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Expression and Characterization of Bryodin 1 and a Bryodin 1-Based Single-Chain Immunotoxin from Tobacco Cell Culture

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Bryodin 1 (BD1) is a potent ribosome-inactivating protein (RIP) isolated from the plant *Bryonia dioica*. It is relatively nontoxic in rodents (LD_{50} > 40 mg/kg) and represents a potential improvement over other RIPs and bacterial toxins that have been used in immunotoxins. Recombinant BD1, expressed in *Escherichia coli*, localizes to insoluble inclusion bodies necessitating denaturation and refolding steps to generate active protein. In this report, BD1 was expressed as a soluble recombinant protein in tobacco cell culture (ntBD1) and purified to near homogeneity with yields of up to 30 mg/(L of culture). The protein synthesis inhibition activity of ntBD1 was identical to that of both native BD1 isolated from the roots of *B. dioica* and recombinant BD1 expressed in *E. coli*. Toxicology analysis showed that ntBD1 was well tolerated in rats at doses that cannot be achieved with most other toxin components of immunotoxins. Additionally, a single-chain immunotoxin composed of BD1 fused to the single-chain Fv region of the anti-CD40 antibody G28-5 (ntBD1−G28-5 sFv) was expressed in tobacco tissue culture as a soluble protein and was specifically cytotoxic toward CD40 expressing non-Hodgkin's lymphoma cells *in vitro*. These data indicate that tobacco tissue culture is a viable system for soluble expression of BD1 and BD1-containing immunotoxins.

INTRODUCTION

Immunotoxins are bispecific proteins composed of an antibody binding domain chemically linked or genetically fused to a protein toxin (*1*−*3*). While the binding moiety directs the immunotoxin to the targeted cell surface antigen, it is the toxin domain that, once inside the cell, is responsible for killing the cell by enzymatically inhibiting protein synthesis. The toxin components of immunotoxins possess unique toxicity profiles that can significantly contribute to the dose-limiting toxicity in patient therapy. The plant ribosome-inactivating proteins (RIPs) ricin, pokeweed antiviral protein, and saporin, and the bacterial ADP-ribosylating toxins *Pseudomonas* exotoxin and diphtheria toxin, have been the most widely utilized toxin components of clinically tested immunotoxins (*3*, *4*). However, even in the absence of a targeting domain, the native or nontargeted forms of these toxins are toxic with LD_{50} values (concentration lethal to 50% of the animals tested) of <5 mg/kg in rodents (*5*, *6*). Dose escalations of immunotoxins in clinical trials to amounts that would be expected to result in significant therapeutic efficacy have been problematic. Even so, immunotoxins containing these toxins have shown some promise in the clinic for indications ranging from cancer to autoimmune disease (*7*−*11*).

The identification of toxins that have potent activity when targeted by an antibody binding domain but reduced nonspecific toxicity profiles could ultimately lead to the development of immunotoxins with broader therapeutic windows. One such toxin is the RIP bryodin 1 (BD1) which has been found to have potent cell-free protein synthesis inhibition activity while exhibiting approximately 10-fold less toxicity *in vivo* (LD_{50} > 40 mg/kg in rodents) than other toxins (*5*). The gene encoding BD1 was recently cloned and expressed in *Escherichia coli* as insoluble inclusion bodies which were refolded to yield protein that had activity *in vitro* and *in vivo* equivalent to that of native BD1 (nBD1) extracted from *Bryonia dioica* roots (*6*).

In this report, we describe the expression of BD1 in soluble form using a transgenic tobacco (*Nicotiania tabacum*) tissue culture system with yields of purified BD1 (ntBD1) of up to 30 mg/L. The *in vitro* and *in vivo* activities of ntBD1 were virtually identical to those of both nBD1 and *E. coli*-expressed rBD1. Additionally, a single-chain immunotoxin containing BD1 and the cloned variable regions of the anti-CD40 mAb G28-5 (ntBD1−G28-5 sFv) was constructed and expressed in transgenic tobacco cultures as a soluble fusion protein. ntBD1−G28-5 sFv was specifically cytotoxic toward CD40-expressing non-Hodgkin's lymphoma cells. These data indicate the utility of tobacco cell culture for the production of BD1 and BD1-based single-chain immunotoxins in soluble form and describe an immunotoxin which is being investigated for potential therapeutic applications.

EXPERIMENTAL PROCEDURES

Construction of Tobacco Culture Expression Plasmids Encoding BD1 and BD1−G28-5 sFv. A previously described rBD1 expression vector, pSE13.0 (*6*), encoding BD1 amino acid residues 1−247 was digested with *Nco*I and *Eco*RI. The *Eco*RI site was prepared as a blunt end using the Klenow fragment of DNA polymerase I, and the resulting 770 bp BD1 fragment was ligated into the plant expression vector WRG2481 that had been digested with *Nco*I and *Sma*I. The resulting plasmid, WRG5074, contained the gene encoding BD1 under control of the cauliflower mosaic virus (CaMV) 35s promoter (*12*) with the extensin leader sequence (*13*) to

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direct secretion of soluble protein into the media and the nopaline synthase poly(A) terminator for efficient transcription termination (*14*). A kanamycin resistance gene under the control of the nopaline synthase promoter was inserted as a selectable marker.

The expression plasmid containing the gene fusion BD1−G28-5 sFv was prepared by first PCR amplifying BD1−G28-5 sFv from pSE151 (*15*) using a 5′ primer that introduced an *Nco*I site and sequences from WRG2481 and a 3′ primer that introduced a *Sma*I site and sequences from WRG2481. Homologous recombination (*16*) assembled the *Nco*I- and *Sma*I-digested plant expression vector WRG2481 and the amplified PCR fragment containing BD1−G28-5 sFv following cotransformation in *E. coli* strain DH5α. The resulting expression plasmid was designated WRG5293. Both WRG5074 (BD1) and WRG5293 (BD1−G28−- sFv) constructs were confirmed by DNA sequencing.

Expression and Purification of ntBD1 and ntBD1−G28-5 sFv in Transgenic Tobacco Cell Culture. *Nicotiana tabacum L.* cell culture line NT-1 was obtained from D. Ellis (University of Wisconsin, Madison, WI). The cells were grown in tobacco suspension medium (TSM) or on solid tobacco culture medium (TCM; J. T. Cooley, J. H. Bathe, J. T. Fuller, L. Zhao, H. P. Fell, and D. R. Russell, in preparation). NT-1 suspension cultures were grown in the dark at 28 °C with constant shaking at 150 rpm. The cells were subcultured once per week.

NT-1 cells were transformed with gold particles coated with plasmid DNA using the Accell microparticle bombardment system (Agracetus, Madison, WI) as previously described (*17*). Briefly, 1 day after bombardment, the cells were transferred to reduced osmoticum (TCM containing 0.1 M mannitol and 0.1 M sorbitol) for 24 h and then transferred to TCM plates containing 350 μg/mL kanamycin sulfate for selection. For growth of suspension cultures, kanamycin resistant calli approximately 1 in. in diameter were placed into TSM media containing kanamycin, shaken in the dark at 28 °C for 48 h, and then homogenized by repeat pipetting. Suspension cultures were maintained by subculturing every 10 days.

For purification, spent medium from suspension culture was filtered and concentrated 4-fold. ntBD1 was purified from the concentrate using Macroprep High S (BioRad, Hercules, CA) cation exchange chromatography. ntBD1−G28-5 sFv was purified by CM Sepharose Fast Flow (Pharmacia, Piscataway, NJ) cation exchange chromatography followed by purification over an immobilized CD40-Ig affinity column (*18*). The affinity eluate, which contained ntBD1−G28-5 sFv as well as free sFv, was subsequently purified by cation exchange chromatography using Poros HS resin (PerSeptive, Cambridge, MA).

Preliminary analysis of N-linked glycosylation was performed using the FACE N-linked oligosaccharide profiling system (Glyko Inc., Novato, CA) according to the manufacturer's instructions.

Cell-Free and Cell-Based Protein Synthesis Inhibition Assays. Cell-free inhibition of protein synthesis was analyzed using a rabbit reticulocyte lysate translation system (Promega, Madison, WI) as previously described (*5*). Various concentrations of ntBD1, ntBD1−G28-5 sFv, and rBD1 as a control were incubated at 30 °C with rabbit reticulocyte lysate, a mixture of amino acids without leucine, 0.5 mCi/mL [^{3}H]leucine (Amersham, Arlington Heights, IL), and brome mosaic virus RNA as a template. The reaction proceeded for 1 h and was terminated by the addition of 1 M NaOH with 2% H_2O_2, and the translation product was precipitated with trichloroacetic acid. The radiolabeled proteins were harvested on glass filters and quantitated using a scintillation counter. Samples were performed in triplicate. IC_{50} was defined as the concentration that inhibited 50% of the cell-free protein synthesis.

Cell-based inhibition of protein synthesis assays was performed using JAR choriocarcinoma cells for ntBD1 and rBD1 and Raji or Daudi non-Hodgkin's lymphoma cells for ntBD1−G28-5 sFv. One hundred microliters of cells, diluted to 1×10^5 cells/mL in leucine-free RPMI 1640, was plated in 96-well cell culture plates and incubated for 48 h at 37 °C with various concentrations of ntBD1, rBD1, or ntBD1−G28-5 sFv. The samples were then pulsed with 1 μCi/well [^{3}H]leucine and incubated at 37 °C for an additional 6 h, and radiolabeled protein was harvested onto filter mats using a Tomtec cell harvester (Orange, CT). The incorporation of [^{3}H]-leucine into cellular proteins was analyzed with an LKB Beta-Plate liquid scintillation counter (Wallac, Gaithersburg, MD).

Toxicity of ntBD1 in Rats. The acute toxicity of ntBD1 was determined in Wistar Furth rats (Harlan-Sprague Dawley, Indianapolis, IN). ntBD1 was administered as bolus intraperitoneal injections at doses of up to 60 mg/kg diluted in PBS. Animals were observed for at least 10 days to determine survival. Selected rats were sacrificed 24 h after administration of ntBD1 to perform comprehensive necropsy analysis.

CD40 Binding Analysis. CD40 binding activity was analyzed by ELISA essentially as previously described (*18*). Briefly, 96-well microtiter plates were coated overnight at 4 °C with 100 μL of CD40-Ig at 0.5 μg/mL. CD40-Ig consists of the extracellular domain of human CD40 fused to the Fc domain of human IgG1 (*19*). The plates were then blocked for 1 h with Specimen Diluent (Genetics Systems, Redmond, WA) and incubated for 1 h with dilutions of ntBD1−G28-5 sFv in the absence or presence of G28−5 IgG or an isotype-matched control antibody. After being washed three times with phosphate-buffered saline (PBS), the plates were incubated with rabbit polyclonal anti-BD1 antiserum followed by horseradish peroxidase-conjugated goat anti-rabbit Ig antiserum. Binding to immobilized CD40 was detected by the addition of TMB chromagen reagent (Genetic Systems) and analyzed on a microtiter plate reader at 450 nm.

RESULTS

Expression and Purification of ntBD1. The expression plasmid WRG5074 was constructed as described in Experimental Procedures and is shown schematically in Figure 1A. The plasmid contains the gene encoding BD1 under the control of the cauliflower mosaic virus (CaMV) 35s promoter (*12*). The extensin leader sequence (*13*) at the amino terminus of BD1 directs the secretion of the recombinant protein into the culture medium. WRG5074 was transfected into the tobacco cell line NT1 using the Accell electric discharge propulsion system (*17*), and callus samples were analyzed by SDS−PAGE and Western blotting to identify clones that expressed ntBD1. One clone which yielded qualitatively higher expression was further expanded, and after 10 days of growth, the culture supernatant was isolated and ntBD1 was purified by cation exchange chromatography. SDS−PAGE of the eluted peak (Figure 1B) revealed that ntBD1 was purified to near homogeneity and migrated at a molecular mass of approximately 28 kDa, slightly higher than rBD1 expressed in *Escherichia coli* (*6*), likely due to glycosylation of the plant-derived protein. Preliminary analysis confirmed the presence of N-linked oligosaccharides (data not shown), although further analysis will be necessary

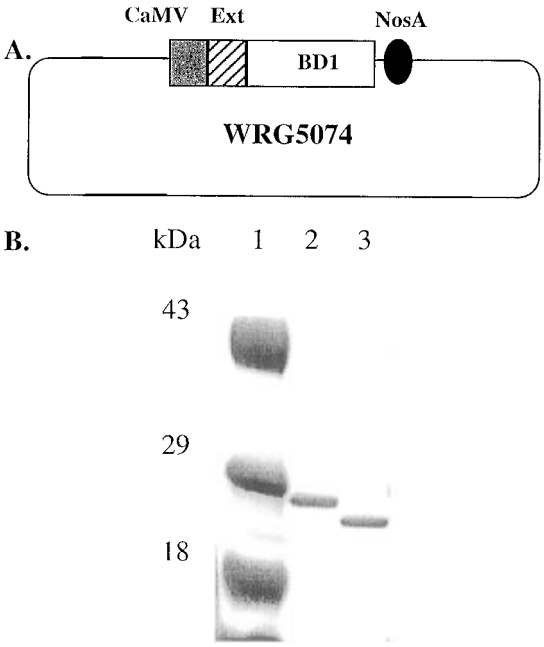

Figure 1. ntBD1 expression plasmid and SDS−PAGE of purified ntBD1. (A) Schematic diagram of the ntBD1 expression plasmid WRG5074. CaMV = the cauliflower mosaic virus 35s promoter. Ext = the extensin leader sequence. NosA = the Nos poly(A) transcription terminator. (B) SDS−PAGE (12%) of ntBD1 (lane 2) and rBD1 (lane 3). The molecular mass markers, in kilodaltons, are shown in lane 1. Samples were electrophoresed under nonreducing conditions.

to determine the extent of N-linked glycosylation as well as to analyze for the presence of O-linked oligosaccharides. The final yield of purified ntBD1 was 30 mg/(L of starting culture).

***In Vitro* Activity of ntBD1.** Both type I and type II RIPs catalytically inactivate the 60S subunit of eukaryotic ribosomes by cleaving the N-glycosidic bond of adenine 4324 in 28S rRNA, thereby halting protein synthesis (*20*). The protein synthesis inhibition activity of ntBD1 was investigated in a cell-free rabbit reticulocyte lysate system and compared to the activity of rBD1. Both ntBD1 and rBD1 inhibited protein synthesis equally with IC_{50} values (concentration that inhibited 50% of protein synthesis) of 0.5 ng/mL (Figure 2A). While BD1 does not contain a cell binding domain, it has previously been shown that type I RIPs such as BD1, saporin, and momorcharin are cytotoxic to trophoblasts and choriocarcinoma cells (*21*). Both ntBD1 and rBD1 were cytotoxic to JAR choriocarcinoma cells with IC_{50} values of 10 ng/mL (Figure 2B). These data indicate that both sources of BD1 were identical in potency and that the glycosylation of ntBD1 did not interfere with the catalytic activity. The difference in IC_{50} values between the cell-free and cell-based protein synthesis inhibition assays reflects the fact that in the cell-based assay the toxin molecule must enter the cell and translocate into the cytosol before it can catalytically inactivate the rRNA.

Table 1. LD_{50} of ntBD1 Rats[a]

amount injected (mg/kg)	no. surviving/no. injected
10	3/3
20	3/3
40	2/3
60	0/3

[a] Groups of Wistar Furth rats were administered the indicated amount of ntBD1 via interperitoneal injection and were observed for 10 days to determine survival.

Toxicity of ntBD1 in Rats. Vascular leak syndrome (VLS) has been the dose-limiting toxicity for a number of immunotoxins tested in the clinic (*8*−*10*, *22*). While immunotoxin-induced VLS has not been seen in mice, it has been observed in rats given either *Pseudomonas* exotoxin-based immunotoxins or binding-defective forms of *Pseudomonas* exotoxin (*23*, *24*). Since it was unknown whether the glycosylated state of ntBD1 would affect its toxicity *in vivo*, experiments were performed to investigate the toxicity of ntBD1 in rats. Pairs of female Wistar Furth rats were administered ntBD1 (up to 60 mg/kg) via intraperitoneal injection. No signs of toxicity were observed at ntBD1 doses of up to 20 mg/kg (Table 1). At 40 and 60 mg/kg, two out of three and zero out of three rats, respectively, survived, indicating that the LD_{50} of ntBD1 in rats was between 40 and 60 mg/kg. This is consistent with previous findings showing that the LD_{50} of rBD1 in rats was >25 mg/kg (*6*). In contrast, the LD_{50} values of PE40 and deglycosylated ricin A chain were 2.0 and 5 mg/kg, respectively (*6*).

Necropsy analysis of the animals 24 h after injection of ntBD1 (60 mg/kg) revealed that the toxicity was localized to the liver and lungs with the gastrointestinal tract, spleen, heart, uterus, brain, kidney, and pancreas all within normal histologic parameters. In the liver, hepatocytes were swollen and some were necrotic. These histologic changes are consistent with the clinical chemistry values obtained showing elevated liver transaminases, indicating liver injury. In the lungs, there was focal edema in the parenchyma and widened perivascular

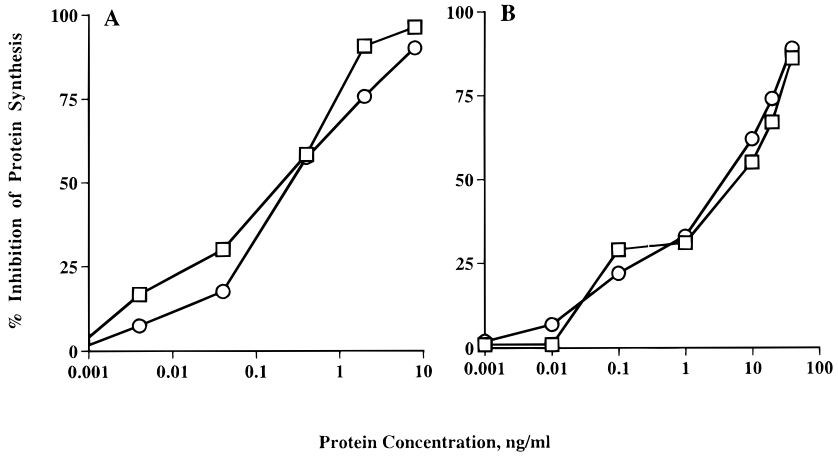

Figure 2. Comparison of the catalytic activity of ntBD1 versus rBD1. (A) Analysis of the cell-free protein synthesis inhibition activity of ntBD1 (○) and rBD1 (□) in the rabbit reticulocyte lysate system. (B) Cytotoxic activity of ntBD1 (○) and rBD1 (□) on JAR choriocarcinoma cells.

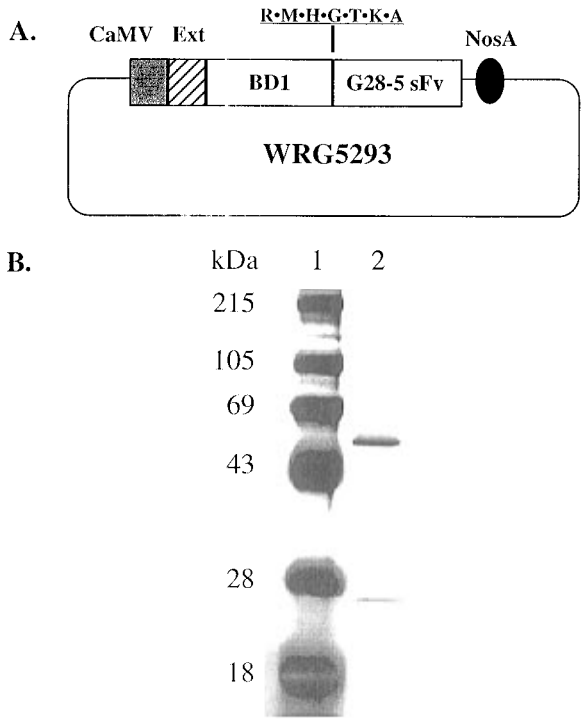

Figure 3. ntBD1−G28-5 sFv expression plasmid and SDS−PAGE. (A) Schematic diagram of ntBD1−G28-5 sFv expression plasmid WRG5293. CaMV = the cauliflower mosaic virus 35s promoter. Ext = the extensin leader sequence. NosA = the Nos poly(A) transcription terminator. The underlined sequence between the BD1 and sFv moieties is the amino acid sequence of the cloning linker. (B) SDS−PAGE (12%) of ntBD1−G28-5 sFv (lane 2) run under nonreducing conditions.

spaces containing scant inflammatory cells. The lung toxicities, indicative of VLS, were similar to those observed in rats 24 h after administration of 2 mg/kg of BR96 sFv-PE40 (*23*) or PE40 alone (*24*). Thus, ntBD1 was approximately 30-fold less toxic to rats than PE40 with the dose-limiting toxicity in both cases being VLS.

Construction, Expression, and Purification of BD1−G28-5 sFv. The use of tobacco cell culture for the production of a single-chain immunotoxin consisting of ntBD1 fused to the sFv region of the G28-5 mAb was investigated. G28−5 recognizes the human CD40 antigen (*24*), and we have previously shown that a single-chain immunotoxin targeted to CD40 (G28-5 sFv−PE40) specifically kills CD40-positive malignant cell lines both *in vitro* and *in vivo* (*18, 26*). Additionally, we have recently shown that BD1−G28-5 sFv expressed in *E. coli* was cytotoxic to CD40-expressing cells *in vitro* (*15*). Since the crystal structure of BD1 indicated that the N terminus was less solvent accessible than the C terminus (*6*), the C terminus of BD1 was fused to the N terminus of G28-5 sFv (BD1−G28-5 sFv) rather than in the opposite orientation (Figure 3A). This was the orientation of the fusion protein that yielded active material when refolded from inclusion bodies isolated from *E. coli*.

After expression of ntBD1−G28-5 sFv, encoded by WRG5293, the fusion protein was purified from tobacco cell culture supernatant by cation exchange followed by affinity chromatography using immobilized CD40-Ig. The affinity eluate was subsequently purified by an additional cation exchange step. Analysis of the purified sample by SDS−PAGE under nonreducing conditions showed two distinct proteins (Figure 3B), one migrating at 55 kDa, the expected size of the immunotoxin, and the second migrating at 27 kDa. Western blot analysis with anti-BD1 monoclonal antibodies and polyclonal anti-G28−5 idiotype antiserum revealed that the 55 kDa protein was ntBD1−G28-5 sFv (data not shown). The 27 kDa protein was recognized only by the anti-G28-5 antiserum and thus represents the sFv fragment, as confirmed by N-terminal protein sequencing (data not shown). The origin of the free sFv is not known, although it is likely the result of proteolysis, either during expression in tobacco cell culture or during purification. However, analysis of the peptide linker between the BD1 and G28-5 sFv moieties did not reveal any known protease cleavage sites that would yield the N-terminal sequence analysis obtained for this protein band (data not shown).

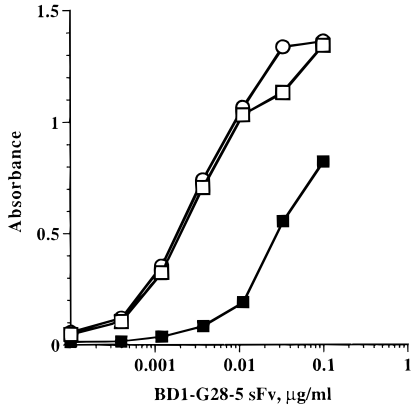

Figure 4. ELISA binding of ntBD1−G28-5 sFv to immobilized CD40-Ig. ntBD1−G28-5 sFv binding was analyzed without competitor (□) or in the presence of 10 mg/mL G28-5 IgG (■) or 10 μg/mL isotype-matched control antibody (○).

The difference in isoelectric points for G28-5 sFv and BD1−G28-5 sFv (theoretical values of 7.0 and 9.0, respectively) should have been sufficient to allow for separation by ion exchange chromatography. The fact that the sFv and ntBD1−G28-5 sFv molecules were readily separated on SDS−PAGE under non-reducing conditions but not by ion exchange chromatography suggests that the two molecules may have been noncovalently associated, although the exact nature of this association is unclear. In addition, the two protein species could not be separated by gel filtration (data not shown). Some sFv molecules have been shown to form dimers in solution with the V_H of one molecule associating with the V_L of a second molecule (*27, 28*), so it is possible that a similar dimerization occurred between the sFv of the fusion protein and the free sFv. Nevertheless, the resulting BD1−G28-5 sFv preparation was >90% pure.

Binding and Cytotoxic Activity of ntBD1−G28-5 sFv. ntBD1−G28-5 sFv bound to immobilized CD40-Ig as determined by an ELISA (Figure 4). This binding was specific since it could be blocked by the addition of the parental antibody G28−5 (10 μg/mL) but not by an isotype-matched control antibody. The ability of ntBD1−G28-5 sFv to inhibit protein synthesis in CD40-expressing cells, and therefore induce cytotoxicity, was examined. For this analysis, the Burkitt's lymphoma cell lines Raji and Daudi and the B lymphoblastoid line T51 were used, all of which were previously shown to express CD40 (*18*). The T cell leukemia line HPB-ALL, which does not express CD40, was used as a control. The three CD40-positive lines were sensitive to the cytotoxic activity of ntBD1−G28-5 sFv with IC_{50} values ranging from 0.2 to 1.0 ng/mL (Figure 5A). HPB-ALL cells were insensitive to this immunotoxin even at 1 μg/mL. ntBD1 by itself was not cytotoxic to any of the cell lines tested, indicating that the cytotoxic effect on the CD40-positive lines was a result of the immunotoxin being targeted to the receptor. Furthermore, the cytotoxic activity was specifically inhibited by the addition of 10 μg/mL G28-5 IgG

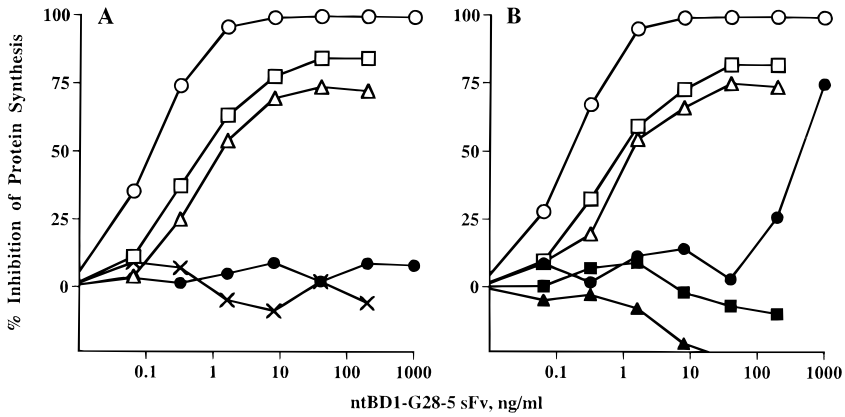

Figure 5. Protein synthesis inhibition activity of ntBD1−G28-5 sFv. (A) Activity of ntBD1−G28-5 sFv on the CD40-positive cell lines Daudi (□), T51 (○), and Raji (△) and the CD40-negative cell line HPB-ALL (×). Also shown is the activity of ntBD1 on T51 cells (●). (B) Activity of ntBD1−G28-5 sFv in the presence of 10 mg/mL G28-5 IgG or isotype-matched control antibody. Daudi: with G28-5 IgG (■) or with control antibody (□). T51: with G28-5 IgG (●) or with control antibody (○). Raji with G28-5 IgG (▲) or with control antibody (△).

but not by the addition of an isotype-matched control antibody (Figure 5B).

DISCUSSION

Immunotoxins can selectively eliminate malignant cells expressing specific surface antigens *in vivo.* However, the clinical utility of immunotoxins has been limited in large part by the nonspecific toxicities that are associated with the toxin moiety rather than cross-reactivities with normal tissues that express the target antigen. The reduced toxicity observed with BD1 (the LD_{50} of BD1 in rodents is 10−30-fold higher than that of ricin A chain and PE40) indicates that this RIP represents an improvement over presently utilized toxins in immunotoxin development.

The use of transgenic tobacco cell culture offers an effective and robust means of producing BD1 and BD1-containing single-chain immunotoxins. Soluble ntBD1 was readily obtained from tobacco cell culture without refolding of denatured inclusion bodies as is required for many products expressed in *E. coli.* Tobacco culture-expressed ntBD1 was indistinguishable from rBD1 in protein synthesis inhibition activity, cytotoxicity, and rat toxicity, although ntBD1 migrated as a slightly larger molecule than rBD1 on nonreducing SDS−PAGE. Additionally, ntBD1−G28-5 sFv, a single-chain immunotoxin fusion protein targeted to the CD40 antigen, was solubly expressed in tobacco tissue culture. This fusion protein was specifically and potently cytotoxic to CD40-expressing non-Hodgkin's lymphoma cell lines *in vitro,* demonstrating that tobacco cell culture is a viable system for the production of RIP-based single-chain immunotoxins.

Plant cell culture offers an attractive alternative to bacterial, yeast, and mammalian cells for the production of heterologous proteins. It may also be possible to express BD1 fusion proteins in transgenic plants such as corn or soybean as a method for large scale production. In this report, we have shown that tobacco cell culture can be utilized to produce soluble forms of a plant ribosome-inactivating protein, ntBD1 and a single-chain immunotoxin containing BD1, ntBD1−G28-5 sFv. It is anticipated that BD1-containing immunotoxins will likewise be less toxic than analogous PE40-containing immunotoxins. Comparative efficacy and toxicity studies of the two single-chain anti-CD40 immunotoxins, ntBD1−G28-5 sFv and G28-5 sFv−PE40, will ultimately allow for determination of their relative therapeutic windows.

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