Construction, Expression, and Characterization of BD1-G28-5 sFv, a Single-chain Anti-CD40 Immunotoxin Containing the Ribosome-inactivating Protein Bryodin 1*

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The major limitation to the use of immunotoxins in the clinic is the toxicity associated with the toxin moiety. BD1-G28-5 single-chain Fv (sFv) is a single-chain immunotoxin targeted to human CD40 and consists of bryodin 1 (BD1), a plant ribosome-inactivating protein that is 20–30-fold less toxic in animals than commonly used toxins, fused to the sFv region of the anti-CD40 monoclonal antibody G28-5. This immunotoxin was expressed in *Escherichia coli* and purified from refolded inclusion bodies. BD1-G28-5 sFv retained the full protein synthesis inhibition activity of recombinant BD1 and specifically bound to CD40 with a binding affinity, $k_D$, of 1.5 nM, within 10-fold of the bivalent parental monoclonal antibody. BD1-G28-5 sFv was potently cytotoxic against CD40-expressing B lineage non-Hodgkin’s lymphoma and multiple myeloma cell lines, with EC$_{50}$ values in the ng/ml range, but not against a CD40-negative T cell line. Interestingly, BD1-G28-5 sFv was not cytotoxic against CD40-expressing carcinoma cell lines that were sensitive to a BD1-based immunotoxin conjugate targeted to the Le$^c$ carbohydrate antigen. These data represent the first report indicating that BD1 can be used in the construction of potent single-chain immunotoxins. Additionally, although BD1-G28-5 sFv effectively killed CD40-expressing hematologic malignancies, its lack of activity against CD40-expressing carcinomas suggests that CD40-mediated trafficking of BD1 differs in the two cancer types.

Single-chain immunotoxins are bifunctional molecules consisting of an antibody binding domain genetically fused to a protein toxin. Once bound to the target cells, immunotoxins internalize into endocytic vesicles where the catalytic portion of the toxin is processed and released into the cytosol. Once in the cytosol, protein synthesis is halted and cell death ensues. A number of immunotoxin conjugates, in which the antibody domain was chemically linked to a protein toxin, have been tested in the clinic for indications ranging from cancer to autoimmune disease (1–4). While there have been indications of therapeutic efficacy in these studies, the maximum tolerated dose has precluded therapeutic effects such as those seen in preclinical studies. The most prevalent dose limiting toxicity encountered in immunotoxin trials has been vascular leak syndrome, as evidenced by pulmonary edema, hypoalbuminemia, and weight gain, although other toxicities including myalgia, thrombocytopenia, and elevations in hepatic transaminases have also been seen (1, 3–6).

A variety of toxin molecules have been utilized in the design of immunotoxins. These include the plant ribosome-inactivating proteins (RIPs)$^*$ saporin, momordin, ricin, and pokeweed antiviral protein and the bacterial toxins diphtheria toxin and *Pseudomonas* exotoxin (7–9). While both the plant RIPs and bacterial toxins inhibit protein synthesis in eukaryotic cells, their catalytic mechanisms are unique; specifically, cleavage of the N-glycosidic bond of adenine 4324 of 28 S rRNA (10) and ADP-ribosylation of elongation factor 2 (11, 12), respectively. However, even in the absence of a targeting domain, the native or non-targeted forms of these molecules are toxic in animals. The identification of novel toxins that are less systemically toxic but maintain potent cytotoxic activity when targeted to malignancies is likely to increase the therapeutic utility of immunotoxins.

Bryodin 1 (BD1) is a type I RIP that was originally isolated from the roots of *Bryonia dioica* (13). Type I RIPs, which include BD1, gelonin, saporin, and trichosanthin, contain an enzymatic domain but no binding domain, while type II RIPs such as ricin contain two domains: a catalytic A chain and cell-binding B chain. BD1 was recently cloned from *B. dioica* leaf mRNA and expressed as a recombinant protein in *Escherichia coli* (14). The crystal structure of recombinant BD1 (rBD1) was resolved to 2.1 Å and indicated structural homology with other type I RIPs as well as the A chain of type II RIPs. BD1 was found to possess potent protein synthesis inhibitory activity in a cell-free system and was 20–30-fold less toxic in rodents than were other plant or bacterial toxins used in immunotoxin construction (14, 15).

In this report, we describe the construction and *in vitro* characterization of a single-chain BD1-containing immunotoxin targeted to the human CD40 receptor. This immunotoxin, BD1-G28-5 sFv, was expressed in *E. coli* as a single polypeptide and consists of BD1 fused to the single-chain Fv (sFv) region of the anti-CD40 monoclonal antibody G28-5. CD40 is highly expressed on a variety of hematologic malignancies (16–18), as well as on certain carcinomas (19, 20). We have shown previously that a *Pseudomonas* exotoxin-based single-chain immunotoxin, G28-5 sFv-PE40, was potently and selectively cytotoxic in *vitro* against CD40-expressing malignant cell lines (21) and was efficacious in treating human non-Hodgkin’s lyn-

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1 The abbreviations used are: RIP, ribosome-inactivating protein; BD1, bryodin 1; ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; IFN, interferon; sFv, single-chain Fv.
Phomax xenografted SCID mice (22). Because BD1 is less toxic than PE40 in mice and rats, it is anticipated that immunotoxins containing BD1 will have larger therapeutic windows. The data presented here indicate that BD1-G28-5 sFv is a potent antitumor agent against CD40-expressing hematologic malignancies.

**EXPERIMENTAL PROCEDURES**

**Construction of the BD1-G28-5 sFv Expression Vector**—To construct the BD1-G28-5 sFv expression plasmid, pSE151, the gene encoding BD1 was PCR-amplified from the BD1 expression plasmid pSE13.0 (14). The PCR primers were designed to include a 5′ Ndel restriction site and a 3′ NcoI site. Subsequently, the gene encoding G28-5 sFv was PCR-amplified from pSE51, a previously described plasmid that encodes G28-5-sFv PE40 (21), to include a 5′ Ncol site and a 3′ stop codon followed by an EcoRI site. The PCR products were digested with the appropriate restriction enzymes and ligated into pBWT.9, which had been digested with Ndel and EcoRI to remove BR96-sFv PE40 (23). After transformation into E. coli strain DH5α, positive clones were identified by restriction enzyme analysis and verified by DNA sequencing. The resulting plasmid encoding BD1-G28-5 sFv contains the T7 promoter (24). A 21-base pair cloning linker encoding the amino acid sequence RMHGTKA separates the BD1 and G28-5 sFv moieties. This is the same linker that was used in the construction of G28-5 sFv-PE40 (21). Refolding for 48 h, the protein was dialyzed extensively against 20 mM phosphate buffer (pH 7.4) containing 0.3 M L-arginine. After addition of 1 M NaOH and 2% H2O2, the translated product was precipitated with 25% trichloroacetic acid containing 2% casamino acids. The inclusion bodies were isolated using a detergent extraction procedure as described previously (21), denatured in 7 M guanidine HCl, and refolded in 20 mM NaH2PO4 (pH 7.4) containing 0.3 M L-arginine. After refolding for 48 h, the protein was dialyzed extensively against 20 mM NaH2PO4 and purified by Blue Sepharose (Pharmacia Biotech Inc.), followed by Poros HS (Perseptive, Cambridge, MA) chromatographies. The purified protein was analyzed by SDS-PAGE under non-reducing conditions and size exclusion chromatography using a TSK 3000SW column (TosoHaas, Philadelphia, PA).

**Cell-free Protein Synthesis Inhibition Assay**—Cell-free inhibition of protein synthesis was determined using an *in vitro* rabbit reticulocyte system essentially as described previously (14). Briefly, various concentrations of BD1 or BD1-G28-5 sFv were mixed with rabbit reticulocyte lysate, a mixture of all 20 amino acids minus leucine, [3H]leucine, and bromo mucus virus RNA as the mRNA template in a final volume of 25 μl. After incubation for 1 h at 30 °C, the reaction was terminated by the addition of 1 M NaOH and 2% H2O2. The translated product was precipitated with 25% trichloroacetic acid containing 2% casamino acids, and the radiolabeled protein was harvested onto glass filters and counted in a liquid scintillation counter.

BD1-G28-5 sFv: Binding Analysis—For ELISA analysis, 96-well microtiter plates were coated with 0.5 μg/ml CD40-Ig (25) diluted in 50 mM carbonate/bicarbonate buffer (pH 9.6) and incubated overnight at 4 °C. Control plates were coated with chimeric BR96 antibody (26), which contains the same immunoglobulin domain as CD40-Ig. The plates were shaken dry, blocked with phosphate-buffered saline (PBS) containing 1% bovine serum albumin, and incubated with various concentrations of BD1-G28-5 sFv. After washing three times with PBS, the plates were incubated with BD1-specific rabbit antisera, washed three times, and then incubated with horseradish peroxidase-conjugated goat anti-rabbit Ig diluted 1:3,000. After five final washes, the plates were incubated with TMB Chromagen reagent (Genetic Systems, Redmond, WA) and the reaction was halted by the addition of 1 M H2SO4. The absorbance of each plate was measured at 450 nm.

**BIAcore binding analysis was performed on BIAcore 1000 and 2000 instruments (Pharmacia Biosensor, Uppsala, Sweden) at 25 °C using PBS containing 0.05% P20 surfactant (Pharmacia Biosensor) as the running buffer. The matrix was coated with CD40-Ig, samples of BD1-G28-5 sFv were injected, and the association and dissociation rates were determined by curve fitting using BIAevaluation 2.1 (Pharmacia Biosensor). The equilibrium constant, Kd, was calculated by dividing the dissociation rate by the association rate.

**Cell Culture,** **Cytotoxicity Analysis,** and **Fluorescence-activated Cell Sorting**—Burkitt’s lymphoma cell lines Raji and Daudi, multiple myeloma lines HS-Sultan and IM-9, breast carcinoma line MCF-7, and monocytic cell line THP-1 were purchased from American Type Culture Collection (Rockville, MD). The multiple myeloma lines RAM and RLO were kindly provided by Dr. Joshua Epstein (University of Arkansas for Medical Sciences, Little Rock, AR). T51 lymphoblastoid cells were a gift from Dr. Paul Gladden (Bristol-Myers Squibb, Seattle, WA). The lung carcinoma line L2987, colon carcinoma H3619, and ovarian carcinoma line H4021 were established at Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, WA. All cell lines except MCF-7, H3619, L2987, and H4021 were maintained in RPMI 1640 media containing 10% fetal bovine serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin. MCF-7, H3619, L2987, and H4021 cells were grown in Iscove’s modified Dulbecco’s medium containing the same supplements as above.

Cytotoxicity analysis was performed essentially as described previously (22). For the adherent lines (MCF-7, H3619, L2987, and H4021), cells were plated into 96-well flat bottom tissue culture plates at 104 cells/well. The cells were allowed to adhere overnight at 37 °C, and the next day the medium was removed and replaced with 100 μl of leucine-free RPMI medium. Samples of BD1-G28-5 sFv, G28-5 sFv PE40, or BR96-BD1 (15) diluted in leucine-free medium were then added to the cells. The plates were incubated for 24 h and then pulsed with 1 μCi/well [3H]leucine for 6 h. After freezing to −20 °C, the plates were harvested onto filters and the incorporation of [3H]leucine into cellular protein was analyzed with an LKB Beta-Plate liquid scintillation counter (Wallace, Gaithersburg, MD). The remaining cell lines were non-adherent; therefore, it was not necessary to allow them time to adhere to the tissue culture plates, nor were they frozen before harvesting. For the study utilizing IFN-γ activation, THP-1 cells were cultured in the presence of IFN-γ (Biosource International, Camarillo, CA) for 48 h prior to cytotoxicity analysis.

Fluorescence-activated cell sorting was performed as described previously (21) using G28-5 or a control antibody at 10 μg/ml.

**RESULTS**

**Construction, Expression, and Purification of BD1-G28-5 sFv**—The BD1-G28-5 sFv expression plasmid, pSE151, was constructed as described under “Experimental Procedures” and is shown in Fig. 1. The crystal structure of BD1 indicated that the N terminus of BD1 is more restricted than the C terminus (14). Thus, the BD1-containing single-chain immunotoxin was constructed with G28-5 sFv fused to the C terminus of BD1. We have found previously that G28-5 sFv with the light chain variable region proceeding the heavy chain variable region, G28-5 sFv(VL-VH) resulted in 10-fold higher antigen binding than PE40 in mice and rats, it is anticipated that immunotoxins containing BD1 will have larger therapeutic windows. The data presented here indicate that BD1-G28-5 sFv is a potent antitumor agent against CD40-expressing hematologic malignancies.
dialysis and purification through a two-step chromatographic procedure described under “Experimental Procedures,” the resulting BD1-G28-5 sFv was 95% pure as determined by non-reducing SDS-PAGE (Fig. 2A). Further analysis of the purified protein by size-exclusion chromatography (Fig. 2B) indicated that BD1-G28-5 sFv was present as a single monomeric protein that eluted at the expected molecular mass (55 kDa). No higher molecular weight aggregates or smaller degradation products were apparent.

**Cell-free Protein Synthesis Inhibition Activity of BD1-G28-5 sFv**—To determine if the catalytic activity of BD1 remained intact in the single-chain immunotoxin, BD1-G28-5 sFv was compared with *E. coli* produced rBD1 (14) in a cell-free protein synthesis inhibition assay. Both molecules inhibited protein synthesis with EC₅₀ values (the concentration of protein that yielded 50% inhibition) of 2 pM (Fig. 3). These values are consistent with those previously reported for rBD1 and for native BD1 isolated from the plants (14, 15).

**Binding of BD1-G28-5 sFv to CD40-Ig**—After ascertaining that the catalytic activity of BD1 was retained, ELISA assays were performed to evaluate the binding activity of the fusion. Dilutions of purified BD1-G28-5 sFv were added to 96-well microtiter plates that had been coated with CD40-Ig or an isotype-matched control immunoglobulin. Wells were then probed with rabbit polyclonal anti-BD1 antisera, followed by horseradish peroxidase-conjugated secondary antibodies. BD1-G28-5 sFv specifically bound the CD40-Ig but not to the control immunoglobulin (Fig. 4). The binding of the fusion protein to CD40-Ig was completely blocked by the addition of 25 μg/ml amounts of the parental anti-CD40 antibody, G28-5, but not by the addition of an isotype-matched control immunoglobulin. BIAcore binding analysis was performed to determine the binding affinity of BD1-G28-5 sFv. These experiments showed that the apparent affinity, kₐ, of this monovalent fusion protein was 1.5 nM. This value is similar to that which we previously reported for G28-5 sFv-PE40 (kₐ = 3 nM) and is within 10-fold of the affinity (0.2 nM) of the parental G28-5 mAb (22).

**BD1-G28-5 sFv Is Cytotoxic to CD40-expressing Hematologic Malignancy Cell Lines**—CD40 is highly expressed on a variety of B-lineage malignancies, including non-Hodgkin’s lymphomas (NHL), multiple myelomas, B-lineage leukemias and Hodgkin’s disease (16–18). The cytotoxic activity of BD1-G28-5 sFv was assessed on the CD40-expressing cell lines Raji and Daudi (NHL), T51 (lymphoblastoid), and RAM, RLO, HS-Sultan, and IM-9 (multiple myeloma). As a control, the immunotoxin was also tested on the T-lineage acute lymphoblastic leukemia cell line HPB-ALL, which does not express CD40.

Raji, Daudi, and T51 cell lines were similarly sensitive to BD1-G28-5 sFv with EC₅₀ values of 5–8 ng/ml (Fig. 5A). These values were comparable to those observed for G28-5 sFv-PE40 (21). The cytotoxicity of BD1-G28-5 sFv was inhibited by the addition of 25 μg/ml G28-5 mAb (●) but not by an isotype-matched control antibody. The multiple myeloma cell lines were also sensitive to BD1-G28-5 sFv-mediated killing (Fig. 5B). RAM, RLO, and HS-Sultan cells had EC₅₀ values ranging from 3 to 20 ng/ml, while IM-9 cells were less sensitive with an EC₅₀ of 200 ng/ml. As with Raji, Daudi, and T51, the killing of the multiple myeloma lines could be blocked by the addition of G28-5 antibody. In contrast to the CD40-positive B-lineage malignancies, HPB-ALL cells were completely insensitive to BD1-G28-5 sFv.
Activation with IFN-γ Sensitizes Monocytic Cells to BD1-G28-5 sFv Cytotoxicity—In addition to its expression on B-lineage malignancies, CD40 is expressed on a variety of normal cell types including monocytes (27) and endothelial cells (28). We have shown previously that monocytic and endothelial cells were insensitive to G28-5 sFv-PE40 under normal culture conditions but could be sensitized to the immunotoxin when activated with IFN-γ or IFN-γ plus tumor necrosis factor-α, respectively (29). The effect of BD1-G28-5 sFv was tested on THP-1 monocytic cells in the absence or presence of IFN-γ activation. Without activation THP-1 cells were unaffected by BD1-G28-5 sFv at 1 μg/ml (Fig. 6). As was previously seen with G28-5 sFv-PE40, activation of the cells with 100 units/ml IFN-γ for 48 h prior to the addition of immunotoxin sensitized them to BD1-G28-5 sFv with an EC₅₀ of 300 ng/ml. A higher concentration of IFN-γ, 1000 units/ml, sensitized the THP-1 cells even more, resulting in an EC₅₀ of 30 ng/ml. Thus, the effect of BD1-G28-5 sFv on THP-1 cells was similar to that of G28-5 sFv-PE40 in that the cells were insensitive to the CD40-targeted immunotoxins unless activated with IFN-γ.

CD40-expressing Carcinomas Are Insensitive to BD1-G28-5 sFv—CD40 is also expressed on a variety of solid tumors including carcinomas (19, 20), melanomas (30), and Kaposi’s sarcoma (31). Fluorescence-activated cell sorting was used to identify four carcinoma cell lines, L2987 (lung), MCF-7 (breast), H3619 (colon) carcinomas, and H4021 (ovarian), that expressed CD40 (Table I). These cell lines were then tested for their sensitivity to both BD1-G28-5 sFv and G28-5 sFv-PE40. All four of the carcinoma lines were insensitive to BD1-G28-5 sFv (Table I). In contrast, these cell lines were sensitive to G28-5 sFv-PE40 with EC₅₀ values ranging from 3 to 600 ng/ml. Activation of these carcinoma lines with cytokines did not sensitize them to BD1-G28-5 sFv (data not shown). Thus, while the effect of BD1-G28-5 sFv on CD40-positive B lineage malignancies was similar to that of G28-5 sFv-PE40, only the latter molecule had a cytotoxic effect on the CD40-expressing carcinoma cell lines.

To determine whether these carcinoma cells were resistant to BD1, cytotoxicity studies were done using a BD1-based anticarcinoma (reactive with the Leα antigen) immunotoxin conjugate, BR96-BD1 (15). All of the tested carcinoma lines, L2987, MCF-7, and H3619, were sensitive to BR96-BD1 with EC₅₀ values of 15, 2, and 45 ng/ml, respectively (Table I). These data indicate that carcinoma cells were not resistant to BD1-mediated cytotoxicity and suggest that CD40 traffics BD1 differently in carcinomas than in hematologic malignancies.

**FIG. 5.** Cytotoxicity of BD1-G28-5 sFv against B-lineage cell lines. A, effect of BD1-G28-5 sFv on Daudi (■) and Raji (▲) non-Hodgkin’s lymphoma lines and T51 (●) B lymphoplastoid cells. The open symbols represent the same cell lines tested in the presence of 25 μg/ml G28-5 mAb. B, effect of BD1-G28-5 sFv on HS-Sultan (▲), RLO (●), RAM (■), and IM-9 (○) multiple myeloma cell lines and HPB-ALL (□) T cell acute lymphoblastic leukemia cell line.

**FIG. 6.** Effect of BD1-G28-5 sFv on THP-1 monocytic cells. THP-1 cells were unactivated (●) or activated for 48 h with 100 units/ml (□) or 1000 units/ml (○) of IFN-γ prior to the addition of immunotoxin.

**TABLE I**

| Carcinoma type | CD40 binding ratioa | EC₅₀ (ng/ml) |
|---------------|---------------------|-------------|
|               | BD1-G28-5 sFv       | G28-5 sFv-PE40 | BR96-BD1 |
| L2987 Lung    | 7.7                 | >1,000       | 4          | 15         |
| MCF-7 Breast  | 1.1                 | <1,000       | 600        | 2          |
| H3619 Colon   | 5.1                 | >1,000       | 3          | 45         |
| H4021 Ovarian | 3.4                 | >1,000       | 10         | ND         |

*a* Binding ratio was determined by FACS and is defined as (mean fluorescence of G28–5 mAb binding)/(mean fluorescence of control mAb). ND, not determined.

**DISCUSSION**

While immunotoxins have proven to be potent and specific cytotoxic agents in vitro and efficacious in treating animal models of human diseases in vivo, the results of clinical trials using immunotoxins have been largely disappointing. This is primarily due to toxicities in humans, most importantly vascular leak syndrome, which have limited dose escalations. The construction of new immunotoxins that have reduced toxicity would improve the clinical prospects for immunotoxins. Here, we have constructed and characterized the in vitro properties of a single-chain immunotoxin consisting of the ribosome-inactivating protein BD1 fused to the sFv region of the anti-CD40 antibody G28-5. This immunotoxin potentially represents an improvement over a previously described immunotoxin, G28-5 sFv-PE40 (21, 22), for targeting CD40-positive hematologic malignancies. While both molecules have similar potency in vivo, it is likely that BD1-G28-5 sFv will be less toxic in vivo than the PE40 construct since BD1 is approximately 30-fold less toxic than PE40 (14, 15).

Because the x-ray crystal structure of BD1 indicated that the N terminus was more spatially constrained than the C terminus (14), BD1-G28-5 sFv was constructed with the C terminus of BD1 fused to the N terminus of G28-5 sFv. This orientation appears to be optimal inasmuch as it yielded a molecule that retained the activities of both of the domains. In contrast, attempts to construct a similar molecule with the sFv domain preceding BD1, G28-5 sFv-BD1, failed to yield any active protein (data not shown). It is interesting to note that the sFv moiety retained a high binding affinity when positioned at
either the C terminus, BD1-G28-5 sFv, or N terminus, G28-5 sFv-PE40 (22), of a bifunctional fusion protein.

It was not originally known if a single-chain immunotoxin constructed with BD1, such as BD1-G28-5 sFv, would be cytotoxic to cells without the introduction of a translocation domain. Other toxins that have been used in the construction of single-chain immunotoxins, such as diphtheria toxoid and PE40, contain translocation domains that are required for the intoxication of target cells (32, 33). Additionally, ricin A chain-based single-chain fusion toxins have been largely ineffective (34). The fact that BD1-G28-5 sFv was cytotoxic to these cells suggests that BD1 contains the function necessary to traffic the catalytic activity into the cytosol. We are presently investigating the role of a putative membrane spanning region found in amino acids 131–156 of BD1 (14) in trafficking and translocation. Studies using the type I RIPs gelonin and saporin have shown that they too can be utilized in single-chain immunotoxins without the introduction of a translocation domain (35, 36).

BD1-G28-5 sFv was at least as potent as G28-5 sFv-PE40 (21), which was efficacious in murine models of disseminated human NHL (22). It was unexpected that BD1-G28-5 sFv would have no cytotoxic effect on CD40-positive carcinomas in light of the fact that G28-5 sFv-PE40 was cytotoxic to these cells. We have found previously that certain cytokines or combinations of cytokines sensitized otherwise insensitive CD40-expressing cells to the cytotoxic effect of G28-5 sFv-PE40. In particular, IFN-γ and the combination of IFN-γ plus tumor necrosis factor-α were able to sensitize monocytes and endothelial cells, respectively, to G28-5 sFv-PE40 (29) and similar results were found in the present study with BD1-G28-5 sFv on monocytic cells (Fig. 6). It has also been shown that, in some cases, lysosomotropic reagents such as NH4Cl and chloroquine can enhance the killing of cells by immunotoxins (37, 38). However, no conditions were found that sensitized carcinoma cells to BD1-G28-5 sFv.

One possible explanation for the ineffectiveness of BD1-G28-5 sFv on carcinomas was that the cells tested were somehow naturally resistant to killing by BD1. However, three of these carcinoma lines were sensitive to an immunotoxin consisting of BD1 conjugated to the anti-carcinoma antibody BR96 (Table I). BD1 conjugated to G28-5 antibody, which was active against NHL and multiple myeloma cell lines, was not cytotoxic to carcinomas (data not shown). Thus, the fusion of BD1 to G28-5 sFv did not affect the ability of BD1 to function against the carcinoma cells since the conjugate form gave the same results as the single-chain immunotoxin. These data suggest that trafficking of BD1 mediated by CD40 differs between carcinomas and B-lineage malignancies by a mechanism that has not yet been elucidated.

This is the first report detailing the utility of BD1 in the construction of a single-chain immunotoxin. It will next be important to compare the in vivo anti-tumor efficacy of BD1-G28-5 sFv with G28-5 sFv-PE40, which, along with monkey and rat toxicology studies, will define the therapeutic window of BD1-G28-5 sFv.

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