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Evidence that Verotoxins (Shiga-Like Toxins) from *Escherichia coli* Bind to P Blood Group Antigens of Human Erythrocytes In Vitro

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The interaction of verotoxins (VTs) with human erythrocytes (RBCs) in vitro was investigated, with particular reference to the role of P blood group glycolipids that are structurally related to the known VT receptors. RBC binding of purified VT1, VT2, VT2c, and VT2e was detected by direct and indirect immunofluorescence. Glycolipids were extracted from defined RBCs, separated by thin-layer chromatography, and assessed for VT binding in an overlay assay by adding toxin and specific antibodies. All VTs bound to P1 phenotype (Pk, P, and P1 antigens) and P2 phenotype (Pk and P antigens) RBCs but not to p phenotype (lacking the Pk, P, and P1 antigens) RBCs. Binding of VT1 and VT2 was approximately 10-fold greater to P1 and the rare Pk2 (Pk antigen but no P1 or P antigen) phenotype cells than to P2 phenotype RBCs, whereas VT2e bound equally well to P1 and P2 phenotype cells. The VT1 and VT2 immunofluorescence results correlated with the detection of P1 and/or increased amounts of Pk (globotriaosylceramide) antigen; VT2e immunofluorescence correlated with the detection of P (globotetraosylceramide) antigen. The Pk band pattern and VT binding observed in the thin-layer chromatogram of human P1 and P phenotype RBC extracts varied from that of human kidney and Pk1 phenotype (Pk and P1 antigens) RBCs. We conclude that each VT binds to human RBCs in vitro by utilizing specific P blood group glycolipids as receptors. On P1 and P phenotype RBCs, the accessibility of the Pk antigen for VTs appeared to be restricted. The occurrence of VT-RBC binding in natural VT-producing *Escherichia coli* disease and its relevance for the pathophysiology of hemolytic uremic syndrome remain to be established.

Evidence is accumulating that verotoxin (VT)-producing *Escherichia coli* (VTEC) is the causative agent of two syndromes of hitherto unknown etiology, the classical, or enteropathic, hemolytic uremic syndrome (HUS) and hemorrhagic colitis (28, 30, 56). Human VTEC isolates were found to produce VT1 [also termed Shiga-like toxin I; type strain C600 (H19J)], VT2 [Shiga-like toxin II; type strain C600(933W)], or VT2c (Shiga-like toxin IIc; comprising a group of closely related, VT2-like toxins; type strains E32511/HSC, B2F1, and 7279). They may be present alone or in various combinations (2, 20, 24, 34, 44, 50, 60, 61, 64) and bind specifically to globotriaosylceramide (Gb_3) (37, 40), classified as CD77 antigen (35). VT2e (also termed VTE or VT2v) has been identified as the cause of edema disease of weaning pigs (10, 38, 41, 68) but has not been associated with HUS in humans. It binds preferentially to globotetraosylceramide (Gb_4) (8, 59). Classical HUS is characterized by the acute onset of hemolytic anemia, thrombocytopenia, and renal failure approximately 1 week after the onset of diarrhea or hemorrhagic colitis (13, 14, 27). Both the VTEC-associated colitis and the acute nephropathy of HUS are associated with characteristic microangiopathic changes in the gut and kidneys, respectively (54). Several lines of evidence support the concept that the thrombotic microangiopathy is the result of selective endothelial cell damage caused by circulating VT (29, 31, 48, 49, 55, 67).

The basis for the rapid, generally profound, hemolytic anemia and thrombocytopenia in enteropathic HUS is not known. Except in rare cases, the reticulocyte count is increased and direct and indirect Coombs' tests are negative (12). Thrombocytopenia coupled with the observation that fibrin is present in the glomerular capillaries and small vessels of other affected organs in HUS (12, 13, 15, 54) and a related condition, thrombotic thrombocytopenic purpura, has led to the concept of a microangiopathic process in which platelets are consumed during the formation of microthrombi (5, 7) and erythrocytes (RBCs) are fragmented by fibrin strands while being squeezed through affected capillaries (6). However, the expression of P blood group glycolipid antigens which carry the VT-specific Galα1-4Gal residue (Table 1) suggests that VTs might bind to human RBCs and that this interaction might have a role in the subsequent hemolytic process.

The P blood group phenotypes are traditionally specified by agglutination with defined human immune sera; RBCs from 80 to 95% of the normal population are agglutinated by anti-P1 and anti-P antisera (termed P1 phenotype), and most of the remainder are agglutinated by anti-P but not by anti-P1 antibody (termed P2 phenotype) (23). The Pk antigen on RBCs is Gb_3. Like Pk/Gb_3, the P1 glycolipid antigen ($IV^4\alpha$-galactosylneolactotetraosylceramide), too, carries a terminal Galα1-4Gal residue (42, 46) (Table 1) and therefore may also bind VT (37).

Taylor et al. described an association between the strength of the P1 antigen on the RBCs (as judged by the strength of the agglutination of RBCs with P1 antiserum) and the outcome of VT-associated, classical HUS (62). These investigators postulated that strong P1 expression of RBCs results in VT binding in VTEC disease, thereby lowering the concentration of circulating toxin and reducing toxin exposure of vulnerable (endothelial) cells (45). However, it has yet to be shown that VTs, implicated in HUS, indeed bind to human RBCs.

The objective for this study was therefore to investigate the binding of VTs to human RBCs in vitro and to determine

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TABLE 1. Structures of P blood group antigens and related glycolipids mentioned in text[a]

Abbreviation	Trivial name	Glycolipid structure[b]
GlcCer	Glucosylceramide	GlcCer
LacCer	Lactosylceramide	Gal β1-4--------------------------GlcCer
Globoseries		
Gb_3 (Pk)	Pk-antigen, globotriaosylceramide	Galα1-4Gal β1-4--------------------------GlcCer
Gb_4 (P)	P-antigen (globoside), globotetraosylceramide	GalNAcβ1-3 Galα1-4Gal β1-4--------------------------GlcCer
Gb_5	Galactosylglobotetraosylceramide (galactosylgloboside, globopentaosylceramide)	Galβ1-3 GalNAcβ1-3 Galα1-4Gal β1-4--------------------------GlcCer
Forssman	Forssman antigen (IV^3-αN-acetylgalactosaminylglobotetraosylceramide)	GalNAcα1-3 GalNAcβ1-3 Galα1-4Gal β1-4--------------------------GlcCer
Neolactoseries		
nLc_4Cer	Paragloboside (neolactotetraosylceramide)	Galβ1-4GlcNAcβ1-3Galβ1-4GlcCer
nLc_5Cer (P1)	P1-antigen (IV^4α-galactosylneolactotetraosylceramide)	Galα1-4Gal β1-4GlcNAcβ1-3Galβ1-4GlcCer
$NeuAcnLc_4Cer$	Sialosylparagloboside (IV^3-N-acetylneuraminosylneolactotetraosylceramide)	NeuAcα2-3----------- Gal β1-4GlcNAcβ1-3Galβ1-4GlcCer
Ganglioseries		
GM_3	Hematoside (II^3-N-acetylneuraminosyllactosylceramide, sialosylgalactosylglucosylceramide)	NeuAcα2-3--------------------------------------Galβ1-4GlcCer

[a] Trivial names and structures as recommended by the Internation Union of Pure and Applied Chemistry-International Union of Biology Commission on Biochemical Nomenclature (22); abbreviations as described in reference 39.

[b] The position of the Galα1-4Gal sugar sequence is underlined.

whether VT binding correlates with the P phenotype of the RBC.

MATERIALS AND METHODS

RBCs. Random blood group A, B, and O donor RBCs were obtained from the blood bank of The Hospital for Sick Children (donor blood stabilized with citric acid and dextrose). Blood samples of the rare Pk1, Pk2, and p [$Tj^{(a-)}$] phenotypes were gifts from the Canadian Red Cross, Toronto. Fresh P1 antigen-positive ($n = 6$) and P1 antigen-negative RBCs ($n = 3$) as defined by agglutination with anti-P1 immune serum (fully typed donors, all with blood group O), were obtained from the Blood Transfusion Laboratory, General Division, Toronto Hospital, Toronto, Canada. All RBC samples were kept in citric acid-dextrose storage solution at 4°C until use. In addition, commercially available typing cells containing 11 donor samples (Resolve Panel A; Ortho Diagnostic Systems Inc., Raritan, N.J.) were used.

Antibodies. Human anti-P1 antibody was obtained from the Canadian Red Cross and Immucor Inc. (Norcross, Ga.), and human anti-P and anti-$Tj^{(a)}$ (anti-P, -P1, -Pk) antibodies were gifts from the Canadian Red Cross. Mouse monoclonal antibody against the VT1 B subunit (PH1, 1 mg of immunoglobulin G per ml) (IgG) (40) and anti-VT2c antibody from rabbits immunized with purified VT2c toxoid (19) (homologous neutralization titer of 1:4,096) were both prepared in our laboratory. The latter antibody also recognizes VT2 and VT2e (4, 18, 20). Fluorescein isothiocyanate (FITC)-linked goat anti-mouse IgG, affinity-purified $F(ab)_2$ fragments (specific activity, 1 mg/ml) were obtained from Cappel, Organon Teknika Corp. (West Chester, Pa.), and goat anti-rabbit IgG (affinity-purified whole IgG; 1 mg/ml) was obtained from Sigma Chemical Co. (St. Louis, Mo.). Horseradish peroxidase (HRP)-conjugated goat anti-mouse and goat anti-rabbit IgG (absorbed with human serum proteins) were obtained from Bio-Rad (Richmond, Calif.); HRP-conjugated goat anti-human IgG and goat anti-human IgM (affinity-isolated whole IgG) were obtained from Tago Inc. (Burlingame, Calif.).

VTs. VT1 was purified from *E. coli* JB28 (21); VT2 was purified from strain DH5α(pJES120), a kind gift from J. E. Samuel; VT2c was purified from strain E32511/HSC (19, 20); and VT2e was purified from strain GT100, a kind gift from G. Tyrrell (65). All VTs were purified in our laboratory by sequential chromatography as described previously (9, 19, 52, 65). The VT1 B subunit was purified from strain TB1 (pJLB120) (a kind gift from J. Brunton, Toronto Hospital) as a stable pentamer (53).

FITC labeling of VTs. For some experiments, VT1 was directly labeled with FITC as described previously (32). Briefly, VT (1 to 10 mg/ml) was incubated with the same amount of FITC (isomer I; Sigma) dissolved in 0.5 M carbonate buffer (pH 9.5) (26) for 2 h at room temperature. Unbound FITC was removed by filter centrifugation (Centricon 30; Amicon, Beverly, Mass.). The FITC-labeled VT was fractionated and stored at −20°C until use; the labeling procedure did not lead to a measurable loss of verocytotoxic activity of the toxin (32).

Purification of GSLs. The glycosphingolipids (GSLs) glucosylceramide (GlcCer), latosylceramide (LacCer), Gb_3, Gb_4, and galactosylglobotetraosylceramide were purified from human kidneys as described previously (3); Forssman antigen was extracted from sheep RBCs (a gift from C. A. Lingwood, Department of Microbiology, The Hospital for Sick Children).

Indirect immunofluorescence assay. RBCs were washed with cold phosphate-buffered saline (PBS) prior to each experiment; the leukocyte-containing buffy coat layer was carefully avoided. Purified VT or VT1 B subunit (0.1 ml), at a concentration of 1 μg/ml unless stated otherwise, was mixed with 0.05 ml of 3% RBCs and incubated for 30 min by using the plastic device (tube) of the SimWash serum-cell separation system (Ortho Diagnostics Systems). Unbound toxin was separated from the RBCs by centrifugation for 2 min at 1,000 × *g* by using a solution based on dextran, bovine serum albumin, and EDTA (SimWash separating solution). Prior experiments had revealed that the gradient centrifugation technique described above yielded brighter fluorescence than did repeated washing and centrifugation in PBS; therefore, this technique was used throughout further experimentation unless stated otherwise.

Washed RBCs were incubated for 60 min with 0.1 ml of PH1 monoclonal antibody or rabbit anti-VT2c antibody (both diluted 1:500). After separation of unbound antibody, the RBCs were incubated with the appropriate FITC-conjugated goat anti-species-IgG antibody. Unbound reagent was removed by additional gradient centrifugation. The pellet was resuspended, fixed with 1% formalin (5 min at room temperature), washed once in PBS, mixed with 1 or 2 drops of glycerol-paraphenylenediamine (pH 8.5) as antifading agent (25), mounted on glass slides, and covered with a coverslip. The fixation step was later simplified by adding glycerol-paraphenylenediamine freshly mixed with 1% formalin (final concentration) without further washing.

All dilutions were made in ice-cold 0.01 M PBS (pH 7.4), and incubations were carried out on ice or at 4°C unless stated otherwise. Rabbit anti-VT2c was heated for 30 min at 56°C to inactivate the complement and absorbed with appropriate test RBCs (1 volume of packed RBCs per volume of rabbit immune serum diluted 1:10) to prevent unspecific binding of the rabbit antibody. Cells were agitated repeatedly during all incubations and thoroughly mixed and resuspended after each washing step. Control incubations were done in each experiment and for each RBC sample under identical conditions, except that the toxin and the primary or secondary antibody were omitted or the primary antibody was replaced by an irrelevant antibody (mouse monoclonal anti-influenza virus antibody or rabbit preimmune serum).

The cell preparations were examined and photographed under incident UV illumination, using a fluorescence microscope (Polyvar; Reichert-Jung, Vienna, Austria). Assay results were recorded blindly from numbered slides, on the basis of (i) the intensity of immunofluorescence, graded as +++ (strongly positive), ++ (moderate to strongly positive), + (weak but clearly positive), or (+) (faint); (ii) the percentage of fluorescent RBCs compared with the number of cells visible under light microscopy; and (iii) the size and number of cell aggregates per microscopic field. Photographs of representative samples were taken by using 36/135, 400 ASA film (Kodak 400 HC).

Direct immunofluorescence. A 2% RBC suspension (0.05 ml) was incubated with FITC-labeled VT1, centrifuged with the dextran gradient solution, and mounted as described above.

Extraction of neutral GSLs from RBCs. Neutral GSLs were extracted from RBCs with 2-propanol–chloroform (57). Briefly, after removal of the buffy coat layer, 0.5 to 2 ml of washed, packed RBCs was suspended in PBS to give a final volume of 3 ml. One volume of RBC suspension was gently mixed with 11 volumes of 2-propanol while being sonicated in a water bath and subjected to extraction for 1 h, followed by the addition of 7 volumes of chloroform and extraction overnight. The filtered extract (Whatman 1 filter; Schleicher & Schuell) was dried by rotor vaporation, redissolved in chloroform-methanol (C/M, 2:1 [vol/vol]), and repeatedly partitioned against water (0.6 vol). The combined lower phases were dried, weighed, resuspended in C/M (2:1), and stored at −70°C. For some experiments, the neutral GSLs were isolated with the aid of a silica column (Rose Scientific Ltd., Edmonton, Alberta, Canada), essentially as described by Ullman and McCluer (66): The acetone-methanol (9:1 [vol/vol]) fraction containing the neutral GSLs was dried, weighed, and redissolved in (C/M, 2:1).

TLC and VT overlay procedure. Appropriate amounts of RBC extracts along with standard GSLs were separated by thin-layer chromatography (TLC) in C/M-water (c/m/w) (60:35:8 or 60:40:9 [vol/vol/vol]), with plastic-backed Polygram silica gel G plates (Machery-Nagel, Düren, Germany) as described previously (40). GSL bands were visualized by orcinol spray; sialylated GSLs were identified by using resorcinol on replicate plates.

The binding of VTs was assayed as described previously (16, 40). Briefly, replicate TLC plates were blocked with 1% gelatin in distilled water at 37°C overnight, thoroughly washed, and incubated for 1 h with VT1 (0.1 μg/ml), VT2 (0.5 μg/ml), VT2c (1 μg/ml), or VT2e (0.5 μg/ml). Bound toxin was detected with either the anti-VT1 monoclonal antibody or rabbit anti-VT2c antibody (both diluted 1/1,000), HRP-conjugated anti-mouse or anti-rabbit antibodies (diluted 1/2,000), and 4-chloro-1-naphthol with peroxidase as the substrate. Color development was stopped by extensive rinsing with distilled water. All dilutions, incubations, and washings were done with 0.05 or 0.1 M (for the monoclonal antibody or rabbit anti-VT antibody, respectively) Tris-buffered saline (pH 7.4) at room temperature.

For immunodetection of the P1 glycolipid, TLC plates with the separated RBC extracts were incubated overnight with human anti-P1 antibody (diluted 1/4), followed by incubation with a mixture of HRP-conjugated goat anti-human IgG and IgM antibodies (1/750, final dilutions).

α-Galactosidase treatment of RBCs. Washed RBC were reacted with α-D-galactoside galactohydrolase (EC 3.2.1.22; Sigma) from green coffee beans, an exoglycosidase that cleaves nonreducing terminal galactose residues linked α1-3 and α1-4. Packed RBCs (0.1 ml) suspended in 1 ml (final volume) of 0.14 M citric acid-Na_2HPO_4 (McIlvaine) buffer (pH 6.4) supplemented with 0.005 M EDTA (CP-EDTA) were incubated with 1.25 U of α-galactosidase for 24 h at 37°C with shaking. Control incubations were carried out with β-D-glucoside glucohydrolase (EC 3.2.1.21; Sigma) or with CP-EDTA buffer alone. RBCs were washed twice with PBS and used immediately or stored in citric acid-dextrose.

Verocytotoxicity assay. Native and FITC-labeled VTs were tested for cytotoxicity on Vero cell monolayers as described previously (30). The cytotoxin titer was defined as the reciprocal of the toxin dilution causing death of 50% of the cells of the monolayer.

RESULTS

Detection of VT binding by immunofluorescence. Initially, human A, B, and O blood group RBCs were examined for the binding of VT. VT binding was defined as detection of immunofluorescence on RBCs preincubated with VT; with the omission of toxin or primary antibody or the use of an irrelevant primary antibody there was no fluorescence. VT binding was demonstrated with the direct assay using FITC-labeled VT1 and with the indirect approach with native VT1 and specific primary and secondary antibodies. The degree of fluorescence varied considerably between RBCs from different donors. Staining differences did not correlate with the ABO blood group phenotype.

VT2, VT2c, and VT2e were also found to bind to these RBCs. As with VT1, binding of VT2 and VT2c was variable, whereas VT2e-binding assays yielded consistently strong immunofluorescence among RBCs from different donors.

With the direct and indirect techniques, the fluorescence outlined the RBC surface entirely with a homogeneous rim. Higher magnification revealed a speckled pattern, possibly indicating the presence of cell surface areas with higher and lower binding capacities (Fig. 1). By focusing carefully through individual RBCs, no intracellular fluorescence was detected, either with the direct or indirect immunofluorescence tech-

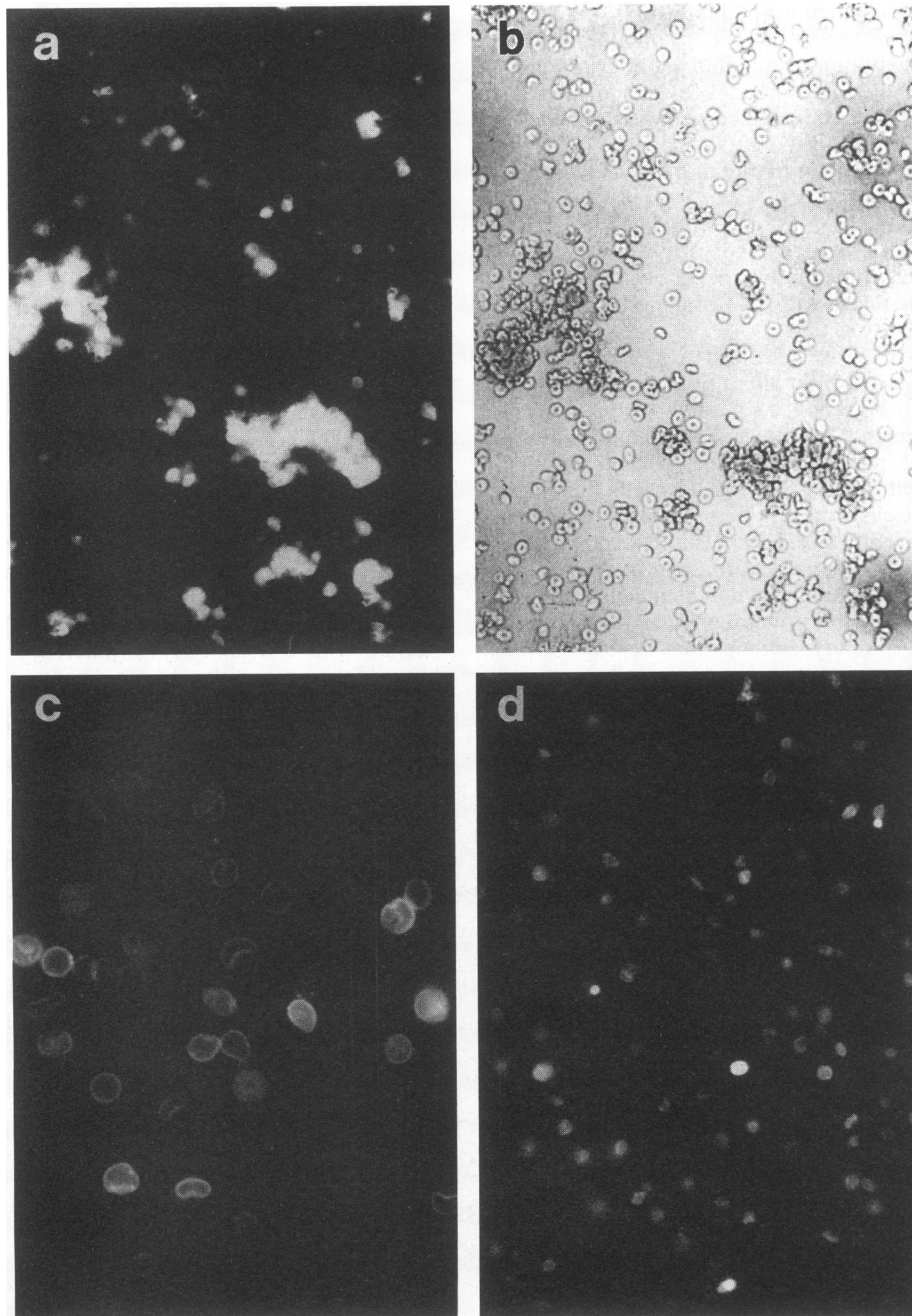

FIG. 1. VT binding to human RBCs detected by immunofluorescence. A 3% suspension of P1 phenotype RBCs (0.05 ml) was incubated with the indicated amount of verotoxin diluted in 0.1 ml. (a) VT1 (100 ng), indirect immunofluorescence; (b) same preparation and microscopic field as described for panel a (light microscopy); (c) FITC-labeled VT1 (1 μg), direct immunofluorescence; (d) VT1 B subunit (400 ng), indirect immunofluorescence. Approximate magnifications, ×250 (a and b); ×1,000 (c); ×400 (d).

niques or after incubation at 37°C with directly labeled VT1. The intensity of the fluorescence varied among individual RBCs in the same microscopic field. Cells with stronger or weaker immunofluorescence did not appear morphologically different, as judged by comparative light microscopy. Incubation with higher toxin concentrations resulted in the formation of increasingly larger RBC aggregates (Fig. 1a and b), which were occasionally noted macroscopically following incubation with the secondary antibody. The presence of aggregates correlated with the intensity of overall immunofluorescence. Aggregates were not observed by using directly labeled VT or in control incubations without toxin or primary or secondary antibody. The binding pattern of the purified VT1 B subunit resembled that of the VT1 holotoxin (Fig. 1d). When VTs were preincubated with anti-VT antibody prior to the addition of RBCs to the assay, no fluorescent signal was obtained, indicating that toxin binding was completely inhibited.

Correlation of VT binding with P blood group antigens. In order to examine the influence of the presence of various P blood group antigens on VT binding, two panels of fully typed

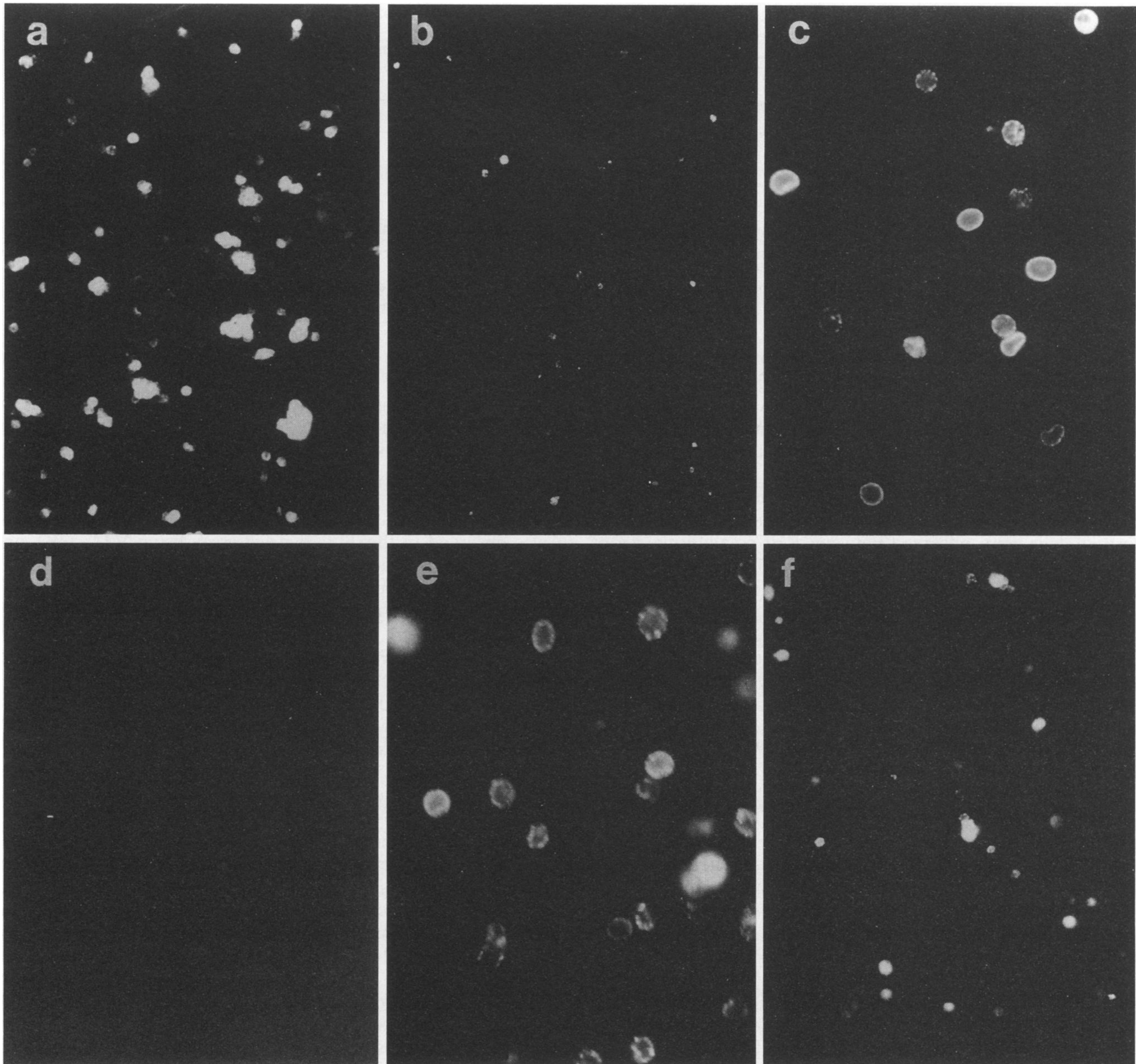

FIG. 2. VT binding to various P blood group phenotype RBCs detected by indirect immunofluorescence, except panel c (direct immunofluorescence). (a) P1 phenotype RBCs with VT2 (30 ng); (b) P2 phenotype RBCs with VT2 (300 ng); (c) Pk2 phenotype RBCs with VT1-FITC (1 μg); (d) P1 phenotype RBCs (control incubation without VT but with primary rabbit anti-VT2c and FITC-conjugated secondary antibody); (e) P2 phenotype RBCs with VT2e (400 ng); (f) P1 phenotype RBCs with VT2c (400 ng). Approximate magnifications, ×250 (a, b, d, and f); ×1,000 (c and e).

RBCs were studied, including 13 P1 antigen-positive and 7 P1 antigen-negative RBC donor samples. The P1 antigen-negative cells were found to be the P2 phenotype, as shown by agglutination with anti-P antibody and the presence of Gb_3 and Gb_4 by TLC (see below). In addition, small amounts of p (from two donors) and Pk1 and Pk2 (one sample each) phenotype cells were available. At a concentration of 3 μg/ml (300 ng per assay tube), VT1, VT2, VT2c, and VT2e consistently bound not only to the P1 but also to the P2 phenotype panel RBCs (Fig. 2). The weakest binding was observed with VT2c. Both Pk phenotypes displayed strong VT1 binding (Fig. 2) but reduced VT2e binding compared with the binding of P1 and P2 phenotype RBCs (Table 2). In contrast, none of the VTs bound to p phenotype RBCs. Thus, the lack of P1, Pk, and P antigens was associated with a lack of VT binding (Table 2).

To determine the effect of the strength of the P1 antigen expression on VT binding, P1 phenotype RBCs (test cells) exhibiting strong (n = 2), moderate (n = 3), or weak (n = 1) agglutination with the anti-P1 antibody and P2 phenotype (P1 agglutination-negative) test RBCs (n = 3) were incubated with limiting dilutions of VT1, VT2, and VT2e (100, 30, and 10 ng/2.5×10^7 RBCs). At a concentration of 1 μg of VT1 or VT2 per ml (100 ng), RBCs of all six P1 phenotypes examined exhibited moderate-to-strong immunofluorescence of almost

TABLE 2. Correlation between P blood group phenotype and VT binding to human RBCs

P phenotype	P blood group antigen(s) on RBCs[a]	VT binding[b]					
		Direct IF (VT1-FITC)	Indirect IF				
			VT1	VT2	VT2c	VT2e	VT1 B subunit
P1	Pk, **P**, P1	+	++	++	+	++	++
P2	Pk, **P**	(+)	+	+	(+)	++	+
Pk1	**Pk**, P1	ND	++	ND	(+)	+	ND
Pk2	**Pk**	+++	++	ND	ND	(+)	ND
p	None	ND	−	−	−	−	ND

[a] Quantitatively predominant antigens printed in boldface.
[b] Similar amounts of VT (300 to 500 ng/0.1 ml) were incubated with approximately 2.5×10^7 RBCs as described in Materials and Methods. The strength of the immunofluorescence (IF) was graded as very strong, +++; moderate to strong, ++; weak but clearly positive, +; faint, (+); or negative, −. ND, not done.

100% of the cells per average microscopic field (Fig. 1a and b), compared with 10 to 15% of the cells of the three P2 phenotypes. At a 10-fold-lower toxin concentration of 0.1 μg/ml (10 ng), none of the P2 phenotype cells but more than 10% of the P1 phenotype cells still exhibited immunofluorescence of at least 1+ (Fig. 3). No differences in the intensity of the fluorescence and the percentage of immunofluorescence-positive cells were observed between RBCs exhibiting strong or weak agglutination with the anti-P1 antibody. VT1 binding to Pk1 and Pk2 phenotype RBCs was at least as strong as the binding to P1 phenotype cells (Table 2).

Similar results were obtained with directly labeled VT1. Compared with P1 phenotype RBCs, detection of VT1-FITC binding to P2 phenotype RBCs was greatly reduced, as determined by the percentage of positive cells and the brightness of fluorescence. On the other hand, Pk phenotype RBCs exhibited brighter immunofluorescence than P1 phenotype cells with VT1-FITC (Table 2; Fig. 2c).

P1 and P2 phenotype RBCs were equally capable of VT2e binding. At a concentration of 0.1 μg of VT2e per ml (10 ng), applying the same primary (anti-VT2c) and secondary antibodies as those used for the VT2 assay, both phenotypes still exhibited 1+ or greater immunofluorescence on almost all RBCs (Fig. 3). Pk2 phenotype RBCs, however, showed little VT2e binding (Table 2).

Blocking experiments. Preincubation of P1 phenotype RBCs with VT1 or VT1 B subunit partially reduced but did not completely block their agglutination by anti-P1 antiserum. The agglutination of P1 phenotype RBCs by anti-P1 immune serum was unaffected after preincubation with VT2e.

Preincubation with excess VT1 B subunit, at a ratio of 10:1 (i.e., 10 versus 1 μg of protein per ml) greatly reduced the binding of VT2, but not of VT2e, to P1 and P2 phenotype RBCs. Detection of VT1 binding by direct and indirect immunofluorescence was not significantly diminished after preincubation of P1 phenotype RBCs with human P1 immune serum. This indicates that the antibody and VT1 recognize different epitopes of the receptor molecule or that the VT binds with higher affinity to the receptor(s) than to the antibody.

Identification of P blood group glycolipids. The distinctive VT binding to different P blood group phenotypes was attributed to the differences in the composition of surface glycolipids. GSLs were therefore extracted from various RBCs and separated by TLC. As expected, the major GSLs of P1 and P2 phenotype RBCs comigrated with Gb_4 purified from human kidneys, while additional bands corresponded to GlcCer, LacCer, and Gb_3 (Fig. 4). The lipid extract from Pk1 phenotype RBCs lacked Gb_4, and p phenotype RBCs lacked both Gb_3 and Gb_4. (Pk2 cells were not available for extraction.)

VT binding to glycolipid extracts from RBCs. VT overlay studies of TLCs of RBC extracts were performed to determine if the VT-binding pattern observed with intact RBCs by immunofluorescence assays correlated with the binding to specific glycolipids. All VTs exhibited binding to Gb_3 (Pk antigen) from P1 and P2 phenotype RBCs, but the strongest reaction occurred with Gb_3 from Pk1 phenotype cells. Only VT2e bound strongly to Gb_4 (P antigen). An additional band was observed in the TLC overlay assay using VT1, VT2, and VT2c, which migrated with or slightly below Gb_4 (Fig. 4 and 5). The VTs did not recognize any of the lipid compounds from p phenotype RBCs (Fig. 4 and 5). Interestingly, low levels of Gb_4, which were not observed after orcinol staining, were detected in the Pk1 phenotype RBC extract by the VT2e overlay assay.

Using VT1 and VT2e, the VT overlay assay revealed a distinct band below Gb_4 in extracts from P1 but not from P2 phenotype RBCs, which migrated below the Forssman antigen control. This band was not visible after orcinol staining but was recognized by the human anti-P1 immune serum and is believed to be the P1 glycolipid (Fig. 6).

Effect of α-galactosidase treatment of RBCs. In order to provide further evidence that the VT-RBC interaction is specific, i.e., mediated by glycolipids with terminal Galα1-4Gal residues, P1 phenotype RBCs were incubated with α-D-galactosidase (which specifically cleaves the terminal galactose residues linked α1-3 and α1-4) prior to the VT-binding assay using VT1-FITC. As a control, P1 phenotype cells were incubated with β-D-glucosidase (which is not specific for terminal α1-4Gal residues). After 24 h of incubation with the respective enzymes, we observed consistently that the fluorescence of the β-glucosidase-incubated RBCs was slightly diminished, while α-galactosidase-treated RBCs showed almost no fluorescence. This is consistent with the loss of VT binding due to digestion of the specific toxin receptor.

DISCUSSION

Using immunofluorescence-labeling techniques, this study clearly demonstrates that VTs bind to human RBCs in vitro and that this binding correlates with the expression of P blood group antigens. The evidence for this is based on the observation that p phenotype RBCs, which lack the Pk (Gb_3), P (Gb_4, globoside) and P1 (nLc_5Cer) antigens, fail to bind VT1, VT2, VT2c, or VT2e. P1 phenotype RBCs revealed at least 10-fold-stronger binding of VT1 and VT2 than did P2 phenotype RBCs which do not express the P1 antigen. Evidence suggesting that the VT-binding specificity on human RBCs was to terminal Galα1-4Gal residues is (i) that the binding was reduced by digestion with α-galactosidase, which is specific for galactosyl residues linked α1-4, and (ii) that the VT1 B

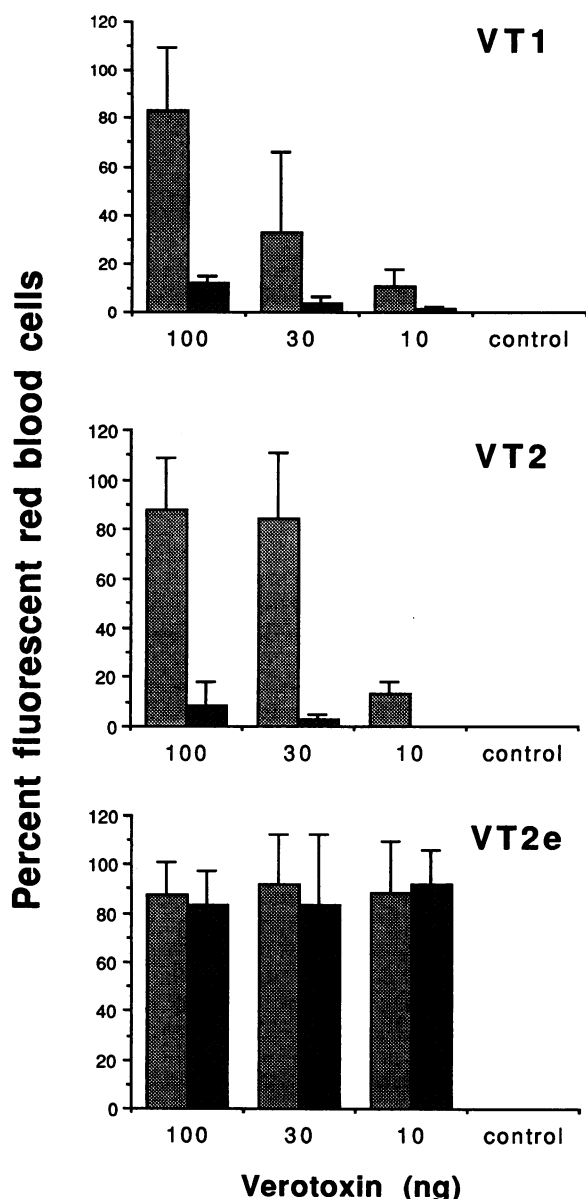

FIG. 3. Comparison of VT binding by P1 (▨) and P2 (▩) phenotype RBCs. Approximately 2.5×10^7 RBCs were incubated with the indicated amount of purified VT (or PBS, as controls) and then with the primary and secondary antibody for detection of bound VT (indirect immunofluorescence) as described in Materials and Methods. The percentage of RBCs (error bars, 1 standard deviation) showing positive (at least 1+) fluorescence, as estimated by comparison with the number of cells visible under light microscopy, is shown.

subunit, which binds specifically to the Galα1-4Gal residue, competitively inhibited VT2 holotoxin binding to RBCs.

Both P1 and P2 phenotype RBCs contain the VT receptor Gb_3 (Pk antigen), although slight differences in the Gb_3 content have been reported (11). The limited binding of VT1, VT2, and VT2c to P2 phenotype cells therefore indicates that the access to the Pk antigen on these cells is restricted. In contrast, no difference was found in the binding of VT2e to P1 and P2 phenotype cells. This is in agreement with the conclusion derived by Lampio, on the basis of galactose oxidase studies, that the Pk/Gb_3 antigen is less accessible than Gb_4 (36). Our findings support the previous hypothesis that the P1 glycolipid, which contains a terminal Galα1-4Gal residue, enhances the binding of VTs to P1 phenotype RBCs (58, 62). Furthermore, the P1 antigen appears to be more favorably exposed to VT than is the Pk antigen on the membranes of P2 and, possibly, P1 phenotype RBCs. Pk2 phenotype RBCs, however, which express only the Gb_3/Pk antigen but lack the P1 antigen, bound VT1 at least as strongly as did P1 antigen-positive RBCs. Furthermore, Pk2 phenotype RBCs exhibited markedly increased binding of directly labeled VT1, compared with P1 phenotype RBCs (Table 2). The binding of VT1 to Pk phenotype cells may be enhanced simply because of the greater content of the Pk/Gb_3 antigen, i.e., because of greater receptor density. Alternatively, the presence of the P antigen on P1 and P2 phenotype cells may sterically hinder the access of the toxin to the Pk antigen on these RBCs. Further, the observation that VT1-FITC binds to P1 and P2 phenotype cells less effectively than to Pk2 phenotype cells raises the possibility that the attached label interferes directly with the binding to the former RBC phenotypes. The weaker binding of VT2c to intact RBCs, compared with that of VT1 and VT2 at the same protein concentration, did not appear to be due to a lack of (homologous) antibody specificity but rather to different binding affinities of the VT2c B subunit (17, 33).

P1 and P2 phenotype RBCs exhibited strong VT2e binding. This appeared to be mediated predominantly by the P antigen; Pk2 phenotype cells expressing only trace amounts of Gb_4 (43) exhibited only weak VT2e binding. This observation mirrors the differential susceptibility of epithelial cell lines to various VTs. For example, the cytotoxic dose of VT2e for Vero cells, which contain Gb_4 in addition to Gb_3, is several orders of magnitude lower than that for HeLa cells which contain only Gb_3 (59).

P1 phenotype RBCs contain only minute amounts of P1 glycolipid (46, 69). Ando et al. (1) and Yamakawa (69) reported a per-weight ratio of extractable Gb_3 to P1 glycolipids in normal human RBCs of 100 to 1. This explains why P1 was not detected on the orcinol-stained TLC of RBC extracts but was detectable only by the overlay technique with VTs or P1 immune serum (Fig. 6). The low P1 antigen content is in marked contrast to its availability for the agglutinating (polyclonal) anti-P1 antibody and its apparent recognition on intact cells by VT. The P1 glycolipid may therefore represent a novel, potent, VT receptor. Our observation that VT2e also recognized solid-phase bound P1 antigen indicates that the P1 glycolipid may function as an adjunct VT2e receptor on P1 and Pk1 phenotype cells.

Unexpectedly, preincubation of P1 phenotype RBCs with human anti-P1 immune serum did not block VT1 binding. However, RBC agglutinates formed by human anti-P1 immune serum are very unstable. The antibody may have lower affinity for the P1 antigen than does the competing VT. Alternatively, VT1 and the antibody may recognize different receptor epitopes. This would be consistent with the results of the reverse experiment, which revealed partial blocking of P1 agglutination following incubation with VT1 or VT1 B subunit. Furthermore, the strength of the agglutination of P1 phenotype RBCs with the anti-P1 immune serum did not appear to correlate with the binding of VT1 or VT2, as judged by the percentage of immunofluorescence-positive cells in the preparation or the brightness of the fluorescent signal.

Gb_3 purified from human kidney or Vero cells is resolved by TLC into two species (doublet), and both bands of the doublet are recognized in the VT overlay assay (3, 51, 59). In contrast, Gb_3 from normal RBCs, in our hands, typically migrated as a single dominant (upper) band, with or without a much weaker

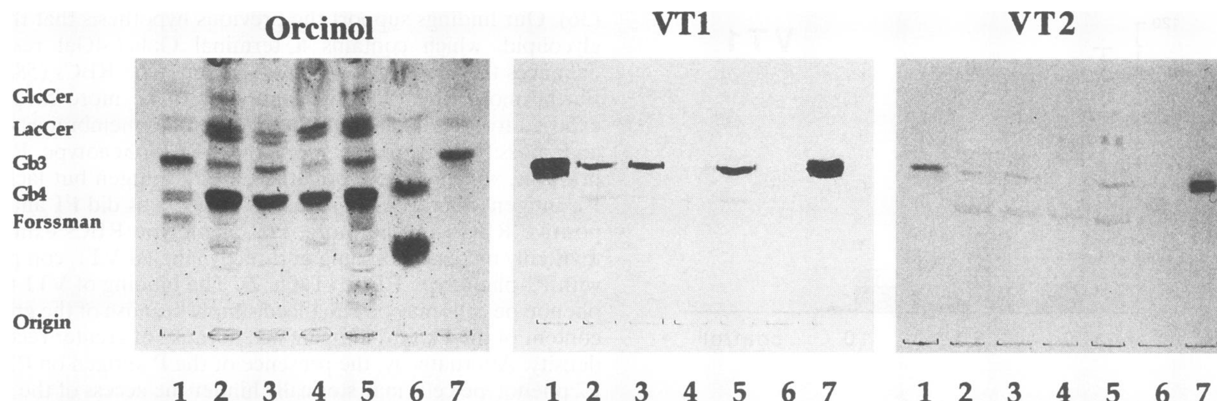

FIG. 4. Binding of VT to human RBC glycolipids. Extracts from the equivalent of approximately 0.03 ml of RBC pellet (120 μg per lane) were separated by TLC in C/M/W (60:35:8 [vol/vol/vol]). Replicate TLC plates were stained with orcinol or overlaid with VT as indicated. Lanes: 1, purified glycolipid standards (GlcCer, 3 μg; LacCer, 1 μg; Gb_3, 1 μg; Gb_4, 0.5 μg; and Forssman antigen, 3 μg); 2 and 3, extracts from P1 phenotype RBCs; 4 and 5, extracts from P2 phenotype RBCs; 6, extract from p phenotype RBCs (the major upper band probably represents GM_3, and the lower band probably represents sialosylparagloboside); 7, extract from Pk1 phenotype RBCs.

lower band. Only the upper band appeared to be recognized in the VT overlay assay. Interestingly, in extracts from Pk1 phenotype RBCs, which are deficient in P/Gb_4 glycolipids, Pk/Gb_3 presented as a doublet, showing strong binding of all VTs in the TLC overlay assay to the upper and lower bands (Fig. 4 and 5). As previously shown by Pellizzari et al., the resolution of Gb_3 into distinct bands correlates with their relative abundance of longer (upper band) and shorter chain fatty acids (lower band) (51). Variations in the lipid portion of Gb_3 are believed to alter the three-dimensional orientation of the carbohydrate moiety for ligand binding (39) and to affect the binding capacity and affinity of VTs (33, 51). Differences in the Gb_3 pattern of RBCs observed by TLC may therefore influence the binding of VT1 and VT2 to the Pk antigen on intact RBCs.

In extracts from P1 and P2 phenotype RBCs, after separation under more-polar conditions, an additional band, which migrated with or slightly below Gb_4, was occasionally observed in the TLC overlay assays (Fig. 4 and 5). In contrast to Gb_4, this band was recognized by VT1, VT2, and VT2c. Its molecular structure, role as potential VT receptor, and degree of exposure on the RBC membrane are unknown.

The binding of VT to RBCs in vivo and its possible clinical significance remain to be established. Taylor et al. reported that the outcome of classical HUS was associated with the RBC P1 antigen expression detected by the agglutination reaction with P1 antiserum (62). It was proposed that in patients with HUS, P1-positive RBCs absorb circulating VT and thus protect VT-sensitive (nucleated) target cells which are susceptible to protein synthesis inhibition (62) and, consequently, that transfusion of P1 phenotype RBCs may be of therapeutic value (45). Newburg et al., without determining the P blood group phenotype of their samples, recently reported that the ratio of Gb_3 to its precursor glycolipid, LacCer, was decreased in RBCs from patients with enteropathic HUS, compared with RBCs from children with VTEC-associated diarrhea who did not develop HUS, and they concluded that the P blood group status is related to the susceptibility to systemic VT disease (47). (The Gb_3/LacCer ratio is up to severalfold higher in P1 than in P2 phenotype RBCs [11]). The presence of P blood group antigens on RBCs may reflect the glycolipid expression by other tissues, such as the intestinal or renal epithelium, with potentially diverse consequences. Boyd et al. (4) have recently shown, that RBC binding of ^{125}I-labeled VT2e, intravenously injected into pigs, indeed resulted in prolonged toxin circulation. However, RBC binding of the

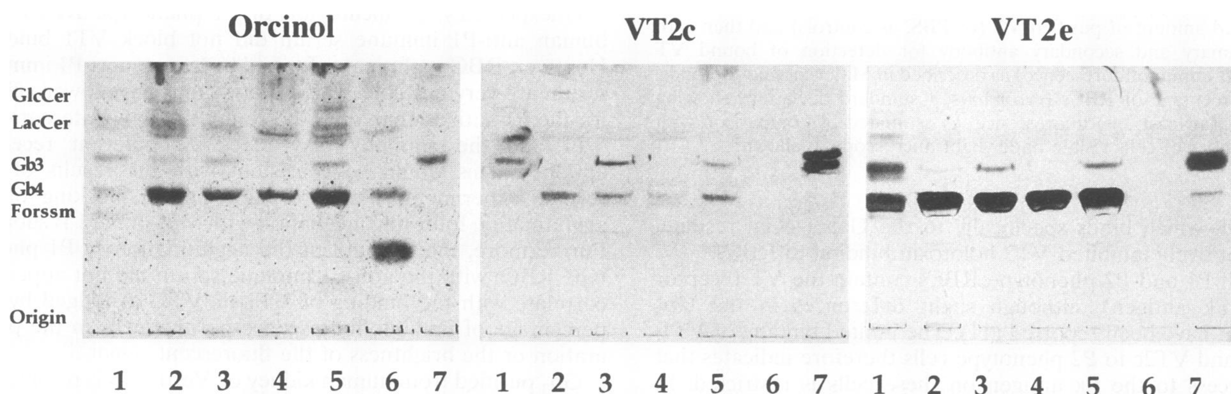

FIG. 5. Binding of VT2c and VT2e to human RBC glycolipids. Extracts from the equivalent of approximately 0.03 ml of RBC pellet (120 μg per lane) were separated by TLC in C/M/W (60:35:8 [vol/vol/vol])and stained with orcinol or overlaid with VT as indicated. Lanes: 1, purified glycolipid standards (GlcCer, 0.5 μg; LacCer, 1 μg; Gb_3, 0.5 μg; Gb_4, 0.5 μg; and Forssman antigen, 2 μg); 2 and 3, extracts from P1 phenotype RBCs; 4 and 5, extracts from P2 phenotype RBCs; 6, extract from p phenotype RBCs; 7, extract from Pk1 phenotype RBCs.

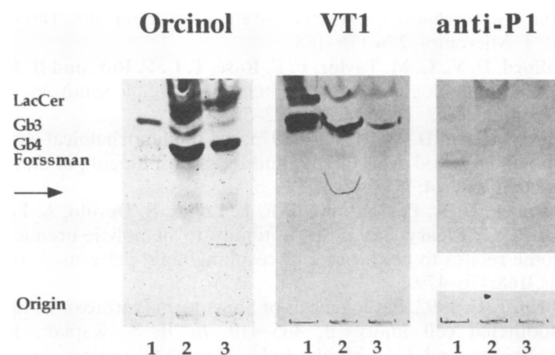

FIG. 6. Detection of P1 glycolipid by VT1 and by human anti-P1 antibody. Identical amounts of RBC extracts (equivalent to approximately 0.08 ml of RBC pellet) were separated by TLC in C/M/W (60:40:9 [vol/vol/vol]), and replicate TLC plates were stained with orcinol or overlaid with VT1 or human anti-P1 and developed with HRP-conjugated secondary antibodies. Lanes 1, standard glycolipids (LacCer, 1 μg; Gb_3, 1 μg; Gb_4, 0.35 μg; and Forssman antigen, 3 μg); 2, P1 phenotype RBC extract; 3, P2 phenotype RBC extract. The arrow indicates the position of the P1 glycolipid.

injected VT2e did not protect the experimental animals from toxin-mediated organ damage (4). Alternatively, it is possible, if VT binds to human RBCs in vivo, that this may contribute to the occurrence of hemolysis in HUS by directly affecting the stability of the RBC membrane or rendering RBCs sensitive to mechanical (6) or oxidative stress (12, 63). To extend our present findings, further studies are warranted to examine the interaction of VTs with RBCs in different stages of maturation, such as (nucleated) erythroblasts or reticulocytes.

In conclusion, we have clearly demonstrated that VTs bind to human RBCs in vitro and that P blood group antigens are the likely receptors. Differential VT binding to various P blood group phenotypes are shown to depend on the availability and accessibility of the Galα1-4Gal residue of the respective P blood group glycolipid. P1 phenotype RBCs exhibited much stronger binding of VT1 and VT2 than did P2 phenotype cells, which lack the P1 antigen, as demonstrated by indirect immunofluorescence, whereas binding of these VTs to Pk1 and Pk2 phenotype RBCs appeared to be mediated primarily by the Pk/Gb_3 antigen. The occurrence of VT binding to RBCs in natural VTEC disease and its relevance for the pathophysiology of HUS, however, remain to be established.

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