Differential tissue targeting and pathogenesis of verotoxins 1 and 2 in the mouse animal model

Niels W.P. Rutjes, Beth A. Binnington, Charles R. Smith, Mark D. Maloney, and Clifford A. Lingwood

Division of Infection, Immunity, Injury and Repair, Research Institute, Hospital for Sick Children, Toronto, Ontario, Canada; Department of Pediatric Laboratory Medicine, Hospital for Sick Children, Toronto, Canada; Spelman College, Atlanta, Georgia; and Departments of Biochemistry and Laboratory Medicine & Pathobiology, University of Toronto, Toronto, Ontario, Canada

Background. Both verotoxin (VT) 1 and VT2 share the same receptor, globotriaosyl ceramide (Gb3). Although VT1 is slightly more cytotoxic in vitro and binds Gb3 with higher affinity, VT2 is more toxic in mice and may be associated with greater pathology in human infections. In this study we have compared the biodistribution of iodine 125 (125I)-VT1 and 125I-VT2 versus pathology in the mouse.

Methods. 125I-VT1 whole-body autoradiography defined the tissues targeted. VT1 and VT2 tissue distribution, clearance, and tissue binding sites were compared. The effect of a soluble receptor analogue, adamantylGb3, on VT2/Gb3 binding and in vivo pathology was assessed.

Results. 125I-VT1 autoradiography identified the lungs and nasal turbinates as major, previously unrecognized targets, while kidney cortex and the bone marrow of the spine, long bones, and ribs were also significant targets. VT2 did not target the lung, but accumulated in the kidney to a greater extent than VT1. The serum half-life of VT1 was 2.7 minutes with 90% clearance at 5 minutes, while that of VT2 was 3.9 minutes with only 40% clearance at 5 minutes. The extensive binding of VT1, but not VT2, within the lung correlated with induced lung disease. Extensive hemorrhage into alveoli, edema, alveolitis and neutrophil margination was seen only after VT1 treatment. VT1 targeted lung capillary endothelial cells. Identical tissue binding sites (subsets of proximal/distal tubules and collecting ducts) for VT1 and VT2 were detected by toxin overlay of serial frozen kidney sections. Glucosuria was found to be a new marker of VT1- and VT2-induced renal pathology and positive predictor of outcome in the mouse. Glucosuria increased a different lipid composition. AdamantylGb3, a soluble Gb3 analogue, competed effectively for Gb3 binding by VT1 and VT2 in vitro. However, the effect in the mouse model (only measured against VT2, due to the lower LD50, a concentration required for 50% lethality) was to increase, rather than reduce, pathology and further reduce the VT2 serum clearance rate. Additional renal pathology was seen in VT2 + adamantylGb3-treated mice.

Conclusions. The lung is a preferential (Gb3) “sink” for VT1, which explains the relatively slower clearance of VT2 and subsequent increased VT2 renal targeting and VT2 mortality in this animal model.

The hemolytic uremic syndrome (HUS) remains the leading cause of acute pediatric renal failure [1]. The syndrome comprises acute renal failure, thrombocytopenia, and microangiopathic hemolytic anemia [2]. Most often HUS is a complication of gastrointestinal infection with verotoxin (VT) producing enterohemorrhagic Escherichia coli (EHEC) [3, 4]. Several serotypes of EHEC bacteria have been implicated in disease, but E. coli O157:H7 is the predominant EHEC serotype associated with HUS in North America and Europe [5, 6]. EHEC bacteria elaborate either or both of two serologically distinct VTs: VT1 and VT2. VTs show a high degree of homology with Shiga toxin, produced by Shigella dysenteriae 1, and hence are also referred to as Shiga toxin 1 and Shiga toxin 2, respectively.

After synthesis in the intestine, the toxins may reach the systemic circulation by transcellular translocation through intestinal epithelial cells [7, 8] aided by neutrophil migration in the opposite direction [9]. Recent investigations in vitro [10] and in vivo [11] have shown that VTs bind to monocytes without inducing cytotoxicity. This is postulated to be a possible mechanism of transport in the circulation. Upon reaching the target organs, VT1 and VT2 bind to a specific receptor, globotriaosyl ceramide (Gb3), also termed CD77 or the p4 blood group...
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antigen) [12]. Various cells in the human body express this receptor, but most common complications of EHEC infection are hemorrhagic colitis, HUS, and neurological dysfunction [13].

VTs are A-B subunit toxins, consisting of five receptor-binding subunits (B subunit) and one enzymatically active subunit (A subunit). Upon binding to Glb, via the B-pentamer, the toxin is internalized via clathrin-coated pits and caveoli-mediated endocytosis [14–16]. Once internalized, the toxin undergoes a process of retrograde transport to the Golgi and/or endoplasmic reticulum [17, 18]. The mechanistic paradigm is that the A subunit is cleaved into an A1 and an A2 fragment and the A1 fragment then targets the 28S RNA portion of 60S ribosomal subunits in the cytosol and by so doing disrupts ribosomal function of protein production resulting in cell death. While inhibition of cellular protein synthesis is clearly established, VT appears to have diverse intercellular actions. VT induces apoptosis, a process requiring protein synthesis, in many cell types [19–25] and can induce specific gene expression [26–31]. VT can depurinate DNA as well as RNA [32, 33]. Intracellular targeting of the holotoxin to the endoplasmic reticulum and nucleus has been associated with high VT sensitivity [18, 34]. Thus, the exact mechanism or combination of mechanisms by which VTs kill cells remains speculative [35, 36].

In human kidneys, Glb is expressed by endothelial cells (and epithelial cells [37]) located primarily in the pediatric glomerulus and, to a lesser extent, in the tubules [38]. Glomerular Glb expression is reduced or lost in the adult kidney [38], despite the fact that the total level of renal Glb is increased [39]. The ontologically regulated glomerular expression of Glb may explain the fact that HUS mainly occurs in young children [38]. This has been recently supported by the detection of VT2 in glomeruli of pediatric, but not geriatric, VT producing E. coli-induced HUS cases [40]. At autopsy, glomerular lesions are seen. Changes noted include glomerular endothelial cell swelling, thromboses, congested glomeruli, arteriolar thromboses, which are common at the hilum of glomeruli and are sometimes seen proximally, including in interlobular arteries, and in the most severe cases, cortical infarction. These changes are assumed to be responsible for the acute renal failure observed [41].

Epidemiological studies have suggested that patients infected with EHEC producing VT1 in combination with VT2 or VT2 alone were more prone to develop serious complications like HUS than patients infected with EHEC producing VT1 alone [42–45]. Tesh et al showed a clear difference between in vivo toxicities of VT1 and VT2 in mice [46]. Their studies showed a 400-fold lower LD₅₀ for VT2 and that VT2 caused greater renal pathology than VT1. It was thought that the difference in toxicity could partially be explained by a difference in toxin stability. These authors also noted that VT1-treated mice died in the absence of excessive renal pathology, suggesting that the fatal effects lay in a different organ system. These studies also showed that the Glb, binding affinity for VT2 was, like VT2c [47], approximately 10-fold lower than that of VT1 [46].

In rabbits, VT1 is more lethal than VT2. Brain lesions can be induced by VT2, but not VT1, whereas VT1-induced spinal cord lesions can be seen [48, 49]. VT2, although generally less cytotoxic than VT1 in vitro [50, 51] shows increased cytotoxicity for cultured human renal microvascular endothelial cells [52]. The A subunit of VT2, but not VT1, was recently found to contain a sequence capable of binding anti-apoptotic partners that prevent Bel-2 action, which may provide a second mechanism for VT2-induced cell killing [53].

In this study we expanded on the mouse model of VT by defining the biodistribution and clearance characteristics of VT1 and VT2. This data show significant differences in tissue targeting and clearance of VT1 and VT2, which could explain the distinct toxicities observed in mice and, perhaps, relate also to the differential pathology in humans.

VT1 and VT2 binding to the receptor Glb plays the primary role in determining tissue targeting and susceptibility in rabbits [54, 55]. Prophylactic attempts to compete with insoluble Glb, analogues within the gastrointestinal tract have proven ambiguous [56], possibly because VT may be transcytosed directly across the intestinal epithelium from the site of bacterial attachment and is receptor independent [9]. A different approach would be to use soluble Glb, analogues to prevent systemic VT tissue targeting. Analysis of the serum Glb levels in VT producing E. coli-infected patients who did or did not develop HUS showed that the level was higher in the non-HUS patients [57], suggesting that soluble Glb, analogues could indeed, prove an effective prophylactic. High-affinity VT/Glb binding requires the lipid moiety [58] and, if administered to compete in such a format, would likely partition into cells to provide additional VT receptors, rather than fewer. The weakly VT-binding galactose (α1-4) galactose disaccharide from Glb, can be appropriately multimerized to provide a structure capable of cross-linking (and hence inactivating) VT B subunits to effectively prevent VT cytotoxicity [59, 60] in vitro. Our approach has been to develop monomeric soluble Glb, analogues that retain high-affinity VT binding [61]. We have now tested the ability of such a species to alter the biodistribution and toxicity of VT2 in the mouse model.

METHODS

Reagents

Monoclonal antibody (mAb) PH1, specific for the VT1 B subunit, has been previously described [62]. PH1 was
purified from culture supernatant using Protein A-sephrose chromatography. Rabbit polyclonal antiserum against the purified VT1 B subunit was prepared, as previously described [63]. Rabbit antiserum raised against VT2e and reactive with VT2 was a generous gift of Dr. Carlton Gyles, University of Guelph, Guelph, Canada. Antibody against the facilitative glucose transporter GLUT-2, selectively expressed in the S1 segment of proximal tubules [64, 65], was obtained from Alpha Diagnostic International, Inc. (San Antonio, TX, USA). Biotinylated Dolichos biflorus agglutinin (which binds α-N-acetyl galactosamine, highly expressed in collecting ducts [66]) and peanut agglutinin (which binds terminal α-galactose and galactosyl (β-1,3) N-acetylgalactosamine; distal tubules) were purchased from Vector Laboratories (Burlingame, CA, USA).

Toxins
VT1 was purified from strain JB28 [67], as previously described [68]. VT2 was purified from E. coli R82pJES1-20DH5a, kindly provided by Dr. J. Samuel (Texas A&M University, College Station, TX, USA). Nucro-Technics, Inc. (Toronto, Ontario, Canada) analyzed purified toxins for endotoxin using the Limulus amoebocyte lysate method. VT1 had <0.1 EU/µg (<10 pg/µg) while VT2 had <12.5 EU/µg (<1.25 ng/µg). These lipopolysaccharides (LPS) levels are far below those shown to cause synergistic or protective effects on VT2 pathology [69].

Purified toxins were labeled with iodine 125 (125I) using iodogen, as described previously [70]. The protein concentration of the isolated iodinated VTs was determined using the bicinchoninic acid (BCA) protein assay (Pierce Chemical Co., Rockford, IL, USA). Greater than 97% of recovered radioactive material was protein-bound as determined by trichloroacetic acid precipitation. Specific activities were typically 8 to 12 × 106 cpm/µg for VT1 and VT2. To verify that radiolabeling of the toxins did not affect activity, a Vero cell cytotoxicity assay was performed to compare labeled and unlabeled toxin. Iodinated VT1 was heat-inactivated by treatment at 90 to 100°C for 30 minutes. Binding of denatured toxin to Vero cells was reduced by >90% compared to native 125I-VT1.

Mice
The mice were 12- to 16-week-old female BALB/c mice (weight range, 20 to 30 grams) that were purchased from Charles River, Canada. Male mice were used in the whole-body autoradiography studies. The mice were maintained under a 12-hour light-dark cycle and fed with standard diet and water ad libitum.

Biodistribution
Whole-body autoradiography. Whole-body autoradiography was performed by Oread Biosafety, Inc. (Memphis, TN, and Pharmacokinetics Division, Lawrence, KS, USA). Mice were pretreated with oral potassium iodide 24 hours prior to injection of 125I-VT1 to block thyroid uptake of any free 125I. 125I-VT1 (1.12 µg) was administered intravenously through the tail vein and the animals were sacrificed by CO2 inhalation 15 minutes (N = 2) or 45 minutes (N = 3) later. Animals were frozen in hexane/dry ice and embedded in carboxymethyl cellulose. Sagittal cryosections (40 µm) using a Richert-Jung Polycut S cryomicrotome (Leica Microsystems, Inc., Bannockburn, IL, USA) were collected on adhesive tape, dried, and exposed to Kodak SB-5 x-ray film. Hematoxylin and eosin (H&E) stained sections used for autoradiography were used for tissue assignment.

Quantitative tissue distribution. Mice were injected via the tail vein with 2 µg/kg 125I-VT1 or 125I-VT2 (about 50 ng/mouse) diluted in 200 µL saline. In these studies, no allowance for the difference in LD50 values for these toxins was made and pathologic-induced changes for VT2 might have occurred. Pilot experiments were performed to examine toxin distribution at 1, 2, or 8 hours post injection and the effects of perfusion prior to tissue collection on the biodistribution data obtained. For final clearance/biodistribution studies, groups of mice (N = 8) were injected with toxin and a 50 µL blood sample was obtained from the lateral saphenous vein at 5 and 30 minutes post injection. At 60 minutes post injection, mice were anesthetized (0.1 mL/g 3.6% chloral hydrate intraperitoneally) and a 200 µL blood sample was obtained from the orbital plexus. Upon taking the last blood sample, mice were sacrificed immediately by cervical dislocation and tissues were removed for analyses of radioactivity using a gamma counter.

Effect of adamantylGb3 on VT2 biodistribution
The semi-synthetic, soluble Gb3 derivative, N-adamantylGb3 (adaGb3), was prepared from human renal Gb3, as previously described [61]. Two groups of mice (N = 8) were injected intravenously with a mixture of 4 mg/kg adaGb3 and 2 µg/kg 125I-VT2 (representing about 100 µg (100 nmol) of adaGb3, and 50 ng VT2 (0.7 pmol) per mouse) in 200 µL. The procedures followed matched those used in the biodistribution studies discussed above.

Clinicopathologic studies with VT2 challenged mice
The LD50 of VT2 in mice is 400-fold lower than that of VT1 [46]. Therefore, VT2 was chosen for in vivo protection studies using adaGb3. Initial studies showed that a dose of 2 ng/mouse (80 ng/kg) caused development of fatal disease in 100% of mice 80 to 105 hours post-injection. This dose represents 1.5 to 4 times the reported LD50 in Balb/c mice [69, 71].

The ability of adaGb3 to alter the VT2 disease course with respect to clinical, biochemical, and histologic symptoms was investigated. In all cases, mice were injected
injected intravenously through the tail vein (N = 5) for each group. Group 1 received 2 ng (80 ng/kg) VT2. Group 2 received a mixture of 2 ng VT2 and 100 μg (4 mg/kg) adaGb3. Group 3 was injected intravenously with 4 mg/kg adaGb3 alone. A fourth group of mice (N = 3) was injected intravenously with sterile saline alone. AdaGb3 or adaGb3 alone. A fourth group of mice (N = 3) was injected intravenously with sterile saline alone. AdaGb3- VT2 mixtures were pre-incubated for 20 to 30 minutes at room temperature prior to injection.

In experiments involving blood collection, 24 hours prior to injection, a 200 μL blood sample was taken from the lateral saphenous vein. At 72 hours post injection, mice were anesthetized with chloral hydrate (3.6% 0.01 mL/g intraperitoneally) and a 300 μL blood sample was taken from the orbital plexus. After obtaining the second blood sample, mice were immediately sacrificed by cervical dislocation and tissues were processed as described under Histopathology below.

All blood samples were collected with heparinized capillary tubes. Blood was centrifuged immediately upon collection, plasma and cells were separated, and plasma was stored at –20°C until analysis. To monitor for signs of renal dysfunction, plasma samples were analyzed for blood urea nitrogen (BUN) and creatinine at the Department of Pediatric Laboratory Medicine, Hospital for Sick Children, in Toronto, Ontario, Canada. Blood glucose levels were measured using a One Touch Basic blood glucose monitoring system (Lifescan Canada, Ltd., Burnaby, B.C., Canada). Urine samples were collected upon spontaneous voiding. Using Albustix (Bayer, Inc., Toronto, Ontario, Canada), urine was analyzed for glucose, protein, pH, specific gravity, erythrocytes, and leukocytes.

Histopathology

Animals treated with VT1, VT2, VT2 + adaGb3, adaGb3, or saline were sacrificed by cervical dislocation. Kidney, lung, brain, spleen, and liver were fixed in neutral-buffered formalin and then paraffin-embedded and sectioned. All sections were stained with H&E and, in addition, kidney sections were stained with periodic acid–Schiff (PAS) to identify the brush-border of proximal tubules.

VT staining of frozen kidney sections

A fresh tissue sample was placed in Tissue-Tek O.C.T. compound (Miles, Inc., Diagnostics Division, Elkhart, IN, USA) and snap-frozen in liquid nitrogen. Serial 6 μm frozen sections were cut and stored at –70°C. The immunoperoxidase VT staining was performed, as described [72]. Sections were dried overnight and then blocked separately for endogenous peroxidase, avidin, and biotin. The sections were incubated with 50 ng/mL of VT1 or VT2 diluted in 1% goat serum/phosphate-buffered saline (PBS) for 30 minutes. After washing, polyclonal anti-VT1 or anti-VT2 was added and bound antibody was detected with a biotinylated goat anti-rabbit IgG-step-tavidin peroxidase system. Sections were counterstained with hematoxylin, dehydrated, and mounted. Serial sections were also stained with PAS, anti-GLUT-2, biotinylated Dolichos biflorus agglutinin, peanut agglutinin, or 1% goat serum/PBS as antibody control.

In situ immunodetection of targeted VT

Mice were injected through the tail vein with 10, 50 or 100 μg of VT1 or VT2 in 200 μL of saline. After 1 hour, the animals were sacrificed by cervical dislocation and organs were collected and divided. Half was placed in O.C.T compound and snap-frozen in liquid nitrogen for preparation of frozen sections. The remainder was placed in neutral buffered formalin for a minimum of 16 hours before being paraffin-embedded and sectioned.

VT was detected in the frozen sections essentially as described above for VT staining of frozen kidney sections, with the following two exceptions. The frozen sections were fixed with 100% acetone for 10 minutes prior to assay rather than dried overnight, and the VT1 binding step was omitted. Tissues from mice injected with saline rather than VT served as controls for non-specific binding of anti-toxin antibodies.

Paraffin sections were de-paraffinized by immersion in xylene, followed by baths of decreasing ethanol content (100%, 95%, 70% ethanol in water), and finally, water. After de-waxing, antigen retrieval was required for detection of VT in kidney sections. Antigen unmasking was performed by heating the de-waxed sections in 0.01 mol/L citrate buffer pH 6, in a pressure cooker [73].

VT TLC overlay

Glycosphingolipids (GSLs) were extracted from lungs and kidneys (pooled from 5 animals) as previously described [39] and equivalent aliquots according to tissue weight were separated by TLC and overlaid with either VT1 or VT2 [74]. Toxin binding was visualized using ECL detection (Amersham Biosciences, Piscataway, NJ, USA).

Statistical analysis

Results are expressed as the mean ± standard deviation.

RESULTS

Whole-body autoradiography after 125I-VT1 administration.

Several illustrative sagittal sections are shown in Figure 1. No additional organs were labeled in other sections. VT1 labeling of the kidney cortex, lung, and the nasal turbinates were the most prominent features of the autoradiogram (Fig. 1). Labeling of the bone marrow of the spine, long bones, and ribs was also extensive. The labeling in the lung could be seen to be non-uniform since bronchi- oles were not labeled and foci within the lung could be
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Fig. 2. Serum clearance of 125I-VT1 and 125I-VT2. Approximately 50 ng 125I-labeled toxin was injected into mice intravenously (N = 8) and counts remaining in circulation over the next hour were measured. The effect of mixing the semi-synthetic soluble Gb3 derivative N-ada-mantylGB3 (adaGb3) with 125I-VT2 prior to injection was also determined. (○), 125I-VT1; (▲), 125I-VT2; (■), 125I-VT2 + adaGb3.

Counts injected. To compare clearances of both VT1 and VT2, the amount of toxin as measured in the blood samples was expressed as a percentage of injected counts recovered in 50 μL blood (Fig. 2). Within 5 minutes, 90% of injected VT1 but only 40% of injected VT2 was cleared. After 60 minutes there were sixfold less counts circulating for VT1 than for VT2. The estimated half-lives for VT1 and VT2 were 2.7 and 3.9 minutes, respectively, based on the decrease in counts between time points 0 and 5 minutes.

Biodistribution. All measurements were at 1 hour post injection. Both groups of mice injected with 125I-VT1 or VT2 showed tissue localization of the toxins primarily in the lungs and nasal turbinates (Fig. 3) consistent with the autoradiography study (Fig. 1). Toxin targeting to these organs has not been previously reported. However, VT1 sections from several mice are shown with labeled tissues indicated. The lower panel highlights the non-uniform labeling in the lung seen on reduced exposure.

distinguished on less exposure (Fig. 1, lower panel). The spleen was significantly labeled, but less than lung. The liver was barely labeled above the background. No labeling of the gastrointestinal tract, central nervous system, brain stem, spinal chord, testes, lymph nodes, thymus, reproductive tract, pancreas, blood, skeletal/cardiac muscle, dermis, or epidermis was seen.

Tissue distribution of 125I-VT1 and 125I-VT2

Clearance. Blood samples were taken at three different time points. Because blood was not collected before 5 minutes, the data for zero time points were calculated from the estimated blood volume for each mouse [75] and the estimated half-lives for VT1 and VT2 were 2.7 and 3.9 minutes, respectively, based on the decrease in counts between time points 0 and 5 minutes.

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Selective VT1 lung targeting and decreased renal pathology

Fig. 3. Biodistribution of $^{125}$I-VT1 and $^{125}$I-VT2. Approximately 50 ng $^{125}$I-labeled toxin was injected into mice intravenously (N=8). After 1 hour, the toxin distribution to various tissues was determined and expressed as the percentage of injected counts per minute recovered per mg of tissue. (□), heat-denatured VT1; (■), VT2; (■■), VT1.

injected (Fig. 4). The VT1 and VT2 immunostaining patterns were similar. Immunostaining was clear in the frozen sections, even at the 10 μg dose, but morphology was better preserved in the fixed, embedded sample. Clear staining of distinct tubule subpopulations was seen for both toxins. No glomerular staining was seen (Fig. 4 A to C). In the paraffin sections, it was clear that VT-targeted tubules were dilated and the epithelial cells flattened, even after this short period (Fig. 4 C, D). This effect was dose dependent. Immunodetection of targeted VT1 in the unmasked paraffin sections was adequate at 100 μg but poor at 50 μg (and not detectable at 10 μg). Nevertheless, the dismorphic tubules could be identified in the H&E section and the corresponding stained tubule identified (for the 50 and 100 μg dosage). Dilated tubules were less apparent in H&E stained sections at the 10 μg dosage (not shown).

By overlay of (untreated) mouse kidney frozen sections, both VT1 and VT2 were found to bind exactly the same tubular structures (Fig. 5). Neither toxin bound to glomeruli (Fig. 5 a, b). Labeling serial sections with the lectin D. biflorus [66] identified some of the renal structures labeled by both VT1 and VT2 as collecting ducts (Fig. 5e), while immunostaining with anti-GLUT-2 [64] identified others as proximal convoluted tubules (Fig. 5f). Some distal tubules labeled with peanut agglutinin [77] were labeled with VT1 or VT2, but most were not (Fig. 5g).

Lung. Frozen-section immunostaining of VT1 accumulated in the lung following intravenous injection shows areas of some intra-alveolar capillaries, and patches of pneumocytes within the alveoli are targeted (Fig. 6). Distinct, sometimes discontinuous, subpopulations of endothelial cells within some capillaries were labeled. Epithelial cells were, for the most part, VT1-negative. VT could not be detected in the paraffin sections perhaps due to a failure to unmask the antigen in this tissue with the conditions used. Gb3 was detected in untreated mouse lung frozen sections by VT1 overlay, but the morphology was not sufficiently retained to allow identification of cell targets.

Pathology

Kidney. Mice injected with 1.5 to 4 LD50 (VT1 or VT2) showed kidney pathology, characteristic of tubular
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**Fig. 4. Immunolocalization of kidney-targeted VT1 and VT2.** Frozen (A, B) or fixed, paraffin-embedded (C, D) sections of kidney were prepared from mice 1 hour after injection with either 50 μg VT1 or VT2 and stained with antiserum against VT1 (A, C) or VT2 (B). A similar subpopulation of tubules are labeled by each toxin but neither bound to glomeruli (arrows). In fixed sections, tubules targeted by VT1 (C) were identified in hematoxylin and eosin (H&E)-stained serial sections as dilated and containing flattened epithelial cells (D).

necrosis as previously reported [46] by day 3. Glomerular morphology appeared normal. A significant subpopulation of proximal and especially distal tubules were dilated in both VT1- and VT2-treated mice. Medullary tubular dilation was more widespread after VT1 treatment. Interstitial hemorrhage was seen for all VT-treated mice. Neutrophil migration to sites of tubular injury was not seen. Apoptosis, as monitored by terminal deoxy transferase uridine triphosphate nick end-labeling (TUNEL) staining, was not increased in the kidneys of VT-treated mice (not shown). Glucose was detected in the urine of VT-treated mice after 3 to 4 days, before the onset of any visible symptoms (Table 1) and was present until the time of death. Glucosuria was an excellent predictor of mortality (Table 1). Co-administration of the neutralizing anti-VT1 mAb PH1 [62] with VT1 completely protected mice from pathology and development of glucosuria (Table 2). VT-treated mice did not become anuric, but did develop a urine-concentrating defect (Table 1). Using urinalysis sticks, no changes in urinary protein concentration, pH, or specific gravity was measured, but erythrocytes and leukocytes were sometimes detected in urine of moribund mice.

**Lung.** Lung sections of 2 LD_{50} [78] VT1-treated mice showed significant hemorrhage (Fig. 7 A, E, J) and marked alveolitis was apparent (Fig. 7 E, H). The lungs had a congested appearance (Fig. 7 H) with thickened edematous alveolar walls showing numerous entrapped erythrocytes (Fig. 7 L). Edema within alveoli was also seen (Fig. 7 L). The edema was often discontinuous, suggesting that some edematous material was lost during tissue preparation/staining (Fig. 7 L). Edema of larger blood vessels was evident (Fig. 7 K). Margination of neutrophils in venules was extensive (Fig. 7 H) and infiltration of type II pneumocytes was evident. In contrast, in VT2-treated animals, there was some slight edema and neutrophil margination evident, but lung histology was, for the most part, indistinguishable from controls (Fig. 7 B, E, H compared with C, F, I).

**Other.** Active acute phagocytosis was present in foci in the spleen after either VT2 or VT1 treatment. TUNEL staining revealed increased apoptosis in spleens of VT1 or VT2 but not in saline-treated mice (not shown). Also, iron pigment from increased red cell turnover was evident in the spleen of toxin-treated mice.

In the liver, lipid vacuoles were seen in VT2-treated animals but were less apparent for VT1-treated animals (not shown).

**Tissue Gb_{3} content**

The Gb_{3} content of glycolipid extracts of lung and kidney was visualized by VT1 and VT2 TLC overlay (Fig. 8). The mouse renal Gb_{3} migrated more slowly than that from lung. VT2/Gb_{3} binding was significantly less than that of VT1. A dose-dependent binding to human renal Gb_{3} standard was seen, and both toxins efficiently recognized adaGb_{3}.

**Treatment with a soluble Gb_{3} analog**

The water-soluble Gb_{3} derivative, adaGb_{3}, was effective to prevent VT1 and VT2 binding to immobi-
be effective in vivo. We mixed 500 VT2 to determine whether this protection in vitro could
worsen necrosis, were seen for VT2
mals. Surprisingly, mice co-injected with VT2
were sacrificed for analysis of histopathology was
Treatment death a
lial cell detachment from the basement membrane is
lumina were occluded (Fig. 10 C, F). Evidence of epithe-
but particularly in the medulla, such that many tubule
were found to die more rapidly than those injected with
VT2 alone (96 hours compared with 72 hours). Indeed,
were seen in both VT2- and VT2 + adaGb3-treated mice. The
epithelial morphology of the affected tubules was more
dysplastic, and more granular eosinophilic spheres in the
tubular lumina (Fig. 10 C, F), typical of acute tubular
carcinosis, were seen for VT2 + adaGb3-treated mice. No
brain pathology was seen for VT1-, VT2- , or VT2 +
adaGb3-treated mice.

Comparison of the biodistribution of125I-VT2 with and
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Table 1. Biochemical parametersa of VT2-treated mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Glucosuria</th>
<th>Blood glucose mmol/L</th>
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</tr>
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<tbody>
<tr>
<td>Baseline</td>
<td>–</td>
<td>4.72 ± 0.6</td>
<td>6.43 ± 1.27</td>
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<td>++</td>
</tr>
<tr>
<td>2 ng VT2</td>
<td>(N = 32)</td>
<td>4.84 ± 0.84</td>
<td>9.92 ± 2.0</td>
<td>27.0 ± 3.24</td>
<td>–</td>
<td>++</td>
</tr>
<tr>
<td>2 ng VT2 + adaGb3</td>
<td>+ (5/5)</td>
<td>4.50 ± 0.43</td>
<td>24.6 ± 4.0</td>
<td>55.0 ± 5.66</td>
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</tr>
<tr>
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<td>5.68 ± 0.26</td>
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</tr>
<tr>
<td>4 ng VT2 + adaGb3</td>
<td>+ (4/5)e</td>
<td>4.22 ± 0.85</td>
<td>24.6 ± 5.8</td>
<td>69.3 ± 5.8</td>
<td>+ (4/6)</td>
<td>+</td>
</tr>
<tr>
<td>adaGb3</td>
<td>– (0/5)</td>
<td>5.04 ± 0.84</td>
<td>6.8 ± 1.0</td>
<td>22.2 ± 4.6</td>
<td>– (0/5)</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Clinical parameters of mice treated with VT1 ± PH1
monoclonal antibodies (mAb)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time of deatha hour</th>
<th>Urine glucose mmol/L</th>
<th>Clinical disease</th>
<th>Color of urine</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.2 μg VT1</td>
<td>58.5</td>
<td>nd</td>
<td>+</td>
<td>nd</td>
</tr>
<tr>
<td>1.2 μg VT1</td>
<td>58.5</td>
<td>14–28</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1.2 μg VT1</td>
<td>68</td>
<td>&gt;14</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>1.2 μg VT1 + PH1</td>
<td>68</td>
<td>+</td>
<td>+ + + + + +</td>
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<td>1.2 μg VT1 + PH1</td>
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Fig. 7. Comparison of VT1 and VT2 induced lung pathology. Mice
were injected with 2 LD50 of VT1 [102] or VT2, or with saline. Toxin
injected mice were sacrificed when moribund and saline-treated
mice at corresponding time points. Hematoxylin and eosin (H&E) stain-
ing of lung of VT1 (A), VT2 (B), and saline (C) treated mice; adjacent
blood vessel and alveoli in VT1 (D), VT2 (E) and saline (F) treated
mice; alveoli of VT1 (G), VT2 (H) and saline (I) treated animals. In
VT1-treated mice, alveolar hemorrhage (J), blood vessel edema (K),
and alveolar edema (L) were widespread.

aParameters monitored at 72 hours post injection
bRuffled fur, inactive
cGlucose was detected at 96 hours
dNo urine obtained from one of five mice

ized Gb3 as monitored by receptor enzyme-linked immu-
nosorbent assay (RELISA) (Fig. 9). The IC50 for VT1
was ~10 μmol/L [61] while that for VT2 was higher (~50 μmol/L). Due to the much lower LD50 in mice, we used
VT2 to determine whether this protection in vitro could be effective in vivo. We mixed 500 μmol/L adaGb3 with
approximately 1 LD50 VT2 and injected it into the ani-
mals. Surprisingly, mice co-injected with VT2 + adaGb3,
were sacrificed when moribund and healthy PH1-protected
animals were sacrificed for analysis of histopathology

Table 1. Biochemical parametersa of VT2-treated mice

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| aVT1-treated mice were sacrificed when moribund and healthy PH1-protected animals were sacrificed for analysis of histopathology

Comparison of the biodistribution of125I-VT2 with and
without adaGb3 showed that, in most tissues, VT2 tissue
targeting was marginally increased, rather than de-
Selective VT1 lung targeting and decreased renal pathology

Fig. 10. Effect of AdamantylGb3 (adaGb3) on VT2-induced renal pathology. Mice (N = 5) were injected intravenously with adaGb3, VT2, or a mixture of VT2 and adaGb3. Animals were sacrificed at 72 hours post injection and hematoxylin and eosin (H&E) stained paraffin sections of renal tissue were inspected for pathology. (A, D, G), adaGb3-treated mice; (B, E, H), VT2-treated mice; (C, F, I), VT2 + adaGb3-treated mice. (A to C), cortex; (D to F), medulla; (G to I), tubules at high power. Glomerular morphology appears unaffected by VT2 (B). The tubular dilation (B and E) are reversed in the VT2 + adaGb3 mice (C, D). The epithelial cell narrowing (E and H) after VT2 treatment is also reversed, but the epithelial cells of many tubules now become enlarged such that their lumina are occluded (F). The epithelial cell layer of tubules of VT2-treated mice was often dismorphic (H) and the frequency of eosinophilic nuclei (in dying cells) increased after VT2 + adaGb3 treatment (I, arrows)

creased, in the presence of adaGb3, (results not shown). This was consistent with the finding that the serum half-life for 125I-VT2 was increased to 8.7 minutes in the presence of adaGb3 (Fig. 1).

DISCUSSION

VT-induced cell cytotoxicity [79, 80] and likely in vivo pathology [38] are mediated by binding to its glycolipid receptor, globotriaosyl ceramide [12]. Can this binding in any way explain the increased sensitivity of mice (and humans?) to VT2? The binding of VTs to Gb3 is differen-

Fig. 6. Immunolocalization of lung-targeted VT1. Frozen sections of lung were prepared from mice 1 hour after injection with 50 μg VT1. (A), Hematoxylin and eosin (H&E) stain; (B), corresponding serial section stained with anti-VT1; (C), anti-VT1 immunostaining; (D), lung section corresponding to A and B stained with non-immune serum. Discontinuous staining of endothelial cells lining a capillary is indicated in (B). Continuous staining of endothelial cells within a capillary is shown in (C). Some staining of pneumocytes is also evident.
Fig. 5. Comparison of VT1 and VT2 binding sites in frozen kidney sections. Serial frozen sections of kidney were stained with VT1 (a, c) or VT2 (b, d), the lectin Dolichos biflorus (e), anti-GLUT2 (f), peanut agglutinin (g) and periodic acid-Schiff (PAS; h). Both toxins label the same collecting ducts and proximal tubules. Comparison staining of the equivalent areas is shown (c to h). A representative glomerulus unstained by VT1 or VT2 is shown (a and b). Four collecting ducts labeled with D. biflorus and VT1, VT2 are indicated (c). The arrowhead indicates a D. biflorus-reactive collecting duct, not labeled with peanut agglutinin (g). Proximal tubules reactive with VT1, VT2 and anti-GLUT2 are indicated (f). Anti-VT2 renal staining after in vivo VT2 administration (j) is compared to PNA staining (i) of serial sections. A VT2-negative, PNA-positive distal tubule is marked (i), while a PNA-negative, VT2-positive proximal tubule is marked (j). Distal tubules labeled by both PNA and VT2 are indicated by arrows.
VT1 and VT2 lung targeting and decreased renal pathology

Fig. 8. VT1/VT2 thin-layer chromatography (TLC) overlay of renal and lung glycolipids. Glycolipids were isolated from mouse lung and kidney and an aliquot representing 20 mg of tissue was separated by TLC and visualized by orcinol (A). VT binding to Gb3 was detected by TLC overlay with either VT1 (B) or VT2 (C) as described [74]. Lane 1, adaGb3; lanes 2, 3, standard glycolipid mixture at 0.25, 0.5 µg each; lane 4, mouse renal glycolipids; lane 5, mouse lung glycolipids. Glycolipid standards are, from the top of the plate, glucosylceramide, galactosylceramide, lactosylceramide, Gb3, Gb4, Forssman glycolipid.

Fig. 9. AdamantylGb3 (adaGb3) inhibition of VT/Gb3 binding. VT1 or VT2 were mixed with the indicated concentration of adaGb3, and tested for residual binding to immobilized Gb3 in a RELISA [61]. (●), VT1; (▲), VT2.

VT2 induces a more severe, generalized metabolic stress such that lipid stores are mobilized for breakdown.

Lung targeting

Some degree of pulmonary symptoms has been reported in a minor fraction of HUS patients [95]. VT1, but not VT2, was detected in lung sections obtained from a patient who died of HUS [22], despite the fact that both VT1 and VT2 were detected in the kidney of the same patient [96]. Reported pulmonary histopathology includes microthrombosis, capillary congestion in the alveolar septa [97], and hemorrhage [98]. Mechanisms responsible for this pathology are thought to be the same as mechanisms causing angiopathy at the renal level. Our biodistribution study shows that the lungs are a major target for both toxins, although 10-fold more VT1 than VT2 was bound.

When detecting injected VT1 in the lung sections, we found the toxin to be localized primarily in capillaries in some of the alveolar septa. Based on the extent of VT1-induced pathology, we expected the lung distribution to be more widespread. However, VT1-induced hemorrhage may initiate the more generalized pathology. Since VT binding is restricted to pulmonary capillaries, high local VT1 levels must accumulate to account for the biodistribution observed. Extensive localized hemorrhage and edema were evident. Margination of polymorphonuclear neutrophils was seen throughout the lung, indicating an active inflammatory response. The high level of VT1 absorbed by the lungs could explain the difference in LD50 for the toxins. The lungs could function as an absorbing buffer, which permits less toxin to reach the kidneys where it could otherwise have caused more life-threatening pathology. However, we did not assess the kidneys of mice given sub-lethal doses of VT1.

A similar effect may be relevant to HUS in humans. Although the renal Gb3 increases with age [39], susceptibility to VT-induced HUS is greatest in the very young when the receptor is expressed in the glomerulus [38]. Thus, the increased expression of tubular Gb3 as a function of age might, to some extent, prevent toxin reabsorption by providing a similar, less crucial VT “sink.”

VT receptor isoforms

The Gb3 species from kidney and lung clearly show differential migration on TLC, indicating differences within the lipid moiety, which may affect VT binding [99]. Mouse renal Gb3 has been reported to contain hydroxylated fatty acids [76], which would be consistent with its reduced TLC mobility. Overall, VT2 binds Gb3 less effectively than VT1 [84] and this was reflected in the TLC overlay both for renal and lung Gb3. This procedure, however, is semiquantitative and not optimum for distinguishing the relative binding of Gb3 isoforms [82]. Kinetic binding analysis and Gb3 characterization has yet be performed to determine whether differential binding to Gb3 lipid isoforms could provide the basis of the differential tissue recognition by VT1 and VT2.

Receptor analogue treatment

It is clear that our first attempts to prevent VT/Gb3 binding in vivo have failed. Despite the fact that adaGb3,
inhibited VT2/Gb3 binding in vitro, co-administration exacerbated, rather than attenuated, VT2-induced disease. This was particularly evident when less than 1 LD50 VT2 was given, when all the VT2-treated mice survived, but all the VT2 + adaGb3 mice died. This correlates with evidence of “novel” renal pathology, despite the fact that some of the VT2-alone pathologies were reduced by adaGb3, co-administration. The additional pathologic features seen in the presence of adaGb3 may result from acceleration of the VT2-induced pathology, rather than new lesions per se. The unusual properties of adaGb3, include that despite its water solubility, the TLC mobility is not greatly affected and VT binding is effectively monitored by TLC overlay. Such solubility properties might result in complex effects in vivo. Our in vivo results are similar to studies attempting to use lyso-Gb3 to protect mice from VT2, which found symptoms were increased rather than alleviated by this treatment [100].

Tissue Gb3 is heterogeneous within its lipid moiety and membrane environment and therefore may bind VTs differentially [81]. We would interpret our results in terms of inhibition of lower affinity VT2/Gb3 interactions (and hence increasing the serum half-life as observed) to allow more VT2 to bind to high-affinity (renal) receptors, thus increasing/altering pathology. The organization of Gb3 at the plasma membrane into lipid microdomains has recently been found to be important in determining VT1 sensitivity [101]. Such organization may affect the relative Gb3-binding affinity of VT1 (and VT2).

At specific Gb3 concentrations, evidence for cooperative VT1-receptor binding has been found [102]. If such binding also occurs for VT2, an alternative explanation for the effect of adaGb3 might be that binding of adaGb3, to a proportion of the Gb3 binding sites within the B subunit pentamer cooperatively increases the Gb3 binding affinity of the residual sites to increase VT2 pathogenesis. However, this explanation would not be consistent with the increased serum half-life seen for VT2 in the presence of adaGb3.

While these results are disappointing, they indicate that adamantyl glycolipids are active in an in vivo system to reveal further complexity of VT2-receptor targeting. These studies must also serve as a caution in the development of receptor analogues for clinical use.

ACKNOWLEDGMENTS

This study was supported by CIHR grant # MT13073 (to C.A.L) and by NIH/MBRS grant # GM08241 and NIH/RMI grant # RR11598 (M.D.M). The authors are indebted to Dr. Ernest Cutz, Department of Pediatric Laboratory Medicine, Hospital for Sick Children, for evaluation of the lung histopathology. We thank Ms. Lily Morikawa and Mr. Davin Chark from the Hospital for Sick Children for preparing and immunostaining some histological sections and Ms. Bonnie Welsh for assistance in animal procedures.

Reprint requests to Clifford A. Lingwood, Ph.D., Division of Infection, Immunity, Injury and Repair, Research Institute, Hospital for Sick Chil-

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