

THE HEMOLYIC UREMIC SYNDROME IN CHILDHOOD

A study of epidemiology and pathophysiology

Stellingen:

- 1 Onderzoek naar serologische antistoffen tegen lipopolysacchariden van verocytotoxine-producerende E.coli stammen is de gevoeligste methode voor het vaststellen van een infectie door verocytotoxine-producerende E.coli in het hemolytisch uremisch syndroom (Hoofdstuk 2).
- 2 De activering van het fibrinolytische systeem vastgesteld in plasma van patiënten met HUS is niet het gevolg van een direct effect van verocytotoxine op endotheelcellen (Hoofdstuk 4).
- 3 Cytokines, zoals tumor necrosis factor α en interleukine-1, induceren de receptor van verocytotoxine in humane endotheelcellen geïsoleerd uit de navelstreng (Hoofdstuk 5).
- 4 De door tumor necrosis factor α geïnduceerde expressie van verocytotoxine receptoren is niet afhankelijk van proteïne kinase C activiteit (Hoofdstuk 6).
- 5 In tegenstelling tot patiënten met de X gebonden vorm van renale diabetes insipidus vertonen patiënten met de autosomaal recessieve vorm een normale stijging van weefsel plasminogeen activator antigeen na het toedienen van 1-desamino-8-D vasopressine (A van Lieburg, aangeboden voor publicatie)
- 6 Kinderen behandeld met continue peritonaal dialyse hebben een toename van de gastro-oesophageale reflux (M Smeulders, aangeboden voor publicatie).
- 7 Ook zonder gen-technologie is het mogelijk vernieuwend onderzoek te verrichten.
- 8 Het door de wereld passief toe laten van oorlogsmisdaden, zoals bijvoorbeeld in Bosnië, maakt het onmogelijk om onschuldigen te vinden die de misdadigers kunnen berechten.
- 9 Het tegenhouden van immigratie valt niet te verdedigen als de slachtoffers worden genegeerd en het Westen van mening blijft dat alle voordelen van het huidige beleid alleen voor onszelf zijn (W Tims, de Volkskrant 11 oktober 1994).

- 10 Een kant van het ontwikkelingsbeleid is naar Nederlandse problemen te kijken met de ogen van mensen uit andere landen (J Pronk, Internationale Samenwerking, oktober 1993).
- 11 Het is een restant van het kolonialisme om te denken dat mensenrechten, feminisme, filosofie, wetenschap en dergelijke, Westerse uitvindingen zijn. Het zijn verworvenheden van de mensheid (Nawal El Saadawi)
- 12 De mensen die zich schuldig voelen bij het genieten van het eten van een bonbon, zijn de oorspronkelijke betekenis van het woord vergeten.

Stellingen behorende bij het proefschrift "The hemolytic uremic syndrome in childhood. A study of epidemiology and pathophysiology".

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Nicole van de Kar

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A study of epidemiology and pathophysiology

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Nicole Catharina Adriana Johanna van de Kar

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Promotor: Prof.dr. L.A.H. Monnens
Co-promotor: Dr. V.W.M. van Hinsbergh (TNO-PG Gaubius Laboratorium, Leiden)

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Aan mijn ouders

CONTENTS

		page
Chapter 1	General Introduction	1
1.1	<i>The hemolytic uremic syndrome</i>	1
1.1.1	<i>Typical or diarrhea-associated (D+) hemolytic uremic syndrome in childhood</i>	2
1.1.2	<i>Atypical or diarrhea-negative (D-) hemolytic uremic syndrome in childhood</i>	9
1.2	<i>Verocytotoxin-producing Escherichia coli infection in the hemolytic uremic syndrome</i>	10
1.3	<i>Outline of the thesis</i>	18
1.4	<i>References</i>	19
Chapter 2	Epidemiological studies in diarrhea-associated hemolytic uremic syndrome in Western Europe.	31
2.1	Serological detection of verocytotoxin-producing <i>Escherichia coli</i> in patients with haemolytic uraemic syndrome in Western Europe. <i>Eur J Clin Microbiol Infect Dis 12; 707-709, 1993</i>	33
2.2	Verocytotoxin-producing <i>Escherichia coli</i> infection in hemolytic uremic syndrome in Western Europe. <i>submitted</i>	39
2.3	Association between the epidemic form of hemolytic uremic syndrome and HLA-B40 in the Netherlands and Flanders. <i>Nephron 59; 170, 1991</i>	51
Chapter 3	Plasma cytokine levels in the hemolytic uremic syndrome. <i>Submitted.</i>	55

Chapter 4	The fibrinolytic system in the hemolytic uremic syndrome: in vivo and in vitro studies. <i>Pediatr Res 36; 257-264, 1994.</i>	63
Chapter 5	Tumor necrosis factor α and interleukin-1 induce expression of the verocytotoxin receptor globotriaosylceramide on human endothelial cells: Implications for the pathogenesis of the hemolytic uremic syndrome. <i>Blood 80; 2755-2764, 1992.</i>	77
Chapter 6	Tumor necrosis factor α induces endothelial galactosyl transferase activity and verocytotoxin receptors. Role of specific TNF receptors and protein kinase C. <i>Submitted.</i>	95
Chapter 7	Summary and perspectives for the future.	113
Samenvatting		123
Abbreviations		127
Dankwoord		129
Curriculum Vitae		131

GENERAL INTRODUCTION

1.1 The hemolytic uremic syndrome

The hemolytic uremic syndrome (HUS) is a clinical syndrome characterized by acute hemolytic anemia with fragmented erythrocytes, thrombocytopenia and acute renal failure [1]. This syndrome was described for the first time in five children by Gasser in 1955 [2]. Since then HUS has been reported from many parts of the world and the documentation of epidemics and sporadic cases of HUS made it possible to identify distinct subgroups of HUS, that differ in their clinical features, epidemiology, prognosis, etiology and pathogenesis. A classification based on etiology of HUS is given in Table 1 and further discussed below [3]. HUS can occur in children and in adults. In childhood it is one of the leading causes of acute renal failure in children in developed countries. HUS in children occurs mostly following an infection and can be divided in the so called typical, post diarrheal (D+) HUS or atypical HUS, which is not preceded with acute diarrhea (D-)[4]. HUS in adults is an uncommon and heterogenous condition [3,5]. In adults it is mostly seen in association with drugs, such as oral anticonceptives [6,7], mitomycin C [8], OKT3 [9] or quinine [10,11]; post-transplantation-associated in combination with or without cyclosporin A [12-17], or in association with pregnancy [18,19]; malignancy-associated in combination with or without chemotherapeutics [20,21], or as a complication of systemic diseases like systemic lupus erythematosus [22] and HIV infection [23,24]. The kidney is predominantly involved in the disease process of HUS. Histo-pathological examination of the kidney by Habib et al [25] reveals swollen endothelial cells which are sometimes detached from the glomerular basement membrane. Thrombi and fibrin depositions can be found in the lumen of glomerular capillaries; fibrin, platelets and amorphous material can accumulate in the subendothelial space. The distribution of damaged vessels in the kidney may vary. Several authors have reported that the thrombotic microangiopathic lesions are mostly confined to the glomeruli in children younger than 2 years [26,27]. In the kidneys of older children and in adults also arterioles and even interlobular arteries can be affected, which results in a worse prognosis.

In the literature, HUS and thrombotic thrombocytopenic purpura (TTP), a disease more common in adults, are often discussed [28]. TTP resembles HUS histopathologically and clinically, but differs by the fact that fever and neurologic signs are more prominent than in HUS [28]. However, the variability of organ dysfunction in TTP and the occasional extra-renal manifestations in HUS can sometimes make it very difficult to distinguish between the

diagnosis TTP or HUS.

1.1.1 Typical or diarrhea-associated (D+) HUS in childhood

HUS in childhood is mostly seen after a prodromal phase of acute, often, bloody gastroenteritis, the so called diarrhea-associated (D+) HUS [4]. D+ HUS is also called "typical", "classical" or "epidemic" form of HUS. This form is mostly diagnosed in children younger than 5 years of age (Figure 1). D+ HUS is rare in adults and often confused with TTP. However, elderly people may be another susceptible group for D+ HUS [29]. In most developed countries, this type of HUS is the leading cause of acute renal failure in children. Population-based studies have shown that the incidence of HUS varies among countries. Incidence rates of 0.27-1.74 per 100.000 children < 15 years and 2.65-3.3 per 100.000 children < 5 years have been reported from studies from Canada, United Kingdom and northern part of the United States [30-34]. The highest incidence of HUS, 21.7 per 100,000 children < 15 years, has been reported from Argentina [35]. HUS is mostly diagnosed in the countries Argentina [36], South Africa [37,38], Canada [33], North-Western part of USA [30,39,40] and the Western part of Europe; Belgium [41], United Kingdom [42,43] and The Netherlands [44]. HUS occurs most frequently during the summer months, roughly from May till September (Figure 2). Boys and girls, who were previously healthy, are equally affected [36]. However, some studies show a slight increase of HUS in females [33,45].

Table 1. The etiology of the hemolytic uremic syndrome

<i>Infectious agents</i>	
Diarrhea-associated (D+) HUS	Verocytotoxin-producing <i>E. coli</i> [168] <i>Shigella dysenteriae</i> [134,143]
Neuraminidase-associated HUS	<i>Streptococcus pneumoniae</i> [145,146]
Others	HIV infection [23,24]
<i>Non-infectious agents</i>	
Idiopathic [5,144]	
Drugs	cyclosporin A [13,14], mitomycin C [8], oral anticonceptiva [6,7], OKT3 [9], quinine [11,12]
Systemic diseases	systemic lupus erythematosus [22]
Pregnancy-associated [18,19]	
Malignancy-associated [20,21]	
Transplantation-associated [12,15-17]	
Inherited	autosomal dominant [147], recessive [151] cobalamin C deficiency [148,149] prostacyclin deficiency [155] complement abnormalities [153,154]

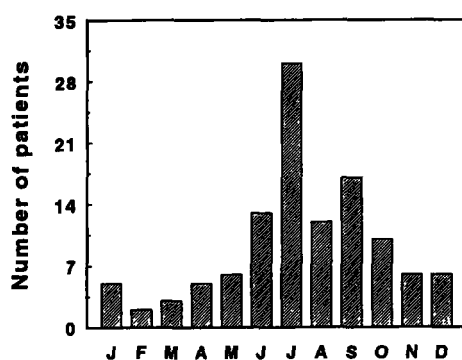
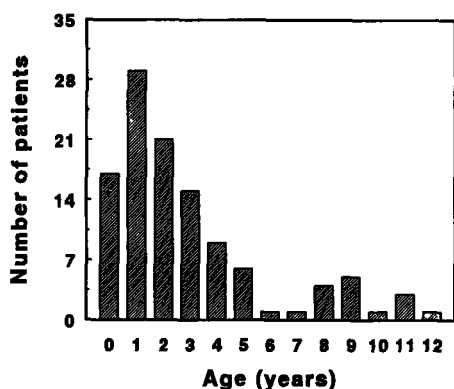


Figure 1. The age of patients with HUS, who were admitted to the Pediatric Nephrology department, University Hospital Nijmegen from 1974 till 1993.

Figure 2. The month of admission of HUS patients, who were admitted to the Pediatric Nephrology department, University Hospital Nijmegen from 1974 till 1993.

Clinical features

Diarrhea precedes the onset of this typical HUS by 3 to 10 days [3]. The diarrhea may be watery and is often bloody and associated with vomiting, cramping abdominal pain and nausea. In most cases the diarrhea is mild, but in some severe cases patients may present with an acute abdomen, severe ileus and even an intestinal perforation. Family-members of the patients can have diarrhea as well. In most cases fever is absent. The acute phase of HUS develops very rapidly; the child appears to be unwell, pale, restless and irritable. In some cases mild jaundice can occur as a consequence of the acute hemolysis. Oliguria is usually present, but polyuria has been described as well. Signs of fluid overload, such as edema may occur. Thrombocytopenia is seen in the majority of patients on admission, but its duration may be brief. Petechiae and bruises are common, but frank bleeding is rare with the exception of the bleeding in the gut. On admission of the patient to the hospital, the liver may be tenderly enlarged. Somnolence and irritability is often considered as mild involvement of the central nervous system. But there are reports which mention severe neurological complications as coma, convulsions and cerebral edema in about 20% of the patients with HUS [46,47]. Occasionally pancreatic involvement has been reported in HUS [48-50]. Dilated cardiomyopathy is a rare but important extra-renal manifestation of HUS [51].

Laboratory features

In patients with HUS, hemolytic anemia, i.e. low hemoglobin levels, reticulocytosis and decreased levels of free haptoglobin, is present. Depending on the degree of the hemolytic process, lactic dehydrogenase and unconjugated bilirubin will be increased. Characteristic are the morphological changes in some erythrocytes, the so called burr cells, helmet cells or

fragmentocytes. The Coomb's test is negative. Elevated numbers of leucocytes, mostly found in the acute phase, have been reported to correlate with the severity of the syndrome [52]. Platelet count is reduced on initial examination in the majority of patients, but occasionally it is found to be normal. The duration of thrombocytopenia may be short and can be missed. The thrombocytes show in vitro a low platelet aggregation. These in vitro studies and the elevation of the platelet proteins β -thromboglobulin, platelet factor 4 and serotonin in serum suggest, that the circulating thrombocytes are 'empty' and therefore functionally exhausted [53,54]. Most of the patients have normal or minimally altered parameters of coagulation. Fibrin(ogen) degradation products are reported to be increased in the serum of HUS patients as well as the levels of fibrinopeptide A, indicating an activation of the intravascular coagulation [55]. Anti-thrombin III levels are usually normal in HUS [56]. Studies have reported an activation of the alternative complement pathway, causing low levels of C3 and the presence of increased degradation products of C3 and factor B [57,58]. Usually the serum-concentrations of urea and creatinine are elevated and may rise rapidly in the acute phase of the disease. Occasionally, elevated levels of bilirubin and hepatic transaminases are found. The urine always contains protein and erythrocytes.

Pathology

In the original cases described by Gasser et al massive bilateral cortical necrosis was seen [2]. In the majority of the kidneys from diarrhea-associated HUS patients the main feature is the alteration of the glomerular capillaries: thickening of the capillary wall which results in narrowing of the lumen. Endothelial cells are swollen and become detached from the basement membrane. Fibrin deposits are seen in the glomerular capillaries and subendothelial spaces [25,59]. Often an expanded mesangium has been described [60]. IgM and C3 can occasionally be found in glomerular mesangium [61]. Although extra-renal manifestations are more commonly associated with TTP and D- HUS than D+ HUS, microthrombi have been found in a wide distribution of organs other than the kidney after post-mortem examination in most cases of HUS [36,62,63]. Gianantonio and co-workers reported that non-renal thrombi in small vessels were found in autopsy materials from 20 out of 47 patients [36]. In a group of 15 children with HUS reported by Upadhyaya and co-workers autopsy was performed in three patients. All three patients had severe neurological symptoms, including coma and uncontrollable seizures [62]. Evidence of myocard involvement was observed in one of the three patients. Microthrombi consisting of fibrin with occasionally enmeshed leucocytes were found in kidney, lung, heart, liver, adrenal glands, brain, thyroid, pancreas, colon, lymph nodes, thymus, and bladder. Focal areas of infarction with surrounding edema and necrosis were seen in the cerebral cortex of all three patients. Non-renal involvement (pancreas, heart, brain, adrenal glands and lungs) has also been described in the pathology reports of ten out of sixteen Dutch patients studied by van Wieringen [63].

Pathogenesis

Although the exact pathogenesis of HUS is still unknown, a lot of progress in understanding has been made in the past decade. Endothelium damage in the kidney plays a central role in

the pathogenesis of HUS, leading to a shift in the hemostatic balance of the kidney towards thrombosis. Microthrombi have also been noted in other organs, such as colon, heart and brain [36,62]. Elevation of von Willebrand factor (vWF) antigen in the plasma has been considered as evidence of endothelial damage. Patients with HUS in the acute phase have high levels of high molecular weight vWF multimers in their plasma [64-66]. These vWF forms can react with activated platelets in the circulation, which in turn leads to platelet aggregation. Rose and co-workers [67] examined the factor VIII-vWF protein and demonstrated that vWF in plasma of HUS and in other vasculitides resembled the endothelial form of vWF in being resistant to reduction by dithiothreitol (DTT), in contrast to vWF from plasma of normal subjects. A number of vasoactive substances stimulate the endothelium to release these multimers of vWF from storage granules. In vitro studies have shown that plasma may be able to correct ultra-large vWF (ULvWF) forms into rather smaller forms of vWF multimers by a "ULvWF reductase" or by neutrophil-derived elastase [68,69]. Recently, Moake hypothesized that in the acute phase in HUS patients, endothelial cell injury or intense stimulation of endothelial cells causing ULvWF release may overwhelm the "reducing capacity" of the plasma. Platelets exposed to the thrombogenic subendothelial matrix bind to ULvWF and become activated [70,71]. Immune-mediated endothelial cell damage is important in the pathogenesis of several vasculitides and may contribute to high molecular vWF in plasma [67]. In both HUS and other vasculitides plasma anti-endothelial cell antibodies have been detected [72]. Leung et al suggested that these antibodies contribute to endothelial damage [72].

Most HUS patients have an increase in the number of neutrophils. A significant relationship in HUS exist between the neutrophil count on admission and adverse outcome [52,73]. Fitzpatrick and co-workers have reported elevated plasma levels of the inactivated product of neutrophils, elastase in complex with α 1-antitrypsin, in the acute phase of the disease. Because it is rapidly bound to cell-surfaces or inhibitors in plasma, free neutrophil elastase is, in contrast to α 1-antitrypsin-complexed elastase, not detectable in plasma [74]. Using a radio-immuno-assay which measures both free and bound human neutrophil elastase, Milford's study has shown that children with a poor outcome had significantly higher plasma elastase concentrations than those who had a good outcome [75]. Forsyth et al demonstrated that neutrophils isolated from plasma of HUS patients taken in the acute phase, adhered more strongly to cultured endothelial cells than control neutrophils [76]. The involvement of neutrophils in the pathogenesis of HUS is also suggested by the elevated plasma levels of the cytokine interleukin-8 in HUS [77]. Interleukin-8, produced mainly by activated macrophages, but also by endothelial cells, is a chemoattractant and activator of neutrophils and is a potential mediator of host response to injury and infection [78-80]. Activated neutrophils and the local release of proteases and peroxides by them may contribute to the endothelial injury seen in HUS [77,81,82].

Several studies have demonstrated abnormal lipid peroxidation in HUS patients [83-85]. Formation of lipid peroxides may occur when oxygen-derived free radicals interact with lipids. Elevated levels of conjugated diene lipids, a marker for lipid peroxidation, have been detected in the plasma of HUS [86]. Decreased concentration of phosphoethanolamine and

a reduction in arachidonic acid content in erythrocyte membranes in HUS may be the consequence of free radical injury [83,87]. In vitro studies have shown that lipid peroxides can damage the endothelium and may have procoagulant activity [88,89]. Recently, Turi et al demonstrated oxidative damage of erythrocytes in HUS patients [85]. The source of oxygen-derived-free radicals is yet not known but a likely candidate is the activated neutrophil.

Normal vascular endothelium has the capacity to prevent thrombosis [90]. This capacity resides in the ability of the endothelium to inhibit the coagulation cascade, in particular by binding of antithrombin III and thrombomodulin-mediated activation of protein C; to reduce platelet activation by the production of prostacyclin, nitric oxide and ADP-degrading ectonucleotidases; and to stimulate fibrinolysis by the production of tissue-type plasminogen activator (t-PA) [91]. A disturbance in the balance between the coagulation and the fibrinolytic system in HUS causes the occurrence of persistent fibrin thrombi in several organs, including the glomerular capillaries of the kidneys [25,36,62]. The formation of fibrin is yet not clearly understood. Elevated levels of fibrinogen- and fibrin split- and degradation products, prothrombin fragments 1+2 and thrombin-antithrombin complexes indicate an active thrombin generation in the acute phase of HUS [55,92]. While t-PA activates the fibrinolytic system, the plasminogen activator inhibitor type I, (PAI-1), reduces its activity. PAI-1 is released from activated blood platelets and newly synthesized PAI-1 can be produced by endothelial cells after exposure to the inflammatory cytokines tumor necrosis factor α and interleukin-1 [91]. Bergstein et al [93] has reported an increase in plasma levels of PAI-1, which could be dramatically reduced by peritoneal dialysis. The removal of the inhibitor from the plasma by peritoneal dialysis was associated with improvement in renal function [93]. The role of the fibrinolytic system in HUS needs to be further investigated. Although there is no experimental model of HUS, rabbit studies of the generalized Shwartzman reaction, resulting in a model of glomerular thrombomicroangiopathy, may lead to a better understanding of the development of intravascular glomerular fibrin deposits in HUS [94,95].

Several interesting studies on the pathogenesis of thrombotic thrombocytopenic purpura (TTP) may be relevant and important for understanding the pathogenesis of HUS. Lian and coworkers have detected and purified a 37kDa platelet agglutinating protein (PAPp37) in the plasma of a subset of patients known with thrombotic thrombocytopenic purpura (TTP) [96,97]. In vitro studies have shown that this protein can agglutinate platelets without any co-factor for its agglutinating activity. PAPp37 binds specifically to the membrane glycoprotein IV (GpIV), present on platelets and endothelial cells [98]. Another important serological factor in the platelet aggregation in TTP may be the presence of calpain activity (calcium-dependent cysteine protease) in sera taken in the acute phase of TTP patients [99]. This calpain, associated with platelet microparticles in plasma from patients with TTP may modify the vWF and induce membrane expression of the vWF receptor GPIIb/IIIa on platelets leading to platelet aggregation [100]. Although no calpain activity has yet been found in patients with HUS (Murphy, personal communication) and no studies so far have looked for the presence of PAPp37 in HUS, earlier studies have shown that platelet aggregating factor

can be present in the plasma of D+ HUS patients [101].

During the last decade various publications with different, often contradictory conclusions about the role of prostacyclin (PGI_2), as platelet anti-aggregatory factor, in HUS have been published [102]. Prostacyclin, a major product of arachidonic acid (the most common poly unsaturable fatty acid in the phospholipids of the cell), is produced by the vascular endothelium. Prostacyclin is an anti-platelet aggregator and a potent vasodilator. Another lipid factor produced by the endothelium but mainly by platelets, is thromboxane A_2 . Thromboxane A_2 is a platelet-aggregator and a potent vasoconstrictor. Both factors can not be measured directly in plasma because of their instability. Inactive 6-keto- $\text{PGF}_{1\alpha}$ and thromboxane B_2 are the measurable stable breakdown products of prostacyclin and thromboxane A_2 , respectively. In 1978 Remuzzi's group was the first who reported that a plasma prostacyclin-stimulating factor was absent in two patients with HUS [103]. This prostacyclin-regulating plasma factor, acting as a reducing co-factor for the cyclo-oxygenase peroxidase, has been partially purified and identified as a stable and very polar molecule of 300-400 Da [104]. Several studies, including Remuzzi's, have been performed by incubating patient's plasma *ex vivo* on aortic rings or cultured endothelial cells [102]. The chosen experimental conditions and preparations of cultured endothelial cells make the interpretation of these *ex vivo* experiments difficult [102,105]. More recently other studies have reported an increased respectively decreased excretion of 6-keto- $\text{PGF}_{1\alpha}$ in the urine during the acute phase of HUS [106,107]. Although, under physiological conditions the presence of the metabolite 6-keto- $\text{PGF}_{1\alpha}$ in urine may reflect renal prostacyclin synthesis, this may be different in renal failure in which the decreased blood-flow may result in an abnormal metabolism and distribution of 6-keto- $\text{PGF}_{1\alpha}$ between the urine and renal venous blood [108,109].

Benigny et al reported significantly elevated urinary levels of platelet aggregating factor (PAF) in children with HUS compared to the urinary PAF levels of healthy control children (measured in ng/mg creatinine)[110]. Control values of urinary PAF of children with chronic renal failure were missing in her study. PAF, a phospholipid, is produced in platelets, endothelial cells, mesangial cells, and other cells. It promotes platelet aggregation, impairs renal function and may play a role in the kidney failure in HUS. Animal models have shown that elevated urinary levels of PAF may reflect increased production in the kidney [111].

Another factor which may play a role in the renal failure observed in HUS is endothelin. Endothelin is a protein produced by endothelial cells as well as mesangial and renal epithelial cells. It is a potent vasoconstrictor and may influence renal blood flow, and glomerular filtration rate. Little circulating endothelin is cleared into the urine and the urinary endothelin is likely of renal origin [112]. In the acute phase an increase in urinary levels of endothelin is observed in HUS patients compared to healthy controls [113]. Both elevated urinary levels of PAF and endothelin return to normal values at convalescence [110,113]. Urinary endothelin-1 is also reported to be elevated in patients with renal diseases [114]. However, in Siegler's study no urinary levels of endothelin have been measured in a control group of children with renal diseases [113]. Therefore good control values are still necessary to interpret the elevated levels of endothelin and PAF in urine of patients with HUS.

Hemolytic anemia, characterized by fragmented erythrocytes, is generally presumed to be the result of mechanical cell injury by passing narrowed, partly occluded capillaries. However, erythrocytes may also be vulnerable to injury by abnormal composition of the erythrocyte membrane phospholipids [85].

Treatment

Since HUS has been reported by Gasser, there has been a reduction in mortality and morbidity of patients. This is due to the fact that HUS is earlier recognized and supportively treated. The risk of involvement of the central nervous system is reduced by the early started supportive treatment, consisting of prompt treatment of acute renal failure by peritoneal dialysis or hemodialysis, correcting of electrolyte imbalance, and by treatment of hypertension. To reverse the microangiopathic process in D+ HUS various controlled and uncontrolled trials with numerous agents have been undertaken [115]. These trials included heparin [116-119], fibrinolytic agents [117,120-122], anti-platelet drugs [119,123], vitamin E [124], infusion of fresh frozen plasma [125,126], gamma-globulin [127,128] or prostacyclin [129,130], and plasmapheresis [131,132]. However, the controlled trials have failed to demonstrate significant improvement [119,125,126].

Prognosis

The clinical course of HUS has been improved, possibly due to a better management in the acute phase of the disease. Although recent reports from Poland [133] and India [134] show mortality rates of 36 and 60% respectively, most series of follow-up studies in Europe and northern America report fatality-rates between 4% and 17% [36,41,135-142]. Most cases of D+ HUS recover spontaneously by supportive treatment, although in various reports 5 to 10% of the cases, in two reports even 16 and 21% [140,141] of the cases, there can still be some residual injury. These abnormalities may be subtle and the long term complications may be unclear, but a number of these patients may develop hypertension or even chronic renal failure in later life [140]. Risk factors for a poor outcome have been reported to be the severity of renal damage [36,137] and high levels of polymorphonuclear leucocytes in the acute phase [43,52]. Nevertheless, the latest published follow-up study in Belgium reported that no variable during the acute phase was predictive of the presence of sequelae after 10 years [142].

Etiology

During the years many different organisms have been described as being the infecting agent causing D+ HUS. Most of the agents suggested as causes have occasionally been implicated and many have no causative role. There is enough evidence that verocytotoxin-producing *E.coli* (VTEC) also called Shiga-like toxin producing *E.coli* (SLT-EC) play the major role in causing D+ HUS in childhood in Western Europe, Canada and USA. The D+ HUS seen in India is mainly due to an infection with *Shigella dysentericae* type 1 and is usually associated with severe renal damage and a high death rate [134,143].

1.1.2 Atypical or diarrhea-negative (D-) HUS in childhood

Hemolytic uremic syndrome has various etiologies (table 1). In children as well as in adults atypical or diarrhea-negative (D-) HUS is a heterogeneous group. D-HUS is rare in childhood and affects children of all ages. In most cases the D- HUS develops without a prodromal illness and has no seasonal variation in time of onset [144]. The D- HUS, who have arteriolar injury have a much worse outcome and prognosis than D+ HUS, who have a predominantly glomerular injury. An infection with *Streptococcus pneumonia* can lead to the neuraminidase-associated form of HUS [145,146]. Circulating neuraminidase derived from the *Streptococcus* removes N-acetylneuraminic acid from glycoproteins in the cell membrane of erythrocytes, thrombocytes and endothelial cells. This leads to an exposure of the antigenic Thomsen Friedreich antigen, allowing the binding of commonly present IgM antibodies to this antigen. This may result in endothelial damage, hemolysis and agglutination of platelets. In contrast to D+ HUS and other forms of D- HUS, the direct Coombs' test is positive in these patients, making transfusions with plasma impossible.

The non-infectious agents in D-HUS in childhood are inherited or idiopathic [3,144]. Recently, published studies demonstrate that there is an association between HUS and cobalamin C deficiency [148,149]. This cobalamin C deficiency is a rare autosomal recessive disorder. Intracellular cobalamin C is a necessary co-enzyme in the conversion of homocysteine to methionine and of methylmalonyl-CoA to succinyl-CoA. Deficiency of cobalamin C will result in elevated levels of homocysteine and methylmalonyl-CoA in plasma and urine. Several in vivo and in vitro reports have demonstrated that elevated levels of homocysteine can damage the endothelium and induce a disbalance in the hemostatic system [150]. Most studies of inherited forms of HUS have been done by Kaplan et al [151,152]. In both children and adults recurrence of HUS may occur in these inherited forms. Several autosomal recessive and dominant modes have been presumed while studying the families with a predisposition of HUS. One study reported an association with HLA-haplotype A3,B7 and hypocomplementemia in a family with predisposition to HUS [153]. In another study by Jorgensen et al a familial deficiency of prostacyclin production stimulating factor was observed [155]. In almost all cases the etiology is idiopathic and the pathogenesis is unknown. HIV associated HUS and other non-infectious agents named in Table 1 occur mainly in adults. In contrast to D+ HUS which rarely occurs in the neonate or within the first few months of life, D- HUS can occur in these first months of life [144,148]. A possible explanation for D+ HUS rarely occurring in neonates or infants might be a result of breastfeeding. Human milk, containing the verocytotoxin receptor GbOse₃cer (see below) which binds verocytotoxin to target cells, may contribute to a protective effect in diarrheal disease by competitive binding of the verocytotoxin [156].

1.2 Verocytotoxin-producing *Escherichia coli* (VTEC) infection in the hemolytic uremic syndrome

1.2.1 Epidemiology of VTEC-associated HUS

It was Konowalchuk, who found, while looking for a cell-assay for heat-labile *E.coli* toxins, that some culture filtrates of *E.coli* strains produced a strange irreversible cytotoxic effect on vero-cells (a cell line derived from the kidney of the African Green monkey) [157]. These *E.coli* strains which produced this cytotoxic effect were isolated from faeces of infants with diarrhea, faeces of a weaning pig and from cheese. The toxin was named after this vero cell-line, verocytotoxin. The major breakthroughs occurred in 1982-1983 with the publication of studies from the United States and Canada which linked VTEC infection to respectively hemorrhagic colitis and HUS [158,159]. In two outbreaks of hemorrhagic colitis in Michigan and Oregon (United States), 47 individuals suffered from severe abdominal pain and grossly bloody diarrhea after having ingested a hamburger from a well-known fast-food restaurant. There was no evidence for an infection by a recognized enteric pathogen. However, in the stools of the patients and the suspected meat an *E.coli* strain serotype O157:H7 was found [160]. Soon, other studies showed that the verocytotoxin-producing *E.coli* O157:H7 was a common isolate from patients with hemorrhagic colitis and bloody diarrhea in which no causal enteric pathogen could be found [158,161,162]. Verocytotoxin-producing *E.coli* strains were first associated with HUS in 1983 by Karmali and co-workers [159]. He isolated these bacteria from the stool from an infant who died after having severe hemorrhagic colitis and in whom after autopsy the diagnosis HUS was made. Since then several reports confirmed the involvement of VTEC in outbreaks and sporadic cases in HUS, and VTEC was strongly implicated with D+ HUS [29,33,43,163-165]. Usually no recurrent HUS follows after VTEC-associated HUS. However, Siegler recently reported a patient who got VTEC-associated HUS, cured and approximately 18 months later again developed HUS upon VTEC infection with *E.coli* serotype O157:H7 [167]. Although in the first episode of HUS serological antibodies to the O157 lipopolysaccharide as well free fecal verocytotoxin were positive, no VTEC O157:H7 strain could be isolated and therefore it is not clear if this patient was infected with the same VTEC O157:H7 strain for the second time.

Until now much information has been gained from the reported outbreaks and sporadic cases of hemorrhagic colitis and HUS in relation to VTEC infection [168]. First, not everybody who is infected with VTEC gets HUS. Infection with VTEC can be asymptomatic, or lead to a mild diarrhea, bloody diarrhea, hemorrhagic colitis or HUS [162,168]. The inoculum size to get ill is not known. Secondly, variability in the frequency of complications may be a function of several factors including young age (< 5 years), prolonged antimotility treatment, presence or absence of antitoxic immunity and the inoculum size [29,169]. Thirdly, the incubation-period of VTEC of 3 to 8 days, is longer than that of most other known enteric pathogens [29,170-172]. Fourthly, most outbreaks have been associated with *E.coli* serotype O157:H7 which is only partly due to the fact that other serotypes are less easy to isolate [29,160,168]. A two-year prospective study by Pai and co-workers in 1988 in Calgary among 5414 Canadian patients with diarrhea, showed that VTEC was the most

commonly isolated enteric pathogen (3.1%) in comparison to *Campylobacter jejuni* (2.7%), *Aeromonas spp* (1.3%), *Shigella spp* (0.5%), *Yersinae enterocolitica* (0.2%) and enteropathogenic *E.coli* (0.15%)[162]. The *E.coli* O157:H7 was isolated in 78% of VTEC strains. Bloody diarrhea was indeed a feature associated with the majority of the VTEC strains. Fifthly, several outbreaks of disease caused by *E.coli* O157:H7 has been linked epidemiologically by the consumption of raw milk and most of all ground beef [29,40,160,173,174]. Using verocytotoxin genotyping, plasmid profiles and DNA restriction fragment length polymorphisms with a bacteriophage probe, Paros and co-workers found that 6 from the 22 examined bovine isolates were generally identical to the isolates from 5 sporadic human cases [175]. Other studies mention the possibility of contaminated, unpasteurized apple-cider [176], or drinking water [177,178]. VTEC infection can also be required via person-to-person transmission [29,172,179]. The principal reservoir of VTEC is the intestinal tract of dairy cattle and perhaps other animals used in the production of food. Doyle et al isolated O157:H7 from 6 (3.7%) of 164 beef, 4 (1.5%) of 264 pork, 4 (1.5%) of 263 poultry and 4 (2.0%) of 205 lamb samples obtained from retail grocery stores [180]. Studies done in faeces from healthy, rather than diseased cattle report the presence of VTEC strains up till 20% of the examined cases. Not all isolated VTEC serotypes in cattle have yet been associated with human diseases. The *E.coli* O157:H7 has been isolated in 0.2-3% of the faeces of cattle in Germany, Spain, United Kingdom and United States [180-184]. Excretion of VTEC by cattle is transient. Because in outbreak investigations, samples from suspected sources have usually been collected a substantial time after the cases have occurred, the source animal may no longer excrete the *E.coli* strain. This probably accounts for the difficulty in recovering the same strain of *E.coli* from human cases and from the incriminated food or animal sources [183].

1.2.2 Biology of VTEC

In literature verocytotoxin-producing *E.coli* associated with human disease are also termed entero-hemorrhagic *E.coli* (EHEC). Although *E.coli* strain O157:H7 is the most commonly isolated VTEC, the list of other O:H serotypes of human VTEC isolates is still growing. Other relatively frequent isolated serotypes isolated from patients with HUS are O26:H11, O111:H8, O111:H-, O21:H19, O145:H-, and O157:H- [168,185-187]. Some of the VTEC serotypes (O26:H11, O111, O119, O127, O128 and O55) strains were already known as enteropathogenic *E.coli* (EPEC), causing infant diarrhea in developing countries [168], but are now classified as VTEC or EHEC. At least three types of verocytotoxins, involved in human disease have been purified and cloned: verocytotoxin-1 (VT1) or shiga-like toxin I (SLTI)[188,189], verocytotoxin-2 (VT2) or shiga like toxin-II (SLTII)[190,191] and verocytotoxin-2 variants (VT2c)[192-195]. Another variant of VT2 (VTe) is associated with edema disease in pigs [196-198]. Studies have shown that most VTEC produce only VT2 or both VT1 and VT2, a small group produces only VT1 or VT2c [43,166,187]. Due to the fact that there is only one amino acid difference in the A-unit between verocytotoxin-1 and shiga toxin produced by *Shigella dysenteriae* type I, verocytotoxins are often called shiga-like toxins [199,200]. Verocytotoxins VT1 and VT2 are encoded by temperate lambda-like

bacteriophages [201,202]. Like several other bacterial toxins such as pertussis toxin, cholera toxin, *E.coli* heat-labile toxin and shiga toxin, verocytotoxin consist of an A subunit, which exhibits the "toxic" activity, and a B subunit, which consists of 5 components and which exhibit the "ligand" activity [203]. In contrast to VT1 and Shiga toxin, which are almost 100% homologous, VT1 and VT2 show a 60% nucleotide sequence homology [204]. The molecular weight of VT is probably about 70,000, with an A subunit of 32,000 and each copy of B subunit of 7,700. Lingwood's group have shown that VT1 and VT2, like shiga toxin, binds specifically to the glycosphingolipid globotriaosylceramide (GbOse₃cer or Gb3, Figure 3a) [205-207]. This verocytotoxin receptor Gb3 has been shown to be present in the human kidney and forms part of the P blood group system (P^k antigen) [205,208]. Both the lipid moiety and the terminal disaccharide moiety (Gal α 1-4Gal) are involved in verocytotoxin-glycosphingolipid binding. Following receptor binding, the toxin is internalized by a receptor-mediated endocytotic event (Figure 3b). The active A unit gets proteolytically nicked, the A1 fragment is released. This A1 fragment acts as a specific N-glycosidase which can depurinate a single adenine residue from the 28S rRNA component of the ribosome. This may result in the blocking the interaction of elongation factor-1 with the ribosome, leading to an inhibition of protein synthesis [210-212] (Figure 3c). Although VT's share the same subunit structure, bind to the same receptor, and inhibit protein synthesis by the same mechanism as Shiga toxin, they fail to cross-neutralize and differ in biological activities in various cell types and animal models [190,192,213].

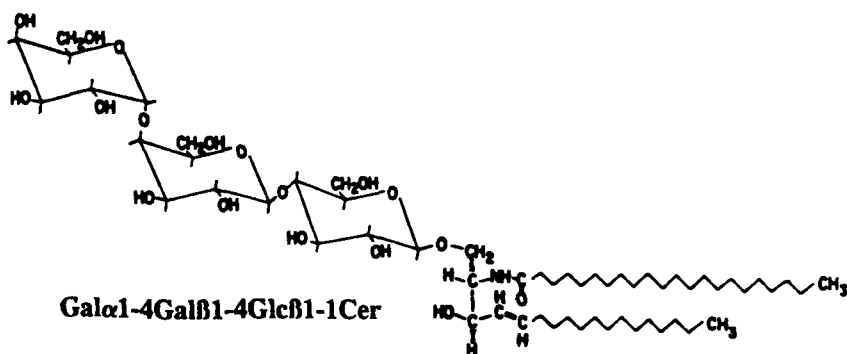


Figure 3a. The biochemical structure of the verocytotoxin-receptor globotriaosylceramide (GbOse₃cer).

1.2.3 Laboratory diagnosis of VTEC

One of the main problems encountered in isolating VTEC from primary media has been the relatively low numbers of VTEC in mixed flora. Tarr et al demonstrated in his study that the recovery of VTEC in the faeces of HUS patients is highly dependent on obtaining stool cultures within 6 days of onset of diarrhea. The detection-rate decreased from 91.7% to 33.3% if stool was cultured for VTEC 3-6 or > 7 days, respectively, after the start of diarrhea [214].

The most common method to examine VTEC in faeces-samples is the use of Sorbitol

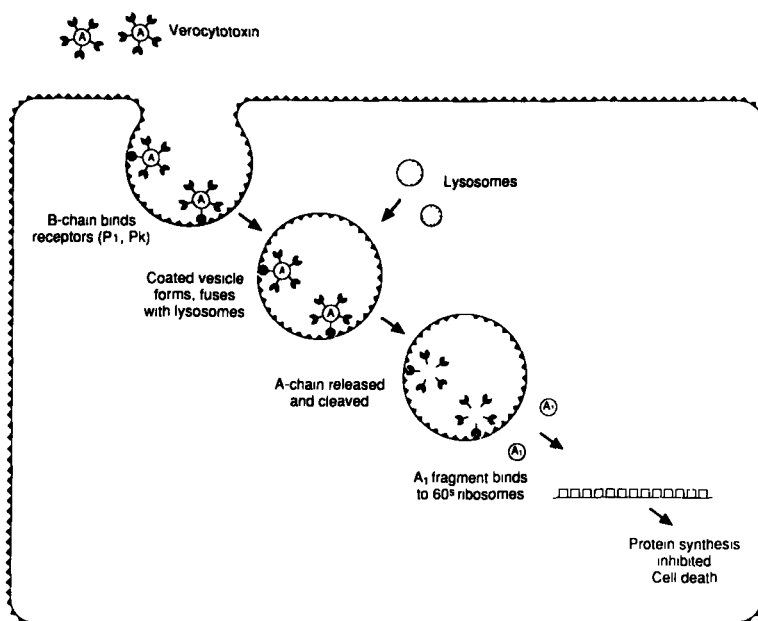


Figure 3b. The action of verocytotoxin on target cells (taken from D. V. Milford *et al* Arch Dis Child 65;713-715, 1990; with permission).

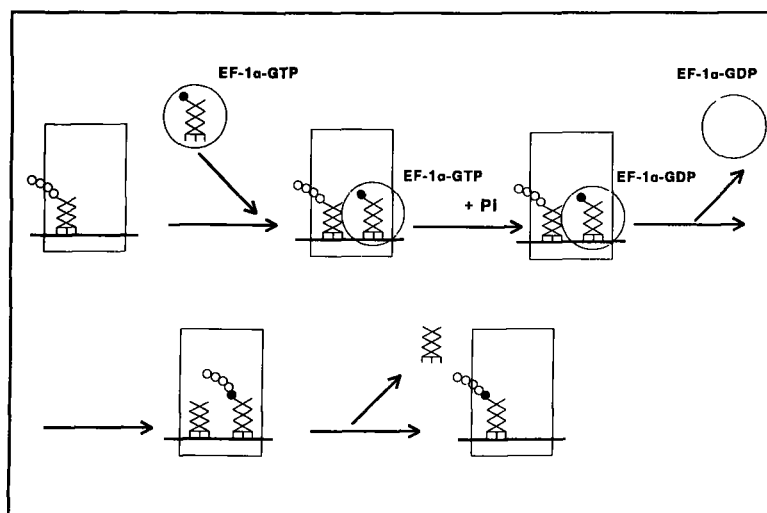


Figure 3c. Peptide elongation on the eukaryotic ribosome. The cytotoxic effect of verocytotoxin on cells is due to a N-glycosidase activity of the A sub-unit, preventing at the end a further binding of the elongation factor-1 (EF-1α) to the ribosome. This results in a inhibition of the protein synthesis, (taken from van de Kar *et al* Behring Inst Mittl 92;202-209,1993, with permission)

McConkey plates. Whereas 95% of the *E.coli* strains are sorbitol fermenters, VTEC strains belonging to serogroup O157; O157:H7 and some O157:H- strains, do not ferment sorbitol in the first 24 hours, giving therefore colourless colonies after a 24 hour incubation-period [174]. These non-sorbitol-fermenting colonies are identified as O157 by using specific O157 antiserum [215,216]. Some sorbitol-negative species of *E.hermanii* can cross-react with this O157 antiserum, so testing for presence of H antigen and presence of verocytotoxins should be added for diagnosing VTEC [217]. It has been reported, that some O157:H- strains isolated from patients with HUS in Germany do ferment sorbitol [166,218]. Moreover, the production of verocytotoxins is closely associated with enterohemolysin in some VTEC [186]. In another recent German study, Beutin and co-workers reported that more different VTEC strains could be identified by using enterohemolysin and, to a lesser extent, the absence of β -glucuronidase activity as epidemiological markers for VTEC infection, than by using the non-fermentation of sorbitol as a criterion [219].

Detection in faeces of VTEC other strains than O157:H7, is possible by using the vero-cell culture assay. In this vero-cell assay, culture filtrates made from a sweep of colonies taken from McConkey agar plate or blood agar plate, are tested for the presence of verocytotoxins [168,220]. The use of polymyxin B to enhance the detachment of verocytotoxin of the *E.coli* and mitomycin C to enhance the production of verocytotoxin in this assay have been reported [220-222]. Detection of free fecal verocytotoxin (FVT) in feces by the vero-cell assay is often more successful in detection VTEC than the culture-filtrate procedure [220,223]. Scotland and co-workers introduced specific DNA probes for detecting verocytotoxins in feces [224]. They found that the DNA hybridization method to be at least as sensitive as the detection of FVT in fecal samples. Other methods, such as colony blot assay with VT monoclonal antibodies and ELISA's to detect verocytotoxin, have been reported [225-227].

Sera of patients with VTEC infection may develop the ability of neutralizing VT. This ability to neutralize VT activity has been used to diagnose VTEC infection in sera of patients without other evidence of infection [220]. Patients infected with *E.coli* O157 and other VTEC appear not always to raise serum antibodies against VT. This may be due to the fact that the first serum sample is mostly taken 3-6 days after the onset of the diarrhea. Another reason might be the fact that VT has shown to bind rapidly to its receptor on endothelial cells [205,228]. A more adequate serological diagnostic method is the detection of antibodies against the lipopolysaccharide of the VTEC by ELISA and immunoblotting. Extensive screening of sera from patients with HUS for antibodies to the lipopolysaccharide of the *E.coli* O157 confirmed the value of immunological test for detecting evidence of infection with *E.coli* O157 [229]. Due to serological cross-reaction, infections caused by *E.coli* O157 can not be distinguished by serology alone from those caused by *Brucella abortus* [230]. Antibody cross-reactions between *E.coli* O157 and *Yersinia enterocolitica* O9 is only a one-way cross reaction. Sera containing antibodies to *Yersinia enterocolitica* O9 were shown to react with the LPS of both *Yersinia* and *E.coli* O157. Antibodies to LPS of *E.coli* O157 did not react with the LPS from *Yersinia enterocolitica* O9, but only with the LPS from *E.coli* O157 [230]. Certain strains of *Citrobacter freundii* spp and *Escherichia hermanii* share also

epitopes with the lipopolysaccharide of *E.coli* O157. Serum antibodies to VTEC antibodies persist for several months following onset of disease enabling both current and retrospective serological testing and are a valuable adjunct to bacteriological procedures for detecting VTEC and VT [229,230].

For epidemiological studies in outbreaks and in food several molecular biological techniques have become available. In feces from cattle or patients and in food samples, different types of verocytotoxins can be detected by using a set of synthetic oligonucleotide primers and the polymerase chain reaction [231,232]. Several characteristics which have been used to differentiate strains have included verocytotoxin genotypes [232-235], plasmid profiles [183,236], bacteriophage typing [237,238], antibiotics susceptibilities [178], restriction enzyme digestion and electrophoresis of plasmid or whole cell DNA [175,239], and multilocus enzyme electrophoresis of bacterial proteins [240]. Plasmid profiles and toxin genotypes have been shown to be less discriminatory than the analysis of restriction enzyme fragments with bacteriophages [175]. Each of these techniques assigns *E.coli* O157:H7 strains to a limited number of subtypes, but due to the fact that they were performed in only a few reference laboratories, more studies, especially in an epidemic setting, are warranted.

1.2.4 Pathogenesis of VTEC infection in HUS

Adhesion of bacteria to the intestinal mucosa is an important factor in the pathogenesis of intestinal infections. In contrast to Shigellae, VTEC strains are not invasive. Verocytotoxins themselves play probably no role in the adhesion of VTEC to the intestines. However not much is known about the presence and distribution of the verocytotoxin-receptor GbOse₃cer in the human intestines [241,242]. During the last two years much has been unravelled about the adherence of VTEC to the intestinal mucosa. Although most information of intestinal colonization and multiplication of VTEC comes from studies in animal models and in tissue-cultures, they may be relevant to the human situation [243,244]. A well-defined pattern of colonization of VTEC infection has emerged from studies of natural and experimental infections in animals. This pattern is consistent with the attaching and effacing adherence (AE lesions) characteristic of human enteropathogenic *E.coli* (EPEC) infection [243,244]. These AE lesions are characterized by extremely close attachment of the bacteria to the intestinal cells, with effacement of the underlying microvilli and accumulation of filamentous actin in the subjacent cytoplasm. Recently, the *E.coli* attaching and effacing (eae) gene, responsible for part of the attaching and effacing lesions in animal models and tissue cultures, has been cloned for EPEC and EHEC [245,246]. Donnenberg et al proposed a three stage model of EPEC adhesion and attaching and effacing (AE) lesion formation [247] (Figure 4). First, an initial adhesion of the bacteria, probably mediated by plasmid factors including the plasmid-encoded bundle-forming pili (bfp) and by additional chromosomal loci (x), is needed. A further intimate adhesion requires the eaeA gene product, a 94-kDa outer membrane protein termed intimin. The eae gene cluster is activated by plasmid encoded regulators (per). The eaeA gene itself is not sufficient for full expression of attaching and effacing (AE) lesions. Other proteins, chromosomally encoded by at least two additional genes, the eae B gene and the cfm (class four mutants) gene(s) (x,y), appear to be necessary for the recruitment of the

host cytoskeletal elements, including filamentous actin, forming the electrodense characteristics adhesion pedestals or cups [247,249,250].

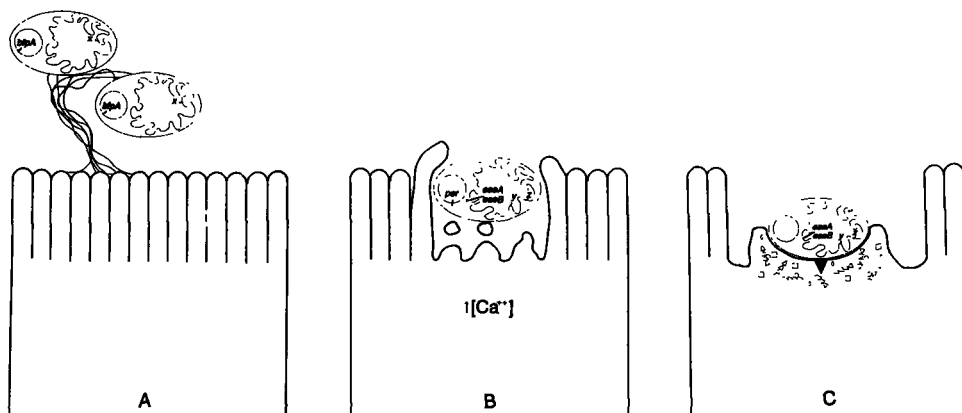


Figure 4. Three stage model of EPEC pathogenesis. Localized adherence (A), the initial interaction between the bacterium and the epithelial cell. This intersection is mediated by BFP's but involves additional chromosomal (gene x) and plasmid loci. In the second stage of infection (B), chromosomal genes (y and z) initiate a signal transduction event that results in increased intracellular calcium levels and effacement of microvilli. Simultaneously, the eae gene cluster is activated by the product of the plasmid (per) locus. The third stage of infection (C) results when intimin (solid triangle), the product of the eaeA locus, and other products of the eae gene cluster mediate close attachment to the epithelial cell. From this proximity, effects on the epithelial are amplified with accumulation of filamentous actin and other cytoskeletal proteins (geometric shapes) (taken from Donnenberg and Kaper, *Infect Immun* 60;3953-3961,1992; with permission).

The adhesion of VTEC in the human intestines may occur in a similar way as observed for EPEC [248]. VTEC strains contain large plasmids of approximately 60 MDa which encode structural and/or regulatory genes for fimbriae that are important in mediating and/or regulating the attachment to human epithelial cells in vitro [251,252]. The formation of the AE lesions, necessary for the intimate contact of the bacteria to the intestinal cells, is mediated by the gene products of VTEC eaeA [248]. The precise nature and site of VTEC colonization in humans remains yet to be established.

Verocytotoxin is thought to be, at least partly, responsible for the systemic manifestations of VTEC disease [228]. After gaining access to the circulation, verocytotoxins bind to specific target cells which are thought to be vascular endothelial cells [1,168]. Capillary endothelial damage is accepted as a central event in the pathogenesis of HUS. Histopathologically studies of glomerular endothelial cells in HUS patients reveal characteristic swelling of endothelial cells accompanied by detachment of the basement membrane. Similar histo-pathology has been described in rabbits challenged with verocytotoxin-1 (VT-1). In a rabbit model Richardson et al demonstrated that intravenously injected ¹²⁵I-labelled VT-1 was rapidly removed from the systemic circulation in minutes. The highest uptake of ¹²⁵I-labelled VT-1 was seen in the spinal cord, brain, cecum, colon, and

small bowel, but not in the kidney of unimmunized rabbits. Immunofluorescent staining of tissue after intravenously administration of VT1 showed evidence of specific vascular endothelial cell binding of the toxin. The localization of ^{125}I -VT1 was consistent with the thrombotic microangiopathy in these tissues. Injection of ^{125}I -VT-1 to rabbits immunized against VT1 prevented disease and binding of the toxin to the target tissues, thus further supporting the specific role of verocytotoxin [253]. Zoja et al demonstrated that the tissue damage due to verocytotoxin was correlated with the presence of the functional receptor of verocytotoxin, the glycosphingolipid GbOse₃cer [254]. No receptors for verocytotoxin were detected in the rabbit-kidney, corresponding with no damage seen in the kidney after administration of verocytotoxin. In mice given intravenously verocytotoxin or orally *E.coli* O157:H7, the development of bilateral tubular necrosis was seen, but no glomerular vascular lesions were observed [255], corresponding with the presence of GbOse₃cer in the tubuli of the mouse kidney [213]. Histological examination of other tissues than the kidney in these mice appeared normal by light microscopy. Very interesting in this regard is the recent observation of Lingwood that GbOse₃cer is present in the glomeruli of the kidney of children younger than 3 years of age and absent in the glomeruli of children older than 3 years and in the glomeruli of adult-kidneys [256].

Unfortunately, there is no experimental animal model of HUS in which the animal develops the classical syndrome following VTEC infection via the natural oral route. However in vitro studies with cultured human endothelial cells, mostly isolated from umbilical cord have elucidated a part of the pathogenesis of HUS. Several in vitro studies have shown that shiga toxin as well as verocytotoxins are able to cause endothelial damage by inhibiting the protein synthesis of the cells [228,257,258]. Recently, one study reported that cultured endothelial cells of human glomeruli are very susceptible for verocytotoxin [259]. First Kavi et al, but also recently Moake and coworkers have shown that endothelial cells isolated from umbilical cord can release a pattern of von Willebrand factor multimers, including ULvWF upon stimulation with verocytotoxin [71,260]. Rose et al reported that filtrates of verocytotoxin-producing *E.coli* are capable of inducing platelet aggregating activity in fresh normal plasma, which is independent of vWF antigen [261]. The study of Karch et al revealed that incubation of purified VT1 or VT2 reduces significantly the production of prostacyclin in rat aortic tissue [262].

The verocytotoxin-receptor GbOse₃cer is part of the P blood group system, namely Gb3 is the P^k antigen (table 2)[263]. The P₁ antigen, which displays the same terminal saccharide structure as P^k antigen, can interfere with the verocytotoxin binding to GbOse₃cer [198]. The antigens of the P system include the P₁ antigen (Gal α 1-4Gal β 1-4GlcNac β 1-3Gal β 1-4GlcCer), P antigen (GalNac β 1-3, Gal α 1-4Gal β 1-4GlcCer, globoside) and the precursor of P antigen, the P^k antigen (Gal α 1-4Gal β 1-4GlcCer, GbOse₃cer). Approximately 75-80% of the normal caucasian population express blood group phenotype P₁ antigen on erythrocytes, but with varying strength. The P^k antigen phenotype is very rare (table 2). P^k antigen is present on all red cells except those of the p phenotype, but can vary in expression [263]. This may be related to the expression of the galactosyl-transferase activities [264]. Erythrocytes, not capable of synthesizing proteins and therefore resistant to the toxic effect of verocytotoxins,

Table 2. The P blood group system

Phenotype	Frequency	Antigens on erythrocytes	Antibodies in serum
P ₁	75 %	P ₁ , P, P ^k	None
P ₂	25 %	P, P ^k	Anti-P
P	very rare	None	Anti-P ₁ , PP ^k
P ^k ₁	very rare	P ₁ , P ^k	Anti-P
P ^k ₂	very rare	P ^k	Anti-P

Structure of the P antigens:

P ^k antigen	Globotriaosylceramide (Gb3)	Gal(α,1-4)Gal(β,1-4)Glc-Cer
P antigen	Globotetraosylceramide (Gb4)	GalNAc(β,1-4)Gal(α,1-4)Gal(β,1-4)Glc-Cer
P ₁ antigen		Gal(α,1-4)Gal(β,1-4)GlcNAc(β,1-3)Gal(β,1-4)Glc-Cer

may protect other susceptible tissues for the effect of this toxin, by 'catching' verocytotoxin out of the circulation. In favour of this hypothesis is the study of Taylor et al, who reported that the erythrocytes of patients with HUS express a weak or absent P₁ antigen [265]. In an other study done by Newburg et al, erythrocytes of patients with HUS had lower Gb3-to-lactosylceramide ratios than did erythrocytes of controls [256]. These studies indicate that there might be a relationship between susceptibility to HUS and the expression of glycosphingolipids on their erythrocytes. A possible involvement of verocytotoxin in red cell changes in HUS has been reported by Rose and his co-workers [267]. In accordance to Butler's study with red cells from *Shigella*-associated HUS, he reported that in vitro red cell vacuolisation evidence may occur upon treatment with sterile culture filtrates of verocytotoxin [268].

1.3 Outline of the thesis

The aim of this thesis is to investigate the incidence of VTEC infection in patients with HUS in the Netherlands, Belgium and Germany. Parallel, in order to elucidate the pathogenesis of HUS further studies were performed in vitro with purified verocytotoxin-1 on cultured human endothelial cells.

The results of the epidemiological study are presented in chapter 2. By using several detection methods, sera and feces from HUS patients were tested for the presence of VTEC. Cytokines or inflammatory mediators, secreted from various cell-types have been implicated in the mediation of host responses to infection and tissue injury. They can induce a wide range of secondary mediators, activate the coagulation and fibrinolytic pathways, and influence the function of endothelial cells. The presence of cytokines in patients with HUS is investigated in chapter 3. Thrombi and fibrin formed in the glomeruli of patients with HUS are a result of endothelial damage. The fibrinolytic system plays an important role in the removal of the intravascular glomerular fibrin deposits. Endothelial cells are not only the main source of the key-enzyme of the fibrinolytic system, tissue type plasminogen activator

(t-PA), but they also produce its inhibitor, the plasminogen activator inhibitor type I (PAI-1). In chapter 4, plasma levels of proteins of the fibrinolytic system are investigated in patients with HUS. The effect of verocytotoxin-1 on the production of fibrinolytic proteins by human cultured endothelial cells is examined. It has been shown that verocytotoxin can damage cultured endothelial cells. Cytokines can change the susceptibility of human cultured endothelial cells to verocytotoxin. The mechanisms of the cytokines, tumor necrosis factor α and interleukin-1, to make endothelial cells more sensitive to verocytotoxin are investigated in chapter 5 and 6.

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Chapter 2

EPIDEMIOLOGICAL STUDIES IN DIARRHEA-ASSOCIATED HEMOLYTIC UREMIC SYNDROME IN WESTERN EUROPE

Chapter 2.1

**SEROLOGICAL DETECTION OF VEROCYTOTOXIN-PRODUCING
ESCHERICHIA COLI IN PATIENTS WITH HEMOLYTIC
UREMIC SYNDROME IN WESTERN EUROPE**

Henrik Chart¹, Nicole van de Kar², Jules Tolboom², Leo Monnens², Bernard Rowe¹

¹Division of Enteric Pathogens, Public Health Laboratory, London, United Kingdom

²Department of Pediatrics, University Hospital, Nijmegen, The Netherlands

Summary

Sera from 45 patients from The Netherlands, Germany and Belgium who had a clinical diagnosis of haemolytic uraemic syndrome (HUS) were screened for antibodies to the lipopolysaccharide (LPS) of *Escherichia coli* producing Verocytotoxin (VTEC). Sera from 43 family contacts and 34 control patients were also examined. Using the techniques of EIA and immunoblotting, antibodies to the LPS of *Escherichia coli* O157 were found in 28 patients, and to the LPS of serogroups O115 and O145 in one patient and one family member respectively. The results of our study suggest that VTEC, and in particular *Escherichia coli* O157, might be a significant cause of HUS in Western Europe.

Introduction

Strains of *Escherichia coli* that produce Verocytotoxin (VTEC) have been identified as a significant cause of the haemolytic uraemic syndrome (HUS) [1, 2]. Verocytotoxin-producing *Escherichia coli* belonging to serotypes O5:H-, O26:H11, O55:H7, O104:H2, O105:H18, O111:H-, O115:H10, O128:H2, O145:H25, O153:H25, O163:H19 and O165:H25 have been isolated from patients with HUS [3]; however, strains belonging to serotype O157:H7 are most frequently isolated [3]. The detection of VTEC or Verocytotoxin in patients' stools identifies the causative organism in cases of HUS. Unfortunately, since VTEC and the toxins they produce are detected in patients' stools for only a short time following onset of disease [4], the infectious agent may go undetected.

Previous studies [5-8] have shown that infection with *Escherichia coli* O5, O115, O145, O157 and O165 can result in the production of serum antibodies to the lipopolysaccharide (LPS) of these VTEC. The value of serology was further illustrated when a serum from a patient with HUS, taken in 1974, was found to contain circulating antibodies to the LPS of *Escherichia coli* O157 [9], making this patient the earliest known case of HUS caused by this organism. Cases of HUS caused by VTEC have been reported in the UK [2], and also in Germany [10] and Italy [11].

In the present study we examined sera from 45 patients with HUS, and in the case of 14 of these patients sera were also taken from family members. Sera from 34 control patients were also examined.

Materials and Methods

Materials

Sera were obtained from 45 patients with HUS, a group with a mean age of 4.2 years ($\delta = 4.5$) comprising 21 males, 23 females and one patient of unknown sex. Patients were from The Netherlands ($n = 29$); Cologne, Germany ($n = 12$), and Leuven, Belgium ($n = 4$).

Patients were characterised by having elevated levels of blood urea and creatinine, and thrombocytopenia. Thirty-two patients had bloody diarrhoea, certain patients exhibited stages of renal failure and some required renal dialysis. Sera were also obtained from 43 family contacts. Thirty-four patients ($\delta = 2.6$ years, $\delta = 2.4$ years) with acute gastroenteritis were used to provide control sera for serological testing. All sera were stored at -10°C in the Laboratory of Enteric Pathogens, PHL, London.

Lipopolysaccharide was prepared from 13 strains of *Escherichia coli* belonging to serotypes O5:H- (E41787), O26:H11 (E36039), O55:H7 (E40230), O104:H2, (E32627), O105:H18 (E43549), O111:H- (E52849), O115:H10 (E47747), O128:H2 (E41509), O145:H25 (E38938), O153:H25 (E31695), O157:H- (E32511), O163:H19 (E31708) and O165:H25 (E40235). With the exception of *Escherichia coli* strain E40235 (O165:H25), all strains had been isolated from patients with HUS. For enzyme immunoassay (EIA) in SDS-PAGE/immunoblotting, LPS was prepared from bacterial outer membranes using the hot-phenol procedure of Westphal and Jahn [6, 12].

Methods

SDS-PAGE was carried out as described previously [5] using the method of Laemmli [13]. Lipopolysaccharide ($10\mu\text{g}$) was applied onto gels comprising a 4.5% stacking gel and a 12.5% separation gel [13], and electrophoresis performed as described elsewhere [6]. SDS-PAGE gels were either used for immunoblotting [6] or fixed for subsequent silver staining [14].

For the EIA [5] plates were coated with $1\mu\text{g}$ of LPS in $100\mu\text{g}$ coating buffer and reacted with sera diluted ($\times 1000$) in phosphate buffered saline (PBS). Antibody-antigen complexes were detected using an alkaline phosphatase conjugated goat-anti-human polyvalent antiserum (Sigma, USA) at a dilution of $1/800$ in PBS. Results were expressed as the absorbance at 405 nm. Sera giving an EIA absorbance value > 0.7 were considered positive for antibody and sera giving an EIA absorbance value of < 0.4 were considered negative for antibody [8]. Sera giving an EIA absorbance value > 0.4 but < 0.7 were examined for antibodies to LPS by immunoblotting. Sera from a previous study [5] known to contain antibodies to the LPS of *Escherichia coli* O157, O5, O115 and O145 were used as positive controls.

Results and Discussion

Forty-five sera from patients with HUS, 43 sera from family contacts and 34 control sera were tested by EIA for antibodies to the LPS from *Escherichia coli* O157. Twenty-eight patients were found to have serum antibodies reacting with LPS from *Escherichia coli* O157. Six of these sera, giving EIA absorbance values ranging from 0.50-0.69, were found to be positive for antibody on immunoblotting (Figure 1, lane 1). A serum giving an EIA absorbance value of 0.43 was found to be negative for antibody on immunoblotting (Figure 1, lane 2). Eleven of the 14 patients for whom sera from family members were available were found to be positive for antibodies to LPS of *Escherichia coli* O157 (Table 1), giving

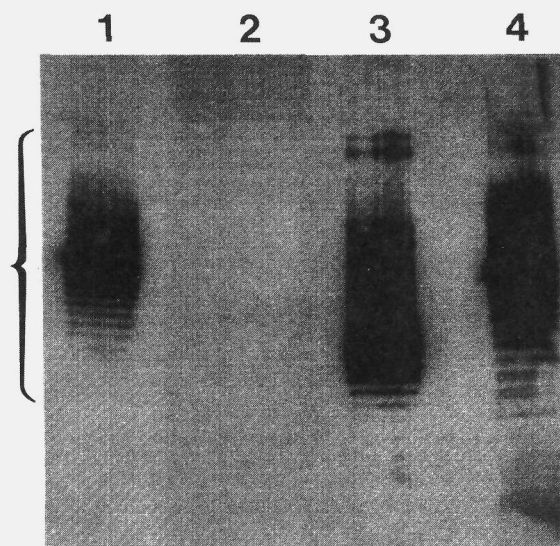


Figure 1. Immunoblotting of sera from patients and contacts to detect antibody to LPS of VTEC. Lane 1: example of the immunoblot in a patient with antibodies reacting with long-chain LPS (bracketed) from *Escherichia coli* O157. Lane 2: example of a negative immunoblot reaction. Lane 3: immunoblot in the one family contact with antibodies to LPS of VTEC O115. Lane 4: immunoblot in the one patient with antibodies to LPS of VTEC O145.

mean EIA absorbance values ranging from 0.52 to 1.46. Three of these patients, with EIA absorbance values of 0.52, 0.57 and 0.69 were confirmed to be positive for anti-O157 LPS antibody on immunoblotting. The three antibody negative patients had mean EIA absorbance values of 0.12, 0.16 and 0.26. These results indicate that more than 60% of patients were suffering from HUS caused by VTEC, and demonstrates the value of serological testing as a means of obtaining evidence of infection with VTEC. Evidence of a causative agent responsible for the remaining 20 cases of HUS was not found and the aetiology of these cases remain unknown. The possibility arises that these cases of HUS may have been caused either by VTEC as yet unassociated with HUS, or by other microbial agents. Alternatively, some of these cases of HUS might have been caused by VTEC without production of O-antigen specific antibodies [5]. None of the family contact had serum antibodies to the LPS of VTEC, giving EIA values considerably lower than the absorbance cut-off value 0.4.

However, one contact from family no. 7 was found to have serum antibodies to the LPS of *Escherichia coli* O115 giving a mean EIA absorbance value of 0.95 and a positive immunoblot result (Figure 1, lane 3). Unfortunately, since bacteriological examination of faeces of this contact was not performed, information concerning carriage of VTEC are not available. One control serum was found to have antibodies to *Escherichia coli* O157 as detected by immunoblotting. Unfortunately there was insufficient information to establish whether this person might have had a previous infection with this organism.

Table 1. Reaction of sera from 14 patients with hemolytic uremic syndrome and their respective family contacts in an EIA to detect antibodies to the LPS of *Escherichia coli* O157. EIA results are expressed as absorbance at 405 nm.

Family No	Mean EAI absorbance value	
	Patient	Family contacts ^b
1	0.16	0.22 (3)
2	0.57 ^a	0.23 (2)
3	1.12	0.23 (2)
4	0.69 ^a	0.18 (2)
5	0.12	0.27 (2)
6	0.95	0.26 (3)
7	1.00	0.14 (9)
8	1.12	0.13 (4)
9	1.46	0.23 (5)
10	0.90	0.23 (5)
11	0.90	0.31 (3)
12	0.52 ^a	0.34 (3)
13	1.18	0.28 (3)
14	0.26	0.16 (2)

^a Confirmed to be antibody-positive on immunoblotting.

^b Number of contacts is shown in brackets.

Patients were also examined for antibodies to the LPS prepared from 12 other VTEC strains. One patient was found to have antibodies to the LPS of *Escherichia coli* O145 (Figure 1, lane 4). Serum antibodies to the LPS of *Escherichia coli* O157 were not detected in the thirty-four control sera. The incidence of cases of HUS caused by VTEC in The Netherlands has been reported only rarely, however in one study 54% of sera from Dutch patients with HUS were found to have circulating antibodies to the LPS of *Escherichia coli* O157 [9]. In the present study more than 60% of patients had evidence of infection with *Escherichia coli* O157 and strains of *Brucella abortus* and *Yersinia enterocolitica* O9 [15], infections caused by these organisms are unlikely to be confused with cases of HUS caused by *Escherichia coli* O157.

When the results of this study are considered together with the findings from other European countries [10,11], HUS caused by *Escherichia coli* and other VTEC appears to be an emerging problem in Europe.

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Chapter 2.2

VEROCYTOTOXIN-PRODUCING ESCHERICHIA COLI INFECTION IN THE HEMOLYTIC UREMIC SYNDROME IN WESTERN EUROPE

Nicole C.A.J. van de Kar¹, Hannie G.R. Roelofs², Harry L. Muijtens²,
Jules J.M. Tolboom¹, Bernhard Roth³, Willem Proesmans⁴,
Willy Chr.C. Reitsma-Bierens⁵, Eric D. Wolff⁶, Mohamed A. Karmali⁷,
Henrik Chart⁸, Leo A.H. Monnens¹

¹Dept of Pediatrics, University Hospital, Nijmegen, The Netherlands

²Dept of Medical Microbiology, University Hospital, Nijmegen, The Netherlands

³Dept of Pediatrics, University Hospital, Cologne, Germany

⁴Dept of Pediatrics, University Hospital, Leuven, Belgium

⁵Dept of Pediatrics, University Hospital, Groningen, The Netherlands

⁶Dept of Pediatrics, University Hospital, Rotterdam, The Netherlands

⁷Dept of Medical Microbiology, Hospital for Sick Children, Toronto, Canada

⁸Central Public Health Laboratory, Divisions of Enteric Pathogens, London, UK

Summary

From September 1989 till September 1993, stool specimens and sera from 113 children with diarrhea-associated hemolytic uremic syndrome (HUS) from the Netherlands, two university hospitals in Belgium and one university hospital in Germany were examined for the presence of verocytotoxin-producing *Escherichia coli* (VTEC) infection. Stool examination for VTEC and for free fecal verocytotoxin combined with serological evidence of verocytotoxins and antibodies to the lipopolysaccharide of O157-antigen, demonstrated a VTEC infection in 88 (78%) patients with HUS and in 2 (3%) of the 65 examined control children with acute gastro-enteritis. The serotype O157 was the causative agent in 86% of these 88 patients with VTEC-associated HUS.

VTEC O157 strains were isolated from the stool of 19 patients with HUS and in one of the controls. Cytotoxicity of the culture filtrates of all isolated strains could be neutralized by antibodies to verocytotoxin-2. The fecal samples tested by a procedure for verocytotoxin detection in polymyxin B extracts of colony sweeps (VT/PECS) and free fecal verocytotoxin (FVT) assays were positive in 27% and 54% respectively in the HUS group as compared to 2% in the control group.

Sera of 71 (65%) of 110 patients with HUS and 1 control serum contained antibodies to O157-antigen. Serological testing for antibodies to O157 O-antigen yielded the highest number of positive results compared to the other test methods. Stool and sera examination for VTEC in 95 family contacts of 28 patients with HUS, demonstrated an evidence for VTEC infection in 33 (35%) family contacts. Serological antibodies to O157 O-antigen were found in only 3 (4%) of 85 family contacts.

We conclude from this study that in this part of Western Europe VT2-producing *Escherichia coli*, mainly those belonging to serogroup O157, are the major cause of HUS in childhood.

Introduction

Hemolytic uremic syndrome (HUS), preceded by an acute, often bloody gastroenteritis, is mostly seen in children. It is a major cause of acute renal failure in childhood [13]. Since the first report by Karmali and coworkers [14], verocytotoxin-producing *Escherichia coli* (VTEC) infections are recognized as an important cause of diarrhea-associated HUS in the United States, Canada, United Kingdom and other countries [3,5,16,21,26]. VTEC strains may belong to different serogroups, but the most commonly isolated VTEC is the serotype O157:H7. A family of at least three verocytotoxins has been identified: verocytotoxin-1 (VT1) or Shiga-like toxin I (SLTI), verocytotoxin-2 (VT2) or Shiga-like toxin II (SLTII) and verocytotoxin 2 variants (VTc)[15]. Not all those infected with VTEC will develop HUS. Infection with VTEC can be asymptotically, lead to a mild diarrhea, bloody diarrhea, hemorrhagic colitis or HUS [19].

In this study, we report the results of a four year prospective study in which we examined the presence of VTEC infection in patients with diarrhea-associated HUS in the Netherlands, two adjacent university hospitals in Belgium and one in Germany at a distance of approximately 150 km from the University of Nijmegen, The Netherlands.

Patients and Methods

Patients

Between September 1989 and September 1993, stool and sera specimens from 113 patients with diarrhea-associated HUS (58 female, 55 male; mean age $\pm$ SD: 46 ± 35 months; range 9-162 months) were received by the Department of Medical Microbiology of the University Hospital Nijmegen for examination of the presence of VTEC infection. Specimens of patients with HUS were obtained from 77 Dutch patients admitted to pediatric nephrology departments of the academic hospitals in the Netherlands, 21 patients admitted to the pediatric nephrology department of the Children's Hospital, Cologne in Germany and 15 patients admitted to the University Hospital of Leuven and Antwerpen in Belgium. A case of HUS was determined by a sudden onset of illness with a prodromal phase of acute gastro-enteritis, and by laboratory evidence of microangiopathic hemolytic anemia, thrombocytopenia and disturbed renal function [10]. All patients with HUS included in this study had a prodromal phase with acute gastro-enteritis; in 73% the gastro-enteritis was reported to be bloody. Stool and serum specimens were also obtained from 95 family contacts (27 fathers, 28 mothers, 35 siblings and two other family contacts) of 28 patients with HUS referred to the pediatric department of the University Hospital, Nijmegen, The Netherlands. The family members included all family contacts who lived with the HUS index case in the same house. Sixteen (17%) family members had diarrhea at time of admission or one week before admission of the HUS case to the hospital. The control group consisted of 65 children (28 females, 37 males, mean age $\pm$ SD; 31 ± 25 months) with acute gastro-enteritis (18% of the cases had bloody diarrhea) and without any evidence of HUS, who were admitted to the pediatric department of the University Hospital Nijmegen and to three referral hospitals in the Netherlands between September 1990 and September 1993.

On admission stools were collected from patients and controls as soon as possible. Blood samples were taken on admission and, when possible, after two to three weeks (convalescent phase). After centrifugation sera samples were frozen at -20°C and transported on dry ice to the Medical Microbiology department, where they were kept frozen at -70°C until the assays were performed.

Stool samples

All stool samples examined for VTEC were plated on sorbitol MacConkey (Oxoid Sorbitol MacConkey agar containing 1% sorbitol) and blood agar. After 24 hours incubation non-sorbitol fermenting, colourless colonies were tested for agglutination with

anti-O157 O-antigen serum (Difco, Detroit, Michigan, USA) and tested for verocytotoxin activity in the Verocell-assay (see below). VTEC O157 strains were serotyped by Dr.W. Jansen, RIVM, Bilthoven, the Netherlands. Furthermore, fecal samples were tested by a procedure for Verocytotoxin (VT) detection in polymyxin B extracts (PE) of colony sweeps (CS) (VT/PECS), as described by Karmali *et al* [14]. Briefly, a colony sweep was taken from a blood agar, inoculated with a fecal sample for 24 hours at 37°C, suspended in Antibiotica Broth No 3 and incubated overnight at 37°C. After incubation and the following centrifugation (10,000 x g for 10 min), the cell-pellet was suspended in 0.1 mg/ml of polymyxin B and incubated for another 30 min at 37°C. After centrifugation (10,000 x g for 10 min), the culture filtrate was tested for cytotoxicity on confluent Verocell monolayers and incubated for three days at 37°C in a 5% CO₂ atmosphere. The titre of the cytotoxic activity was expressed as the reciprocal of the highest dilution inducing 50% cytotoxic effect (CD50) after incubation for three days at 37°C. Positive cytotoxicity by verocytotoxin was neutralized by using a polyclonal antibodies to VT1 and VT2c and the monoclonal antibody BC5 BB12 to VT2.

To detect free fecal verocytotoxin (FVT), fecal filtrates were made from fecal samples as follows: equal volumes of the fecal specimen and sodium phosphate buffer (pH 7.2) were thoroughly mixed by using a vortex mixer and then centrifuged (10,000 x g for 10 min); the fecal filtrate was tested for cytotoxicity in the Verocell assay as described above [14]. The stools of all patients with HUS and patients with acute gastro-enteritis were also tested for the most common enteric pathogens.

Serum samples

Paired serum samples, collected on admission and after 14 days were used to detect neutralizing ability to VT1 and VT2 or VT2c with the Verocell assay [14]. Briefly, two CD50 of the verocytotoxin preparations, obtained from culture supernatants of reference strains H30 (VT1-producing *E.coli*) and E32511 (VT2 and VT2c-producing *E.coli*)[10], were incubated for one hour with twofold dilutions of patient's serum. After preincubation the mixture was added to a confluent monolayer of verocells. The highest serum dilution that caused inhibition of cytotoxic effect after three days of incubation of cell-culture, was taken as the end point. Paired samples were always tested in the same Verocell-microtitre plate. A fourfold or more rising titre to VT1, VT2 and/or VT2c in the sera was regarded as positive for recent VTEC infection. Serum antibodies to the lipopolysaccharide of *E.coli* O157 were analyzed by ELISA and immunoblotting, as described previously [6]. A case was defined positive for VTEC infection when one or more of the above described detection methods were positive.

Statistical analysis

The significance of differences between the groups was determined by using the Fisher's exact test (two tailed).

Results

The results are shown in Table 1. Evidence for VTEC infection was found in 88 (78%) out of 113 patients with HUS. Only two patients (3%) from the control group of 65 children with acute gastroenteritis demonstrated a VTEC infection.

Table 1. Verocytotoxin-producing *Escherichia coli* infection in patients with hemolytic uremic syndrome and controls with acute gastro-enteritis.

Methods	No of patients with HUS positive for VTEC (n=11)		No of controls positive for VTEC (n=65)	
<i>Feces</i>				
Isolation of VTEC O157	19/90	(21%)	1/57	(2%)
O157:H7	18/19	(95%)	0/57	(0%)
O157:H-	1/19	(5%)	1/57	(2%)*
VT/PECS	24/90	(27%)	1/57	(2%)
Free fecal verocytotoxin	49/90	(54%)	1/56	(2%)
One or more methods	54/90	(60%)	1/57	(2%)
<i>Serology</i>				
VT-neutralizing ability	4/76	(5%)	0/19	(0%)
Antibodies to O157-antigen	71/110	(65%)	1/53	(2%)
One or both methods	72/110	(65%)	1/50	(2%)
<i>Total VTEC infection</i>	88/113	(78%)	2/65	(3%)

* The other isolated *E.coli* O157:H- strain in the control group did not produce verocytotoxin and is not mentioned in this table. All methods, except the assay for VT-neutralizing ability, had $p < 0.001$ compared with controls.

Examination of stool was possible in 90 patients with HUS. By using Sorbitol MacConkey plates (VTEC serotype O157 isolation), the VT/PECS method and FVT-assay evidence for VTEC infection was provided in 54 (60%) patients with HUS. VTEC strains could be isolated in 19 HUS patients. Strains of *E.coli* belonging to serotype O157:H7 were isolated from eighteen cases and a strain belonging to serotype O157:H- in one case. All strains demonstrated cytotoxicity in the Verocell-assay which could be neutralized by polyclonal antibodies to VT2. Infection with *Shigella dysenteriae* I occurred in one patient with HUS. No *Campylobacter*, *Salmonella* or *Yersinia* species were isolated in the stool of the HUS group. Evidence for *Clostridium difficile* infection was found in three patients with HUS. One HUS patient had both *E.coli* O157:H7 and *Clostridium difficile* in the stool. The FVT assay proved to be more sensitive than the VT/PECS method. The observed cytotoxicity observed in the Verocell assays for both culture- and fecal filtrate was in all, except two, cases neutralizable with antibodies to VT2. Antibodies to VT1 neutralized the cytotoxic effect in the filtrates of two HUS

patients.

E.coli O157:H- was isolated in two control cases. One strain *E.coli* O157:H- was isolated from the non-bloody stool of a patient with coeliac disease. This isolated strain could not be classified as VTEC because culture filtrates of the strain did not demonstrate any cytotoxicity in the Verocell assay. The other *E.coli* O157:H- strain was found in the stool of a patient with acute, bloody gastro-enteritis. The cytotoxin produced by this strain could not be neutralized by the antibodies to VT1 or VT2 used in our Verocell assay. Nevertheless, using polymerase chain reaction in amplifying the genomic DNA of the strain and specific primers for VT, a positive result was obtained for VT1 and VT2 genes (Ir A Heuvelink, personal communication). Other enteric pathogens isolated from the stool of control cases were *Salmonella spp* (11x), rota-virus (9x), adeno-virus (3x), entero-virus (3x), *Campylobacter jejuni* (3x), *Giardia lamblia* (2x) and coxsackie virus, *Yersinia* and *Clostridium difficile* (1x).

The Verocell-assay for neutralizing ability to verocytotoxins was positive in paired sera samples from 4 of the 76 patients with HUS and in none of the 19 control children with acute gastroenteritis (Table 1). All detected neutralizing ability to VT were against VT1. Antibodies to VT2 or VT2c were not detected in any of the examined sera. Antibodies to O157 O-antigen were present in 71 of the 110 tested HUS patients and in one of the 53 control cases. By using both serological methods evidence for VTEC infection was present in 72 (65%) of the 110 tested HUS patients and in 1 (2%) of the 53 controls.

To investigate which of the used detection methods was the most sensitive for diagnosing VTEC infections, we examined the data of 69 patients with HUS in which all assays could be performed (Table 2). Presence of serological antibodies to O157 O-antigen was found in 70% and yielded the highest number of positive results compared to the fecal assays (59%). The neutralizing Verocell assay for VT was positive in only 3% of test cases. No further investigation was done to identify the VTEC strains with serotypes other than serogroup O157 which were responsible for the positive results in the VT/PECS and FVT assays. However, the results of VTEC O157:H7 isolation by Sorbitol McConkey agar and the positive serological tests for antibodies to the O157 O-antigen demonstrated that VTEC O157 infection was present in 76 (86%) of the 88 patients with HUS having a VTEC infection.

Stool and sera examination were performed in 95 family contacts of 28 patients with HUS admitted to one of the hospitals. In all families two or more members could be examined. Sixteen family contacts had an acute gastro-enteritis: none of the ten adults had bloody diarrhea, in four of the six siblings the diarrhea was bloody. The results are shown in Table 3. In 17 families one or more members were found to have a VTEC infection at the time the patient was admitted to the hospital. In all these seventeen families, VTEC infection was present in the respective patient as well. Nine families with a VTEC positive HUS patient had at least two family members positive for VTEC infection. Thirty three (35%) family members had evidence of VTEC infection. Evidence for VTEC infection was found in 50% of the family members with diarrhea. VTEC strain

Table 2. Combined microbiological and serological assays for VTEC detection tested in 69 patients with HUS.

Methods	No of patients with HUS positive for VTEC (n=69)	
<i>Feces</i>		
Isolation of VTEC O157	13	(19%)
VT/PEC	17	(25%)
FVT	36	(52%)
Total	41	(59%)
<i>Serology</i>		
VT-neutralizing ability	2	(3%)
Antibodies to O157	48	(70%)
Total	48	(70%)

All methods (Sorbitol MacConkey agar, VT/PECS assay, FVT assay, VT neutralization assay and ELISA and immunoblotting for antibodies to O157 O-antigen) could be used in the examination of feces and sera of 69 patients with HUS. Serological examination for the presence of antibodies to the *E.coli* O157 O-antigen gave the highest number of positive results.

O157 was isolated in 16 family members. O157:H7 and O157:H- could be isolated from the stool of 14 respectively 2 family contacts. In the stools of one family, the VTEC serotype O157:H7 was isolated in all members including the patient. *E.coli* O157:H7 was isolated on six occasions and once *E.coli* O157:H- was isolated from family members of

Table 3. Evidence for VTEC infection in 28 patients with HUS and their family contacts.

Methods	No of patients with HUS positive for VTEC (n=28)	No of family members positive for VTEC [#] (n=95)	No of families positive for VTEC (n=28)
<i>Feces</i>			
Isolation of VTEC strain	5/26 (19%)	16/81 (20%)	10/25
VT/PEC	10/26 (26%)	23/81 (28%)	16/25
FVT	18/26 (69%)*	25/75 (33%)	15/25
<i>Serology</i>			
VT-neutralizing ability	1/25 (4%)	2/54 (4%)	2/28
Antibodies to O157-antigen	21/28 (75%)*	3/85 (4%)	3/28
Total VTEC infection	26/28 (93%)	33/95 (35%)	17/28

[#] Evidence for VTEC infection in family contacts was most prominent in the siblings of the patient (49%); Evidence for VTEC infection in mothers or fathers of the patient was present in 18% and 26% of the parent respectively. * p < 0.01 , ** p < 0.001 compared with family-members.

seven HUS patients who had evidence for VTEC infection, but in whom's stools VTEC strain were not isolated. All isolated VTEC O157 strains of family members gave cytotoxicity in the Verocell assay which could be neutralized by antibodies to VT2 alone. Verocell-cytotoxicity of culture and fecal filtrate, positive in 28% respectively 33% of the samples tested, could in all cases be neutralized by antibodies to VT2 and not by antibodies to VT1. Only three of the 85 family members, children of 1, 4, and 6 years of age, had serum antibodies to the O157 O-antigen. All three children had diarrhea. Two of the three children had an acute bloody gastro-enteritis one week before the symptoms started in the patient.

Discussion

Combined microbiological and serological procedures provided evidence for VTEC infection in 78% of the patients with HUS compared with 3% in the control group of children with acute gastro-enteritis. Infection by VT-producing *E.coli* O157 were the predominant cause of diarrhea-associated HUS in our patients. These results are comparable with other published epidemiological studies in Europe in which combined microbiological and serological tests were performed [3,5,6,16]. Cytotoxicity of the culture filtrates made from the isolated VTEC O157 strains was neutralizable with antibodies to VT2 only. Population studies in the United States and United Kingdom have reported that almost all isolated strains of *E.coli* O157:H7 possessed the VT2 gene. Approximately two-thirds of these strains also contained the VT1 gene and only less than 3% of *E.coli* O157:H7 strains had only VT1 [18,23,26,28]. VT2-producing strains are associated with a higher frequency of bloody gastro-enteritis and systemic complications in human diseases [4,18,26,28]. Although the isolated *E.coli* O157 strains from HUS patients in other European studies are shown to be mainly VT2 producers [3,5,16], a recently published Canadian study reported that 22 out of the 26 VTEC O157:H7 isolated from HUS patients were VT1 and VT2 producers [21].

Not much is known about the occurrence of VTEC, especially strains belonging to serotype O157, in the human population in Europe. During an 18 month screening study for VTEC O157 in Belgium, VTEC O157:H7 was isolated in 8 (0.2%) of 3940 stool specimens [20]. The isolation rate for all VTEC in this study was 0.3%. A higher isolation rate of *E.coli* O157:H7 was found in the population studies performed in Canada (1.9%, 2.5%) and United States (2.9%) [4,11,19]. Tarr and coworkers have shown that the recovery rate for *E.coli* O157:H7 depended on the interval of days between the onset of symptoms and the collection of stool [27]. This rate decreased from 100% to 33% if the stool was cultured within two days or respectively one week after the diarrhea began. In our study, patients were hospitalized 7 ± 3 (SD) days after the onset of diarrhea. In the samples of 69 HUS patients in which all microbiological and serological methods could be performed, serological testing of antibodies to the O157 O-antigen was the most successful method for demonstrating VTEC infection. Serological testing for antibodies to

O157 O-antigen can provide evidence of VTEC infection for several weeks after the onset of diarrhea and has been proven a valuable method in VTEC detection [6]. In accordance to other studies, no neutralizing activity against VT2 or VT2c could be detected in the sera samples of patients with HUS and control cases [1,7,12]. Neutralizing activity to VT1 were found in only 4 patients with HUS. This is surprising because in this study the cytotoxicity of filtrates could be mainly neutralized by antibodies to VT2. It has been suggested that the discrepancy of no serological response to VT2 and the VT2 positive results of the fecal tests, might be due to a different ability of VT1 and VT2 to elicit a specific antibody response during the disease or the contact time to the toxin in the blood to establish an immune response may be too short [5]. The observation that the cytotoxicity of the culture filtrate of the O157:H- strain isolated from one control patient could not be neutralized by the used antibodies to VT, although the polymerase chain reaction assay of chromosomal DNA demonstrated the presence of VT1 and VT2 genes, has also been observed by others [23]. Smith *et al* observed that in the neutralization test with VT1 and VT2 producing strains, cytotoxicity could not be neutralized by antibodies to VT1 whereas partial neutralization was observed in some cases with antibodies to VT2 [23]. This might be due to a difference in VT-production of the VTEC or maybe some of these strains who have the VT1 and VT2 sequence, do not produce VT1 in contrast to VT2.

VTEC isolation, VT/PECS, FVT assay and serological tests demonstrated a high percentage (35%) of VTEC infection in the stool of mostly asymptomatic family members of HUS patients. Caprioli *et al* demonstrated a VTEC infection by using combined microbiological and serological assays in only three of the 110 family members [5]. In contrast to the study of Lopez *et al* [17], who reported that 75% of the family members of HUS patients had neutralizing serological antibodies to VT1 and or 2, in our study only 2 of the 54 family members had antibodies to VT1 and nobody had antibodies to VT2, the same percentage as in the HUS group (Table 3). Although fecal tests demonstrate that VTEC infection is often present in family members, it is remarkable that the presence of serum antibodies to O157 O-antigen were significantly lower in the family members as compared to the patients. The three family members who were positive for serum antibodies to O157 LPS, were children. Two of them had bloody diarrhea. Bloody diarrhea was a more prominent feature of VTEC infection in patients with HUS than in the family-members and correlated significantly with the presence of antibodies to O157 antigen ($p < 0.05$). Assuming that a VTEC infection in a family was due to the same strain of *E.coli* O157, these family studies might indicate that other, yet unknown, host factors in the intestine are involved in making it more easier for VTEC lipopolysaccharides to cross the mucosal surface. A difference in adherence of VTEC to the intestinal epithelium of adults and children might be an explanation for the occurrence of HUS in mostly children as compared to adults. Cimolai *et al* reported that a patient's age and prolonged use of an antimotility agent might be a risk factor for the progression of gastro-enteritis and HUS [25]. No antimotility agents were used in these three families.

Several outbreaks of VTEC infection by strains of *E.coli* of serotype O157:H7 in the

United States and Canada have revealed that the major route of acquisition of VTEC seems to be the consumption of contaminated meat [2,15], unpasteurized milk [9], exposure to contaminated water [25], and through person-to-person transmission [22]. Although there is some indication that in our family study VTEC transmission through person-to-person might be very important, this study was not designed to detect the source of VTEC. Further studies to characterize strains of *E.coli* O157 and to establish the source of these strains are currently going on.

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Chapter 2.3

ASSOCIATION BETWEEN THE EPIDEMIC FORM OF HEMOLYTIC UREMIC SYNDROME AND HLA-B40 IN THE NETHERLANDS AND FLANDERS

N. van de Kar¹, P. Reekers¹, K. van Acker², R. Donckerwolcke³, S. Ploos van Amstel⁴,
W. Proesmans⁵, E. Wolff⁶, L. Monnens¹.

Depts of Pediatrics, University Hospitals of Nijmegen¹ (NL), Antwerpen² (B),
Utrecht³ (NL), Amsterdam⁴ (NL), Leuven⁵ (B), Rotterdam⁶ (NL).

Dear Sir,

Evidence for an autosomal recessive or autosomal dominant mode of inheritance is present in a small minority of patients with hemolytic uremic syndrome (HUS) [1].

In one report of a familial relapsing autosomal dominant form, the disease was associated with HLA-A3, B7 haplotype [2]. In the Netherlands and the Flemish part of Belgium, the epidemic or classical form is most frequently observed. An infection by verocytotoxin-producing *Escherichia coli* was a proven frequent cause of this epidemic form during recent years [unpubl. study]. Not all infants and children infected by verocytotoxin-producing *E. coli* develop HUS. In one outbreak of *E. coli* O157 only 3 (8.3%) of the symptomatic children developed HUS. In another outbreak, HUS became evident in 3 (7%) of 42 symptomatic children [3]. A genetic predisposition was suspected.

Sheth et al. [4] evaluated HLA-A, B, C, DR, DQ antigens in 31 children, who had classical HUS previously. They reported a relative risk of 5 of developing HUS with the presence of HLA-B 40 alone. The severity of HUS was not related to the genetic predisposition.

The distribution of HLA-A, B and DR antigen was studied in 31 patients with chronic renal failure due to the epidemic form of HUS. All patients were on dialysis or transplanted. One patient was excluded because he was of Turkish origin. All other patients were of caucasian origin and were cared for in six transplantation centres.

Compared with the control population, there was no significant difference in the distribution of HLA-A, B, and DR antigens with one exception. An odds ratio of 2.5 (X^2 , $p=0.039$) could be calculated in our group of patients for HLA-B40 (Table 1). In contrast to Sheth et al. [4], who found that all their patients had at least one of the following HLA-B types: 40,13,7,44, which share a distinct amino acid sequence of α_1 domain, this was found in only 23 of our 31 patients (4 patients had more than 1 of the 4 HLA-B types).

We may conclude that in the severe form of epidemic HUS, as seen in the six transplantation centres there is a significant association with HLA-B 40.

Table 1. Comparison of HLA frequencies in Sheth's study compared with our study.

	Study of Sheth (n=31)			Our study (n=31)		
	patients		random population	patients		random population
	%	n		%	n	%
B40	45	14	14	25	8	12
B13	13	4	6	0	0	3.3
B44	23	7	23	34.4	11	22.1
B7	23	7	23	25	8	28.9

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Chapter 3

PLASMA CYTOKINE LEVELS IN THE HEMOLYTIC UREMIC SYNDROME

Nicole C.A.J. van de Kar^{1,5}, Robert W. Sauerwein³, Pierre N.M. Demacker²,
Georges E. Grau⁴, Victor W.M. van Hinsbergh⁵, Leo A.H. Monnens¹.

¹Dept of Pediatrics, University Hospital, Nijmegen, The Netherlands.

²Dept of Internal Medicine, University Hospital, Nijmegen, The Netherlands

³Dept of Medical Microbiology, University Hospital, Nijmegen, The Netherlands

⁴Centre Medical Universitaire, Geneva, Switzerland

⁵Gaubius Laboratory, TNO-PG, Leiden, The Netherlands

Submitted.

Summary

The cytokines tumor necrosis factor α (TNF α) and its soluble TNF receptors 55 and 75 (sTNFR55, sTNFR75), interleukin-1 β (IL-1 β) and interleukin-6 (IL-6) were measured in the plasma from 13 patients with the hemolytic uremic syndrome (HUS) on admission. No significant changes in the plasma-levels of TNF α and IL-1 β were detected in the HUS patients as compared to the plasma-levels of the control groups. Levels of IL-6 were significantly elevated in the plasma of those HUS patients, who had extra-renal manifestations, consisting of seizures, loss of consciousness, coma and pancreatic necrosis. Although the exact function of IL-6 in the plasma of HUS patients is still unknown and the group of HUS patients is small, plasma IL-6 is associated with the severity and outcome of the disease. Plasma levels of sTNFR55 and sTNFR75 were significantly elevated in all HUS patients compared to the healthy controls, but they were also elevated in the children with chronic renal failure. This indicates that elevated levels of circulating sTNFR should be carefully interpreted when kidney failure exist.

Introduction

Hemolytic uremic syndrome (HUS) is characterized by hemolytic anemia, thrombocytopenia and acute renal failure. The most common form of HUS seen in children is the epidemic form, which is preceded by an acute, often bloody, gastro-enteritis [1]. In the last decade it has become clear that infections with a verocytotoxin-producing *E.coli* strains are the main cause of HUS in childhood [2,3]. Although the exact pathogenesis of HUS is still unknown, it is generally accepted that endothelial cell activation and damage play a central role. Cytokines such as tumor necrosis factor α (TNF α), interleukin-1 β (IL-1 β) and interleukin-6 (IL-6) are known to play an important role in infectious and inflammatory diseases, and have been extensively studied in diseases such as sepsis, severe infectious purpura and Kawasaki disease [4-6]. Elevated levels of cytokines are also found in plasma taken in the acute phase of patients with thrombotic thrombocytopenic purpura (TTP), a syndrome which is in many aspects familiar with HUS [7].

The aim of this study was to determine whether circulating cytokines are detectable in the blood of HUS patients. For this purpose, we measured TNF α and its soluble receptors sTNFR55 and sTNFR75, IL-1 β and IL-6 in the plasma of 13 patients admitted to the hospital with the epidemic form of HUS.

Patients and Methods

Patients

Thirteen children (4 females / 9 males, mean age 38 ± 28 months; range 6 - 112 months),

were diagnosed as having HUS, because of the presence of hemolytic anemia with burr cells in the peripheral blood smear, renal failure and thrombocytopenia [8]. HUS was preceded by mostly blood-stained diarrhea. Antibodies against the most common verocytotoxin-producing *E.coli* (VTEC) serotype O157, which indicates an infection with VTEC, were detected in the plasma of seven out of twelve patients [9].

The main laboratory indexes of the patients on admission are displayed in Table 1. On the day of admission to the hospital, before any treatment was given, blood from these thirteen children was collected into EDTA containing tubes, centrifuged and the plasma was stored in small aliquots at -70°C until assays were performed. Plasma of eight age-matched healthy control children and eight children known with chronic renal failure taken before dialysis, served as controls.

Table 1. Laboratory indices of patients with HUS on the day of admission.

Number of patient	Age (months)	Hb (mmol/l)	Platelets (10 ⁹ /l)	WBC (10 ⁹ /l)	Urea (mmol/l)	Creatinine (μmol/l)
1.	14	3.9	276	22.3	20.2	106
2.	21	4.9	45	20.4	33	295
3.	6	4.7	25	25.5	29.6	350
4.	53	5.3	56	31.5	36	770
5.	112	5.5	55	10.9	67	1020
6.	36	4.2	138	17.7	40	640
7.	54	3.7	203	8.8	63.2	928
8.	10	4.1	56	23.2	61.6	514
9.	41	3.7	130	12	45	178
10*.	22	3.9	37	19.5	26.9	288
11*®.	36	9.2	304	24.1	6.8	54
12*.	22	7.5	11	53.6	68.2	335
13*.	40	5.7	30	33.6	17.2	157
Normal values children		6-9	140-440	4-11	3-7	30-90

The laboratory indexes are given for each HUS patient, individually indicated by numbers. ® On the second day after admission the laboratory parameters of patient 11 were as following: Hb 4.5 mmol/l, platelets 25 x 10⁹/l, urea 10.5 mmol/l and creatinine 130 μmol/l. * Patients 10,11,12 and 13 had, in contrast with the other HUS patients, severe extra-renal manifestations, consisting of seizures, loss of consciousness, coma. Pancreatic necrosis was also diagnosed in patient 10.

Methods

TNFα was assessed by radioimmunoassay (RIA), as previously described [10]. This RIA measures total TNFα (both free and complexed to its receptors), as demonstrated by the lack of interference of the addition of up to 5 ng/ml recombinant sTNFR55 and sTNFR75) to sera containing known amounts of TNFα. The sensitivity of this assay is 100 pg/ml. IL-1β assay was also performed with a RIA [11]. Before application, both RIA's (TNFα and IL-1β) were thoroughly validated towards linearity and determination

of matrix effects. When 100 μ l of plasma was tested, recovery experiments of the respective cytokine resulted in recovery values exceeding 90%, both at the low and the high concentration range tested. IL-6 was measured by an ELISA as described by Barrera et al [12]; materials were a gift from Dr.J.Wijdenes (Innotherapy, Besancon, France). The sensitivity of this ELISA is 20 pg/ml.

Soluble TNF receptors (sTNFR55 and sTNFR75) were measured by ELISA (Hoffmann-La Roche, Basel, Switzerland). These assays measure total circulating (both free and bound) receptor concentrations, since addition of up to 10 ng/ml recombinant TNF α to plasma containing known amounts of sTNFR does not influence these assays. The sensitivity of these assays is 100 pg/ml.

Statistical analysis

Results are expressed as mean $\pm$ SD. The significance of differences between the various groups of patients was determined by using the Mann-Whitney test. The Spearman's ranks test was used for determining the correlation between cytokine levels and the laboratory indexes in the HUS group.

Results

The individual data of cytokine levels in the plasma of the HUS patients are displayed in Table 2. Only the patients 10,11,12 and 13 had severe extra-renal manifestations, consisting of seizures, loss of consciousness and coma. Patient 10 also developed necrosis of the pancreas. The latter three patients (11-13) died due to cerebral complications in the acute phase of the disease. No significant increased levels of TNF α and IL-1 β were found in the plasma of patients with HUS as compared to the control groups (Table 2). Only the four patients, who had extra-renal manifestations (severe form of HUS), had significantly elevated levels of IL-6 (277 pg/ml $\pm$ 103), as compared to the control group of healthy children, whereas the IL-6 levels in the plasma of the other HUS patients (<30 pg/ml, mild form of HUS) was not significantly elevated. Significant increased levels of both soluble TNF receptors were found in all HUS patients (sTNFR55 22 ng/ml $\pm$ 12 and sTNFR75 21 ng/ml $\pm$ 13) as compared to the control group of healthy children (sTNFR55 1.4 ng/ml $\pm$ 1 and sTNFR75 4.2 ng/ml $\pm$ 1). There was no difference in the plasma concentrations of sTNFR55 and sTNFR75 between the mild and severe forms of HUS. However, both sTNFRs were also increased in the plasma of the children with chronic renal failure (17 ng/ml $\pm$ 6. for sTNFR55 and 27 ng/ml $\pm$ 7 for the sTNFR75), which suggest that renal failure per se, and associated with a decrease in clearance causes high levels of sTNFR in the plasma.

Plasma levels of TNF α , IL-1 β or sTNFR75 did not correlate with hemoglobin, platelets, white blood cell count, urea or creatinine. Only sTNFR55 and IL-6 showed a mild correlation with hemoglobin ($r=0.56$ respectively $r=0.52$) and white blood cell count (both $r=0.60$, Spearman's correlation) in the total HUS group.

Table 2. Plasma cytokine levels in patients with the hemolytic uremic syndrome.

Number of patient	TNF α ng/ml	IL-1 β ng/mL	IL-6 pg/ml	sTNFR55 ng/ml	sTNFR75 ng/ml
1.	0.15	< 0.10	< 20	3.5	9.3
2.	0.10	< 0.10	< 20	13.2	9.0
3.	0.14	< 0.10	30	45.1	6.4
4.	0.15	< 0.10	< 20	38.5	39.8
5.	0.15	< 0.10	< 20	17.2	10.9
6.	0.12	< 0.10	< 20	26.4	35.7
7.	0.13	< 0.10	< 20	11.1	4.3
8.	0.15	< 0.10	20	nm	nm
9.	0.13	< 0.10	< 20	10.1	20.6
10 ^a .	0.15	< 0.10	275	22.0	24.7
11 ^a .	0.13	< 0.10	160	28.2	34.0
12 ^a .	nm	nm	262	32.1	33.2
13 ^a .	0.14	< 0.10	410	20.2	22.4
Patients 1-9	0.13 $\pm$ 0.02	< 0.085	< 30	20 $\pm$ 16 ^{##}	17 $\pm$ 14 [#]
Patients 10-13	0.13 $\pm$ 0.05	< 0.10	277 $\pm$ 103 [*]	26 $\pm$ 6 [#]	29 $\pm$ 6 [#]
Control (n=8)	0.12 $\pm$ 0.02	0.07 $\pm$ 0.02	< 20	1.4 $\pm$ 1	4.2 $\pm$ 1
CRF (n=8)	0.14 $\pm$ 0.04	0.08 $\pm$ 0.05	< 20	17 $\pm$ 6 ^{##}	27 $\pm$ 7 [#]

Plasma cytokine levels were determined on admission in patients with HUS. ^a Patients 10,11,12 and 13 had, in contrast with the other HUS patients, severe extra-renal manifestations, consisting of seizures, loss of consciousness, coma. Pancreatic necrosis was also diagnosed in patient 10. The data are expressed individually and as the mean $\pm$ SD of the various groups. To compare the data of the different groups of patients and healthy controls the Mann-Whitney test was used. [#] $p < 0.05$, ^{##} $p < 0.001$ as compared to the control group; ^{*} = $p < 0.01$ as compared to HUS patients (and the control group). nm means not enough material for the test available.

Discussion

The role of circulating cytokines and soluble TNF receptors in the pathogenesis of HUS was investigated in this study. No elevated levels of TNF α , IL-1 β and IL-6 were found in the plasma of HUS patients, who had no extra-renal manifestations during the disease. In the study done by Fitzpatrick et al, in which IL-8 and TNF α were measured in the plasma of 16 HUS patients, only one patient had an elevated concentration of TNF α in the plasma [13]. However, the occurrence of these cytokines in the plasma during the initial phase of the disease can not be excluded, because these cytokines have a short half-life (less than 1 hour) and may therefore no longer be detectable in the plasma taken on admission in the hospital.

On the other hand, Siegler et al [14] reported that, although TNF α is not detectable in plasma, it is elevated in the urine of HUS patients, suggesting that TNF α may be locally produced in the kidney and have a local effect in the kidney of HUS. Elevated levels of urinary cytokines have also been found in other kidney diseases and are believed to play a role in local inflammatory and infectious processes in the kidney [15]. Evidence for locally

produced cytokines in the kidney has also been obtained by immuno-staining and in situ hybridization [16] and from in vitro experiments with renal cells [17,18]. Evidence for a local role of $\text{TNF}\alpha$ in HUS was recently given by Harel et al [19], who gave shiga like toxin I to transgenic mice bearing a chloramphenicol acetyltransferase (CAT) reporter gene with the $\text{TNF}\alpha$ promoter. When given shiga like toxin I to these mice, CAT activity, which reflects the degree of the activation of $\text{TNF}\alpha$ synthesis, was induced within the kidney, but not in other tissues. Increased production of the cytokines $\text{TNF}\alpha$, $\text{IL-1}\beta$ and IL-6 can also be found in the media of cultured human monocytes after stimulation with verocytotoxin-1 [20]. A possible role for the locally produced $\text{TNF}\alpha$ in the kidney, could be to increase the susceptibility of the endothelial cells locally for verocytotoxin by inducing more verocytotoxin receptors on the cell surface [21].

Elevated levels of IL-6 were only found in the plasma of those HUS patients, who had extra-renal manifestations, consisting of seizures, loss of consciousness, coma and pancreatic necrosis. In inflammatory conditions IL-6 is synthesized by a wide variety of cell-types (macrophages, endothelial cells and fibroblasts) upon stimulation with $\text{TNF}\alpha$, IL-1 and other agents. The major effects of IL-6 in an infection, are the induction of acute phase proteins in hepatocytes and the final differentiation of B-cells into antibody-producing cells. We have shown before, that IL-6 did not induce the receptor of verocytotoxin on cultured human endothelial cells and therefore did not increase the susceptibility of the endothelial cells for verocytotoxin, in contrast to $\text{TNF}\alpha$ and IL-1 [21]. Although the exact function of IL-6 in the plasma of HUS patients is still unknown and the group of HUS patients is small, plasma IL-6 is associated in our study with the severity and/of outcome of the disease. Closely related to HUS is thrombotic thrombocytopenic purpura (TTP), a syndrome which in many aspects is familiar to HUS (micro-angiopathic hemolytic anemia, thrombocytopenia and renal impairment), but in which always generalized symptoms (fever and neurological complications) are present. In the plasma of all the thirteen patients of TTP, examined by Wada et al [7], elevated circulating levels of $\text{TNF}\alpha$, $\text{IL-1}\beta$ and IL-6 were found. These data suggest that in patients with TTP and in severe cases of HUS, who develop also extra-renal manifestations, circulating cytokines may play a role in the pathogenesis of the disease.

Recently, two immunologically distinct soluble receptors sTNFR55 and sTNFR75 have been identified [22]. These soluble receptors represent forms of the extracellular domain of the cell surface receptors for $\text{TNF}\alpha$ and studies done in healthy volunteers indicate that $\text{TNF}\alpha$ itself is involved in the induction of sTNFR release [22,23]. Recent studies have shown that in sepsis and in malaria elevated levels of sTNFR are present [22,24,25]. Although both sTNFR were elevated in the plasma of HUS patients, they were also elevated in the group of children with chronic renal failure. This implies that these elevated sTNFR levels can not only be explained by increased activity of $\text{TNF}\alpha$ or by other substances with proteolytic activity, like elastase, but may be caused by insufficient kidney function. In favor of this hypothesis is the recently report by Bemelmans et al [26], who demonstrated in a murine model that kidney malfunction induces an increase in the amount of sTNFRs in the plasma.

In conclusion, no elevated levels of circulating $\text{TNF}\alpha$, $\text{IL-1}\beta$ and IL-6 were detected in

the mild form of HUS. On the other hand, elevated levels of IL-6 in the plasma were detected in HUS patients who displayed severe extra-renal manifestations. Probably locally produced cytokines may be more important in the pathogenesis of HUS than the circulating concentration. However, when in HUS extra-renal manifestations occur, circulating cytokines may have played or play a role in the pathogenesis. Results of elevated circulating sTNFR as shown in this study, should be carefully interpreted when kidney failure exist.

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Chapter 4

THE FIBRINOLYTIC SYSTEM IN THE HEMOLYTIC UREMIC SYNDROME: IN VIVO AND IN VITRO STUDIES

Nicole C.A.J. van de Kar^{1,2}, Victor W.M. van Hinsbergh¹,
Emile J.P. Brommer¹, Leo A.H. Monnens²

¹Gaubius Laboratory TNO-PG, Leiden, The Netherlands,

²Department of Pediatrics, University Hospital Nijmegen, The Netherlands.

Summary

Fibrinolytic parameters and von Willebrand factor (vWF) antigen were measured in the plasma of 10 patients with hemolytic uremic syndrome (HUS). Samples were taken at presentation and again two weeks later, before and after infusion of 1-desamino-8-arginine vasopressin (DDAVP). Compared with the plasma values of healthy control children, levels of tissue-plasminogen activator (t-PA) antigen, plasminogen activator inhibitor type I (PAI-1) activity, vWF as well as fibrin(ogen) degradation products were significantly elevated in the plasma of HUS patients on admission. No response of the fibrinolytic parameters and vWF were seen when DDAVP-infusion was given on admission. After two weeks, t-PA antigen and vWF had partially returned to basal values, and t-PA antigen increased rapidly again after DDAVP-infusion.

In order to investigate whether verocytotoxin contributes to the alteration of the fibrinolytic system found in HUS patients, purified verocytotoxin-1 (VT-1) was added to the media of cultured human endothelial cells. Addition of VT-1 alone did not change the production of t-PA, PAI-1 and vWF antigen in these cells. However, when the endothelial cells were pre-incubated with tumor necrosis factor α (TNF- α) to increase the number of VT-1 receptors, VT-1 induced a marked decrease of the synthesis of t-PA, PAI-1 and vWF. This was due to a decrease in overall protein synthesis in the TNF- α - and VT-1-treated endothelial cells.

We conclude from this study that the systemic fibrinolytic parameters measured in the plasma of HUS patients, are probably not a direct effect of VT-1 on the endothelium, but are sequelae of the disease in which the intestine and the kidney are predominantly affected.

Introduction

Hemolytic uremic syndrome (HUS) is characterized by the triad microangiopathic hemolytic anemia, thrombocytopenia and acute renal failure. The most common form of HUS seen in children is the epidemic form, which is preceded by an acute, often bloody, gastro-enteritis. Although the exact pathogenesis is not known, it is evident that the endothelium plays a central role. Histopathological studies of the kidney show swollen endothelial cells, detached from the glomerular basement membrane and bulging into the lumen of the capillaries, thrombi and fibrin depositions. These thrombotic obstructions contribute to the acute renal failure in HUS patients [1]. Since the beginning of the eighties several studies have demonstrated that verocytotoxin-producing *E.coli* are the main cause of the epidemic form of HUS [2]. In vitro studies on human endothelial cells have shown that verocytotoxins are able to damage endothelial cells and may lead to inhibition of the protein synthesis in the cell [3,4].

The endothelial cells play an important role in the maintenance of the hemostatic balance in the blood [5]. Besides having anticoagulant properties, the endothelial cells also prevent

the aggregation of platelets and stimulate fibrinolysis. Endothelial cells are the principal source of tissue plasminogen activator (t-PA) in the blood. In addition, they synthesize and secrete plasminogen activator inhibitor type 1 (PAI-1) [6]. PAI-1 can also be liberated from platelets, but the platelet PAI activity is rather low and only contributes to a minor degree to a prolonged elevation of PAI activity in the circulating blood [7]. Endothelial cells not only provide circulating t-PA under resting conditions, but they can also rapidly release t-PA upon stimulation by various agents including 1-desamino-8-D-arginine vasopressin (DDAVP). This results in a marked increase in the fibrinolytic capacity of the blood [8] and is also accompanied by a marked, transient, release of von Willebrand factor (vWF) antigen from endothelial storage sites [9]. In addition to the increase in circulating t-PA and vWF, the urokinase (u-PA) concentrations also rapidly increase in plasma after DDAVP-infusion [10]. The mechanisms by which DDAVP causes increases in t-PA, u-PA and vWF are not completely understood, but it is likely that extrarenal V2-like receptors are involved in the effects of DDAVP [11].

The role of the fibrinolytic system in HUS has recently been highlighted by the report of Bergstein et al. [12], who suggest that a decrease in PAI-1 antigen during peritoneal dialysis in HUS patients contributes to the recovery of the patients. In order to get a better insight into the fibrinolytic system in HUS patients and to see if the regulation of the production of the fibrinolytic proteins by the endothelial cell is disturbed, we have measured fibrinolytic parameters in plasma samples taken on admission and after two to three weeks (convalescent phase), before and after stimulation with DDAVP. In addition we have investigated whether verocytotoxin contributes to the change in the circulating fibrinolytic proteins and vWF. To that end, we have examined whether human endothelial cells in culture change their synthesis of fibrinolytic proteins when they are incubated with purified verocytotoxin-1 (VT-1).

Materials and Methods

Patients

Ten children (5 females/5 males, mean age 27.2 ± 16 months; range 6.5 - 52.5 months), were diagnosed as having HUS, based on the presence of hemolytic anemia with burr cells in the peripheral blood smear, renal failure and thrombocytopenia [13]. HUS was preceded by diarrhea, usually blood-stained. Seven patients needed peritoneal dialysis, which ranged from one to 10 days (mean 5.3 ± 3.3 days). In the blood of eight of these 10 patients, antibodies were detected against the most common verocytotoxin-producing *E.coli* (VTEC) serotype O157, indicating an infection with VTEC [14]. In the faeces of one of the other two patients a VTEC serotype O157:H7 was isolated. The main laboratory indexes of the patients are displayed in Table 1. In the first morning after admission to the hospital, before dialysis was undertaken, $0.3 \mu\text{g/kg}$ body weight DDAVP, diluted in 100 ml saline, was administered intravenously over a period of 30 minutes. The same DDAVP-infusion was given to nine of the 10 HUS patients in the convalescent phase, ranging from 10 to 21 days after admission

Table 1. Laboratory indexes of patients known with HUS.

	On admission (n=10)		Convalescent phase (n=9)	
	mean	± SD (range)	mean	± SD (range)
Hemoglobin (mmol/l)	4.5	± 1.0 (3.4-6.5)	5.4	± 0.7 (4.5-6.4)
Platelets (x 10 ⁹ /ml)	63	± 59 (23-203)	398	± 159 (171-652)
Leucocytes (x 10 ⁶ /l)	17.2	± 8.2 (7.6-33.8)	8.4	± 2.5 (5.5-12.5)
Urea (mmol/l)	38	± 17 (12-63.9)	6.3	± 1.7 (3.9-9.6)
Creatinine (μmol/l)	386	± 265 (139-928)	60	± 14 (44-84)
Fibrinogen (g/l)	3.1	± 1 (1.5-4.6)	not determined	
Presence of VTEC infection	9 patients			

(14 ± 4 days, mean ± SD). A 4.5 ml blood sample was collected immediately before and after the DDAVP-infusion. Blood samples were transferred to plastic tubes, containing 0.5 ml 3.8% sodium citrate, and immediately cooled on melting ice. Platelet-poor plasma, obtained by centrifugation at 4°C for 10 minutes at 3000 g, was quickly frozen and stored at -70°C until assays were performed. In the same way, control blood samples were taken from ten children with chronic renal failure (CRF); from six children immediately before the hemodialysis session and 4 children during peritoneal dialysis. Blood samples from thirteen age-matched children in good clinical condition and with no underlying hematological or renal disease were used as controls. This study was approved by the local Institutional Committee of the Department of Pediatrics and carried out according to the guidelines of the Institutional Review Board of the Sint Radboud Hospital in Nijmegen, the Netherlands.

Material

1-desamino-8-D-arginine vasopressin (DDAVP), Minrin[®] was obtained from Ferring Pharmaceuticals AB (Malmö, Sweden). Purified verocytotoxin-1 (VT-1) was prepared in the laboratory of Dr. Karmali (1.2 mg protein/ml; CD₅₀ vero-cells: titer 10⁻⁸-10⁻⁹M) [15]. Endotoxin content of the VT-1 preparation was < 0.05 EU/ml by Limulus amoebocyte lysate assay (E-Toxic, Sigma Chemicals Co (St. Louis, MO); at detection level of 0.05-0.1 EU/ml). Monoclonal antibodies PH1 against VT-1 was a generous gift from Dr. C. Lingwood, Dept. of Microbiology, Hospital for Sick Children, Toronto, Canada. M199 medium supplemented with 20 mmol/l HEPES was obtained from Flow Laboratories (Irvine, Scotland); tissue culture plastics were from Costar (Cambridge, MA). A crude preparation of endothelial cell growth factor was prepared from bovine brain as described by Maciag et al. [16]. Human serum was obtained from the local blood bank and was prepared from fresh blood of healthy donors, pooled, and stored at 4°C; it was not heat-inactivated before use. Newborn calf serum (NBCS) and fetal calf serum (FCS) were from GIBCO (Grand Island, NY) and Boehringer Mannheim (Mannheim, Germany), respectively; they were heat-inactivated before use (at 56°C for 30 min). Heparin was purchased from Leo Pharmaceuticals (Weesp, the

Netherlands). Penicillin/streptomycin was from Boehringer Mannheim (Mannheim, Germany). Human fibronectin was a gift of J.A. van Mourik, Central Laboratory of the Red Cross Blood Transfusion Service (Amsterdam, the Netherlands). Human recombinant tumor necrosis factor α (TNF- α) was a gift from Jan Tavernier (Biogent, Ghent, Belgium). The TNF- α preparation contained 2.45×10^{-7} U/mg protein and less than 40 ng LPS per mg protein. ^{35}S - methionine was purchased from Amersham (Amersham, UK).

Isolation and culture of cells

Endothelial cells from human umbilical vein were isolated by collagenase treatment [17], cultured and characterized as previously described [18]; endothelial cells from human foreskin were isolated and characterized as described [19]. The tissues for isolation of endothelial cells were obtained according the guidelines of the Institutional Ethical Committee of the Academic Hospital and St.Elisabeth Hospital in Leiden, the Netherlands. The endothelial cells were seeded in fibronectin-coated 10 cm² wells and cultured in M199 medium supplemented with 20 mmol/l HEPES (pH 7.4), 10% human serum (HS), 10% newborn calf serum (NBCS), 2 mmol/l L-glutamine, 5 U/ml heparin and 150 $\mu\text{g}/\text{ml}$ crude preparation of endothelial cell growth factor under 5%CO₂ and 95% air at 37°C. When the cells reached confluency, they were detached by trypsin/EDTA and seeded in 2 cm² fibronectin-coated dishes with a split ratio of 1:3. The medium was renewed every two or three days. The cells in the experiments were used after 2-4 passages. Experiments of endothelial cells from umbilical cord and foreskin used in this study, were isolated from three different donors.

In vitro experiments

For the experiments cells were released with trypsin/EDTA and seeded in a split-ratio 1:3 in 2 cm² fibronectin-coated plates and cultured in complete culture medium (400 $\mu\text{l}/\text{well}$). In the culture medium, 20% FCS was used instead of 10% human serum and 10% NBCS, because neutralising activity of NBCS was found against VT-1 and because of potential neutralising activity against VT-1 in human pooled serum. When confluency was reached, medium was renewed and the cells were pre-incubated for 24 hours with or without TNF- α . After this pre-incubation period media were renewed and media with or without VT-1 (17 pmol/l - 17 nmol/l) were added. 24 hours later the media above the cells were collected, centrifuged and the supernatant was stored at -20°C until fibrinolytic assays were performed. The cells were washed with M199 medium and released with trypsin/EDTA. The cells were diluted with 1:1 trypan blue dye solution and counted in a hemocytometer. Those cells, that did not absorb trypan blue dye, were recognized as viable cells. The experiments were done in duplicate wells.

Protein synthesis was determined by assay of the incorporation of ^{35}S -labelled methionine (0.25 $\mu\text{Ci}/\text{ml}$ added to complete culture medium) in ^{35}S -proteins during an 24- hour incubation period. After incubation the cells were washed and the cellular ^{35}S -labelled proteins were precipitated by adding 10% trichloroacetic acid. Precipitated radioactivity was dissolved in 0.3 ml 0.3 mol/l NaOH and counted in a liquid scintillation counter. Protein was

determined according to the method of Lowry using bovine serum albumin as a standard [20].

Fibrinolytic assays

Assay of t-PA antigen was performed with the ELISA Thrombonostika t-PA from Organon Teknika (Turnhout, Belgium) as described by Bos et al. [21]. PAI-1-antigen in the patients' sera was determined using the commercially available TintElize PAI-1 kit from Biopool (Umeå, Sweden). For the in vitro assays the Immulyse ELISA from Biopool was used. For the u-PA antigen determination a sandwich ELISA was used as described by Binnema et al. [22]. The assay measures the u-PA antigen present in plasma, irrespective of molecular form i.e. pro-u-PA, active u-PA and u-PA in complex with inhibitors. The plasmin-activable single-chain u-PA (scu-PA) was measured with a biological immuno-assay [23]. PAI activity was determined by a titration method with purified t-PA. Residual activity of t-PA was measured with a spectrophotometric assay and the inhibitor activity was calculated from the amount of t-PA inhibited as described by Verheijen et al. [24]. vWF antigen was measured with a modified ELISA adapted from Ingerslev, using pooled plasma from healthy donors as a reference (= 100%) [25]. Fibrinogen was measured with the chronometric assay as described by Clauss [26]. Total fibrin(ogen) degradation products (TDP), fibrin degradation products (FbDP) and fibrinogen degradation products (FgDP) were measured with the sandwich-type enzyme immunoassays Fibrinostika TDP, Fibrinostika FbDP and Fibrinostika FgDP respectively, from Organon Teknika (Boxtel, the Netherlands). The data of TDP, FbDP and FgDP are expressed as mg fibrinogen equivalent unit/l [27]. All measurements were done in duplicate.

Statistical analysis

Results are expressed as mean $\pm$ SD. The significance of differences between the different groups of patients was determined by using the Mann-Whitney test. The Wilcoxon-test was performed for paired data when the fibrinolytic parameters before and after DDAVP-infusion in HUS-patients were compared. The Pearson's coefficient correlation-test was used to determine the correlations between fibrinolytic parameters, - clinical severity and laboratory indexes.

Results

Patients

Table 2 summarizes fibrinolysis parameters and vWF antigen values in the blood of children with HUS, children with chronic renal failure and of children with no underlying hematological or renal disease (control children). Basal values of t-PA antigen were lower in control children ($2.8 \pm 1.5 \mu\text{g/l}$) than in healthy adults ($6.4 \pm 2.3 \mu\text{g/l}$) [21]. These observations resemble the recently published fibrinolytic parameters measured in plasma of children by Andrew et al. [28]. Basal values of u-PA antigen were higher in control children

Table 2. Fibrinolytic parameters and vwf measured in plasma of patients with HUS.

	HUS (n=10) Admission	HUS (n=9) Convalescent	CRF (n=10)	Control (n=13)
t-PA antigen ($\mu\text{g/l}$)	9.2 $\pm$ 3.9	4.5 $\pm$ 1.3 [#]	2.5 $\pm$ 2.3 ^{***}	2.8 $\pm$ 1.5 ^{***}
PAI-1 antigen ($\mu\text{g/l}$)	68 $\pm$ 75	21 $\pm$ 6	27 $\pm$ 21	29 $\pm$ 13
PAI activity ($\text{IU} \times 10^3/\text{l}$)	14.8 $\pm$ 11.4	5.2 $\pm$ 1.8 [#]	5.9 $\pm$ 2.6 ^{***}	3.4 $\pm$ 0.8 ^{***}
u-PA antigen ($\mu\text{g/l}$)	5.2 $\pm$ 1.8	5.8 $\pm$ 1.2	5.6 $\pm$ 1.7	4.9 $\pm$ 1.2
scu-PA ($\mu\text{g/l}$)	2.5 $\pm$ 0.7	3.2 $\pm$ 0.5 [#]	3.1 $\pm$ 1.3	3.2 $\pm$ 0.6
vWF				
(% of pooled plasma)	311 $\pm$ 92	166 $\pm$ 87 [#]	180 $\pm$ 69 [*]	105 $\pm$ 62 ^{***}
TDP (mg/l)	11 $\pm$ 8	2.7 $\pm$ 4.7 [#]	0.9 $\pm$ 1.3 [*]	0.26 $\pm$ 0.20 ^{***}
FbDP (mg/l)	6.7 $\pm$ 6.7	0.9 $\pm$ 1.3 ^{##}	0.9 $\pm$ 1.9 [*]	0.07 $\pm$ 0.05 ^{***}
FgDP (mg/l)	3.9 $\pm$ 1.9	1.5 $\pm$ 2.9	0.5 $\pm$ 1.0 ^{***}	0.07 $\pm$ 0.09 ^{***}

The data of HUS patients on admission (mean $\pm$ SD) are compared with data from the same patients two or three weeks after admission (convalescent phase), with those of patients with chronic renal failure (CRF) and those of the control group with no hematological and nephrological problems (control). To determine significant changes in the HUS patients, paired data obtained on admission and in the convalescence phase were compared with the Wilcoxon test. [#] = $p < 0.05$, ^{##} $p < 0.01$ as compared to data on admission. To compare the data of different groups of patients, the Mann-Whitney test was used. ^{*} = $p < 0.01$, ^{***} = $p < 0.001$ as compared to the HUS group on admission

(4.9 $\pm$ 1.2 $\mu\text{g/l}$) than in adults (3.5 $\pm$ 1.2 $\mu\text{g/l}$) [23]; basal values of vWF antigen and PAI-1 antigen are comparable to adults [29]. In accordance with other studies [30] elevated vWF concentrations were found in children with acute and chronic renal failure.

The plasmas of HUS patients on admission had significantly elevated levels of t-PA antigen, vWF antigen and PAI activity compared with control children. PAI activity correlated very well with PAI-1 antigen ($r=0.71$, Pearson's coefficient correlation). t-PA antigen levels measured from plasma taken on admission, showed moderate correlation with PAI activity and resp vWF ($r=0.67$ resp $r=0.59$, Pearson's coefficient correlation). No correlation was found for the fibrinolytic parameters and the clinical severity, white blood cell count, platelets or leucocytes. Only vWf antigen correlated with the amount of white blood cells on admission.

After two to three weeks hospitalization, the increases in t-PA antigen and vWF antigen were reduced by about 75% and the PAI activity levels had been normalized (Fig. 1). In contrast to children with HUS, children with chronic renal failure showed similar plasma t-PA antigen and PAI-1 antigen levels as control children; PAI activity and vWF antigen were higher in these patients than in the control group. Elevated levels of TDP, FbDP and FgDP were detected in the plasma of HUS patients on admission. A decline in these degradation products was seen in the convalescent phase, but the values were not yet entirely normalized (Fig. 1).

The concentration of circulating t-PA, u-PA and vWF can rapidly be increased by infusion of DDAVP. When DDAVP infusion was given upon admission to the HUS patients, we observed no significant increase in t-PA antigen or vWF antigen in the plasma (Table 3).

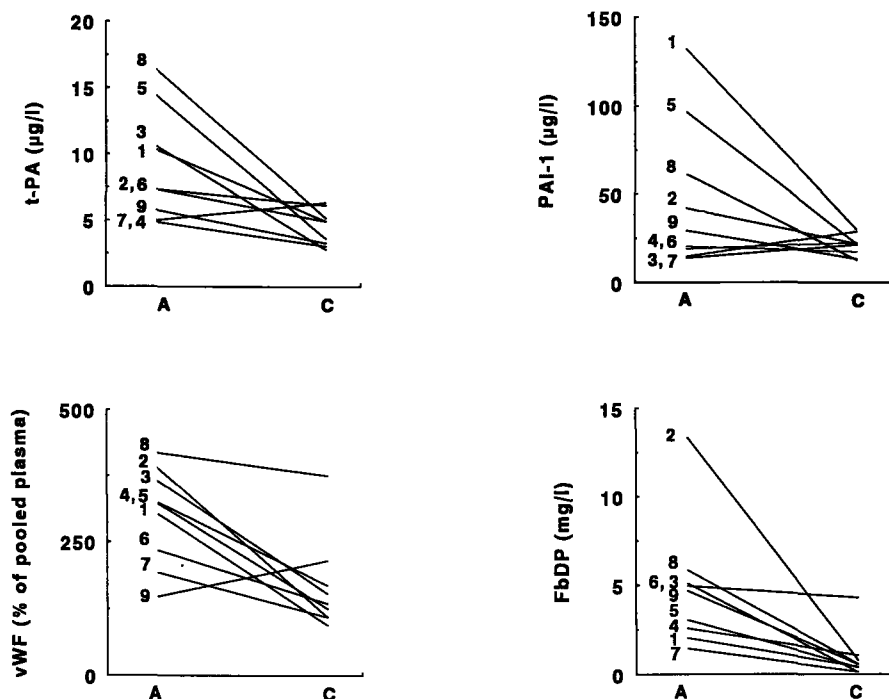


Figure 1. T-PA antigen, PAI activity, vWF antigen and fibrin degradation products (FbDP) measured in the plasma of nine HUS patients, individually indicated by numbers. Measurements were done in plasma taken on admission (A) and in the convalescent phase of HUS (C).

Neither did the plasma concentration of u-PA antigen change in HUS patients during DDAVP infusion. When DDAVP was given to the same patients two to three weeks later during the convalescent phase, an increase in t-PA antigen was found in all patients, whereas no increase was seen for vWF antigen or u-PA antigen (Table 3).

In vitro experiments

It is generally assumed that endothelial damage by verocytotoxins plays a key role in the pathogenesis of the epidemiological form of HUS. As the prolonged elevations of the plasma t-PA, PAI-1 and vWF levels suggest that the synthesis of these proteins in endothelial cells is enhanced, we examined whether verocytotoxin-1 (VT-1) changes the production of t-PA and PAI-1 by human endothelial cells in vitro. Fig. 2A shows that VT-1 did not significantly affect the production of t-PA and PAI-1 in non-stimulated confluent umbilical vein endothelial cells. To enhance the sensitivity of human umbilical vein endothelial cells for VT-1, we also studied these parameters in endothelial cells that had been preincubated for 24 h with TNF α . This preincubation causes a marked increase in the expression of VT-1 receptors [4]. Under these conditions VT-1 markedly reduced the synthesis of t-PA and PAI-1 (Fig. 2B). In agreement of the known inhibitory effect of VT-1 on the cellular protein synthesis, a marked decrease in the synthesis of cellular proteins and in the number of cells was

Table 3. Effect of DDAVP on fibrinolytic parameters and vwf measured in plasma of patients with HUS.

	HUS on admission (n=10)		HUS convalescent phase (n=9)	
	before DDAVP	after DDAVP	before DDAVP	after DDAVP
t-PA antigen ($\mu\text{g/l}$)	9.2 $\pm$ 3.9	10.9 $\pm$ 5.0	4.5 $\pm$ 1.3	8.0 $\pm$ 3.3*
PAI-1 antigen ($\mu\text{g/l}$)	68 $\pm$ 75	44 $\pm$ 51	21 $\pm$ 6	17 $\pm$ 7
PAI activity ($\text{IU} \times 10^3/\text{l}$)	15 $\pm$ 11	20 $\pm$ 27	5.2 $\pm$ 1.8	4.0 $\pm$ 1.4
u-PA antigen ($\mu\text{g/l}$)	5.2 $\pm$ 1.8	5.6 $\pm$ 1.4	5.8 $\pm$ 1.2	5.0 $\pm$ 0.9
scu-PA antigen ($\mu\text{g/l}$)	2.5 $\pm$ 0.7	2.5 $\pm$ 0.6	3.2 $\pm$ 0.5	2.9 $\pm$ 1.1
vWF (% of pooled plasma)	311 $\pm$ 92	373 $\pm$ 172	166 $\pm$ 87	211 $\pm$ 100

The effect of DDAVP on fibrinolytic parameters and vWF was measured before and after treatment with DDAVP in the acute and in the convalescent phase. The data are expressed as mean $\pm$ SD. Statistical analysis of the paired data obtained on admission and in the convalescent phase was performed with the Wilcoxon test.

* = $p < 0.05$.

observed after incubation with VT-1. Similarly, the production of vWF antigen decreased after exposure of the cells to $\text{TNF}\alpha$ and VT-1 (not shown). These effects were not found when VT-1 was inactivated by heat-treatment before being added to the cells or when VT-1 was preincubated with monoclonal antibody to VT-1. Similarly, in $\text{TNF}\alpha$ -treated human microvascular endothelial cells VT-1 induced a concentration-dependent decrease in cell number, PAI-1 production and accumulation of t-PA (Fig. 3). These experiments indicate that VT-1 does not induce t-PA, PAI-1 or vWF production, but rather decreases their synthesis in endothelial cells by its inhibitory effect on protein synthesis.

Discussion

In this study we report on the fibrinolytic parameters and vWF antigen in plasma of HUS patients, patients with chronic renal failure and healthy children.

Elevated levels of t-PA antigen and PAI activity were found in the plasma of HUS patients on admission. These findings correspond with the recent observations in thrombotic thrombocytopenic purpura and HUS patients by Monteagudo et al. [31]. Simultaneously the plasma vWF concentration was enhanced. Elevated fibrin(ogen) degradation products found in the plasma on admission, indicate that there is a systemic fibrinogenolysis and fibrinolysis secondary to an activated state of coagulation.

Nowadays it has become clear that verocytotoxin-producing *E.coli* infection is the major cause of the epidemic form of HUS. In nine HUS patients we found evidence for a verocytotoxin-producing *E.coli* infection [14]. Verocytotoxin can cause endothelial cell-damage, which is the predominant feature of the glomeruli in the kidney of patients with HUS. We demonstrated that addition of VT-1 to human endothelial cells that contained sufficient VT-1 receptors (as induced here by $\text{TNF}\alpha$ -pretreatment [4]) causes a decrease in the production of t-PA and PAI-1 antigens likely due to an inhibitory effect of VT-1 on

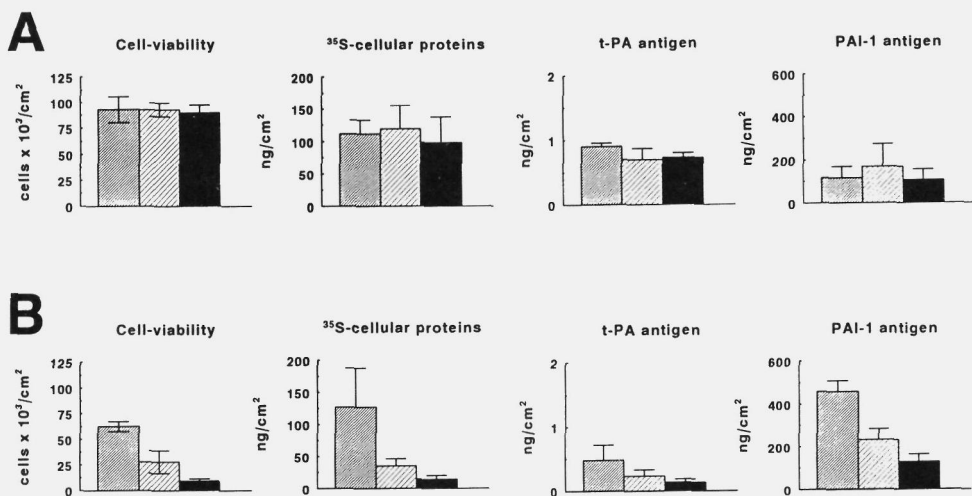


Figure 2. Confluent human umbilical vein endothelial cells in 2 cm² wells, were pre-incubated for 24 hours without (A) or with (B) 500 U/ml TNF- α and subsequently incubated in 0.4 ml M199-HEPES medium supplemented with 20% fetal calf serum and the same amount of TNF- α , together with 17 pmol/l VT-1 (hatched bars) and 17 nmol/l VT-1 (black bars) and without VT-1 (double hatched bars). The number of cells at the start of the preincubation was 9×10^4 cells/cm² for all wells. After preincubation the number of cells was 9.3×10^4 cells/cm² for control cells (A) and 6.3×10^4 cells/cm² for TNF α -treated cells (B). Because VT-1 caused cell death during the incubation with VT-1 and TNF α , by which the number of viable cells changed during the incubation period, the production of t-PA and PAI-1 antigens are expressed per cm². Viability of the cells was determined by trypan blue exclusion test. Other experimental details are given in the Material and Methods section. Data are expressed as mean $\pm$ SD of three experiments with endothelial cells from different donors.

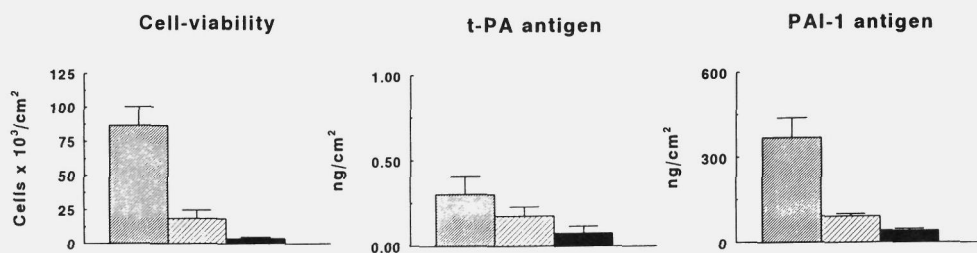


Figure 3. Confluent human foreskin microvascular endothelial cells in 2 cm² wells were pre-incubated for 24 hours with 500 U/ml TNF- α and subsequently in 0.4 ml M199-HEPES medium supplemented with 20% fetal calf serum and the same amount of TNF α , together with 17 pmol/l VT-1 (hatched bars) and 17 nmol/l VT-1 (black bars) or without VT-1 (double hatched bars). The number of cells after 24 hours preincubation with TNF α was 8×10^4 cells/cm² for all variables. Because VT-1 caused cell death during the incubation with VT-1 and TNF, by which the number of viable cells changed, the production of t-PA and PAI-1 antigens are expressed per cm². Data given are expressed as mean $\pm$ SD of three independent experiments.

overall protein-synthesis. Such inhibition of protein-synthesis has also recently been observed in renal endothelial cells [32].

The t-PA, PAI-1 and vWF levels were elevated in the plasma of HUS patients on admission. This suggests that - in addition to the hemorrhagic colitis and the kidney failure- HUS patients display a general activation of the vascular endothelium. A similar pattern of elevated levels of t-PA, PAI-1 and vWF has been observed in the plasma of patients with sepsis, during which endotoxins and endogenous mediators, in particular the inflammatory mediators $\text{TNF}\alpha$ and interleukin-1 (IL-1) contribute to these alterations [33,34]. $\text{TNF}\alpha$, IL-1 and IL-6 were not elevated in the plasma of patients with a mild form of HUS [35,36] and own unpublished observations), but we observed that they were elevated in patients with a severe form of HUS, i.e. in patients who also developed neurological complications and pancreas involvement (N.van de Kar, manuscript in preparation). These data suggest that circulating cytokines may play a role in HUS, but are unlikely to be significant in mild forms of HUS. On the other hand, locally produced inflammatory mediators in the kidney of HUS patients may escape detection in plasma, because of their short biological short half-life [37]. In favour of a local effect of inflammatory mediators, Siegler et al. reported that $\text{TNF}\alpha$ was elevated in the urine of HUS patients [36]. Even, if inflammatory mediators may contribute locally to the increase of the t-PA, PAI-1 and vWF levels in plasma, it is likely that other factors which are generated in the injured intestines and/or kidney, are an important cause of the sustained elevation of the t-PA, PAI-1 and vWF concentration in the blood of HUS patients [38-40]. Activated leucocytes, which can be found in the peripheral blood in HUS [41], contribute to endothelial activation, and also other factors such as activated complement and coagulation factors may be involved [42-45].

In our patients no release of t-PA, u-PA and vWF could be evoked by DDAVP-infusion on admission. Unresponsiveness to DDAVP cannot entirely be explained by the renal problems seen in HUS, because patients with terminal renal insufficiency and anephric patients can still react to DDAVP-infusion [46,47]. However, it is well-known that patients with kidney diseases respond less pronouncedly to DDAVP than healthy volunteers, and some are non-responders [46,47]. Unresponsiveness to DDAVP-infusion of t-PA and vWF antigen release has also been observed in patients with inflammatory bowel disease (M. Crohn, ulcerative colitis), who have a normal level of t-PA and vWF before DDAVP-infusion [48]. It is therefore possible that the lack of response to DDAVP in HUS patients is caused by a simultaneous disturbance of the kidney and the intestine. On the other hand, it is unlikely that the lack of response of t-PA and vWF to DDAVP-infusion is due to the depletion of endothelial 'storage pools', because the biological half-life of t-PA is very short and depletion of the t-PA should result in a drop in circulating t-PA. After two weeks of hospitalization not only the plasma levels of t-PA, PAI-1 and vWF antigen were normalizing, but also t-PA antigen increased upon DDAVP-infusion. This indicates that the activation of the endothelium had decreased and that the response to DDAVP was recovering. It is of interest to note that a DDAVP-induced increase in t-PA antigen was also found in patients recovering from M.Crohn or ulcerative colitis. Recently, Bergstein et al. reported that elevated levels of PAI-1 during the clinical course was related to a poor outcome [12]. In our

study, all patients, including those with a high PAI-1 level on admission, completely recovered.

While VT-1 causes a decrease of the production of fibrinolytic proteins by endothelial cells in vitro, the plasma levels of these proteins in HUS patients changed in the opposite direction. Although these data appear to be paradoxical, they do not really conflict. We hypothesize that the following events may occur in HUS. After a period of hemorrhagic colitis, the main organ affected in HUS is the kidney. The human kidney contains receptors for verocytotoxin [49], the toxin is generally assumed to contribute to the damage of the endothelium in the glomeruli in the kidney. This damage can lead to a local procoagulant situation. In this condition thrombin and inflammatory mediators are generated and polymorphonuclear leucocytes are activated, which probably cause a general activation of the endothelium at sites distal of the affected areas. This is reflected in the elevated levels of t-PA, PAI-1 and vWF found in the plasma of the HUS patients in the acute phase of the disease. Further studies will be necessary to prove this hypothesis.

We conclude from this study, that the systemic fibrinolytic parameters measured in plasma of HUS patients are probably not a direct effect of VT-1 on the endothelium, but are sequelae of the disease in which the kidney and the intestine are predominantly affected.

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Chapter 5

TUMOR NECROSIS FACTOR AND INTERLEUKIN-1 INDUCE EXPRESSION OF THE VEROCYTOTOXIN RECEPTOR GLOBOTRIAOSYLCERAMIDE ON HUMAN ENDOTHELIAL CELLS Implications for the pathogenesis of the hemolytic uremic syndrome

Nicole C.A.J. van de Kar^{1,2}, Leo A.H. Monnens²,
Mohammed A. Karmali³, Victor W.M. van Hinsbergh¹.

¹Gaubius Laboratory TNO-PG, Leiden, The Netherlands.

²Department of Pediatrics, University Hospital, Nijmegen, The Netherlands.

³Department of Microbiology, Hospital for Sick Children, Toronto, Canada.

Summary

The epidemic form of the hemolytic uremic syndrome (HUS), beginning with an acute gastro-enteritis has been associated with a verocytotoxin producing *E. coli* infection. The endothelial cell is believed to play an important role in the pathogenesis of HUS. Endothelial cell damage by verocytotoxin-1 (VT-1) in vitro is potentiated by the additional exposure of inflammatory mediators, such as tumor necrosis factor α (TNF α). Preincubation of human umbilical vein endothelial cells with TNF α resulted in a ten- to hundred-fold increase of specific binding sites for ^{125}I -VT-1. Furthermore, interleukin-1 (IL-1), lymphotoxin (TNF β) and lipopolysaccharide (LPS) also markedly increase VT-1 binding. An exposure of several hours to TNF α was enough to enhance the number of VT-1 receptors on the endothelial cells for 2 days. The TNF α -induced increase in VT-1 binding could be inhibited by simultaneous addition of the protein synthesis inhibitor cycloheximide. Glycolipid-extracts of TNF α -treated cells tested on thin layer chromatography demonstrated an increase of globotriaosylceramide (GbOse₃cer), a functional receptor for VT-1, suggesting that preincubation of human endothelial cells with TNF α leads to an increase in GbOse₃cer synthesis in these cells.

We conclude from this study that TNF α and IL-1 induce one (or more) enzyme(s) that is (are) rate-limiting in the synthesis of the glycolipid VT-1 receptor, GbOse₃cer. These in vitro studies suggest that in addition to verocytotoxin-1 inflammatory mediators play an important role in the pathogenesis of the hemolytic uremic syndrome.

Introduction

The hemolytic uremic syndrome (HUS) is a clinical syndrome consisting of the triad hemolytic anemia, thrombocytopenia and acute renal failure. Although the exact pathogenesis is still unknown, it is believed that endothelial cell damage plays a central role in this syndrome and that one of the requirements for an agent to cause HUS is its ability to injure endothelial cells. The epidemic form of HUS starting with an acute, often bloody, gastro-enteritis is mostly seen in children [1]. In 1985 Karmali associated this form of HUS with an infection of verocytotoxin or shiga-like toxin producing *E. coli* infection [2]. Since then several other investigators across the world have been able to confirm this association. A family of three verocytotoxins or shiga-like toxins has been described: verocytotoxin-1, verocytotoxin-2 (shiga-like toxin II) and verocytotoxin-2 variant [3,4]. Recently, the functional receptor for these toxins has been identified as the glycosphingolipid, globotriaosylceramide [5,6]. This receptor has also been found in the human kidney [7] and on cultured human endothelial cells [8].

In most HUS patients, the endothelial damage is primarily concentrated in the kidney endothelium, whereas in severe HUS patients the endothelial damage extends to other organs, such as pancreas and brain [9]. When we investigated whether local factors generated during coagulation or inflammation can make the endothelium more vulnerable to verocytotoxins,

we observed that endothelial damage by verocytotoxin-1 (VT-1) depends on the additional presence of the inflammatory mediator $\text{TNF}\alpha$ [10]. Similarly, two other groups have recently found that endothelial cells become more sensitive to other related toxins, shiga-like toxin II and shiga toxin when they are simultaneously exposed to $\text{TNF}\alpha$ [11,12]. The inflammatory mediators $\text{TNF}\alpha$ and IL-1 are produced and released by monocytes [13] and by mesangial cells [14,15] and hence may play a local role in the kidney.

In this report we extend our observations and demonstrate that the inflammatory mediators $\text{TNF}\alpha$ and IL-1 make the endothelial cells sensitive to VT-1 by the induction of VT-1 receptors on their surface.

Materials and Methods

Materials

Purified verocytotoxin-1 (VT-1) was prepared in the laboratory of Dr. Karmali (1.2 mg protein/ml; CD_{50} vero-cells: titer 10^{-8} - 10^{-9}) [16]. Endotoxin content of the VT-1 preparation was < 0.05 EU/ml by Limulus amoebocyte lysate assay (E-Toxic, Sigma Chemicals Co (St. Louis, MO); at detection level of 0.05-0.1 EU/ml). Monoclonal antibodies PH_1 against VT-1 was a generous gift from Dr. C. Lingwood, Dept. of Microbiology, Hospital for Sick Children, Toronto, Canada. M199 medium supplemented with 20 mM HEPES was obtained from Flow Laboratories (Irvine, Scotland); tissue culture plastics were from Costar (Cambridge, MA). A crude preparation of endothelial cell growth factor was prepared from bovine brain as described by Maciag et al [17]. Human serum was obtained from the local blood bank and was prepared from fresh, healthy donors, pooled, and stored at 4°C ; it was not heat-inactivated before use. Newborn calf serum (NBCS) and fetal calf serum (FCS) was from GIBCO (Grand Island, NY) and Boehringer Mannheim (Mannheim, Germany), respectively; they were heat-inactivated before use (at 56°C for 30 min). Heparin was purchased from Leo Pharmaceuticals (Weesp, The Netherlands). Penicillin/streptomycin was from Boehringer Mannheim (Mannheim, Germany). Human fibronectin was a gift of J.A. van Mourik, Central Laboratory of the Red Cross Blood Transfusion Service (Amsterdam, the Netherlands). Pyrogen-free human serum albumin was purchased from the Central Laboratory of the Red Cross Blood Transfusion Service (Amsterdam, the Netherlands). α -Thrombin was obtained from Sigma Chemical Co. (St. Louis, MO). Human recombinant tumor necrosis factor α ($\text{TNF}\alpha$) and lymphotoxin ($\text{TNF}\beta$) were gifts from Jan Tavernier (Biogent, Gent, Belgium). The $\text{TNF}\alpha$ preparation contained 2.45×10^{-7} U/mg protein and less than 40 ng LPS per mg protein; the specific activity of lymphotoxin ($\text{TNF}\beta$) was 1.6×10^6 U/mg protein. Human recombinant IL-6 (expressed in yeast cells) was prepared in the Laboratory of Molecular Biology, State University, Gent, Belgium: it was biologically active and had a specific activity of 1.4×10^8 U/mg protein as tested on 7TD1 cells. Human recombinant interleukin- 1α (IL- 1α) and interleukin- 1β (IL- 1β) were a gift from S. Gillis (Immunex Corporation, Seattle, WA): they had a specific activity of 10^8 U/mg. Lipopolysaccharide (LPS) of *E.coli* serotype O128-B12 was obtained from Sigma Chemical

Co. (St. Louis, MO). ^{35}S -methionine and Na^{125}I -iodine were purchased from Amersham (Amersham, UK). Iodo-gen, iodination reagent was obtained from Pierce (Rockford, IL). Anti-Tj^a serum [18], serum which contains antibodies against P^k(Globotriaosylceramide: GbOse₃cer), P (Globotetraosylceramide: GbOse₄cer) and P₁ blood group antigens, was obtained from DiaMed (Morat Murten, Switzerland). Chloroform, methanol, and hexane was obtained from Merck (Darmstadt, Germany). Plastic-coated silica gel F1500 TLC-plates came from Schleicher and Schuell (Dassel, Germany). Polyisobutylmethacrylate was obtained from Polysciences Inc (Washington, MD). A standard mixture of pure neutral glycolipids containing Gal β 1-1Ceramide(Cer)(CMH), Gal β 1-4Glc β 1-1Cer(CDH), Gal α 1-4Gal β 1-4Glc β 1-1Cer(GbOse₃cer), GalNac β 1-3Gal α 1-4Gal β 1-4Glc β 1-1Cer(GbOse₄cer), GalNac α 1-3GalNac β 1-3Gal α 1-4Gal β 1-4Glc β 1-1Cer (Forssman pentasaccharide) was from BioCarb AB (Lund, Sweden). X-OMAT X-ray film came from Eastman Kodak Co (Rochester NY).

Cell culture

Endothelial cells from human umbilical vein and from human femoral vein were isolated by collagenase treatment [19], cultured and characterized as previously described [20,21]. The endothelial cells were seeded in fibronectin-coated 10 cm² wells and cultured in M199 medium supplemented with 20mM HEPES (pH 7.4), 10% human serum (HS), 10% newborn calf serum (NBCS), L-glutamine, 5 U/ml heparin and 150 $\mu\text{g}/\text{ml}$ crude preparation of endothelial cell growth factor under 5%CO₂ and 95% air at 37°C. When the cells reached confluency, they were detached by trypsin/EDTA and seeded in 2 cm² fibronectin coated dishes with a split ratio of 1:3. Umbilical vein endothelial cells in the experiments were used after 2-4 passages; femoral vein endothelial cells after 6 or 7 passages. The medium was renewed every two or three days.

Cytotoxicity-Assay

For these experiments cells were released with trypsin/EDTA and seeded in confluent density in 2 cm² fibronectin-coated plates and cultured in complete culture medium (400 $\mu\text{l}/\text{well}$). 20% FCS was used instead of 10% human serum and 10% NBCS, because neutralising activity of NBCS was found against VT-1 and because of potential neutralising activity against VT-1 in human pooled serum. When confluency was reached, medium was renewed and the cells were preincubated for 24 hours with TNF α (500 U/ml). The next day the medium was renewed, and purified VT-1 (17pM-17nM) with or without TNF α or α -thrombin, was added to the wells. After 24 hours incubation the cells were washed with M199 medium, the viable cells released with trypsin/EDTA and counted in a hemocytometer. The experiments were done in duplicate.

Other assays

Protein synthesis was determined by assay of the incorporation of ^{35}S -labelled methionine (0.25 $\mu\text{Ci}/\text{ml}$ added to complete medium) in ^{35}S -proteins during an 8 or 24-hour incubation-period. After incubation, the cells were washed and cellular ^{35}S -labeled proteins were precipitated by adding 10% trichloroacetic acid. Precipitated radioactivity was dissolved in

0.3 ml 0.3 M NaOH and counted in a liquid scintillating counter. Protein was determined according to the method of Lowry et al [22] using bovine serum albumin as a standard. Cell counts were performed in triplicate with a hemocytometer.

Binding of ^{125}I -VT-1 to human endothelial cells

VT-1 was radiolabeled with Na^{125}I according to the Iodogen-procedure [23]. Four preparations of purified VT-1 were iodinated to specific activities of 2.7 μCi , 4.1 μCi , 21 μCi and 37 $\mu\text{Ci}/\mu\text{g}$ of protein. All preparations gave similar results.

The binding-assay was carried out as follows: after the incubation period at 37°C with or without inflammatory mediators, the endothelial cell cultures in 24-well plates were washed with M199 medium + 0.1% human serum albumin (HSA). Subsequently the cells were incubated for 3 hours with 0.3 or 1 nM ^{125}I -VT-1 in M199 medium + 0.1% HSA at 0°C. After this incubation, the supernatant fluid was aspirated, the cells were washed five times with M199 medium + 0.1% HSA, and total cell protein was solubilized in 400 μl 1M sodium hydroxide at room temperature. Radioactivity of the endothelial cells was measured in a gamma-counter. Non-specific binding was determined by assay of ^{125}I -VT-1 binding in the presence of a 100-fold excess of unlabelled VT-1. Cellular specific binding was determined by subtracting the non-specific binding from the cellular binding of ^{125}I -VT-1 determined in the absence of unlabelled VT-1. To study the effect of anti-Tj^a serum (blocking of the binding of VT-1 to GbOse₃cer), anti-Tj^a serum in M199 medium + 0.1% HSA was added 2 hours prior to and during the binding assay with ^{125}I -VT-1.

Extraction of Glycolipids

Confluent human umbilical vein endothelial cells (HUVEC) in 162 cm² flasks were incubated for 24 h with or without TNF α (500 U/ml). Subsequently the glycolipids were extracted as described by Lingwood et al [6]: in short, the cells were trypsinized, harvested with ice-cold PBS and spun down by 3 min centrifugation (3000 rpm) at 4°C. The pellet was washed 3 times with PBS. The pellet was finally resuspended in PBS, and 20 volumes of chloroform/methanol (2:1, v/v) were added. Cell debris was removed by filtration through glass-wool. One volume of water was added and partitioned. The lower phase was dried and incubated at 37°C for 2 hours in 0.4 M KOH in ethanol; two volumes (v/v) of chloroform were added and the mixture was partitioned against two volumes of water. The lower phase was separated and frozen at -20°C until TLC-studies were performed.

Thin Layer Chromatography

The lower phase from the extraction above was dried and resuspended in chloroform/methanol (2:1). Samples were separated on a silica gel TLC plate using chloroform: methanol:water (65:25:4). After separation the plate was soaked 3 times for 1 minute in 0.01% polyisobutylmethacrylate in hexane and air dried, followed by overnight incubation in PBS supplemented with 1% BSA and 0.05% Tween 20. Subsequently, the plate was incubated with 50 ml VT-1 solution (15 nM unlabelled and 1.5 nM ^{125}I -VT-1 in 1% BSA and 0.05% Tween in PBS) during 4 hours at 4°C. The plate was extensively washed with

0.05% Tween 20 and 1% BSA in PBS, air dried and exposed overnight to X-OMAT X-ray film.

Results

Cytotoxicity of VT-1 towards human endothelial cells

Verocytotoxin-1 (VT-1) caused a decrease in cell viability of endothelial cells when they had been preincubated with the inflammatory mediator $\text{TNF}\alpha$ (Figure 1a). VT-1 had no significant effect on the cell viability of untreated cells. Only in two out of seven independent cell cultures of umbilical vein endothelial cells, 24 h incubation with 17 nM VT-1 alone caused a small degree of cell detachment (21 and 24%). Even upon prolonged incubation with 17 nM VT-1 alone (72 h) we found in only four out of six independent cell-cultures a moderate cell detachment ($32\% \pm 17\%$; mean $\pm$ SD). In all conditions, no cytotoxicity was found when VT-1 was inactivated by heat-treatment prior to addition or when the VT-1 was preincubated with monoclonal antibodies to VT-1. Preincubation of the cells with thrombin (0.1 to 1 U/ml) did not change the sensitivity of endothelial cells for VT-1 (3 independent experiments; not shown). Similar results were obtained when endothelial cells from adult human vein (v.femoralis) were used (Figure 2a).

Verocytotoxin may inhibit eukaryotic protein synthesis by depurination of a single adenine residue from the 28S RNA component of the ribosome [24], which may result in the inhibition of the interaction of elongation factor-1 with the ribosome. Cellular protein

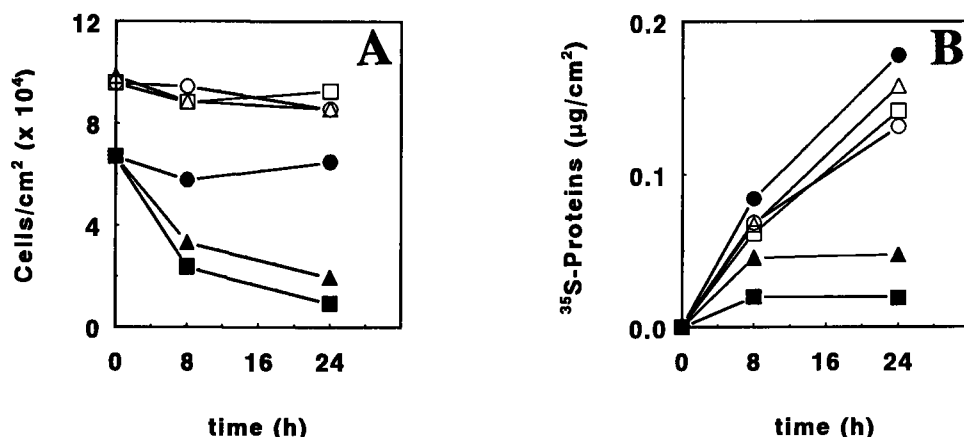


Figure 1. Toxicity of verocytotoxin-1 (VT-1) for confluent human umbilical vein endothelial cells (HUVEC) preincubated for 24 h without or with $\text{TNF}\alpha$ 500 U/ml. A: Number of viable cells after an 8 and 24-hour incubation period with VT-1. After a preincubation period of 24 hours without (open symbols) or with (closed symbols) $\text{TNF}\alpha$, cells were incubated without VT-1 (circles), with 17pM VT-1 (triangles) or with 17nM VT-1 (squares) for 8 and 24 h, and the number of viable cells was counted in a hemocytometer. B: Incorporation of ^{35}S -methionine in ^{35}S -proteins was determined by trichloroacetic acid precipitation after an 8- or 24-h incubation period with the various concentrations of VT-1 given above.

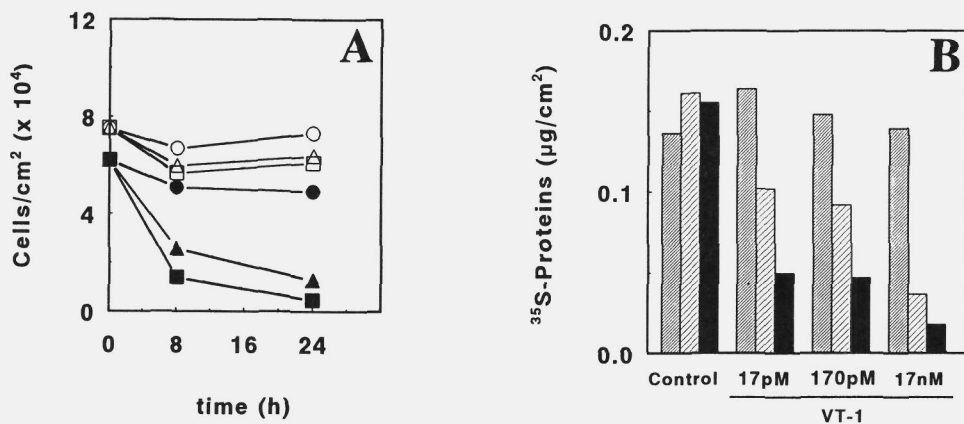


Figure 2. Toxicity of VT-1 for confluent human femoral vein endothelial cells preincubated with TNF α 500 U/ml for 24 hours.

A: After a preincubation period of 24 hours without (open symbols) or with (closed symbols) TNF α , cells were incubated without VT-1 (circles), with 17pM VT-1 (triangles) or with 17nM VT-1 (squares) for 8 and 24 h, and the number of viable cells was counted.

B: Incorporation of 35 S-methionine in 35 S-proteins. Cells were preincubated for 24 h without TNF α (double hatched bars), with 50 U/ml TNF α (hatched bars) or with 500 U/ml TNF α (black bars). After preincubation, cells were incubated for 24 hours with 17pM, 170pM or 17nM VT-1 or without VT-1 (control).

synthesis was measured by assaying the incorporation of 35 S-methionine into total cellular protein. Incubation of the cells with VT-1 alone did not affect the protein synthesis over a 24 h period (Figure 1b,). However, when the cells were preincubated for 24 h with TNF α , the protein synthesis rate was decreased dramatically by VT-1, whereas the protein synthesis was not decreased by TNF α alone. As is shown in Figure 2b, the observed decrease depended on the concentrations of both VT-1 and TNF α .

Binding of VT-1 to human endothelial cells

To evaluate whether the enhanced VT-1 sensitivity of endothelial cells caused by TNF α was due to an increase in VT-1 receptors on the cell surface, binding experiments with 125 I-VT-1 were performed. TNF α induced an increase in VT-1 binding after a 6 to 8 hour lag period; this increase continued for up to 48 hours (Fig. 3). The increase was due to an increase in specific binding of 125 I-VT-1, as nearly all 125 I-VT-1 binding could be displaced by excess of unlabelled VT-1. No change in VT-1 binding was observed in non-treated cells (Fig. 3). In six independent experiments with confluent HUVEC of different donors, 24-hour incubation with 500 U/ml TNF α resulted in a 38-fold increase in specific VT-1 binding (range 13- to 90-fold).

Subconfluent HUVEC displayed a higher basal VT-1 binding than highly confluent cells, as is shown in Fig. 4 for a representative experiment. When the same cells were incubated for 18 hours with TNF α , the binding of VT-1 increased for all cells, but the relative increase was larger in highly confluent HUVEC. In further experiments highly confluent cells have

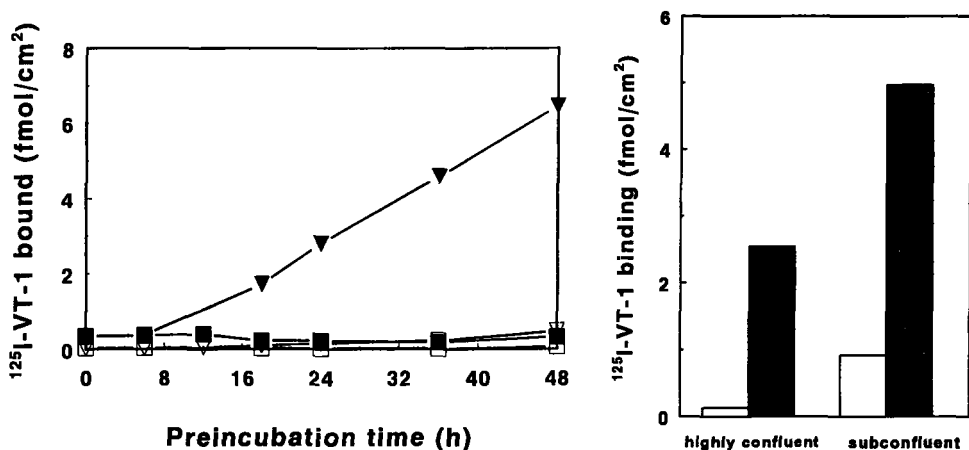


Figure 3 (left). Effect of TNF α pretreatment on the binding of VT-1 to confluent HUVEC.

After preincubation of the cells with 500 U/ml TNF α (triangles) at 37°C for the indicated period of time, the cells were washed and binding of 0.3 nM ^{125}I -VT-1 was assayed at 0°C in the presence (open symbols) or absence (closed symbols) of 30 nM unlabeled VT-1. Binding of VT-1 to control cells that were preincubated without TNF α are indicated in squares.

Figure 4 (right). Effect of cell density on the specific binding of VT-1 to HUVEC.

During the assay of VT-1 binding, subconfluent cells had a 70% to 80% cell density of that of confluent cells. Highly confluent cells were maintained for three days at confluent density before the start of the experiment. Before assay of the binding of 0.3 nM ^{125}I -VT-1, the cells were preincubated with 500 U/ml TNF α (black bars) or without it (open bars). Non-specific binding (determined in the presence of 30 nM unlabeled VT-1) was 5% or less in all conditions.

been used.

The effect of TNF α on ^{125}I -VT-1 binding was concentration dependent (Fig. 5a). The binding saturated at about 10 nM VT-1 (Fig. 5b). From analysis of Scatchard plots of the binding curves (Fig. 5c, Table 1) it was concluded that the number of specific VT-1 binding sites increased by one or two orders of magnitude after 24 hours of incubation with TNF α , whereas the apparent affinity of VT-1 binding to HUVEC did not significantly change (Table 1). Similarly, in human femoral vein endothelial cells the number of VT-1 binding sites increase 14-fold after a 24 h incubation period with 100 U/ml TNF α from 2×10^5 VT-1 binding sites/cell in control cells to 29×10^5 VT-1 binding sites in TNF α -treated cells.

In addition to TNF α , interleukin-1 α (IL-1 α), interleukin-1 β (IL-1 β), lymphotoxin (TNF β) and bacterial lipopolysaccharide (LPS) induced a more than ten-fold increase in specific VT-1 binding to HUVEC (Fig. 6). Thrombin induced only a three-fold increase, whereas IL-6 (5 or 50 U/ml) did not significantly alter VT-1 binding. The increase induced by 10 $\mu\text{g}/\text{ml}$ LPS was two-fold less than that induced by 500 U/ml TNF α . LPS increased VT-1 binding in a concentration-dependent way (Fig. 7). As can be seen in Fig. 7, 24 h incubation with small amounts of TNF α and LPS resulted in an additional rise in the binding of VT-1 to HUVEC.

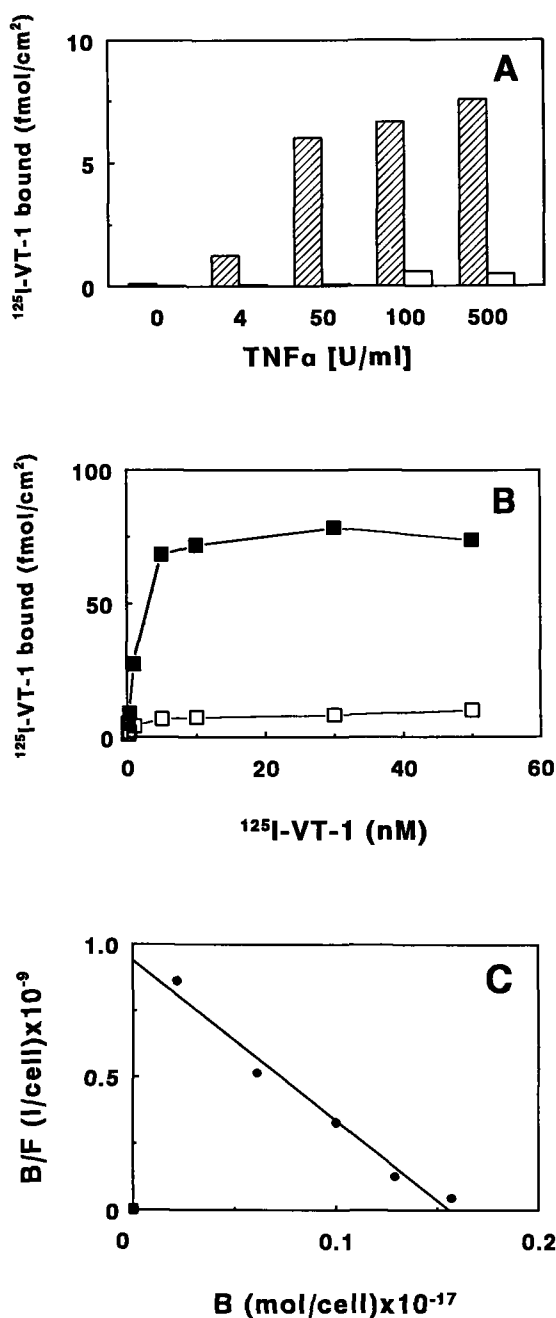


Figure 5. Effect of $\text{TNF}\alpha$ on the binding of VT-1 to HUVEC.

A: After preincubation of confluent HUVEC for 24 hours with varying concentrations of $\text{TNF}\alpha$, binding of 0.3 nM ^{125}I -VT-1 was assayed in the absence (hatched bars) or presence of 30 nM unlabeled VT-1 (open bars). B: Binding of 0.15-50 nM ^{125}I -VT-1 to human endothelial cells treated for 24 hours without (open squares) or with $\text{TNF}\alpha$ (dark squares). C: Scatchard plot of ^{125}I -VT-1 binding to human endothelial cells preincubated with 500 U/ml $\text{TNF}\alpha$ for 24 h (circles). Control cells are indicated by the square.

As the half-life time of $\text{TNF}\alpha$ in plasma is relatively short (several minutes) [25], we have evaluated how long after exposure of HUVEC to $\text{TNF}\alpha$ an effect on the VT-1 binding can be established. To that end, HUVEC were incubated for various time periods with 20 U/ml $\text{TNF}\alpha$, vigorously washed and subsequently incubated for another period without $\text{TNF}\alpha$, after which the VT-1 binding was assayed. The results of two independent experiments are

Table 1. Binding of verocytotoxin-1 (VT-1) to human umbilical vein endothelial cells (HUVEC).

Cells	Addition	Binding sites/cell	Apparent Kd (M)
HUVEC 1	None	0.03×10^5	0.6×10^{-9}
	TNF α (500 U/ml)	9.4×10^5	1.7×10^{-9}
HUVEC 2	None	0.8×10^5	1.2×10^{-9}
	TNF α (500 U/ml)	11.3×10^5	1.9×10^{-9}

Binding of ^{125}I -VT-1 was performed at various concentrations (0.15 to 50 nM) and with excess of unlabeled VT-1, in two independent HUVEC cultures. The number of binding sites for the holotoxin VT-1 and the apparent Kd were calculated by analysis of Scatchard plots.

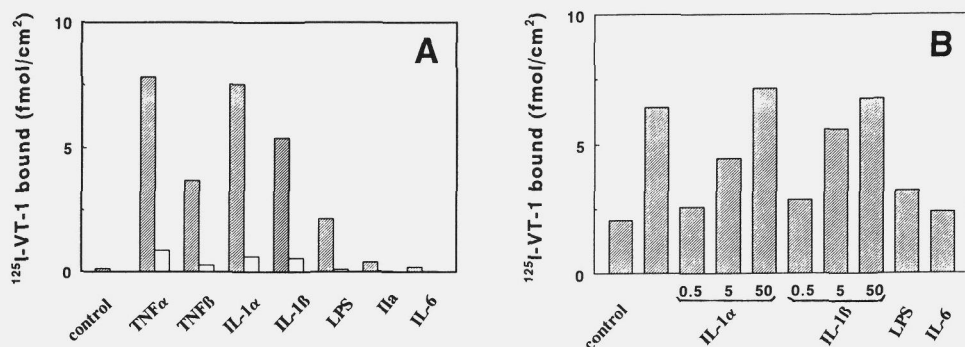


Figure 6. Binding of VT-1 to HUVEC after preincubation with various inflammatory mediators and thrombin. A: Binding of 0.3 nM ^{125}I -VT-1 to confluent HUVEC was determined in the absence (hatched bars) or presence of 30 nM unlabeled VT-1 (open bars) after a 24-hour preincubation period with 500 U/ml TNF α , 500 U/ml TNF β , 50 U/ml IL-1 α , 50 U/ml IL-1 β , 50 U/ml IL-6, 10 $\mu\text{g/ml}$ LPS or 1 U/ml α -thrombin (IIa). B: Specific binding of ^{125}I -VT-1 to subconfluent HUVEC after 24-hour preincubation with 500 U/ml TNF α , varying concentrations of IL-1 α and IL-1 β (0.5, 5 and 50 U/ml), 10 $\mu\text{g/ml}$ LPS or 50 U/ml IL-6.

given in Table 2. Six hours of incubation with TNF α was enough to detect an increase in VT-1 binding after a 24- or 48-hours incubation. This suggests that the increased sensitivity of endothelial cells for VT-1 can last for at least one day after the disappearance of inflammatory mediators.

The nature of the TNF α -induced VT-1 binding to endothelial cells

Globotriaosylceramide (GbOse₃cer) has been identified as a receptor for VT-1 in various cells [26]. To evaluate whether the increase in specific VT-1 binding was due to an increase in the exposure of GbOse₃cer to the cell surface, we have incubated HUVEC with anti-Tj^a serum (which contains antibodies against the P^k(GbOse₃cer), P (GbOse₄cer) and P₁ blood group antigens). The addition of anti-Tj^a serum inhibited the binding of VT-1 in a concentration

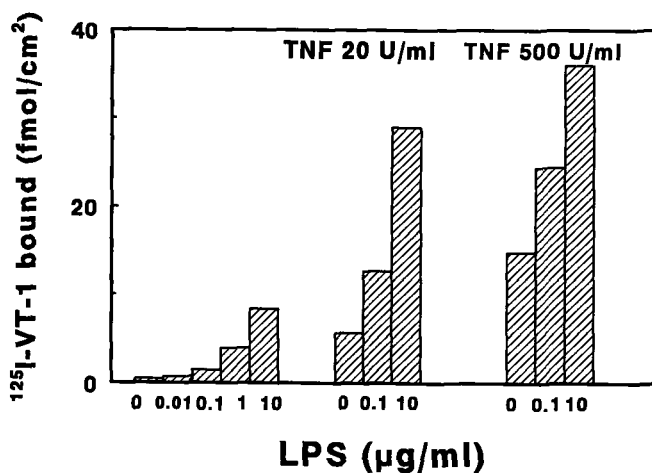


Figure 7. Specific binding of 1 nM ¹²⁵I-VT-1 to HUVEC after preincubation for 24 hours with varying concentrations of LPS, and in combination with 20 or 500 U/ml TNFα.

dependent way (Fig. 8). This finding was confirmed in three other experiments of HUVEC, as well as in femoral vein endothelial cells. Seven different human serum preparations from healthy donors did not decrease the binding of VT-1 to the endothelial cells. To ascertain whether the increase in VT-1 binding resulted from an increase in cellular GbOse₃cer synthesis or from a change in the availability of GbOse₃cer at the cell surface, HUVEC were

Table 2. Duration of the effect of TNFα on the binding of VT-1 to human endothelial cells.

TNFα preincubation (hours)	Subsequent incubation without TNFα (hours)	Binding of ¹²⁵ I-VT-1 (fmol/10 ⁵ cells)
<i>Experiment 1</i>		
0 (control)	24	0.68
1	23	1.36
6	18	3.31
12	12	7.10
24	0	8.75
<i>Experiment 2</i>		
0 (control)	48	1.87
1	47	1.79
6	42	3.39
12	36	5.38
24	24	10.76
48	0	10.76

To evaluate how long confluent umbilical vein endothelial cells have to be exposed to TNFα to express an increase in VT-1 binding after 1 or 2 days, the cells were incubated for various times with 20 U/ml TNFα, washed vigorously three times, and subsequently incubated without TNFα for a residual time period until 24 h (experiment 1) or 48 h (experiment 2) after addition of TNFα. Binding of 1nM ¹²⁵I-VT-1 was determined with two different endothelial cell cultures, which had a threefold difference in basal VT-1 binding. Binding assay was performed as described in Materials and Methods.

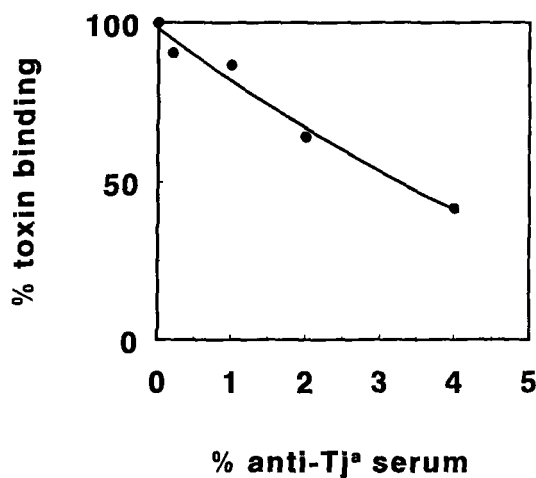


Figure 8. Inhibition of 1nM ^{125}I -VT-1 binding by anti-Tj* serum (concentration range 0.2-4%). Cells were incubated for 24 hours without or with $\text{TNF}\alpha$ 500 U/ml. Anti-Tj* serum was added 2 hours before and during incubation with ^{125}I -VT-1. Similar inhibition by 4% anti-Tj* serum was obtained in three other endothelial cell cultures ($45 \pm 9\%$; mean $\pm$ SD).

incubated with and without $\text{TNF}\alpha$, and glycolipid-extracts of cells were subsequently prepared. After thin layer chromatography of these glycolipids, an increase in VT-1 binding at the spot of GbOse_3cer was clearly demonstrated in the $\text{TNF}\alpha$ -treated cells (Fig. 9). Occasionally, a second VT-1 binding band was observed, as demonstrated in Fig. 9. Because the GbOse_3cer in our standard sample sometimes appeared as two bands after mixture with the cellular extract, it is likely that the second band here is also (derived from) GbOse_3cer . But the involvement of $\alpha\text{OH-GbOse}_3\text{cer}$, which has a similar R_f -value as the second band and also can bind VT-1 [5], can not be excluded. When standard glycolipids were run together with glycolipid extracts of the $\text{TNF}\alpha$ -treated cells, VT-1 binding to the GbOse_3cer of the standard mixture coincided precisely with the induced VT-1 binding glycolipids in the $\text{TNF}\alpha$ -treated cells (not shown). These experiments indicate that the number of GbOse_3cer molecules in/on the cells has been markedly increased after preincubation with $\text{TNF}\alpha$.

Requirement of protein synthesis for $\text{TNF}\alpha$ -induced VT-1 binding.

The protein synthesis inhibitor cycloheximide was used to evaluate whether protein synthesis was required for the appearance of VT-1 receptors on the cell surface induced by $\text{TNF}\alpha$. The simultaneous addition of high concentrations of $\text{TNF}\alpha$ and cycloheximide was toxic for the cells. However, it has been reported that partial protection for $\text{TNF}\alpha$ cytotoxicity can be mediated by plasminogen activator inhibitor-2 [27], the mRNA of which is rapidly induced in endothelial cells by $\text{TNF}\alpha$ itself (own unpublished data). Therefore, we have added various concentrations of cycloheximide 3 hours after addition of 20 U/ml $\text{TNF}\alpha$, and incubated the cells for another 21 hours. Subsequently, the cells were counted and specific VT-1 binding was determined in parallel wells and calculated on a per cell basis. Whereas cycloheximide did not change the number of VT-1 binding sites per cell in control cells, it prevented the $\text{TNF}\alpha$ -induced increase in VT-1 binding in a concentration-dependent way (Fig. 10). Similarly, in human umbilical vein endothelial cells 0.5 and 2 $\mu\text{g/ml}$ cycloheximide reduced the $\text{TNF}\alpha$ -induced increase in VT-1 binding sites per cell by 58% and

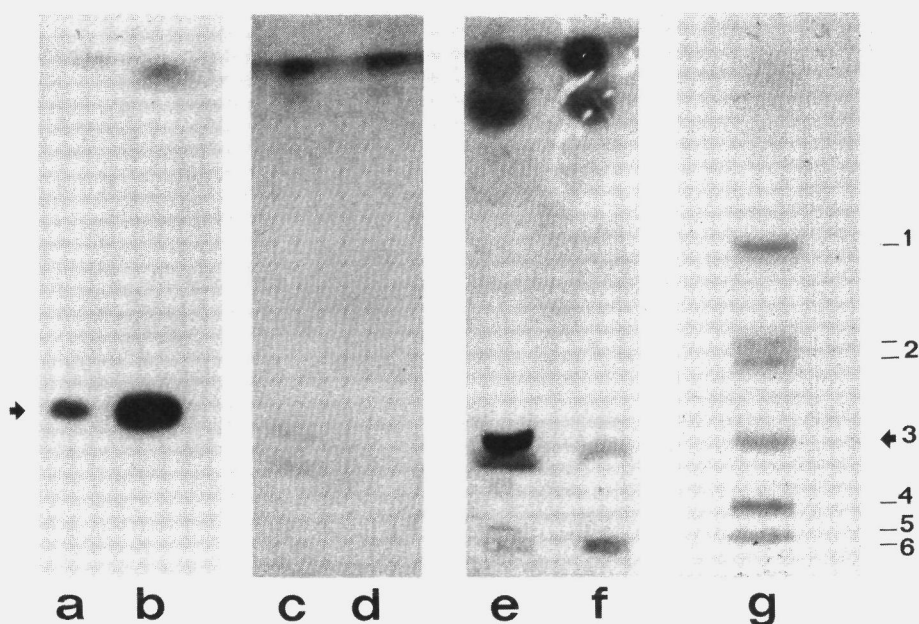


Figure 9. Verocytotoxin-1 binding to HUVEC glycolipids. Glycolipids from endothelial cells were extracted, separated by TLC and assayed for ^{125}I -verocytotoxin-1 binding. Lane a-f: Autoradiographs of ^{125}I -VT-1 binding to separated standard or endothelial glycolipids. Lane a: standard neutral glycosphingolipids 0.2 μg of each glycolipid, lane b: idem, 2 μg of each glycolipid. Lane c and d: glycolipid extracts of 1.6×10^5 HUVEC, lane c: 500 U/ml $\text{TNF}\alpha$ -treated cells, lane d: control cells. Lane e and f: glycolipid extracts of 1.44×10^6 cells, lane e: $\text{TNF}\alpha$ -treated cells, lane f: control cells. Lane g: standard neutral glycosphingolipids 2 μg of each glycolipid, stained by orcinol spray. 1: galactosylceramide, 2: lactosylceramide, 3: globotriaosylceramide, 4: globotetraosylceramide, 5: Forssman pentasaccharide, 6: Origin of the lane.

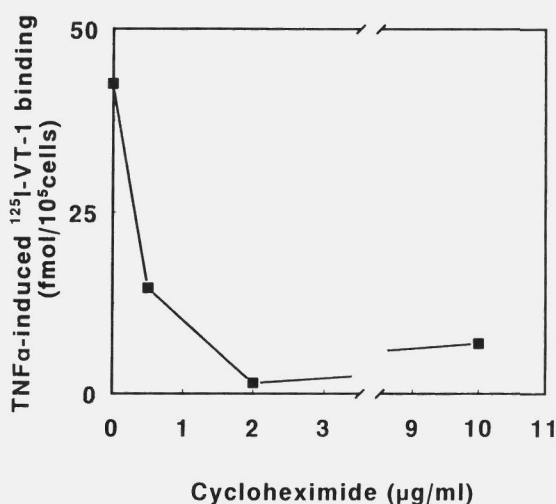


Figure 10. Effect of cycloheximide on the $\text{TNF}\alpha$ -induced increase in VT-1 binding to human femoral vein endothelial cells. Various concentrations of cycloheximide were added 3 h after addition of 20 U/ml $\text{TNF}\alpha$ and the cells were incubated for another 21 h. Subsequently, the cells were counted and specific VT-1 binding was determined in parallel wells and calculated on a per cell basis. The number of $\text{TNF}\alpha$ -treated cells decreased by 13%, 19% and 46% after simultaneous incubation with 0.5, 2 and 10 $\mu\text{g/ml}$ cycloheximide as compared to cells incubated with $\text{TNF}\alpha$ but without cycloheximide.

73%, respectively (not shown). These data suggest that the TNF α -induced increase in VT-1 receptors involves the induction of de novo synthesis of protein(s), that is (are) involved in the synthesis pathway of the glycolipid VT-1 receptor.

Discussion

Infections with verocytotoxin-producing *E.coli* are associated with the hemolytic uremic syndrome and verocytotoxins are believed to play a role in the etiology of HUS [28,29]. A functional receptor (GbOse₃cer) for VT-1 has been demonstrated on human umbilical vein endothelial cells (HUVEC), but it is present in a very low amount [8]. We have demonstrated here that incubation of human endothelial cells with TNF α , IL-1 or LPS markedly enhances the binding of VT-1 to these cells. Furthermore, we have demonstrated that the TNF α -induced increase in VT-1 binding is accompanied by an increase in the cellular content of GbOse₃cer, a molecule that has been recognized as a functional receptor for VT-1 [5,6].

Two recent papers [11,12] have demonstrated that human endothelial cells also become more sensitive to other related toxins, shiga-like toxin II and shiga toxin, when they are simultaneously exposed to TNF α . Tesh et al. [11] has suggested that this may be due to a higher TNF α -susceptibility of endothelial cells whose protein synthesis was inhibited by VT-1. However, in our study, VT-1 was ineffective in untreated confluent endothelial cells, and only reduced protein synthesis in these cells when they were pre- or co-incubated with TNF α . As the incubation with TNF α resulted in a ten- to hundred-fold increase in VT-1 receptors, it is likely that the reduction of protein synthesis and the increased cytotoxicity caused by VT-1 is primarily the consequence of enhanced VT-1 binding to endothelial cells. Once the VT-1 binding sites are increased on the cell surface, the toxicity of TNF α itself may act synergistically to the effect of VT-1.

Obrig et al [30] have observed that shiga toxin has a higher cytotoxicity for subconfluent cells than for confluent cells. Our observation that subconfluent cells bind more VT-1 may correspond with this finding. However, it should be noted that the subconfluent cells were studied 24 hour after exposure to a strong proteolytic agent (trypsin, during passage of the cells), by which the cells certainly have been activated. Furthermore the half-life time of endothelial cells in the normal adult human body is in the order of magnitude of hundred to thousand of days [31], the patho-physiological relevance of VT-1 cytotoxicity towards subconfluent cells will be limited to areas of neovascularisation. Although our confluent cells displayed a low basal VT-1 binding, the amount of VT-1 binding was apparently not sufficient to interfere with the viability and protein synthesis of the endothelial cells.

An exposure of several hours of endothelial cells to TNF α was enough to induce a detectable increase in VT-1 binding to these cells for two days. Therefore, an increase in endothelial VT-1 receptors may persist, even though inflammatory mediators are no longer detectable. No increase in concentrations of TNF α and IL-1 could be established in the plasmas of HUS patients upon arrival in the hospital [32] (and our own unpublished results). However, Siegler [32] recently reported that TNF α was elevated in the urine of HUS

patients. This suggests that $\text{TNF}\alpha$ may indeed have an effect in the kidney of HUS patients. Possible local sources of $\text{TNF}\alpha$ in the kidney are glomerular macrophages [33] and mesangial cells [14,15]. It is of interest to note that a study in mice on the toxicity of a related toxin, verocytotoxin-2 (shiga-like toxin II), has suggested a role of $\text{TNF}\alpha$ in the toxicity of this toxin [34]. Mice defective in macrophage response to LPS, including a defective production of $\text{TNF}\alpha$, showed a consistently longer mean time to death than mice who were normal responsive. In addition, Barrett found that verocytotoxin-2 induced normal peritoneal macrophages to produce $\text{TNF}\alpha$ [34]. This may indicate that verocytotoxins act at various levels, i.e. in activating macrophages to produce $\text{TNF}\alpha$ and in damaging endothelial cells after the induction of their toxin receptors by $\text{TNF}\alpha$. It would be of great interest to know whether mesangial cells can also be induced to release $\text{TNF}\alpha$ upon exposure to verocytotoxins.

In addition to $\text{TNF}\alpha$, other inflammatory mediators, in particular IL-1, lymphotoxin ($\text{TNF}\beta$) and LPS also enhance VT-1 binding sites on endothelial cells. The parallel effects of these mediators on the synthesis of many endothelial proteins is well known [13]. Co-incubation of LPS with Shiga toxin resulted in one recent study [35] in more sensitive endothelial cells than with Shiga toxin alone, whereas in another study [11] no additional effect was observed. Louise and O'Brig [12] reported that co-incubation of IL-1 in combination with Shiga toxin did not result in an enhanced cell death. In contrast, unpublished experiments in our laboratory demonstrate that after 24 h preincubation of HUVEC with IL-1 α or IL-1 β (5 or 50 U/ml) the endothelial viability becomes more sensitive towards VT-1, albeit less dramatical than towards 500 U/ml $\text{TNF}\alpha$. The variation in these data suggests that, IL-1 and LPS, similar to $\text{TNF}\alpha$, can induce VT-1 receptors on endothelial cells, necessary to make these cells vulnerable to verocytotoxin, and that they act in concert with VT-1 on endothelial cell viability, but less profound than high concentrations of $\text{TNF}\alpha$. The combination of low concentrations of both LPS and $\text{TNF}\alpha$, which, in our study, caused a larger increase in the binding of VT-1 on the endothelial cells than did incubation with these mediators separately, may be of interest in HUS, because the patient may become exposed to very low concentrations of LPS during the prodromal phase of acute gastroenteritis.

Because of the fibrin seen in the histopathology of the kidney in HUS and the presence of thrombin-antithrombin complex (TAT), elevated levels of prothrombin fragment F_{1+2} (marker of thrombin formation) [36], and increased concentrations of fibrinopeptide A [37] which are seen in the plasma of HUS patients on admission, we wondered whether thrombin might make HUVEC more sensitive to VT-1. This was not the case. The small increase in VT-1 binding caused by thrombin might be explained by the fact that thrombin can induce HUVEC to produce IL-1 α , which in turn will induce more VT-1 binding sites on the cell surface [38].

The endothelial binding of VT-1, induced by $\text{TNF}\alpha$, was inhibited by the presence of anti-Tj^a serum, which indicates that at least a major part of the specific VT-1 binding is a glycosphingolipid (GbOse₃cer). From VT-1 binding to cellular glycolipid extracts, which were separated by thin layer chromatography, a large increase in the GbOse₃cer content of

TNF α -treated endothelial cells was established. The increase must be due to an increase in GbOse₃cer synthesis rather than to a change in the availability of existing GbOse₃cer on the cell surface, because the amount of GbOse₃cer increased in the cellular extracts and the increase in binding could be prevented by simultaneous condition of cycloheximide. Therefore, we conclude that TNF α induces one (or more) enzyme(s) that is (are) rate-limiting in the synthesis of GbOse₃cer.

It is believed that the presence of verocytotoxins is necessary but not sufficient for getting HUS [1]. Our study suggests that one of the additional stimuli needed to develop the syndrome is the exposure of kidney endothelial cells to one or more of the inflammatory mediators TNF α , IL-1 and LPS. We cannot yet discriminate whether the local availability of inflammatory mediators causes the preferential kidney damage in HUS in children, or whether the sensitivity of the endothelium in human glomeruli is larger than that of other endothelia. Only recently, endothelial cells of glomeruli have been isolated from animal kidneys [39] and isolation of these cells from human glomeruli is still in its infancy. However, in severe cases of HUS, not only kidney endothelial cells are affected, but also the endothelium in many other organs, including brain and pancreas [8]. We therefore believe that our observations on the induction of verocytotoxin receptors on human vein endothelial cells bear impact for understanding the etiology of HUS, as well as for a better understanding of the biological actions of inflammatory mediators.

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Chapter 6

TNF α INDUCES ENDOTHELIAL GALACTOSYL TRANSFERASE ACTIVITY AND VEROCTOTOXIN RECEPTORS. ROLE OF SPECIFIC TNF RECEPTORS AND PROTEIN KINASE C.

Nicole C.A.J. van de Kar^{1,2}, Teake Kooistra¹, Mario Vermeer¹,
Werner Lesslauer³, Leo A.H. Monnens², Victor W.M. van Hinsbergh¹.

¹Gaubius Laboratory TNO-PG, Leiden, The Netherlands.

²Department of Pediatrics, University Hospital, Nijmegen, The Netherlands.

³Hoffmann-La Roche Ltd, Basel, Switzerland

Summary

Infections with verocytotoxin (VT) producing *E.coli* have been strongly implicated in the epidemic form of hemolytic uremic syndrome (HUS). Endothelial damage plays a central role in the pathogenesis of HUS. In vitro studies have shown that VT can damage endothelial cells after interaction with its cellular receptor globotriaosylceramide (GbOse₃cer). Cytokines, such as TNF α and IL-1 can potentiate the toxic effect of VT by inducing a protein-synthesis dependent increase in VT receptors on endothelial cells. In this study the mechanisms underlying the increase in endothelial VT receptors induced by TNF α were studied in more detail.

To investigate which proteins were involved in this induction, endothelial cells were incubated with and without TNF α in the presence of ¹⁴C-galactose or ¹⁴C-glucose. TLC analysis of the glycolipid extracts of these cells demonstrated a markedly enhanced incorporation of ¹⁴C-galactose in GbOse₃cer and other galactose-containing glycolipids, suggesting that TNF α enhanced galactosyl-transferase activity(ies).

To examine the role of the two recently cloned TNF-receptors (TNFR-p75 and TNFR-p55) in the TNF α -induced increase in GbOse₃cer in human endothelial cells, cells were incubated with TNF α , the TNFR-p55 selective R32W-S86T-TNF α -mutant or the TNFR-p75 selective D143N-A145R-TNF α -mutant. The effect of TNF α activation, determined by binding-experiments with ¹²⁵I-VT-1, could be largely, but not completely mimicked by R32W-S86T-TNF α . Although incubation of cells with D143N-A145R-TNF α did not show an increase in VT-1 binding, the monoclonal antibody utr-1, which prevents binding to TNFR-p75 decreased the TNF α -induced VT-1 binding.

Activation of protein kinase C by phorbol ester increases the expression of VT-1 receptors; this effect was prevented by the protein kinase C inhibitor Ro31-8220 and by homologous desensitization by pretreatment with phorbol ester. In contrast, the presence of the protein kinase inhibitor Ro31-8220 or desensitization of protein kinase C activity reduced the TNF α -induced increase in VT-1 receptors maximally by 50% and 24%, respectively. Comparable reductions in overall proteine synthesis and the synthesis of E-selectin and PAI-1 were observed. This suggests an effect on general protein synthesis rather than a specific effect of protein kinase C in the signal transduction pathway, by which TNF α induces VT-1 receptors.

Our results indicate that TNF α can increase the VT-1 receptors on endothelial cells by inducing galactosyl-transferase activity(ies); that this action of TNF α mainly occurs via the TNFR-p55; and that protein kinase C activation increases expression of VT-1 receptors by a separate mechanism that acts additively to the TNF α -induced increase in VT-1 receptors.

Introduction

The epidemic form of the hemolytic uremic syndrome (HUS) is characterized by hemolytic anemia, thrombocytopenia and acute renal failure. It is mostly seen in young children and

has a prodromal phase of acute, often bloody, gastro-enteritis [1]. Since the beginning of the eighties, it has become clear that verocytotoxin- or Shiga-like toxin-producing *E.coli* infections are the main cause of this form of HUS [2]. A family of three verocytotoxins has been described: verocytotoxin-1 (VT-1) or shiga-like toxin I (SLTI), verocytotoxin-2 (VT-2) or shiga-like toxin II (SLTII) and verocytotoxin-2 variant (VT-2c) [3]. Although the exact pathogenesis is still unknown, endothelial cell damage, predominantly seen in the glomeruli in the kidney, is believed to play a central role [1].

Several *in vitro* studies have shown that purified verocytotoxins can damage the endothelium [4-7]. The functional verocytotoxin receptor, the glycosphingolipid globotriaosylceramide (GbOse₃cer), plays a crucial role in the endothelial cell damage [8]. This receptor has been found in the human kidney [9] and on cultured endothelial cells [10]. Recently, we have reported that inflammatory mediators, such as tumor necrosis factor α (TNF α) and interleukin-1 (IL-1), can potentiate the toxic effect of verocytotoxin-1 to human endothelial cells by inducing an increase in the GbOse₃cer synthesis in these cells [7]. Inflammatory mediators are produced and released by monocytes [11] and mesangial cells *in vitro* [12,13], and may play a local role in the kidney. A recent report from Harel et al [14] demonstrated that shiga-like toxin I can specifically induce TNF α activity in mouse kidney. Increased production of the cytokines TNF α , IL-1 β and IL-6 can also be found in the media of cultured human monocytes after stimulation with verocytotoxin-1 [15]. In this report, we extend our observations and demonstrate that TNF α and IL-1 induce specific galactosyl-transferase(s), which is (are) necessary for the synthesis of GbOse₃cer in the endothelial cells.

Recently, two TNF receptors (TNFRs) have been identified and cloned, a 55 kDa receptor TNFR-p55 and a 75 kDa receptor TNFR-p75 [16]. Both receptors are present on human endothelial cells [17,18]. The TNFR-p55 has been demonstrated to be involved in the signal transduction of TNF α during the induction of several products of endothelial cells, such as E-selectin and VCAM-1 [17]. The involvement of both TNF receptors in the TNF α -induced increase in GbOse₃cer synthesis and the role of protein kinase C (PKC) in the induction of verocytotoxin receptors in human endothelial cells were studied.

Materials and methods

Materials

Purified VT-1 was prepared in the laboratory of Dr Karmali (1.2 mg protein/mL; CD₅₀ verocells: titer 10⁻⁸ to 10⁻⁹) [19]. Endotoxin content of the VT-1 preparation was less than 0.05 EU/mL by Limulus amoebocyte lysate assay (E-Toxic, Sigma Chemicals, St Louis, MO) at detection level 0.05 to 0.10 EU/mL. M199 medium supplemented with 20 mmol/L HEPES was obtained from Flow Laboratories (Irvine, Scotland); tissue culture plastics were from Costar (Cambridge, MA). A crude preparation of endothelial cell growth factor was prepared from bovine brain as described by Maciag et al [20]. Human serum was obtained from a local blood bank and was prepared from fresh blood of healthy donors, pooled and stored

at 4°C; it was not heat-inactivated before use. Newborn calf serum (NBCS) was from GIBCO (Grand Island, NY), it was heat-inactivated before use (at 56°C for 30 minutes). Heparin was purchased from Leo Pharmaceuticals (Weesp, the Netherlands). Penicillin/streptomycin was from Boehringer Mannheim (Mannheim, Germany). Human fibronectin was a gift from J.A. van Mourik, Central Laboratory of the Red Cross Blood Transfusion Service (Amsterdam, the Netherlands). Pyrogen-free human serum albumin (HSA) was purchased from the Central Laboratory of the Red Cross Blood Transfusion Service (Amsterdam, the Netherlands). Human recombinant TNF α was a gift from Jan Tavernier (Biogent, Ghent, Belgium). The TNF α preparation contained 2.45×10^7 U/mg protein and less than 40 ng lipopolysaccharide (LPS) per mg protein. Human recombinant IL-1 β was a gift from S. Gillis (Immunex, Seattle, WA); it had a specific activity of 10^8 U/mg. The mutant R32W-S86T-TNF α , which selectively binds and activates the human TNFR-p55, and does not react with the human TNFR-p75; and the mutant D143N-A145R-TNF α , which specifically recognizes the TNFR-p75, were previously reported [21]. The antagonistic monoclonal antibody utr-1, specific for the TNFR-p75, and the agonistic monoclonal antibody htr-9, specific for the TNFR-p55 were previously reported [22].

The specific PKC-inhibitor C3 (Ro-31-8220) [23] was a gift from Dr G.Lawton (Hoffmann La Roche, Welwyn Garden City, UK). The inhibitors H-7 and HA-1004 were purchased from Seikagaku, Tokyo, Japan). Phorbol 12-myristate 13-acetate (PMA), bovine serum albumin (BSA) and Tween 20 were obtained from Sigma Chemical Co (St Louis, Mo). Na¹²⁵I-iodine, ¹⁴C-glucose (50-60 mCi/mmol) and ¹⁴C-galactose (50.3 mCi/mmol) were purchased from Amersham (Amersham, UK). Iodo-gen iodination reagent was obtained from Pierce (Rockford, IL).

Chloroform, methanol, and hexane were obtained from Merck (Darmstadt, Germany). Plastic coated silica gel F1500 thin-layer chromatography (TLC) plates were from Schleicher and Schüll (Dassel, Germany). Polyisobutylmethacrylate was obtained from PolySciences (Washington, MD). A standard mixture of pure neutral glycosphingolipids containing Gal β 1-1Ceramide(Cer) (CMH), Gal β 1-4Glc β 1-1Cer (CDH), Gal α 1-4Gal β 1-4Glc β 1-1Cer (GbOse₄cer), GalNac β 1-3Gal α 1-4Gal β 1-4Glc β 1-1Cer (GbOse₄cer) and GalNac α 1-3GalNac β 1-3Gal α 1-4Gal β 1-4Glc β 1-1Cer (Forssmann pentasaccharide) was from Biocarb (Lund, Sweden). X-OMAT x-ray film was from Eastman Kodak (Rochester, NY).

Cell culture

Endothelial cells from human umbilical vein (HUVEC) and from human foreskin were isolated by collagenase treatment, cultured, and characterized as previously described [24-27]. The endothelial cells were seeded in fibronectin-coated 10-cm² wells and cultured in M199 medium supplemented with 20 mmol/L HEPES (pH 7.4), 10% (v/v) human serum (HS), 10% (v/v) heat-inactivated newborn calf serum, 2 mmol/L L-glutamine, 5 U/mL heparin, and 150 μ g/mL crude preparation of endothelial growth factor under 5% CO₂ and 95% air at 37°C. When the cells reached confluency, they were detached with trypsin/EDTA and seeded in 2-cm² fibronectin coated dishes with a split ratio of 1:3. HUVEC in the experiments were used after one to three passages. Human foreskin microvascular endothelial

cells were used after four to six passages. The medium was renewed every 2 or 3 days.

Binding of ^{125}I -VT-1 to human endothelial cells

VT-1 was radiolabeled with Na^{125}I according to the Iodogen procedure [28]. Five preparations of purified VT-1 were iodinated to specific activities ranging from $16.2\ \mu\text{Ci}/\mu\text{g}$ till $26.8\ \mu\text{Ci}/\mu\text{g}$ of protein. All preparations gave similar results. For the experiment, confluent HUVEC, cultured in 2-cm^2 wells, were incubated for indicated times with medium M199 to which the appropriate concentration of the test-compound(s) was (were) added. The inhibitory antibody utr-1 and the PKC-inhibitor Ro31-8220 were added one hour before the start of the experiment.

The binding assay was performed as follows: after the incubation period at 37°C with the indicated compounds, the endothelial cell cultures in 24-well plates were washed with M199 medium plus 0.1% HSA (w/v). Subsequently, the cells were incubated for 3 hours with $1.0\ \text{nmol/L}$ ^{125}I -VT1 in M199 plus 0.1% HSA (w/v) at 0°C . After the incubation, the supernatant fluid was aspirated, the cells were washed five times with M199 plus 0.1% HSA, and total cell protein was solubilized in $400\ \mu\text{L}$ $0.5\ \text{mol/L}$ sodium hydroxide at room temperature. Radioactivity of the endothelial cells was measured in a gamma-counter. Non-specific binding was determined by assay of ^{125}I -VT1 binding in the presence of 50-fold excess of unlabeled VT-1. Cellular specific binding was determined by subtracting the non-specific binding from the cellular binding of ^{125}I -VT-1 determined in the absence of unlabeled VT-1.

Extraction of glycolipids

Confluent endothelial cell cultures were incubated for 24 hours with or without $20\ \text{ng/mL}$ $\text{TNF}\alpha$ or $0.5\ \text{ng/mL}$ $\text{IL-1}\beta$. Six hours after the start of the incubation, $0.5\ \mu\text{Ci/mL}$ ^{14}C -glucose or ^{14}C -galactose was added to the media. Subsequently, after the 24 hours incubation period, the glycolipids were extracted as described by Lingwood et al [8]. In short, the cells were trypsinized, harvested with ice-cold phosphate-buffered saline (PBS), and spun down by 3-minute centrifugation ($3,000\ \text{rpm}$) at 4°C . The pellet was washed three times with PBS. The pellet was finally suspended in PBS, and 20 vol of chloroform/methanol (2:1, vol/vol) was added. Cell debris was removed by filtration through glass-wool. One volume of water was added to obtain phase separation. The lower phase was dried and incubated at 37°C for 2 hours in $1\ \text{mL}$ $0.4\ \text{mol/L}$ KOH in ethanol; 2 vol of chloroform was added and the mixture was partitioned against 2 vol of water. The lower phase was separated and frozen until TLC studies were performed.

Thin layer chromatography (TLC)

The lower phase from the extraction above was dried and resuspended in chloroform/methanol (2:1, v/v). Samples were separated on a silica gel TLC plate using chloroform:methanol:water (65:25:4, v/v/v). Standard neutral glycosphingolipids, $2\ \mu\text{g}$ of each glycolipid, together with an equal volume of unlabeled glycolipid cell-extract, were run on the same TLC and afterwards stained by orcinol-spray. After separation, the plate was air-dried, and exposed to X-OMAT x-ray film.

After developing the film, the plate was soaked three times for 1 minute in 0.01% polyisobutylmethacrylate in hexane and air-dried, followed by overnight incubation in PBS supplemented with 1% BSA and 0.05% Tween 20. Subsequently, the plate was incubated with 50 mL VT-1 solution (15 nmol/L unlabeled, 1.5 nmol/L ^{125}I -VT-1 in PBS supplemented with 1% BSA and 0.05% Tween 20) for 4 hours at 4°C. The plate was extensively washed with 0.05% Tween 20 and 1% BSA in PBS, air-dried, and exposed to X-OMAT x-ray film.

Assays

Levels of PAI-1 antigen in endothelial cell-conditioned medium were assayed by ELISA (IMULYSE™ PAI-1) obtained from Biopool (Umea, Sweden), according to the manufacturer's description.

The presence of E-selectin was determined by cell-ELISA in triplicate wells with HUVEC cultured in fibronectin-coated 96-multiwell dishes. After 5 h incubation with 20 ng/mL TNF α (inhibitors added 1 h before addition of TNF α), the amount of E-selectin was determined on fixed cells by using an anti-E-selectin monoclonal antibody Ena-2 [29] (gift of Jet Leeuwenberg, Maastricht, The Netherlands) and a rat peroxidase-labeled anti-mouse IgG. After development of the assay, the optical density was recorded.

Statistics

Experiments were done with at least three different cultures of HUVEC, unless otherwise mentioned. Data are given as mean $\pm$ SEM. Statistical analysis was performed with the Wilcoxon-test. Statistical significance was accepted for $p < 0.05$.

Results

TNF α increases the activities of glycolipid galactosyl transferases

Our earlier report showed that the incubation of human endothelial cells with the cytokines TNF α or IL-1 β causes a protein synthesis-dependent increase in the number of VT-1 receptor molecules, the glycosphingolipid globotriaosylceramide (GbOse₃cer, Gal α 1-4Gal β 1-4Glc β 1-1Cer)⁷. The biosynthesis of glycosphingolipids occurs via sequential transfer of sugar moieties from nucleotide sugar donors to ceramide. Specific glucosyl- and galactosyltransferases are involved in this process.

To investigate whether the increase in GbOse₃cer molecules by the cytokines TNF α or IL-1 β is due to the induction of glucosyl- or galactosyltransferases, HUVEC were incubated with or without the cytokines TNF α or IL-1 β together with ^{14}C -glucose or ^{14}C -galactose for a period of 24 hours. After the incubation-period, the ^{14}C -labeled neutral glycosphingolipids were extracted and separated on thin layer chromatography. In Figure 1, it is demonstrated that the incorporation of ^{14}C -galactose in GbOse₃cer and other neutral glycosphingolipids is markedly enhanced after incubation of the cells with TNF α and, to a less extent, with IL-1 β (lanes b-d). The presence of GbOse₃cer was confirmed by the standard sample of neutral glycosphingolipids (lane a) and by incubation of the thin layer chromatography with ^{125}I -VT-

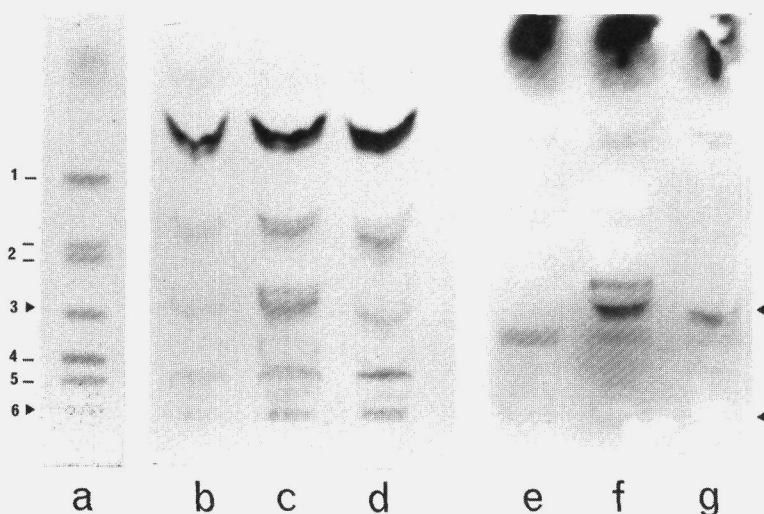


Figure 1. Increase of galactose-containing glycolipids extracted from 1.6×10^6 HUVEC. Confluent HUVEC were incubated for 24 hours with or without 20 ng/mL TNF α or 50 U/mL IL-1 β . Six hours after addition of TNF α , 0.5 μ Ci/mL ¹⁴C-galactose was added to the medium. After the incubation-period, glycolipids of 1.6×10^6 cells were extracted, separated by TLC. Lane a: standard neutral glycosphingolipids, 2 μ g of each glycolipid, visualized by orcinol-spray. Lane b-d: Autoradiograms of ¹⁴C-galactose containing endothelial glycosphingolipids. Separated glycolipid-extracts of 1.6×10^6 cells treated with no inflammatory mediator (lane b), with 20 ng/mL TNF α (lane c) and with 50 U/mL IL-1 β (lane d). Lanes e-g: The same TLC was assayed for ¹²⁵I-VT-1 binding. Autoradiographs of ¹²⁵I-VT-1 binding to glycolipid-extracts of control cells (lane e), treated with 20 ng/mL TNF α (lane f), or with 50 U/mL IL-1 β (lane g). 1, galactosylceramide; 2, lactosylceramide; 3, globotriaosylceramide (GbOse₃cer); 4, globotetraosylceramide; 5, Forssman pentasaccharide; 6, origin of the lane.

1, which specifically binds to GbOse₃cer (lane e-g). The increase in the incorporation of ¹⁴C-galactose in GbOse₃cer is paralleled by an increase in ¹²⁵I-VT-1 binding to the GbOse₃cer position on the thin layer chromatography. A similar TNF α -induced increase in ¹⁴C-galactose incorporation in GbOse₃cer and other neutral glycosphingolipids was also observed in human foreskin microvascular endothelial cells (not shown). Subsequent experiments, in which the incorporation of ¹⁴C-glucose and ¹⁴C-galactose were compared, demonstrated that incorporation ¹⁴C-glucose in glycosphingolipids in the TNF α -treated HUVEC were comparable to that in control cells (Figure 2).

Involvement of the two TNF receptors in the TNF α -mediated increase in verocytotoxin receptors

To investigate which of the recently cloned TNF receptors, TNFR-p55 and TNFR-p75, is involved in the induction of GbOse₃cer in human endothelial cells by TNF α , the binding of ¹²⁵I-VT-1 was determined after incubation of HUVEC with wild-type TNF α , the mutant R32W-S86T-TNF α which recognizes and stimulates TNFR-p55 only, or the mutant D143N-

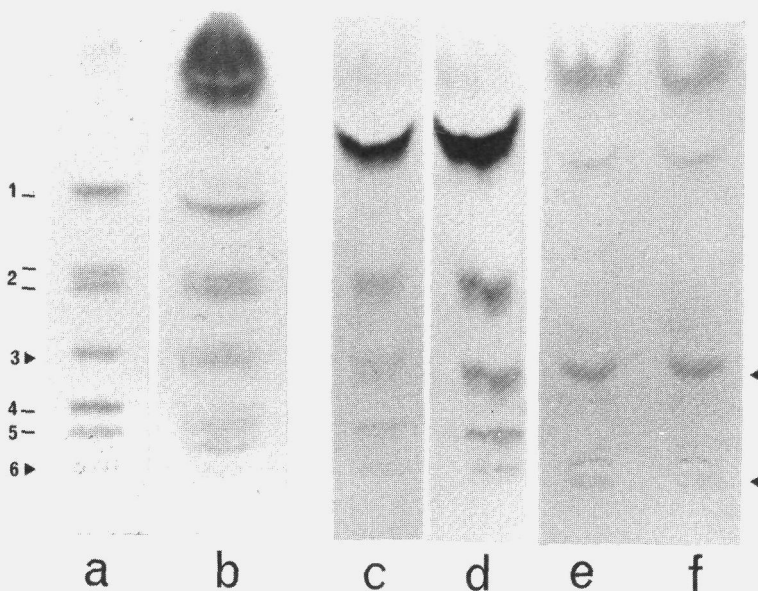


Figure 2. Autoradiogram of ^{14}C -glucose- or ^{14}C -galactose-containing glycolipid-extracts separated on TLC. Confluent HUVEC treated for 24 hours without or with 20 ng/mL $\text{TNF}\alpha$ together with ^{14}C -glucose or ^{14}C -galactose. Glycolipids of 1.6×10^6 cells were extracted and separated on TLC. Lane a: Standard mixture of 2 μg of each neutral glycosphingolipids visualized by orcinol-spray. Lane b: Standard mixture of neutral glycosphingolipids mixed with unlabeled glycosphingolipid cell-extract, visualized with orcinol spray. ^{14}C -galactose containing glycolipid-extract of control cells (lane c) and of $\text{TNF}\alpha$ -treated cells (lane d). ^{14}C -glucose containing glycolipid-extract of control cells (lane e), ^{14}C -glucose containing glycolipid-extract of $\text{TNF}\alpha$ -treated cells (lane f). 1, galactosylceramide; 2, lactosylceramide; 3, globotriaosylceramide (GbOse_3cer); 4, globotetraosylceramide; 5, Forssman pentasaccharide; 6, origin of the lane.

A145R- $\text{TNF}\alpha$, which specifically interacts with the TNFR-p75 . With concentrations up to 20 ng/mL, R32W-S86T- $\text{TNF}\alpha$ induced a concentration-dependent increase in VT-1 binding, whereas D143N-A145R- $\text{TNF}\alpha$ had no effect (Table 1, Figure 3a-c). Thus, the sole stimulation of the TNFR-p55 is sufficient to induce VT-1 receptor synthesis in endothelial cells. This was confirmed with the agonistic monoclonal antibody htr-9, that specifically activates the TNFR-p55 (Table 1). However, when the effects of wild-type $\text{TNF}\alpha$ and R32W-S86T- $\text{TNF}\alpha$ were compared, the mutant reached $77 \pm 5\%$ of the effect of the wild-type $\text{TNF}\alpha$ (mean $\pm$ SEM, $P < 0.05$; paired data of 7 cultures, incubated for 24 h with 20 ng/ml of both $\text{TNF}\alpha$ forms). Furthermore, it was observed in several time course experiments that, at low concentrations of $\text{TNF}\alpha$ and its mutant (0.2 ng/ml), the initial increase of VT-1 receptors induced by the R32W-S86T- $\text{TNF}\alpha$ was detectable one or two hours later than that by wild-type $\text{TNF}\alpha$.

These observations suggest that wild-type $\text{TNF}\alpha$ provides a signal additional to stimulation of the TNFR-p55 , probably via the TNFR-p75 . This may occur by activation of the TNFR-p55 via TNFR-p75 -mediated signal transduction, or by concentration of $\text{TNF}\alpha$

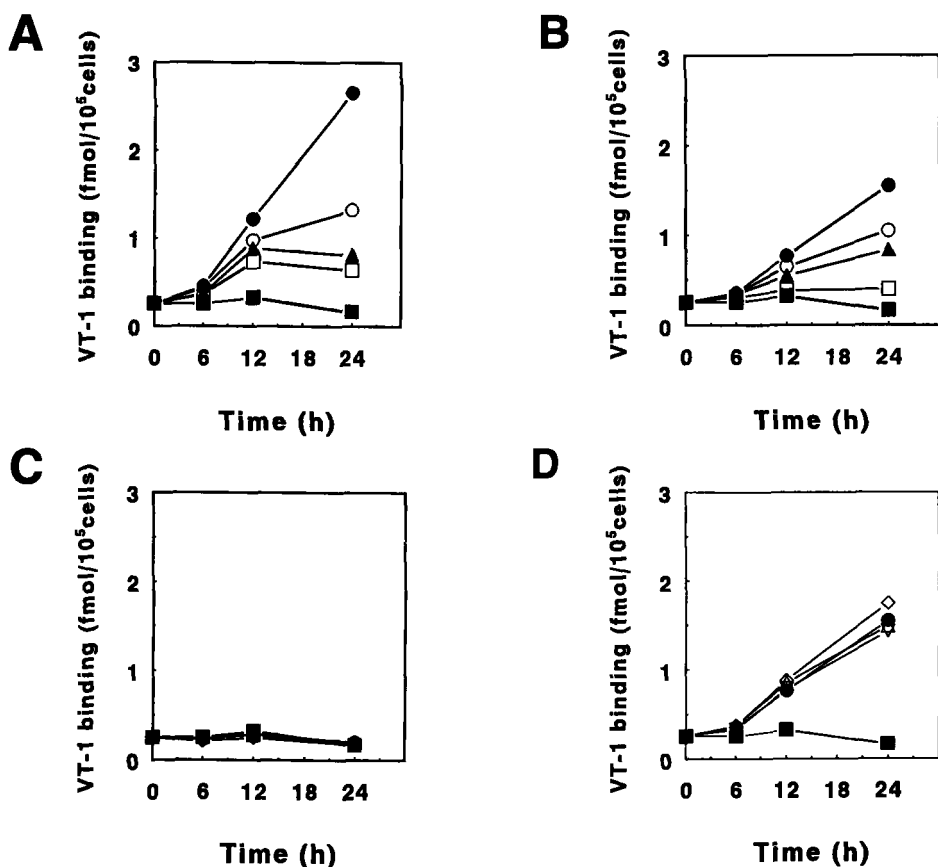


Figure 3. Effect of various concentrations of wild-type TNF α (A), the TNFR-p55-specific mutant R32W-S86T-TNF α (B) and the TNFR-p75-specific mutant D143N-A145R-TNF α (C) on the specific 125 I-VT-binding to HUVEC. Cells were incubated with a low concentration range wild-type TNF α or TNF α mutant for respectively 6, 12 or 24 hours (■ control cells; □ 0.2 ng/mL; ▲ 0.4 ng/mL; ○ 0.8 ng/mL; ● 2 ng/mL). Concentrations of 20 or 200 ng/mL D143N-A145R-TNF α showed the same binding as 2 ng/mL D143N-A145R-TNF α . D. 125 I-VT binding to endothelial cells incubated for 6, 12 or 24 hours with 2 ng/mL R32W-S86T-TNF α (●) together with D143N-A145R-TNF α at 0.4 ng/mL (▲), 4 ng/mL (◇) or 20 ng/mL (▽); (■ control cells).

molecules on the cell surface by the TNFR-p75, so that the binding to the TNFR-p55 is facilitated by ligand passing [30]. When we added the TNFR-p75-stimulating mutant together with the TNFR-p55-stimulating mutant to the cells, no further increase in VT-1 binding was observed as compared to the TNFR-p55 mutant alone (Figure 3d). Therefore, TNFR-p75-mediated enhancement of the TNFR-p55 activity is unlikely. Similarly, when the TNFR-p75-binding mutant D143N-A145R-TNF α was added simultaneously with wild-type TNF α , no reduction of the VT-1 binding was observed (not shown). However, when the cells were preincubated with the monoclonal antibody utr-1, that blocks the TNFR-p75, a reduction in the effect of TNF α on VT-1 binding was observed after 9 h of incubation (Table 2). After

Table 1. Effect of TNF-receptor agonist on VT-1 binding to human endothelial cells.

Addition	¹²⁵ I-VT-1 binding (fmol/10 ⁵ cells)	
	9 h incubation	24 h incubation
Control	2.3 ± 0.8 (9)	1.7 ± 0.6 (10)
Wild-type TNFα (20 ng/mL)	7.7 ± 2.6 (9)*	23.2 ± 5.8 (10)**
R32W-S86T-TNFα (20 ng/mL)	5.8 ± 3.8 (5)*	19.7 ± 7.2 (7)*
D143N-A145R-TNFα (20 ng/mL)	2.4 ± 0.2 (3) [#]	2.2 ± 0.1 (4) [#]
D143N-A145R-TNFα (200 ng/mL)	2.2 ± 0.1 (2) [#]	2.0 ± 0.1 (3) [#]
MoAb htr-9 (10 μg/mL)	n.d.	8.5 ± 1.6 (3)

Specific binding of 1 nmol/L ¹²⁵I-VT to confluent human umbilical vein endothelial cells was determined after 9 or 24 h incubation with wild-type TNFα, the mutant R32W-S86T-TNFα, which only activates TNFR-p55, or the mutant D143N-A145R-TNFα, which only activates TNFR-p75, or with the monoclonal antibody htr-9 (moAb htr-9), which activates the TNFR-p55. Data are expressed as the mean ± SEM of the number of independent experiments indicated between parentheses. Statistically significant difference was evaluated by the Wilcoxon-test for paired data * p < 0.05, ** p < 0.01 as compared to control cells. [#] The control data of these experiments were normalized to the mean control value to aid comparability. N.D.: not determined.

Table 2. Effect of the TNFR-p75 blocking antibody utr-1 on the TNFα-induced increase of VT-1 receptors.

Addition	¹²⁵ I-VT-1 binding (fmol/10 ⁵ cells)			
	culture 1	culture 2	culture 3	% effect utr-1
None	0.6	0.4	6.5	100
utr-1 (10 μg/mL)	0.6	0.5	6.7	108 ± 6
TNFα (2 ng/mL)	3.2	3.1	19.4	100
TNFα (2 ng/mL) + utr-1 (10 μg/mL)	1.6	2.2	15.4	66 ± 9
TNFα (20 ng/mL)	4.9	4.7	26.1	100
TNFα (20 ng/mL) + utr-1 (10 μg/mL)	2.7	3.6	22.6	73 ± 9
R32W-S86T-TNFα (20 ng/mL)	2.6	2.9	20.9	100
R32W-S86T-TNFα (20 ng/mL) + utr-1 (10 μg/mL)	2.3	2.6	21.5	93 ± 5

Effect of the antagonistic monoclonal antibody utr-1, specific for TNFR-p75 on the ¹²⁵I-VT-1 binding to three different cultures of confluent HUVEC. Cells were treated for 9 hours with TNFα or R32W-S86T-TNFα in the absence or presence of the antibody utr-1. The antibody utr-1 was added to the cells 1 hour before addition of TNFα or its mutant. After the 9 h incubation period, the media above the cells were removed and 1 nmol/L ¹²⁵I-VT-1 was added to the cells as described in material and methods. Data are also expressed as the percentage toxin binding as compared to their counterparts, which were not incubated with utr-1 (mean ± SEM for the three experiments).

24 h of incubation, this effect was still observed at moderate concentrations of TNF α (2 ng/mL), but not at high concentrations (20 ng/mL TNF α) (not shown). This is compatible with a "ligand passing" effect, which vanishes at saturating TNF concentrations. The utr-1 monoclonal antibody had no effect on the ability of the TNFR-p55-stimulating R32W-S86T-TNF α to induce VT-1 receptors (Table 2). This makes an aspecific effect of the utr-1 antibody unlikely.

Involvement of protein kinase C in the induction of VT-1 receptors

The protein kinase C activator phorbol myristate acetate (PMA) and the protein kinase C inhibitors Ro31-8220 and H-7 were used to study the role of protein kinase C in the induction of the VT-1 receptor GbOse₃cer. Stimulation of protein kinase C by PMA enhanced the specific binding of VT-1 to a moderate extent in comparison with TNF α (Figure 4a). In six different HUVEC cultures 10 nmol/L PMA stimulated specific ¹²⁵I-VT-1 binding 4 $\pm$ 1 -fold, while 20 ng/mL TNF α induced a 16 $\pm$ 3 -fold increase in the same cells (mean $\pm$ SEM, 24 h incubation). When HUVEC were incubated with PMA and TNF α or R32W-S86T-TNF α simultaneously, the increase of VT-1 receptors was considerably larger than that obtained by TNF α or R32W-S86T-TNF α alone, and the induction occurred faster than after stimulation by TNF α , R32W-S86T-TNF α or PMA alone (Figure 4a). No additional increase in specific VT-1 binding was seen when PMA was given together with D143N-A145R-TNF α , as compared with PMA alone (not shown). For comparison, the production of PAI-1 was assayed in the conditioned medium of the same cells. As expected, PMA did not alter PAI-1 production; addition of 10 nM PMA had a slight stimulatory effect on the TNF α -induced increase in PAI-1 production (Figure 4b).

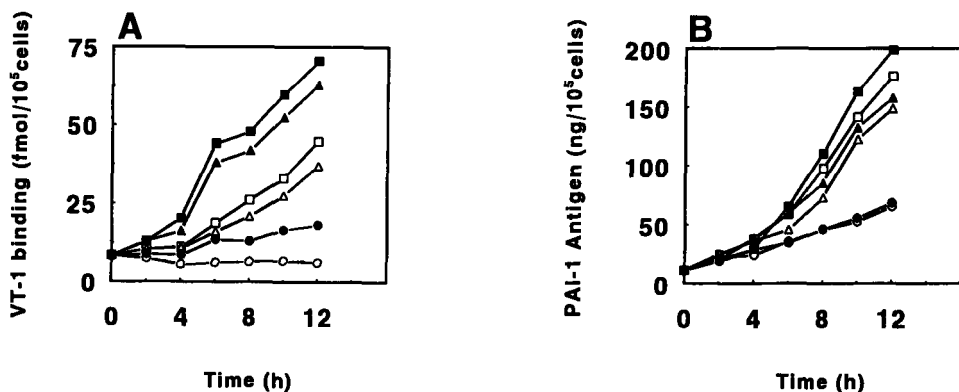


Figure 4. Specific binding of ¹²⁵I-VT-1 (A) and production of PAI-1 antigen (B) by confluent human endothelial cells incubated with 10 nmol/L phorbol ester PMA (closed symbols) or in its absence (open symbols). HUVEC were simultaneously incubated with 20 ng/mL TNF α (■, □) or 20 ng/mL R32W-S86T-TNF α (▲, △) for the indicated time intervals. The control cells are indicated with circles. No difference in ¹²⁵I-VT-1 binding and PAI-1 production was observed when D143W-A145R-TNF α was incubated together with 10 nmol/L PMA as compared to PMA alone (data not shown).

To evaluate the role of protein kinase C activity in the PMA- and $\text{TNF}\alpha$ -induced increases in VT-1 receptors, HUVEC were preincubated for 20 h with 10^{-6} mol/L PMA, washed three times and subsequently incubated for another 24 h with 10 nmol/L PMA or 2 ng/mL $\text{TNF}\alpha$. Whereas PMA enhanced the induction of VT-1 receptors under control conditions, its effect disappeared after homologous desensitisation (Figure 5). Desensitization of protein kinase C activity by PMA pretreatment reduced $\text{TNF}\alpha$ -induced increase in VT-1 receptors only by $24 \pm 9\%$. A similar decrease ($22 \pm 11\%$) was seen in $\text{TNF}\alpha$ -increased PAI-1 synthesis of these cells (302 ± 19 vs 392 ± 18 ng PAI-1/24 h/ 10^5 cells in $\text{TNF}\alpha$ -stimulated cells pretreated with 10^{-6} mol/L PMA or control medium, respectively; 3 independent cultures).

Comparable results were obtained with protein kinase C inhibitors. While the inhibitor Ro31-8220 at $3 \mu\text{mol/L}$ completely suppressed the effect of PMA on VT-1 binding (Figure 6, inset), it reduced the induction of VT-1 receptors by the single addition of $\text{TNF}\alpha$ or

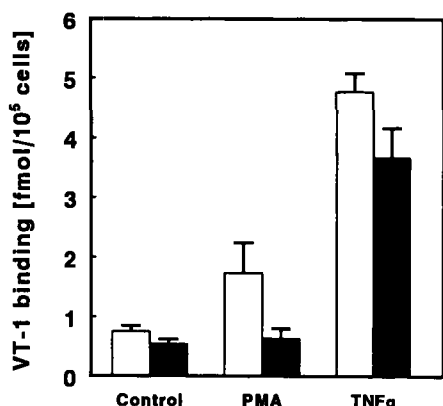


Figure 5. Effect of protein kinase C desensitization on the induction of VT-1 receptors by $\text{TNF}\alpha$ and phorbol ester PMA. HUVEC were preincubated for 20 h in culture medium supplemented with 10^{-6} M PMA (black bars) or in the same medium without PMA (open bars). Subsequently, the cells were washed three times and incubated for 24 h in culture medium supplemented with 20 ng/mL $\text{TNF}\alpha$, 10 nM PMA or without addition (control), after which specific binding of ^{125}I -VT-1 to the cells was determined. The data are the mean $\pm$ SEM of 3 different HUVEC cultures.

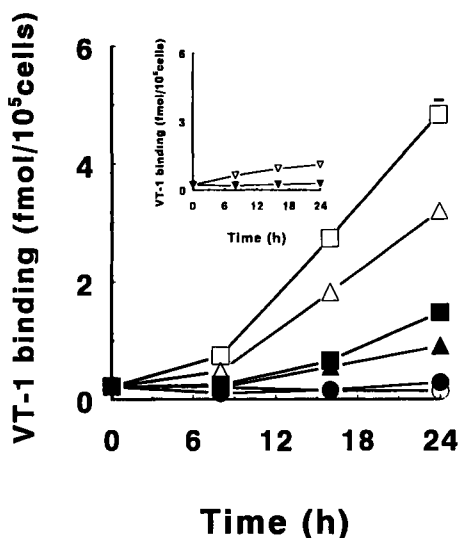


Figure 6. Specific binding of ^{125}I -VT-1 to HUVEC incubated for 8, 16 or 24 hours with 20 ng/mL $\text{TNF}\alpha$ (□), 20 ng/mL R32W-S86T- $\text{TNF}\alpha$ (Δ) or without addition (○). The protein kinase C inhibitor Ro31-8220 ($3 \mu\text{mol/L}$) was added one hour before the start of the experiment to the cells and remained present during the incubation period (closed symbols). Results are the mean $\pm$ SEM of three independent experiments. Inset: Specific binding of ^{125}I -VT-1 to confluent HUVEC treated with 10 nmol/L PMA in the presence of $3 \mu\text{mol/L}$ Ro31-8220 (▼) or without inhibitor (▽).

R32W-S86T-TNF α by 48 ± 9 % (Figures 6, 7a). Incubation of the cells with another protein kinase C inhibitor, H-7 (30 μ mol/L), gave the same results as obtained with Ro31-8220, whereas a structural homologue of H-7, HA-1004 (30 μ mol/L), which has a similar protein kinase A-inhibiting capacity as H-7, but much less protein kinase C-inhibiting activity, was inactive in this respect (Figure 7a). However, Ro-31-8220 (3 μ Mol/L) and H-7 (30 μ mol/L), but not HA-1004 (30 μ mol/L), inhibited TNF α -induced expression of PAI-1 and E-selectin, a protein of which the TNF α induction is not dependent on protein kinase C activity [31,32], to a comparable extent (Figure 7b,c). Furthermore, the reduction in TNF α -induced

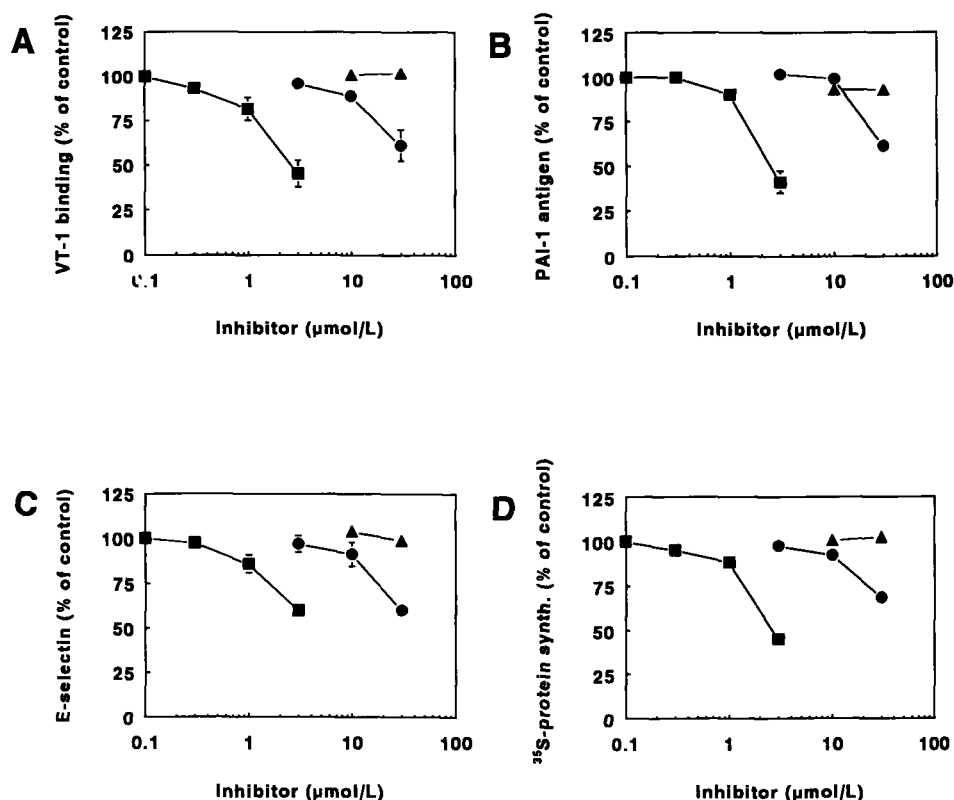


Figure 7. A. Concentration dependency of the inhibition of TNF α -induced 125 I-VT-1 binding to HUVEC by protein kinase C inhibitors. HUVEC were preincubated for 1 h with various concentrations of Ro31-8220 (■), H-7 (●) or HA-1004 (▲) and incubated for 24 h in the presence of these inhibitors and 20 ng/mL TNF α . Subsequently, the binding of 1 nmol/mL 125 I-VT-1 was determined. B. Production of PAI-1 antigen by the same cells during the 24 h incubation with inhibitors. C. Expression of E-selectin by HUVEC after 5 h exposure to 20 ng/mL TNF α and the indicated inhibitors. E-selectin was assayed by cell-ELISA as described in Materials and methods. D. Incorporation of 35 S-methionine in 10%-TCA-precipitable proteins⁷ during a 24 h incubation with TNF α and the various inhibitors, indicated in A. The values represent the mean $\pm$ SEM of 3 to 5 independent HUVEC cultures.

VT-1 receptors by these inhibitors was paralleled by a comparable reduction in overall protein synthesis, as estimated from the incorporation of ^{35}S -methionine in proteins (Figure 7d). In the absence of $\text{TNF}\alpha$, these inhibitors affected protein synthesis by less than 10% (not shown).

These findings indicate that activation of protein kinase C underlies the stimulation of VT-1 receptors by PMA. They suggest that protein kinase C is not directly involved in the $\text{TNF}\alpha$ -dependent signalling pathway causing induction of VT-1 receptors, but can contribute via another pathway additionally to the effect of $\text{TNF}\alpha$ on the expression of VT-1 receptors.

Discussion

The inflammatory mediators $\text{TNF}\alpha$ and IL-1 increase the toxicity of verocytotoxin and the closely related shiga toxin for human endothelial cells [5-7]. Previously, we have demonstrated that $\text{TNF}\alpha$ and IL-1 increase the number of VT-1 receptors known to be globotriaosyl-ceramide (GbOse_3cer) on endothelial cells, and that protein synthesis was necessary for this induction [7]. Here, we have demonstrated that the $\text{TNF}\alpha$ -induced increase in VT-1 receptors is due to an increase in galactosyl-transferase activity(ies) in the endothelial cell. This induction occurs predominantly via the TNFR-p55 by a mechanism distinct from the induction of VT-1 receptors by protein kinase C activation.

The kidney contains a relatively high amount of glycosphingolipids [8,33]. GbOse_3cer is in particular found in the tubular epithelial cells [33,34]. It is also encountered in the glomeruli of children younger than two years old [35], but the cellular distribution in the glomeruli has not been resolved yet. In the glomeruli of kidneys of adults and children older than two years no significant expression of GbOse_3cer was found [35]. This suggests a developmental shift in the synthesis of glycosphingolipids in glomerular cells. In cultured cells glycosphingolipids play a role in cell growth and cell differentiation [33], but little is known about the physiological role of these glycosphingolipids in various cell types of the intact kidney. Bacterial exotoxins use specific glycolipids as receptors to enter eukaryotic cells where they interfere with the metabolism of the cell [36,37]. In the case of verocytotoxin the toxicity is primarily caused by inhibition of the interaction of elongation factor-1 with the ribosome, which results in a complete inhibition of protein synthesis [38]. Previous studies have demonstrated that the sensitivity of endothelial cells for the toxin is determined by the number of toxin receptors, i.e. GbOse_3cer [5-7], and that the number of the VT-1 receptors is markedly increased after exposure of endothelial cells to inflammatory mediators [7]. We have suggested that (local) generation of inflammatory mediators may increase the sensitivity of the kidney and, in severe cases of HUS, also that of the endothelium of other organs to verocytotoxin.

Our present data demonstrate that $\text{TNF}\alpha$, and also IL- 1β , induce an enhanced production of neutral galactose-containing glycolipids by an increase in galactosyl-transferase activity. This explains the increase in GbOse_3cer molecules found on $\text{TNF}\alpha$ -stimulated endothelial cells [5-7]. To our knowledge, this is the first report indicating an inductive effect of

inflammatory mediators TNF α and IL-1 on the synthesis of neutral cellular glycolipids. A confirmation of the induction of galactosyl-transferase(s) at the mRNA level is not yet possible, because ceramide glycosyl-transferases have not been cloned, with the exception of a brain-specific ceramide UDP-galactosyl transferase, which was reported very recently [39]. The physiological meaning of the induction of galactosyl-transferase(s) in inflammation is not known. On the basis of sequence homologies of verotoxins and the α -interferon receptor, Lingwood et al [40] has suggested that GbOse₃cer may act as an accessory molecule for the α -interferon receptor. Hence, the altered synthesis of glycosphingolipids may play a role in the modulation of the inflammatory process. In this respect it is of interest to note that another inflammatory mediator, γ -interferon shifts the cellular distribution of glycosphingolipids towards the surface of endothelial cells [41].

TNF α acts on cells via two receptors, TNFR-p55 and TNFR-p75, to which it binds with similar affinity [16]. Both receptors are expressed on unstimulated HUVEC [17,18], but this study shows that activation of TNFR-p55 by TNF α is sufficient for the induction of GbOse₃cer in human endothelial cells. This finding corresponds well with the TNF α -induced expression in endothelial cells of E-selectin, VCAM-1, ICAM-1, interleukin-8, interleukin-6 and GM-CSF, which are under TNFR-p55 control [17,18,42]. However, the TNFR-p55 selective mutant was always slightly less potent than the wild type TNF α . While activation of TNFR-p75 by D143N-A145R-TNF α mutant had no effect on GbOse₃cer synthesis, blocking of TNFR-p75 by the monoclonal antibody utr-1 reduced the TNF α -induced increase in VT-1 receptors, in particular at low TNF α concentrations and at early time points. Because simultaneous stimulation of both TNF receptor types by two TNF α mutants did not enhance VT-1 receptor expression more than obtained by stimulation of the TNFR-p55, it is unlikely that an intracellular signal generated via the TNFR-p75 enhanced TNFR-p55 activity or TNFR-p55-mediated signal(s). Our findings are consistent with the hypothesis of Tartaglia et al [30], who proposed that TNFR-p75 can concentrate the TNF α molecules at the cell-surface, thereby facilitating the TNF α molecule to be passed on to the TNFR-p55. Similar observations have been made regarding the TNF α induced expression of α 2-integrins [17] and the TNF α -induced synthesis of E-selectin [18].

A complex cascade of signal transducing events, including activation of the nuclear transcription factor NF- κ B is probably involved in the induction of various proteins by TNF α in endothelial cells [43]. Protein kinase C activity has been reported to be needed for the induction of some TNF α -induced proteins. The TNF α -induced synthesis of urokinase [44] and the adhesion molecule VCAM-1 [31] can be reduced by inhibitors of protein kinase C, whereas these inhibitors do not affect the TNF α -induced synthesis of E-selectin [31,32], ICAM-1 [32] and PAI-1 [44,45]. Our data indicate that the TNF α -induced increase of the synthesis of VT-1 receptors does not require protein kinase C activity. This conclusion is based on the observations that heterologous desensitisation by PMA did not specifically reduce the TNF α -dependent increase in VT-1 binding, while homologous desensitization entirely prevented an increase of VT-1 receptors by PMA. In the presence of TNF α , the protein kinase C inhibitors Ro31-8220 and H-7, but not the structural analogue HA-1004, reduced VT-1 receptor expression to a similar extent as that of E-selectin and PAI-1,

probably as the result of a generally reduced protein synthesis. This suggests that under our experimental conditions protein kinase C activity can become a limiting factor in protein synthesis in endothelial cells exposed to $\text{TNF}\alpha$.

Activation of protein kinase C by itself causes a moderate increase in VT-1 receptors, and as such adds to the $\text{TNF}\alpha$ -induced increase in VT-1 receptors. It is not known yet whether the effect of PMA on VT-1 binding is caused by an increased insertion of GbOse_3cer -containing caveolae in the plasma membrane after activation of protein kinase C,[46] or by an increased synthesis of VT-1 receptors, similar as happens after activation of endothelial cells by $\text{TNF}\alpha$. In the latter case, the induction of galactosyl transferase activity, which underlies the increase in VT-1 receptors, behaves similarly as the induction of E-selectin, which can also be induced by protein kinase C activation by a pathway that is different from the $\text{TNF}\alpha$ -induced expression [31,32].

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SUMMARY AND PERSPECTIVES FOR THE FUTURE

Summary

The hemolytic uremic syndrome (HUS) preceded by an acute gastro-enteritis (D+ HUS) is predominantly seen in childhood. Since the first report by Karmali's group in 1983 [1], several studies from other parts of the world have shown that verocytotoxin-producing *Escherichia coli* (VTEC) infection are the main cause of D+ HUS [2]. In this thesis we have studied the epidemiology and pathophysiology of VTEC infection in D+ HUS.

In chapter 2 the results are presented of a four year retrospective and prospective study of the epidemiology of VTEC infection in patients with HUS and children with acute gastro-enteritis. In the first retrospective study, sera of HUS patients, family members and children with acute gastro-enteritis were examined for the presence of antibodies to the lipopolysaccharide (LPS) of the most common O-serotypes of VTEC. Antibodies to the LPS of O157 antigen were found in sera from 28 of the 45 patients examined. Antibodies to the LPS of serotypes O115 and O145 were detected in the sera from one patient respectively one family member. In the prospective study evidence for VTEC infection was obtained by performing combined microbiological and serological procedures. In total 77% of the 113 HUS patients, 35% of the 95 family members and 3% of the 65 children with acute gastro-enteritis had a VTEC infection. Serum antibodies to O157-antigen yielded the highest amount of positive results compared to the other test methods. Although fecal tests demonstrated that VTEC infection was often present in family members, it is remarkable that the presence of serum antibodies to O157-antigen was significantly lower in the family members as compared to the patients with HUS. Assuming that a VTEC infection in a family was due to the same genotypic O157 strains, a hypothesis confirmed by the recent findings of A Heuvelink et al [3], these family studies might indicate that in cases of diarrhea, yet unknown, host factors in the intestine are involved in determining the pathogenicity of VTEC. The hypothesis that there is a difference in adherence of VTEC between the intestinal epithelium of the young children and adults has to be investigated. These epidemiology studies show that VTEC infection, mainly serotype O157, is the main cause of HUS in childhood in the Netherlands and parts of Germany and Belgium. The fact that not all infants and children, who are infected by VTEC develop HUS, suggested that there might be a genetic predisposition for getting D+ HUS. Therefore, the frequency of HLA antigens of 31 children with chronic renal failure due to D+ HUS from the Netherlands and Flanders were compared to the HLA

frequencies of a control population. A significant association with HLA-B40 was observed in this severe form of D+ HUS as compared with the control population.

The cytokines tumor necrosis factor α (TNF α), interleukin-1 (IL-1) and interleukin-6 (IL-6) are known to play an important role in several infectious diseases and in acute inflammation. The role of inflammatory mediators in the pathogenesis of HUS was investigated in chapter 3. TNF α and its soluble receptors, IL-1 β and IL-6 were measured in the plasma of patients with HUS, children with chronic renal failure and healthy children. No elevated levels of TNF α , IL-1 β and IL-6 were found in the plasma of HUS patients, who had no extra-renal manifestations during the disease. Only in plasma of patients with HUS who had extra-renal manifestations, consisting of seizures, loss of consciousness, coma and pancreatic necrosis, IL-6 was significantly elevated. The fact that inflammatory mediators have a short half-life (less than one hour) may be a reason why they are no longer detectable in the plasma taken on admission in the hospital. On the other hand, several studies have suggested that TNF α may play a local role in the kidney. Siegler et al reported that, although TNF α was not elevated in the plasma of HUS patients, it was significantly elevated in the urine of these patients [4]. In an animal model with transgenic mice Harel and coworkers [5] demonstrated that injected shiga toxin induced TNF α synthesis within the kidney, but not in other tissues. Although the exact function of IL-6 in plasma of HUS patients is still unknown and the number of patients in our study is small, IL-6 in plasma was associated with the severity and outcome of the disease. Studies performed in volunteers have shown that TNF α is involved in the induction of soluble TNF α receptors release [6]. Soluble TNF α receptors were elevated in the plasma of patients with HUS. However, they were also elevated in the plasma of children with chronic renal failure. This implies that elevated soluble TNF receptor levels may be the consequence of the decreased clearance by the disturbed kidney function in HUS patients.

Normal vascular endothelium has the capacity to prevent thrombosis by producing proteins that retard or inhibit the coagulation pathway, proteins that interfere with platelet aggregation and vasoconstriction, and the release of tissue type plasminogen activator (t-PA), which triggers fibrinolysis. Endothelial damage causes to a disturbance in the balance between the coagulation and fibrinolytic system resulting in thrombi and fibrin depositions. Endothelial damage in HUS is mainly seen in the kidney. Histopathological studies show swollen endothelial cells detached from the glomerular basement membrane, and in the lumen of the glomerular capillaries, thrombi and fibrin depositions are seen. The results of plasma levels of fibrinolytic parameters and von Willebrand factor (vWF) antigen in patients with HUS and the effect of purified verocytotoxin-1 (VT-1) on cultured human endothelial cells are reported in chapter 4. Elevated levels of t-PA, plasminogen activator inhibitor type I (PAI-1) activity, vWF as well as fibrin(ogen) degradation products were observed in the plasma of HUS patients on admission as compared to the control groups of children with chronic renal failure and healthy control children. After two to three weeks, t-PA antigen and vWF had partially returned to basal values and PAI-1 activity had been normalized. On admission no response of the t-PA, vWF and urokinase (u-PA) were seen upon DDAVP infusion, but after two weeks t-PA increased again after DDAVP infusion. These data

suggest that on admission- in addition to the hemorrhagic colitis and the kidney failure- HUS patients display a general activation of the vascular endothelium. In order to investigate the role of verocytotoxin in the alteration of the fibrinolytic system found in HUS, purified verocytotoxin-1 (VT-1) was added to the media of cultured endothelial cells. No change in the production of t-PA, PAI-1 and vWF was observed when VT-1 alone was added to endothelial cells. However, when VT-1 was added to endothelial cells that contained sufficient VT-1 receptors, a decrease in production of t-PA, PAI-1 and vWF was seen likely due to an inhibitory effect of VT-1 on overall protein-synthesis. While VT-1 causes a decrease of the production of fibrinolytic proteins by endothelial cells in vitro, the plasma levels of these proteins in HUS patients changed in the opposite direction. Although these data appear to be paradoxical, they do not really conflict. We hypothesized that after a period of hemorrhagic colitis, the main affected organ in HUS is the kidney. It is known that the human kidney contains the receptor for verocytotoxin and the toxin is assumed to contribute to the damage the endothelial damage in the glomeruli. This damage can lead to a local prothrombotic condition. Then, thrombin and inflammatory mediators are generated and leucocytes become activated, which cause to a general activation of the endothelium at sites distal of the affected areas.

The effect of purified VT-1 on cultured human umbilical vein endothelial cells (HUVEC) was examined in chapter 5. By investigating whether local factors generated during coagulation or inflammation can make the endothelium more vulnerable to verocytotoxins, we observed that endothelial cell damage by VT-1 depends on the additional presence of the inflammatory mediator $\text{TNF}\alpha$ or IL-1. Studies with ^{125}I -VT1 demonstrated that the preincubation of the endothelial cells with $\text{TNF}\alpha$ led to an increase in VT-1 binding. Although confluent non-stimulated HUVEC demonstrated a low basal ^{125}I -VT-1 binding, the amount of VT-1 binding was apparently not sufficient to interfere with the viability and protein synthesis of the endothelial cells. The effect of $\text{TNF}\alpha$ on ^{125}I -VT-1 binding was concentration-dependent and saturated at approximately 10 nmol/l. From Scatchard-plot analysis it was concluded that the apparent affinity of VT-1 binding to HUVEC did not change, but the number of specific binding-sites increased after incubation with $\text{TNF}\alpha$. In addition to $\text{TNF}\alpha$, other inflammatory mediators, in particular interleukin-1 and $\text{TNF}\beta$, and LPS, also enhance the binding-sites on endothelial cells. The $\text{TNF}\alpha$ -induced VT-1 binding sites on endothelial cells were identified as the glycolipid GbOse_3cer . This $\text{TNF}\alpha$ -induced increase in VT-1 receptors involved de novo synthesis of protein(s). This study suggests that the presence of verocytotoxins is necessary but not sufficient for getting HUS. Inflammatory mediators, such as $\text{TNF}\alpha$ and IL-1 might be one of the additional stimuli needed to develop the syndrome.

The mechanisms underlying the $\text{TNF}\alpha$ -induced increase in VT-1 receptors in human endothelial cells were studied more in detail in Chapter 6. The biosynthesis of glycosphingolipids occurs via sequential transfer of sugar moieties from an activated sugar donor, usually nucleotide sugars, to ceramide which involves glycosyltransferases. The specificity of the enzymes for their donor and acceptor substrates constitutes the primary basis for determining the structures of the sugar chains produced by a cell. The level of

glycosyltransferase transcription may change during tissue morphogenesis and embryonic development, cellular differentiation, disease or tumor metastasis [7,8]. The data presented in chapter 6 demonstrate that the inflammatory mediators $\text{TNF}\alpha$ and IL-1, induce an enhanced production of the neutral galactose-containing glycosphingolipids as the verocytotoxin receptor Gb3 by an increase in galactosyl-transferase activity(ies). The physiological meaning of the induction of galactosyl-transferase(s) in inflammation is yet not known. $\text{TNF}\alpha$ acts on endothelial cells via two receptors, a 55KDa receptor (TNFR-p55) and a 75KDa receptor (TNFR-p75), to which it binds with similar affinity. Both receptors are expressed in unstimulated HUVEC. By using specific $\text{TNF}\alpha$ mutants and monoclonal antibodies we have shown in this study that activation of the TNFR-p55 by $\text{TNF}\alpha$ is sufficient for the induction of the VT-1 receptor GbOse₃cer in human endothelial cells. A role for TNFR-p75 was demonstrated at low $\text{TNF}\alpha$ concentrations and during the first eight to ten hours of $\text{TNF}\alpha$ incubation, probably by facilitating the $\text{TNF}\alpha$ molecule to be passed on to the TNFR-p55. Protein kinase C activity, as signalling pathway, was reported not to be required for the induction of the $\text{TNF}\alpha$ -induced increase in VT-1 receptors in HUVEC.

Perspectives for the future

Although the recognition of verocytotoxin-producing *E.coli* infections as the main cause of the diarrhea-associated (D+) HUS has given an impulse to a better understanding of the pathophysiology of HUS, the treatment has yet not changed. Patients with HUS are still only treated supportively. In Canada a new therapeutical agent, SYNSORB-Pk, has recently been developed. SYNSORB-Pk is a porous particle to which synthetic oligosaccharide receptors for verocytotoxin are attached [9]. These particles appear to bind verocytotoxins effectively and make them inactive. A three year Canadian multicentre-study has just started to evaluate the efficacy of this agent in preventing HUS in children with bloody diarrhea. Until now prevention of VTEC infection is still the best method to reduce the incidence of HUS. The most commonly isolated *E.coli* strain O157:H7 is generally associated with dairy cattle and their products, milk and beef; but it has also caused outbreaks associated with water and apple-cider. Handling guidelines for meat processors, retailers and food-service establishments should be given and controlled by National Food Services. Research to elucidate the ecology of *E.coli* O157:H7 should include identification and control of sources of the pathogen in the farm environment and the development of rapid diagnostic assays for identifying the animals and products that harbour these pathogens.

After ingestion of food products of animal origin contaminated with VTEC or the acquireance of a VTEC infection via person-to-person transmission, the bacteria adhere to and colonize in the human intestine. By using cultured epithelial cells and animal models a lot of progress has been made in the understanding the mechanisms of the adherence of VTEC in the last four years. This pattern demonstrates similar characteristics as the adhesion pattern of enteropathogenic *E.coli* (EPEC). Important in the initial adhesion of VTEC strains to the intestinal epithelium are the plasmid encoded structural and/or regulatory genes for fimbriae

[10]. In a study by Windsor et al [11], it was reported that mannose can partially inhibit the adherence of VTEC strains belonging to serotype O157:H7 to cultured human colon carcinoma cells and rabbit colonocytes. The adherence to these cells appeared to be related to the amount of pili present and not to the serotype [11]. In several animal studies it has been demonstrated that after the initially localized adherence VTEC strains colonize the terminal ileum, cecum and colon by the attaching-and effacing (AE) lesions, which are similar to that produced by certain strains of enteropathogenic *E.coli* (EPEC) [12-14]. These AE lesions are characterized by extremely close attachment of the bacteria to the intestinal cells, with effacement of the underlying microvilli and accumulation of filamentous actin in the subjacent cytoplasm. Jerse et al reported the identification of a locus in EPEC necessary for the intimate attachment to epithelial cells in vitro [15]. This locus, now referred to as *eaeA* (for *E.coli* attaching and effacing), is part of a gene cluster on the EPEC chromosome necessary for intimate attachment. The product of the *eaeA* locus is intimin, a 94 KDa outer membrane protein. The *eae* locus of VTEC strains has recently been cloned and sequenced [16,17]. A role of the *eae* gene in the characteristics of the AE lesions was for the first time demonstrated in vivo by Donnenberg et al [18]. He constructed an *eae* mutant of EHEC, which was unable of attaching intimately to colonic epithelial cells in a newborn piglet model. The intimate attachment was restored in vivo when the VTEC *eae* gene or the EPEC *eae* gene was introduced into the mutant on a plasmid. The complementation achieved by the EPEC locus indicates that the *eae* gene of VTEC and the EHEC *eae* gene are functionally homologous. The intestinal ligands of the protein products produced by VTEC and necessary for adherence on the intestinal epithelium are yet unknown. Diarrhea-associated HUS is rarely seen in adults and occurs mostly in children younger than 5 years of age. Although fecal tests demonstrate that VTEC infection is often present in family-members of the HUS index case, the presence of serum antibodies to the O157 O-antigen is significantly lower in the family-members as compared patients with HUS. This may indicate that other, yet unknown host factors in the intestines are involved in facilitating VTEC lipopolysaccharides to cross the mucosal surface.

Mobassaleh and co-workers [19] demonstrated in a rabbit model that quantitative differences in the Shiga toxin receptor globotriaosylceramide (GbOse₃cer), in the small intestine are the underlying basis for the age-specific differences in functional responsiveness of rabbit intestinal tissue to Shiga-toxin. Intestinal microvillus membranes were prepared from the small intestines of rabbits at various ages and glycolipids were quantitated by high-performance liquid chromatography. In these rabbit studies a marked increase in GbOse₃cer with age, ranging from 0.02 to 16.2 pmol/ μ g microvillus protein in neonates and adults, correlated with the secretory (enterotoxic) effects of the toxin [20]. Whether the age-related increase in rabbit intestinal GbOse₃cer content is mediated through the induction of the specific galactosyltransferase responsible for the α 1-4 addition of a terminal galactose residue to lactosylceramide, the precursor of GbOse₃cer, remains to be investigated. If similar developmentally regulated changes in GbOse₃cer occur in the human intestine as well, this could explain the relative resistance of human neonates to clinical shigellosis and D+ HUS. Developmentally regulated changes in the amount of receptors have also been reported for the

toxins from *Clostridium difficile*, *Vibrio cholerae* and enterotoxigenic *E.coli* [21,22]. Not much is known about the presence and distribution of the verocytotoxin-receptor GbOse₃cer in human intestines and therefore no data are available for an assessment of the possible developmental regulation of intestinal GbOse₃cer content in the microvillus membranes. Björk et al [23] observed no GbOse₃cer in the epithelial cells isolated from resections of the ileum of four human adults, but Keusch et al could detect GbOse₃cer in proximal small-bowel samples with normal histology which were surgically removed from adult patients [20].

Verocytotoxin is thought to be, at least partly, responsible for the systemic manifestations seen in HUS. After gaining access to the circulation, verocytotoxin binds to its specific functional receptor GbOse₃cer, which is present on endothelial cells. The use of cultured human endothelial cells has made it possible to study in vitro the direct effect of verocytotoxin. Although endothelial cell damage in HUS is mainly located in the capillaries in the glomeruli, most of the data provided about the effect of verocytotoxin on endothelial cells are based on the findings in cultures using human vein endothelial cells derived from the umbilical cord [24-26]. Recently, Obrig and coworkers reported that human renal endothelium is more sensitive to verocytotoxin than the endothelium derived from the umbilical cord [27]. This increase in sensitivity was due to an increase in the verocytotoxin-receptor, GbOse₃cer in human renal endothelial cells. The observed differences in GbOse₃cer in various cultured endothelial cells may be due to specific mechanisms for controlling the receptor expression by regulation of individual glycosyltransferases responsible for addition of receptor sugar sequences to the glycolipids. For example, administration of the hormone testosterone alters the glycosphingolipid synthesis, including GbOse₃cer, in the kidney of mice, resulting in marked increases in the synthesis of glucosylceramide by inducing the ceramide-UDP-glucose glucosyl transferase [28]. Not only the absolute amount of GbOse₃cer determines the sensitivity of the various endothelial cells towards verocytotoxin, also the heterogenous fatty acid composition of the GbOse₃cer molecule can influence the binding and the toxicity of verocytotoxin to the endothelial cells [29]. Recently, Jacewicz et al [30] demonstrated that GbOse₃cer, expressed at the cell surface, is necessary but not sufficient to confer sensitivity of mammalian cells to Shiga toxin. When liposomal GbOse₃cer was introduced in natural resistant GbOse₃cer deficient CHO (Chinese hamster ovary) cells, no change was observed in the sensitivity of CHO cells, even though there was an increase in Shiga toxin binding. This raises the possibility that CHO cells may lack a necessary postbinding translocation mechanism for GbOse₃cer-bound toxin [30]. Not enough is known about the role and regulation of neutral glycosphingolipids, including GbOse₃cer, in human endothelial cells [31]. The fact that endothelial cells from human glomeruli can be cultured nowadays [ref!!!] and the fact that the sensitivity of various types of human endothelial cells to verocytotoxin can be compared, will be helpful in studying the pathogenesis of HUS.

Although the culture of endothelial cells has the advantage to study endothelial-specific processes without the interference of other cells, it is obvious that the culture process per se may also introduce artifacts. Cultured endothelial cells may lose specific functions and may acquire new metabolic characteristics, which are not present in vivo. In the in vivo situation blood cells and plasma are flowing over the endothelial cells. Platelets, leucocytes and other

renal cells (mesangial cells) may contribute to the pathology seen in HUS. An animal model would be ideal to study HUS. Several animal studies have been performed with purified verocytotoxin, but no typical HUS pathology was observed. Diarrhea and neurological symptoms were present in gnotobiotic piglets after oral inoculation of *E.coli* strain O157:H7 [32]. Verocytotoxin injected i.v. or i.p. in mice leads to bilateral renal cortical tubular necrosis [33,34]. Intravenously given verocytotoxin has been demonstrated to cause abnormalities in the central nervous system and the gastrointestinal tract in the rabbit without producing signs of renal dysfunction [35,36]. The lesions seen in mice and rabbits correlated with the amount of expression of the verocytotoxin receptor, GbOse₃cer in the damaged organs. Glomerular thrombotic microangiopathy seen in patients with HUS, was not observed in any of the verocytotoxin-treated animal model (mice, rabbits). In an attempt to induce glomerular thrombotic microangiopathy with verocytotoxin, we have recently performed a pilot-study in three rhesus monkeys. Briefly the protocol was as following: To upregulate the expression of GbOse₃cer on vascular endothelial cells lipopolysaccharide (LPS) i.v. was given 24 hours before the addition of verocytotoxin. Verocytotoxin-1 (VT-1) was infused into the left arteria renalis and saline was infused into the right arteria renalis. Histopathology and immunohistopathology of the both kidneys were performed 48-72 hours after VT-1 administration. The glomerular thrombotic microangiopathy as observed in patients with HUS was not seen in the verocytotoxin-treated kidney of the rhesus-monkeys. Until now an appropriate animal model of human thrombotic microangiopathy occurring in HUS might be the modified Schwartzman reaction induced by a 5h endotoxin infusion in rabbits [37]. During endotoxin infusion (40µg/kg/h) the animals developed signs of endotoxemia, transient leucopenia and thrombocytopenia as well as acute renal failure. By light microscopy a marked polymorphonuclear cell infiltration was a prominent finding within 1 hour from the start of the infusion. At 5 h fibrin deposits and thrombi were present in the kidney of all animals. Endothelial damage was detected few minutes after the beginning of endotoxin infusion followed by leucocyte and platelet infiltration in glomerular capillaries. Occasionally, occlusive fibrin thrombi were seen. The abnormalities were reversible. Later on, i.e. at hour 48 after endotoxin infusion, no more fibrin was detectable in glomerular capillaries. Recently another rabbit model to study glomerular thrombotic microangiopathy was described by Faraco et al [38]. Rabbits receiving cyclosporin A i.m. for 10 days and on the 11th day an endotoxin infusion for 5 h developed more fibrin depositions and thrombi in the glomeruli than rabbits treated with one of the vehicles alone (Faraco 1991). Although these animal studies can be considered as an example for human thrombotic microangiopathy, it does not fully represent HUS. First, the reaction of rabbit endothelium towards endotoxin might be different than that of human vascular endothelium. Secondly, the fixed amount of endotoxin given is different from that of patients developing HUS, who are presumably exposed to a lower amount of endotoxin and/or verocytotoxin but for considerably longer periods. Loirat et al treated 33 children with HUS in a multicentre, controlled study either with urokinase and heparin (n=15) or symptomatically (n=18) [39]. No differences were observed in terms of renal histological changes and in long-term renal function between the two groups. In four patients, hemorrhage resulted in interruption of the antithrombotic therapy after 14-31 hours,

and therefore, only 11 patients had no modification of the treatment protocol. No conclusions can be drawn about the effect of fibrinolytic treatment in this study. First, urokinase was not given as a sole treatment, but it was given together with heparin. Secondly, whether the administered doses of urokinase was correct, can not be concluded from this study, because no control-values of fibrinolytic activity, such as fibrin(ogen) degradation products, fibrinogen and thrombin time, were measured during treatment with urokinase-heparin infusions. One can wonder whether the duration of urokinase administered was adequate. Still no good controlled study in patients with HUS has been performed to examine the effect of fibrinolytic agents in the treatment of HUS, but the above mentioned rabbit model receiving cyclosporin and LPS might be a good candidate to study the effectivity and kinetics of fibrinolytic treatment of the glomerular thrombotic microangiopathy. A better understanding of the pathogenesis of HUS might lead to a improvement of the treatment and outcome of the patients with HUS, especially for those with the D- form of HUS, who still have a worse prognosis compared to the D+ HUS [40]. It might be interesting to know whether plasma of children with D- HUS contain the 37 kDa platelet agglutinating protein which was reported to be present in the plasma of patients with TTP [41]. Lian et al observed that IgG present in human adult plasma could inhibited the TTP plasma induced agglutinating ability in vitro [42]. It has yet to be examined whether therapy with IgG infusions might contribute to a better treatment of D- HUS.

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SAMENVATTING

In dit proefschrift worden de resultaten beschreven van epidemiologisch en pathofysiologisch onderzoek van het hemolytisch uremisch syndroom (HUS). HUS, voorafgegaan door een periode van acute, vaak bloederige, diarree, wordt met name gezien bij kinderen onder de vijf jaar en wordt gekenmerkt door een hemolytische anemie, thrombocytopenie en een acute nierinsufficiëntie. Sinds het begin van de tachtiger jaren wordt een infectie met verocytotoxine producerende *E.coli* gezien als de voornaamste oorzaak van deze vorm van HUS.

Hoofdstuk 1 vormt een introductie tot de verschillende aspecten van HUS en de verocytotoxine-producerende *E.coli*. De verschillende vormen van HUS worden hier genoemd en uitgebreider wordt ingegaan op HUS voorafgegaan door een acute gastro-enteritis (D+ HUS) en op de belangrijkste veroorzaker van D+ HUS, de verocytotoxine-producerende *E.coli*.

Gedurende vier jaren werd retrospectief en prospectief onderzoek verricht naar de aanwezigheid van een infectie met verocytotoxine-producerende *E.coli* (VTEC) in feces en sera van patiëntjes met D+ HUS afkomstig uit Nederland en de academische ziekenhuizen te Leuven, Antwerpen en Keulen. De resultaten van dit onderzoek worden beschreven in hoofdstuk 2. In het eerste retrospectieve deel van dit hoofdstuk werden sera afgenomen bij opname van patiëntjes met HUS, familieleden van patiëntjes met HUS en patiëntjes met acute gastro-enteritis onderzocht op de aanwezigheid van antilichamen tegen de lipopolysaccharide O-antigenen van de 13 meest voorkomende serotypen van de met HUS geassocieerde VTEC stammen. Antilichamen tegen het lipopolysaccharide O157-antigeen werd aangetroffen in het serum van 28 van de 45 (62%) patiëntjes met HUS. Antilichamen tegen het O115-antigeen waren aanwezig in het serum van een patiëntje met HUS. In het serum van één van de 43 onderzochte familieleden werden antilichamen tegen het O145 antigeen aangetroffen. Geen van de 34 sera van de controle groep van patiëntjes met acute gastro-enteritis was positief. De resultaten van het prospectieve deel staan beschreven in het tweede deel van dit hoofdstuk. Feces-onderzoek naar de aanwezigheid van VTEC en/of vrij verocytotoxine gecombineerd met serologisch onderzoek naar antilichamen tegen verocytotoxine en tegen het O157-antigeen toonden aan dat er in 88 (78%) van de 113 patiëntjes met HUS en 2 (3%) van de 65 patiëntjes met acute gastro-enteritis sprake was van een VTEC infectie. Aanwijzingen voor een infectie met VTEC serotype O157 werden in 76% van de 88 HUS patiëntjes gevonden. Het testen op de aanwezigheid van antilichamen tegen het O157-antigeen was de meest sensitieve methode. Tevens werd feces- en serologisch onderzoek verricht bij 95 familieleden van de 28 HUS patiëntjes. VTEC infectie werd aangetoond in 33 (35%) van de familieleden. Hoewel VTEC en/of vrij verocytotoxine vaak werd aangetroffen in de feces van familieleden, werden antilichamen tegen het O157-antigeen maar in de sera van 3 familieleden aangetroffen. Daar het genotype van de geïsoleerde VTEC stammen niet

verschilde per familie, zou dit kunnen betekenen dat andere, nog onbekende factoren in het maag-darm kanaal van de gastheer een rol spelen in de invasiviteit van VTEC. Een mogelijk verschil in adhesie van VTEC aan het darm-epitheel van kinderen ten opzichte van volwassenen dient te worden onderzocht. Deze epidemiologische studies laten zien dat VTEC infectie, met name het serotype O157, de belangrijkste oorzaak is van HUS bij kinderen in Nederland en delen in België en Duitsland. Niet alle kinderen die geïnfecteerd worden met VTEC krijgen HUS. In het derde deel van dit hoofdstuk werd onderzocht of er een genetische predispositie is voor het verkrijgen van HUS. HLA types van 31 patiëntjes met een chronische nier-insufficiëntie ten gevolge van HUS werden vergeleken met de HLA types van de Nederlandse populatie. Er werd een significante associatie gezien tussen HLA-B40 en de ernstige vorm van D+ HUS.

Cytokines spelen een belangrijke rol in de pathogenese van infectie ziekten en inflammatoire processen. Verhoogde spiegels van cytokines zijn o.a. beschreven bij sepsis, het syndroom van Kawasaki en thrombotische thrombocytopenische purpura. Om inzicht te krijgen in een mogelijke rol van cytokines in de pathogenese van D+ HUS, werden de spiegels van tumor necrosis factor α (TNF α), vrij circulerende TNF receptoren (sTNFR55 en sTNFR75), interleukine-1 β (IL-1 β) en interleukine-6 (IL-6) bepaald in het plasma van patiëntjes met HUS bij opname en in de controle groepen van patiëntjes met chronisch nierfalen (CRF) en gezonde kinderen. De resultaten zijn beschreven in hoofdstuk 3. TNF α en IL-1 β waren niet verhoogd in het plasma van HUS patiëntjes vergeleken met de controle groepen. Echter plasma spiegels van IL-6 waren significant hoger in de HUS patiëntjes met extra-renale manifestaties (277 ± 103 pg/ml) vergeleken met de spiegels van HUS patiëntjes zonder extra-renale manifestaties (milde vorm) en de beide controle groepen (< 30 pg/ml). Hoewel de exacte functie van IL-6 in HUS niet bekend is en de alhier onderzochte groep klein is, blijkt uit deze studie dat IL-6 geassocieerd is met de ernstige vorm van HUS. Plasma spiegels van sTNFR55 en sTNFR75 waren significant verhoogd in alle HUS patiëntjes in vergelijking met de sTNFR spiegels in gezonde kinderen. Echter hoge sTNFR spiegels werden ook aangetroffen in het plasma van patiëntjes met CRF. Plasma spiegels van sTNFR dienen voorzichtig te worden geïnterpreteerd indien er sprake is van een verstoorde nierfunctie.

Activatie van het fibrinolytische systeem leidt tot afbraak van fibrine en vervolgens tot desintegratie van een stolsel. De vaatwand speelt een belangrijke rol in de productie van de fibrinolytische parameters. Beschadiging van de endotheelcellen leidt tot een verstoring in het evenwicht tussen het stolling- en het fibrinolytische systeem. In HUS wordt de endotheelschade met name gezien in de nier: glomerulaire endotheelcellen laten los van de basaal-membraan en thrombi en fibrine deposities worden aangetroffen in het lumen van de glomerulaire capillairen. In hoofdstuk 4 worden de plasma spiegels van fibrinolytische parameters en van Willebrand factor bij patiëntjes met D+ HUS beschreven. Tevens werd het effect van verocytotoxine-1 (VT-1) op de humane endotheelcellen onderzocht. De spiegels van tissue plasminogeen activator (t-PA), diens remmer de plasminogeen-activator inhibitor type 1 (PAI-1), von Willebrand factor (vWF) en de fibrin- en fibrinogeen degradatie producten waren significant verhoogd in het plasma afgenomen bij opname. In de

convalescente fase, twee tot drie weken na opname, werden geen verhoogde plasma-waarden van t-PA, PAI-1 en vWF spiegels gevonden. Intraveneuze toediening van DDAVP bij opname gaf geen verandering van de t-PA, urokinase (u-PA) en vWF concentratie in het bloed, echter DDAVP-toediening twee weken na opname liet een toename zien in de t-PA secretie. De verhoogde fibrinolytische parameters en vWF bij opname suggereren dat er in D+ HUS, naast de hemorrhagische colitis en de gestoorde nier-functie, sprake is van een gegeneraliseerde activatie van het endotheel. Door middel van in vitro studies met humane endotheelcellen werd de rol van VT-1 in het fibrinolytische systeem onderzocht. Humane endotheelcellen gepreïncubeerd met VT-1 gaven geen verandering in de produktie van t-PA, PAI-1 en vWF. Echter wanneer VT-1 gegeven werd aan met $\text{TNF}\alpha$ voorbehandelde endotheelcellen zodat er voldoende VT-1 receptoren aanwezig waren, was er een duidelijke afname van zowel t-PA, PAI-1 als vWF. Deze afname was het gevolg van het inhibitoire effect van VT-1 op de algehele eiwit-synthese. Hoewel de uitslagen van het in vivo en in vitro onderzoek paradoxaal lijken, zouden ze middels de volgende hypothese verklaarbaar kunnen zijn: Na een periode van acute, vaak bloederige diarree is de nier het meest aangedane orgaan in HUS. Het is aangetoond dat de humane nier de receptor voor VT bezit en VT wordt mede verantwoordelijk geacht voor de endotheelschade in de glomeruli. Deze schade leidt tot het ontstaan van fibrine deposities en thrombus vorming. Het in deze situatie ontstane thrombine, ontstekingsmediatoren en activatie van leucocyten kunnen er toe leiden dat het endotheel distaal van de aangedane gebieden geactiveerd wordt.

In hoofdstuk 5 werd het effect van verocytotoxine in humane endotheelcellen verder onderzocht. Een toename in de gevoeligheid voor VT-1 werd gezien wanneer humane endotheelcellen werden gepreïncubeerd met $\text{TNF}\alpha$. Binding-studies met ^{125}I -VT-1 lieten zien dat deze preïncubatie met $\text{TNF}\alpha$ zorgde voor een toename in de ^{125}I -VT-1 binding aan endotheelcellen. Het effect van $\text{TNF}\alpha$ was concentratie afhankelijk en verzadigbaar bij ongeveer 10 nmol/l VT-1. Analyse van de Scatchard plot wees uit dat de affiniteit van VT-1 voor de endotheelcellen niet veranderde, maar dat $\text{TNF}\alpha$ een tien- tot honderdvoudige toename in specifieke VT-1 receptoren per cel induceerde. Niet alleen $\text{TNF}\alpha$, maar ook IL-1, lymphotoxine en lipopolysaccharide lieten een toename zien in VT-1 binding. Deze toename in VT-1 binding was het gevolg van een toename in de functionele receptor van VT, het glycosphingolipide globotriaosylceramide (GbOse_3cer). De novo eiwitsynthese was nodig voor deze inductie van GbOse_3cer door $\text{TNF}\alpha$. Uit deze studie bleek, dat $\text{TNF}\alpha$ een (of meer) enzym(en) indiceert, die noodzakelijk zijn voor de synthese van de VT-1 receptor, GbOse_3cer . Tevens laten deze in vitro data zien dat naast VT-1, ontstekingsmediatoren een belangrijke rol zouden kunnen spelen in de pathogenese van HUS.

De biosynthese van glycosphingolipiden geschiedt door dat geactiveerde suikers, gebonden aan nucleotiden, worden gekoppeld aan het ceramide. Glycosyltransferases zijn nodig voor deze overdracht. De data in hoofdstuk 6 laten zien dat de inductie van GbOse_3cer door $\text{TNF}\alpha$ en IL-1 geschiedde door een stijging in galactose bevattende glycosphingolipiden ten gevolge van een toename in galactosyl transferase activiteit(en). Humane veneuze endotheelcellen geïsoleerd uit de navelstreng (HUVEC) hebben twee $\text{TNF}\alpha$ receptoren, een 55 kDa receptor (TNFR-p55) en een 75 kDa receptor (TNFR-p75). Beide receptoren zijn

aanwezig in ongestimuleerde endotheelcellen. In dit hoofdstuk laten we zien dat activatie van de TNFR-p55 door $\text{TNF}\alpha$ voldoende is voor de inductie van de VT-1 receptor GbOse_3cer . Een rol voor de TNFR-p75 kon worden aangetoond bij lage $\text{TNF}\alpha$ concentraties en gedurende de eerste 8 tot 10 uur van de incubatie periode. Proteïne kinase C activiteit was niet noodzakelijk voor de inductie van GbOse_3cer door $\text{TNF}\alpha$ in de humane endotheelcellen.

ABBREVIATIONS

BSA	bovine serum albumin
DDAVP	1-desamino-8-arginine vasopressin
D+ HUS	diarrhea-associated hemolytic uremic syndrome
D- HUS	diarrhea-negative hemolytic uremic syndrome
EHEC	enterohemorrhagic <i>Escherichia coli</i>
EIA	enzyme immunoassay
ELISA	enzyme linked immunosorbent assay
EPEC	enteropathogenic <i>Escherichia coli</i>
FVT	free verocytotoxin
GbOse ₃ cer	globotriaosylceramide
HUS	hemolytic uremic syndrome
HUVEC	human umbilical vein endothelial cells
HSA	human serum albumin
IL-1	interleukin-1
IL-6	interleukin-6
LPS	lipopolysaccharide
PAI-1	plasminogen activator inhibitor type I
PBS	phosphate buffered saline
PKC	protein kinase C
PMA	phorbol myristate acetate
SEM	standard error to the mean
SD	standard deviation
SLT	shiga-like toxin
TLC	thin layer chromatography
sTNFR	soluble tumor necrosis factor receptor
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
t-PA	tissue-type plasminogen activator
TTP	trombotic thrombocytopenic purpura
u-PA	urokinase
VT	verocytotoxin
VTEC	verocytotoxin-producing <i>Escherichia coli</i>
VT/PECS	verocytotoxin detection in polymyxin B extracts of colony sweeps
vWF	von Willebrand factor

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Liefste Ivo, alhoewel dit onderzoek onze plannen om samen in het buitenland te leven en werken tijdelijk in de war schopte, was jij altijd vol vertrouwen. Een grotere steun, ook al waren we niet altijd samen (maar toch ook weer wel), is ondenkbaar.

CURRICULUM VITAE

Nicole van de Kar werd op 28 maart 1963 geboren te Ubach over Worms. In 1981 behaalde zij het diploma Gymnasium β aan het Katholiek Gymnasium Rolduc te Kerkrade. In 1981 begon zij de studie Geneeskunde aan de Katholieke Universiteit te Nijmegen. Het doctoraal examen en artsexamen werden behaald in 1986 respectievelijk 1989. In 1988 was ze vijf maanden werkzaam in de 1^e lijns gezondheidszorg te Nicaragua. In deze periode heeft ze met de gemeenschap van Pikin Guerero samengewerkt aan de opbouw van een school en een soja verwerkings fabriek. Van februari 1990 tot en met februari 1994 was zij als arts-onderzoeker in dienst van de afdeling Kindergeneeskunde van het Academisch Ziekenhuis Sint Radboud te Nijmegen (hoofd: Prof.Dr. R.C.A. Sengers). In de eerste 4 maanden verrichte zij haar onderzoek bij Prof.Dr. M.A. Karmali op de afdeling Medische Microbiologie, Hospital for Sick Children, Toronto, Canada. Terug in Nederland werd het epidemiologische onderzoek voortgezet op de afdeling Medische Microbiologie, Sint Radboud Ziekenhuis Nijmegen (hoofd: Prof.Dr. J. Meeuwissen en Prof.Dr. J.A.A. Hoogkamp-Korstanje). Het fundamentele gedeelte van dit promotie-onderzoek werd uitgevoerd in het Gaubius Laboratorium TNO-PG te Leiden (hoofd: Prof.Dr. P. Brakman en Prof.Dr. Knook). Financiële ondersteuning werd in het eerste jaar verkregen van het Ter Meulen Fonds van de commissie geneeskunde van de Koninklijke Nederlands Academie van Wetenschappen. Dit project werd de daarop volgende drie jaren gesubsidieerd door de Nier Stichting Nederland (project 90.1021).

Vanaf augustus 1994 is zij in verband met de opleiding tot kinderarts, werkzaam als arts-assistent Kindergeneeskunde op de afdeling Neonatologie in het Sint Radboud Ziekenhuis Nijmegen (hoofd: Dr. L. Kollee).

De auteur is getrouwd met Ivo de Blaauw.