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Preclinical Assessment of an Anti-EpCAM Immunotoxin: Locoregional Delivery Provides a Safer Alternative to Systemic Administration

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Abstract

VB4-845 is a recombinant immunotoxin that is comprised of a truncated form of *Pseudomonas* exotoxin A (ETA) genetically-linked to a humanized scFv fragment, (4D5MOCB), specific to epithelial cell adhesion molecule (EpCAM). EpCAM is overexpressed on a wide variety of human tumors and thus represents a suitable target antigen for immunotoxin therapy. Preclinical studies were used to evaluate the benefit of locoregional administration of an ETA-based immunotoxin versus systemic delivery. Repeated subcutaneous (s.c.) administration of VB4-845 (up to 77.8 μg/kg) in rats resulted in minimal adverse effects, except for injection-site reactions, while repeated systemic administration elicited symptoms consistent with vascular leak syndrome. S.c. weekly doses of the drug in cynomolgus monkeys resulted in minimal adverse effects limited to injection-site reactions and a transient elevation of liver enzymes in 1 animal. Toxicokinetics showed rapid clearance of the drug, with the development of an immune response by day 14 following repeated injections. These results argue that the local administration of VB4-845 has advantages with respect to safety over systemic administration and may be an effective alternative method for targeting those cancers that are amenable to local routes of administration.

Key words: immunotherapy, cancer, targeted therapy, antibody

Introduction

Immunotoxins offer a promising alternative approach for the treatment of cancer with reduced toxicity and greater therapeutic potential. Immunotoxins are recombinant proteins comprised of a monoclonal antibody portion that binds to its target antigen on the surface of tumor cells and a potent cytotoxic moiety that, when internalized, causes cell death. VB4-845 is an antiepithelial cell adhesion molecule (EpCAM) immunotoxin comprised of the humanized single-chain antibody fragment (scFv) 4D5MOCB fused to a truncated form of *Pseudomonas* exotoxin A (ETA) that lacks the cell-binding domain.[1,2] Upon internalization, an activated form of ETA, released by furin cleavage, causes ADP-ribosylation of elongation factor-2 that prevents protein synthesis, thus leading to cell death.[3–5] Although ETA immunotoxins are extremely potent in killing tumor cells *in vitro*, drug-related manifestations from systemic dosing with ETA compounds have limited this therapeutic approach. Patients treated with ETA-based immunotoxins often present with the classical symptoms of vascular leak syndrome (VLS) characterized, in part, by increased vascular permeability, hypotension, pulmonary insufficiency, and, in its most severe form, multiple organ failure.[6] Deleting the cell-binding domain (residues 1-252) in second-generation ETA-immunotoxins has reduced binding to normal tissue; however, VLS symptoms are still encountered.[7–10] Being a bacterial protein, the immunogenicity of ETA limits the number of treatment cycles due to the production of antitoxin antibodies.[6,11,12] These dose-limiting side-effects represent significant obstacles on the path to clinical efficacy, in particular with epithelial-derived tumors.

EpCAM, is an epithelial cell marker to which various normal cellular functions have been ascribed. Overexpression of EpCAM in tumors, though, is associated with

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disease progression, enhanced proliferation, and the development of malignancy.[13] EpCAM is present in all stages of tumor development and is highly expressed in a variety of carcinomas, including bladder, breast, colon, gastric, and head and neck cancer, with comparatively less expression in most normal epithelial tissues.[14–16] Several antibody therapies have been developed for EpCAM-positive tumors utilizing systemic administration for drug delivery; but, none have proven overly effective in the treatment of solid tumors in patients.[17–20] In one instance, where an anti-EpCAM-ricinA immunotoxin was delivered via systemic administration to breast cancer patients, drug-related symptoms indicative of VLS and neuropathy were detected.[21]

Preclinical efficacy with intravenously i.v. administered VB4-845 has been demonstrated in animal models of EpCAM-positive human tumor xenografts in mice.[1] Also, peritumoral treatment of EpCAM-positive head and neck tumor xenografts showed a significant decrease in tumor volume and a concomitant increase in survival, as compared to the untreated controls. No animal toxicity was observed with VB4-845, and the biodistribution showed preferential accumulation in EpCAM-positive tumor cells, as compared to EpCAM-negative tumor cells,[1] indicating that VB4-845 has the therapeutic potential to treat EpCAM overexpressing tumors. In contrast, systemic delivery of a radiolabeled, full-length anti-EpCAM antibody in mice with SCCHN xenografts resulted in low tumor-specific accumulation and significant toxicity in normal tissue, emphasizing the requirement for smaller antibody fragments for better tumor penetration.[22]

Most solid tumors develop resistance to conventional treatments, resulting in relapse and metastatic progression. Increasing the dose of systemically delivered drugs leads to unacceptable side-effects; thus, the design of alternative therapies is of paramount importance. Locoregional regimens with chemotherapeutics have proven, in some instances, to be more efficacious while also reducing side-effects.[23–25] This approach has proven effective with antibody-based therapeutics in early-stage clinical trials for the treatment of glioblastoma and breast cancer with few adverse events.[26,27] Given the limitations of systemically administered ETA immunotoxins, this study assessed the toxicokinetic and immunologic parameters of the anti-EpCAM-ETA immunotoxin, VB4-845, administered both regionally and systemically in two different animal species. The results support that the local administration of VB4-845 is a safer alternative treatment option for accessible EpCAM-positive tumors.

Materials and Methods

Construct

The VB4-845 template was placed under the transcriptional control of the *ara*B (L-arabinose-inducible) promoter from *Salmonella typhimurium*, using splice overlapping extension polymerase chain reaction (SOE-PCR). The internal *Eco*RI and *Xho*I restriction sites of the original 4D5MOCB-ETA DNA template were disabled from using four primer sets.[1] A *Pel*B leader sequence was added to the full-length immunotoxin by consecutive PCR reactions, using two primer sets.[28] Two primers were used to introduce *Eco*RI and *Xho*I restrictions sites to facilitate subcloning into the pING3302 vector (Xoma, Berkeley, CA). Upon sequence confirmation, the VB4-845 template was purified, digested with *Eco*RI and *Xho*I, and ligated into the pING3302 expression vector. The completed vector was transformed into *Escherichia coli* E104 (Xoma, Berkeley, CA).

Expression and purification. Cultivation of transformed *E. coli* E104 was carried out by using a seed-expansion culture in shake-flasks followed by fed-batch cultivation in a bioreactor. Briefly, a 120-L bioreactor (TB medium) was inoculated with 1.2 L of the seed culture (OD_{600nm} of 2.0–2.5) and VB4-845 protein expression induced at an OD_{600nm} of 20 ± 1, using a glycerol solution containing 17% L-arabinose, for a period of 46 hours at 25°C. Following centrifugation of the culture, the supernatant containing the VB4-845 protein was clarified by crossflow filtration (Hydrosart™ cassette [0.6 m^2]; Sartorius, Edgewood, NY).

The VB4-845 protein (Fig. 1) was purified by initial capture directly from the supernatant on a Ni^{2+} affinity column (Chelating Sepharose FF; GE Healthcare, Buckinghamshire, UK) and eluted in a buffer containing yeast extract (24 g/L), 72 mM K_2HPO_4, 17 mM KH_2PO_4, and 500 mM of imidazole (pH 7.0). The Ni^{2+} column eluate was diluted in elution buffer without imidazole (pH 9.0), allowed to stand at room temperature, and filtered through a Sartobran P (0.2 μm) filter (Sartorius). The filtrate was applied to a DEAE Sepharose column (FF resin; GE Healthcare, Buckinghamshire, UK), and the flow-through material was collected and diafiltered to reduce the levels of imidazole. The filtrate was applied to a second nickel-affinity column and the column washed with 20 mM NaH_2PO_4, 20 mM of imidazole, 150 mM NaCl, and 0.5% Triton X-100 (pH 7.2) and eluted with 20 mM NaH_2PO_4, 500 mM of imidazole, and 150 mM NaCl (pH 7.2). The VB4-845 fraction was diluted 5-fold with 20 mM NaH_2PO_4 (pH 7.2) and loaded onto a Q Sepharose chromatography column (HP resin; GE Healthcare). The column was washed (20 mM NaH_2PO_4 and 90 mM NaCl; pH 7.2) and the purified VB4-845 eluted with 20 mM NaH_2PO_4 and 500 mM NaCl (pH 7.2). VB4-845 identity was confirmed by immunoblotting, using anti-ETA (Sigma-Aldrich, St. Louis, MO) and anti-His (GE Healthcare), followed by anti-rabbit-IgG-HRP and anti-mouse-IgG-HRP (The Binding Site, Birmingham, UK), respectively.

Bioassays

Cell lines. Bladder (T-24), breast (ZR-75-1), head and neck (CAL27), lung (NCI-H69), melanoma (A-375), and prostate (LNCaP) tumor cell lines (American Type Culture Collection, Manassa, VA) and bladder (HMVEC-bd), breast (HMEC), lung (NHLF), and prostate (PrEC) primary normal

FIG. 1. Schematic representation of VB4-845. The VB4-845 protein contains the EpCAM-specific 4D5MOCB scFv moiety at the N-terminus and a truncated form of ETA at the C-terminus that induces cell death upon internalization. The two moieties are flanked at either end by two His-tags (H). A KDEL signal sequence is located at the C-terminus to facilitate intracellular trafficking of ETA.

cell lines (Lonza Walkersville, Inc., Walkersville, MD) were cultured according to specification and maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Flow cytometry. The cell-surface reactivity of VB4-845 with human normal and tumor epithelial cell lines was assessed by flow cytometry. Briefly, cells (0.3×10^6) were incubated with VB4-845 (10 μg/mL) on ice for 2 hours. Anti-EGFR (epidermal growth-factor receptor mouse monoclonal antibody (1 μg/mL; EMD Biosciences, San Diego, CA) was used as a positive control and 4B5-ETA (10 μg/mL), a His-tagged scFv version of the human anti-Id genetically linked to ETA, was used as a negative control.[29] Binding was detected with either anti-His-Tag antibody (1:800; GE Healthcare) or biotin-conjugated antimouse IgG for anti-EGFR (1:200; Pierce, Rockland, IL) followed by either fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (1:100; The Binding Site) or Streptavidin-Cy-Chrome (1:120; BD Bioscience Pharmingen, San Diego, CA). The cells were washed and resuspended in buffer containing propidium iodide (0.6 μg/mL; Molecular Probes, Eugene, OR), and binding was analyzed with a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA). Head and neck EpCAM-positive (CAL27) and melanoma EpCAM-negative (A-375) tumor cell lines were included as controls. Binding was considered positive if antibody-treated cells exhibited a positive shift in fluorescence resulting in >30% positive cells over the negative control.

Cell potency assay. The ability of VB4-845 to inhibit cell growth was determined by using a standard MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] assay. Tumor and normal cells (5000 cells/well) in culture medium containing 10% fetal calf serum (FCS) were incubated with VB4-845 or the negative control, scFv 4B5-ETA, over a range of concentrations (0.00005–500 pM) for 72 hours at 37°C in the presence of 5% CO_2. The MTS assay was performed, according to the manufacturer's instructions (Promega, Madison, WI) and the OD_{450nm} read (Molecular Devices Corp., Sunnyvale, CA), and an IC_{50} value was calculated for each cell line.

Animal toxicology studies

Sprague-Dawley rats and cynomolgus monkeys were obtained from Charles River Canada (Montreal, Quebec, Canada) and housed and maintained according to the AAALAC International Guide for the Care and Use of Experimental animals. Rats were fed commercial rodent chow (8728C; Teklad, Harlan, Haslett, MI). Monkeys were housed with two or three animals of the same sex and same dosing group together and fed commercial primate food (2050C, Harlan) twice daily and allowed other supplements.

Single-dose toxicology. A dose-ranging study was conducted in rats, using local and systemic administration of VB4-845. Sprague-Dawley rats (2/gender/dose) were administered single intradermal (i.d.) injections of VB4-845 into the shoulder at dose levels of 1.14, 4.29, 8.57, 17.10, 42.90, or 85.7 μg/kg. I.v. injections of VB4-845 were administered as a single bolus via the lateral tail vein at doses of 429.0, 643.5, and 858.0 μg/kg, diluted in the vehicle or the vehicle alone as control (20 mM Tris and 500 mM NaCl; pH 7.4). Clinical signs, body weight, hematology, coagulation, clinical chemistry, and urinalysis were examined. Animals were euthanized at 24 hours upon completion of dosing and subjected to gross pathologic examinations, organ-weight measurements, and histopathologic examination.

A dose-escalation study of VB4-845 in cynomolgus monkeys was conducted to determine the maximum tolerated dose (MTD). Two (2) male animals were alternately dosed s.c. (right scapular region) with 15–350 μg/kg/day, with a wash-out period of 72 hours between doses. Another 2 animals received doses of 1000 or 3500 μg/kg/day. Animals were monitored for clinical signs and clinical biochemistry, and pathological examinations were conducted, including organ weights and macroscopic evaluations.

Repeated-dose toxicology. Sprague-Dawley rats were administered VB4-845 by s.c. injections (1.0, 5.0, 35.0, or 77.8 μg/kg) in the shoulder or i.v. bolus injections (77.8 μg/kg) via the lateral tail vein as a daily dose for 7 days, with a 14-day observation period. Control animals received the drug vehicle. Cynomolgus monkeys received s.c. weekly doses of 35, 175, or 350 μg/kg/day over 4 weeks, with an observation period of 2 weeks. Different sites of injection were used for each dose (right and left scapular and lumbar regions). Control animals in all studies received the drug vehicle (20 mM NaH_2PO_4; pH 7.4). Parameters monitored during both studies included clinical signs, mortality, changes in body weight and food consumption, and ophthalmologic examinations. Hematology, clinical chemistry, and urinalysis were performed on days 8, 14, and 21 for rats. Similar analyses were done on samples from monkeys taken prior to dosing and during weeks 4 and 6. Baseline hematology/clinical biochemistry data were established, based upon a historical database for normal Spraque-Dawley rats, as well as published information.[30,31] Normal ranges for the monkeys were obtained during dosing with either the pretreatment values, the control group animal values, or historical data gathered from control cynomolgus monkeys ($n=159$) over an extended period of time (Charles River Laboratories, Preclinical Services, Montreal, Quebec, Canada). In addition to the clinical pathology and hematology, the monkeys also underwent neurologic assessments and electrocardiograms. Histopathologic examinations were conducted on tissue samples from necropsied animals taken on days 8, 14, and 21 for the rat study and at the end of dosing and the end of the recovery period for the monkey study.

Pharmacokinetics. The pharmacokinetic profile of VB4-845, following s.c. administration, was evaluated as part of the repeated-dose study in cynomolgus monkeys, using a sandwich enzyme-linked immunosorbent assay (ELISA). Briefly, plasma samples from animals at all doses (0, 35, 175, and 350 μg/kg/day) were collected prior to dosing on days 1 and 22 and postdose at 0.25, 0.50, 1, 2, 4, and 24 hours. ELISA plates were coated with affinity-purified rabbit polyclonal anti-ETA (Sigma Aldrich) and incubated overnight at 2–8°C. Plates were treated with blocking buffer phosphate-buffer saline (PBS) with 5% bovine serum albumin (BSA) for 1 hour at 22°C, and plasma samples were added. Commercial monkey plasma (Biomeda, Foster City, CA; or Bioreclamation, Hicksville, NY) was used as a negative control, and

standard curves were prepared by spiking VB4-845 (0–50 ng/mL) in the commercial plasma. ELISA plates were incubated for 2 hours at 22°C and washed. A biotinylated affinity-purified rabbit polyclonal anti-4D5MOCB scFv detector antibody (Viventia Biotechnologies, Inc., Winnepeg, Manitoba, Canada) was added, plates incubated for 1 hour (22°C), and then washed. Streptavidin-HRP (horseradish peroxidase conjugate (Pierce) was added for 30 minutes at 22°C, followed by TMB substrate (KPL Gaithersburg, MD), and the absorbance was read at 450 nm (V_{max}; Molecular Devices Corp.). The concentration of VB4-845 was interpolated from the standard curve ($SOFT_{max}$; Molecular Devices Corp.).

Immunogenicity. Serum samples from rats receiving repeated doses (0.0, 1.0, 5.0, 35.0, or 77.8 μg/kg) were collected on day 0 and day 21 (end of recovery period) to analyze the immune response to VB4-845. Monkey plasma samples were taken on day 0 and prior to dosing on days 7, 14, 28, and at the end of the recovery period (day 42). All samples were analyzed by ELISA for the presence of antibodies against full-length VB4-845, as well as the human scFv moiety (4D5MOCB scFv) and ETA. For both the rat and monkey samples, ELISA plates were coated with ETA_{1-608} (Sigma-Aldrich), 4D5MOCB scFv, or VB4-845 at equimolar concentrations (14 nM) in carbonate buffer at pH 9.6. After incubation with blocking buffer (PBS, 1% BSA), serially diluted monkey plasma or rat-serum samples were added and incubated for 2 hours at 22°C. Rat and cynomolgus monkey pretreatment samples were used as negative controls and rabbit anti-4D5MOCB scFv and rabbit anti-ETA antibodies included as positive controls. For rat samples, following incubation, the plates were washed and HRP-conjugated rabbit antirat IgG and IgM antibodies were added (Southern Biotech, Birmingham, AL). Positive controls (rabbit anti-ETA or rabbit anti-4D5MOCB scFv) were incubated with HRP-conjugated goat antirabbit IgG (The Binding Site, San Diego, CA). For monkey samples, HRP-conjugated goat anti-monkey IgG, IgM, and IgA (H+L) antibodies (Rockland Immunochemicals, Gilbertsville, PA) were used. Secondary antibody incubation (1 hour at 22°C) was followed by the addition of the TMB substrate (KPL), and absorbance at 450 nm was determined (V_{max}; Molecular Devices Corp.). Samples were scored as positive when the OD_{450nm} was >0.1 after subtracting the value of the negative control. The antibody titer was defined as the reciprocal dilution at 0.1 OD_{450nm} + 4×standard deviation (SD).

Neutralizing antibody. Plasma samples were collected from monkeys in the repeated-dose toxicology study (35, 175, and 350 μg/kg/day) prior to dosing on days 0, 7, 14, 28, and day 42. Neutralizing antibodies were detected in animal plasma by measuring the inhibition of VB4-845-mediated cytotoxicity of EpCAM-positive CAL27 cells by the MTS-potency assay. CAL27 cells were seeded at a density of 5000 cells/well in a 96-well microplate in a total volume of 50 μL of culture medium per well and incubated for 2 hours at 37°C in the presence of 5% CO_2. Plasma samples were serially diluted, starting at 1/1000, and VB4-845 was spiked at a final concentration of 0.15 ng/mL. The negative control was commercial cynomolgus plasma spiked with VB4-845 (0.15 ng/mL), a drug level representing maximum cytotoxicity. The positive control was commercial cynomolgus monkey plasma spiked with VB4-845 (0.15 ng/mL) and an affinity-purified rabbit anti-ETA polyclonal antibody (Sigma-Alrich) known to neutralize VB4-845. Maximum growth was determined with cells grown in commercial cynomolgus plasma. After 72 hours of incubation at 37°C under 5% CO_2, the MTS reagent was added and the absorbance read. The inhibition or neutralizing effect was expressed as the percent increase in viability over the negative control, as previously described.[32] The neutralizing titer was determined as the reciprocal dilution of the plasma sample required to neutralize 50% of the activity of VB4-845 (0.15 ng/mL).

Results

VB4-845 biological activity

To facilitate downstream purification and thereby ease scale-up production, the original VB4-845 template was placed under the control of the *ara*B promoter, leading to the soluble expression of VB4-845 protein into the supernatant. The relative protein size and identity were confirmed by Coomassie staining and Western blotting, respectively, and showed a single product migrating at the expected molecular weight of M_r 70,000. The identity was further verified by mass spectrometry (data not shown).

VB4-845 specificity for EpCAM was demonstrated by cell-surface reactivity and potency testing against various epithelial-derived tumor and normal cell lines originating from different tissue types (Table 1). VB4-845 showed strong reactivity to all tumor cell lines tested (28- to 134-fold increase over the control), except for the EpCAM-negative cell line, A-375. Cell-surface binding was observed with the normal prostate cell line, PrEC, with binding of approximately 6-fold less than to that detected against the prostate tumor cell line, LNCaP. As predicted by the binding data, all the EpCAM-positive tumor cell lines were sensitive to killing with VB4-845, with IC_{50} values ranging from 0.001 to 1.84 pM. No IC_{50} (>500 pM) was determined for two of the normal cell lines

Table 1. Cell-Surface Reactivity and Potency of VB4-845 Versus Normal and Tumor Epithelial Cell Lines

Tissue of origin	*Cell line*	*Binding[a] (MF increase ± SEM)*	*Cytotoxicity IC_{50} pM*
Tumor			
Bladder	T-24	134.1 ± 35.9	0.001 ± 0.00
Breast	ZR-75-1	37.7 ± 8.5	0.084 ± 0.036
Lung	NCI-H69	28.2 ± 4.0	1.84 ± 1.17
Prostate	LNCaP	78.4 ± 6.7	0.18 ± 0.12
Head and Neck	CAL-27	87.0 ± 3.0	0.29 ± 0.1
Melanoma	A-375	1.1 ± 0.1	>500
Normal			
Bladder	HMVEC-bd	1.0 ± 0.0	>500
Breast	HMEC	2.2 ± 0.1	395 ± 25.0
Lung	NHLF	1.1 ± 0.0	>500
Prostate	PrEC	13.4 ± 1.2	10.7 ± 6.3

[a]Binding was determined as the mean fold (MF) increase in median fluorescence above the scFv 4B5 ETA negative control from 2 independent experiments.
SEM, standard error of the mean.

Table 2. Toxicology Profile of Local Subcutaneous (s.c.) and Systemic Intravenous (i.v.) Repeated Daily Administration of VB4-845 in Rats

	Drug dose (μg/kg/day)					
	s.c.					*i.v.*
	0	*1.0*	*5.0*	*35.0*	*77.8*	*77.8*
Clinical Observations (daily)	$n=40$	$n=40$	$n=40$	$n=40$	$n=60$	$n=60$
Injection site						
erythema/edema	0	0	39	40	60	0
ulceration	0	0	2	2	8	0
alopecia	0	0	2	16	22	0
scratches	0	0	4	4	2	0
scab	0	0	7	20	40	0
necrosis	0	0	6	40	60	0
skin thickening	0	0	0	18	40	0
Dyspnea	0	0	0	0	0	50
Rough pelage	0	0	0	0	8	44
Wobbly gate	0	0	0	0	0	27
Piloerection	0	0	0	0	0	4
Lethargy	0	0	0	0	0	9
Passivity	0	0	0	0	0	1
Tremor	0	0	0	0	0	2
Hyperactivity	0	0	0	0	0	1
Opthamology	0	0	0	0	0	0
Body-weight loss (day 8)	0	0	0	0	0	28
Clinical Pathology[a]						
Hematology (day 8)[b]	$n=20$	$n=20$	$n=20$	$n=20$	$n=20$	$n=14$
WBC count	2	2	0	5	9	3
Neutrophil	0	1	1	17	20	10
Platelet	0	0	2	18	17	4
Serum Chemistry (day 8)[b]	$n=20$	$n=20$	$n=20$	$n=20$	$n=20$	$n=14$
Hyperfibrinogenemia	0	1	13	19	18	7
Hypoproteinemia	0	0	0	6	5	0
Hypoalbuminemia	0	0	0	11	20	0
Hyperglobulinemia	0	0	0	0	0	0
Albumin/globulin	0	0	1	20	20	0
BUN	0	0	0	0	0	12
Creatinine	0	0	0	0	0	3
AST	3	4	8	11	13	7
ALT	0	0	0	1	7	0
Urinalysis (day 8)[b]	$n=20$	$n=20$	$n=20$	$n=20$	$n=20$	$n=14$
Proteinuria	0	0	2	0	0	12
Leucocytes	0	2	0	0	0	12
Hematuria	0	0	2	2	4	3
Histopathology (day 8)[c]	$n=20$	$n=20$	$n=20$	$n=20$	$n=20$	$n=14$
Injection site reaction	0	9	18	16	20	0
Kidney inflammation	NR	NR	NR	NR	1	13
Lung inflammation	NR	NR	NR	NR	0	4
Mortality[d]	0	0	0	0	0	14

Values represent the number of animals showing the adverse effect; male and female results were combined.

s.c., subcutaneous injection; i.v., intravenous injection; WBC, white blood cell; BUN, blood urea nitrogen; AST, aspartate aminotransferase; ALT, alanine aminotransferase; NR, not reported.

[a]Baseline hematology/clinical biochemistry data were established based upon a historical database for normal Spraque-Dawley rats as well as published information.[30,31] Values were considered normal/abnormal if they fell within/outside baseline mean ± 2 standard deviations for control animals.

[b]Individuals outside of normal range.

[c]Histology was completed on tissue samples from adrenals, aorta (thoracic), brain, cecum, colon, duodenum, epididymes, esophagus, eyes, femur and marrow, heart, ileum, jejunum, kidneys, liver, lungs, mandibular and mesenteric lymph nodes, mammary gland, optic nerves, ovaries, pancreas, pituitary, prostate, salivary glands, seminal vesicles, sciatic nerve, skeletal muscle, skin, spinal cord, spleen, sternum and marrow, stomach, testes, thymus, thyroid/parathyroid, tongue, trachea, urinary bladder, uterus, and vagina. Tissues showing gross pathologic changes (i.e., injection-site reactions) were also assessed. Only those tissues showing markedly different histologic findings from controls are indicated.

[d]Death or moribund sacrifice by day 8.

(bladder and lung). However, where an IC_{50} was measured, as in the case of prostate and breast, the normal cell lines were 59- and 4.7×10^3-fold less sensitive to VB4-845 than their tumor counterparts, respectively.

Animal toxicology studies

In order to establish a pharmacologically relevant species for use in toxicology studies, several animal species (mouse, rat, dog, cynomolgus and Rhesus monkeys, and chimpanzee) were screened for binding cross-reactivity with VB4-845 through immunohistochemistry analysis. Only chimpanzees had similar cross-reactivity to humans (data not shown); however, due to growing ethical concerns and a lack of available colonies, toxicology studies were not performed with these animals. It is well documented that i.v. administration of ETA immunotoxins to rats results in symptoms that resemble VLS, as seen in human immunotoxin trials.[6,33] Thus, the Sprague-Dawley rat was chosen for toxicologic testing, and the effects of local administration (s.c.) were compared to systemic administration (i.v.). Additional studies in a second animal model of the cynomolgus monkey, with more clinical relevance to humans, were carried out to establish an MTD for s.c. administration and to monitor for any adverse effects.

Dose-ranging study in rats. Clinical signs noted in the dose-ranging study in Sprague-Dawley rats administered VB4-845 locally (1.14–85.70 μg/kg i.d.) and systemically (429.0–858.0 μg/kg i.v.) were limited to injection-site lesions that exhibited a dose-dependent effect. There were no other adverse effects in animals locally dosed. Animals that were systemically dosed had an increase in red blood cell parameters, total red blood cell counts, hemoglobin, and hematocrit and a decrease in albumin, total protein, and albumin-globulin ratio. While all these findings were dose dependent, these variations remained within the normal physiologic range.

Repeat-dosing study in rats. Local and systemic effects of VB4-845 in rats were evaluated after repeated doses of VB4-845 administered by local s.c. (1.0, 5.0, 35.0, or 77.8 μg/kg) or systemic IV (77.8 μg/kg) injections. Due to anticipated difficulties with repeated i.d. dosing, based on single injection-site reactions, the s.c. route of administration was used as another representative route of local administration. Although animals were assessed on days 8, 14, and 21, adverse events related to dosing were maximal upon completion of dosing, so only day 8 results are shown (Table 2). No effects attributable to the local administration of VB4-845 were noted in clinical signs, body weight, temperature, urinalysis, ophthalmologic examinations, or organ weights. Injection-site reactions (i.e., slight erythema, edema, superficial necrosis, ulcerations, and scab formation), attributable to VB4-845, were dose related and noted at or above 5.0 μg/kg and, in most cases, had resolved by the end of the observation period. Dose-dependent, but transient, changes were noted in hematology (e.g., increased white blood cells, platelets, and neutrophils), coagulation parameters (elevated fibrinogen), and serum chemistry (mild hypoalbuminemia) that were most likely due to acute tissue injury and inflammation at the injection sites. The liver enzyme levels, aspar-

Table 3. Toxicology Profile of Repeat Subcutaneous Dosing of VB4-845 in Cynomolgus Monkeys (Week 4)

	Dose (μg/kg/day)			
	0 n = *10*	*35* n = *6*	*175* n = *10*	*350* n = *10*
Clinical Observations[a]				
Injection-site skin flaking[b]	3	15	30	32
Injection-site redness[b]	4	10	21	25
Injection-site lesion[b]	0	1	2	4
Injection-site scab[b]	10	3	9	12
Injection-site discolor[b]	0	1	2	4
Reduced appetite	1	1	3	7
Limited forearm use	0	0	3	6
Opthamology[c,d]	0	0	0	0
Body weight[c]	0	0	0	0
Neurology[c,e]	0	0	0	0
Electrocardiography[c,f]	0	0	0	0
Hematology[c,g]				
WBC	2	0	1	1
% Neutrophils	1	1	1	3
Serum Chemistry[c,h]				
ALT	0	0	0	1
AST	0	0	0	1
hypobilirubinemia	0	0	2	3
Urinalysis[c,i]	0	0	0	0
Histopathology[c,j]	0	0	0	0
Mortality	0	0	0	0

Male and female results were combined in each case.

[a]All animals were observed twice-daily (once on the day of arrival) for mortality and signs of ill health and/or reaction to treatment.

[b]Total number of events reported for 4 injection sites per animal.

[c]Number of animals with values outside of normal range.

[d]Animals were subjected to funduscopic (direct and indirect ophthalmoscopy) and biomicroscopic (slit-lamp) examinations.

[e]Neurologic examinations were performed twice prior to the start of treatment and near the end of the week 4. Examinations included general attitude and behavior, postural reactions (assessments of proprioceptive positioning, visual and tactile placing reactions), cranial nerve function (assessments of head movement/symmetry, head muscle tone, eye reactions, eye symmetry, vestibular nystagmus, eye position, corneal reflex, pupillary light reflex, and nasal septum), and spinal nerve function (assessments of muscle tone, patellar reflex, flexor reflex, and perineal reflex).

[f]Electrocardiogram recordings were performed prior to treatment and on days 1 and 28 and at the end of the recovery period.

[g]Parameters examined: activated partial thromboplastin time, blood cell morphology, erythrocyte indices (MCV, MCH, MCHC, and RDW), hematocrit, hemoglobin, mean platelet volume, platelet count, prothrombin time, red blood cell count, reticulocyte count (absolute and percent), white blood cell count (total, absolute, and percent differential).

[h]Serum chemistry parameters measured were: A/G ratio (calculated), ALT, albumin, alkaline phosphatase, aspartate aminotransferase, blood urea nitrogen, calcium, chloride, cholesterol, creatinine, globulin (calculated), glucose, inorganic phosphorus, potassium, sodium total, direct and indirect bilirubin, total protein, and triglycerides.

[i]Parameters examined included: bilirubin, blood, color and appearance, glucose, ketones, microscopy of centrifuged deposit, nitrite, pH, protein, specific gravity, urobilinogen, and volume.

[j]A total of 43 different tissue types were assessed.

AST, aspartate aminotransferase; ALT, alanine aminotransferase; WBC, white blood cell count.

TABLE 4. SUMMARY OF PHARMACOKINETIC PARAMETERS WITH LOCAL ADMINISTRATION OF VB4-845 IN CYNOMOLGUS MONKEYS

Dose level (μg/kg)	*Day*	C_{max} *(ng/mL)*	T_{max} *(hr)*	$AUC_{(0\text{-}last)}$ *(hr ng/mL)*
35	1	46.7 ± 12.6	3.0 ± 1.10	298 ± 269
	22	0	0	0
175	1	336 ± 244	3.80 ± .63	4560 ± 3110
	22	0	0	0
350	1	279 ± 117	3.50 ± 1.08	2810 ± 1390
	22	0	0	0

Data for males and females are combined. Values are means ± standard deviation. C_{max}, maximum observed drug concentration in plasma; T_{max}, time of maximum observed drug concentration in plasma; $AUC_{(0\text{-}last)}$, area under the drug concentration – time curve from time 0 to time t, where t is the time of the last measurable plasma concentration.

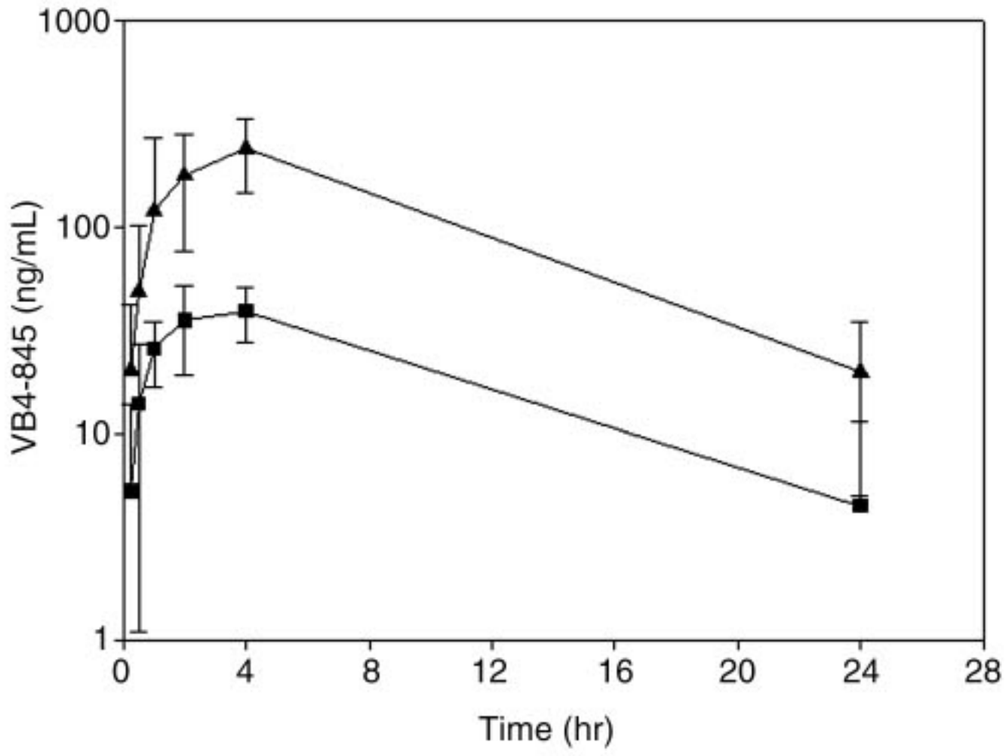

FIG. 2. Plasma concentrations of VB4-845 in cynomolgus monkeys on day 1 following subcutaneous administration. Values for both sexes were combined. Error bars represent standard deviation. ■, 35 μg/kg; ▲, 350 μg/kg.

tate aminotransferase (AST) and alanine aminotransferase (ALT), were marginally elevated in rats at upper dose levels but returned to the normal physiologic range by the end of the observation period. These were thought to be associated with the increased number of necroinflammatory foci in the liver, secondary to the bacterial shedding from the infected skin wounds, a common clinically insignificant inflammatory disease in rats. Alternatively, this may be due to the increased clearance of foreign protein in the liver, causing elevated liver enzymes.

Repeat systemic administration of VB4-845 in rats resulted in microvascular injury and pulmonary edema, with subsequent hypoxia (data not shown). A total of 7 of 30 male and 7 of 30 female rats died or were moribund-sacrificed at the 77.8 μg/kg i.v. dose of VB4-845 within 3–10 days following the initiation of dosing. Animals that died within the first 6 days of dosing exhibited hydrothorax and hemorrhaging plus edema of the lungs, consistent with symptoms of VLS.[34] The main effects on animals that died after 6 days were dehydration, cachexia, and stomach ulcers. Most animals exhibited respiratory difficulties (e.g., dyspnea) and neurologic symptoms (i.e., behavior) (Table 2). Reduced food consumption and decreased body weight were noted; however, surviving animals improved during the observation period. Transient changes were noted in hematology (increased neutrophil and platelet counts) and serum chemistry (increased blood urea nitrogen and creatinine) at the completion of dosing. Serum chemistry changes correlated with increased kidney weights and were consistent with histologic findings of nephrosis and papillary necrosis. During the recovery period, serum chemistry returned to normal and renal histopathology scores improved. Urinalysis evaluation revealed proteinuria and the presence of leukocytes; mild hematuria was also found in a small number of animals. Gross and histopathologic findings of ischemic necrosis in all major organs, including the kidney, heart, brain, and liver in the animals that died, were consistent with those previously observed in rats exposed to ETA-based immunotoxins.[35,36]

Dose-ranging study in cynomolgus monkeys. Single s.c. doses (15 to 3500 μg/kg/day) were administered to cynomolgus monkeys on an alternating basis to determine the MTD. Doses up to 350 μg/kg/day did not result in clinical changes at any dose level and all clinical biochemistry was within the normal range. Animals dosed at 1000 and 3500 μg/kg/day displayed lesions at the injection site associated with other localized or systemic effects suggestive of inflammation (i.e., limited usage of affected limb, elevated body temperature, and neutrophilia). Reduced appetite was also noted and a body weight loss was recorded for the animal dosed with 3500 μg/kg/day. Marginally elevated AST levels were also observed in the animal dosed with 1000 μg/kg/day. These findings had no macroscopic correlate. Based on this initial study, the MTD was determined to be 1 mg/kg, as doses of 1000 and 3500 μg/kg/day were considered too irritant for repeated-dose administration. Therefore, dosing levels for repeated s.c. injections were set at 35, 175, and 350 μg/kg/day to represent up to 10 times the intended clinical dose, with weekly administration to reflect the intended clinical schedule.

Repeat-dosing study in cynomolgus monkeys. Repeated weekly s.c. dosing of VB4-845 in cynomolgus monkeys over a 4-week period resulted in minimal aberrant clinical observations. As was noted in the repeat-dosing rat study, the drug-related effects were maximal immediately following the last dose (week 4) and subsided during the recovery period (week 6). As such, only week 4 data are shown, (Table 3). Most events were associated with the administration of the drug (e.g., skin flaking, redness, and lesions) that were limited to the injection site and observed at all dose levels. As was observed in the rats, these findings were transient and not seen at the end of the recovery period. At higher doses of the drug, reduced appetite and limited forearm usage were also apparent. At week 4, no significant changes were found in body weight, neurology, electrocardiography, urinalysis, organ weight, gross pathology, and histopathology. Although 2 animals had white blood cell counts outside the normal range at the 175 and 350 μg/kg doses, they were within the range of the control group. Increased values for % neutrophils outside of normal were also indicated for animals across all doses. These

Table 5. Immunogenicity of VB4-845, ETA, and 4D5MOCB scFv on Day 14 Following Local or Systemic Administration of VB4-845 In Sprague-Dawley Rats

		Males		Females	
Dose (μg/kg)		*Titer (±SEM)*[a]	*Responders*	*Titer (±SEM)*[a]	*Responders*
Anti-VB4-845					
Local[b]	0	0	0/10	0	0/10
	1	100	1/10	1200 ± 611	3/10
	5	11,667 ± 3249	8/10	11,100 ± 3164	9/10
	35	57,600 ± 4267	10/10	81,600 ± 13,587	10/10
	77.8	83,200 ± 9776	10/10	102,400 ± 10,451	10/10
Systemic[c]	77.8	11,000 ± 2995	10/10	11,200 ± 2894	10/10
Anti-ETA					
Local[b]	0	0	0/10	0	0/10
	1	0	0/10	300 ± 153	3/10
	5	5111 ± 1670	8/9	4000 ± 1145	9/10
	35	24,000 ± 3373	10/10	45,200 ± 11,471	10/10
	77.8	36,000 ± 6667	10/10	33,600 ± 3733	10/10
Systemic[c]	77.8	4,500 ± 980	10/10	2500 ± 522	10/10
Anti-4D5MOCB scFv					
Local[b]	0	0	0/10	0	0/10
	1	0	0/10	100	1/10
	5	667 ± 289	4/9	1200 ± 416	6/10
	35	18,400 ± 2400	10/10	23,200 ± 3029	10/10
	77.8	24,000 ± 2667	10/10	64,000 ± 11,685	10/10
Systemic[c]	77.8	5400 ± 1485	10/10	4800 ± 952	9/10

Values represent the mean titer of the responders (± standard error of the mean; SEM) within each group.
[a]Titer is defined as the reciprocal of the greatest dilution of sera with a positive OD (OD blank + 4×SD).
[b]Subcutaneous administration.
[c]Intravenous administration.

changes were not considered treatment related, as similar changes were seen in control animals. One (1) female in the 350 μg/kg group had increased levels of ALT (2-fold) and AST (1.5-fold), when compared to pretreatment values, control group animal values, and historic data. However, these findings had no macroscopic or microscopic correlate and returned to normal ranges during the recovery period. Hypobilirubinemia occurred at doses of 175 and 350 μg/kg/day in 2 and 3 animals, respectively. During the recovery period, the bilirubin levels in these animals remained low. However, recovery levels in the control group were also lower than the normal range and experimental levels were within the control-group range. These results indicate that weekly s.c. injections of VB4-845 of up to 350 μg/kg/day were well tolerated in cynomolgus monkeys, with no systemic inflammation and minimal and inconsequential microscopic changes. The no-adverse-effect level (NOAEL) was determined to be 350 μg/kg/day.

Pharmacokinetics. Plasma samples were taken from cynomolgus monkeys prior to s.c. dosing on days 1 and 22 and 0.25, 0.5, 1, 2, 4, and 24 hours after dosing with VB4-845 (Table 4) and the pharmacokinetic profile determined. High variability was seen among individuals within groups. The mean VB4-845 concentration on day 1 peaked at the 4-hour time point in all dose levels (Fig. 2). Concentrations of VB4-845 were below the limit of quantification (<10 ng/mL) in all samples on day 22 and were most likely due to the clearance of the drug by the immune response. Total exposure (AUC_{last}) demonstrated dose proportionality in the lowest and highest dose groups, with a 10-fold increase in dose corresponding to a 9.4-fold increase in AUC. Statistical analysis was not possible due to the limited number of animals and high level of variability. VB4-845 half-life ($T_{1/2}$) and elimination rate (K_{el}) could not be evaluated due to insufficient time points following the T_{max}.

The pharmacokinetics of VB4-845 administered locally and systemically has been previously reported for rats.[37] Similar to the monkey pharmacokinetics, the maximum VB4-845 plasma concentration (50 ng/mL) in rats with local administration occurred at 4 hours postinjection. Systemic administration in rats resulted in a maximum plasma concentration of 1000 ng/mL after 10 minutes, with a half-life of 2.3 hours. A decrease in plasma concentrations was apparent with systemic injections from days 1–7 but was not observable in local injections.

Immunogenicity. An immune response to VB4-845 was detected in both the rats and monkeys. On day 14 postinjection, a dose-dependent antidrug antibody response was observed in rats dosed by local and systemic administration with VB4-845, with the majority of animals showing a response when injected with ≥5 μg/kg (Table 5). In general, both portions of the construct (scFv and ETA moiety) induced a similar strength antibody titer irrespective of gender or route of administration. S.c. injection of proteins usually elicits a better immune responses than i.v. delivery; thus, not surprisingly, the VB4-845 titer following local administration was significantly higher ($p < 0.001$) than that observed after systemic administration at the same dose level.[38]

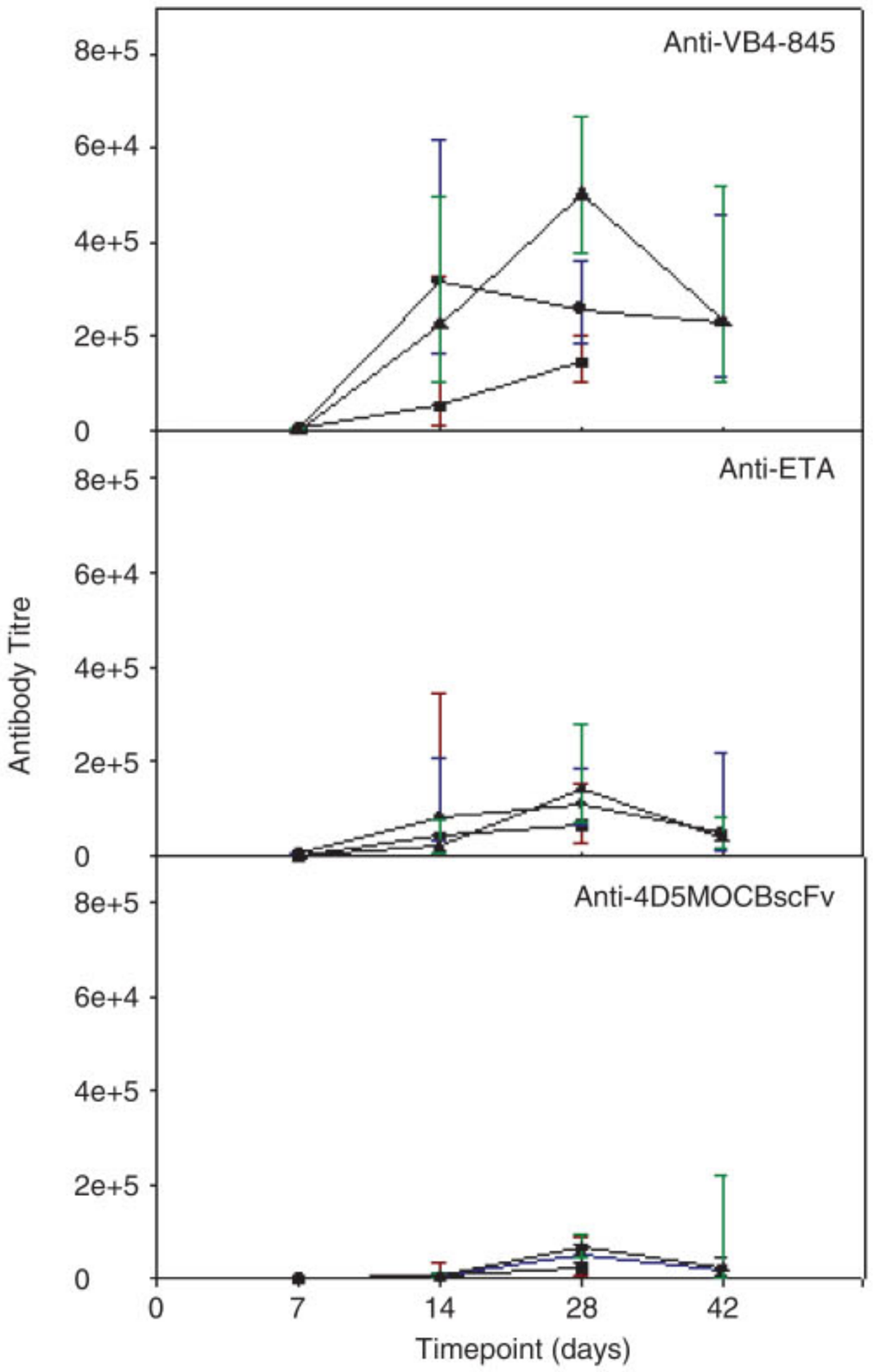

FIG. 3. Antibody response to VB4-845, ETA, and 4D5MOCB scFv in cynomolgus monkeys following weekly repeated subcutaneous administration. Values represent the geometric mean ± 95% confidence interval. Values for both genders were combined. ■, 35 μg/kg; ●, 175 μg/kg; ▲, 350 μg/kg.

Antibody titers in monkeys to VB4-845 and component molecules, ETA, and 4D5MOCB scFv were observed at day 14, with a maximum response seen by day 28 (Fig. 3). Again, there was a high degree of variability in the responses among animals within the same dose, which is expected of non-in-bred animals.

Neutralizing antibodies. Neutralizing antibodies were not detected in cynomolgus monkeys until day 14 (Table 6). Of the samples analyzed, 8 of 12 (67%) animals presented with neutralizing antibodies on day 14, and at day 28, 20 of 26 (77%) animals had a detectable titer. Titers generally decreased during the recovery period at day 42. A large variability in neutralizing antibody production was apparent within groups across all doses. No linear correlation between neutralizing antibody production and immunogenicity of VB4-845 was observed.

Discussion

Recombinant immunotoxins have proven to be an effective treatment in humoral cancers, but significant barriers still exist in the systemic treatment of solid tumors, such as unwanted toxicities, limited penetration into the tumor bed, and immunogenicity.[39] Moreover, systemic delivery of recombinant ETA immunotoxins commonly induces VLS, a side-effect resulting in microvascular injury, leading to edema, hypoxia, and, potentially death.[36] Although modifications and use of truncated forms of the toxin molecules have significantly reduced VLS, higher concentrations of immunotoxins may still induce these symptoms.[40,41] The effectiveness of the intratumoral injection for the delivery of an anti-ErbB2/HER2 ETA-based immunotoxin has been evaluated for the treatment of cutaneous lesions in patients with breast cancer, colorectal carcinoma, and melanoma.[27] Not only was there a more favorable safety profile reported for these patients, in that the drug-related reactions were limited to inflammation at the injection site, but the patients also showed a higher response rate to the treatment, in comparison to similar patients treated with the same drug intravenously.[42]

In this study, we performed a preclinical assessment on an anti-EpCAM-ETA immunotoxin, VB4-845, intended for local treatment. A significantly favorable difference in toxicity was observed between local and systemic administration in the rat repeated-dose study. Animals administered systemic doses of VB4-845 displayed symptoms of VLS that resulted in deaths or the requirement for moribund sacrifice with multiple organ ischemic necrosis and changes in histopathology. In marked contrast, rats that received 7 consecutive days of locally administered VB4-845 showed no adverse clinical signs, except for injection-site reactions that resolved by the end of the observation period. The transient changes observed in hematology and serum chemistry were likely

TABLE 6. NEUTRALIZING ANTIBODY PRODUCTION IN CYNOMOLGUS MONKEYS FOLLOWING SUBCUTANEOUS INJECTIONS OF VB4-845

Dose level (μg/kg)	*Neutralizing antibody titer*			
	Day 7	*Day 14*[a]	*Day 28*	*Day 42*[a]
35	0 (0)	2278 (0–26,904)	7124 (0–27,616)[b]	NA
175	0 (0)	35,600 (14,963–65,763)	25,920 (0–97,790)[c]	10,220 (4520–14,491)
350	0 (0)	18,980 (0–70,503)	7924 (0–99,449)[c]	8038 (3103–36,411)

Values represent the geometric mean. Values in parentheses indicate the range.
[a] $n = 4$/dose level.
[b] $n = 6$/dose level.
[c] $n = 10$/dose level.
NA, not applicable.

due to inflammatory responses associated with the aforementioned injection-site reactions.

The toxicologic profile of the local administration of VB4-845 in cynomolgus monkeys was similar to that in rats, with effects of the drug limited to transient localized injection-site reactions. Hepatocyte toxicity has been associated with the release of tumor necrosis factor-alpha (TNFα) from Kupffer cells in response to an ETA-based immunotoxin administered i.v.[43] However, we observed no symptoms of VLS or liver toxicity with local administration and the drug was well tolerated. Based on these toxicology findings, VB4-845 has a high safety profile when administered locally. For animals treated by local injection, it would be reasonable to suspect that the resident macrophages involved in the physiologic clearance of the drug would be responsible for the localized skin reactions at the site of injection.

By comparison, the local administration of ETA immunotoxins offers several advantages over systemic delivery. First, direct administration to the tumor ensures the highest concentration of drug at the site of injection with low systemic exposure, thus minimizing effects on the vascular system and nontarget tissues. In fact, lower amounts of systemically bioavailable VB4-845 were demonstrated in rats following local injection, as compared to systemic delivery. Second, recombinant immunotoxins are foreign proteins and will elicit an immunogenic response.[44] Circulating antibody titers to an immunotoxin has been a significant impediment to systemic drug delivery, as an immune response reduces systemic drug exposure by increasing the rate of drug clearance.[45] Moreover, immune responses can result in the inhibition of the therapeutic effect of the drug and loss of efficacy.[46] Ensuring a higher concentration of drug in the vicinity of the targeted tumor through local injection evades the neutralizing effect and postulated rapid elimination of the immunotoxin by circulating antibodies. This possible outcome was described for scFv(FRP5)-ETA, where a patient had a complete clinical response to a second intratumoral treatment, even though an immune response was generated that completely neutralized the circulating immunotoxin.[27] In light of the possible reactivity of VB4-845 with some normal tissues, the presence of circulating antibodies may actually prove to be clinically beneficial, as they would minimize systemic drug exposure, thus further enhancing its safety profile.

The specificity and safety profile of locally administered VB4-845 indicates that it has the potential to be an effective, safe drug for the treatment of EpCAM-positive solid tumors amenable to locoregional delivery. The high dose achieved in the monkeys and the capacity to repeat dose, and thereby avoid the immune response, suggests that a therapeutic window exists for VB4-845 and, thus, supports this concept. Two cancer indications amenable to local administration of VB4-845 are squamous cell carcinoma of the head and neck (SCCHN) and transitional cell carcinoma (TCC) of the bladder. Current statistics surrounding these cancers indicate a lack of effective treatments and unmet clinical needs. Head and neck cancers are the ninth most common cancer in males in the United States, with the majority being SCCHN.[47,48] The current prognosis for patients with SCCHN is poor, with a 5-year survival rate, ranging from 10% to 50% for patients with locally advanced disease.[49] Bladder TCC is the fourth most common cancer in males and the tenth most common cancer in females.

Conclusions

Supported by the safety profile, clinical trials have been conducted in patients with SCCHN and patients with TCC of the bladder, using local administration strategies for the delivery of VB4-845. Based upon the MTD in cynomolgus monkeys and the therapeutic dose in the SCCHN clinical trials (700 μg), the therapeutic index of 28.6 indicates a high safety margin for VB4-845.[50]

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Disclosure Statement

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