell lines was recently suggested by Mir *et al.* (12), who demonstrated that constitutive NF $\kappa$ B activation in HD cells rendered them resistant to CD30-induced apoptosis. Despite their ability to identify the disease, other CD30 mAbs had no therapeutic effect on HD cells

(11), and prompted alternative strategies, including immunotoxins and bispecific antibodies, for CD30-targeted treatment of HD (28, 29).

In contrast to these findings, we have observed that the anti-CD30 mAb AC10 and its chimeric form SGN-30 were effective at inhibiting the growth of HD lines *in vitro* and in xenograft models. Recent studies indicate that, as with other receptor-mediated responses, the strength of the CD30 signal varies, depending on the use of soluble or immobilized mAb, and that this may determine sensitivity to growth arrest or apoptosis (13, 14). To parallel earlier studies (11), we examined the effect of the murine AC10 on HD cells using immobilized mAb. The effects were stronger when AC10 or SGN-30 were immobilized to the tissue culture wells as compared with solution. Similarly, as shown for SGN-30, the effects were enhanced when cross-linked using a secondary antibody. The significance of receptor cross-linking has been described previously for other TNF superfamily members including TNF- $\alpha$  and CD40, which require receptor multimerization to bring about TNF-R-associated factor (TRAF)-mediated signaling (30–32). It may be possible that cross-linking, rather than immobilization of mAbs, could reflect the situation *in vivo* in which Fc receptor-mediated clustering of SGN-30 bound to CD30 could induce a similar effect. In this manner, CD30 clustering could then provide transduction of growth arrest and apoptotic signals that, in addition to the mAb accessory functions of antibody-dependent cell-mediated cytotoxicity and complement-mediated cytotoxicity, would promote antitumor activity.

Importantly, the activities of SGN-30 observed on HD cells in culture were reflected by its efficacy in the treatment of xenograft models of solid and disseminated HD at doses ranging from 1 to 4 mg/kg. These doses of SGN-30 were well tolerated with no signs of toxicity and single doses of up to 100 mg/kg have been administered to mice without any toxicity (data not shown). A significant advantage to mAb therapy for the treatment of diseases in man is the potential to specifically target diseased cells with little or no systemic toxicity. This should be especially true with target antigens such as CD30, which are highly expressed on diseased cells but have limited expression in normal tissues. SGN-30 has been administered i.v. to cynomolgus monkeys at 100 mg/kg with no signs of toxicity.<sup>5</sup> On the basis of the antitumor activities reported here, the restricted nature of CD30 expression on normal cells, a favorable tolerance profile in nonhuman primates, and the need for additional therapies for patients with relapsed HD, SGN-30 is being developed for clinical trials.

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