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Detergent-resistant globotriaosyl ceramide may define verotoxin/glomeruli-restricted hemolytic uremic syndrome pathology

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Verotoxin binding to its receptor, globotriaosyl ceramide(Gb_3) mediates the glomerular pathology of hemolytic uremic syndrome, but Gb_3 is expressed in both tubular and glomerular cells. Gb_3 within detergent-resistant membranes, an index of glycolipid-cholesterol enriched lipid rafts, is required for *in vitro* cytotoxicity. We found that verotoxin 1 and 2 binding to human adult renal glomeruli is detergent resistant, whereas the strong verotoxin binding to renal tubules is detergent sensitive. Verotoxin binding to pediatric glomeruli was detergent resistant but binding to adult glomeruli was enhanced, remarkably for some samples, by detergent extraction. Detergent-sensitive glomerular components may provide age-related protection against verotoxin glomerular binding. Mouse glomeruli remained verotoxin unreactive after detergent extraction, whereas tubular binding was lost. Cholesterol extraction induced strong verotoxin binding in poorly reactive adult glomeruli, suggesting cholesterol can mask Gb_3 in glomerular lipid rafts. Binding of the human immunodeficiency virus (HIV) adhesin, gp120 (another Gb_3 ligand) was detergent sensitive, tubule-restricted, and inhibited by verotoxin B subunit pretreatment, and may relate to HIV nephropathy. Our study shows that differential membrane Gb_3 organization in glomeruli and tubules provides a basis for the age- and glomerular-restricted pathology of hemolytic uremic syndrome.

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The more rigid membrane domains of the liquid ordered phase are easily demonstrable in model systems[1] but the relevance of such models in terms of normal cellular physiology have frequently been questioned,[2,3] primarily due to the difficulty of isolation of such microdomains (or 'lipid rafts') from living cells. This is commonly achieved by resistance to Triton detergent solubilization at 4°C and sucrose gradient separation.[4,5] These extracted detergent-resistant membrane (DRM) domains are enriched in cholesterol, glycosphingolipids (GSLs), sphingomyelin and glycophosphoinositol-linked membrane proteins,[6,7] but represent a mixed pool, not representative of potential individual domains in cell membranes. Although the exact cell physiological parameter measured by detergent resistance has yet to be precisely defined,[8,9] this procedure provides a useful tool for investigation of membrane dynamics in living cells.[10] Domains, which may be equivalent to the l_o phase in model membranes, have been shown in living cells by a variety of staining procedures.[11–15] Whatever their physiological basis, DRMs are central in many transmembrane signaling pathways[16] and, in relation to the present studies, play important roles in many aspects of microbial–host cell interactions.[17–19]

Verotoxins are an *Escherichia coli* elaborated family of subunit toxins comprising a single A subunit, which inactivates ribosomal protein synthesis and a pentamer of B subunits responsible for binding the GSL, globotriaosyl ceramide Galα1-4Galß1-4 glucosyl ceramide (Gb_3) also known as the B cell marker CD77,[20] and the p^k blood group antigen.[21] Gb_3 is primarily found in the kidney of man and mice.[22] Verotoxins are vasotoxins that target endothelial cells. Infection in humans is gastrointestinal, and submucosal blood vessels are the site of primary lesions following such infections, which result in hemorrhagic colitis. Such infections and lesions spontaneously resolve in most cases, but in a minor subset of individuals, primarily in the very young and old, a second phase of pathology may occur.[23] This is termed hemolytic uremic syndrome (HUS),[24] whereby systemic verotoxemia targets the renal microvasculature to

induce infarcts within the glomerulus and the ensuing thrombocytopenia and hemolysis can yet be fatal.[25] In the mouse, no verotoxin-binding sites within the glomerulus can be detected [26] and this is consistent with the fact that systemic verotoxemia in the mouse results in tubular necrosis rather than HUS.[27]

Human immunodeficiency virus (HIV) infection has been associated with HUS susceptibility,[28] and HIV transgenic mice show nephropathy and abnormal renal Gb_3 metabolism.[29] The HIV envelope glycoprotein, gp120, binds several GSLs, including Gb_3,[30] and a soluble Gb_3 mimic inhibits HIV infection and gp120-dependent membrane fusion.[31] Nephropathy is common in HIV/AIDS, and renal epithelial cells can be infected by HIV[32] to provide a viral reservoir.[33]

Gb_3 can be found in DRMs in the plasma membrane of cultured cells and mediate transmembrane signals.[34–36] VT cytotoxicity in cell culture is dependent on Gb_3 presentation within a DRM format.[37–40] Cytotoxicity depends on the retrograde transport of VT1 or VT2 from the cell surface to the Golgi/ER[40] for cytosolic transit of the A subunit.[41] Non-DRM plasma membrane Gb_3 mediates binding and subsequent traffic to lysosomes for VT degradation.[37,38] Our present studies indicate that differential DRM expression of Gb_3 also plays a major age-related role in VT-induced renal pathology in man.

The role played by Gb_3 expression on renal glomerular endothelial cells in the susceptibility to HUC following gastrointestinal verocytotoxin-producing *E. coli* infection remains to be clearly defined. Our early studies using direct labeled VT1 showed selective binding of pediatric as opposed to adult renal glomeruli,[42] which correlates with the incidence of HUS. However, later studies using indirect immunostaining showed that VT1 stained some, and VT2 all, adult glomeruli and that cholesterol depletion induced VT1 glomerular staining.[43] We now confirm these results but show that the differential glomerular/tubular VT binding is a function of resistance to detergent extraction as a function of age. These studies imply that the membrane organization of Gb_3 is different in tubular epithelial and glomerular endothelial cells and that this changes with age such that HUS susceptibility may be lipid raft-based.

RESULTS

Double labeling of human pediatric renal frozen tissue with VT1 and VT2 showed strong VT2 glomerular binding with (Figure 1a), or without (Figure 1b and c), coincident VT1 staining, according to sample. Samples with selective VT2 glomerular staining showed pronounced VT1 tubular binding. VT2 blood vessel staining was more prominent (arrows) than VT1. Similar results were obtained by separate VT1 and VT2 staining of serial pediatric renal sections (Figure 2). Although VT1 glomerular was greater than tubular staining (Figure 2A, a), VT2 glomerular staining was more pronounced and VT2 tubular staining was also more significant (cf. Figure 2A, a vs c).

Comparison of pediatric renal section staining with VT1 or VT2 before and after Triton extraction at 4°C (cf. Figure 2A, a, c vs b, d), clearly showed that glomerular VT1 and VT2 staining are marginally affected by detergent extraction (Figure 2A, b and d). In the serial sections shown, the same two glomeruli are apparent (arrows) in each section. VT2 staining of both glomeruli is more pronounced than VT1. For VT1 only, the right glomerulus is more extensively stained than the left, consistent with the differential VT2 glomerular staining observed by double labeling in pediatric (Figure 1) and adult[43] renal sections. In untreated sections, VT1 glomerular staining is particularly prevalent as we reported.[42] VT1 and VT2 blood vessel staining is seen (*). VT2 tubular staining is greater than VT1. Following Triton extraction, VT1 staining of the left glomerulus is enhanced and staining of the right is unaffected (that is, detergent resistant) but blood vessel staining is lost. The left glomerulus, less well stained with VT1, is strongly stained by VT2. VT2 glomerular staining is largely resistant to detergent extraction but, similar to VT1, VT2 blood vessel staining is lost. VT2 tubular binding is detergent sensitive.

In adult renal sections (Figure 2B), VT1 glomerular staining is significantly less than that seen for pediatric sections (though some pediatric glomeruli are less VT1-reactive than others). Adult tubular VT1 staining is remarkably increased relative to pediatric sections (Figure 2B, a and c). This strong VT1 tubular staining is virtually eliminated in detergent-treated sections (Figure 2B, b and d) but glomerular staining is increased. In serial sections, it can be seen that the same glomerulus initially VT1/2 reactive can become intensely bound by VT1 and VT2 following detergent extraction (Figure 2B, b and d). Some adult samples contained glomeruli preferentially reactive with VT1 (cf. Figure 2C, a vs c). In this case, detergent extraction enhanced VT2 staining (cf. Figure 2C, c vs d). In contrast, tubular VT1 and VT2 staining was entirely detergent sensitive (Figure 2C, a, c vs b, d). In the more common samples in which VT2

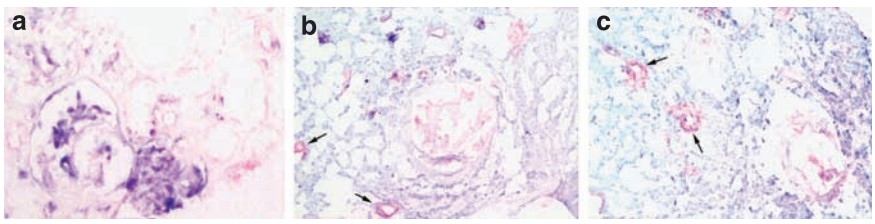

Figure 1 | Comparison of VT1 and VT2 staining of human renal sections. Pediatric renal cortex frozen section double labeled with VT1 (blue) and VT2 (pink). (**a**) Glomerulus stained with both VT1 and VT2; (**b**, **c**) glomeruli and blood vessels (arrows) preferentially VT2 labeled.

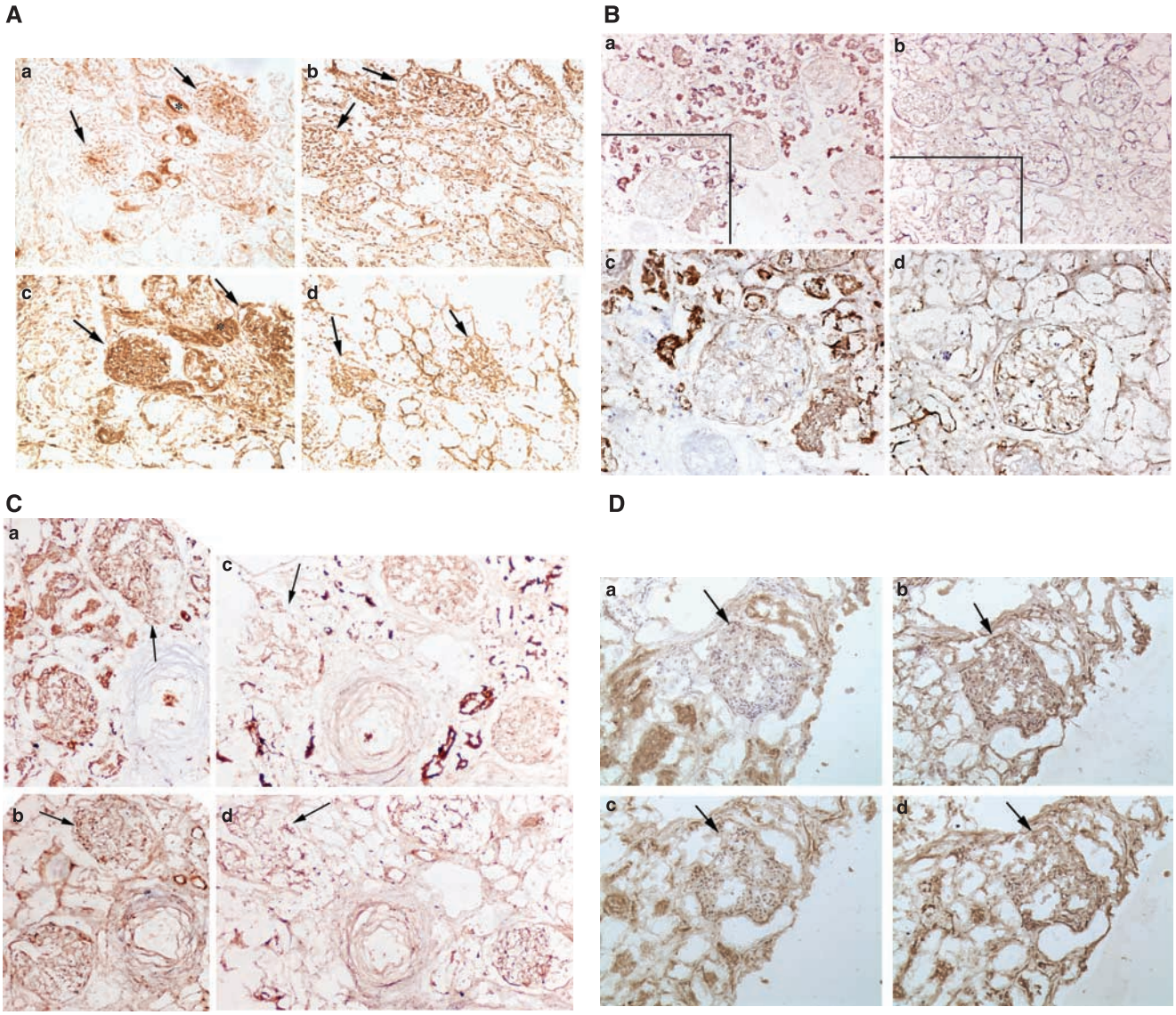

Figure 2 | Effect of tissue section Triton extraction on VT1 and VT2 human renal section staining. (**A**) Pediatric serial sections (**a**) VT1 staining, (**b**) VT1 staining of serial section after detergent extraction, (**c**) serial section VT2 staining, (**d**) serial section VT2 staining after detergent extraction. The same two glomeruli are marked with arrows in each section and a small blood vessel marked with an asterisk in each unextracted section. (**B**) Adult (83 years) frozen renal cortical sections stained with VT1. (**a**, **c**) Serial sections were stained without or (**b**, **c**) with prior detergent extraction. Boxed glomerulus in **a**, **b** is expanded in **c**, **d**. Glomerular staining is enhanced but tubular staining was removed after extraction. Image analysis showed glomerular staining was increased by 260 ± 103% ($n = 9$), while tubular staining was reduced to 23.6 ± 5.6% ($n = 7$) by detergent extraction. (**C**) Renal cortex (60 years). Serial section staining: (**a**, **b**) VT1 or (**c**, **d**) VT2. (**a**, **c**) Untreated or (**b**, **d**) after detergent extraction counterstained with hematoxylin and eosin. Arrows indicate the same glomerulus in each panel. In this case VT1 shows stronger glomerular staining than VT2. Tubular, but not glomerular, staining is lost after detergent extraction. Image analysis showed VT1 glomerular staining was increased by 117 ± 21.9% ($n = 5$) and VT2 staining was increased by 179 ± 49% ($n = 8$), while VT1 tubular staining was reduced to 37.7 ± 11.5% ($n = 5$) and VT2 to 18.2 ± 7.7% ($n = 5$) by detergent extraction. (**D**) Adult (24 years) serial renal sections were stained with (**a**, **b**) VT1 or (**c**, **d**) VT2. (**a**, **c**) Untreated or (**b**, **d**) after detergent extraction. The same glomerulus (arrow) is seen in each panel. This glomerulus is more strongly stained by VT2, but after detergent extraction both VT1 and VT2 staining is markedly enhanced (371.6 ± 210% ($n = 8$), 159.6 ± 52% ($n = 9$), respectively).

glomerular staining was predominant (Figure 2D), serial sections showed that detergent extraction enhanced both VT1 and VT2 glomerular staining.

For all adult sections tested, tubular staining was lost following detergent extraction and glomerular staining was increased (to a variable degree).

We had shown that treatment with β-methylcyclodextrin (β-MCD) to extract cholesterol enhanced VT1 glomerular binding.[43] This was therefore compared with detergent extraction (Figure 3). As expected, MCD extraction had no effect on tubular VT1 staining and detergent extraction eliminated tubular, and enhanced glomerular VT1 staining.

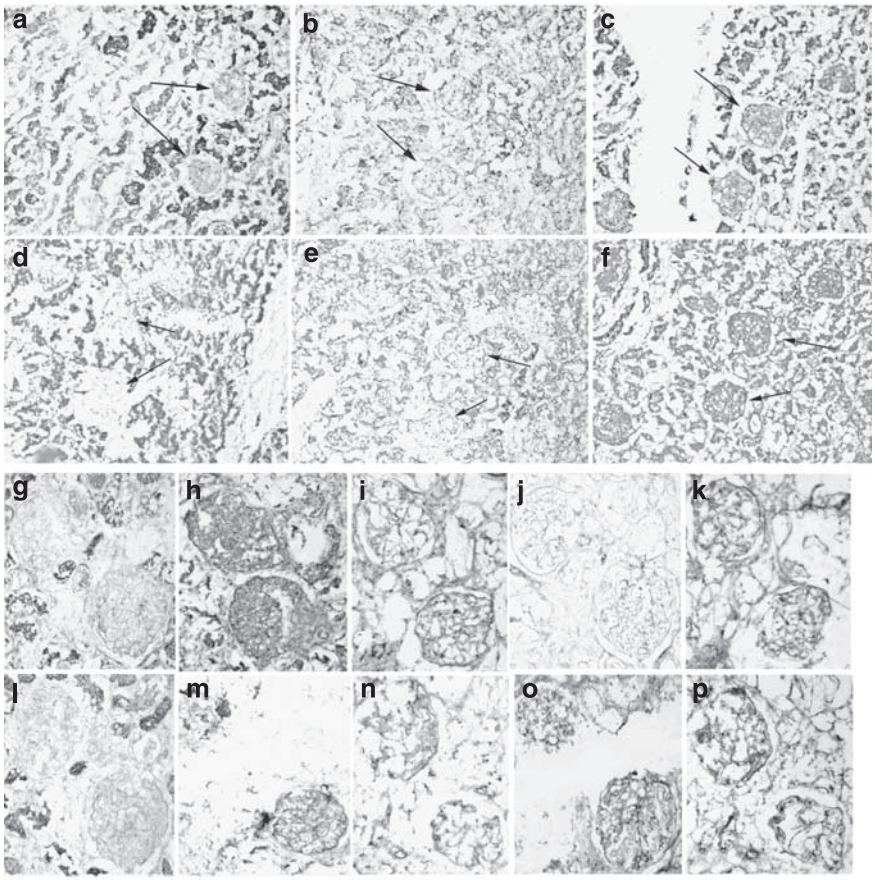

Figure 3 | Comparison of βMCD and Triton extraction on VT1/VT2 adult renal section staining. (**a**–**f** and **g**–**p**) Serial frozen sections (**a**–**c**) VT1 staining, (**a**) untreated, (**b**) detergent extracted, and (**c**) β-MCD extracted. (**d**–**f**) VT2 staining, (**d**) untreated, (**e**) detergent extracted, (**f**) β-MCD extracted. Arrows indicate the same two glomeruli. Renal glomeruli show low VT1 and no VT2 binding. After detergent extraction VT1/VT2 tubular staining is lost and glomerular staining is slightly enhanced. After β-MCD extraction, strong VT1/VT2 staining of all glomeruli is seen but tubular staining is unaffected. Sequential extraction of serial sections: (**g**–**k**) VT1 staining, (**l**–**p**) VT2 staining; (**g**) untreated, (**h**) β-MCD extracted, (**i**) detergent extracted after β-MCD extraction, (**j**) detergent extracted, (**k**) β-MCD extracted after detergent extraction, (**l**) untreated, (**m**) β-MCD extracted, (**n**) detergent extracted after β-MCD extraction, (**o**) detergent extracted, (**p**) β-MCD extracted after detergent extraction. MCD induces strong VT1 and VT2 glomerular binding, which is significantly detergent resistant. Detergent extraction is less effective to induce VT1 than VT2 binding, but MCD is more effective for VT1 even after detergent extraction.

Surprisingly however, MCD extraction increased VT1 glomerular staining more than detergent extraction (cf. Figure 3c vs b and). In sequential extractions of serial sections (Figure 3g–p), MCD-induced glomerular staining was largely detergent resistant. After detergent extraction, MCD induction of VT1, but not VT2, glomerular binding was still evident. Thus, even after detergent extraction, Gb_3-binding sites within the glomerulus can be hidden by cholesterol.

As mouse renal glomeruli do not bind VT1 or VT2,[26,44] we assessed the effect of detergent extract on mouse glomerular binding (Figure 4). The intense VT1 and VT2 tubular binding was largely eliminated by detergent extraction, but the unstained glomeruli (Figure 4 a, c and e) remained completely VT1/VT2 unreactive after detergent extraction (Figure 4b, d and f).

Study of the renal binding of the HIV adhesin gp120, which also binds Gb_3[45] (among other GSLs[46]) served to further emphasize the complex regulation of Gb_3 receptor function within the kidney. Gp120 bound exclusively to the renal tubular epithelium (Figure 5A) and this binding was significantly reduced when the sections were pretreated with VT1 B subunit, suggesting that a large fraction of the gp120 renal binding is Gb_3 mediated. Following detergent extraction, the gp120 tubular binding was lost (as for VT1, VT2). However, unlike for VT1/2 staining, there was no induction of gp120 glomerular binding (Figure 5b). Serial section staining confirmed that while detergent extraction increased VT1 staining of a glomerulus (Figure 2B) gp120 staining of the same glomerulus remained negative. Thus, detergent extraction has a selective effect to 'unmask' VT1 (VT2) glomerular Gb_3 binding.

DISCUSSION

Aglycone regulation of GSL receptor function, both by the lipid moiety of the GSL itself, and by the membrane

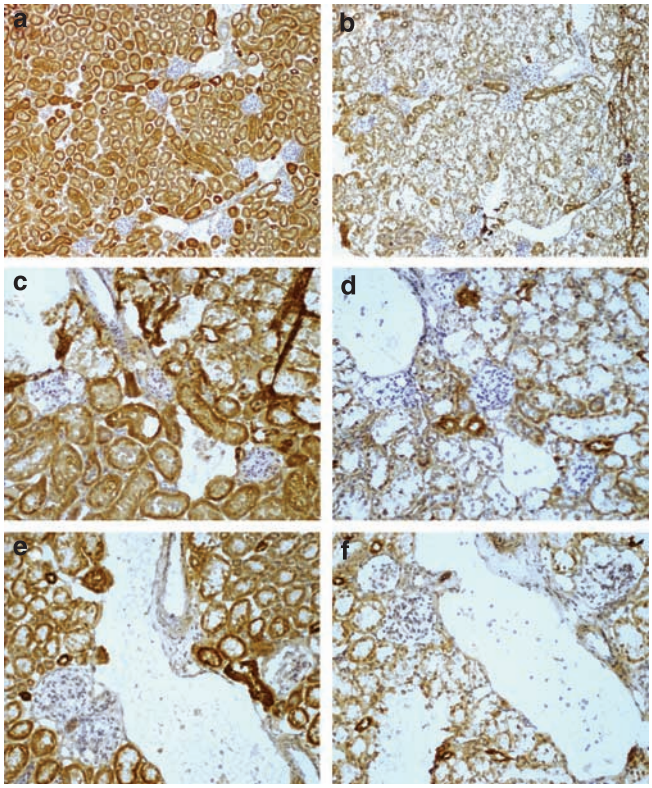

Figure 4 | Effect of tissue section Triton extraction on VT1 and VT2 mouse renal section staining. Serial frozen sections were stained with VT1 (**a–b**, **c–d**) or VT2. (**e–f**) Untreated (**a, c, e**) or after detergent extraction (**b, d, f**). Extensive VT1/2 tubular but not glomerular staining is seen. After extraction the tubular staining is largely lost and glomeruli remain unstained. By image analysis, VT1/VT2 tubular staining was reduced to 18.4 ± 1.4% ($n = 4$)/ 46.7 ± 11.7% ($n = 7$) following detergent extraction.

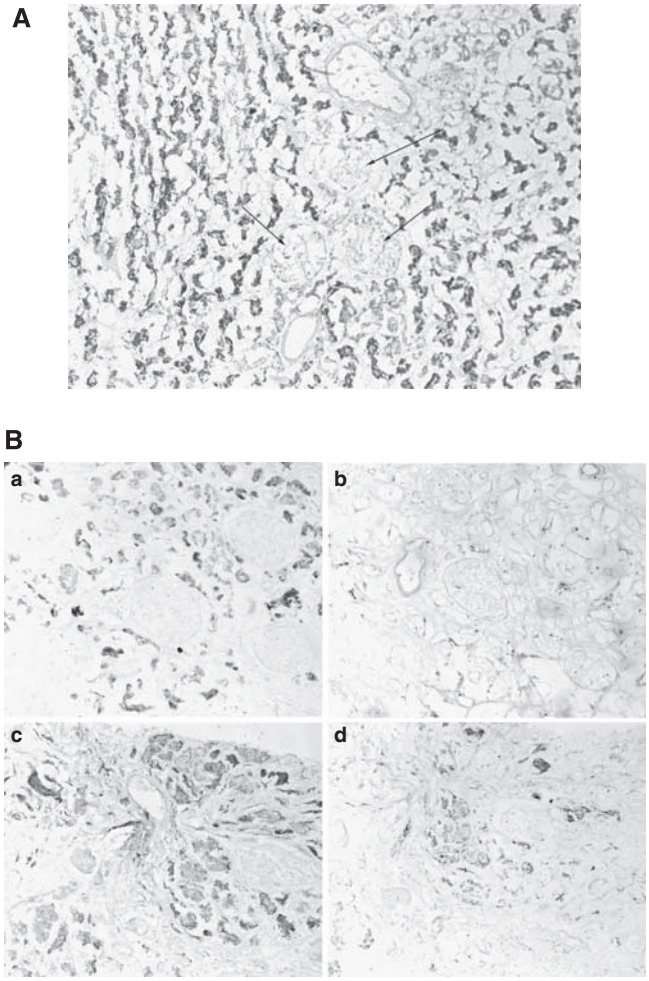

Figure 5 | HIV gp120– human renal tubular staining– effect of Triton extraction and VT1B. (**A**) gp120 staining of adult kidney frozen sections. Extensive tubular but not glomerular (arrows) staining is seen. (**B**) Serial sections (**a**) gp120 immunostaining, (**b**) gp120 immunostaining of serial section after Triton extraction, (**c**) gp120 immunostaining, (**d**) gp120 immunostaining of serial section after prior VT1B blockage. Same sample as in Figure 2B. By image analysis, gp120 tubular staining was reduced to 15.3 ± 11.7% ($n = 6$) following detergent extraction.

environment has been described in many reports.[47] Although Gb_3 is the only functional receptor for VT1 and VT2,[48] VT1 and VT2 can preferentially bind different Gb_3 'lipid assemblies' *in vitro*, and following internalization, undergo differential membrane retrograde trafficking to the Golgi and ER in cultured cells.[40] VT1 and VT2 recognize overlapping epitopes within the Gb_3 carbohydrate sequence,[43] and VT1 (but not VT2) binding to Gb_3 within the renal glomerulus[43] and to Gb_3 vesicle constructs[40] can be inhibited by excess cholesterol.

The relevance of sucrose gradient separated DRMs in normal cellular physiology remains a matter of debate. Such DRMs, although useful to assess changing parameters of cell membrane physiology, represent an amalgum of potentially separate microdomains, which might be originally present in the cell plasma membrane.[10] However, detergent extraction/ resistance of tissue sections potentially represents a more physiologically relevant probe of cellular/tissue DRMs, as the residual antigens are, by definition, detergent resistant and therefore, more representative of their original cellular presentation and location. The use of this approach to investigate differences in membrane domains in tissue sections has not been previously used, but examination of intracellular membranes of cultured cells according to detergent resistance has been reported.[37,40] Detergent extraction of tissue sections *in situ* may provide a new tool to examine the role of lipid rafts in cellular physiology, and perhaps more importantly, in disease. In simple model Gb_3/ cholesterol DRM vesicles, we have found that a significant fraction of the Gb_3 is unavailable for ligand binding.[49,50] This may provide a model of the undetected glomerular Gb_3 that we have found to be exposed following MCD treatment.

Resistance of membrane lipids to mild nonionic detergent extraction is a measure of the relative strength of lateral interaction within the membrane, as opposed to forming mixed micellar complexes with the detergent. The interaction of cholesterol with sphingolipids[51] increases such lateral

interaction to promote resistance to detergent extraction. Thus in glomerular endothelial cells, Gb_3 shows stronger lateral interactions within the membrane, likely with cholesterol.

Gb_3 can be found in cultured cell DRMs together with tyrosine kinases involved in transmembrane signaling.[34,35,52,53] VT1 binding Gb_3 within plasma membrane DRMs results in the intracellular retrograde translocation of the toxin to the Golgi/ER and cell killing, whereas binding to non-DRM Gb_3 results in the intracellular targeting of the toxin to endosomes/lysosomes and degradation of the toxin without cell killing.[37,38] Thus, membrane organization of Gb_3 is an important risk factor for VT cellular intoxication *in vitro*. Our data suggest that it may also be an important risk faction for the pathology of HUS.

In HUS, tubular necrosis is not a primary feature,[54] but rather glomerular occlusion/infarct. Children under 2 years are at greatest risk following gastrointestinal verocytotoxin-producing *E. coli* infection.[55] We had previously shown that pediatric renal glomeruli bind fluorescein isothiocyanate-labeled VT1, whereas no binding to adult glomeruli was seen,[42] implying that receptor distribution could be a primary risk factor for HUS.

This age-related binding was subsequently questioned by VT1 immunohistochemical renal staining showing equal glomerular staining of adult and pediatric renal samples.[56] However, this binding was at 4°C and was lost at 37°C. Binding at 4°C provides a bias toward a membrane lipid rigidity, which might not reflect physiological Gb_3 presentation. We previously confirmed that fluorescein isothiocyanate-labeled VT1 did not bind adult glomeruli[43] and find that Alexa-labeled VT1 is also unreactive with adult glomeruli (not shown). Previously,[43] and in this study, we find that native VT1 does bind to some adult glomeruli. Thus, chemical fluorescent labeling of VT1 alters adult glomerular reactivity.

In our immunohistochemical studies using unmodified toxin ([43] and this study), we find VT2 (and less VT1) binding to adult glomeruli but the staining is less than that for pediatric glomeruli. This supports a role for glomerular Gb_3 binding in the incidence of HUS. In addition, adult glomerular staining is variable, particularly for VT1, suggesting that this could remain a basis for HUS susceptibility. Our finding that detergent extraction can significantly increase adult glomerular VT1 and VT2 binding, while depleting tubular binding indicates that (i) VT binding to non-DRM Gb_3 in renal tubules may explain the relative lack of tubular pathology in HUS and (ii) that the adult glomerular VT1 and VT2 binding sites are significantly protected from toxin binding by component(s) readily susceptible to detergent extraction. Whether this is due to interference by another GSL, such as the blocking by sulfogalactosyl ceramide, we have shown for VT1 binding to model Gb_3 DRM membranes,[57] or a less specific exposure mechanism, remains to be determined. Detergent extraction has a similar effect on VT1 glomerular Gb_3 binding as we have reported for β-MCD,[43] but our studies show that these effects, although overlapping, are distinct in that MCD increased VT1 glomerular staining more effectively than detergent extraction and is effective even after detergent extraction. Cholesterol is largely detergent resistant and glomerular Gb_3 within DRMs must still, in part, be unavailable to bind VT until some cholesterol is removed. Whatever the mechanism, this provides new insight into the age-related incidence of HUS. As the degree of enhanced VT1/VT2 glomerular binding, though always observed, was variable between adult renal specimens, the protection afforded by the non-DRM fraction may also be a variable to consider in the incidence of VT-induced HUS, particularly the elderly.

Human immunodeficiency virus infection has been proposed as an HUS risk factor,[28] and renal tubular Gb_3 is increased in HIV transgenic mice.[29] Focal glomerulosclerosis/tubulointerstitial disease is associated with HIV/AIDS(HIVAN)[58] and gp120 binding to cultured glomerular epithelial, mesangial, and endothelial cells can modulate growth.[59–61] However, such glomerular interactions must be less prevalent or less robust than the tubular epithelial gp120 binding we observe. Gp120 binding can induce inflammatory cytokines,[62] but the relationship of the non-DRM tubular Gb_3/gp120 binding with HIVAN has yet to be established.

The lack of glomerular gp120 binding, as compared with VT1/VT2, emphasizes the selectivity of aglycone modulation of Gb_3 receptor function, and the unique character of VT1/VT2 glomerular Gb_3 binding.

In conclusion, Gb_3 is expressed in human renal tubules to the same, or even greater[63] levels than glomeruli. Strong VT tubular binding *in vitro* is seen, yet VT-induced pathology is restricted to the glomerulus. The marked distinction between renal tubular non-DRM and glomerular DRM VT1/VT2-Gb_3 binding provides a potential basis for this discrepancy, and together with the crucial role we show glomerular cholesterol to play, suggests that the glomerular nephropathy of HUS may be lipid raft-based.

MATERIALS AND METHODS

Antibodies

Monoclonal antibody PH1, specific for the VT1 B subunit, has been previously described.[64] Rabbit anti-VT2e was supplied by C Gyles (University of Guelph). Polyclonal rabbit anti-VT1 B 6869 was prepared in our laboratory. R5 HIV-1 gp120, monoclonal antibody (F425 B4a1) were obtained through the NIH AIDS Research and Reference reagent program, Division of AIDs, NIAID, NIH. Biotin conjugated goat anti-rat IgM serum and biotin conjugated goat anti-rabbit Ig were from Jackson Immunoresearch Laboratories (West Grove, PA, USA). Anti-human IgG peroxidase (raised in goat) was purchased from Sigma, St Louis, MO, USA.

Ligands

VT1 was purified from recombinant *E. coli* strain JB28, as previously described.[65] VT1 B subunit was purified from *E. coli* strain JB120 as described.[66] VT2 was purified from *E. coli* strain R82pJES1-20DH5a, kindly provided by Dr J Samuel (Texas A&M University).[67] HIV-1

Bal gp120 recombinant was obtained through the NIH AIDS Research and Reference reagent program, Division of AIDs, NIAID, NIH.

Tissue preparation

Human renal cortical tissue was harvested 5–10 h postmortem (due to non-renal, non-infectious cause) with University of Toronto Ethics Committee approval. Grossly normal appearing tissue was excised, embedded in Tissue Tek OCT Compound (Sakura Finetek, Torrence, CA, USA), and snap-frozen in liquid nitrogen. Frozen tissue was then cryo sectioned (6 μm), and serial sections were stored at −70°C. Sections to be stained were dried overnight at room temperature and all subsequent steps were performed in a humid chamber. Mouse renal tissue was prepared as above immediately after killing.

Triton extraction on renal tissue sections

For Triton X-100 extraction on renal tissue sections, the tissue sections were kept on ice, washed once with 1% phosphate-buffered saline (PBS), and incubated for 1 min in 1% Triton/PBS buffer. The buffer was then removed. Sections were then immuno stained as stated bellow.

VT staining of frozen kidney sections

Immunoperoxidase VT staining was performed at room temperature, as described[43,68] with some modifications. Nine renal samples were studied, but results are presented primarily for four samples in which sufficient serial cryo sections allowed direct staining comparison. Both Triton treated and untreated sections were blocked for endogenous peroxidase using universal block (KPL) for 30 min, sections were then blocked for Avidin/Biotin blocker (Vector, Burlingame, CA, USA) for 20 min followed by 1% (v/v) normal goat serum (NGS) (Jackson Immunoresearch Laboratories) in 1% PBS for 20 min in room temperature. Sections were then successfully incubated with 1.5 μg/ml VT1 or 10 μg/ml VT2, mouse anti-VT1 (PH1)(1:1000), rabbit anti-VT1B 6869 (1:2000) or rabbit anti-VT2e (H245) (1:500) and biotinylated goat anti-mouse IgG (1:250) or biotinylated goat anti-rabbit IgG (1:500), and Vecstatin ABC elite reagent (Vector Burlingame). Each step consisted of a 30 min incubation followed by extensive washing with PBS. All the toxins and antibodies were diluted in 1% NGS/PBS reagent. VT binding was visualized with diaminobenzidine, DAB substrate (Vector Burlingame). Sections were counterstained with Mayer's hematoxylin, Lillies modification (Dako, Carpintera, CA, USA), washed with water and then dehydrated, cleared and mounted with Permount (Sigma). Control slides were treated identically but not exposed to the toxin.

Gp120 renal section staining

Both Triton treated and untreated human renal sections to be stained with HIV gp120 were blocked with Universal block for endogenous peroxidase as above. Sections were blocked with 1% NGS/PBS for 20 min. Sections were stained with HIV-1 Bal gp120 at 1 μg/ml for 30 min at room temperature, extensively washed then incubated with HIV-1 gp120, monoclonal antibody (F425 B4a1) (1 mg/ml), human IgG (1:2000), and Vecstatin ABC elite reagent as above. Gp120 binding was visualized with DAB substrate and counterstained with hematoxylin as above. To block Gb_3 receptor, the sections were treated with 10 μg/ml VT1B for 30 min and washed before staining with gp120.

Image analysis was performed with Image J,[69] and the effect of detergent extraction on staining was quantitated as a percentage of untreated control ± s.d. for n measurements.

DISCLOSURE

All the authors declared no competing interests.

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