Alteration of the Glycolipid Binding Specificity of the Pig Edema Toxin from Globotetraosyl to Globotriaosyl Ceramide Alters In Vivo Tissue Targetting and Results in a Verotoxin 1-like Disease in Pigs

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Summary

All members of the verotoxin (VT) family specifically recognize globo-series glycolipids on the surface of susceptible cells. Those toxins that are associated with human disease, VT1, VT2, and VT2c, bind to globotriaosyl ceramide (Gb_3) while VT2e, which is associated with edema disease of swine, binds preferentially to globotetraosyl ceramide (Gb_4). We were recently able to identify, using site-directed mutagenesis, amino acids in the binding subunit of these toxins that are important in defining their glycosphingolipid (GSL) binding specificity (Tyrrell, G. J., K. Ramotar, B. Boyd, B. W. Toye, C. A. Lingwood, and J. L. Brunton. 1992. *Proc. Natl. Acad. Sci. USA.* 89:524). The concomitant mutation of Gln_{64} and Lys_{66} in the VT2e binding subunit to the corresponding residues (Glu and Gln, respectively) found in VT2 effectively converted the GSL binding specificity of the mutant toxin from Gb_4 to Gb_3 in vitro. We now report that the altered carbohydrate recognition of the mutant toxin (termed GT3) has biological significance, resulting in a unique disease after intravascular injection into pigs as compared with classical VT2e-induced edema disease. The tissue localization of radiolabeled GT3 after intravascular injection was elevated in neural tissues compared with VT2e accumulation, while localization of GT3 to the gastrointestinal tract was relatively reduced. Accordingly, the pathological lesions after challenge with GT3 involved gross edema of the cerebrum, cerebellum, and brain stem, while purified VT2e caused hemorrhage and edema of the cerebellum, and submucosa of the stomach and large intestine. In addition, both radiolabeled toxins bound extensively to tissues not directly involved in the pathology of disease. VT2e, unlike GT3 or VT1, bound extensively to red cells, which have high levels of Gb_4. The overall tissue distribution of VT2e was thus found to be influenced by regional blood flow to each organ and not solely by the Gb_4 levels of these tissues. Conversely, the distribution of GT3 (and VT1), which cleared more rapidly from the circulation, correlated with respective tissue Gb_3 levels rather than blood flow. These studies indicate the primary role of carbohydrate binding specificity in determining systemic pathology, suggest that the red cells act as a toxin carrier in edema disease, and indicate that red cell binding does not protect against the pathology of systemic verotoxemia.

Verotoxins (VTs)[1] are a unique family of *Escherichia coli*-elaborated subunit toxins that recognize globo-series glycolipids on the surface of susceptible cells (1–4). Certain serotypes of VT-producing *E. coli* (VTEC), most notably O157:H7, are strongly associated with hemorrhagic colitis and hemolytic uremic syndrome (5, 6). The pathogenesis of these infections, however, is poorly understood. The edema

[1] *Abbreviations used in this paper:* Gb_3, globotriaosyl ceramide; Gb_4, globotetraosyl ceramide; Gb_5, galactosylglobotetraosyl ceramide; GSL, glycosphingolipid; HUS, hemolytic uremic syndrome; VT, verotoxin.

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disease toxin (VT2e) is produced by *E. coli* serotypes specific for the pig. This toxin is closely related to VT2 (Shiga-like toxin II [SLT II]) and has therefore also been termed SLT IIv (variant) (7). A recent consensus on nomenclature has agreed to the term VT2e or SLT IIe (8). This toxin is responsible for a disease in pigs typified by edema, perivascular hemorrhage, and necrosis of small blood vessels (9). The lesions are primarily restricted to the gastric and colonic submucosa, the colonic mesentery, the eyelids, and cerebellar folia. Unlike other members of the VT family, which bind solely to globotriaosyl ceramide (Gb_3), VT2e is able to bind to globotetraosyl ceramide (Gb_4) in the solid phase (3, 4).

Based on the close homology of VT2 and VT2e and the lesser degree of homology between VT2 and VT1 in the B subunit, we have used site-directed mutagenesis to define amino acids in the B subunit responsible, at least in part, for the discrimination of binding of Gb_3 and Gb_4 (10). Thus, concomitant alteration of amino acids Gln_{64} and Lys_{66} in VT2e to the corresponding Glu and Gln found in VT2 renders the resulting mutant toxin (termed GT3) unable to recognize Gb_4 and only bind Gb_3 in a manner similar to VT1 (10). It is likely that this alteration in glycosphingolipid (GSL) binding is due to the direct interaction of these amino acid residues with the receptor: the recently determined crystal structure of the VT1 B subunit places the corresponding residues in a solvent-exposed cleft that is proposed to be the carbohydrate-binding domain (11). Thus, the mutant toxin provides an excellent tool for examining the role of protein–glycosphingolipid interactions in toxin-induced pathology. Compared with VT2e, the mutant GT3 was found to show an altered cytotoxic specificity for cells in culture, typified by VT1, which correlated with their GSL content (10). We now report on the tissue localization and pathology of this mutant toxin in comparison with the wild-type VT2e and VT1 after intravascular injection into pigs.

Materials and Methods

Reagents. VT1, VT2e, and the mutant toxin GT3 were purified as described previously (10, 12). Carrier-free $Na^{125}I$ was purchased from Amersham Corp. (Arlington Heights, IL). ^{57}Co microspheres (15-μm diameter) were obtained from DuPont Co.-New England Nuclear (Wilmington, DE). Iodogen and Iodobeads were from Pierce Chemical Co. (Rockford, IL). Plastic-backed Sil-G silica TLC plates were obtained from Brinkmann Instruments (Westbury, NY). Horseradish peroxidase–conjugated goat anti-rabbit IgG was purchased from Sigma Chemical Co. (St. Louis, MO).

Glycolipid Extraction and Quantitation. Weanling Yorkshire pigs (12–16 kg) were anesthetized with pentobarbitol (35 mg/kg) and killed by exsanguination. Representative tissue samples were collected and neutral glycolipids were isolated as previously described (13). The Gb_3 and Gb_4 levels in each tissue sample were quantitated by benzoylation and HPLC analysis (13).

Toxin Radiolabeling. VT2e and GT3 were radiolabeled with ^{125}I using Iodogen (10). VT1 was iodinated using Iodobeads as previously described (14).

Toxin Binding to Tissue Glycolipids In Vitro. Glycolipid extracts (20 mg tissue equivalent) were applied to silica plastic-backed TLC plates and separated in chloroform/methanol/water (65:25:4 [vol/vol/vol]). Gb_3 and Gb_4 present in the extracts were identified by comparison with standards purified from human kidney (13). Total glycolipids were visualized using orcinol spray. Toxin binding to individual glycolipids was determined by TLC overlay procedure (15). Polyclonal rabbit antiserum prepared against VT2c (16) was used to detect VT2e and GT3 binding. Toxin binding was visualized using peroxidase-conjugated goat anti-rabbit IgG followed by development with 4-chloro-1-naphthol peroxidase substrate.

Measurement of Radiolabeled Toxin or Microsphere Distribution in Pigs. To determine the sites of localization of toxin in vivo, pigs were anesthetized with pentobarbitol (35 mg/kg) and injected via the ear vein with purified, iodinated toxin. In an initial experiment one pig each was injected with VT2e and GT3 (200 μg in 3 ml of PBS; sp act, 5×10^4 cpm/μg). After 2 h, the animals were killed by anesthetic overdose, the various tissues were weighed, and the radioactivity of representative samples was determined. Due to the significant binding of VT2e to red cells, the experiment was repeated, including ^{125}I-VT1 for comparison, with the exception that the pigs were killed by exsanguination. In the case of each toxin, the amount localized to each organ was determined by extrapolating to the total organ weight.

It was of importance to determine whether toxin distribution was influenced by blood flow to various tissue sites. Blood flow measurements were conducted in the laboratory of Dr. H. M. Clarke (Department of Surgical Research, Hospital for Sick Children). Two pigs were anesthetized with pentobarbitol (35 mg/kg) and injected with ^{57}Co-labeled microspheres via the left ventricle (3.3×10^7 and 6.4×10^7 cpm, respectively). After 2 min, at which time the microspheres were trapped in the capillary beds, the pigs were killed by anesthetic overdose. Tissues were collected for weight and radioactivity determinations as described above for toxin distribution measurements. The blood flow (ml/min/100 g) to each organ was then calculated (17).

Glycolipid Reconstitution of Toxin Receptor–negative Cultured Cells. The generation of a mutant Daudi lymphoma cell line deficient in Gb_3 and Gb_4 has been previously described (18). Briefly, a Daudi cell line selected for resistance to growth inhibition by IFN-α (19) was found to be deficient in Gb_3 and crossresistant to verotoxin (18). The cell line was mutagenized and then further selected for resistance to 500 ng/ml verotoxin. The resulting cell line (termed VT_{500}) was found to have a greatly reduced Gb_3 content (>95%) relative to wild-type cells.

VT_{500} cells were reconstituted with Gb_3 or Gb_4 by liposome-mediated delivery as previously described (20). After glycolipid incorporation, the cells were washed, cultured overnight, and resuspended in media with or without VT2e or GT3 (100 ng/ml). The cells were maintained at 37°C, 5% CO_2 for 4 d, at which time the percentage of viable cells in each group was determined by dye exclusion.

Determination of the Clinical and Gross Pathological Effects of VT2e, GT3, and VT1 in Pigs. Weanling pigs were injected via the ear vein with 24 ng/kg of VT1 or VT2e (four animals each) or increasing doses of GT3 (two animals received 12 ng/kg, while four animals each received 24 or 48 ng/kg body weight). The pigs were then observed for clinical signs of toxemia (9). Animals that began to experience debilitating illness were killed, while those that recovered from initial signs of verotoxemia and became asymptomatic were killed from 3 to 7 d postinjection. Clinical symptoms were classified into three groups: nervous disorders (including incoordination, confusion, inappatence, limb paralysis, and tremors), edema of the eyelids and forehead, and respiratory difficulties. A postmortem examination was performed on all animals immedi-

ately after death. Tissue samples were collected and examined as described previously to determine the gross and histopathological effects of each toxin (9).

Results

Gb_3 but Not Gb_4 Is a Functional Receptor for GT3. Binding kinetics performed in vitro have indicated that wild-type VT2e binds predominantly to Gb_4 and to a lesser extent to Gb_3. In contrast, mutant GT3 shows a selective loss of Gb_4 binding and increase in Gb_3 binding affinity (10). These findings were reflected in vitro, as the Gb_3/Gb_4 content of several cell lines determined the relative cytotoxicity of VT2e and GT3 (10). However, a cell line expressing Gb_4 but lacking Gb_3 was not available. To confirm that Gb_4 showed no residual functional receptor activity for GT3, we used a previously described technique for delivering individual GSLs to viable cells (20). The mutant Daudi cell line (VT_{500}) lacks Gb_3 (and Gb_4) and is resistant to VT1 (18). As expected, the cells were found to be crossresistant to VT2e and GT3 (Fig. 1). We have previously shown that reconstitution of cells with Gb_3 but not with Gb_4 restored VT1 sensitivity (20). Reconstitution of VT_{500} cells with Gb_3 or Gb_4 induced sensitivity to VT2e (Fig. 1). However, induction of GT3 toxicity was only achieved by reconstitution with Gb_3 (Fig. 1). These results demonstrate that Gb_4 cannot serve as a functional receptor for GT3, and thus, this allowed the unambiguous assessment of the role of Gb_4 binding in the pathology of pig edema disease.

Tissue Distribution of Radiolabeled Toxin. The most significant observation with regard to the tissue distribution of VT2e after intravenous administration was the extensive binding observed to blood cells. 2 h after administration of radiolabeled toxin, 30% of VT2e was still circulating, bound to red cells (Fig. 2). This is entirely unlike previous experiments with VT1 distribution in rabbits, which showed rapid clearance of toxin from the circulation and accumulation in target organs (central nervous system [CNS] and cecum) (21). These experiments were therefore repeated in comparison with VT1, and in a manner similar to the rabbit model, binding of VT1 to pig red cells is minimal (Fig. 2). To compare the toxin dose delivered to various tissues, the distributions of VT2e, GT3, and VT1 were normalized for tissue weight (Fig. 3). Significant differences between the localization of GT3 and the wild-type VT2e were the reduced binding for GT3 observed in the gastrointestinal tract (Fig. 3 *A*) and the increased binding of GT3 relative to VT2e for neural tissues (brain stem, cerebellum, and cerebrum; Fig. 3 *B*). All the differences observed for the tissue localization of GT3 as opposed to VT2e were duplicated for VT1 localization (Fig. 3). Thus, the tissue distribution correlated with the restricted binding specificity of GT3 and VT1 for Gb_3 as compared to Gb_3 and Gb_4 for VT2e.

Exsanguination had no effect on the relative distribution of VT2e and GT3 for all organs examined with the exception of the spleen. In this case, exsanguination before tissue collection reduced the amount of VT2e bound (by fourfold) to levels observed for GT3 and VT1. Results from the latter experiment, in which animals were exsanguinated before tissue collection, are presented here.

Quantitation of Tissue Levels of Gb_3 and Gb_4. Total Gb_3 and Gb_4 levels for each tissue were quantitated by HPLC (13) (Table 1). Tissue localization of VT1 and GT3 was found to correlate directly with the level of Gb_3 in the target tissues (R^2 = 0.68 and 0.81, respectively; Fig. 4 *A*). Tissue localization of VT2e, however, did not correlate with the

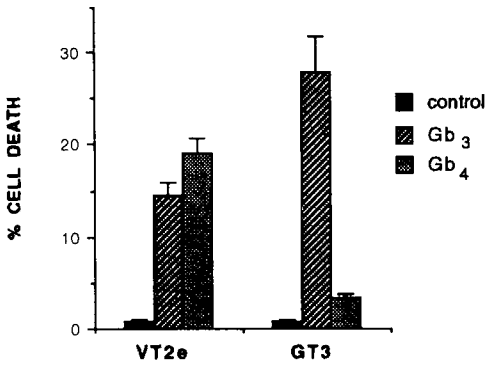

Figure 1. Demonstration of the functional receptor specificity of wild-type VT2e and mutant GT3. The ability of Gb_3 and Gb_4 to act as functional receptors for VT2e and GT3 was examined using a glycolipid-deficient mutant Daudi cell line. VT_{500} cells were reconstituted with either Gb_3 or Gb_4 by liposome-mediated delivery (20) and then incubated in media containing 100 ng/ml VT2e or GT3. Reconstituted cells that were incubated in media alone served as controls (zero cell death). Cell viability was assayed after 4 d.

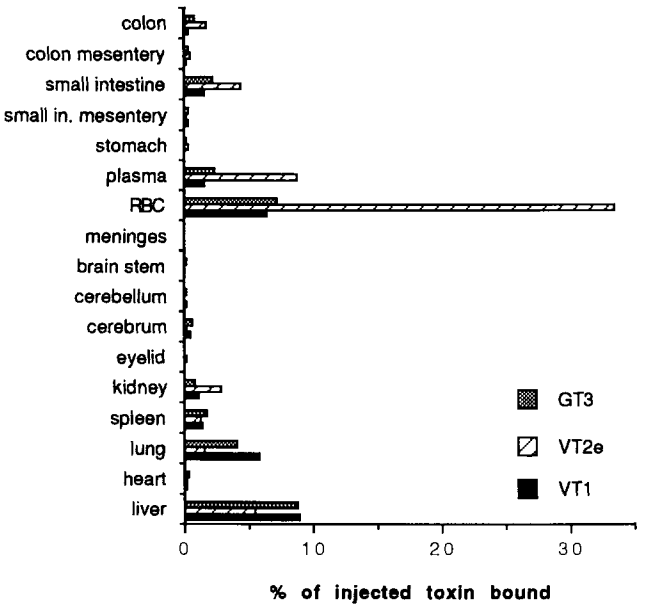

Figure 2. Tissue binding distribution of radiolabeled VT2e, GT3, and VT1. ^{125}I-labeled verotoxins were individually injected via the ear vein into weanling pigs and allowed to circulate for 2 h. At this time the animals were killed by exsanguination and the various tissues were weighed and a portion subjected to gamma counting.

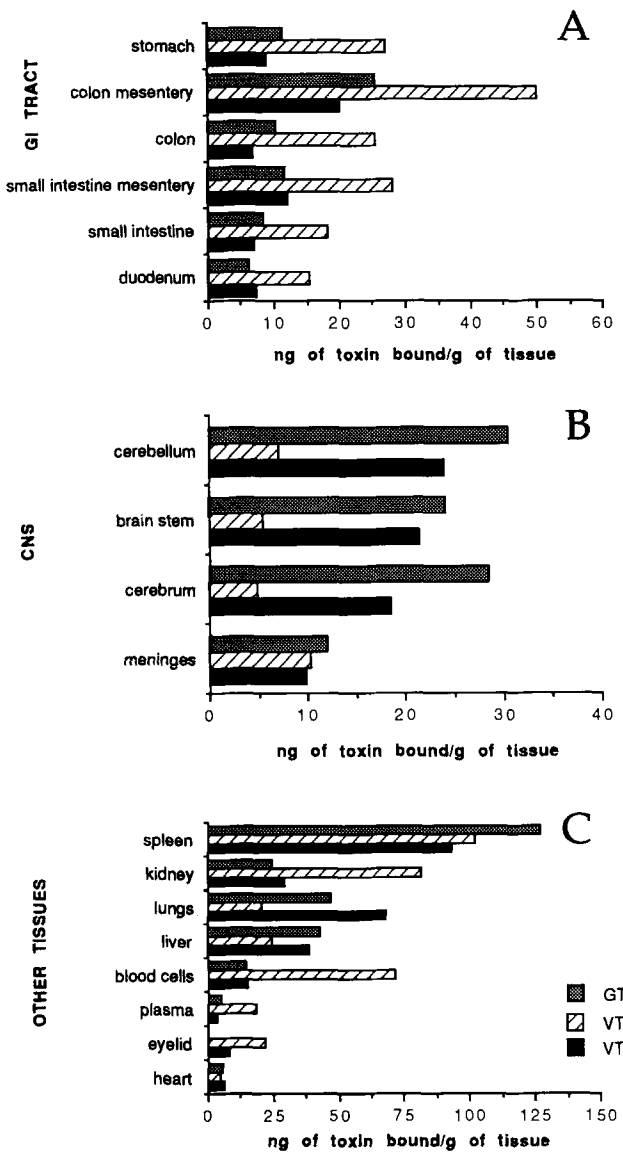

Figure 3. Comparison of toxin binding normalized for tissue weight. To compare the relative binding of VT2e, GT3, or VT1 to each organ, the toxin distribution was normalized for tissue weight. The data have been organized according to the sites that show pathology after challenge with purified VT2e (9) (*A*) gastrointestinal tract; (*B*) central nervous system. Toxin binding to other tissues is shown in *C*.

tissue levels of Gb_4 or Gb_4 + Gb_3 (R^2 = 0.21 for both; Fig. 4 *B*).

Binding of VT2e and GT3 to Glycolipids Extracted from Pig Tissues. Binding of VT2e and GT3 to lipids extracted from pig tissues was performed to determine whether other toxin-binding GSL species were present. It has been shown that in addition to Gb_3 and Gb_4, VT2e recognizes galactosyl-globotetraosyl ceramide (Gb_5) (4). As shown in Fig. 5, GT3 bound only to Gb_3 in tissue extracts while VT2e recognized Gb_3 and Gb_4. Gb_5 was not detected in the lipid extracts of the tissues examined (Fig. 5).

Table 1. *Concentration of Gb_3 and Gb_4 in Pig Tissues*

Tissue	Gb_3	Gb_4
	nmol/g	*nmol/g*
Liver	11.1	ND
Heart	23.7	43.5
Lung	38.0	27.2
Spleen	89.7	138.4
Kidney medulla	20.2	52.5
Kidney cortex	18.0	13.9
Stomach fundus	14.1	14.3
Stomach antrum	13.6	10.9
Red cells*	0.70	18.0
Eyelid	14.2	32.2
Colon	21.9	18.8
Colon mesentery	25.4	18.5
Cerebrum	ND	ND
Cerebellum	ND	ND

* nmol GSL/ml blood.

The Localization of VT2e but Not of GT3 or VT1 Is Influenced by Differential Tissue Blood Flow. Gb_3 and/or Gb_4 were detected at some level in all tissues examined, yet the accumulation of VT2e generally did not correlate with the glycolipid levels observed. Since VT2e bound extensively to red cells, we considered whether differential blood flow to various organs could be a factor affecting toxin distribution. The cardiac output and blood flow to various tissues were determined in two pigs using the radiolabeled microsphere technique (17). VT2e, but not VT1 or GT3, distribution was found to be related to blood flow (Fig. 6). When the tissue localization data was replotted allowing for blood flow (Fig. 7), it was apparent that the eyelids bound a significantly greater fraction of available toxin than other tissues. It was now evident that the specific binding to the stomach fundus was greater relative to the antrum. Furthermore, the considerable toxin binding to kidney and spleen was likely a result of high blood flow to these sites.

In Vivo Action of the Mutant Toxin Reflects Its Altered Receptor Specificity and Tissue Localization. Histological and gross analysis of the pathology induced after intravenous administration of VT2e, GT3, and VT1 was found to segregate remarkably according to the ability of the toxins to bind to Gb_3 or Gb_4 (Table 2). Thus, VT1 and GT3 were found to cause identical lesions, particularly notable in that of brain edema; high doses of GT3 caused more marked, gelatinous edema than was observed for VT2e. In contrast, the wild-type VT2e caused microangiopathic hemorrhage and edema in the stomach fundus and large intestine (not seen for GT3 or VT1). Despite the effect of GSL-binding specificity on toxin localization and sites of pathology, the basic histological lesion observed was the same for all three toxins. Fig. 8 shows the

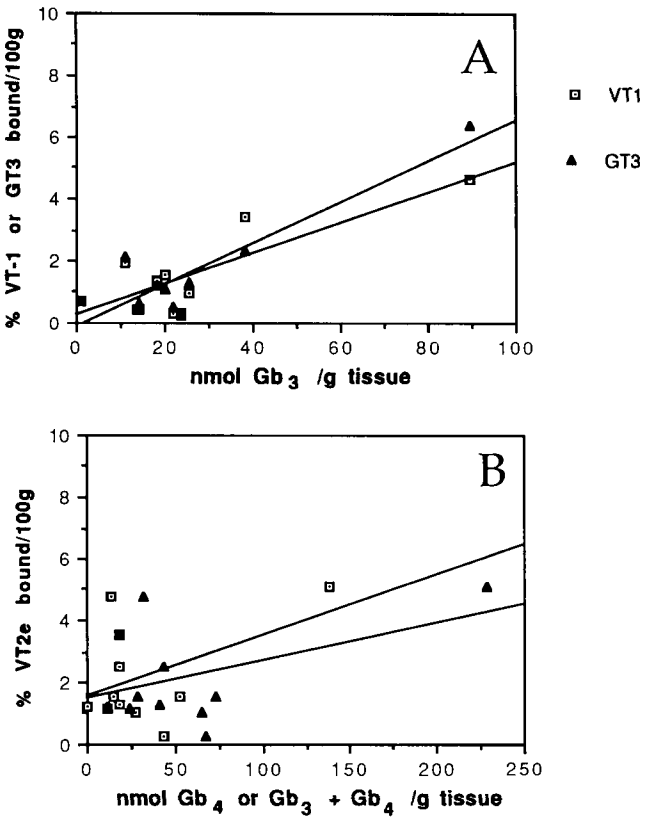

Figure 4. Correlation of toxin distribution with receptor concentration. ^{125}I-labeled VT1, GT3, or VT2e were injected via the ear vein of weanling pigs and allowed to circulate for 2 h. The toxin localization to various tissues was determined and plotted against the total GSL content of that tissue determined by HPLC analysis. Neural tissues, in which Gb_3 and Gb_4 were not detected, are not represented above. (*A*) VT1 (□) (R^2 = 0.68) and GT3 (▲) (R^2 = 0.81) bound is plotted against Gb_3 concentration. (*B*) VT2e bound is plotted against Gb_4 (□) (R^2 = 0.21) or Gb_4 + Gb_3 concentration (▲) (R^2 = 0.21).

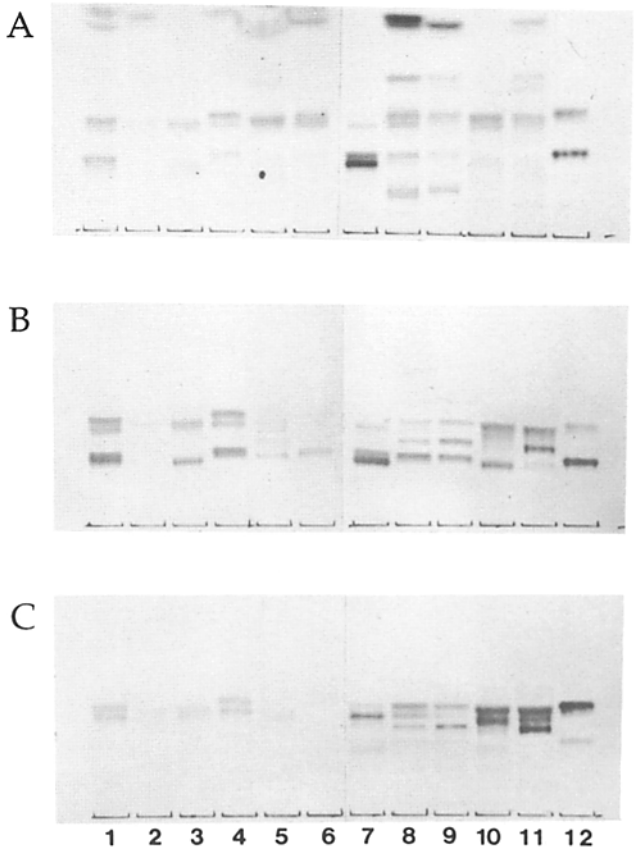

Figure 5. Binding of VT2e and GT3 to glycolipid extracts of pig tissues by TLC overlay. Neutral glycolipids were isolated from pig tissues as described in Materials and Methods. Glycolipid extracts were applied to plastic-backed TLC plates (20 mg tissue equivalent per lane, with the exception of lane 7, which represents 50 μl of blood) and separated (chloroform/methanol/water, 65:25:4 [vol/vol/vol]). Tissue extracts are: lane *1*, spleen; lane *2*, liver; lane *3*, heart; lane *4*, lung; lane *5*, kidney cortex; lane *6*, kidney medulla; lane 7, red blood cells; lane *8*, stomach fundus; lane *9*, stomach antrum; lane *10*, colon mesentery; lane *11*, colon; lane *12*, standard Gb_3 and Gb_4. (*A*) Total glycolipid detection by orcinol spray. (*B*) An equivalent set of TLC plates was incubated with VT2e (0.15 μg/ml), followed by a cross reactive polyclonal antisera raised against VT2c (1:1,000 dilution). The plates were finally incubated with a peroxidase-conjugated goat anti-rabbit antibody, and binding was visualized using 4-chloro-1-naphthol (15). (*C*) Plates were treated as in *B* but were incubated with GT3 instead of VT2e.

distinct pathology of the colonic mucosa after exposure to VT2e compared with the unaffected colonic histology of GT3-treated pigs. Significant edema of the submucosa and damage to the epithelium was observed only for VT2e-treated pigs (Fig. 8). Neither gross nor microscopic lesions were observed in lung, liver, or spleen of pigs to which VT2e, GT3, or VT1 had been administered. A detailed histological analysis of lesions observed in VT2e-, GT3-, and VT1-treated pigs will be presented elsewhere (Gyles et al., manuscript in preparation).

It was interesting to note that both animals that received a dose of 12 ng/kg and half of those receiving 24 ng/kg of GT3 recovered fully from the neurological manifestations of toxemia (see Table 2; mean recovery time in each group was 40 and 37 h, respectively). In the latter group, recovery was associated with the failure to develop respiratory difficulties at the clinical level. Furthermore, the pathology of CNS, eye, and forehead edema had normalized at postmortem (Table 2). This characteristic of GT3 toxicity has not been observed in the case of VT2e (9) and may relate to the fact that although the generalized edema of the brain was more marked and gelatinous for GT3, hemorrhage of the cerebellar folium (9) was not observed for GT3-treated pigs (Table 2).

Discussion

The disease in pigs caused by VTs is quite distinct from the hemolytic uremic syndrome (HUS) that may be observed in humans after infection by VT1- or VT2-producing *E. coli*. Nevertheless, the pig provides a more amenable model to study the molecular basis of the cytopathology, and pig edema disease is a significant veterinary problem in its own right. Studies with VT1 in the rabbit model have shown that the lesion sites correlate with the expression of glycolipid receptors for

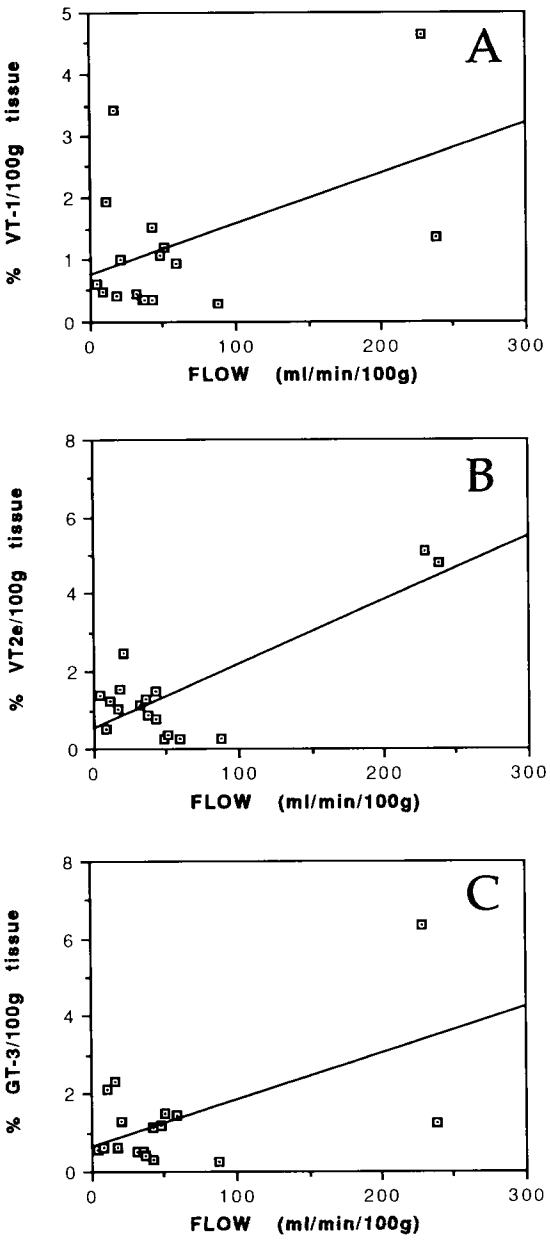

Figure 6. Correlation of toxin distribution with regional blood flow. The relationship between radiolabeled toxin localization and regional blood flow was examined. Blood flow measurements were performed in two animals under the conditions used in the toxin distribution studies. The percentage of each toxin delivered to each organ was plotted against the average blood flow (ml/min/100 g) to that tissue. The correlation of toxin distribution with blood flow was as follows: ^{125}I-VT1 (*A*, R^2 = 0.33), ^{125}I-VT2e (*B*, R^2 = 0.64), ^{125}I-GT3 (*C*, R^2 = 0.23).

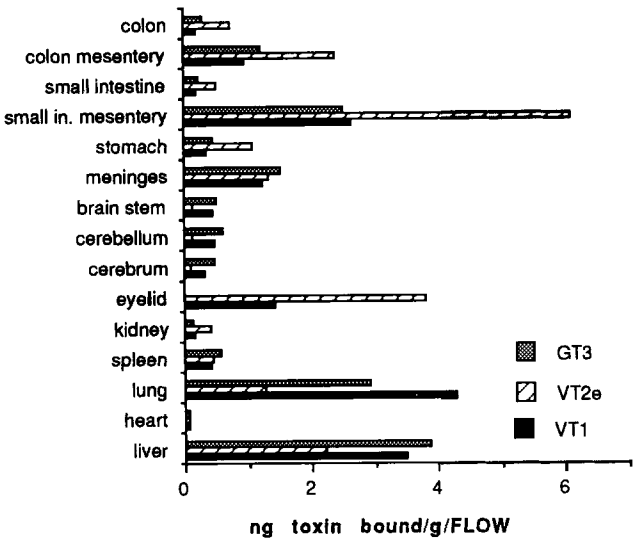

Figure 7. Distribution of radiolabeled toxins normalized for blood flow. The radiolabeled toxin distribution data expressed in Fig. 3 was replotted as a function of the blood flow to the respective tissue as measured by microsphere entrapment to determine the "specific" tissue distribution of toxin.

this toxin in the rabbit tissues (21, 22). Our present studies more definitively illustrate the role of glycolipid binding in verotoxemia and allow us to draw some interesting conclusions.

First, the tissue distribution of GT3 was distinct from that of VT2e. For each tissue, GT3 localized in a manner virtually identical to VT1, which also shows a binding specificity restricted to Gb_3. The major differences in the distribution of GT3/VT1 relative to VT2e included dramatically reduced red cell binding, decreased binding to the gastrointestinal tract, and increased binding to the CNS. These alterations correlated with differences in pathology, most notably the lack of GT3- or VT1-induced lesions in the colon and stomach.

Second, GT3 and VT-1 generally distributed in vivo according to the concentration of Gb_3 in each tissue. This binding correlation was less marked for the TLC overlay, particularly for GT3 binding renal glycolipids, but solid phase assays do not necessarily reflect glycolipid receptor function in vivo. However, lesions were not observed at some of the sites where extensive toxin binding was found. Thus, it appears that some toxin-binding sites are relatively resistant to toxin action while other sites are highly sensitive. The same might be true in the case of humans, where Gb_3 is present in many tissues (23), yet glomerular thrombotic microangiopathy is the major lesion of HUS (24). The nature of the toxin-insensitive sites might relate to differential routing of bound toxin in different cells. A cell line was recently described (25) that binds and internalizes VT but that delivers it to lysosomes and is thus not intoxicated. Sensitive cells deliver toxin to the ER and Golgi complex (25, Khine, A. A., and C. A. Lingwood, manuscript submitted for publication), which may reflect a specialized role of Gb_3 to target the ER in these cells (Khine, A. A., and C. A. Lingwood, manuscript submitted for publication). It is also of relevance to note that sensitivity of cultured cells to VT is a function of cell growth (26). We have found that stationary, in contrast to log-phase–cultured, cells are resistant to verotoxin. This altered sensitivity to VT was found to be a function of Gb_3 surface exposure, but not of changes in total Gb_3 content during the cell cycle (26). However, since the binding of VT1 and GT3 was found to correlate with total tissue Gb_3 content, such variations may only be relevant at the level of individual cells and not affect Gb_3 availability as a whole. Our finding that liver and lung contain significant levels of Gb_3,

Table 2. *Pathological Effects of GT3, VT2e, and VT1 in Pigs*

Toxin	Dose*	*n*	Clinical symptoms: Avg. time to onset	Nervous disorders: *n*	Nervous disorders: ‡Severity	Edema: *n*	Edema: Severity	Respiratory difficulties: *n*	Respiratory difficulties: Severity	Recovery from symptoms	Postmortem edema (*n*): CNS	Eye/forehead	Stomach	Large intestine
	ng/kg		*h*											
GT3	12	2	17	2	+ + + +	0		0		Yes (2/2)	0	0	0	0
	24	4	14	4	+ + + +	2	+ +	2	+ +	Yes (2/4)§	2‖	2	0	0
	48	4	8	4	+ + + +	4	+ + + +	4	+ + + +	No	4	4	0	0
VTE	24	4	7	4	+ + + +	4	+ +	4	+ + +	No	4	4	4	4
VT1	24	4	8	4	+ + + +	0		4	+ +	No	4	4	0	0

* All animals received the indicated dose of toxin by intravenous injection via the ear vein.
‡ Number of animals affected. The severity of symptoms was similar for affected animals at each dose and is scored from mild (+) to severe (+ + + +) in each case.
§ Two of four of the pigs that received 24 ng/kg of GT3 did not develop respiratory difficulties or edema and fully recovered from their symptoms within 48 h postinjection.
‖ Marked gelatinous edema of the CNS with GT3. More watery fluid with VT2e as well as extensive hemorrhage of the cerebellar folia.

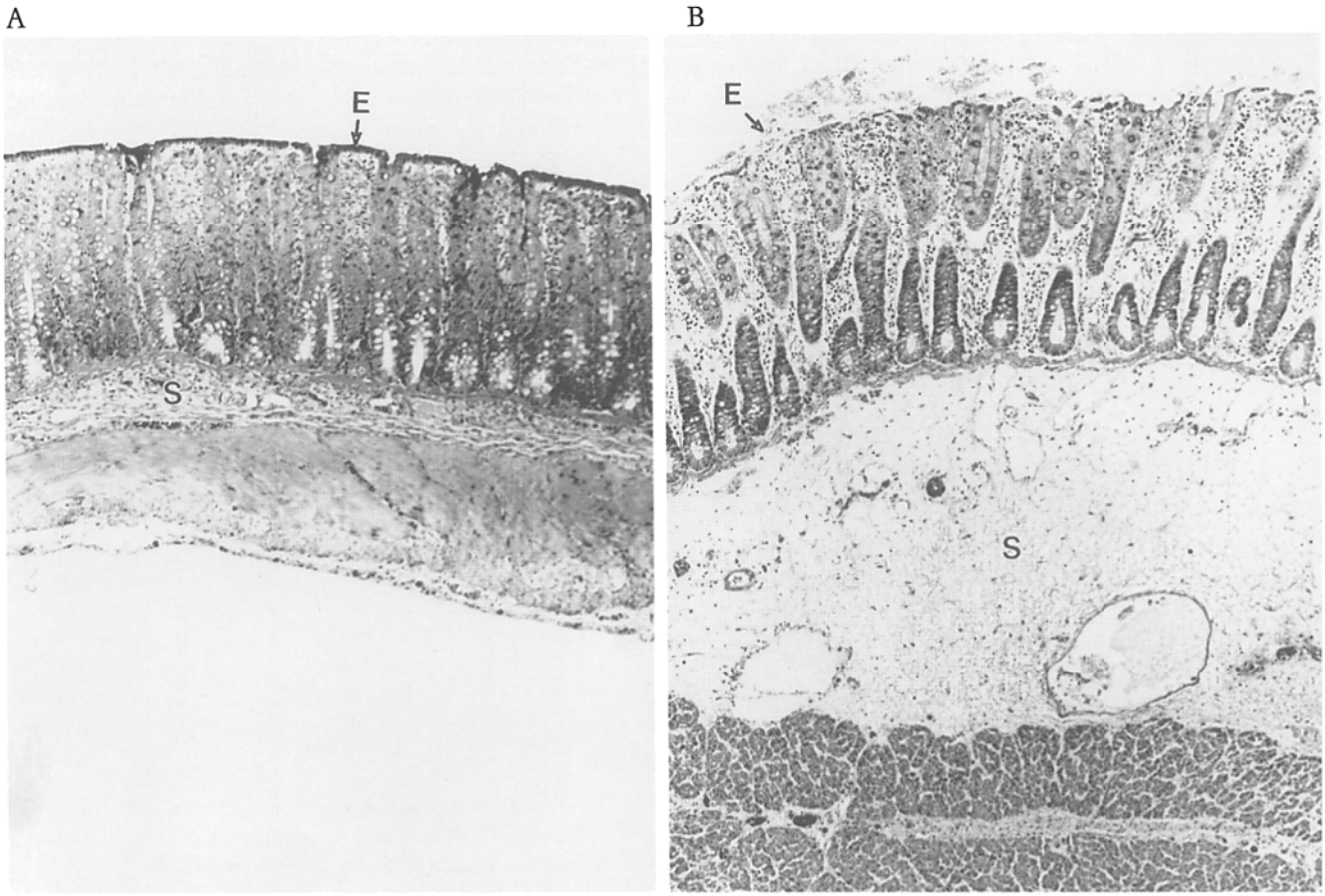

Figure 8. Comparison of microscopic appearance of the colon of pigs inoculated with 24 ng/kg GT3 (*A*) or VT2e (*B*). (*A*) The colon of pigs inoculated with GT3 appeared essentially normal; the epithelium (*E*) is intact and the submucosa (*S*) is not distended. (*B*) The colon of pigs inoculated with VT2e showed loss of epithelial covering (*E*) and marked edema of the submucosa (*S*), causing an increase in thickness of the intestine (hematoxylin-eosin stain; ×73).

bind VT1 and GT3 (and VT2e to a lesser extent), and yet show no evidence of lesions illustrates the considerable gap in our understanding of Gb_3-related pathology of VTs.

Third, pig red cells bind significant levels of VT2e yet do not protect animals from toxin-induced pathology. The prolonged retention of VT2e in the circulation may actually be a risk factor, as susceptible tissues may effectively be subjected to prolonged toxin exposure as compared with GT3, which rapidly clears from the circulation. It has recently been proposed that humans with lower levels of red cell–associated Gb_3 and P1 antigen may be at increased risk for developing HUS during VTEC infection (27). The P1 glycolipid has a terminal Galα1-4Gal dissacharide required for VT binding and is present on the red cells of most humans (27). However, our results indicate that the ability of red cells to bind VT is not protective in pigs. The same may be true in humans. It is possible that glycolipid content of human red cells is indicative of GSL levels in other tissues, some of which may be resistant to toxin action and thus reduce the toxin load to sensitive sites.

Unlike VT1 and GT3, the distribution of VT2e was not a function of its receptor distribution but rather of blood flow. This may indicate that the administered dose was unable to saturate the initial target (red blood cells) and that the observed tissue distribution was a result of red blood cell delivery and a slower toxin exchange from this carrier to target organ receptors. When the normalization factor for blood flow was included in the calculation of distribution, it became obvious that the eyelids bound a considerably larger fraction of the blood-delivered VT2e toxin than other tissues. This indicates that this tissue has a higher affinity for VT2e, perhaps due to increased receptor exposure. This correlates with the observation that the eyelids are one of the primary and earliest sites of VT2e-induced lesions in pigs (9).

Although the gross tissue levels of Gb_3 correlated well with GT3 and VT1 localization, toxin binding is really indicative of vascular GSL content. Thus, the negative correlation of VT2e distribution with total organ Gb_4 levels may merely reflect the nonvascular distribution of Gb_4 in various tissues.

In comparing the in vivo pathogenesis of VT2e with that of the mutant GT3, we have shown that Gb_4 binding is necessary for the action of VT2e on gastrointestinal tissue, despite the fact that these tissues contain significant levels of both Gb_3 and Gb_4. GT3 showed greatly reduced binding and none of the morphological lesions in the colon and colonic mesentery typical of VT2e action. The surface exposure of Gb_4 in the gastrointestinal mucosa and vasculature must therefore be far greater than that of Gb_3. In contrast, the binding of VT2e, relative to GT3 and VT1, was reduced in brain tissues. These results indicate a differential distribution or function of Gb_3 and Gb_4 in these tissues. We were unable to detect these glycolipids in pig brain lipid extracts (by TLC overlay or HPLC) but they may be present in high concentrations in discrete blood vessels of the nervous system. This was found to be the case in rabbits, where intravenously administered VT1 was found to bind to a few specific blood vessels of the brain, as detected by indirect immunofluorescence (21). However, galabiosyl ceramide could be detected by TLC overlay in rabbit brain (22). Nevertheless, the present studies establish that the glycolipid binding specificity of VTs is a primary determinant in the site of toxin localization and toxin-induced lesions in vivo.

The lack of lesions in the gastrointestinal tract and cerebellar folia after administration of GT3 and the finding that at low doses all pigs recovered suggests that GT3 may represent a viable route for the immunoprotection of pigs against the edema disease toxin.

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