ACTIVATION OF COAGULATION IN THE
CHILDHOOD HAEMOLYTIC URAEMIC SYNDROME

Thesis submitted in accordance with the requirements of the
University of Leicester for the degree of Doctor of Medicine
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<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
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<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
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<td>AT III</td>
<td>antithrombin III</td>
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<td>D+ HUS</td>
<td>diarrhoea associated haemolytic uraemic syndrome</td>
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<td>E. coli</td>
<td>Eschericia coli</td>
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<td>ELISA</td>
<td>enzyme linked immunosorbant assay</td>
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<td>F1+2</td>
<td>prothrombin fragment 1+2</td>
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<tr>
<td>Factor VIIa</td>
<td>activated factor VII</td>
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<tr>
<td>Factor Xa</td>
<td>activated factor X</td>
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<tr>
<td>Factor XIIa</td>
<td>activated factor XII</td>
</tr>
<tr>
<td>FFP</td>
<td>fresh frozen plasma</td>
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<tr>
<td>Gb3</td>
<td>globotriaosylceramide</td>
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<tr>
<td>GMP</td>
<td>guanidine monophosphate</td>
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<tr>
<td>HD</td>
<td>haemodialysis</td>
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<td>HRP</td>
<td>horseradish peroxidase</td>
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<td>HUS</td>
<td>haemolytic uraemic syndrome</td>
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<td>HUVEC</td>
<td>human umbilical vein endothelial cells</td>
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<tr>
<td>ICAM-1</td>
<td>intercellular adhesion molecule 1</td>
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<tr>
<td>IL-1</td>
<td>interleukin 1</td>
</tr>
<tr>
<td>OPD/H_{2}O_{2}</td>
<td>o-phenylenediamine dihydrochloride / hydrogen peroxide</td>
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<td>PAI-1</td>
<td>plasminogen activator inhibitor</td>
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<td>PAI-1:Ag</td>
<td>plasminogen activator inhibitor antigen</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PD</td>
<td>peritoneal dialysis</td>
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<tr>
<td>PE</td>
<td>plasma exchange</td>
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<tr>
<td>PET</td>
<td>PBS/EDTA/Tween</td>
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<td>PGI2</td>
<td>prostacyclin</td>
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<td>sE-Selectin</td>
<td>soluble E selectin</td>
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<td>sP-Selectin</td>
<td>soluble P selectin</td>
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<td>TAT</td>
<td>thrombin-antithrombin III</td>
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<td>sTF</td>
<td>soluble tissue factor</td>
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<td>sTM</td>
<td>soluble thrombomodulin</td>
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<td>t-PA</td>
<td>tissue plasminogen activator</td>
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<td>t-PA:AG</td>
<td>tissue plasminogen activator antigen</td>
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<tr>
<td>TF/VIIa</td>
<td>tissue factor/activated factor VII complex</td>
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<tr>
<td>TFPI</td>
<td>tissue factor pathway inhibitor</td>
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<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
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<td>TTP</td>
<td>thrombotic thrombocytopenic purpura</td>
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<tr>
<td>u-PA</td>
<td>urokinase plasminogen activator</td>
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<tr>
<td>VCAM-1</td>
<td>vascular cell adhesion molecule 1</td>
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<tr>
<td>VT</td>
<td>verotoxin</td>
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<td>VTEC</td>
<td>verotoxin producing Eschericia coli</td>
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Unless stated otherwise, all of the work in this thesis was undertaken by myself.
CHAPTER 1
INTRODUCTION
Renal failure is traumatic at any age, but when it occurs suddenly in childhood, the effects are even more overwhelming. Haemolytic uraemic syndrome (HUS) is the commonest cause of acute renal failure in childhood and is particularly distressing for the child and family. The disease develops within days and makes the child extremely fractious, at a time when daily blood tests are needed, and when he may be immobilised by attachment to a dialysis machine. The disease affects about 150 children a year in Britain (Milford, DV et al. 1990) and occurs in outbreaks which often generate considerable public interest. The incidence may be increasing (Subcommittee of the PHLS Working Group on Verocytotoxin producing Eschericia coli, 1996) and during 1996 alone there were at least two widely publicised outbreaks. The first, in Japan in 1996 affected nearly 10,000 people (Featherstone, C 1997) and is the largest outbreak yet recorded. The second occurred in Scotland in November 1997, was fatal in eighteen cases (Featherstone, C 1997), and has been described as the worst outbreak of food poisoning in this country to date. Despite this publicity and considerable research into the disease, the pathogenesis is still incompletely understood and no specific treatment has been shown to be effective. It was the aim of this thesis to shed a little more light on the pathogenesis of this disease by investigating changes in haemostasis.

HUS does in fact cover a group of diseases characterised by a triad consisting of haemolytic anaemia, thrombocytopenia and acute renal dysfunction. Until relatively recently, the heterogeneous nature of the various diseases comprising HUS (see table 1) was not fully appreciated. Furthermore, it is difficult to distinguish between some types of HUS and the closely related syndrome of thrombotic thrombocytopenic purpura (TTP), characterised also by thrombocytopenia and haemolysis but with predominantly neurological rather than renal symptoms (Remuzzi, G 1987 ), (Moake, JL 1991). Because of this, the conclusions of many earlier studies of HUS which include a heterogeneous mix of adults and children with a variety of forms of HUS and TTP, comprising different aetiologies, symptoms and prognoses, need to be interpreted with caution. Here, work relating to the pathogenesis of one form of HUS, which accounts for 90% of all cases of HUS (Moake, JL 1994) and is especially common in childhood, is discussed.
Table 1 - Types of Haemolytic Uraemic Syndrome

- **Infectious Aetiology**
  - Diarrhoea associated
    - Verotoxin producing *Eschericia coli*
    - Shiga toxin producing *Shigella dysenteriae*
  - Neuraminidase associated
    - *Streptococcus pneumoniae*
  - Other
    - HIV

- **Non-infectious Aetiology**
  - Drug / Hormone associated
    - Cyclosporin A / Mitomycin C
    - Pregnancy
    - Oral contraceptives
  - Malignancy associated
  - Transplant associated
  - Inherited
    - autosomal dominant / autosomal recessive
    - prostacyclin deficiency
    - complement abnormalities
    - cobalamin C deficiency
  - Idiopathic

**Diarrhoea-Associated Haemolytic Uraemic Syndrome (D+ HUS)**

This form of HUS, which stands in clear distinction to the others listed has been called “typical HUS” (Kaplan, BS *et al.* 1990, Remuzzi, G *et al.* 1995) “epidemic HUS” (Levin, M *et al.* 1984), “enteropathic HUS” (Fitzpatrick, MM *et al.* 1991) “diarrhoea associated HUS” (Kaplan, BS *et al.* 1990) and “D+ HUS” (Fitzpatrick, MM *et al.* 1991, Milford, DV *et al.* 1990). The latter term has been used in this thesis. The
renal dysfunction is typically preceded by a bloody diarrhoeal prodrome and most commonly affects children under the age of 5 years. There is an epidemic pattern to the disease, with clusters occurring most commonly in the Spring and Summer months. The prognosis in terms of mortality and morbidity is generally better than for other forms of HUS, (Milford, DV et al. 1990, Remuzzi, G et al. 1995, Spizzirri, FD et al. 1997).

Clinical Features

Most, but not all (Caprioli, A et al. 1992) cases of D+ HUS are, as the name suggests, preceded by a diarrhoeal prodrome which usually contains blood and is associated with severe abdominal cramps. After a variable period, typically 2-10 days (Taylor, CM 1995), between 9 and 30% of infected children develop HUS (Remuzzi, G et al. 1995), becoming non specifically unwell with lethargy and irritability. Significant fever is unusual. Pallor secondary to anaemia and mild jaundice secondary to haemolysis may be detectable clinically. Thrombocytopenia may lead to petechiae and bruising, but rarely frank bleeding except from the gut. Although polyuria has been described, oligo- or anuria is the rule, often leading to symptoms of fluid overload, namely raised jugular venous pressure, displaced apex beat and hypertension (Frishberg, Y et al. 1994). Dialysis is required to treat hyperkalaemia or fluid overload in many of the cases referred to tertiary referral centres in Britain (Milford, DV et al. 1990, Trompeter, RS et al. 1983). 20-30% of children exhibit severe acute neurological dysfunction (Siegler, RL 1994), usually in the form of grand mal seizures. These often resolve on correction of hypertension or electrolyte imbalance, but occasionally the symptoms persist and indicate a poor prognosis (Sheth, KJ et al. 1986).

Epidemiology

Verotoxin producing Escherichia coli (VTEC) is the infectious agent responsible for over 80% of cases of D+ HUS in Britain (Chart, H et al. 1989), (Taylor, CM 1995) and was first linked to two outbreaks of haemorrhagic colitis by Riley et al. (Riley, LW et al. 1983). Subsequently Karmali et al (Karmali, MA et al. 1983) demonstrated VTEC infection in 11 of 15 children with HUS and since that time, many reports have confirmed the association between VTEC and D+ HUS (Karmali, MA et al. 1985, Neill, MA et al. 1987, Gransden, WR et al. 1986, Kleanthous, H et al. 1990, Bell, BP
Transmission of VTEC is usually via food, particularly undercooked ground beef (Bell, BP et al. 1994). Several other modes of transmission including unpasteurised milk (Chapman, PA et al. 1993), swimming pools (Hildebrand, JM et al. 1996) and person to person spread (Spika, JS et al. 1986, Belongia, EA et al. 1993) have also been described.

Verotoxin

Verotoxin (VT) consists of two subunits, the A-subunit which contributes toxic activity, and the pentameric B-subunit which exhibits ligand activity (O'Brian, AD et al. 1987, Sandvig, K et al. 1996). The B-subunit binds specifically to globotriasylceremide (Gb3) via a terminal disaccharide, galactose α 1- 4 galactose (Lingwood, CA et al. 1987). Verotoxin binding to Gb3 has been demonstrated on glomerular endothelial cells in biopsy specimens from children under the age of 2, but not those from adults (Lingwood, CA 1994). Verotoxin has also been shown to bind to human renal tubular (Lingwood, CA 1994) and mesangial (Mahan, JD et al. 1995) cells, intestinal epithelial cells (Jacewicz, MS et al. 1995), red blood cells (Bitzan, M et al. 1994) and monocytes (van Setten, PA et al. 1996). Once bound, the toxin is endocytosed and the A-subunit cleaved to reveal an active A1 fragment. This acts as a specific N-glycosidase which can depurinate a single amino acid residue from the 28S rRNA component of the ribosome, blocking the interaction of elongation factor-1 with the ribosome and leading to inhibition of protein synthesis (Obrig, TG et al. 1987, Endo, Y et al. 1988, Sandvig, K et al. 1996).

Treatment and Prognosis

Since HUS was first described by Gasser in 1955 (Gasser, C et al. 1955), the acute mortality rate has fallen to under 5% in developed countries (Trompeter, RS et al. 1983, Taylor, CM 1995). Despite attempts to find a specific therapy, supportive care, including dialysis remains the mainstay of treatment. The literature contains many reports of controlled and uncontrolled trials in search of a more specific treatment for the disease. Attempts to treat glomerular thromboses using heparin and/or anti-platelet agents (Vitacco, M et al. 1973, van Damme-Lombaerts, R et al. 1988, O'Regan, S et al. 1980) were found to be ineffective, and even streptokinase therapy did not appear to alter the course or outcome of disease (Monnens, L et al. 1978, Jones, RWA et al. 1981). Following the theory that a plasma prostacyclin stimulating
factor might be absent in HUS (Remuzzi, G et al. 1981), a series of studies on the
effect of plasma infusion (Rizzoni, G et al. 1988, Loirat, C et al. 1988), plasma
exchange (Gianviti, A et al. 1993) and prostacyclin infusion (Beattie, TJ et al. 1981,
Defreyn, G et al. 1982) were reported, but none showed significant improvement in
prognosis. In an attempt to reduce cell damage by oxygen free radicals, vitamin E
therapy was tried (Powell, HR et al. 1984) but was ineffective, as were
immunoglobulin infusions, given in an attempt to neutralise verotoxin (Sheth, KJ et
al. 1990, Robson, WLM et al. 1991). The most recent treatment trial attempted to
bind verotoxin in the gut using Pk-trisaccharide receptors coupled to Chromosorb
(Rowe, PC et al. 1997). Although this did not reduce the incidence of HUS following
infection with VTEC in the study group as a whole, there was a slight reduction in
incidence of HUS in the subset of children treated within 4 days of developing
diarrhoea.

Although 85-90% of affected patients have a normal GFR at one year post disease,
in the long term a significantly reduced GFR (below 80 ml/min/1.73m²) has been
reported in approximately 30% patients (Siegler, RL et al. 1991, Fitzpatrick, MM et al.
1991). A reduced renal reserve has also been described in approximately 30%
children 6-8 years following D+ HUS (Perelstein, EM et al. 1990, Tufro, A et al.

Laboratory Diagnosis

The diagnosis is usually clear when a child presents with the clinical features noted
above, but should be confirmed by stool culture on sorbitol-MacConkey agar looking
for non-fermenting Escherichia. coli (E. coli) colonies that can then be assayed for
the 0157 antigen (Boyce, TG et al. 1995), or by detection of IgM antibody to the
lipopolysaccharide of E. coli serotype 0157 in an acute blood sample. These tests
may be negative if the child presents late (Milford, DV et al. 1990), in which case
stool cultures are often negative, or if E. coli of a different serotype are involved
(Chart, H et al. 1993).

As would be expected from the clinical presentation, blood urea and creatinine
concentrations are raised in relation to the degree of renal failure, which is often
severe (Frishberg, Y et al. 1994). Hyponatremia secondary to inappropriate fluid
replacement prior to arrival at a tertiary referral centre is common and may contribute to neurological dysfunction (Bos, AP et al. 1985).

Examination of a blood film reveals haemolysis and thrombocytopenia. Leucocytosis is common, and it has been suggested that a high neutrophil count at presentation may indicate a poor outcome (Walters, MDS et al. 1989, Milford, DV et al. 1991). Thrombin time and activated thromboplastin time are commonly normal (Katz, J et al. 1971). These findings previously led to the theory that the disease is caused by platelet abnormality without significant involvement of coagulation.

Platelets

Studies on platelets in D+ HUS have shown decreased half life (Katz, J et al. 1973) suggesting increased destruction or platelet consumption. The remaining platelets are functionally defective, having a reduced ability to aggregate under various stimulating conditions (Fong, JSC et al. 1982, Walters, MDS et al. 1988). Furthermore, these platelets contain reduced levels of serotonin (Walters, MDS et al. 1988) and β-thromboglobulin (Fong, JSC et al. 1982) whereas plasma levels of serotonin, β-thromboglobulin and platelet factor 4 are increased (Walters, MDS et al. 1988, Appiani, AC et al. 1982). These findings suggest that the platelets have been activated, releasing serotonin and β-thromboglobulin into the plasma.

Coagulation

In the 1970s, work investigating activation of coagulation in D+ HUS measured changes in thrombin time, activated thromboplastin time, levels of several coagulation factors and soluble circulating fibrin and showed no change in these parameters (Katz, J et al. 1971, Kisker, CT et al. 1975, Sanchez Avalos, J et al. 1970). Studies using radiolabelled fibrinogen showed little increase in fibrinogen turnover (Katz, J et al. 1973, Harker, LA et al. 1972). On the basis of these results, it was suggested that there was no activation of coagulation in childhood HUS (Kisker, CT et al. 1975). However, these findings are also compatible with activation of coagulation providing the consumption of coagulation factors is being adequately compensated. Furthermore, increased levels of fibrin degradation products in urine (Taira, K et al. 1989) and plasma (Uttley, WS 1970, Katz, J et al. 1971), and raised
levels of fibrinopeptide A (Monnens, L et al. 1982) suggest significant activation of coagulation does occur.

Histopathology

D+ HUS is characterised histologically by a glomerular thrombotic microangiopathy i.e. glomerular endothelial cell swelling and the presence of thrombi containing both fibrin and platelet elements within the capillary lumen (Habib, R 1992). Histologically, other forms of HUS differ in that the arterioles and small arteries tend to be affected, resulting in glomerular ischaemia rather than thrombosis (Barratt, TM et al. 1987, Neild, GH 1994, Kaplan, BS 1995). This may have prognostic relevance (Kaplan, BS 1995). In TTP, the thrombi are slightly different from normal in that they consist of an abundance of platelets and von Willebrand factor (vWF) with little fibrin (Asada, Y et al. 1985).

The histological appearances of endothelial cell swelling associated with thrombus seen in D+ HUS suggest activation of the glomerular endothelium causing these cells to become pro-thrombotic, resulting in localised thrombosis.

ENDOTHELIAL CELL ACTIVATION

Perturbation of endothelial cells, for example by tissue necrosis factor (TNF), interleukin 1 (IL-1) or endotoxin leads to endothelial cell activation, a status distinct from actual cell damage and defined by Pober as “the capacity (of the endothelial cell) to perform new functions without evidence of cell injury or cell division.... the result of quantitative changes in certain gene products”, allowing the endothelial cell to act as a mediator of inflammation (Pober, JS et al. 1990). He describes four new functions of the activated endothelium: cell shape change, which can be detected by light microscopy as swelling and rounding up of cells, and increased permeability of the endothelial monolayer; release of further cytokine such as IL-1, which amplifies the response; expression of cell adhesion molecules which promote interaction between endothelial cells and leukocytes; and change from an anti- to a pro-coagulant phenotype (Pober, JS 1988). The latter two functional changes are of particular relevance to this thesis. They are depicted in figure 1 and described in more detail below.
Endothelial cell activation

- Thrombomodulin
- selectins & adhesion molecules (e.g., E-selectin, P-selectin, ICAM, VCAM)
- cytokines (e.g., IL1)
- PAF, vWF
- Tissue factor
- fibrin
- endotelial cell
- platelet
- tPA, PAI-1
- fibrinolysis
Expression of Adhesion Molecules

Activated endothelium communicates with leukocytes via two types of adhesion molecule expressed on the endothelial cell surface. These are selectins and members of the immunoglobulin superfamily. The former group includes E-selectin, expressed only on endothelium, and P-selectin which is expressed by both endothelial cells and platelets. Both selectins are expressed early in the course of endothelial cell activation and mediate tethering and rolling of leukocytes, particularly neutrophils and monocytes. Intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), both members of the immunoglobulin superfamily, then mediate closer adhesion, flattening and transmigration of the leukocytes through the endothelial monolayer to the underlying tissues (Scholz, D et al. 1996). Soluble forms of these molecules exist in normal plasma and are increased in several disease states associated with endothelial cell activation (Gearing, AJH et al. 1992). Inward (Inward, CD et al. 1995) has demonstrated an increase in soluble VCAM-1 and a fall in soluble ICAM-1 in children with D+ HUS. She found a trend towards raised levels of soluble E-selectin levels but this was not significant.

Changes in Thromboresistance

Quiescent endothelial cells are thromboresistant, expressing mechanisms that inhibit both platelet adhesion and the coagulation cascade, in addition to pro-fibrinolytic properties. A series of interactions between adenosine metabolites and endothelium regulate platelet function (Pearson, JD et al. 1985). Prostacyclin (PGI₂) released from endothelium (Jaffe, EA 1987) activates adenylate cyclase, increasing intra-platelet concentrations of cyclic adenosine monophosphate (AMP) thus inhibiting adhesion of stimulated platelets (Moncada, S 1982). Nitric oxide released by endothelial cells inhibits platelet aggregation by a mechanism synergistic with prostacyclin but dependent on cyclic guanosine monophosphate (GMP) to increase the concentration of intra-platelet cyclic AMP (Moncada, S et al. 1993). Endothelial release of PGI₂ and nitric oxide is induced by release of adenosine diphosphate (ADP) from stimulated platelets. ADP itself is pro-aggregatory, recruiting platelets into the developing platelet plug (Petty, RG et al. 1989) but ecto-ADPase enzymes residing on the surface of the endothelial cell catalyse the conversion of ADP to adenosine, a strong platelet inhibitor (Jaffe, EA 1987).
Endothelial cells also express the important physiological anticoagulants tissue factor pathway inhibitor (TFPI), thrombomodulin and heparan sulphate on the cell surface (Shireman, PK et al. 1996). As the name suggests, TFPI blocks tissue factor, the main physiological initiator of coagulation. TFPI initially complexes with activated factor X which then combines with the tissue factor/activated factor VII (TF/VIIa) complex, forming an inactive quaternary complex (Osterud, B et al. 1995). Thrombomodulin binds thrombin and changes the specificity of the active site such that its major procoagulant ability to cleave fibrinogen is reduced. Instead, thrombin bound to thrombomodulin is able to activate protein C, which binds to protein S also expressed on the endothelial cell (Preissner, KT 1988). This complex catalyses the proteolysis of activated coagulation factors V and VIII, thus inactivating them (Marlar, RA et al. 1981). Activated protein C also inhibits the activity of plasminogen activator inhibitor (PAI) thereby indirectly augmenting fibrinolytic activity (van Hinsbergh, VWM et al. 1985). Heparan sulphate, a glycosaminoglycan expressed on the endothelial cell surface acts as an endogenous catalyst for the anticoagulant actions of antithrombin III (ATIII) (Shireman, PK et al. 1996). ATIII, produced in the liver, is the major plasma inhibitor protein of thrombin, irreversibly binding thrombin, forming thrombin-antithrombin III complexes (TAT) (Pelzer, H et al. 1988). In addition to these anti-platelet and anti-coagulant functions, resting endothelial cells release tissue plasminogen activator (t-PA), which activates plasminogen, the enzyme responsible for cleaving insoluble fibrin to soluble fibrin degradation products (van Hinsbergh, VWM 1988).

Upon activation, these active anti-thrombotic properties are downregulated, for example endothelial cells exposed to endotoxin, IL-1 or TNF express less thrombomodulin than unstimulated cells (Moore, KL et al. 1989) and have decreased thrombomodulin dependent activation of protein C (Nawroth, PP et al. 1986), thus inactivation of factors V and VII, and anti-coagulant change in specificity of thrombin which normally result from thrombomodulin expression are reduced. Furthermore, latent procoagulant properties are upregulated. Endothelial cells activated by exposure to cytokine release platelet activating factor, a potent stimulant of platelet aggregation (Shireman, PK et al. 1996). Benigni (Benigni, A et al. 1992) has reported an increase in platelet activating factor in the urine of children with D+ HUS. Activation also results in release of vWF from Wiebel-Palade bodies, storage sites within endothelial cells (Jaffe, EA 1977). vWF monomers released from this source are linked by disulphide bonds into multimers which, having a collection of closely
related active sites, are particularly effective at binding clumps of platelets, both to each other and the basement membrane exposed by the change of cell shape (Moake, JL 1994). Rose (Rose, PE et al. 1984) has reported the presence of vWF multimers in plasma samples of children with D+ HUS. Tissue factor which is absent from the surface of the resting endothelial cell is expressed on activated endothelial cells (Petty, RG et al. 1989, Pober, JS et al. 1990, Colucci, M et al. 1983). This initiates the coagulation cascade (Edgington, TS et al. 1991) resulting in fibrin deposition as described in the next section. Furthermore, excess PAI is released (Petty, RG et al. 1989, Pober, JS et al. 1990), reducing fibrinolytic potential as discussed in the section following that.

These changes are distinct from actual cell damage. A soluble form of thrombomodulin is known to exist, and although the exact structure is not clearly known, it is thought to represent a cleaved form of the tissue bound thrombomodulin, as it lacks the transmembrane domain and cytoplasmic tail (Cucurull, E et al. 1997). Soluble thrombomodulin (sTM) present in plasma is thought to represent actual endothelial cell damage, since this form of thrombomodulin is not secreted by cells under physiological conditions (Ishii, H et al. 1991). In a similar manner, soluble tissue factor (sTF) is thought to be cleaved and released into the circulation upon cellular damage, but expressed locally, remaining attached to the cell membrane upon cell activation (Koyama, T et al. 1994). Thus increased levels of circulating sTM and sTF are markers of endothelial cell damage rather than activation.
COAGULATION

Under physiological conditions, coagulation factors circulate in blood in an inactive or precursor form. The coagulation cascade consists of a series of stepwise reactions in which these inactive precursors are converted to enzymes and cofactors. Traditionally, the coagulation cascade is considered in terms of extrinsic and intrinsic pathways which converge at the activation of factor X to a final common pathway comprising the generation of thrombin and conversion of soluble fibrinogen to insoluble fibrin (Davie, EW et al. 1964, Macfarlane, RG 1964).

The extrinsic pathway is activated by tissue factor. This is constitutively expressed on all nonvascular cells which would only normally come into contact with blood following injury (Edgington, TS et al. 1991, Nemerson, Y 1988), thus the system acts as a protective mechanism against severe haemorrhage following the breach of a blood vessel. It is also expressed on endothelial cells and monocytes, but only following their activation (Carson, SD et al. 1993). Tissue factor is a high affinity membrane receptor which binds to and activates factor VII. The TF/VIIa complex avidly binds and activates factor X which converts prothrombin to thrombin, triggering the formation of fibrin. The intrinsic (or contact) pathway is initiated by the exposure of contact factors, namely factor XII, high molecular weight kininogen and prekallikrein to negatively charged subendothelial components such as collagen. This leads to the sequential activation of factors XI, IX, X and prothrombin with consequent thrombin generation and fibrin formation (Halkier, T 1991).

Since patients with deficiency of factors XI or VIII, who have a defective intrinsic but intact extrinsic pathway, and those with factor VII deficiency, who have a defective extrinsic but intact intrinsic pathway, have clinically evident haemostatic problems (Halkier, T 1991), both pathways must be required for adequate haemostasis. Patients with hereditary deficiencies of factor XII however, have no bleeding problems (Coleman, RW et al. 1975). These facts challenge the traditional view that coagulation is initiated via one of two independent pathways which converge into a final common pathway, and a revised model of blood coagulation has now been proposed (Davie, EW et al. 1991, Broze, GJ 1995). This revised hypothesis proposes that coagulation is initiated when blood is exposed to tissue factor, either in the extravascular environment, or on activated endothelial cells or monocytes. Factor
VII or factor VIIa present in plasma binds to tissue factor, and the TF/VIIa complex activates both factor X and factor IX. The generation of factor Xa activates TFPI, initiating a feedback loop blocking further activity of TF/VIIa. Additional Xa can then only be produced through the pathway initiated by thrombin and involving factors XI and VIII. This revised model is depicted in figure 2, with initiating reactions shown in red.

Activated factor X converts prothrombin to thrombin, releasing prothrombin fragment (F1+2) which can be measured to quantify the amount of thrombin generated (Bauer, KA et al. 1987). Once formed, thrombin is inactivated by binding to ATIII, forming TAT. The quantity of TAT complexes formed can also be used to measure thrombin generation (Pelzer, H et al. 1988). Measurement of activated factor XII and VII levels can be used to determine the predominant pathways involved in thrombin generation (Dempfle, CE et al. 1995).

Activation of coagulation in Childhood D+ HUS
Thus the debate as to whether there is significant activation of coagulation in this disease can now be readdressed using more sensitive markers of activation of coagulation, namely TAT and F1+2 (Mannucci, PM 1994). Raised TAT levels have been described in a heterogeneous group of children and adults with a mixture of HUS and TTP (13 patients, including a single case of D+ HUS) (Monteagudo, J et al. 1991) but there is no similar study of a large group of children with D+ HUS.
A Modern View of the Coagulation Cascade

endothelial cell

endothelial cell

endothelial cell

endothelial cell

tissue factor

VII ▶ VIIa

VIIa ▶ VIIIa

VIIIa ▶ VIII

VIII ▶ X

X ▶ Xa

Xa ▶ prothrombin

prothrombin ▶ thrombin

thrombin ▶ thrombin-antithrombin III (TAT)

antithrombin III

TAT fragment 1.2

IXa ▶ IX

IX ▶ XI

XI ▶ XII

XIIa ▶ XII
FIBRINOLYSIS

Endothelial cell activation not only promotes a pro-coagulant phenotype but also changes the fibrinolytic potential if the cell. The fibrinolytic pathway is controlled via enzymes which catalyse conversion of inactive plasminogen to the active enzyme plasmin resulting in cleavage of insoluble cross linked fibrin by plasmin, producing soluble fibrin degradation products (FDP) as depicted in figure 3. Raised levels of FDP in plasma therefore indicate fibrinolytic activity (Gaffney, PJ et al. 1994).

The principal endogenous activator of plasminogen is t-PA which is present in resting endothelial cells, including those of the human glomerulus (Angles-Cano, E et al. 1985). Urokinase type plasminogen activator (u-PA) is not thought to play a significant role in the lysis of intravascular thrombus but seems instead to be involved in tissue fibrinolysis and is found in glomerular epithelial rather than endothelial cells (Angles-Cano, E et al. 1985). Upon stimulation, endothelial cells release more t-PA but also release relatively more plasminogen activator inhibitor type-1 (PAI-1) (Petty, RG et al. 1989, Pober, JS et al. 1990). Thus PAI-1 activity is increased despite increased levels of t-PA.

PAI-1 is considered to be the most physiologically important of the three known regulatory plasminogen activator inhibitors (Sprengers, ED et al. 1987). It is produced by a number of cells including hepatocytes and endothelial cells. It is also stored in platelets, but the platelet PAI-1 activity is low and only contributes to a minor degree to a prolonged elevation of PAI activity in circulating blood (Booth, NA et al. 1988). PAI-1 binds to and inactivates t-PA (Kruithof, EKO et al. 1987). The overall effect on fibrinolysis depends on the balance between PAI-1 and t-PA rather than the actual levels of either enzyme. Total PAI-1 antigen (PAI:Ag) and t-PA antigen (t-PA:Ag) levels can be measured, but more information regarding fibrinolytic potential is gained by measuring unbound t-PA (t-PA activity) or unbound PAI-1 (PAI-1 activity). Functional studies of euglobulin fractions such as the euglobulin clot lysis time or a fibrin plate assay assess the net available fibrinolytic activators.
Fibrinolysis in Childhood D+ HUS

Recent studies looking at the fibrinolytic changes associated with D+ HUS have produced conflicting results. van de Kar et al. (van de Kar, NCAJ et al. 1994) demonstrated increased levels of t-PA:Ag and PAI-1 activity but no increase in PAI-1:Ag in 10 children with D+ HUS, whereas Bergstein et al. (Bergstein, JM et al. 1992) showed increased PAI-1:Ag levels in D+ HUS and an association between the duration of elevation of PAI-1:Ag and disease outcome. Chant et al. (Chant, ID et al. 1994) suggested PAI-1 activity may have prognostic value for long term outcome and be an acute marker for dialysis requirement in acute D+ HUS.

Figure 2 The Fibrinolytic Pathway
IN VITRO STUDIES

The clinical experiments undertaken suggested that the hypothesis that verotoxin caused local endothelial cell activation leading to activation of coagulation was correct. However, other events known to occur in HUS including the acute phase response, diarrhoea, renal failure, haemolysis and platelet destruction complicated interpretation of results. Furthermore, the fact that presentation inevitably occurred several days following infection precluded investigation of the earliest stages of the disease process. No further studies on patient plasma samples could have elucidated the mechanism of activation of coagulation in D+ HUS in any more detail. It is not now considered necessary, ethical or safe to biopsy children in the acute phase of this disease, so it was not possible to investigate this hypothesis further using in vivo methods. Therefore, in order to investigate whether verotoxin caused activation of coagulation in this disease via induction of tissue factor expression on endothelial cells, a series of in vitro experiments were conducted.

Animal Models

It would be usual to turn to an animal model in these circumstances, but unfortunately there is no adequate animal model for HUS, although the Shwartzmann reaction has been proposed. Here, rabbits pre-treated with endotoxin produce self limiting thrombotic lesions on exposure to a second dose of endotoxin. Although the renal lesions are similar to those in HUS (Bergstein, JM et al. 1974), other organs not normally involved in HUS, such as the skin are also affected (Bergstein, JM 1977). Furthermore, this model is one of endotoxaemia rather than a response to verotoxin. No animal other than the human appears to respond to oral administration of verotoxin by development of predominant renal thrombosis, though in some animals, thrombi may be formed in other organs (Karpman, D et al. 1995, Tzipori, S et al. 1988).

Cell Culture Models

Cell culture models have therefore been used to study the disease in vitro. The advantage of such an approach is that the system is simple and can be easily manipulated. However, there are several drawbacks of this technique. Firstly, cells in culture may not display the same characteristics as they would in vitro when they have structural and functional relationships with other cells, and a homeostatic balance provided by other organs. Secondly, there may be organ-specific differences
among endothelia (Drake, TA et al. 1993, Hewett, PW et al. 1993) which are retained by cultured endothelial cells. This is a problem if the cultured cell (eg. human umbilical vein endothelium) is from a source different to that of interest (eg. the renal glomerular endothelium). Lastly, specific endothelial cell differences may be lost during the process of isolation of cells for culture or by the propagating conditions (Pober, JS et al. 1990).

Human umbilical vein endothelial cells (HUVEC) have been most commonly used to study HUS (Louise, CB et al. 1992, Morigi, M et al. 1995, van de Kar, NCAJ et al. 1992, van de Kar, NCAJ et al. 1994). These have the benefit of human endothelial although not renal glomerular origin, and are also easily obtained and grown. van de Kar et al. (van de Kar, NCAJ et al. 1992) have established that the number of verotoxin receptors on HUVEC is low but can be upregulated by pre-treatment with TNF. They propose that endotoxaemia in the early phase of HUS may result in a similar upregulation of receptors on renal glomerular endothelial cells in vivo. Using such a model, they demonstrated reduction of t-PA and increase of PAI production by these cells on stimulation with verotoxin, although the cell viability under these conditions was low (60%).

As mentioned above, use of HUVEC to study a pathology affecting renal glomerular endothelial cells is not ideal. The renal glomerular endothelial cell would be the best cell model but these cells have been extremely difficult to propagate, and source cells are in limited supply. Two groups have published work using human glomerular endothelial cells (Louise, CB et al. 1994, van Setten, PA et al. 1997). Louise et al. (Louise, CB et al. 1994) suggest that renal microvascular cells produce excess PAI and u-PA rather than t-PA in response to verotoxin when compared with HUVEC. They also found their cells produced tissue factor constitutively (Louise, CB et al. 1994) which is incompatible with the cells being of endothelial origin. van Setten et al. succeeded in reliably culturing carefully characterised human endothelial cells from glomeruli (van Setten, PA et al. 1997) a few months prior to this research being completed. The number of verotoxin receptors on these cells was expected to be greater than on HUVEC. It was possible to perform one experiment using human glomerular endothelial cells from this laboratory in order to compare the response of the two cell types to incubation with verotoxin.
**HYPOTHESIS/ AIMS**

The hypothesis tested was that binding of verotoxin to renal glomerular endothelium caused endothelial cell activation resulting in a change of cell phenotype from anti-thrombotic to pro-thrombotic, producing localised glomerular thrombosis via localised expression of tissue factor.

The specific aims of were:

- to assess activation of coagulation and fibrinolysis in a large cohort of children with D+ HUS.

- to explore further evidence for endothelial cell activation and damage in this disease.

- to determine whether verotoxin upregulated tissue factor expression on cultured endothelial cells.

Serial plasma samples were collected from 30 children with well documented D+ HUS, excluding those with atypical HUS in order to achieve homogeneity with respect to the actual disease studied. Because of the possible effects of renal dysfunction requiring dialysis and acute diarrhoea on the systemic markers measured, children with these complications were included in control groups.

In view of the clinical events which complicated interpretation of the markers measured, HUVEC and human renal glomerular endothelial cell models of this disease were used to examine possible pro-coagulant effects of verotoxin.
CHAPTER 2
CLINICAL STUDIES
METHODS
PATIENTS AND SAMPLE COLLECTION

This study was approved by the Lewisham and North Southwark Committee on Ethical Practice and the Ethical Practices Sub-Committee of the Royal Free Hampstead NHS Trust, London.

Over the period 1st July 1994 - 31st December 1995, 25 patients presenting with D+ HUS to the Paediatric Nephrology Department, Guy's Hospital, London and 5 patients presenting to the Paediatric Nephrology Department at the Royal Free Hospital, Hampstead, London were recruited. Diagnosis was based on the presence of haemolytic anaemia on peripheral blood film, raised creatinine and urea, thrombocytopenia and a diarrhoeal prodrome.

The treatment regimen usually followed by each hospital was not altered. The treatment protocol was as therefore as follows:

- **Continuous cycling peritoneal dialysis (PD)** was commenced if the patient was fluid overloaded with a history of severe oliguria or anuria, or hyperkalaemic.

- **Haemodialysis (HD)** was commenced in cases of technical difficulties with peritoneal dialysis or if plasma exchange was necessary. Frequency and timing of dialysis was determined by the clinical and biochemical state of the patient. The dose of heparin prescribed was determined by presence of coagulation in the lines and the filter, venous pressure and regular activated clotting times during the dialysis session. In most cases, no heparin was required early in the disease course but gradually increasing doses (10-30 U/kg/hr) were needed as the platelet count normalised.

- **Plasma exchange (PE)** was initiated if the patient developed severe neurological symptoms (usually grand mal seizures). Daily exchanges of 1-2 times patient plasma volume were undertaken until the neurological symptoms receded.

- **Repeated transfusions of packed cells** were given to maintain a haemoglobin level greater than 10 g/dl.
The clinical practice at Guy's Hospital was to infuse one unit of fresh frozen plasma (FFP) daily until the platelet count had reached >100x10⁸ for two consecutive days. The 5 patients treated at the Royal Free Hospital, Hampstead did not receive FFP infusions.

The author was contacted by airpage as soon as it was known that a child was to be transferred from another hospital, or had presented to the casualty department of either of the hospitals involved in the study with a diagnosis of suspected D+ HUS. A visit was made to the relevant hospital as soon as possible and all but three cases were seen within 60 min of admission and prior to any treatment being prescribed. The parents (and the child if of sufficient age to understand) were counselled regarding the study protocol, and if willing to take part, a blood sample was collected at the time of initial clinical blood sampling. In the first 17 cases samples were collected on inpatient days 3, 7 and 14 following admission. Plasma samples were collected during the first week of disease in 18 cases and during the second week of disease in 27 cases, defining day one as the first day of diarrhoea. Most patients were discharged after 2 weeks.

In ten cases, daily morning samples were collected. Five of these patients required peritoneal dialysis and five did not. None required haemodialysis or plasma exchange therapy. On 11 occasions, samples were collected prior to and within 12 h of blood transfusion, and on 13 occasions prior to and within 12 h of FFP infusion. Three of the five children who required plasma exchange therapy were bled prior to and immediately following 16 exchange sessions and then again on 11 occasions following subsequent haemodialysis. Samples of 11 of the FFP infusions given were also analysed.

Further samples were collected on three occasions after discharge. These samples were taken at approximately two weeks, one month and three months following discharge from hospital to coincide with routine follow up appointments. In 12 cases follow up samples were not collected because follow up was undertaken at a different hospital. Creatinine was normal by three months following discharge in all but two of the 19 cases from whom this last sample was available.

In order to assess the prevalence of acute infection with VTEC, stool samples were collected as soon as possible after admission and sent to the relevant hospital.
microbiology laboratory with a request to culture specifically for this organism. Acute blood samples were also collected and sent to the Public Health Services Laboratory, Colindale for detection of IgM antibody to the lipopolysaccharide of *E. coli* serotype 0157. In order to determine over what period the serological test used became negative, levels of this antibody were also determined in blood samples taken at 1-3 months and at 1 year post disease onset.

For each patient, the number of days diarrhoea, anuria and dialysis, and type of dialysis started were noted, as were admission levels of haemoglobin (g/dl), neutrophil count (x10⁹/l), platelet count (x10⁹/l), urea (mmol/l) and creatinine (µmol/l). Glomerular filtration rate (GFR) was calculated from the height and creatinine using the Schwarz formula (40 x ht (cm)/ creatinine) (Schwartz, GJ et al. 1976).

**Control cases**

Control patients were of four types:

1. Twelve normal children with no previous or current renal disease
   A single sample was collected prior to elective surgery.

2. Nine children receiving chronic ambulatory or overnight cycling peritoneal dialysis
   A single sample was collected from each child at the time of routine blood sampling during a morning clinic visit.

3. Nine children receiving chronic haemodialysis
   A single sample was collected as each child was connected to the dialysis machine, and a second sample at the termination of dialysis. All children were on a dialysis regime of 3 sessions per week, each session lasting 3-4 h.

4. Four children with severe acute diarrhoea
   Each of these children had been admitted to hospital on the day prior to sample collection, and all were receiving intravenous rehydration. Samples were collected simultaneously with morning clinical blood samples.
Blood sampling
Venous blood samples were taken in a ratio of 9:1 into 0.109 M trisodium citrate. In each case two 2.5 ml (final volume) samples were collected. One sample, to be used for fibrinolytic studies (PAI activity and fibrin plate) was immediately placed on ice and centrifuged at 4°C, the other was collected and centrifuged at room temperature. Platelet poor plasma was prepared by centrifugation for 20 min at 2500 x g. Samples were split into 200 μl aliquots and stored at -70°C until time of assay.

ASSAY METHODS

a) MARKERS OF ACTIVATION OF COAGULATION

i) Thrombin-antithrombin III complex (TAT)
The sandwich enzyme immunoassay (ELISA) Enzygnost® TAT micro (Behringwerke, Marburg, Germany) was used to determine the amount of TAT in each sample collected. The inter-assay and intra-assay variabilities of the test were <10% and <7% respectively.

Lyophilised standard plasmas containing 2, 6, 20, and 60 ng/ml TAT were reconstituted. Sample buffer (Tris/Tween/Ethyldiaminetetracetic acid(EDTA)) and test plasma or TAT standard plasma were added to microtitre plate wells coated with rabbit antibodies against human thrombin. A plate plan was constructed and the plate was briefly shaken to ensure mixing of the well contents. The plate was covered with self adhesive foil and incubated for 15 min at 37°C. During this time the TAT in the sample quantitatively bound to the antithrombin antibodies coating the wells.

The contents of the wells were then discarded and each well washed three times with wash buffer (Phosphate buffered saline (PBS)/Tween) to remove unbound substances, and then slap-blotted onto absorbent material. Horseradish peroxidase (HRP) conjugated rabbit anti-human AT III was added to each well and the strips incubated for 15 min at 37°C. During this time the peroxidase labelled antibody bound to the TAT from the samples which had bound to the thrombin antibodies.
immobilised on the wells, forming a sandwich of coat antibody:TAT:conjugate antibody.

Following incubation, the contents of the wells were discarded and each well washed three times with wash buffer to remove unbound material, and then slap-blotted onto absorbent material. Freshly prepared o-phenylenediamine dihydrochloride/hydrogen peroxide (OPD/H₂O₂) was added to each well. The strips were incubated for 30 min in the dark at room temperature during which time the OPD was converted to a yellow coloured product proportional to the amount of HRP-conjugated antibody bound and hence the amount of TAT present. The reaction was then stopped by the addition of 0.5 M sulphuric acid. The plate was gently tapped to mix the contents of the wells and the absorbance read at 492 nm. An ANTHOS HTII microtest spectrophotometer was used for this and all subsequent absorbance readings.

A standard curve was constructed from the absorbance of each standard on the y axis against the log of its concentration on the x axis. The TAT concentration of the test plasma was determined by interpolation from the standard curve. A standard curve was constructed each time the assay was performed. If values over 60 ng/ml were obtained, the test was repeated with an appropriately diluted plasma sample.

ii) Prothrombin Fragment F1+2 (F1+2)
Enzygnost® F1+2 micro enzyme immunoassay (Behringwerke AG, Marburg, Germany) was used to quantitatively determine the prothrombin fragment F1+2 (F1+2) concentration in the patient plasma samples. The inter-assay and intra-assay variabilities were 6-13% and 5-7.5% respectively.

The test plate consisted of wells coated with rabbit antibody to human F1+2. Sample buffer (Tris/NaCl/Tween) was mixed with either test plasma, F1+2 standard containing 0.04, 0.2, 2.0 or 10 nmol/l F1+2, or a control plasma in the wells of the test plate which was then shaken briefly on a microtest plate shaker. A plate plan of sample position was constructed and the plate was incubated at for 30 min at 37°C. During this time the F1+2 in the sample quantitatively bound to the antibody coating the wells.

The contents of the wells were then discarded and each well washed three times with wash buffer (PBS/Tween) to remove unbound material, and then slap-blotted
onto absorbent material. HRP-conjugated rabbit-anti human prothrombin was added to each well and the plate incubated at 37°C for 15 min. During this time the HRP-conjugated antibody also bound to the F1+2 molecule forming a sandwich of mouse anti-F1+2 coating antibody:F1+2:HRP-conjugated antibody. The contents of the wells were then discarded and each well washed three times with wash buffer to remove unbound conjugate, and then slap-blotted onto absorbent material.

OPD/H₂O₂ was added to each well using an 8-channel pipette and the plate was incubated at room temperature for 15 min in the dark, during which time it was converted to a yellow-coloured product proportional to the amount of HRP-conjugate bound and hence the amount of F1+2 present. The reaction was stopped by the addition of 0.5 M sulphuric acid to each well and the absorbance at 492 nm was measured.

A standard curve was constructed from the absorbance of each standard on the y axis against its concentration on the x axis. The F1+2 concentration of the test plasma was determined by interpolation from the standard curve. A standard curve was constructed each time the assay was performed. If values over 10 nmol/l were obtained, the test was repeated with an appropriately diluted plasma sample.

### iii) Activated Factor XII (Factor XIIa)

Factor XIIa levels were measured using Shield Activated Factor XII enzyme immunoassay kit (Shield Diagnostics Ltd., Dundee). The inter-assay and intra-assay variability were <7% and <5% respectively.

Test plasma or a factor XIIa standard containing 0, 1, 5, 10 or 20 ng/ml factor XIIa were added to microtitre strip wells coated with mouse monoclonal antibody directed against factor XIIa. A plate plan of sample position was constructed as the strips were incubated for 60 min at room temperature. During this time the factor XIIa in the sample quantitatively bound to antibodies coating the wells.

The contents of the wells were then discarded and each well washed five times with borate buffer to remove unbound material, and then slap-blotted onto absorbent material. Alkaline phosphatase-labelled sheep anti-human factor XIIa antibody was added to each well and the strips incubated for 60 min at room temperature. During
this time the alkaline phosphatase-labelled antibody bound to the factor XIIa from the samples which had bound to the factor XIIa antibodies immobilised on the wells, forming a sandwich of coat antibody:factor XIIa:conjugate antibody. The contents of the wells were then discarded and each well washed five times with borate buffer to remove unbound material, and then slap-blotted onto absorbent material.

Substrate containing phenolphthalein monophosphate and Mg$^{2+}$ as enzyme cofactor in buffer solution was added to each well and the strips incubated for 15 min at room temperature during which time the phenolphthalein was converted to a pink coloured product proportional to the amount of alkaline phosphatase-conjugate bound and hence the amount of factor XIIa present. The reaction was then stopped by the addition of sodium hydroxide/ EDTA in carbonate buffer pH >10. The plate was gently tapped to mix the contents of the wells and the absorbance read at 550 nm.

A standard curve was constructed from the absorbance of each standard on the y axis against its concentration on the x axis. The XIIa concentration of the test plasma was determined by interpolation from the standard curve. A standard curve was constructed each time the assay was performed.

iv) **Activated Factor VII (Factor VIIa)**

Factor VIIa levels were determined by a novel ELISA developed by Dr Helen Philippou and Professor David Lane. This utilises as capture antibody a sequence-specific polyclonal antibody raised against the new C-terminal generated at the factor VII activation site Arg152-Ile 153. Using this assay, the factor VIIa antigen levels in healthy normal adults (n=14, mean age 35, range 21-51 years) was 0.025+/− 0.010 ng/ml (mean +/- SE) (Philippou, H et al. 1997). Dr Philippou kindly performed this assay on selected samples.

v) **Fibrinogen**

This assay was kindly performed on selected samples by Dr Andrew Blann from the Department of Surgery, University Hospital of South Manchester using the method of Clauss (Clauss, VA 1957) on a KC10 coagulometer (Ameling). Dade bovine thrombin reagent was obtained from Baxter Diagnostics Inc., Deerfield, USA. The intra-assay CV was <5% and inter-assay CV was <10%.
b) MARKERS OF FIBRINOLYSIS / ENDOTHELIAL CELL ACTIVATION

i) D-Dimer
Coaliza® D-dimer solid phase enzyme immunoassay (Chromogenix, Sweden) was used to assay the concentration of D-dimer in the plasma samples. Intra and inter assay variabilities were <5% and <7% respectively.

All samples were thawed at 37°C and then centrifuged at 2000 x g for 20 min before testing to remove all insoluble material. The 2000 ng/ml D-dimer stock solution was diluted with phosphate-EDTA buffer to produce standards containing 2000, 1000, 500, 250, 125 and 62.5 ng/ml D-dimer. Phosphate-EDTA buffer was added to wells pre-coated with an anti-D-dimer monoclonal antibody. Standard or test plasma was added to each well and a plate plan of sample position constructed. The plate was agitated for 1 min using a microtitre plate shaker and then incubated for 2 h at room temperature during which time D-dimer present in the test sample or standard bound to the immobilised antibody. The well contents were then discarded and the wells washed four times with phosphate buffer. After blotting the microtitre plate onto paper towels, HRP-conjugated monoclonal antibody against D-dimer was added to each well and the plate incubated at room temperature for a further 60 min during which time a sandwich consisting of coat antibody:D-dimer:HRP-conjugated anti D-Dimer antibody was formed. The well contents were then discarded and the wells washed four times with phosphate buffer. After blotting the microtitre plate onto paper towels, tetramethylbenzidine in dimethyl sulphoxide was added to each well and incubated at room temperature for 30 min. During this period, it was converted to a blue-coloured product proportional to the amount of HRP-conjugate bound and hence the amount of D-Dimer present.

The reaction was stopped by addition of 1.5 M sulphuric acid and the plate tapped to mix the well contents. The absorbance was read at 450 nm. A standard curve was constructed from the absorbancies given by the D-Dimer standards and the D-Dimer concentration of the test plasma was determined by interpolation from the standard curve. Where values higher than 2000 ng/ml were measured, the assay was repeated on an appropriately diluted test sample.
ii) Plasminogen Activator Inhibitor Type 1 Antigen (PAI-1:Ag)

PAI-1:Ag levels were measured using Tintelize® PAI-1 enzyme immunoassay kit (Biopool, Sweden). The inter-assay and intra-assay variabilities were 2-3% and 2.5-3.3% respectively.

The test plate consisted of two types of well to exclude falsely elevated results: A-wells contained monoclonal antibodies against PAI-1 immobilised on the well surface in addition to soluble antibodies against PAI-1; and N-wells contained the same immobilised antibodies but non-immune soluble antibodies. PBS/EDTA/Tween (PET) buffer was added to each well using an 8-channel pipette taking care not to contaminate the N-wells with the contents of the A-wells. The plate was then agitated on a plate shaker for 1 min. PAI-1 standard plasma 40 ng/ml was diluted with PAI-1 depleted plasma to produce a series of standard concentrations of 40, 20, 10 and 0 ng/ml.

PAI-1 standard or test plasmas were added separately to an N-well and the adjacent A-well and a plate plan of sample position constructed. HRP-conjugated anti-PAI-1 was added to each well and the plate was incubated on a microtest plate shaker for 2 h at room temperature. During this time the PAI-1:Ag in the sample quantitatively bound to antibodies coating the N-wells but not the A-wells (prevented from binding by the soluble anti-PAI-1 antibodies). The HRP-conjugated anti-PAI-1 antibodies also bound the PAI-1 molecule forming a sandwich of coat antibody:PAI-1:conjugate antibody.

The contents of the wells were then discarded and each well washed three times with PET buffer to remove unbound conjugate and other unbound material, and then slapped blotted onto absorbent material. OPD/H$_2$O$_2$ was added to each well using an 8-channel pipette and the plate was incubated on a microtest plate shaker for 15 min, during which time it was converted to a yellow-coloured product proportional to the amount of HRP-conjugate bound and hence the amount of PAI-1 present. The reaction was stopped after 15 min by the addition of 3 M sulphuric acid to each well. The plate was stored for 10 min in the dark to allow colour stabilisation before measurement of the absorbance at 492 nm. The difference in absorbance between the N-well and the corresponding A-well was calculated for each standard and test sample (the specific PAI-1 response).
A standard curve was constructed from the absorbance of each standard on the y axis against its concentration on the x axis. The PAI-1:Ag concentration of the test plasma was determined by interpolation from the standard curve. A standard curve was constructed each time the assay was performed. If values over 40 ng/ml were obtained, the test was repeated with an appropriately diluted plasma sample.

iii) **Plasminogen Activator Inhibitor Type 1 Activity (PAI-1 activity)**

Chromolize® PAI-1 activity test (Biopool, Sweden) was used to measure PAI-1 activity in the plasma samples. The inter-assay and intra-assay variabilities of this test were 4% and 5% respectively.

The test plate consisted of lyophilised active t-PA immobilised on the plate well surface. Lyophilised plasma samples containing 0 IU/ml and 50 IU/ml PAI-1 activity were reconstituted. One unit of PAI-1 activity is defined as the amount of PAI-1 that inhibits one international unit of human single chain t-PA as calibrated against the International Standard for t-PA, lot 86/670 distributed by the NIBSC, Holly Hill, London, England. The 50 IU/ml PAI-1 standard plasma was diluted with the PAI-1 depleted plasma to produce PAI-1 standards containing 50, 30, 15 and 0 IU/ml PAI-1 activity.

PET buffer was added to each well using an 8-channel pipette and the plate was agitated on a plate shaker for 2 min. PAI-1 standard or test plasma were added to the wells and a plate plan of sample position constructed. HRP-conjugated monoclonal anti-PAI-1 antibody was added to each well and the plate was incubated on a microtest plate shaker for 30 min at room temperature. During this time the active PAI-1 in the sample quantitatively bound to the t-PA coating the wells. PAI-1 already complexed with t-PA in the sample (inactivated PAI-1) was unable to bind. The HRP-conjugated anti-PAI-1 antibodies also bound the PAI-1 molecule forming a sandwich of t-PA coat:PAI-1:conjugate antibody.

The contents of the wells were then discarded and each well washed four times with PET buffer to remove unbound conjugate and other unbound material, and then slap-blotted onto absorbent material. OPD/H₂O₂ was added to each well using an 8-channel pipette and the plate was incubated on a microtest plate shaker for 5 min, during which time it was converted to a yellow-coloured product proportional to the
amount of HRP-conjugate bound and hence the amount of active PAI-1 present. The reaction was stopped by the addition of 1.5 M sulphuric acid to each well and briefly tapping the plate to mix the contents of the wells. The absorbance at 492 nm was measured.

A standard curve was constructed from the absorbance of each standard on the y axis against its concentration on the x axis. The PAI-1 activity of the test plasma was determined by interpolation from the standard curve. A standard curve was constructed each time the assay was performed. If values over 50 lU/ml were obtained, the test was repeated with an appropriately diluted plasma sample.

iv) **Tissue Plasminogen Activator Antigen (t-PA:Ag)**

A Tintelize® t-PA kit (Biopool, Sweden) was used to measure t-PA:Ag levels in the samples. Intra and inter assay variabilities were <6.5% and <5.5% respectively.

The 30 ng/ml t-PA:Ag standard and t-PA depleted plasma were reconstituted and the standard diluted with the depleted plasma to achieve t-PA concentrations of 0, 10, 20 and 30 ng/ml. PET buffer was added to each well pre-coated with goat anti-human t-PA IgG antibody and containing non-immune IgG antibody, and agitated gently for 1 min. Standard or test plasmas were added to the wells and placed on a microtitre plate shaker for 1 h at room temperature during which time t-PA antigen present in the test sample or standard bound to the immobilised IgG antibody. After this time HRP-conjugated goat anti-human t-PA IgG was added to each well and the plate incubated on a microtitre plate shaker at room temperature for a further 15 min during which time a sandwich consisting of coat antibody:t-PA:HRP-conjugated anti t-PA antibody was formed.

The well contents were then discarded and the wells washed four times with PET buffer. After blotting the microtitre plate onto paper towels, freshly prepared OPD/H$_2$O$_2$ substrate was added to each well and incubated at room temperature for 15 min on a microtitre plate shaker. During this period, it was converted to a yellow-coloured product proportional to the amount of HRP-conjugate bound and hence the amount of t-PA antigen present. The reaction was stopped by addition of 1.5 M H$_2$SO$_4$, and after the plate had been stored in the dark for 10 min to allow colour stabilisation, the absorbance was read at 492 nm. A standard curve was constructed from the
absorbancies of the t-PA standards against their concentrations and test values were interpolated from this curve. If values over 30 ng/ml were obtained, the test was repeated with an appropriately diluted plasma sample.

v) Fibrin Plate
The fibrin plate test measures the fibrinolytic activity of the euglobulin fraction of plasma. This fraction, prepared by acidification contains fibrinogen, plasminogen, and t-PA but not the inhibitors α-1-antiplasmin or α2-macroglobulin. As excess fibrinogen and plasminogen are added to the system, the degree of fibrin lysis depends predominantly on the t-PA activity. Free PAI-1 is partitioned between the euglobulin fraction and the supernatant so also affects the result to some degree.

8 ml fibrin plate buffer (75 mM Tris/22 mM NaCl, pH 7.8) were mixed with 2 ml 1% human fibrinogen (Quadratech) and 200 μl of 10 U/ml thrombin (Diagnostic reagents) in a universal container. This was then gently inverted to mix and the solution poured into a petri dish on a level surface. The dish was swirled gently to distribute the solution evenly without forming air bubbles, and placed in an incubator at 37°C for 30 min to allow the fibrinogen to be converted to a fibrin clot.

An euglobulin fraction of the standard and plasma samples was prepared by the addition of 0.5 ml of standard or test plasma to 9.5 ml of cold 0.27 M acetic acid. This was mixed and left on ice for 15 min. After centrifugation at 4°C for 10 min at 1000 x g, the supernatant was discarded and the tubes inverted onto a paper towel to drain. The insides of the tubes were dried with tissue, taking care not to touch the precipitate at the base. 0.5 ml of ice cold 0.04 M phosphate buffer at pH 7.4 was added to each tube and the precipitate was resuspended by drawing up and down with a plastic pipette tip.

Duplicate 30 μl aliquots of each redissolved euglobulin fraction were placed on the fibrin surface and the plate was incubated for 22 h at 37°C. Fibrinolytic activity of the plasma was assessed by measuring the diameter of the zone of lysis at the end of this incubation period. Pooled normal plasma acted as a quality control and duplicate applications of normal plasma were made for each plate.
c) OTHER MARKERS OF ENDOTHELIAL CELL ACTIVATION

i) von Willebrand Factor antigen (vWF:Ag)

ii) Soluble P-selectin (sP-selectin)

These assays were kindly performed on selected samples by Dr Andrew Blann from the Department of Surgery, University Hospital of South Manchester. vWF:Ag was measured using reagents from Dako A/S Producktionsvej 42, Denmark in an in house assay (Woolf, AD et al. 1987) and sP-selectin was measured using an immunoassay kit (Takara Shuzo Co. Ltd., Japan). For both assays, the intra-assay CV was <5% and inter-assay CV was <10%.

iii) von Willebrand Multimers

The pattern of von Willebrand multimers was determined by Dr Andrew Laurie, Haemostasis Research, University College Hospital, London on samples taken during weeks one and two, and following discharge in four patients.

iv) Soluble E-selectin (sE-selectin)

Parameter human sE-selectin enzyme immunoassay kit (R&D Systems, Abingdon, Oxfordshire) was used to measure the sE-selectin present in the plasma samples. The inter-assay and intra-assay variabilities were <10% and <5% respectively.

The test plate consisted of wells coated with murine antibody to human E-selectin. Lyophilised plasma samples containing 0, 0.51, 2.55, 4.88, 7.74, 10.20 ng/ml E-selectin and a control serum sample were reconstituted. All samples and the control serum were diluted 1 in 20 with sample diluent provided.

Diluted test plasma or standard were added to the wells of the microtitre plate. HRP-conjugated antibody to human E-selectin was then added to each well using an 8-channel pipette. A plate plan of sample position was constructed and the plate was incubated at room temperature for 1.5 h. During this time the sE-selectin in the sample quantitatively bound to the E-selectin antibody coating the wells. The HRP-conjugated anti-human E-selectin antibody also bound the E-selectin molecule forming a sandwich of mouse anti E-selectin coating antibody:E-selectin:HRP-conjugated antibody. The contents of the wells were then discarded and each well
washed six times with wash buffer provided to remove unbound conjugate and other unbound material, and then slap-blotted onto absorbent material.

Tetramethylbenzidine was added to each well using an 8-channel pipette and the plate was incubated at room temperature for 30 min, during which time a blue-coloured product was formed proportional to the amount of HRP-conjugate bound and hence the amount of sE-selectin present. The reaction was stopped by the addition of acid provided to each well and the absorbance at 450 nm was measured.

A standard curve was constructed from the absorbance of each standard on the y axis against its concentration on the x axis. The sE-selectin concentration of the test plasma was determined by interpolation from the standard curve, multiplying by 20 to correct for the sample dilution. A new standard curve was constructed each time the assay was performed.

d) MARKERS OF ENDOTHELIAL CELL DAMAGE

i) Soluble Thrombomodulin (sTM)
Dr Andrew Blann from the Department of Surgery, University Hospital of South Manchester kindly measured sTM in selected samples using Asserchrom® Thrombomodulin enzyme immunoassay kit (Diagnostica Stago, France). The intra-assay CV was <5% and inter-assay CV was <10%.

ii) Soluble Tissue Factor (sTF)
Imubind® Tissue Factor ELISA Kit (American Diagnostica, Greenwich, USA) was used to quantitatively determine the sTF concentration in the patient plasma samples. The inter-assay and intra-assay variabilities were <10% and <5% respectively.

Lyophilised sTF standards containing 1000, 500, 200, 100 and 50 pg/ml were reconstituted. Plasma samples were diluted 1:10 in PBS. sTF standard or diluted test plasma were added to each well of the test plate coated with murine anti-human tissue factor and incubated at room temperature for 3 h. During this time the sTF in the sample quantitatively bound to the antibody coating the wells. A plate plan of sample position was constructed. Following incubation, the contents of the wells
were discarded and each well was washed four times with PBS to remove unbound material, and the plate was then slap-blotted onto absorbent material.

Biotinylated anti-human tissue factor fragment (ab')₂ was added to each well and incubated at room temperature for 1 h. During this time the biotinylated antibody also bound to the tissue factor molecule forming a sandwich of mouse anti-tissue factor coating antibody:sTF:biotinylated antibody. The contents of the wells were then discarded and each well washed four times with PBS to remove unbound conjugate, and the plate was then slap-blotted onto absorbent material.

Streptavidin-HRP was added to each well using an 8-channel pipette and the plate incubated at room temperature for 1 h, during which time a blue-coloured product was formed proportional to the amount of biotin bound and hence the amount of sTF present.

The reaction was stopped by the addition of 0.5 M sulphuric acid to each well and the absorbance at 450 nm was measured.

A standard curve was constructed from the absorbance of each standard on the y axis against its concentration on the x axis. The tissue factor concentration of the test plasma was determined by interpolation from the standard curve. A standard curve was constructed each time the assay was performed.

STATISTICAL ANALYSIS

Data were assessed for normality using a Normal plot and Shapiro-Wilk Test of Normality. In no case was raw or logged data normally distributed, hence all results are expressed as median and range. The significance of differences between the different patient and control groups were determined using the Mann-Whitney U test. Paired serial measurements on patient samples were compared using Wilcoxon Signed Rank Test. Pearson's Product Moment Correlation was used to determine the correlation between results, measures of clinical severity, renal function and haematological parameters.

Statistical analyses were performed using Unistat® statistical package, version 4 for MS Windows™ (London, England).
RESULTS
PATIENT DETAILS

i) Demographics
The median (range) age of the patients with D+ HUS was 4.1 (0.46-16.0) years. Two of the control groups were of similar age, however, chronic dialysis control patients were significantly older. The median and range of ages of the different control groups are shown in Table 2.

ii) Diagnosis
In 25/30 (83%) of cases, there was microbiological or serological evidence for infection with verotoxin producing E. coli. Of those with detectable antibodies to the lipopolysaccharide of E. coli serotype 0157 on admission, three months following disease onset, 78% of cases had no detectable antibody, rising to 100% of those tested at one year post disease onset.

Table 3 shows the haematological and biological parameters of patients on admission. None had received blood products or dialysis prior to sampling. In several cases, the haemoglobin level was only slightly reduced on admission, but all had typical appearances of haemolysis on blood film, and in most cases, haemoglobin fell over the subsequent hours. In one case (no. 29), the platelet count on admission was above 150 x10^9/l. This patient presented with a typical history, during an epidemic, and had evidence of E. coli serotype 0157 infection and documented oliguria. He was included in the study as he had severe anaemia (Hb 6.5 g/dl) and a typical history suggesting that he was presenting at a time when thrombocytopenia was resolving.

iii) Clinical Details
Table 4 shows details of the number of days each patient suffered diarrhoea. Two patients (nos. 7 and 25) suffered from severe gastrointestinal symptoms which became chronic. It was therefore not possible to determine the length of the acute diarrhoeal prodrome. For the remaining patients, the median (range) time that diarrhoea lasted was 7 (2-25) days. Patients were admitted a median (range) of 6 (2-17) days from onset of diarrhoea.
Table 2 - Ages of Control Groups Compared with Ages of Children with D+ HUS.

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Table 3 - Haematological and Biochemical Parameters of D+ HUS Patients on Admission

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<th>Platelet (x10^9/l)</th>
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Table 4 - Clinical Details of D+ HUS Patients.

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KEY
PD - peritoneal dialysis
HD - haemodialysis
PE - plasma exchange
* - gastrointestinal complications (see text for details)
Table 4 also details the median and range of days of anuria including all patients in the analysis. The median duration of anuria on this basis was 3 days, but when only the seventeen patients who became anuric were analysed, the median (range) duration was 7 (1-26) days with anuria commencing median 5 (range 1-14) days from the onset of diarrhoea.

iv) Treatment given
The dialysis requirements are also detailed in table 4. Twenty patients (66%) required dialysis lasting a median (range) of 9.5 (1-56) days and commencing median 5 (range 2-14) days from onset of diarrhoea. Most patients were dialysed because of anuria, although three patients with severe oliguria were also judged to require dialysis. Fourteen patients (70% of those dialysed) received peritoneal dialysis and six (30%) were haemodialysed.

Five of the children who received haemodialysis also underwent plasma exchange therapy on the basis of severe neurological dysfunction, mainly grand mal seizures. There were no long term neurological sequelae. Twenty one (70%) patients received daily infusions of one unit of fresh frozen plasma until the platelet count rose to over 100 x10⁹/l and the remaining four patients received no fresh frozen plasma.

v) Outcome
One patient (no. 7) died of gastrointestinal obstruction on day 56 after the onset of diarrhoea. Patient 25 did not recover normal renal function and had a calculated glomerular filtration rate (GFR) of 30 ml/min/1.73m² one year after disease onset. All other patients made a good clinical recovery and had a normal GFR (>80 ml/min/1.73m²) one year post disease onset.
ASSAY RESULTS

Figs 5 - 23 and tables 5 - 9 summarise the levels of the parameters measured in patients with D+ HUS and in each group of control children, and can be found at the end of this chapter. Defining day one as the first day of diarrhoea, the median and interquartile range of each parameter have been plotted at three timepoints over the course of disease: within one week of disease onset (median 5 days, range 4-7 days, n=18); between one and two weeks of disease onset (median 10 days, range 9-14 days, n=27); and at 1-3 months post disease onset (median 77 days, range 40-128 days, n=19). The results of normal control children, control children on chronic dialysis and children with acute diarrhoea are also shown. The significance of changes in results of paired samples taken between weeks one and two of disease are documented in the text. The figures depict not only the paired samples, but also results of the children from whom samples were available for only one of the two timepoints. Where measured, the results of the patients with acute diarrhoea are mentioned in the text, but statistical analysis was not undertaken as the group was too small (n=4).

Tables 5 and 6 summarise the results and statistical differences between admission and 1-3 month post discharge samples of children with D+ HUS. The haemodialysis and peritoneal dialysis control groups have been amalgamated to give data on all control patients receiving chronic dialysis. Results of normal control children are also shown. Significance of differences between the groups are as detailed in the key.

Although the pattern of data was suggestive of a log normal distribution, logging the results did not produce a normal distribution. Hence data is shown as median and range or interquartile range and non parametric statistical tests have been used.

a) MARKERS OF ACTIVATION OF COAGULATION

i) Thrombin-antithrombin III (TAT)

The data summarising the levels of TAT are shown in figures 8 and 20, and in table 5. Median (range) TAT levels in the first week of disease were 15.0 (7.2-48.0) ng/ml, significantly elevated compared to normal controls (median difference (95% confidence limits) 12.6 (8.9-21.0) ng/ml, (p<0.001)). Median (range) levels during the
second week were 12.0 (3.9-43.2) ng/ml, still significantly elevated compared with control values (2.6 (0.0-4.2) ng/ml, p<0.001). Post discharge, the values were still significantly raised, but to a much lesser degree, with a median difference (95% confidence limits) of 1.5 (0.5-3.2) ng/ml, p<0.001. Figure 20 shows in more detail the change in TAT levels in patients over the course of D+ HUS. Median and interquartile range of all samples collected were plotted against day from onset of diarrhoea, omitting days on which less than four samples were available. Day one is the first day of diarrhoea. TAT levels of children with D+ HUS were significantly higher than those in normal children, and comparing the two groups, p<0.001 until day 15, and were still just significantly higher than normal even by day 95 post disease onset (p=0.04).

Median (range) TAT levels of patients on haemodialysis were 3.6 (2.7-9.3) ng/ml, elevated compared with normal controls with a median difference of 1.17 and 95% confidence limits 2.24-0.38 ng/ml (p=0.008). Levels were, however far lower than TAT levels of patients in the first week of D+ HUS (median difference of (95% confidence limits) 11.63 (8.49-18.65) ng/ml, p<0.001). Children receiving peritoneal dialysis had levels similar to normal controls (p=0.2) and significantly lower than patients in week one of D+ HUS (p<0.001). Levels of TAT in children with acute diarrhoea were similar to those of children on chronic haemodialysis. They were slightly higher than levels in normal controls but much lower than the levels measured during the acute phase of D+ HUS.

ii) Prothrombin fragment 1+2 (F1+2)
Results of measurements of F1+2 are summarised in figure 9 and table 5. Median (range) F1+2 levels were 9.2 (3.8-13.3) and 6.8 (3.0-9.9) nmol/l during the first and second weeks of illness respectively. F1+2 levels were significantly elevated on admission when compared with normal or dialysed controls (p<0.001 compared with either control group). There was a significant fall in F1+2 level from the first to the second weeks of disease when paired samples taken at these times were compared (p=0.007), and levels were within normal limits post discharge.

Median (range) F1+2 levels of peritoneal dialysis controls were 4.8 (4.4-6.7) ng/ml and of haemodialysis controls 5.8 (1.8-6.8) ng/ml. These were not significantly
different from each other (p=0.91) but dialysis controls as a whole had significantly higher F1+2 levels than normal controls (p=0.012).

F1+2 levels correlated with TAT levels. Spearman coefficient (95% confidence intervals was 0.51 (0.17-0.73), p=0.002.

iii) **Factor Xlla**
Factor Xlla level results are summarised in figure 10 and table 5. Median (range) levels during the first and second weeks of disease were 7.4 (0.6-20.0) ng/ml and 6.8 (1.3-15.3) ng/ml respectively, significantly elevated when compared with normal control children median (range) 0.95 (0.6-1.9) ng/ml; p=0.002 and p=0.0006 respectively. After discharge, D+ HUS patient factor Xlla levels were not significantly different from normal.

Median (range) levels of Xlla in samples from controls receiving haemodialysis were 9.4 (4.2-20.0) ng/ml and from those receiving peritoneal dialysis were 10.75 (4.2-20.0) ng/ml. Levels in the two groups were not significantly different from each other (p=0.3) or the acute D+ HUS patients (p=0.21 and p=0.09) but were significantly higher than normal controls, p=0.001 compared with haemodialysed and p=0.002 compared with peritoneally dialysed controls.

Factor Xlla levels were negatively correlated with calculated GFR and positively correlated with number of days anuric and number of days on dialysis. Spearman coefficient (95% confidence limits) were: -0.75 (-0.88 - -0.52), p<0.001; 0.54 (0.2-0.76), p=0.002 and 0.63 (0.33-0.82), p<0.001 respectively.

iv) **Factor VIIa**
Factor VIIa levels are summarised in figure 11 and table 5. Levels were not significantly altered during D+ HUS, with median (range) levels being 0.053 (0.021-0.254) ng/ml during week one and 0.047 (0.006-0.186) ng/ml during week two of disease. Levels in the dialysed control groups were not significantly different from those of the normal control group. The median (range) level in the haemodialysed group of children (0.065 (0.055-0.076) ng/ml) was higher than that of the normal (0.033 (0.000-0.066 ng/ml) or peritoneally dialysed children (0.049 (0.000-0.094
ng/ml), but the differences were not significant. Kruskal-Wallis testing showed no significant difference between the group medians.

v) Fibrinogen.
Fibrinogen levels in patients with D+ HUS and in the control groups are shown in figure 5 and table 5. Median (range) fibrinogen levels in D+ HUS patients at week one and week two were 3.6 (2.5-4.8) g/l and 3.4 (1.4-5.8) g/l respectively. At each timepoint, levels were significantly higher than normal controls (p=0.002 and 0.01 respectively), yet not different from dialysed controls (p=0.36). There was no significant difference between paired results at week one and two (p=0.3) or between admission and post discharge levels (p=0.14). Levels post discharge were still significantly higher than the normal control group (p<0.001).

Median (range) levels of children receiving chronic peritoneal dialysis were 4.8 (3.8-6.2) g/l and of children receiving chronic haemodialysis were 3.0 (2.8-5.6) g/l. These levels were significantly higher than normal control children with median differences (95% confidence limits) of 2.2 (1.4-3.6) g/l, p=0.003 and 0.65 (0.3-3.0) g/l, p=0.003 respectively. Although there was a trend towards higher levels in patients receiving peritoneal rather than haemodialysis, this did not reach significance (p=0.06). Fibrinogen levels of children with acute diarrhoea were similar to those of the D+ HUS patients.

b) MARKERS OF FIBRINOLYSIS / ENDOTHELIAL CELL ACTIVATION

i) D- Dimer
Results of measurements of D-dimer level in plasma samples from children with D+ HUS and control children are shown in figure 6 and table 5. Median (range) D-dimer levels of patients with D+ HUS were 6,300 (600-47,000) ng/ml during the first week and 3,000 (200-33,000) ng/ml during the second week of disease. These were increased with median differences (95% confidence limits) of 6,300 (2,600-8,200) ng/ml, and 2,900 (1,400-5,100) ng/ml respectively, p<0.001 in each case. Levels returned to normal by convalescence. Levels of D-dimer in the acute phase of D+ HUS were significantly higher than those of either dialysis control group (p<0.001). There was no significant difference in D-dimer level comparing the two modes of
dialysis (p=0.13), though the D-dimer levels of the dialysis group as a whole were just significantly different from normal controls (p=0.0498).

There was a significant negative correlation between admission D-dimer level and degree of renal failure as measured by GFR (Spearman coefficient (95% confidence limits) -0.05 (-0.74 - -0.15), p=0.004 and a positive correlation between admission D-dimer level and number of days on dialysis or days of anuria (Spearman coefficients (95% confidence limits) 0.64 (0.35-0.82), p<0.001; 0.58 (0.26-0.79), p<0.001 respectively).

ii) Plasminogen activator inhibitor type 1 (PAI-1)

The data regarding PAI-1:Ag and PAI activity levels in the patient and control groups is summarised in figures 12, 13 and 21, and in table 5. During the first two weeks of D+ HUS, levels of PAI-1:Ag and activity were significantly increased when compared with healthy controls. PAI:Ag median (range) was 30.2 (6.9-104.6) ng/ml during week one and during week two was 16.8 (7.0-99.4) ng/ml. The median differences compared with normal controls were 22.1 (10.2-34.2) ng/ml, p<0.001 and 10.5 (3.6-17.5) ng/ml, p<0.001 respectively. The changes in PAI-1:Ag level over the course of the disease are shown in more detail in Figure 21. Median and interquartile range of all samples collected were plotted against day from onset of diarrhoea, omitting days on which less than four samples were available. Day one is the first day of diarrhoea. PAI:Ag levels were significantly higher than normal children until day 24.

PAI activity followed a similar pattern. Median (range) PAI activity during week one was 21.2 (1.8-80.0) IU/ml and during week two was 6.6 (1.6-80.0) IU/ml. At each acute timepoint, PAI activity was greater than normal but as with PAI:Ag, there was a trend towards normal with time (median difference (95% confidence limits) 19.3 (11.8-25.6) IU/ml for week one and 4.2 (1.5-12.9) IU/ml for week two, p<0.001 in each case). Comparison of paired samples taken on week one and week two showed a significant fall in PAI activity (p=0.009) with time. Levels of PAI:Ag and activity returned to normal post discharge.

Median (range) levels of PAI:Ag in samples from haemodialysed control children were 14.8 (7.7-26.3) ng/ml and from peritoneally dialysed children were 7.8 (4.2-27.8) ng/ml, not significantly different from each other (p=0.18). Dialysed patients as
a whole had similar PAI:Ag levels to normal children (p=0.15), although the haemodialysed subgroup had slightly higher levels than normal (p=0.04). The median (range) levels of PAI activity were 3.6 (1.6-4.6) IU/ml and 6.1 (1.4-8.5) IU/ml for samples from the haemodialysed and peritoneally dialysed controls respectively. PAI activity in the combined group was significantly higher than normal (p=0.02) although the median difference (95% confidence limits) of the dialysed group as a whole compared with normal children was 2.1 (4.1-0.9) IU/ml, much smaller than that seen comparing acute D+ HUS with normal controls (median difference (95% confidence limits) 15.7 (8.8-23.9) IU/ml).

Children with acute diarrhoea had similar levels of PAI:Ag and activity to normal, although one child had a PAI activity level of 10.6 IU/ml, which was outside the normal control range (1.5-3.3 IU/ml).

Although there was a significant correlation between PAI activity and PAI-1:Ag (Spearman coefficient = 0.69 (0.42-0.85), p<0.001), admission PAI-1:Ag correlated significantly with number of days of anuria (p=0.006) but not days of dialysis (p=0.1) and PAI activity did not correlate significantly with either.

iii) Tissue plasminogen activator antigen (t-PA:Ag)
Plasma levels of t-PA:Ag in patients with D+ HUS and the control children are shown in figures 14 and 22, and in table 5. Median (range) levels during week one of disease were 9.5 (3.5-28.8) ng/ml and during week two were 7.1 (1.0-19.6) ng/ml. At each of these timepoints, levels were significantly higher than normal (median differences (95% confidence limits) 7.1 (5.9-9.4) ng/ml and 4.7 (3.0-6.2) ng/ml respectively, p<0.001 in each case). Comparing paired samples, there was a significant fall in t-PA:Ag level from week one to week two of disease (p=0.002). Figure 22 shows the changes in t-PA:Ag level over the course of the disease in more detail. Median and interquartile range of all samples collected were plotted against day from onset of diarrhoea, omitting days on which less than four samples were available. Day one is the first day of diarrhoea. t-PA:Ag levels were significantly higher than those of normal children until day 26.

Levels of t-PA:Ag in the two groups of dialysed patients were not significantly different from each other (p=0.07) and the group as a whole was not significantly
different from normal (p=0.2). Levels were significantly lower than those in patients with D+ HUS during both weeks one and two of disease (p<0.001 at both timepoints). Children with acute diarrhoea had slightly higher t-PA:Ag levels than normal, but the range was similar to that of the D+ HUS children post discharge, and considerably lower than levels of children in the acute phase of D+ HUS.

iv) Fibrin Plate

Figure 4 shows a typical fibrin plate. Fibrin plate results are summarised in figures 15 and 23, and in table 5. In D+ HUS, fibrinolytic activity as measured by fibrin plates was decreased compared with healthy controls. The median differences were 10.5 (6.7-12.4) mm, p<0.001 during week one and 4.5 (7.7-1.0) mm, p=0.005 during week two. Comparing paired samples, there was a significant improvement in fibrinolytic activity between weeks one and two (p=0.01). After discharge, fibrinolytic activity as measured by fibrin plate was similar to the normal control group (p=0.7). Figure 23 shows the time course over which fibrin plate results normalised in greater detail. Median and interquartile range of all samples collected were plotted against day from onset of diarrhoea, omitting days on which less than four samples were available. Day one is the first day of diarrhoea. Fibrin plate levels were significantly higher than those of normal children until day 9.

Although there was a trend for fibrin plate results to be lower in children receiving haemodialysis when compared with children receiving peritoneal dialysis, this was not significant (p=0.18). The dialysed group as a whole did have significantly less fibrinolytic activity as measured by fibrin plate than normal (p=0.02) though more than patients during the first week of D+ HUS (p=0.002). Children with acute diarrhoea had levels similar to normal.

There was no significant correlation between t-PA:Ag and fibrin plate result but there was an inverse correlation between PAI activity and fibrin plate result (Spearman coefficient (95% confidence limits) -0.73 (-0.87 - -0.47), p<0.001). t-PA:Ag did not correlate significantly with PAI:Ag.
Figure 4 - An Example of a Fibrin Plate
c) OTHER MARKERS OF ENDOTHELIAL CELL ACTIVATION

i) von Willebrand Factor (vWF)
Levels of vWF in plasma samples are summarised in figure 16 and table 6. Median (range) vWF levels of children with D+ HUS during week one of disease were 165 (137-192) IU/dl and during week two were 156 (102-193) IU/dl. At both acute timepoints, vWF levels were increased when compared with normal controls (p<0.001 in each case). The median difference (95% confidence limits) was 74 (52-90) IU/dl, p<0.001 for samples taken in week one and 68 (46-89) IU/dl, p<0.001 for samples taken in week two. Levels did not fall significantly between the first and second weeks of illness (p=0.22), but were within normal limits by the time the disease had resolved clinically. von Willebrand multimer patterns in the four cases measured were not different from normal in either the acute or convalescent phases of disease.

Children receiving dialysis also had raised vWF levels compared with normal children: median difference 56 (26-77) IU/dl, p=0.003 for patients on peritoneal and 51 (13-72) IU/dl, p=0.008 for patients on haemodialysis. The levels in the peritoneally dialysed children did not differ significantly from those in haemodialysed children. Median (range) vWF levels of the combined dialysed controls were 140 (122-163) IU/dl, significantly lower than those taken from patients in the first week of D+ HUS (p=0.002). Children with acute diarrhoea had raised levels of vWF compared with normal, but levels were similar to those measured in plasma samples from children with D+ HUS and dialysed controls.

There was no significant correlation between platelet count and vWF:Ag level (Spearman coefficient (95% confidence limits) -0.075 (-0.44-0.31), p=0.36).

ii) Soluble P-selectin (sP-selectin)
Levels of sP-selectin are shown in figure 17 and table 6. Median (range) sP-selectin levels of children with D+ HUS during week one were 293 (88-436) ng/ml and during week two 288 (33-488) ng/ml. The median difference (95% confidence limits) for samples taken in week one were 184.5 (78-264) ng/ml, p=0.001 and for samples from week two 169 (75-250) ng/ml, p=0.002). Comparison of paired samples showed no significant change in sP-selectin levels between the first and second weeks of
illness (p=0.06). Levels were not significantly different from normal post discharge (p=0.2).

Median (range) sP-selectin levels of peritoneally dialysed children were 254 (113-300) ng/ml and of haemodialysed children were 148 (88-257) ng/ml. Although levels were higher in the peritoneally dialysed group this difference was not statistically significant (p=0.2). sP-selectin levels of the dialysed group as a whole were also not significantly higher than the normal control group (p=0.04). The levels of sP-selectin in the children with acute diarrhoea were within the range of the convalescent D+ HUS and normal control patients.

There was no significant correlation between platelet count and sP-selectin (Spearman coefficient 0.0043 (-0.38-0.38), p=0.49) or sP-selectin and vWF:Ag (Spearman coefficient 0.19 (-0.2-0.53), p=0.17).

### iii) Soluble E-selectin (sE-selectin)

Levels of sE-selectin in plasma samples are summarised in figure 7 and table 6. There was no increase in sE-selectin level during the acute phase of D+ HUS. The median (range) during week one was 111 (49-288) ng/ml and during week two 96 (47-239) ng/ml, not significantly different from the normal control median (range) levels of 81 (53-110) ng/ml. sE-selectin levels of the control groups on dialysis were also not significantly different from normal. The levels of children with acute diarrhoea also fell within the same range as the normal children. Using the Kruskal-Wallis test, no significant difference was demonstrated between the medians of the patient and control groups.

### d) MARKERS OF ENDOTHELIAL CELL DAMAGE

#### i) Soluble Thrombomodulin (sTM)

Figure 18 and table 6 summarise the levels of sTM measured in the plasma of D+ HUS patients and controls. D+ HUS patients had elevated sTM levels (median (range) 130 (60-250) ng/ml) when compared with normal children (median (range) 70 (50-90) ng/ml). Separating the D+ HUS results by time from disease onset, the
median (range) of sTM levels in week one was 142 (60-250) ng/ml and in week two was 120 (60-300) ng/ml. At each acute timepoint sTM levels were significantly higher than normal controls with median differences (95% confidence limits) of 70 (35-105) ng/ml, p<0.001 in week one and 50 (21-120) ng/ml, p<0.001 in week two. There was no significant difference in paired samples taken at each of these two timepoints (p=0.93). Levels returned to normal with resolution of the disease. The dialysed control group sTM levels were significantly higher still. Peritoneally dialysed children had higher levels than haemodialysed children, median levels being 420 and 290 ng/ml respectively but the difference was not significant (p=0.28). sTM levels were significantly higher in samples from dialysed children than samples taken during the first week of D+ HUS. The median difference (95% confidence limits) was 210 (170-300) ng/ml, p<0.001. There was a significant negative correlation with admission GFR (Spearman coefficient (95% confidence limits) -0.82 (-0.91 -0.63), p<0.001) and a positive correlation with days on dialysis and days anuria (Spearman coefficients (95% confidence limits) 0.74 (0.51-0.88), p<0.001 and 0.665 (0.38-0.83), p<0.001 respectively).

ii) Soluble tissue factor (sTF)
Levels of sTF are shown in figure 19 and table 6. Median (range) levels of sTF in children with D+ HUS during week one of disease were 171 (28-421) pg/ml and during week two were 206 (21-498) pg/ml. At neither timepoint were levels significantly different from normal control children. Control children receiving peritoneal and haemodialysis had median (range) sTF levels of 471 (190-875) pg/ml and 490 (398-705) pg/ml respectively, in each case significantly higher than the median (range) 171 (10-521) pg/ml level of the normal control group (p<0.002). There was no difference in sTF levels comparing the two modes of dialysis and no significant correlation between sTF and calculated GFR.
EFFECT OF TREATMENT

i) Peritoneal dialysis
TAT, PAI-1:Ag, t-PA:Ag and fibrin plate assays were performed on daily samples taken from ten patients over the first five days following admission. Five of these patients were receiving peritoneal dialysis over this period and five did not require dialysis during this period or subsequently. Table 7 shows the median and range of admission levels (taken prior to any treatment) and rate of change of each parameter over the first five days following admission calculated separately for those requiring dialysis and those who did not require dialysis. Although there was a trend towards higher levels of TAT, PAI-1:Ag and t-PA:Ag, and towards lower fibrin plate result on admission, this was not significant for any parameter. Furthermore, the rate of change in each parameter over the first five days of admission was not significantly different when the dialysed and non-dialysed groups were compared.

ii) Blood transfusion / Fresh Frozen Plasma (FFP) infusion
Levels of TAT, PAI-1:Ag, t-PA:Ag and fibrin plate assay result of 11 paired samples taken before and after administration of blood and 13 paired samples taken before and after administration of FFP revealed no significant change in any parameter following either blood product (data shown in table 8). Levels of TAT, t-PA:Ag and PAI-1:Ag in 11 samples of FFP infusion are also shown in table 8 and were not raised when compared with the plasma levels.

iii) Plasma Exchange/Haemodialysis
The median results of TAT, PAI-1:Ag, t-PA:Ag and fibrin plate assay of samples taken from patients prior to and following 16 plasma exchange sessions, and on 9 occasions, following a subsequent haemodialysis session are shown in table 9. The results of the chronic dialysis control children are also shown.

Following plasma exchange alone there was a significant increase in level of TAT (p<0.001) and PAI-1:Ag (p<0.001) and less significant reductions in t-PA:Ag level (p=0.02) and fibrinolytic potential as measured by fibrin plate (p=0.01).
When paired samples taken post plasma exchange (pre haemodialysis) were compared with those taken following the dialysis session there was no further change in TAT, PAI-1:Ag or fibrin plate result, although the lowered t-PA:Ag levels increased slightly (p=0.01), becoming similar to levels present prior to plasma exchange. Paired pre and post haemodialysis plasma samples from the 9 haemodialysis control patients showed a significant increase in TAT (p=0.01) but no change in the fibrinolytic parameters.
Table 5 - Median (Range) of Markers of Coagulation and Fibrinolysis in Plasma Samples from Children with D+ HUS and from Controls.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>HUS (acute)</th>
<th>HUS (discharged)</th>
<th>Normal controls</th>
<th>Dialysed controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n 30</td>
<td>19</td>
<td>12</td>
<td>18</td>
</tr>
<tr>
<td>TAT (ng/ml)</td>
<td>12.7 (6.9 - 48.0)**^A</td>
<td>3.6 (1.7 - 38.3)*</td>
<td>2.6 (0.0 - 4.2)</td>
<td>3.2 (1.7 - 9.3)*</td>
</tr>
<tr>
<td>F 1+2 (nmol/l)</td>
<td>8.4 (3.5 - 13.3)**^A</td>
<td>3.0 (2.0 - 5.2)</td>
<td>2.4 (2.1 - 5.0)</td>
<td>5.3 (1.8 - 6.8)*</td>
</tr>
<tr>
<td>Factor Xlla (ng/ml)</td>
<td>7.4 (0.6 - 20.0)*</td>
<td>1.9 (0.1 - 5.8)</td>
<td>0.95 (0.6 - 1.9)</td>
<td>10.1 (4.2 - 20.0)**</td>
</tr>
<tr>
<td>Factor Vlla (ng/ml)</td>
<td>0.05 (0.02 - 0.25)</td>
<td>0.05 (0.01 - 0.10)</td>
<td>0.03 (0.00 - 0.07)</td>
<td>0.05 (0.00 - 0.09)</td>
</tr>
<tr>
<td>Fibrinogen (g/l)</td>
<td>3.5 (1.5 - 5.8)**</td>
<td>3.3 (2.3 - 4.6)*</td>
<td>2.6 (2.3 - 2.7)</td>
<td>3.9 (3.8 - 6.2)**</td>
</tr>
<tr>
<td>D-dimer (ng/ml)</td>
<td>4689 (398 - 47380)**^A</td>
<td>62 (0.0 - 390)</td>
<td>78 (0.0 - 130)</td>
<td>190 (5.6 - 1300)*</td>
</tr>
<tr>
<td>PAI-1:Ag (ng/ml)</td>
<td>29.6 (6.9 - 104.6)**^A</td>
<td>11.2 (5.0 - 28.5)</td>
<td>11.2 (3.0 - 13.6)</td>
<td>12.0 (4.2 - 27.8)</td>
</tr>
<tr>
<td>PAI-1 activity (IU/ml)</td>
<td>17.7 (1.8 - 80.0)**^A</td>
<td>1.9 (1.3 - 8.8)</td>
<td>2.0 (1.5 - 3.3)</td>
<td>4.3 (1.4 - 8.5)*</td>
</tr>
<tr>
<td>t-PA:Ag (ng/ml)</td>
<td>9.4 (1.0 - 28.8)**^A</td>
<td>3.0 (0.9 - 4.7)</td>
<td>2.5 (0.04 - 4.2)</td>
<td>3.2 (1.7 - 9.3)</td>
</tr>
<tr>
<td>Fibrin plate (mm lysis)</td>
<td>2.0 (0.0 - 18.8)**^A</td>
<td>11.5 (0.0 - 14.0)</td>
<td>12.2 (4.0 - 13.0)</td>
<td>9.8 (0.0 - 13.5)*</td>
</tr>
</tbody>
</table>

** p<0.001 compared with normal controls
^A p<0.001 compared with dialysed controls
* p<0.05 and >0.001 compared with normal controls
^ p<0.05 and >0.001 compared with dialysed controls
Table 6 - Median (Range) of Markers of Endothelial Cell Activation and Injury in Plasma Samples from Children with D+ HUS and from Controls.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Children with D+ HUS</th>
<th>Control Children</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(on admission)</td>
<td>(post discharge)</td>
</tr>
<tr>
<td>n</td>
<td>30</td>
<td>19</td>
</tr>
<tr>
<td>vWF (IU/dl)</td>
<td>165 (118 - 193)**A</td>
<td>101 (72 - 146)</td>
</tr>
<tr>
<td>sP-selectin (ng/ml)</td>
<td>294 (88 - 436)**A</td>
<td>131 (8 - 720)</td>
</tr>
<tr>
<td>sE-selectin (ng/ml)</td>
<td>98 (49 - 288)</td>
<td>108 (46 - 212)</td>
</tr>
<tr>
<td>sTM (ng/ml)</td>
<td>130 (60 - 250)**A^A</td>
<td>70 (55 - 200)</td>
</tr>
<tr>
<td>sTF (pg/ml)</td>
<td>171 (28 - 498)^A^A</td>
<td>282 (75 - 505)</td>
</tr>
</tbody>
</table>

KEY

** p<0.001 compared with normal controls
A A p<0.001 compared with dialysed controls
* p<0.05 and >0.001 compared with normal controls
^ p<0.05 and >0.001 compared with dialysed controls
Figures 5 - 7. Median and Interquartile Range of Fibrinogen, D-dimer and sE Selectin Levels in Children with D+ HUS Against Time Since Disease Onset and in Control Children.

**Figure 5. Fibrinogen**

- **Y-axis:** Fibrinogen (g/l)
- **X-axis:** Week (wk1, wk2)
- **Legend:**
  - D+ HUS patients
  - Well
  - N (Normal)
  - PD (Chronic Peritoneal Dialysis)
  - HD (Chronic Haemodialysis)
  - AD (Control with Acute Severe Diarrhoea)

**Figure 6. D-dimer**

- **Y-axis:** D-dimer (ng/ml)
- **X-axis:** Week (wk1, wk2)
- **Legend:**
  - D+ HUS patients
  - Well
  - N (Normal)
  - PD (Chronic Peritoneal Dialysis)
  - HD (Chronic Haemodialysis)

**Figure 7. sE-Selectin**

- **Y-axis:** sE-selectin (ng/ml)
- **X-axis:** Week (wk1, wk2)
- **Legend:**
  - D+ HUS patients
  - Well
  - N (Normal)
  - PD (Chronic Peritoneal Dialysis)
  - HD (Chronic Haemodialysis)
  - AD (Control with Acute Severe Diarrhoea)

**KEY TO FIGURES 5-19**

- **WK1:** D+ HUS CHILDREN 4-7 DAYS POST DISEASE ONSET
- **WK2:** D+ HUS CHILDREN 9-14 DAYS POST DISEASE ONSET
- **WELL:** D+ HUS CHILDREN >40 DAYS POST DISEASE ONSET
- **N:** NORMAL CONTROL CHILDREN
- **PD:** CHRONIC PERITONEAL DIALYSIS CONTROL CHILDREN
- **HD:** CHRONIC HAEMODIALYSIS CONTROL CHILDREN
- **AD:** CONTROL CHILDREN WITH ACUTE SEVERE DIARRHOEA
Figures 8-11. Median and Interquartile range of Coagulation Parameters in Children with D+ HUS Against Time Since Disease Onset and in Control Children.

Figure 8. Thrombin-antithrombin III

Figure 9. Prothrombin fragment 1+2

Figure 10. Activated factor XII

Figure 11. Activated factor VII

**Figure 12.** Plasminogen Activator Inhibitor Antigen

**Figure 13.** Plasminogen Activator Inhibitor Activity

**Figure 14.** Tissue Plasminogen Activator Antigen

**Figure 15.** Fibrin Plate
Figure 20. Median and Interquartile Range TAT Level in Children with D+ HUS By Day Since Disease Onset.
Figure 21. Median and Interquartile Range PAI:Ag Level of Children with D+ HUS By Day Since Disease Onset.
Figure 22. Median and Interquartile Range tPA:Ag Level By Day Since Disease Onset in Children with D+ HUS.
Figure 23. Median and Interquartile Range Fibrin Plate Result By Day Since Disease Onset in Children with D+ HUS.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Median (range) levels prior to treatment</th>
<th>Median (range) rate of change (units/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dialysed (n=5)</td>
<td>Non - Dialysed (n=5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TAT (ng/ml)</td>
<td>22.0 (7.4 - 35.6)</td>
<td>12.8 (8.9 - 29.0)</td>
</tr>
<tr>
<td></td>
<td>0.6 (-2.7-6.0)</td>
<td>-0.1 (-3.1-2.3)</td>
</tr>
<tr>
<td>PAI-1:Ag (ng/ml)</td>
<td>30.0 (17.2 - 99.4)</td>
<td>17.7 (7.0 - 47.3)</td>
</tr>
<tr>
<td></td>
<td>-0.4 (-1.7-0.5)</td>
<td>-1.1 (-7.2-5.3)</td>
</tr>
<tr>
<td>tPA:Ag (ng/ml)</td>
<td>9.2 (3.5 - 11.2)</td>
<td>8.4 (1.0 - 13.4)</td>
</tr>
<tr>
<td></td>
<td>-0.4 (-1.7-0.5)</td>
<td>-0.8 (-1.3- -0.7)</td>
</tr>
<tr>
<td>Fibrin plate (mm lysis)</td>
<td>2.5 (0.1 - 6.0)</td>
<td>6.0 (0.1 - 18.7)</td>
</tr>
<tr>
<td></td>
<td>1.4 (1.2-2.3)</td>
<td>0.5 (-1.0-1.5)</td>
</tr>
</tbody>
</table>

Table 7 - Comparison of Median (Range) of Markers of Haemostasis in Daily Plasma Samples from Dialysed and Non Dialysed Children with D+ HUS.
Table 8 - Effect of Blood Transfusion and Infusion of Fresh Frozen Plasma (FFP) on Median (Range) of Markers of Coagulation and Fibrinolysis in Children with D+ HUS

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Blood transfusion (n=11)</th>
<th>FFP Infusion (n=13)</th>
<th>FFP sample (n=11)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pre</td>
<td>post</td>
<td>pre</td>
</tr>
<tr>
<td>TAT (ng/ml)</td>
<td>12.8 (7.1-29.0)</td>
<td>9.6 (6.0-26.6)</td>
<td>13.7 (6.0-35.6)</td>
</tr>
<tr>
<td>PAI-1:Ag (ng/ml)</td>
<td>24.3 (6.9-99.4)</td>
<td>37.3 (11.2-65.5)</td>
<td>30.0 (9.2-366.4)</td>
</tr>
<tr>
<td>tPA:Ag (ng/ml)</td>
<td>9.5 (5.6-34.4)</td>
<td>7.2 (6.4-13.1)</td>
<td>9.3 (5.7-34.4)</td>
</tr>
<tr>
<td>fibrin plate (mm lysis)</td>
<td>6.0 (0.1-18.7)</td>
<td>3.5 (0.1-12.7)</td>
<td>0.5 (0.0-12.2)</td>
</tr>
</tbody>
</table>
Table 9 - Effect of Plasma Exchange (PE) and Haemodialysis (HD) on Median (Range) of Markers of Coagulation and Fibrinolysis in Patients with D+ HUS and Haemodialysed Control Children

<table>
<thead>
<tr>
<th>Parameter</th>
<th>D+ HUS patients (n=16)</th>
<th>D+ HUS patients (n=9)</th>
<th>controls (n=9)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pre PE</td>
<td>post PE</td>
<td>pre HD</td>
</tr>
<tr>
<td>TAT (ng/ml)</td>
<td>7.0 (0.3-22.2)</td>
<td>31.2 (6.7-152)**</td>
<td>27.7 (13.7-152)</td>
</tr>
<tr>
<td>PAI-1:Ag (ng/ml)</td>
<td>38.3 (9.0-311)</td>
<td>98.8 (41.7-362)**</td>
<td>97.8 (45.1-362)</td>
</tr>
<tr>
<td>tPA:Ag (ng/ml)</td>
<td>5.1 (2.9-19.8)</td>
<td>3.4 (1.7-9.6)*</td>
<td>3.3 (1.9-7.1)</td>
</tr>
<tr>
<td>fibrin plate (mm lysis)</td>
<td>2.4 (0.0-14.0)</td>
<td>0.5 (0.0-4.0)*</td>
<td>0.25 (0.0-2.75)</td>
</tr>
</tbody>
</table>

KEY
** Wilcoxon Coefficient of Corellation pre vs. post p<0.001
* Wilcoxon Coefficient of Corellation pre vs. post p<0.05
SUMMARY

Markers of activation of coagulation showed thrombin was being actively produced during the acute phase of childhood D+ HUS. The source of thrombin generation was unclear. Factor VIIa levels were not raised so the early phase of activation by tissue factor was not proven to be involved. Levels of factor XIIa were also unhelpful for, although raised in children with D+ HUS, they were significantly higher in children on chronic dialysis who did not have evidence of thrombin generation.

Markers of fibrinolytic potential were changed in a pattern typical of endothelial cell activation. Both PAI-1 and t-PA antigen levels were raised, but the net effect was that of plasminogen activator inhibition as demonstrated by raised levels of PAI-1 activity. Consistent with this, fibrin plate results were reduced. During the period of admission, fibrin plate result recovered more rapidly than the other fibrinolytic parameters.

Other, more commonly used markers of endothelial cell activation were less helpful in confirming endothelial cell activation. Although both vWF and sP-selectin were increased, sE-selectin levels were not different from normal.

Markers of endothelial cell damage were not raised in acute childhood D+ HUS. sTF was not increased, and although sTM was raised in the patients with D+ HUS, the rise seen in children on chronic dialysis was much greater, suggesting that renal dysfunction rather than D+ HUS was the cause.

Peritoneal dialysis was shown to have no effect on levels of four markers of haemostatic activation. Infusion of blood or FFP also had no effect on these. As expected, haemodialysis and plasma exchange increased TAT, and in addition, plasma exchange reduced fibrinolytic potential as measured by fibrin plate.
DISCUSSION
Patients Studied

D+ HUS is the commonest cause of acute renal failure in childhood. The disease is traumatic and in some cases life threatening. There was one death in this series of 30 patients. Many different diseases are covered by the term HUS, so in order to study a relatively homogeneous population, only children with well documented diarrhoea-associated HUS were included. Although five children had no evidence of verotoxin producing E. coli infection, all suffered a typical illness. The clinical course and assay results were not clearly different when the children without evidence of E. coli infection were compared with the 25 cases with definite evidence of infection. The majority of children with antibodies against the 0157 lipopolysaccharide on admission had no detectable antibody at 1-3 months post disease, so it can be assumed that the presence of antibody related to an acute infection. The group as a whole were typical of the patients with D+ HUS seen at these two tertiary referral centres. There was a 3% (1/30) mortality and 3% (1/30) incidence of severe morbidity (failure to regain a normal GFR). In 14/30 (66%) cases dialysis was required and 5/30 (17%) children required plasma exchange.

Confounding Variables

Interpretation of any systemic marker in patients with acute D+ HUS, could potentially have been complicated by the acute phase response, platelet activation and acute renal failure seen in this disease. Control groups therefore included children with renal failure and with acute diarrhoea. Treatment may also have altered the levels of systemic markers so in order to control for this, initial samples were collected prior to any intervention. Samples were also collected to investigate the acute effect of fresh frozen plasma infusion, blood transfusion, dialysis and plasma exchange on markers of activation of coagulation and fibrinolysis.

Definition of Disease Onset

The time of admission in relation to the duration of disease was not consistent for all children, although in cases where anuria occurred, it was usual for children to be admitted around the time of onset of anuria, so time of admission did bear some relation to time of critical renal dysfunction. An alternative definition of time of onset of disease was sought in order to relate the levels of markers measured to duration of disease. Some children did not suffer anuria, and although oliguria was
demonstrated in almost all cases, defining the day of onset was almost impossible. Therefore onset of diarrhoea was used as the definition of onset of disease, for it could be determined to the nearest day and was present in all cases.

Analysis of Results

Four markers of haemostatic activity were measured in all 351 samples collected in order to examine the relationship with day since disease onset in detail. Other markers were not measured in all samples because of financial and time constraints. Instead, samples were allocated to three subsets, two covering acute timepoints (the first and second weeks) of the disease, and the third reflecting results following disease resolution. No patient was represented more than once at each timepoint. The first sample collected from patients presenting within 7 days of onset of diarrhoea was used to represent week one. The samples representing second week of disease included admission samples of those patients presenting 7-17 days from onset of diarrhoea in addition to second samples from those patients who had presented within the first week of disease.

These points complicated statistical analysis of these groups so the results have simply been displayed graphically in order that the obvious trends can be seen. Statistical analysis relating to the differences between week one and week two was confined to those samples where paired data were available, accepting the provisos that this described only the subgroup of patients presenting in the first week of disease and that the changes documented between weeks one and two may have been a treatment effect. Because of these restrictions, the data from all admission patient samples regardless of time since disease onset were also analysed and statistical comparisons made with data following disease resolution and control group data.

The normal control data was comparable with published values for t-PA:Ag and PAI:Ag (Andrew, M et al. 1992), vWF and sE-selectin (Nash, MC et al. 1996) levels in children. As there is a lack of published data regarding normal levels of the other markers in childhood, the levels measured in the normal control children, who were of similar age to the patients with D+ HUS were taken to be the normal range. Ideally, the chronic renal failure control children would also have been of similar age. Although samples were taken from the youngest dialysed children available, the
chronic peritoneal and haemodialysis control groups were both significantly older than the D+ HUS children. It is possible, therefore, that some of the differences discovered were age-related, but in the instances where a large difference between patient or normal groups and chronic dialysis groups was seen, renal failure or the dialysis process itself was more likely to be the cause.

The following sections review in detail the results of the different markers measured.

a) **ACTIVATION OF COAGULATION**

The marked rise in both TAT and F1+2 level in the acute phase of D+ HUS was clear evidence of thrombin generation, which had not previously been clearly demonstrated in this disease. Active thrombin generation occurred particularly in the first week of disease. Between the first and second weeks, there was a significant fall in F1+2 but not TAT, although the trend for the latter was downwards. The daily TAT measurements showed a gradual fall over the first 30 days following onset of diarrhoea. The levels fell more slowly than the markers of fibrinolysis, suggesting an ongoing activation of coagulation at a time when the endothelium had returned to the resting state. This would be compatible with the current model of haemostasis which would predict continuing production of thrombin via a pathway which does not involve tissue factor. After discharge, the significantly higher TAT levels yet normal F1+2 levels are difficult to explain as both measure generation of thrombin, and levels did correlate as would be expected. The anomaly may be secondary to differences in sensitivity of the two assays, if TAT is a more sensitive test, revealing ongoing low grade coagulation.

The activation of coagulation appeared to be a consequence of the disease itself, for increased TAT and F1+2 levels were present on admission, prior to any treatment. Furthermore, there was no change in TAT level following infusion of blood or FFP and the use of peritoneal dialysis was also shown to have no significant effect on TAT level. Haemodialysis is known to increase TAT levels (Yamazaki, M *et al.* 1992) as was demonstrated in the control group.

Examination of the source of thrombin generation was inconclusive. Factor XIIa levels have not previously been reported in this disease. If elevated, they are said to
reflect activation of the intrinsic coagulation pathway (Dempfle, CE et al. 1995). Factor XIIa levels were significantly elevated in the control patients on dialysis. As levels in D+ HUS were not significantly higher than the control dialysis group, it is not clear whether ongoing factor XII activation contributes to the pathophysiology of D+ HUS.

A newly developed ELISA method was used to measure factor VIIa levels (Philippou, H et al. 1997). There was no significant elevation of factor VIIa level in the D+ HUS group as might be expected during activation of the tissue factor pathway following endothelial cell activation. However, if glomerular endothelial cell activation occurred as a result of verotoxin binding, this event would have happened prior to hospital admission, as would the resultant tissue factor upregulation and activation of factor VIIa. TFPI blockade of continuing activation of VIIa (Broze, GJ 1995), would explain why levels of factor VIIa might no longer be elevated by the time patients with D+ HUS reached hospital. A second possibility is that this assay was not sensitive enough to detect a localised increase in VIIa in this disease, although it has been shown to be effective in detecting raised levels of VIIa in adults with myocardial infarction (Philippou, H et al. 1997).

Fibrinogen levels were increased rather than decreased in patients with D+ HUS, confirming that hepatic production was able to compensate for fibrinogen consumption. Fibrinogen is known to be an acute phase reactant (Wardle, EN 1994), with levels rising in conditions such as sepsis. Levels were also increased in the dialysed controls in agreement with data described by others (Bostom, AG et al. 1996). Like TAT, fibrinogen levels remained significantly higher than normal even following apparent recovery of renal function, suggesting again a continued low grade healing after this time.

b) MARKERS OF FIBRINOLYSIS/ ENDOTHELIAL CELL ACTIVATION

The increased D-dimer levels confirm results of several previous studies (Uttley, WS 1970, Taira, K et al. 1989, van de Kar, NCAJ et al. 1994) showing raised levels despite reduced plasma fibrinolytic potential. Renal failure was shown to increase D-dimer level as dialysed controls had higher levels than normal controls and there was a positive correlation with GFR. However, D-dimer levels were so much higher in the
patients studied that the pathology of D+ HUS itself can be assumed to influence D-dimer levels to a greater degree than the renal failure induced by the disease.

The PAI-1 activity and t-PA data support that published to date showing raised levels in this disease (van de Kar, NCAJ et al. 1994, Chant, ID et al. 1994), and although there was no significant correlation between PAI-1 activity and need for dialysis, there was a correlation with number of days anuria. This suggests that although there is some relation between severity of renal disease and PAI-1 activity, the relationship is not close enough to use clinically. Unlike van de Kar (van de Kar, NCAJ et al. 1994), but in agreement with Bergstein (Bergstein, JM et al. 1992), levels of PAI-1:Ag were significantly elevated. There have been no previous functional studies of t-PA activity in D+ HUS. At the time the study was planned, the fibrin plate test was chosen as such a measure, for it is cheap and relatively simple to undertake. It is, however, not an ideal test, being technically difficult to reproduce as the result is affected in some degree by the amount of free PAI-1 partitioned within the euglobulin fraction. An ELISA test measuring free t-PA is now available but financial constraints precluded use of this kit in this instance. Measurement of both antigen level and activity of t-PA and PAI in this study revealed a clear pattern of simultaneously raised PAI:Ag and t-PA:Ag levels with predominance of PAI activity. Although activated platelets (Fong, JSC et al. 1982) may have contributed to the elevation in PAI levels (Kruithof, EKO et al. 1986), this would not explain raised levels of t-PA. Furthermore, there was a lack of correlation of PAI:Ag or PAI activity with platelet number. As expected, there was a negative correlation between fibrin plate result and PAI-1 activity but there was no significant correlation with t-PA:Ag. The pattern of fibrinolytic markers was classical of endothelial cell activation (Pober, JS et al. 1990) and supports the theory that endothelial cell activation is integral to the pathogenesis of this disease.

The time course over which markers of fibrinolysis change during the acute phase of this disease has not previously been described. PAI:Ag and t-PA:Ag both fell gradually over the 25 days following onset of diarrhoea. It is therefore surprising that the two did not correlate statistically. Fibrin plate results normalised much more quickly, with levels being within normal limits by day 9. Renal failure occurred within 14 days of onset of diarrhoea in all cases requiring dialysis, and dialysis was required for a median (range) of 8.5 (3-56) days. The duration of raised antigen levels therefore mirrors the usual time course of renal failure in D+ HUS.
Although dialysed controls showed a small but significant elevation of PAI activity and reduction of fibrin plate results with increase in D-dimer levels, these changes were minor compared with those seen in acute D+ HUS and there was no correlation with GFR in any case. It is therefore unlikely that renal failure itself was fully responsible for the changes in the fibrinolytic parameters of D+ HUS patients.

c) OTHER MARKERS OF ENDOTHELIAL CELL ACTIVATION

The increased levels of vWF:Ag found in D+ HUS in this study are in agreement with previously published data (Rose, PE et al. 1990, Milford, DV et al. 1991). vWF is used clinically as a marker of endothelial cell activation, for example as a marker of disease activity in the systemic vasculitidies (Nusinow, SR et al. 1984). More specifically, multimers of vWF are thought to represent vWF lost from endothelium. The lack of abnormal vWF multimer patterns in the four plasma samples tested was at variance with published data in children with D+ HUS (Rose, PE et al. 1984). It is possible that the absence of a protease inhibitor in the citrate used to collect the blood samples allowed breakdown of the largest multimers. Since the samples tested were collected on ice and frozen within 30 minutes of collection, this is unlikely but the possibility cannot be excluded. Current methods of measurement of multimer levels are more specific than those employed by Rose et al. and it would be interesting to re-investigate levels of vWF multimers in children with D+ HUS after collecting plasma samples in a more appropriate manner.

The increase in vWF may be due to other factors. Firstly, vWF levels rise as part of the acute phase response (Pottinger, BE et al. 1989, Wardle, EN 1994), and levels were shown to be increased in children with acute diarrhoea but no HUS. The lack of correlation between fibrinogen and vWF:Ag levels suggests that this is not the major factor underlying the increase in vWF. Secondly, vWF levels were increased in dialysed control patients consistent with other studies reporting raised levels in patients in stable renal failure (Kario, K et al. 1995, Warrell, RP et al. 1978). However, levels of vWF:Ag in the first week of D+ HUS were significantly higher than those of dialysed patients, suggesting that factors other than renal failure were responsible for the increase in vWF in D+ HUS. Thirdly, vWF levels are also increased as a result of platelet activation which is known to occur in HUS (Walters,
MDS et al. 1988, Fong, JSC et al. 1982). The lack of correlation between vWF and platelet number suggested that this was not the predominant factor responsible for the raised levels seen in D+ HUS. In conclusion, the increase in vWF in D+ HUS was probably multifactorial, and the relative contributions from different sources could not be established by this study.

Like vWF, sP-selectin is considered to be a marker of both platelet and endothelial cell activation for it is a component of alpha granules of platelets and Weibel-Palade bodies of endothelial cells, and is expressed on the cell membrane on activation of these cells. Previous studies of sP-selectin in HUS have only been conducted in small groups of mainly adult patients with a mixture of TTP or HUS, and the levels were increased (Chong, BH et al. 1994, Katayama, M et al. 1993). In this study, an increase in sP-selectin levels was shown in children with D+ HUS with normal levels in dialysed control patients. As there was no correlation between platelet count and sP-selectin level, the source of increase in sP-selectin in D+ HUS was perhaps not only activated or damaged platelets but also activated endothelium. The normal levels in dialysed controls and children with acute diarrhoea suggested sP-selectin levels were not affected by renal dysfunction or the acute phase response.

Plasma sE-selectin was not significantly altered in the hospitalised phase of D+ HUS. Inward (Inward, CD et al. 1995) found a non significant rise in sE-selectin level, which was confirmed. She also found raised sVCAM which is further evidence for endothelial cell activation in this disease. Plasma sE-selectin is considered to be a marker of endothelial cell activation for it is increased in various inflammatory vascular conditions (Carson, CW et al. 1993, Pall, AA et al. 1994, Denton, CP et al. 1995), and in patients in septic shock (Newman, W et al. 1993). The absence of an increase in sE-selectin was surprising but it is known that E-selectin is expressed only transiently following endothelial cell activation (Newman, W et al. 1993). Therefore, expression of the molecule may have resolved by the time the patients were admitted to hospital median (range) 7 (2-17) days following disease onset. Alternatively, E-selectin may not be present on the cells involved in D+ HUS for it is expressed predominantly on the post-capillary venules (Munro, M et al. 1989) and the verotoxin receptor expressing renal endothelial cells are glomerular capillary cells, not venule endothelial cells (Lingwood, CA 1994).
d) MARKERS OF ENDOTHELIAL CELL DAMAGE

Thrombomodulin is an endothelial cell surface glycoprotein necessary for activation of the protein C pathway. Since it is endocytosed during endothelial cell activation, increased levels of sTM are thought to represent endothelial cell injury rather than activation (Ishii, H et al. 1991, Moore, KL et al. 1987). Levels of sTM were increased in adults with TTP (Kobayashi, M et al. 1995) but have not previously been measured in D+ HUS. There were raised levels of sTM in D+ HUS, but levels were significantly greater still in the dialysed controls. It is therefore likely that the increased levels reflected renal failure as found by others (Kario, K et al. 1995, Hergesell, O et al. 1993, Takano, S et al. 1990) rather than endothelial cell damage.

sTF levels were not elevated during the acute phase of D+ HUS. Tissue factor is absent from the unperturbed endothelial cell and monocyte surface, but is upregulated by cytokines such as IL-1 and TNF creating a local stimulus for fibrin formation and thrombosis (Pober, JS 1988). Increased levels of sTF have been demonstrated in the plasma of patients with DIC, TTP, diabetic microangiopathy and vasculitis with collagen diseases (Koyama, T et al. 1994). Kobayashi, however, found normal levels in patients with TTP (Kobayashi, M et al. 1995). The normal levels of sTF in D+ HUS were surprising for the dialysed patients had significantly increased levels as reported by others (Koyama, T et al. 1994, Kario, K et al. 1995). It is possible that sTF accumulates in chronic but not acute renal failure, though there is no precedent or obvious explanation for this phenomenon.

d) EFFECT OF TREATMENT

Peritoneal Dialysis
Peritoneal dialysis did not significantly alter any parameter, contrasting with the marked fall in PAI-1:Ag level during peritoneal dialysis reported by Bergstein (Bergstein, JM et al. 1992). This was an important finding as many units in Britain currently advocate early dialysis in an attempt to remove PAI-1:Ag. If, as suggested by these results, PAI-1:Ag or more importantly functional fibrinolytic potential is not altered by peritoneal dialysis, the treatment should only be advocated on clinical grounds. These data also suggest that changing to haemo- instead of peritoneal dialysis as first line dialysis therapy would not favourably alter fibrinolytic parameters.
Blood/FFP Infusion
Most children with D+ HUS require blood transfusion. This would not be expected to activate thrombosis or alter fibrinolytic potential, but as there was no published evidence to this effect, haemostatic parameters were measured in samples taken pre and post transfusion and confirmed no change in any parameter at 12 hours post transfusion. Infusion of FFP is not practised in all centres in Britain. The rationale behind this treatment was based on two trials, both of which demonstrated normal biopsy findings in children with D+ HUS treated with plasma compared with areas of cortical necrosis (Loirat, C et al. 1988) or thickening of the laminar rara interna and arteriolar damage (Rizzoni, G et al. 1988) in those not given this treatment. In neither trial was any significant change in acute clinical, haematological or biochemical parameters seen. Although both trials report important histological differences, the different findings in the untreated groups is difficult to explain. Furthermore, unnecessary use of FFP is expensive and exposes children to infection risks. This study has added to the argument against FFP treatment since no effect on markers of haemostasis was demonstrated.

Plasma Exchange
Plasma exchange is used in severe cases of D+ HUS with cerebral involvement, for no clear scientific reason other than that removal of some toxin or replacement of some missing factor (possibly a prostacyclin stimulating factor (Remuzzi, G et al. 1981)) may be achieved. There was a significant rise in TAT and PAI-1:Ag and a fall in t-PA:Ag and fibrin plate result following plasma exchange therapy. Activation of coagulation was also seen following haemodialysis and was probably due to the extracorporeal circuit. It is interesting to note that patients with D+ HUS having haemodialysis following plasma exchange did not show a further increase in TAT level but the reason for this is unclear. The basis of the changes in markers of fibrinolysis is also not obvious. If the extracorporeal circuit were responsible, one would expect changes following haemodialysis. The pattern is not classical of endothelial cell activation since t-PA:Ag falls, and as none of the parameters measured were raised in samples of FFP, addition of significant amounts of PAI cannot be the explanation.
SUMMARY

This study investigated the activation of coagulation and alteration in fibrinolytic parameters in childhood D+ HUS. Evidence for endothelial cell activation was also examined.

Coagulation was clearly shown to be active during the acute phase of this disease however the path leading to thrombin generation could not be established by measurement of factor XIIa and factor VIIa levels. The elevation in factor XIIa levels may have been due to renal dysfunction. The normal levels of factor VIIa did not support the hypothesis that activation of coagulation was initiated via the tissue factor pathway on the surface of activated endothelial cells, but current views of haemostasis would postulate that the activity of tissue factor and hence activation of factor VII is quickly blocked by TFPI. It is therefore possible that by the time these children reached hospital, the tissue factor coagulation pathway had been switched off and factor VII was no longer being activated. If this were the case, examination of plasma samples following admission to hospital would not be helpful in the further investigation of this hypothesis.

The fibrinolytic changes were consistent with the hypothesis that the pathophysiology of D+ HUS involves endothelial cell activation for an increase in both t-PA and PAI-1 levels with a predominance of inhibitor activity was demonstrated. Measurements of some of the more commonly used markers of endothelial cell activation and of markers of endothelial cell injury were less helpful in confirming this theory. Increased levels of vWF:Ag, sP-selectin and sTM in the acute phase of D+ HUS were demonstrated. However, vWF and sP-selectin might have been released by activated or damaged platelets and sTM and vWF may have been elevated because of renal dysfunction. There was no significant change in sTF or sE-selectin levels when compared with normal controls.

Finally, none of the commonly used treatments for this disease were shown to have a favourable effect on coagulation or fibrinolysis.
As it is not now considered ethical to biopsy children with acute D+ HUS, it was necessary to turn to in vitro methods to examine the effects of verotoxin on endothelial cells. For the reasons outlined in Chapter 1, a cell culture model was used. The aim of the experiments was to test the hypothesis that verotoxin induces thrombin generation via induction of tissue factor expression on glomerular endothelial cells.

Cells were stimulated for variable periods with varying concentrations of verotoxin. Verotoxin has been shown to cause cell death (Louise, CB et al. 1992, van de Kar, NCAJ et al. 1992), so the viability of cells under the experimental conditions used was also investigated. Initially a standard trypan blue test was used, but later an adaptation of this method was developed for use with fewer cells, allowing viability testing to be undertaken concurrently with the experiments examining tissue factor expression.
CHAPTER 3
IN VITRO STUDY
MATERIALS AND METHODS
MATERIALS

All materials used were obtained from Sigma-Aldrich Company, Gillingham, Kent unless stated below:

Human glomerular endothelial cells were provided by Dr Petra van Setten, working in Professor Monnens' Laboratory, Nijmegen, Holland.

Purified verotoxin (VT-1) was prepared and kindly donated by the laboratory of Dr M.A. Karmali, Department of Paediatric Laboratory Medicine, Hospital for Sick Children, Toronto, Canada.

Endothelial cell culture medium (ECM) was obtained from Life Technologies, Paisley, Scotland.

Tissue culture plastics were obtained from Greiner Labortechnik, Dursley, Gloucester.

Thermanox® coverslips were obtained from Intermed, Naperville, USA.

Normal goat serum was supplied by Dr Stewart Abbott, St Thomas' Hospital.

Mouse anti-human von Willebrand factor antibody was obtained from Dako, Denmark.

Mouse anti-human CD-31 antibody was supplied by R&D Systems, Abingdon, Oxfordshire.

Biotinylated goat anti-rabbit antiserum and Vectastain® Elite ABC reagent (ABC) were obtained from Vector, Peterborough.

S-2765 was obtained from Quadratech, Epsom, Surrey.

Factors VIIa and X were obtained from Enzyme Research Laboratories, Swansea, Wales.
METHODS

Preparation of tumour necrosis factor and verotoxin

1) Tumour Necrosis Factor (TNF)
Lyophilised TNF was reconstituted under sterile conditions to produce a 1000 ng/ml stock solution which was kept frozen in aliquots at -70°C until use. Before each assay, a 100 ng/ml solution was prepared by dilution with DMEM 10% FCS. The endotoxin content was <0.1 ng/μg TNFα (Sigma datasheet).

2) Verotoxin (VT)
580 μg aliquots of lyophilised verotoxin were stored at 4°C until reconstitution. In order to reconstitute, 50 μl sterile endotoxin free water was added to produce a 8285 nM solution. DMEM was added under sterile conditions, to produce a 1000 nM stock solution which was frozen at -70°C until use. Prior to each experiment, this stock solution was diluted with DMEM 10% FCS, to produce concentrations of 100 nM, 10 nM, 1 nM and 0.1 nM verotoxin.

Determination of endotoxin content of verotoxin

In order to exclude contamination with endotoxin, a 100 nM preparation of each batch of verotoxin used was tested using the E-Toxate Limulus amebocyte lysate assay. This assay uses E-Toxate, prepared from a lysate of the circulating amebocytes of the horseshoe crab, Limulus polyphemus, which increases in opacity and viscosity on exposure to minute quantities of the biologically active portion of endotoxin.

Using endotoxin free plastics and glassware, a stock solution of endotoxin standard was prepared by reconstitution with endotoxin free water. This stock solution was diluted further with endotoxin free water to produce endotoxin standards of 400, 40, 4, 0.5, 0.25, 0.125, 0.06, 0.03 and 0.015 endotoxin units (EU)/ml. The stock and each dilution was vortexed for 30-60 s prior to further dilution. E-Toxate working solution was reconstituted using endotoxin free water.

Nine endotoxin free non-siliconized 10 x 75 mm glass test tubes were prepared by autoclaving at 121°C for 1 h followed by heating in an oven at 175°C for 3 h. 0.1 ml of endotoxin standards containing 0.5, 0.25, 0.125, 0.06, 0.03 and 0.015 EU/ml were
added directly to the bottom of 6 of the tubes. 0.1 ml 100 nM verotoxin was added directly to the bottom of two further tubes and 0.1 ml endotoxin free water to the last tube. 0.1 ml 4 EU/ml endotoxin standard was then added directly to the bottom of one of the verotoxin containing tubes. 0.1 ml E-Toxate working solution was added to each tube by inserting the pipette to just above the contents and allowing the lysate to flow down the side of the tube, avoiding contact and possible cross contamination.

The tubes were placed in a water bath and incubated undisturbed at 37°C for 1 h. They were then gently removed one at a time and slowly inverted 180 degrees whilst observing for evidence of gelation. Formation of a hard gel was considered a positive result.

**HUVEC Cell harvest and culture**

Umbilical cords less than 24 h old that had been kept at 4°C were transported in Hanks balanced salt solution. In a laminar flow hood, a sterile blunted 16 gauge needle was inserted into one end of the umbilical vein and securely fastened with string. 20 ml Hanks balanced salt solution was syringed through this needle twice to clear the vein of blood. A second 16 gauge needle was inserted into the other end of the vein and similarly secured. 20-25 ml DMEM containing 1.75 collagen digestion units of type II collagenase was then instilled via one needle, the other being blocked by an empty syringe. After 10 min, the cord was gently massaged and the contents, along with any detached endothelial cells were aspirated into the empty syringe and collected into a sterile universal container. 20-25 ml DMEM were then instilled and the cord gently massaged. This was aspirated with a further harvest of endothelial cells and collected in a second sterile universal container.

The collected cell suspensions were centrifuged for 5 min at 1000 x g and the pellets combined and resuspended in 5 ml endothelial cell medium (ECM) containing 10% FCS, 100 IU/ml penicillin and 100 µg/ml streptomycin. The cells were seeded into a 25 cm² culture flask coated with 1% gelatin and incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. The medium was replaced every 24-36 h.

When the cells reached confluence, they were released with trypsin/EDTA, seeded in a split ratio of 1:2 into either 1% gelatin coated 6-well tissue culture plates or 1% gelatin coated wells of a 96 flat bottomed well tissue culture plate. Cells were
cultured in ECM with 10% FCS (ECM 10% FCS) using culture volumes of 2 ml per well of a 6 well plate and 200 μl per well of a 96 well plate. Once confluence was reached, the medium was changed and cells were incubated for a further 24 h before experiments were performed.

**Human Glomerular Endothelial Cell Harvest and Culture**

Human kidneys were collected in Hanks balanced salt solution. The cortex was dissected off and cut into small pieces. A serial sieving procedure was then employed, collecting glomeruli from the top of screens with opening sizes 180, 125, 108, 90 and 53 μm. The material collected was centrifuged for 5 min at 200 x g and the pellet digested with 0.1% collagenase for 2 h at 37 °C. The pellet was shaken vigorously to loosen the glomeruli and culture medium containing serum was then added to terminate collagenase activity. The material was re-centrifuged for 5 min at 200 x g, and the pellet resuspended in culture medium. The glomerular remnants were then seeded onto gelatin coated 6 well plates and incubated at 37°C in a humidified atmosphere of 5% CO₂ in air. The medium was replaced every 24-36 h.

Outgrowing cells were monitored daily using phase-contrast microscopy and once a 50:50 ratio of endothelial to epithelial cells was present, selective trypsinisation was undertaken. Cells were incubated with trypsin/EDTA at room temperature until visual inspection of the cell layer by phase-contrast microscopy revealed rounding up of endothelial but not epithelial cells. Serum containing culture medium was then added in order to terminate digestion, and the trypsinized cells were then filtrated through a 38μm sieve to remove glomerular remnants.

The filtrate was centrifuged for 5 min at 200 x g, the pellet washed twice and then resuspended in Hanks balanced salt solution/10% fetal calf serum supplemented with 10μg/ml anti-CD31 antibody (CLB, Amsterdam). The suspension was washed three times to remove unbound antibody and then dynabeads coated with goat anti-mouse antibody (Dynal, Oslo) were added and incubated for 15-30 min on ice. The immunomagnetic positive fraction was washed five times using a magnetic particle collector (Dynal, Oslo), resuspended in culture media and seeded onto gelatin coated 6 well plates.
Cell characterisation

i) HUVEC
In order to ensure that cells at passage one were of endothelial origin, and of adequate purity, they were seeded at 300 cells/cm² onto 1% gelatin coated 13 mm Thermanox® coverslips and grown to confluence under the above conditions. The cells were then fixed by immersion in ice cold 80% acetone for 10 min. Endogenous peroxidase activity was quenched by incubating the cells for 5 min in 0.6% hydrogen peroxide in methanol at room temperature. Cells were then washed once in distilled water and once in Tris buffered saline (TBS), following which they were incubated for 20 min at room temperature in a 1:70 dilution of normal goat serum in TBS. Cells were subsequently washed in 2 changes of TBS over 10 min before each coverslip was incubated for 30 min at room temperature with a different primary antibody.

Five coverslips covered with cells were processed. The primary antibodies used were:

i) 1:50 and 1:100 dilutions of mouse anti-human vWF in TBS

ii) 1:500 and 1:2000 dilutions of mouse anti-human CD-31 in TBS

One coverslip had no primary antibody to act as a negative control. All coverslips were washed in 3 changes of TBS over 20 min and then incubated for a further 30 min at room temperature with a 1:2000 dilution of biotinylated goat anti-rabbit antiserum in TBS.

Coverslips were then washed in 3 changes of TBS over 20 min and incubated with Vectastain® Elite ABC reagent for 30 min at room temperature. After a further three washes in TBS, coverslips were incubated for 5 min in a 1% diaminobenzidine / 6% hydrogen peroxide solution and then rinsed in tap water.

The cells were lightly counterstained with haematoxylin for 30 s and then dehydrated in graded alcohol solutions of 70%, 90% and 100% followed by xylene before being mounted under DPX mountant. Seven fields of view (approx. 200 cells per field) were examined using an Olympus BX50 photomicroscope to assess the purity of the cell population.
ii) Human Glomerular Endothelial Cells

Charaterisation of human glomerular endothelial cells was undertaken by Dr Petra van Setten. The presence of endothelial cell specific antigens was determined using direct immunofluorescence studies with a panel of endothelial cell specific monoclonal antibodies (vWF, EN-4, PECAM-1 and V,E-cahedin) and antibodies to control for contamination with epithelial (anti-cytokeratin 8) and muscle cells (anti-α smooth muscle actin) (van Setten, PA et al. 1997).

Cell viability following stimulation with verotoxin

To determine the degree of cytotoxicity occurring under the conditions to be studied, a series of measurements of cell viability were undertaken. Initially, a standard trypan blue test was performed on cells following a 5 h incubation with varying concentrations of verotoxin or under negative (medium alone) or positive (100 ng/ml TNF) control conditions.

Confluent HUVEC in a six well plate were washed twice with fresh DMEM 10% FCS. 0.5 ml of one of the following stimulants was added to each well:

1. 100 nM verotoxin
2. 10 nM verotoxin
3. 1 nM verotoxin
4. 0.1 nM verotoxin
5. 100 ng/ml TNF (positive control)
6. DMEM 10% FCS alone (negative control)

The plate was incubated for 5 h at 37°C in a humidified atmosphere of 5% CO₂ in air. The cells were washed twice with Hanks balanced salt solution and then released with trypsin/EDTA. The cells were resuspended in 2 ml DMEM 10% FCS and centrifuged at 1000 x g for 5 min. The pellet was resuspended in 50 μl Hanks balanced salt solution and 50 μl Trypan Blue solution was added to the cell suspension and mixed well. After standing for 5-15 min, 10 μl of the mixture was transferred to a haemocytometer and the viable (dye excluding) and non-viable (blue coloured) cells contained in ten 1 mm squares were counted. The % cell viability was then calculated.
The experiment was performed on three occasions, using cells from different umbilical cords on each occasion. A modification which used fewer cells was then developed. As only one well of a 96 well plate was needed for each single result, it was possible to run this assay concurrently with the tissue factor activity assay so that the exact conditions being assessed were the same for the viability and tissue factor expression assays. This cell viability method is described alongside the relevant tissue factor experimental method.

Experiments to establish whether verotoxin induces tissue factor expression on human endothelial cells

The main aim of this in vitro work was to establish whether verotoxin induced tissue factor expression on human endothelial cells in culture. The following experiments were performed in order to answer this question using a chromogenic assay for factor X activity on cellular models of the disease.

A functional tissue factor assay was chosen as this had the advantage over methods looking for tissue factor antigen that non-functional tissue factor complexed with TFPI would not be measured. The assay used was adapted from the work of Colucci et al. (Colucci, M et al. 1983). Instead of BaSO₄ serum eluate, pure preparations of factor VIIa and factor X were used at concentrations equivalent to those of factor VII and X in plasma under physiological conditions. Calcium was added at a concentration found to be effective in studies undertaken by Dr K. Jurd (personal communication). The quantity of tissue factor present was assumed to be proportional to the activation of factor X. The dependence of the reaction on factor VIIa and factor X was established by omission of these factors. Activation of factor X was determined by a colour change in the chromogenic substrate for factor Xa, S-2765. Paranitroanalide is cleaved from the end of S-2765 by factor Xa, resulting in the formation of a yellow colour, the rate of formation of which is proportional to the amount of Xa. The rate of development of colour was determined by measuring absorbance at 405 nm after a set period of time using a photometer. Because TNF was known to induce tissue factor expression on HUVEC (Colucci, M et al. 1983), it was used as a positive control. A negative control consisting of cells with no added stimulant was also run concurrently in each set of experiments. In all cases, confluent cells were used as although sub-confluent cells were known to bind more verotoxin (van de Kar, NCAJ
et al. 1992), reproducibility would be harder to achieve, and the \textit{in vivo} situation would be less closely modelled.

As cultured HUVEC have been shown to express tissue factor following incubation for 4-6 h with TNF (Colucci, M \textit{et al.} 1983), the initial incubation time chosen was 5 h. In a subsequent experiment, the model developed by van de Kar \textit{et al.} (van de Kar, NCAJ \textit{et al.} 1992) was used. This involved pre-stimulating HUVEC for 24 h with TNF in order to increase the number of Gb3 receptors on the cell surface, and subsequently incubating with verotoxin. IL-1, which is released by activated HUVEC also increases the number of Gb3 receptor sites on the cell surface (van de Kar, NCAJ \textit{et al.} 1992). We therefore incubated cells in a third experiment for 24 h to investigate whether auto-activation occurred following upregulation of Gb3 by IL-1 or similar substances produced by the cells themselves.

\textbf{i) HUVEC incubated for 5 h with verotoxin alone}

20 wells of a 96 well tissue culture plate containing confluent cells were used. The cells were washed twice with DMEM 10\% FCS. One of the following stimulants was then added to each of three wells:

1. 100 nM verotoxin
2. 10 nM verotoxin
3. 1 nM verotoxin
4. 0.1 nM verotoxin
5. 100 ng/ml TNF (positive control)
6. DMEM 10\% FCS alone (negative control)

TNF was added to a further two wells and a plate plan was constructed. The plate was incubated for 5 h at 37\(^\circ\)C in a humidified atmosphere of 5\% CO\(_2\) in air. The wells were then washed twice with Hanks balanced salt solution and the following were added to each well:

50 \(\mu\)l Hanks balanced salt solution
50 \(\mu\)l 15 mM calcium chloride in Hanks balanced salt solution
20 \(\mu\)l 2.5 mg/ml S-2765
15 \(\mu\)l 10 U/ml Factor VIIa

The plate was incubated for 2 min at 37\(^\circ\)C followed by the addition of 15\(\mu\)l 10 U/ml Factor X. Factor VIIa was omitted in one of the additional positive control wells and
factor X was omitted in the other to demonstrate factor VIIa and factor X dependence respectively. The plate was incubated for 35 min at 37°C. The reaction was then stopped by the addition of 50 μl 50% v/v acetic acid to each well. The absorbance of each well at 405 nm was determined.

The experiment was performed on five separate occasions using HUVEC from different umbilical cords on each occasion.

ii) Human glomerular endothelial cells stimulated for 5 h with verotoxin
The experiment was repeated using human glomerular endothelial cells in place of HUVEC. The experiment was conducted four times, using cells from two different donors in duplicate.

iii) HUVEC pre-stimulated with tumour necrosis factor for 24 h followed by a 5 h stimulation with verotoxin
The above experiment was repeated using confluent HUVEC which had been incubated with 10 ng/ml TNF for 24 h in a humidified atmosphere of 5% CO₂ in air at 37°C. On this occasion, the 100 nM concentration of verotoxin was omitted.

Concurrently, nine extra wells of confluent cells were used to assess cell viability under the three conditions listed below in triplicate. The following modification of the trypan blue viability test was used.

One of the following were added to each of three wells:
1) 10 nM verotoxin
2) 100 ng/ml TNF (positive control)
3) DMEM 10% FCS alone (negative control)

The plate was incubated for 24 h at 37°C in a humidified atmosphere of 5% CO₂ in air, following which the cells were washed twice with Hanks balanced salt solution and then released with trypsin/EDTA. The cells were resuspended in 2 ml DMEM 10% FCS and centrifuged at 1000 x g for 5 min. The supernatant was carefully tipped off, and the sides of the centrifuge tubes dried with tissue, taking care not to disturb the pellet. The pellet was resuspended in 5 μl neat Trypan Blue solution,
making a total volume of approx. 10 μl. After standing for 5-15 min, 10 μl of the mixture was transferred to a haemocytometer and the viable (dye excluding) and non-viable (blue coloured) cells contained in ten 1 mm squares were counted. The % cell viability was then calculated.

The experiment was performed on four occasions using HUVEC from different umbilical cords on each occasion.

iv) HUVEC stimulated for 24 h with verotoxin
The above experiment, including the modified cell viability assay, was repeated on HUVEC which had not been pre-stimulated with TNF. Cells were incubated for 24 h rather than 5 h and the 100 nM concentration of verotoxin was again omitted.

The experiment was performed on four occasions using HUVEC from different umbilical cords on each occasion.

STATISTICAL ANALYSIS

The distribution of this data was found to be normal, so ANOVA testing was used to compare the means of each of the groups tested, Where a difference was found, Tukey HSD testing was undertaken in order to determine which of the groups were responsible for the difference. Where only two groups were to be compared, student T-test was used.
RESULTS
Endotoxin content of verotoxin

Hard gels were formed in tubes containing endotoxin standards 0.5, 0.25, 0.125 and 0.06 EU/ml but not in the 0.03, 0.015 EU/ml standards or the negative control. Thus the detection level of this assay was 0.06-0.5 EU/ml. Hard gels were not formed in any of the 100 nM VT solutions.

The endotoxin content of the verotoxin preparations used was therefore <0.06 EU/ml.

Cell characterisation

i) HUVEC

Photomicrographs of cells stained with antibody to vWF or CD-31, and the negative control cells can be seen in Figure 24.

98% of cells exhibited discrete granular cytoplasmic von Willebrand factor immunoreactivity and the same proportion of cells exhibited positive cytoplasmic staining for CD-31. The negative control cells were not stained.

ii) Human Glomerular Endothelial Cells

Indirect immunofluorescence microscopy undertaken by Dr Petra van Setten revealed a distinct granular pattern of von Willebrand factor, and the endothelial cell specific antigens EN-4, PECAM-1 and V,E-cahedrin were present at regions of intercellular contact. Furthermore, there was no immunoreactivity to the anti-cytokeratin 8 antibody or to the anti-α smooth muscle actin antibody.
Figure 24 - Photomicrographs of HUVEC stained with antibody to vWF or CD-31, and the negative control cells

vWF Antibody

CD-31 Antibody

Negative Control Cells
Cell Viability (HUVEC)

Viability of HUVEC under the different conditions used is shown in table 10.

i) 5 h incubation
Following 5 h stimulation, ANOVA testing showed no significant difference in viability according to stimulant used when the means of five cords tested in triplicate were compared (p=0.65). Mean cell viability was 87%.

ii) following pre-stimulation with 10 ng/ml TNF
The cells pre-stimulated for 24 h with 10 ng/ml TNF and subsequently incubated for 5 h with 10 nM verotoxin had significantly lower cell viability (28%) than those subsequently incubated with 100 ng/ml TNF or DMEM 10% FCS alone (viability 83% and 89% respectively). ANOVA testing showed that this difference was highly significant (p<0.0001). Tukey HSD test showed that the difference was due to the verotoxin treated cells. Taking the other two groups as a homogeneous subset, there was a difference between the means of 58% (95% confidence limits 28%-89%).

iii) 24 h incubation
Similarly, only 22% of the cells incubated for 24 h with verotoxin were viable compared with 89% of those incubated with DMEM 10% FCS alone and 88% of those incubated with 100 ng/ml TNF. ANOVA testing revealed a significant difference in the means (p=0.001). Again, the positive and negative controls formed a homogeneous subset (p=1.0) and the outlying group was that treated with verotoxin.
Table 10. Viability of HUVEC Exposed to Verotoxin under various conditions

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>5 h</th>
<th>5 h</th>
<th>24 h</th>
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<tr>
<td>Medium alone</td>
<td>90%</td>
<td>89%</td>
<td>89%</td>
</tr>
<tr>
<td>100 ng/ml TNF</td>
<td>91%</td>
<td>83%</td>
<td>88%</td>
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<td>100 nM VT</td>
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<td>10 nM VT</td>
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<td>1 nM VT</td>
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<tr>
<td>0.1 nM VT</td>
<td>83%</td>
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</table>
Tissue Factor Expression on Endothelial Cells

The mean absorbance at 405 nm is shown in table 11. The four experiments were conducted in triplicate on 4-5 occasions as documented, and the mean of all wells tested is given. Mean absorbancies of the positive control wells missing factor VIIa or factor X are also shown.

i) Assay Controls
Mean absorbance of wells containing cells incubated with 100 ng/ml TNF on which the assay was performed omitting factor X was 0.08 in all cases. Omitting factor VIIa, the mean absorbancies were between 0.12 and 0.16. In all cases the values were significantly lower than the positive controls on which a complete assay had been performed, demonstrating dependence on VIIa and X for the change in colour of the chromogenic substrate and hence involvement of tissue factor in the reaction. The mean absorbance of four wells without cells on which the assay for tissue factor was performed was 0.08, identical to that obtained on cells when factor X was omitted.

ii) 5h incubation (HUVEC and renal glomerular cells)
After 5 h stimulation with verotoxin, both HUVEC and glomerular endothelial cells reacted in a similar manner. There was a highly significant difference in tissue factor expression when cells treated with TNF, verotoxin and medium alone were compared (p<0.0001 for each type of endothelial cell). Tukey HSD test revealed that the difference was due to the effect of the positive control TNF. Excluding cells treated with TNF, ANOVA testing revealed no significant difference in absorbance between those cells treated with any concentration of verotoxin and the negative control cells (p=0.37 for HUVEC and p=0.67 for glomerular endothelial cells).
Table 11. Tissue Factor Expression (absorbance at 405 nm) of HUVEC and Human Glomerular Endothelial Cells exposed to Verotoxin under various conditions

<table>
<thead>
<tr>
<th>Time stimulated</th>
<th>5 h</th>
<th>5 h</th>
<th>5 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prestimulated?</td>
<td>not</td>
<td>not</td>
<td>24 h</td>
<td>10 ng/ml TNF</td>
</tr>
<tr>
<td>Cell Type</td>
<td>HUVEC</td>
<td>Glom. Cells</td>
<td>HUVEC</td>
<td>HUVEC</td>
</tr>
<tr>
<td>n</td>
<td>5</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Stimulant</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Medium alone</td>
<td>0.31</td>
<td>0.19</td>
<td>0.32</td>
<td>0.24</td>
</tr>
<tr>
<td>100 ng/ml TNF</td>
<td>0.66</td>
<td>0.58</td>
<td>0.39</td>
<td>0.37</td>
</tr>
<tr>
<td>100 nM VT</td>
<td>0.30</td>
<td>0.18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 nM VT</td>
<td>0.31</td>
<td>0.18</td>
<td>0.98</td>
<td>0.42</td>
</tr>
<tr>
<td>1 nM VT</td>
<td>0.34</td>
<td>0.19</td>
<td>0.86</td>
<td>0.39</td>
</tr>
<tr>
<td>0.1 nM VT</td>
<td>0.34</td>
<td>0.19</td>
<td>1.07</td>
<td>0.36</td>
</tr>
<tr>
<td>no X</td>
<td>0.08</td>
<td>0.08</td>
<td>0.08</td>
<td>0.08</td>
</tr>
<tr>
<td>no VIIa</td>
<td>0.12</td>
<td>0.16</td>
<td>0.15</td>
<td>0.12</td>
</tr>
</tbody>
</table>

**KEY**
- **HUVEC** human umbilical vein endothelial cells
- **Glom. cells** human renal glomerular cells
- **no X** assay performed omitting factor X
- **no VII** assay performed omitting factor VII
Comparing the results of HUVEC with renal glomerular endothelial cells, the latter expressed significantly less tissue factor than HUVEC following incubation with medium alone (p<0.001) but following stimulation with 100 ng/ml TNF, both types of cell responded by expression of similar amounts of tissue factor (p=0.37).

ii) following pre-stimulation with 10 ng/ml TNF (HUVEC)
When HUVEC were pre-stimulated with TNF for 24 h before a 5 h incubation with verotoxin, fresh TNF or medium alone, there was again a difference between the groups (ANOVA p<0.001) but Tukey-HSD test showed that on this occasion, the difference was due to the verotoxin treated wells. Cells pre-stimulated with TNF and then incubated with more TNF expressed similar amounts of tissue factor to cells incubated with medium alone following pre-stimulation with TNF and were counted as one subgroup. Cells treated with verotoxin expressed significantly more tissue factor but ANOVA testing showed no difference between the means of the wells treated with different concentrations of verotoxin (p=0.12) i.e. there was no dose effect. All cells treated with verotoxin were therefore considered as a second subgroup. The difference between the means of the two subgroups was 0.59 (95% confidence limits 0.49-0.69, p<0.00001).

Comparing tissue factor expression in pre-stimulated for 24 h with 10 ng/ml TNF and then incubated with verotoxin for 5 h with the amount of tissue factor expressed by cells incubated with 100 ng/ml TNF for 5 h alone showed the cells pre-treated with TNF followed by verotoxin expressed significantly more tissue factor (p=0.0001) even though the concentration of TNF was less.

iii) 24 incubation (HUVEC)
The cells incubated for 24 h with verotoxin alone reacted differently again. ANOVA testing showed a significant difference when all of the stimuli were compared (p=0.005). Tukey HSD testing revealed that the significantly different groups were those treated with 10 nM and 1 nM concentrations of verotoxin. Comparing the two control groups (those cells incubated with medium alone against those treated with 100 ng/ml TNF), student-T test showed that the TNF treated cells were expressing significantly more tissue factor with a difference between the means of 0.13 (95% confidence limits 0.2-0.05, p=0.003).
Comparing the tissue factor expression of cells stimulated with verotoxin alone for 24 h and cells stimulated with verotoxin for 5 h following a 24 h pre-incubation with 10 ng/ml TNF, there was significantly more tissue factor expression in the pre-stimulated group of cells. The difference between the means was 0.56 with 95% confidence limits of 0.47-0.64 (p<0.00001). Comparison of the cells treated with medium alone (negative controls) also showed a significant difference, with pre-stimulated cells expressing more tissue factor, although the difference was smaller (0.07 with 95% confidence limits 0.015-0.13, p=0.02).
SUMMARY

- HUVEC incubated for 5 h
  - 80-90% cell viability
  - no expression of tissue factor following incubation with VT
  - tissue factor expression following incubation with TNF

- Human glomerular endothelial cells incubated for 5 h
  - 80-90% cell viability documented in published work (van Setten, PA et al. 1997)
  - less baseline tissue factor expression than HUVEC
  - no expression of tissue factor following incubation with VT
  - tissue factor expression following incubation with TNF similar to HUVEC

- HUVEC pre-stimulated with TNF for 24 h followed by incubation for 5 h
  - 80-90% cell viability without VT; 30% cell viability following VT
  - more tissue factor expression following incubation with VT than with TNF alone
  - no increase in tissue factor expression following continued incubation with TNF compared with medium alone

- HUVEC incubated for 24 h
  - 90% cell viability without VT, 20% cell viability with VT
  - some tissue factor expression following incubation with VT or TNF compared with negative control cells but less than that obtained following prestimulation with TNF
  - no tissue factor expression following incubation with medium alone
DISCUSSION
All experiments were carried out on pure populations of endothelial cells. HUVEC were used at passage one in order to achieve consistency within the study system and to preserve as far as possible the in vivo characteristics of these cells, for it is known that age-related changes in structure and function occur in normal cell lines (McAteer, JA et al. 1994). Glomerular endothelial cells were used at passage 7 as prior to this adequate purity of the cells could not be guaranteed. The verotoxin effects seen were not caused by contamination with endotoxin as each batch of verotoxin used was tested to confirm absence of such contamination.

**Cell Viability**

Significant cytotoxicity was demonstrated following incubation of cells with verotoxin. Those pre-treated with TNF and subsequently incubated with 0.1-10 nM verotoxin were significantly less viable than cells incubated with TNF alone and cells incubated with medium alone. Significantly fewer cells incubated for 24 h with 0.1-10 nM verotoxin alone were viable when compared with cells incubated with TNF or medium alone for 24 h. TNF alone was not significantly cytotoxic at the concentrations used. These results were comparable with previously published work (Louise, CB et al. 1992, van de Kar, NCAJ et al. 1994).

Viability of human glomerular endothelial cells following 8 h incubation with TNF or verotoxin has been shown to be 80-90% (van Setten, PA et al. 1997). and because of the difficulty in culturing these cells, this experiment was not repeated.

**Tissue Factor Expression**

The main aim of these in vitro experiments was to determine whether verotoxin induced tissue factor expression on human endothelial cells. The assay used involved activation of factor X in the presence of factor VIIa, which is dependent on tissue factor. Dependence on both factors X and VIIa and dependence on a factor present on the cells (presumed to be tissue factor) was demonstrated by negative results in omission experiments. The slight colour change in the presence of factor X
alone was probably due to presence of a small amount of activated factor X in the preparation of factor X used, although a high quality preparation was used in order to minimise such contamination. The results, however are still valid as cells under experimental conditions showed significantly higher absorbance values.

The assay was shown to be sensitive enough to detect tissue factor which was known to be expressed on HUVEC incubated for 5 h with 100 ng/ml TNF (Colucci, M et al. 1983). Incubation on HUVEC with verotoxin alone at concentrations ranging from 0.1-100 nM for 5h did not induce detectable amounts of tissue factor expression, although increasing the incubation time to 24 h did result in some tissue factor expression. Pre-stimulation of HUVEC for 24 h with 10 ng/ml TNF followed by a 5 h incubation with 0.1-10 nM verotoxin induced significantly more tissue factor activity.

Human glomerular endothelial cells did not express tissue factor following incubation with verotoxin for 5 h, although levels of tissue factor expression following incubation with 100 ng/ml TNF for 5 h were similar to those obtained with HUVEC under the same conditions.

HUVEC express a limited number of verotoxin binding Gb3 receptors. It has been shown that pre-treatment of HUVEC with TNF has been shown to induce increase binding of radiolabelled verotoxin to HUVEC after a 6-8 h lag period for up to 48 h (van de Kar, NCAJ et al. 1995). After 24 h, the number of receptors was increased mean 38-fold (range 13-90 fold). Although it is possible that TNF primes the endothelial cell in some other way, the different response of cells pre-treated with TNF is most likely to be due to the increased number of Gb3 receptors. Because of this, 10 fold higher concentrations of verotoxin (100 nM) than used in published work (van de Kar, NCAJ et al. 1995) were used with HUVEC in the resting state in an attempt to saturate binding of the smaller number of Gb3 receptors present. There was, however, no significant response even with this amount of verotoxin suggesting that an increase in the number of receptors is needed before a response can be initiated. Since there was no increase in tissue factor expression with increasing concentrations of verotoxin, the response seemed to be an on/off effect. As no dose effect was detected in these experiments, the highest concentration of verotoxin used in subsequent experiments was 10 nM in order to avoid wastage of the toxin. A small dose response was in fact seen following 24 h incubation of cells with verotoxin.
alone. It would therefore be interesting to repeat this experiment using 100 nM verotoxin.

Pre-incubation of HUVEC for 24 h with IL-1 has also been shown to increase the number of surface Gb3 receptor sites (van de Kar, NCAJ et al. 1992). A 24 h incubation period was therefore included to investigate whether release of IL-1 by activated cells incubated with verotoxin alone might induce a subsequent increase in Gb3 receptors and therefore an escalating response to verotoxin. In support of this hypothesis, there was expression of tissue factor on the cells stimulated with verotoxin which was most marked in cells treated with 1-10 nM concentrations of verotoxin. There was also expression of tissue factor but to a lesser degree on cells incubated for 24 h with 100 ng/ml TNF.

In summary, verotoxin was shown to induce tissue factor expression on HUVEC. Tissue factor was expressed to a small degree on cells incubated for 24 h with verotoxin, but to a much greater degree on cells pre-stimulated with TNF. It is likely that the number of Gb3 receptors on the cell surface determines the response to verotoxin.

HUVEC are not the ideal model for D+ HUS which specifically affects the kidney. Because of this, human glomerular endothelial cells were also investigated. These cells have been particularly difficult to culture, but recently a reproducible method has been developed, producing pure cell cultures by passage 7-15 (van Setten, PA et al. 1997). Histological investigations would suggest that there are increased numbers of Gb3 receptors on human glomerular endothelial cells, although this has only been demonstrated in biopsies from children under the age of 2 y (Lingwood, CA 1994). It was therefore hoped that this cell line might express a higher concentration of Gb3 receptor in the resting state than HUVEC. There was, however, no difference in the response of these cells following 5 h incubation with verotoxin when compared with HUVEC. Subsequently, it has been shown that the number of Gb3 receptors on these cells is in fact equivalent to the number on HUVEC, and there is a similar upregulation following incubation with TNF (van Setten, PA et al. 1997). It would therefore be interesting to investigate the response of renal glomerular cells to a 24 h incubation with verotoxin, and to a 24 h incubation with TNF followed by a 5 h incubation with verotoxin.
Verotoxin is thought to act via inhibition of protein synthesis by depurination of a single adenine residue from the 28S RNA component of the ribosome, leading to inhibition of interaction of elongation factor-1 with the ribosome (Endo, Y et al. 1988). van de Kar showed that HUVEC pre-stimulated with TNF and subsequently incubated with verotoxin have reduced viability, protein synthesis, and release reduced amounts of t-PA:Ag and PAI-1:Ag, but cells incubated with verotoxin alone are not affected in this way (van de Kar, NCAJ et al. 1994). In contrast to these results, pre-stimulation of HUVEC with TNF followed by incubation with verotoxin favoured the greatest tissue factor expression, although viability was shown to be significantly reduced in agreement with the work of van de Kar (van de Kar, NCAJ et al. 1994).

If verotoxin does reduce protein synthesis, then the production of tissue factor on the surface of the cell must also be initiated following inhibition of protein synthesis, and cannot occur via the same mechanism as that initiated by endotoxin which requires RNA and protein synthesis (Colucci, M et al. 1983). A possible mechanism would involve an intracellular messenger such as Nuclear factor κB (NFκB) which is activated in response to signals which represent a threat to cells, including agents that reduce protein synthesis (Baeuerle, PA 1991). It is likely that NFκB is involved for the tissue factor gene is preceded by a κB promoter region (Edgington, TS et al. 1991). This is an area requiring further investigation.

Thus the hypothesis that verotoxin induces tissue factor expression on the surface of human endothelial cells is supported. Whether verotoxin induces endothelial cell activation as would be suggested by this finding, or whether it does not, as suggested by the reduction in t-PA:Ag and PAI-1:Ag release requires further study looking at the effect of verotoxin on several markers of endothelial cell activation simultaneously. Furthermore, the effect of verotoxin on intracellular messengers requires study. Further clarification of the pathogenesis of this disease might allow new therapeutic strategies to be devised.
CHAPTER 4
OVERVIEW
The work described in this thesis tested the hypothesis that the pathogenesis of childhood D+ HUS involves activation of coagulation and endothelial cell activation. Levels of systemic markers of endothelial cell activation and activation of coagulation were measured in serial plasma samples taken from children with D+ HUS and from normal and dialysed control groups.

At the time of initial testing, the clinical picture of most children investigated was one of resolving intestinal symptoms and worsening renal dysfunction. A variety of markers were measured as many were known or found to be affected by concurrent events occurring in the disease, such as platelet activation/damage, renal failure and acute phase response. All markers of activation rather than injury, except sE-selectin were significantly raised in children with D+ HUS when compared with normal children, although in several instances renal failure was shown to contribute to raised levels. Taken as a whole, these results suggested that endothelial cell activation was occurring in this disease and although there was no proof that these changes were of glomerular endothelial cell origin, histological studies (Lingwood, CA 1994) and the clinical picture would favour this supposition.

Levels of both TAT and F1+2 were significantly raised in the patients with D+ HUS but not in the control children with renal failure, demonstrating thrombin generation in this disease. The source of thrombin was investigated by measurement of levels of activated factor VII and XII. Factor XIIa levels were raised, but were significantly higher still in control children with renal failure suggesting the renal failure rather than the specific disease pathogenesis caused the raised levels. Factor VIIa was not raised which did not support the theory that the endothelial activation was inducing activation of coagulation via tissue factor expression. As the children studied did not present to hospital until around a week following onset of diarrhoea, it was possible that by the time of admission, TFPI released by activated endothelial cells was inactivating cell surface expressed tissue factor, preventing further activation of factor VII.

As children could not be studied at an earlier phase of disease, cell culture models were used to investigate further the hypothesis that verotoxin induced activation of coagulation via activation of endothelial cells. HUVEC incubated with TNF for 24 h in order to increase the number of Gb3 receptors were shown to express tissue factor following incubation with verotoxin. Although not conclusive proof, this result
supports the hypothesis that the pathogenesis of D+ HUS involves expression of tissue factor on endothelial cells leading to activation of coagulation.

Cultured human glomerular endothelial cells did not express tissue factor upon stimulation with verotoxin alone which was surprising as it was expected that these cells would express more GB3 receptor and thus be more sensitive to the effects of verotoxin. However, concurrent with the research reported here, it became known that these cells in fact have the same number of Gb3 receptors as HUVEC, and that the receptors can be upregulated by pre-incubation with TNF in a similar manner (van Setten, PA et al. 1997). Further experiments examining the response of human glomerular endothelial cells pre-stimulated with TNF are therefore indicated.

There were some differences noted when results from in vivo and in vitro experiments were compared. The in vivo findings are in agreement with the thrust of findings of several other workers (Bergstein, JM et al. 1992, Chant, ID et al. 1994, van de Kar, NCAJ et al. 1994, Rose, PE et al. 1990) suggesting that endothelial cell activation is occurring in this disease. It is not possible to determine the exact source of activated endothelium in vivo. These results are at variance with the proposed mode of action of verotoxin which is via reduction of protein synthesis. However, endothelial heterogeneity is well described (Pober, JS 1988) and some types of endothelium may not have the capacity to express Gb3 receptors, and therefore might not be affected by verotoxin in the same manner as HUVEC and human glomerular endothelium. It is therefore possible that only certain types of endothelium, such as that in the renal glomerulus and the umbilical vein, respond to verotoxin with a reduction in protein synthesis, yet other types of endothelium, lacking the specific receptor needed to induce a response to verotoxin, nevertheless become activated in response to the raised levels of cytokines found in the acute phase of the disease (van de Kar, NC et al. 1995, Karpman, D et al. 1995). In order to test this theory, comparative experiments using various types of human endothelium (in particular gastrointestinal endothelium) could be undertaken.

The cytotoxic activity of verotoxin on HUVEC shown in other in vivo work (van de Kar, NCAJ et al. 1994) was confirmed, but is at variance with the lack of rise in markers of endothelial cell damage seen in plasma samples from children with D+ HUS and the typical clinical picture of early renal recovery. It may be that the amount of endothelium affected was too small to effect a significant systemic rise in these
markers. Alternatively, verotoxin may cause cell death by apoptosis as in the case of vero cells (Inward, CD et al. 1995). If so, it is possible that thrombomodulin and tissue factor might not be released upon cell death.

In summary, the in vitro model used did suggest that verotoxin could induce tissue factor expression on cells that had been pre-treated with TNF to increase the number of Gb3 receptors expressed. This finding was compatible with the in vivo evidence for activation of coagulation. It would be interesting to investigate whether HUVEC and human glomerular endothelial cells simultaneously released PAI-1:Ag and t-PA as would be expected if they had been stimulated with TNF, or whether protein synthesis was in fact reduced as found by other workers (van de Kar, NCAJ et al. 1994). Finally, the intracellular messenger involved in this response to verotoxin should be investigated. The prime candidate would be NFκB as there is a site for NFκB in the promoter region of the tissue factor gene and this messenger is known to respond to signals that reduce protein synthesis.

**IMPROVEMENTS AND FUTURE PLANS**

This study has provided useful insights into the pathogenesis of D+ HUS in childhood, although a few changes may have yielded more information. Recognising that it would be best to analyse results according to time from disease onset rather than admission to hospital, it would have been easier to analyse the results had samples been collected taking day of onset of diarrhoea rather than the day of admission as the baseline time from which serial samples were collected. The information gained from the ten cases from whom daily samples were collected was helpful in establishing the influence of treatment. These results would have been more significant had daily samples been available from all children.

Plasma samples from control groups of children with acute renal failure of different aetiology would have been informative, and plans were made to include such a control group, but unfortunately these patients are rare, and in no case was permission obtained to collect a sample in the acute phase of disease.
I did consider pioneering a method for the isolation and culture of human glomerular endothelial cells but it quickly became clear that this would have been an entire project in itself. I was most fortunate to be able to collaborate with Dr Petra van Setten and use cells she had cultured. Had these been available earlier in the course of my project, I would have performed all experiments on both these cells and HUVEC.

The most obvious direction in which to develop this work would involve repeating the full set of HUVEC experiments on the human glomerular endothelial cells. Were tissue factor expression shown, it would be interesting to examine the intracellular messenger involved, the most obvious candidate being NFκB.
CHAPTER 5
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