HAEMOLYTIC URAEMIC SYNDROME OF CHILDHOOD - HETEROGENEITY,

OUTCOME AND NEUTROPHIL INVOLVEMENT IN PATHOGENESIS

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ABSTRACT

This thesis involves clinical and laboratory studies undertaken to elucidate the different clinical manifestations and prognosis of the haemolytic uraemic syndrome (HUS) of childhood, and the role of polymorphonuclear leucocytes in the pathogenesis of this disorder.

An historical review, a classification and views on pathogenesis, treatment and outcome of childhood HUS are elaborated in Chapter 1. The primary subdivision of HUS in children is assumed to be those cases with prodromal diarrhoea (D+ HUS) and those without (D- HUS). An overall summary of the children presenting to the Hospital for Sick Children, Great Ormond Street, London, with a diagnosis of HUS between 1966 and 1991 is presented in Chapter 2. The clinical and laboratory features, response to treatment, and outcome of idiopathic D- HUS is reviewed in Chapter 3. In Chapter 4 a retrospective case control study to identify risk factors at presentation for the development of neurological disease in D+ HUS is described. A long-term follow-up study to evaluate the outcome of renal function in infants and children after an episode of D+ HUS (1966-85), and a prospective single blind study using duplex Doppler ultrasound in the
investigation of occult nephropathy following D+ HUS are presented in Chapter 5.

In the second half of the thesis 3 laboratory studies examining the role of polymorphonuclear leucocytes (PMNLs) in the pathogenesis of D+ HUS are described. The first study examines PMNL adherence and subsequent injury to the endothelium with PMNLs and plasma from children with D+ HUS (Chapter 6). PMNL activation in HUS is evaluated in Chapter 7 by the measurement of complexed elastase in plasma. Possible mechanisms of PMNL involvement are explored in Chapter 8 with the measurement of cytokines interleukin-8 and tumour necrosis factor α, together with complexed elastase, in HUS plasma.

These studies emphasize the heterogeneity of the disorder, implicate PMNLs as having a role in pathogenesis and raise the possibility that agents which suppress PMNL recruitment, activation and adhesion to endothelium have a potential therapeutic role in the more severe forms of this disorder.
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My thanks go to Dr Kevin Forsyth from the Department of Immunology at the Institute, with whom I collaborated on the work regarding neutrophil mediated endothelial injury and to Vanita Shah, without whose technical advice and help in the laboratory studies this work would not have been possible. Finally my sincere thanks are due to the children with the haemolytic uraemic syndrome and their parents for kindly agreeing to participate in the studies.
1.1 Historical Review

1.1.1 Description of the Syndrome

The haemolytic uraemic syndrome (HUS) occurs throughout the world and is the commonest cause of acute renal failure of childhood in North America and Western Europe. In this section the early reports of the syndrome, the recognition of the renal histopathological features and the definition of the concept of microangiopathic haemolytic anaemia are described.

(a) Early Reports. The first description of the haemolytic uraemic syndrome (HUS) is usually credited to Gasser and his colleagues Gautier, Steck, Siebenman, and Oeschlin, who delineated the features of what they initially called the hemolytic uremic syndromes (Hämolytisch-urämische Syndrome) in 1955 (1). Gasser was a Swiss haematologist who, while examining numerous peripheral blood films as part of a thesis on the haemolytic anaemias, noted that some contained bizarre shaped erythrocytes. Five cases were described who in addition to evidence of haemolysis were severely ill with
thrombocytopaenia, acute renal failure with cortical necrosis, and cerebral symptoms. The features of these cases were published under the heading of the haemolytic uraemic syndromes.

There may have been earlier reports of unrecognized cases; Joan Wagner reported such a case under the heading 'Acute Tubular Necrosis with Anaemia from the Hospitals for Sick Children, Great Ormond Street in 1954 (2). Although the patient described had haemolytic anaemia, thrombocytopaenia, acute renal failure, and seizures, there is no mention of the presence or absence of fragmented erythrocytes. Fluge and Moe (3) reviewed the records of infants with acute gastroenteritis who presented during the years 1950-55 and retrospectively diagnosed 2 of 104 as having had HUS. In 1954 McKay and Wahle in The Lancet (4) described an epidemic of severe gastroenteritis in neonatal infants caused by Escherichia coli 0-111,B4, in which thrombi were noted in many organs and 'in the more severely affected kidneys every glomerular tuft was completely occluded by a fibrin thrombus.'

(b) Renal Histopathology. Between 1957 and 1960, Habib and her colleagues wrote 5 papers on HUS (5-9). Their first case was reported in la Presse Medicale in 1957 (5). While working in the pathology laboratory of
Professor Debre, Habib undertook a retrospective survey of 60 infants who had died with acute renal failure, 6 of whom had the same pathological features she had observed in her first case. The findings were published under the title *Maladie Thrombotique Arteriolo-capillaire du Rein* (6). Habib first used the term *microangiopathie thrombotique du rein* in 1959 (8) and she has continued to define and redefine the pathology of HUS in subsequent publications (10,11). Habib and her colleagues described 3 main groups of renal injury: cortical necrosis, predominance of glomerular injury, and predominance of arteriolar injury; a mixture of glomerular and arteriolar lesions also occur. They demonstrated that the severity of the disease and its prognosis correlated with this classification (11).

(c) **Microangiopathic Haemolytic Anaemia.** It was soon realised that there was a relationship between HUS and the Moschowitz syndrome of thrombotic thrombocytopenic purpura (TTP) first described in 1924 (12), in which, additionally, fever and neurological features are present. It was recognized that erythrocyte fragmentation and haemolysis may be present in patients with TTP, or with renal failure from a variety of causes. Monroe and Strauss (13) observed erythrocyte fragments in the blood vessels and tissues in a fatal case of TTP, and suggested that the
abnormal vessels might be the site of erythrocyte fragmentation. This concept was extended by Brain and his co-workers (14), who postulated that this process took place in a variety of diseases in which there was partial occlusion of small blood vessels by fibrin or platelets, and might account for the presence of fragmented erythrocytes in patients with acute renal failure, including the haemolytic uraemic syndrome of infancy, in malignant hypertension and in severe pre-eclampsia or eclampsia. Brain and colleagues (14) suggested that the term microangiopathic haemolytic anaemia might be used to describe the characteristic blood picture with erythrocyte fragmentation observed in patients found to have small-blood-vessel disease in biopsies of the kidneys or other organs, or at autopsy; and later proposed that HUS might be a consequence of disseminated intravascular coagulation (DIC), perhaps triggered by a generalized Shwartzman reaction (15,16).

1.1.2 Epidemiology

(a) HUS in Argentina. Shortly after Gasser's description of HUS in 1955 (1), it became apparent that this rare disease was in fact extraordinarily common in a few locations. By the early 1960s, more cases of HUS were seen in Argentina than anywhere else. In a series of
studies published from 1964 (17-22), Gianantonio and colleagues defined the spectrum of the illness, suggested possible causes, speculated about the pathophysiology, made observations on the pathology, and increased worldwide awareness and interest, in what was until then thought to be a rare disease of childhood. Among Gianantonio's important contributions is the recognition that some patients with HUS who recover from the acute episode may go on to develop end-stage renal failure (ESRF) many years later (19). In 1973, Gianantonio et al. described the clinical and pathological characteristics of the acute stage of HUS drawn from a series of 678 patients (21).

(b) Epidemics of HUS. The first report of a small epidemic of HUS came from a hospital serving a rural community in North Wales (23), in which McClean et al described nine children from a small area who developed HUS over a period of eight weeks. No enteric pathogens were identified. Since then a further epidemic has been reported in the UK (24,25); 11 children and 2 adults from the same locality in the West Midlands presented over a period of 2 weeks in the summer of 1983. The first major epidemiological studies on HUS were reported in 1965 from southern Africa by Kibel and Barnard (26,27), and these workers were the first to note that HUS was an uncommon occurrence in blacks.
1.1.3 Heterogeneity of HUS

(a) Familial Occurrence and Genetic Factors. The familial occurrence of HUS in siblings was first reported in an article entitled 'Acute Glomerular Nephritis in Infancy', by Fison in 1956 (28). HUS in twins was first reported in 1965 (29); they were monozygotic twins aged 5 months at the onset of their illness, in which the prodrome was a respiratory tract infection with vomiting, and both children died. In 1969 Chan et al. (30) described the occurrence of HUS in 2 unrelated adopted siblings neither of whom died. Kaplan et al (31) recognized in 1975, that there were 2 patterns of familial involvement of HUS, in that some sibs seemed to have developed HUS as a result of exogenous exposure to an infective agent, and a second group of sibs seemed to have had a genetic predisposition to develop HUS. Subsequent studies indicate that familial occurrence of HUS, particularly of the first pattern, is not unusual (32-38).

It has also become clear that some patients inherit HUS in an autosomal dominant mode (39-44).

(b) Recurrent HUS. In Brain's review of HUS in 1969 (16) he noted that 'very occasionally, recurrent episodes of the haemolytic-uraemic syndrome have been associated
with a seemingly wide variety of infective episodes', but it was not until 1977 that the importance of recurrent HUS was recognized by Kaplan et al. (45).

(c) \textit{D+ and D- HUS.} By the 1980s the concept of heterogeneity of HUS was established, and in 1984 Levin and Barratt (46) proposed a simple classification into those cases with antecedent diarrhoea (D+ HUS) and those without (D- HUS). D+ HUS synonymous with typical, prototypic, epidemic or enteropathic HUS and D- HUS with atypical or sporadic disease.

1.1.4 Infection and HUS

(a) \textit{Neuraminidase-producing Organisms} Although epidemiologic and family studies suggested a noninfectious cause for most cases of D- HUS, an important subgroup caused by infection with neuraminidase-producing organisms was first recognized by Klein et al in 1977 (47). Several organisms, including pneumococci (48,49), Clostridia species (50), and some viruses have since been shown to produce the enzyme neuraminidase. This cleaves sialic acid from the cell membranes of red blood cells, platelets and endothelial cells, so exposing the Thomsen-Friedenreich antigen that is normally hidden. Antibodies against this antigen are normally present in human sera, probably in response to cross reacting intestinal bacterial
polysaccharide antigens. These antibodies agglutinate red blood cells with exposed antigens and possibly bind to endothelial and platelet membranes leading to haemolysis, thrombocytopenia and renal failure. This form of HUS has been most commonly reported after pneumococcal infection (49).

(b) Shigella Dysenteriae Type 1 In 1978 Koster and co-workers (51) reported data supporting the hypothesis that severe colitis in shigellosis was associated with circulating endotoxin from the colon producing coagulopathy, renal microangiopathy and haemolytic anaemia. HUS was found to complicate some cases of Shigella dysenteriae type 1 infection late in the course of the disease, and a case control study by Butler et al. in 1987 (52) identified a high peripheral white cell count and the administration of antibiotics, particularly ampicillin, as factors associated with the development of HUS.

(c) Verocytotoxin (Shiga-like Toxin). The discovery by Karmali et al in 1983 (53) that Verocytotoxin producing Escherichia coli (VTEC) were implicated in cases of D+ HUS was a major breakthrough, they reported 8 of 15 patients with D+ HUS with evidence of infection with Escherichia coli, which produced a cytotoxin toxic to Vero cells. In a
subsequent prospective study (54), evidence of infection with verotoxin-producing organisms was found in 30 (75%) of 40 patients with D+ HUS but in none of 40 age matched control patients. No evidence of infection with verotoxin-producing organisms was found in 5 patients with D- HUS. The link between infection with verotoxin-producing organisms and D+ HUS was soon confirmed by other workers in Canada (55), the United States (56-8), and the United Kingdom (25).

1.2 Current Classification

HUS can be classified primarily into 2 major subgroups on the basis of the presence (D+ HUS) or absence (D- HUS) of a diarrhoeal prodrome.

1.2.1 D+ HUS

(a) Verocytotoxin-producing Enteric Pathogens. The most common subgroup of HUS is usually referred to as typical childhood HUS (46,59) or D+ HUS. This form of HUS affects infants and young children following a diarrhoeal prodrome (21,25). The disorder often occurs in epidemics, usually in the summer (25,60). Despite an acute presentation the prognosis is good; most patients recover with supportive treatment alone and without relapses (21,60).
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Following Karmali's discovery that VTEC were implicated in cases of D+ HUS (53), verotoxin-producing enteric pathogens, in particular VTEC, are now thought to be responsible for most cases of D+ HUS.

(b) *Shigella Dysenteriae*. HUS following *Shigella dysenteriae* infection is another form of D+ HUS and occurs frequently in less developed countries (51). *Shigella*-associated HUS differs from typical D+ HUS by its worse prognosis and its pathologic and pathophysiologic features (51). Unlike enterohaemorrhagic *E coli*, *Shigella* invades the bowel wall (61), perhaps thereby allowing larger quantities of Shiga toxin or other toxins to enter the blood stream. It is of interest that endotoxaemia is often detected in *Shigella*-associated HUS but not in VTEC-associated D+ HUS.

1.2.2 D- HUS

(a) *Idiopathic*. Idiopathic D- HUS affects children of all ages and develops insidiously, either with or without a prodromal illness or with a respiratory prodrome (27,46,62,63). This form of HUS may be familial (31) and frequently follows a relapsing or progressive course (45,60). Severe hypertension, end stage renal disease, and multisystem involvement may occur. The ultimate prognosis
is poor (31,45,60). Many cases of D- HUS are indistinguishable from adult TTP, and consequently the literature on the 2 conditions is often confused and overlapping (59,62,64).

The cause of most cases of D- HUS remains unknown. The occurrence within families, relapsing course, and absence of evidence of infection suggest that these forms of HUS are related to inherited or acquired defects in the interaction between the vascular endothelium and the circulating blood cells (46,62,64). Reduced prostacyclin production and platelet aggregating factors may be involved in the pathogenesis of HUS in some of these patients.

(b) Neuraminidase-associated. This is an important though rare subgroup of D- HUS; it is associated with neuraminidase-producing organisms, notable pneumococci, but also Clostridia species. The pathophysiology has been discussed in section 1.1.4.(a). The illness may be associated with a pneumococcal pneumonia or bacteraemia, or clostridial sepsis and the prognosis is significantly worse than for D+ HUS. Treatment is difficult and involves supportive management with dialysis, antibiotics, washed red blood cells, and possibly exchange transfusion. Fresh plasma infusions should be avoided as they may be deleterious due to the further supply of anti Thomsen-Friedenreich antibody (48-50).
(c) **Oral Contraceptives.** The occurrence of HUS in women using oestrogen-containing oral contraceptives led to the belief that there was a causal relationship between oral contraceptives and HUS (65-67). Renal histopathological changes in postpill HUS were considered compatible with the diagnosis of malignant nephrosclerosis (68) or with that of thrombotic microangiopathy (69). This association however remains hypothetical as firm evidence in support of oral contraceptives as a cause of HUS is lacking.

(d) **Pregnancy.** The triad of microangiopathic haemolytic anaemia (MAHA), acute renal failure and often thrombocytopenia may develop during pregnancy or in the postpartum period. HUS occurs most commonly in the immediate postpartum period and severe acute renal failure is the prominent finding. TTP develops in the second or third trimester, renal involvement is generally mild, whereas extrarenal features predominate. Both HUS and TTP are characterised by a similar basic histopathological lesion, thrombotic microangiopathy (TMA). Haemolytic anaemia with red cell fragmentation, thrombocytopenia and acute renal failure are not uncommon findings in severe preeclampsia/eclampsia.

The current estimate of the incidence of acute renal failure necessitating dialysis during pregnancy is less
than 0.01% (70,71). Among the causes of pre and postpartum renal failure the incidence of HUS is unknown. Sixty-two cases were identified by Weiner et al in 1989 (72), and in Grunfeld's 1957-1979 series, idiopathic postpartum acute renal failure, including HUS, represented approximately 9% of the cases.

(e) Malignancy and Chemotherapy. There are 2 conditions in the patient with malignancy in which TMA and MAHA are characteristic features. The first was initially described by Brain et al in 1962 (14), in patients with widely metastatic carcinoma. MAHA associated with carcinoma may be considered a variant of disseminated intravascular coagulation (DIC) in which TMA and MAHA are the prominent findings, often overshadowing the coagulopathy (74-76). The other syndrome is a more recently recognised complication of chemotherapy that resembles HUS and occurs most frequently in patients who have been treated with mitomycin (mitomycin C; MMC) (75,77,78).

(f) Transplantation. HUS has been reported in renal (79-81), liver (82,83) and particularly bone marrow transplant recipients (84-90). The actual risk of recurrence of HUS in the renal allograft of a patient whose primary disease was HUS cannot be calculated, and
'de novo' HUS may occur in the renal transplant of a patient whose original disease was not HUS. Cyclosporin A has been implicated as a possible cause of HUS in renal allograft recipients (91), either as recurrent disease or as de novo HUS in liver (82,83) and bone marrow recipients (84-90).

The risk of recurrence of HUS post renal transplant is linked to the heterogeneity of the syndrome and it would appear that recurrent disease is less common in children than adults, and children with D+ HUS rather than D- HUS are less likely to suffer recurrence postengraftment.

1.3 Pathogenesis

1.3.1 Endothelial Cell Injury

Morphological evidence suggests that endothelial damage represents a key step in the development of microangiopathic lesions (10,92). The microvascular lesion is central to the pathogenesis of this syndrome (93). Morphological studies performed in the best available model of HUS indicate that endothelial damage precedes the deposition of PMNLs and platelets within the glomerular capillary lumen (94). It is of interest that all the proposed causative agents of HUS are toxic to vascular
endothelium: VTs (95,96), bacterial endotoxins (97-99), neuraminidase (47), circulating Abs directed against endothelial cells (100), and drugs eg Cyclosporin A and mitomycin C (82,84,91,101), may all induce endothelial cell injury. The properties of normal endothelium then become altered after the initial insult. It is possible that abnormalities in haemostatic mediators of endothelial cell origin, including prostacyclin (PGI\(_2\)), von Willebrand factor (vWF) and tissue plasminogen activator (t-PA) are part of the reactive process that follows endothelial cell damage (64). Such complex abnormalities may then contribute to the development of microangiopathic lesions.

1.3.2 Verocytotoxin

(a) Structure and Mechanism of Action. The importance of verocytotoxin in the pathogenesis of D+ HUS is now generally accepted (102,103). Konowalchuk et al (104) are credited with identifying this novel exotoxin from \textit{E coli} which proved lethal to vero cells, an epithelial cell line derived from kidney of the green monkey. Two distinct verotoxins (VTs) have been isolated; the first Verocytotoxin 1 (VT1) is identical to Shiga toxin produced by \textit{Shigella dysenteriae} type 1 (105), and the second, VT2, exhibits 58% genetic homology and 55-60% amino acid homology with VT1 (106). The toxins consist of
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A central A-subunit and 5 B-subunits which bear receptors for the terminal disaccharide galactose-(α1-4β)-galactose which requires a sphingoside base for successful VT binding (107). In man this conformation occurs in the cell membrane glycoprotein Gb3 (the blood group antigen Pk), and in P1, another component of the P blood group system. After binding to the cell surface endocytosis takes place and the A subunit is released into the cytosol. It is then proteolytically cleaved to an A1 fragment which binds to 60s ribosomes and blocks RNA transcription, preventing protein synthesis and killing the cell. The A1 fragment acts enzymatically and thus has the potential to be highly injurious at very low dosage (108).

(b) Verotoxin-Endothelial Cell Interaction

Glomerular endothelium is the main site of injury in HUS. VT is lethal to human umbilical vein endothelium in vitro, but this is best seen when cells are proliferating (95,96). VT causes the release of endothelial cell factor VIII observed during the acute phase of D+ HUS (109). The injection of VT into laboratory animals does not provoke the disease, probably because they lack the appropriate toxin receptors in the kidney, unlike human kidney cortex which is known to express Gb3 (110). The erythrocyte expression of blood group P1 is heterogeneous in man. Taylor et al (111) found a larger than expected proportion
of patients with D+ HUS to be either PI negative or only weakly positive, those with the most severe disease having the least expression. This suggests that the capacity of red blood cells to bind VT may serve as a buffer by reducing endothelial exposure to the toxin and consequent renal damage, and indirectly supports the role of VT.

1.3.3 Coagulation, Platelets and Fibrinolysis

(a) Coagulation. Levels of coagulation factors are typically normal in HUS (112-115), although diminished levels of 11, V, V11, V111, IX and XI have been described (21,115). Fibrinogen levels and fibrinogen turnover studies are generally normal (112,115,116), suggesting that fibrinogen is no longer being consumed, and that fibrin deposition has ceased by the time the patient presents. The detection of elevated levels of fibrin breakdown products indicates activation of the fibrinolytic system (20,112,117,118).

(b) Platelets. Thrombocytopenia and reduced platelet survival are typical in HUS (116) and may result from platelet adherence to intravascular thrombi or injured endothelium and to damage acquired during passage through the thrombosed microvasculature, with subsequent removal from the circulation by the reticulo endothelial
system. Adherent platelets may undergo the release reaction, inducing further platelet aggregation and vasoconstriction through the release of thromboxanes. In vitro, platelet aggregation is reduced during the acute phase of HUS (119-122), suggesting that the remaining circulating platelets may have already undergone the release reaction. The presence of elevated plasma levels of β-thromboglobulin (123), platelet factor 4 (123), serotonin (122), factors that aggregate platelets (122,124,125), and platelet derived growth factors (126), in certain patients, supports the hypothesis of intravascular platelet activation in HUS. Intravascular platelet activation continues for several weeks after the platelet count has returned to normal in both D+ and D- HUS (122). In relapsing D- HUS platelet activation is present during each relapse but not during remission (122). Whether these platelet abnormalities are involved in the pathogenesis of the disease, or whether they result from the development of the disease, remains to be determined.

(c) **Fibrinolysis.** Inhibition of glomerular fibrinolytic activity may play a role in the pathogenesis and outcome of HUS. Patients with D+ HUS have a circulating inhibitor of glomerular fibrinolysis (127), which has recently been shown to be plasminogen-activator
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inhibitor type 1 (PAI-1) (128,129). Bergstein et al have shown that normalization of plasma PAI-1 levels, for example by peritoneal dialysis, is correlated with improvement in renal function (129). However, the possibility that increased plasma levels of PAI-1 are either causes or effects of HUS is not established.

1.3.4 Prostacyclin

Abnormalities in prostacyclin (PGI₂) production may play a role in the pathogenesis of HUS. Produced primarily by vascular endothelial cells, PGI₂ is a potent vasodilator and inhibitor of platelet aggregation (130). Some patients with HUS demonstrate decreased vascular prostaglandin production and diminished plasma levels of PGI₂ metabolites (122,131-135), sometimes in association with the absence of a plasma factor that stimulates endothelial cell PGI₂ release (132,134,136-138) or the presence of an inhibitor of vascular PGI₂ regeneration (131). However, other patients have normal or elevated levels of PGI₂ metabolites (134,139,140) and show no evidence for an inhibitor of vascular PGI₂ production (141).
1.3.5 Polymorphonuclear Leucocytes (PMNLs)

In recent years, there has been increasing evidence that PMNLs play a central role in the pathogenesis of HUS induced by both VTEC and *Shigella dysenteriae* type 1. In shigellosis neutrophilia, endotoxaemia and complement activation correlate with the development of HUS (51). A neutrophil leucocytosis is characteristic of D+ HUS (17,51) and there is an association between the height of the PMNL count acute mortality and residual nephropathy (142-145). There is evidence that activated PMNLs cause endothelial cell damage (146-148) and that their release products can damage the glomerular basement membrane (149). PMNLs harvested from children during the acute phase of HUS exhibit increased adhesion to human umbilical vein endothelial cells (HUVEC) in vitro (150), and α1-antitrypsin complexed elastase (α1-AT) levels are raised at presentation reflecting PMNL activation and degranulation (151).

1.4 Treatment

A wide variety of therapeutic approaches have been attempted but most of the therapeutic trials have been uncontrolled or have used historical controls. Treatment regimes have tended to follow current theories on
pathogenesis and have included heparin (152-155), urokinase (154,156), antiplatelet agents (153,157), vitamin E (158), plasma exchange (159,160), and infusions of fresh frozen plasma (FFP) (161-164), PGI₂ (165), or immunoglobulins (166,167).

1.4.1 General Measures

(a) Supportive Management. The cornerstone of treatment remains prompt and vigorous treatment of renal failure, hypertension, and electrolyte imbalance, and careful correction of anaemia. Inappropriate administration of fluid or blood when oliguria is wrongly suspected to be prerenal may produce hyponatraemia, pulmonary oedema and hypertension. Early dialysis should therefore be instituted as soon as the patient with HUS becomes oligo-anuric. Control of circulating volume thereby allows blood transfusion to proceed with less risk of fluid overload. The haemoglobin should be maintained above 8-9 g/dl by infusing small quantities of red blood cells frequently.

(b) Hypertension. Hypertension is most often due to volume overload (168) and if so responds to fluid depletion, but it may be renin mediated (169). Hypertension persisting after control of volume overload should be treated with antihypertensives as haemolysis and
microvascular damage are more likely in the presence of raised blood pressure.

(c) **Gastrointestinal Disease.** In D+ HUS gastrointestinal involvement may be severe with profuse diarrhoea, haemorrhagic colitis, ileitis, abdominal distension and rectal prolapse, in such cases intravenous alimentation should be given until the gastrointestinal symptoms resolve and enteral feeding be contemplated.

(c) **Central Nervous System (CNS) Disease.** Control of hypertension, early dialysis, and gradual correction of uraemia lessen the risks of CNS disease but severe neurological dysfunction may still occur as a consequence of microvascular disease. Anticonvulsants should be given if required and elective ventilation instituted should the patient be comatose or the level of consciousness become impaired. Computed tomography and cerebral perfusion scanning with intracranial pressure monitoring may be important adjuncts to effective treatment as either cerebral oedema or vascular involvement with normal intracranial pressure may be present. Hyperventilation, mannitol to be given cautiously if the patient is anuric, and fluid restriction should be instituted in the presence of raised intracranial pressure (ICP) whereas such measures may impair cerebral perfusion should a cerebral
vasculopathy be present. If ICP is found to be low, there is no indication for hyperventilation or fluid depletion, and treatment of the vascular pathology with PGI$_2$ could be considered.

1.4.2 Specific Treatments

(a) $D^+$ HUS. The benefit of any treatment in this form of HUS must be measured against the natural history of the disease. The claims of a beneficial effect of anticoagulants, antiplatelet agents, fresh plasma, plasma exchange, and fibrinolytic therapy have been difficult to interpret because of the spontaneous improvement seen in the majority of patients. Rizzoni et al (164) conducted a prospective randomized controlled trial on the use of FFP in $D^+$ HUS and concluded that infusions of plasma did not influence the short or medium term clinical outcome. In a second similar study, Loirat et al (163) also showed no apparent long-term benefits but seven patients in the control group, and none in the FFP group, had extensive cortical necrosis diagnosed by renal biopsy. The results of controlled trials with antithrombotic agents (153,154) have been disappointing. In view of the complications of haemorrhage and the lack of any clear benefits, there seems to be no indication for anticoagulation or fibrinolytic agents in this disease.
(b) **D- HUS.** In contrast to D+ HUS, patients with idiopathic or familial D- HUS have a poor prognosis unless specific treatment is used. Anecdotal impressions of beneficial effects of fresh blood, fresh plasma or plasma exchange have been reported. Patients with deficient PGI₂ stimulating factors and some of those with platelet aggregating factors respond to fresh plasma infusions (170). Patients with inhibitors of PGI₂ production or platelet aggregating factors have not responded to plasma but have improved after plasma exchange (171,172). Plasma exchange now has an established position in the management of adults with TTP; a controlled trial by the Canadian Apheresis Group comparing plasma exchange with plasma infusion found plasma exchange to be more effective than plasma infusion in reducing both mortality and morbidity (173).

(b) **Consensus on Treatment.** In typical, uncomplicated D+ HUS it is generally agreed that careful supportive management, without specific treatment, should be carried out until the results of prospective trials to assess the usefulness of other specific treatments are available. In D- HUS, which has a poor prognosis, a more aggressive approach to treatment is justified; this involves infusions of FFP and PGI₂ followed by a programme of plasma exchange.
1.5 Outcome

1.5.1 Prognostic Indicators

The prognosis for HUS is known to depend on several factors, most significantly the subtype and predominant histopathological lesion (11,46,174). Children with D+ HUS who have a predominant glomerular injury have a much better outcome than those with D- HUS who have arteriolar injury or cortical necrosis. Other factors suggesting a poor prognosis include older age at onset (60,175,176), a family history of HUS, recurrence, anuria for more than 14 days, intestinal gangrene and pneumococcal infection (177).

1.5.2 D+ HUS

(a) Mortality. Before the introduction of dialysis, a high proportion of children with any form of HUS died of fluid overload, metabolic derangement, and uraemia (1,27,21,178,179). Once short-term dialysis became a widely available form of treatment, the prognosis for HUS generally improved, and most recent series from Europe and North America report an acute fatality rate between 4 and 12% (60,143,144,180,181). High mortality rates, up to 60%, have been reported in post-dysenteric D+ HUS (182-185). The poor outcome in this form of HUS appears to be because
of more profound renal injury. In a recent series from Northern India (185), the mortality rate was higher in those with prolonged anuria and total cortical necrosis.

(b) Morbidity. The long-term prognosis following D+ HUS remains incompletely documented. Follow-up studies published over the years, from different countries, have produced variable results for renal outcome ranging from 15-40% of survivors with renal sequelae (21,143,144,186). This subject will be discussed in more detail in Chapter 5.

1.5.3 D- HUS

The prognosis for D- HUS is poor with a high mortality (25-30%) and morbidity (31,45,60,174). This subject will be discussed in more detail in Chapter 3.
CHAPTER 2 RETROSPECTIVE REVIEW OF HUS 1966-1992

2.1 Introduction

This chapter provides an overall summary of the principal epidemiological and clinical features of children presenting to the Hospital for Sick Children, Great Ormond Street (HSC,GOS), between 1966 and 1992 with a diagnosis of HUS. It is from these patients that the clinical and laboratory studies elaborated in later chapters in this thesis emanate.

2.2 Patients

A total of 208 children presented to the HSC,GOS between 1966 and 1992 with a diagnosis of HUS defined as the simultaneous occurrence of a microangiopathic haemolytic anaemia, thrombocytopenia and acute renal failure; this excluded cases of septicaemia with associated microangiopathy and malignant hypertension. One hundred and eighty six (89%) of these children had a 'typical' enteropathic illness with a diarrhoeal prodrome (referred to as \( D^+ \) \textit{HUS}) and 22 (10.6%) had an 'atypical' presentation without diarrhoea (\( D^- \) \textit{HUS}).
2.3 D+ HUS

2.3.1 Epidemiology

(a) **Year and Season.** Annual totals of cases of D+ HUS referred to the HSC, GOS are shown in Fig. 2.1. This demonstrates an increase in the number of new cases presenting after 1980 and a further significant and sustained increase in the mid 1980s. An epidemiological study in the West Midlands region of the United Kingdom (144) demonstrated a similar abrupt increase in the incidence of D+ HUS from 1981 onwards which they postulated was associated with the emergence of verocytotoxin producing *Escherichia coli* in mainland Britain. In keeping with other studies (144, 145, 174), the incidence of D+ HUS showed a marked seasonal variation, with most cases presenting between May and September inclusive (Fig. 2.2). Of the 186 cases 121 (65%) were admitted over these 5 months.

(b) **Race, Gender and Age.** In this cohort, 177 (95%) children with D+ HUS were white Caucasians, and nine were Asians from the Indian subcontinent. Although the current ethnic distribution of the British population under 16 years of age is unknown, there may be an excess of white
Caucasians in the D+ HUS patients and a deficiency of Caribbeans; they formed 91% and 1.3% respectively of the Great Britain population under 16 in 1985 (187). There was a slight female preponderance with an overall female to male ratio of 101:85 (1.2:1). The incidence by age is diagramatically shown in Fig. 2.3. with a median age at presentation of 2.5 years and range of 0.2 to 14.5.

(c) Familial Incidence. There was only one sib pair admitted, Asian girls with HUS secondary to 
*Shigella dysenteriae* type 1 and the details of these cases will be given later in the chapter. A concurrent family history of a diarrhoeal illness was however very much more common and reported in 58 (31%) of these cases, 36 of the contacts were siblings and 22 were parents.

2.3.2 Clinical Features

(a) Renal. Not all children developing D+ HUS require dialysis as oligoanuria is not invariable. Of the 186 children admitted to the HSC,GOS reported to have oliguria or anuria 150 (81%) required dialysis, 123 of these (82%) were managed by peritoneal dialysis, 16 required a combination of peritoneal and haemodialysis and 11 needed to be haemodialysed from the start. Thirty six
children (19%) never required dialysis and this represents an underestimation of the number of such cases because of the referral bias to the HSC,GOS with a greater number of more severely affected children being referred.

(b) Neurological. Thirty seven (20%) of the patients presented with significant neurological involvement defined as generalized disturbances such as encephalopathy, coma and convulsions, and/or focal signs including hemiplegia, hemiparesis, visual defects and extrapyramidal signs. In Chapter 4 the spectrum of neurological manifestations in children with D+ HUS presenting to the HSC,GOS between 1980 and 1992 are examined in the context of a case control study to identify risk factors at presentation for the development of neurological disease.

2.3.3 Microbiological Findings

(a) Verocytotoxin-producing Escherichia Coli. From 1985 faecal samples from children with D+ HUS were obtained as soon as possible after presentation and forwarded to the Division of Enteric Pathogens, Central Public Health Laboratory, London, for DNA probe tests for the presence of VTEC and examination for free neutralisable Verocytotoxin. These tests were in addition
to routine stool bacteriological analysis performed at the HSC, GOS. Stool samples or rectal swabs were obtained from 69 of 104 patients presenting from 1985 onwards. Evidence for VTEC infection was found in 16 (23%) of these cases. The study looking at the association of D+ HUS and VTEC in the British Isles reported by Milford et al (145) found evidence for VTEC infection in 33% of D+ cases whose stools were analysed and that up to 62% of stools obtained early in the course of the disease were positive for Verocytotoxin or VTEC. As the excretion of VTEC is brief and the diarrhoea has often reduced or ceased by the time the patients are admitted to the HSC, GOS, the findings from this series underestimate the true association with VTEC.

(b) *Shigella Dysenteriae Type 1*. There were 6 cases of HUS associated with *Shigella dysenteriae type 1* infection. Five of the patients were from Bangladesh and their illness started whilst in Bangladesh or within 1 week of returning to the UK following holidays there. The fifth patient had an Egyptian father and this child's diarrhoea started following a visit to Cairo. In all cases there was profuse watery diarrhoea occurring in association with severe haemolysis and thrombocytopenia. There were 4 females and 2 males with a mean age of 5.7 years (range 2-
The mean presenting haemoglobin was 5.8 ±1.7 g/dl, polymorphonuclear leucocyte count 52.4 ±24.3 x 10^9/l and platelet count 47.6 ±37.6 x 10^9/l. The clinical and laboratory features of the 6 cases are detailed in Table 2.1. Three of the children did not require dialysis, despite deteriorating renal function they never became anuric and with conservative management and appropriate rehydration renal function improved. The other 3 children became anuric and were dialysed. These all had severe gastrointestinal disease with ongoing profuse bloody diarrhoea associated with abdominal distension and tenderness, colitis and functional obstruction. In one case a peritoneal dialysis catheter was inserted under direct vision and the child was successfully dialysed for 15 days prior to the recovery of renal function. The other 2 were haemodialysed and because of associated neurological disease in one and severe gastrointestinal disease with malabsorption and carbohydrate intolerance in the other they were also treated with a course of plasma exchange and infused prostacyclin. Four of the children made a complete recovery, one child still has moderate carbohydrate intolerance but is not diabetic and one is on anticonvulsants. All have normal renal function.
(c) **Salmonella Typhimurium.** There was one case of D+ HUS associated with salmonella septicaemia. The patient was an 11 year old boy who developed severe bloody diarrhoea with very frequent bowel motions up to 10 to 20 a day. There was no contact history and the rest of the family were well. He presented in renal failure with a urea of 69 mmol/l and creatinine of 1027 µmol/l; his haemoglobin was 6.4 g/dl, PMNL 23.7 x10⁹/1 and platelet count 61 x 10⁹/1. Salmonella typhimurium species were detected in his faeces and blood at the time of admission. The salmonella was treated with ceftazidine and he was dialysed using both peritoneal and haemodialysis for a total of 11 days. He made a full recovery from this illness.

(d) **Yersinia Pseudotuberculosis.** There was one case of D+ HUS associated with evidence of infection with Yersinia pseudotuberculosis. The patient was a 1.3 year old male referred with a diagnosis of pseudomembranous colitis made following a sigmoidoscopy for suspected intussusception. On admission his haemoglobin was 8.8 g/dl, PMNL 16.9 x 10⁹/1 and platelet count 51 x 10⁹/1,; he was in renal failure with a urea of 19.1 mmol/l and creatinine of 362 µmol/l. His blood showed evidence of infection with Yersinia pseudotuberculosis type 111 with a
titre of 1:1,280. In view of abdominal distension and dilated bowel loops a peritoneal dialysis catheter was inserted under direct vision and he was dialysed for a total of 24 days and received in addition intravenous vancomycin, penicillin and metronidazole. He did not recover full renal function and 5 years after presentation has a GFR of 60 ml/min/1.73m² SA and significant proteinuria with a urinary albumin/creatinine ratio of 151.8 mg/mmol.

(e) *Pseudomonas Aeruginosa.* There was one case of D+ HUS associated with pseudomonas septicaemia. This was a one year old boy who was referred from a hospital in Khartoum, Sudan extremely unwell with a high swinging fever and persistent diarrhoea. He was in renal failure with a urea of 17.9 mmol/l and creatinine of 370 μmol/l; his haemoglobin was 10.6 g/dl, PMNL 31 x10⁹/l and platelet count 58 x 10⁹/l. Pseudomonas aeruginosa was detected in his faeces and cultured from his blood. He was peritoneally dialysed and treated with intravenous carbenicillin and intraperitoneal gentamicin. His acute illness was complicated by the development of a persistent pleuro-peritoneal canal and recurrent pleural effusions. He never regained renal function, went into end stage renal failure and was managed by intermittent haemodialysis. He was successfully transplanted but died 7 years after presentation following a septicaemic illness.
2.3.4 Specific Treatments

There have been numerous attempts to reverse the microangiopathic process in D+ HUS by specific treatments which have tended to reflect the current theory on pathogenesis. In this series only 2 specific treatments have been used in any significant number of patients with D+ HUS, albeit in an uncontrolled way; pre 1978 - heparin by infusion, and post 1980 - plasma exchange (PEx).

(a) Heparin. Between 1966 and 1978 nineteen children with D+ HUS were treated with infused heparin from the time of admission to the Renal Unit. No specific criteria were required for the initiation of this treatment. There were 12 females and 7 males with a median age of 1.1 year (range 0.3 to 11 years). Five did not require dialysis. The heparin was infused at between 20 to 40 units/kg/hour and the dose adjusted according to the thrombin time. The median duration of infusion was 8 days (range 1 to 21). The only complications to this therapy recorded were local bruising (n=5) and mild haematemesis (n=2). The outcome for this group was however poor, but it must be remembered that they presented at at time when the overall supportive management was suboptimal and the mortality rate higher than seen after 1980. Three died within 6 months of presentation of faecal peritonitis, cerebral and cardiac complications respectively. Of the remaining 16, four
developed residual nephropathy with GFRs of less than 80 ml/min/1.73 m²SA and proteinuria.

(b) Plasma Exchange. Since 1980 PEx with purified protein fraction and an infusion of fresh frozen plasma at the end of each exchange has been used routinely in the management of children with D- HUS (see Chapter 3 for details of PEx). It has now also been used in a number of children with D+ HUS associated with neurological and/or severe gastrointestinal disease presenting with high PMNL counts, in an attempt to improve the prognosis for these more severely affected patients. Between 1980 and 1992 20 children with D+ HUS have received PEx. There were 11 females and 9 males with a median age of 4.8 years (range 0.3 to 11.1). Fifteen had neurological involvement, 2 had HUS associated with Shigella dysenteriae type 1 infection one of whom also had neurological complications. In 3 cases the patients plasma either failed to support, or showed inhibition to prostacyclin production and it was thought, at that time, they might therefore benefit from therapeutic PEx. The median PMNL count at presentation was 30.2 x10⁹/l (range 6.6 to 60.9). All these patients were dialysed with a median duration of dialysis of 16 days (range 6 to 35) and 13 with neurological disease also required assisted ventilation. The median number of PExs was 7 (range of 2 to 13) and in addition to PEx 12 also
received infused prostacyclin at a dose of 2 to 20 ng/kg/min. The outcome for this group, which represents those with the most severe disease and worst prognosis was not surprisingly poor (Fig 2.4). There were 4 deaths as a consequence of cerebral and gastrointestinal complications. Two of the children have neurological sequelae, one of whom also has an impaired GFR and proteinuria. Four have residual nephropathy alone with a GFR < 80 ml/min/1.73m²SA and proteinuria. Three of the children were diabetic during the acute phase of their illness and one remains insulin dependent 2 years post presentation while the other 2 have resolving carbohydrate intolerance. Six of the group have apparently made a full recovery. In the absence of any controlled data or prospective trials it remains impossible to evaluate the usefulness of PEx in the treatment of this difficult subgroup of D+ HUS and the management of these patients remains controversial.

2.3.5 Mortality

The total number of deaths in children with D+ HUS presenting between 1966 and 1992 was 15, giving an overall mortality rate of 8%. The mortality during the acute phase of the disease was 3/35 (9%) before 1980 and 9/151 (6%) after 1980. The characteristics of the 15 patients with D+ HUS who died are presented in Table 2.2.
2.3.6 Morbidity

The morbidity associated with D+ HUS is discussed in detail in Chapter 5.

2.4 D- HUS

Between 1966 and 1992 twenty two children were admitted to the HSC, GOS with a diagnosis of 'atypical', non-diarrhoea associated or D- HUS, constituting 10.6% of the total HUS population. In 20 of these children the illness was not associated with any specific infection, and in Chapter 3 the clinical and laboratory features of this group, defined as idiopathic D- HUS, are elaborated. Two of the patients did however have documented infections in association with their disease and details of these 2 cases are discussed below.

2.4.1 Infection-associated D- HUS

(a) *Streptococcus Pneumoniae*. There was one case of D- HUS associated with neuraminidase producing *Streptococcus pneumoniae*. The patient was a 2.7 year old boy admitted with a severe disseminated coagulation-like illness and renal failure. The prodromal illness was
associated with fever and attributed to a non-specific viral infection. A chest X-ray at the referring hospital showed right upper and middle lobe consolidation and he was treated with intravenous antibiotics. On admission to the HSC, GOS he was extremely unwell, poorly perfused and obtunded. He was cyanosed in air, grunting and had intercostal recession. His haemoglobin was 7.9 g/dl and platelet count 22 x10^9/l. He was in renal failure with a urea of 24.9 mmol/l and creatinine of 201 µmol/l. Blood cultures and pleural effusion were positive for pneumococcus. He was ventilated, peritoneally dialysed and treated with appropriate antibiotics and anti fungal agents. His red blood cells were found to be revealing Thomsen-Friedenreich antigen and he was transfused with washed red cells and human derived plasma products were avoided. His illness was complicated by the development of adult respiratory distress syndrome. Following an initial improvement there was a sudden deterioration in his condition associated with CT scan evidence of extensive intracranial haemorrhage and brain stem compression and he died 11 days following his admission to the Intensive Care Unit.
(b) Group A β-Haemolytic Streptococcus. There was one case of D-HUS associated with group A β-haemolytic streptococcal septicaemia. This was a 2.5 year old girl who was referred following a prodromal illness associated with an upper respiratory tract infection, vomiting and a petechial rash for which she had received antibiotics. At the referring hospital she had a convulsion and was noted to be hypertensive. On admission to the HSC, GOS her haemoglobin was 7.8, PMNL count 7.4 x10⁹/l, platelets 50 x 10⁹/l and clotting screen was normal. She was in renal failure with a urea of 36.5 mmol/l and creatinine of 304 μmol/l. Blood and cerebro spinal fluid co-agglutination tests were positive for Group A β streptococci. She had an abnormal electroencephalogram with multifocal distribution maximal over a wide area of the right hemisphere. She required both peritoneal and haemodialysis and was dialysed for 3 weeks. She made a full neurological recovery but has a GFR of 64 ml/min/1.73 m²SA and is on antihypertensive medication.
Table 2.1. Clinical and Laboratory Features of Children Presenting with HUS Associated with *Shigella dysenteriae* type 1 Infection.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Year</th>
<th>Age (yr)</th>
<th>PMNL x10^9/l</th>
<th>Microbiology</th>
<th>Complications</th>
<th>Treatment</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>1982</td>
<td>3.0</td>
<td>56.3</td>
<td>+ve</td>
<td>Shigella dysentery</td>
<td>nil</td>
<td>PD</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IV feeding</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IV antibiotics</td>
</tr>
<tr>
<td>F</td>
<td>1987</td>
<td>7.0</td>
<td>28.2</td>
<td>+ve</td>
<td>nil</td>
<td>IV fluids</td>
<td>Good</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IV antibiotics</td>
</tr>
<tr>
<td>F</td>
<td>1988</td>
<td>2.0</td>
<td>27.0</td>
<td>+ve</td>
<td>Shigella HUS</td>
<td>nil</td>
<td>IV fluids</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IV antibiotics</td>
</tr>
<tr>
<td>F</td>
<td>1988</td>
<td>4.5</td>
<td>94.9</td>
<td>+ve</td>
<td>Shigella HUS</td>
<td>nil</td>
<td>IV fluids</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>IV antibiotics</td>
</tr>
<tr>
<td>M</td>
<td>1989</td>
<td>10.0</td>
<td>38.6</td>
<td>+ve</td>
<td>Rectal prolapse</td>
<td>HD/PEx</td>
<td>Carbohydrate intolerance</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Malabsorption</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Diabetes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>1992</td>
<td>8.0</td>
<td>69.4</td>
<td>+ve</td>
<td>CNS vasculitis</td>
<td>HD/PEx</td>
<td>Convulsions</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>blood +ve</td>
<td>Pericarditis</td>
<td>IV antibiotics</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Ileostomy</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

CNS: Central nervous system; HD: Haemodialysis; HUS: Haemolytic uraemic syndrome; IV: Intravenous; PD: Peritoneal dialysis; PEx: Plasma exchange; PMNL: Polymorphonuclear leucocytes.
Table 2.2 Characteristics of the 15 Patients with D+HUS who Died.

<table>
<thead>
<tr>
<th>Sex</th>
<th>Year</th>
<th>Age (yr)</th>
<th>PMNL x10^9/l</th>
<th>Time after presentation (days)</th>
<th>Cause of death</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>1966</td>
<td>0.6</td>
<td>20.7</td>
<td>14</td>
<td>CNS/cardiac</td>
</tr>
<tr>
<td>F</td>
<td>1966</td>
<td>1.1</td>
<td>24.7</td>
<td>45</td>
<td>Peritonitis</td>
</tr>
<tr>
<td>F</td>
<td>1969</td>
<td>0.2</td>
<td>21.3</td>
<td>6</td>
<td>CNS infarct/RVT</td>
</tr>
<tr>
<td>F</td>
<td>1971</td>
<td>0.5</td>
<td>11.5</td>
<td>183</td>
<td>CNS</td>
</tr>
<tr>
<td>F</td>
<td>1978</td>
<td>11.0</td>
<td>16.0</td>
<td>165</td>
<td>Cardiac</td>
</tr>
<tr>
<td>M</td>
<td>1978</td>
<td>1.0</td>
<td>30.1</td>
<td>2500</td>
<td>Septicaemia</td>
</tr>
<tr>
<td>M</td>
<td>1980</td>
<td>2.0</td>
<td>25.8</td>
<td>6</td>
<td>GI</td>
</tr>
<tr>
<td>F</td>
<td>1985</td>
<td>3.0</td>
<td>18.3</td>
<td>11</td>
<td>CNS/GI</td>
</tr>
<tr>
<td>M</td>
<td>1986</td>
<td>2.1</td>
<td>36.0</td>
<td>3</td>
<td>CNS/GI</td>
</tr>
<tr>
<td>F</td>
<td>1986</td>
<td>3.4</td>
<td>52.3</td>
<td>14</td>
<td>CNS</td>
</tr>
<tr>
<td>M</td>
<td>1987</td>
<td>2.1</td>
<td>24.5</td>
<td>3</td>
<td>CNS</td>
</tr>
<tr>
<td>M</td>
<td>1989</td>
<td>10.9</td>
<td>28.9</td>
<td>4</td>
<td>CNS/GI/cardiac</td>
</tr>
<tr>
<td>F</td>
<td>1990</td>
<td>1.6</td>
<td>17.8</td>
<td>2</td>
<td>CNS/GI</td>
</tr>
<tr>
<td>M</td>
<td>1990</td>
<td>11.1</td>
<td>43.0</td>
<td>5</td>
<td>CNS/ARDS</td>
</tr>
<tr>
<td>F</td>
<td>1990</td>
<td>2.0</td>
<td>17.1</td>
<td>3</td>
<td>CNS/GI</td>
</tr>
</tbody>
</table>

ARDS: Adult respiratory distress syndrome; CNS: Central nervous system; GI: Gastrointestinal (ischaemic ulcerative proctocolitis); PMNL: Polymorphonuclear leucocytes; RVT: Renal venous thrombosis.
Fig. 2.1: Annual totals of D+ HUS cases referred to HSC,GOS (1966-92)
Fig. 2.2: D+ HUS by month of presentation (1966-92).
Fig. 2.3: Age distribution of D+ HUS patients (1966-92).
Fig. 2.4: Diagrammatic representation of outcome of D+ HUS cases treated with plasma exchange (1980-92).
CHAPTER 3: IDIOPATHIC D-HUS

3.1 Introduction

This chapter describes the clinical and laboratory features of twenty children who presented to the Hospital for Sick Children, Great Ormond Street (HSC,GOS), over the past 20 years with idiopathic non-diarrhoea associated HUS. This subgroup is itself heterogeneous; some cases are familial and some follow a relapsing or progressive course.

3.2 Patients

3.2.1 Epidemiology

Idiopathic D- HUS is a rare disorder. Between 1966 and 1992 two hundred and eight children with a diagnosis of HUS were referred to the HSC,GOS. Of these, 186 (89%) had prodromal diarrhoea and 22 (10.5%) did not. Two of the patients with D- HUS had documented infections in association with their disease which excluded them from the idiopathic group and details of these 2 cases have been given in Chapter 2. The clinical and laboratory features of the remaining 20 children with idiopathic D- HUS, constituting 9.6% of the total HUS population, are listed in Tables 3.1 a, b, c and d.
(a) Gender, Age and Race. Ten of the 20 children were male and 10 female with a median age of 4.5 years (range 0.2-13.5). Seven of the group (35%) were under the age of one year and 6 (30%) were over the age of 5 years at presentation (Fig. 3.1). Five (25%) were from Asian (n=4) or African (n=1) families, all but one of whom were resident in the United Kingdom at the time of onset of the illness.

(b) Familial Incidence. Only one of the patients (Fig. 3.2) had affected siblings; this case (no. 10) and one other (no. 6) had consanguinous parents, and in none was there a history of the disorder in preceding generations.

(c) Season. In contrast to D+ HUS, which is more common in the summer months (60,174,188), there was no seasonal variation in the incidence of D- HUS with 9 (45%) of the cases presenting between May and September inclusive.
3.2.2 Clinical and Laboratory Features

(a) **Prodrome.** There were various prodromal symptoms in these children (Table 3.1) which included vomiting (n=15), anaemia (n=8), convulsions (n=6), fever (n=6), a maculopapular rash (n=6), apparent upper respiratory tract infection (n=6), malaise (n=6) and jaundice (n=4). One child (no.1) had tuberculous adenitis and was on treatment with rifampicin, and 2 (nos.13 & 18) had *Escherichia coli* urinary tract infection.

(b) **Renal Involvement.** The median plasma creatinine concentration at onset was 215 μmol/L (range 51 - 2310). Five of the children did not require dialysis at any stage; the remaining 15 were dialysed for management of their renal failure but in 5 of this group dialysis was delayed for a mean of 14 days (range 3-31) from the time of admission, as the development of renal failure was insidious. Of the 15 who required dialysis 3 died whilst still being dialysed, on days 49 (no.3), 56 (no.2), and 120 (no.4); 2 progressed to end stage renal failure without any recovery of renal function (nos. 8 & 9) and for the remaining 10 the mean duration of dialysis was 20 days (range 4-46).
Chapter 3: Idiopathic D-HUS

(c) Blood Pressure. The mean ±SD systolic blood pressure (BP) standard deviation score (SDS), derived by reference to age and sex matched 1987 Task Force standards (189), at the time of admission to the Hospital for Sick Children was 2.5 ±0.9. Fifteen were hypertensive with a systolic BP SDS > 2.0. The mean diastolic BP SDS was 1.9 ± 1.1; 11 had a diastolic BP SDS > 2.0. Thirteen of these 15 required antihypertensive medication in addition to volume depletion to control their blood pressure, suggesting that the hypertension was renin mediated. Data on plasma renin activity (PRA) were available in only 12 children. The mean PRA SDS, calculated by comparing PRA with age-matched control values (190), was 2.5 ±1.4, 6 of them had a PRA SDS > 2.0. There was no correlation between the systolic BP SDS and PRA SDS in the 12 children for whom both results were available (p>0.05, Spearman rank correlation coefficient). In 5 of the 11 children with 'relapsing' disease, recurrence was associated with an exacerbation of hypertension. Of the 14 children followed, excluding 2 who had successful renal transplants and 2 who are currently on continuous ambulatory peritoneal dialysis, 8 are significantly hypertensive and require substantial antihypertensive medication.
(d) **Neurological Involvement.** Six (30%) of the children had central nervous system involvement at the time they were first seen, with convulsions and alteration in level of consciousness. In all these cases the symptoms persisted despite correction of hypertension and the metabolic complications of renal failure.

(e) **Haematology.** These patients had a marked degree of anaemia at presentation with a microangiopathic haemolytic anaemia and erythrocyte fragmentation. The mean haemoglobin concentration was 6.3 ±1.5 g/dl, but there was no correlation between the haemoglobin at onset and the severity of renal failure as expressed by the plasma creatinine concentration (r=-0.02), p=0.90). In contrast to D+ HUS, which is associated with a raised peripheral PMNL count at presentation (142-145), the mean circulating PMNL count was 7.1 ±3.6 x 10⁹/L. There was no apparent difference in PMNL count between those who died (8.1 ±3.7 x 10⁹/L; n=5), those who were in end stage renal failure (6.2 ±3.1; n=4), those with residual nephropathy (6.3 ±3.9; n=8), and the 2 who recovered (8.6,13.6). The mean platelet count at presentation was 67.9 x 10⁹/L (range 15-136). There was no correlation between the severity of thrombocytopenia and the plasma creatinine concentration (r=0.30, p=0.30). Data on the coagulation profile was obtained at admission in 14 of the children. The mean
prothrombin time (PT) was 13.5 ±1.2 (sec ±SD), kaolin partial thromboplastin time (KPTT) 34.5 ±4.9, and thrombin time (TT) 19.8 ±8.6. The KPTT was significantly shorter than control values (paired t-test; p=0.02), and the TT significantly longer (p<0.001), but the PT was not significantly different from control values (p=0.60).

(f) Renal Pathology. Histological studies were available in 7 of the 20 children, all of whom had relapsing disease. The predominant histological findings were extraglomerular arteriolar proliferative changes involving endothelial and smooth muscle cells producing changes resembling the 'onion skin' proliferative lesions of malignant hypertension (191-193). In all cases blood vessels showed marked muscular medial hypertrophy with intimal proliferation. In the 4 biopsied later in the disease, many glomeruli were sclerosed and there were widespread areas of tubular necrosis and atrophy. Patient no.15 had a renal biopsy following a deterioration of renal function with a relapse and the biopsy findings are illustrated in Figs 3.3 a and b: on light microscopy there was tubular atrophy, interstitial chronic inflammation and fibrosis, and on electron microscopy the capillary walls showed marked widening of the subendothelial space and one capillary loop contained numerous platelets.
3.3 Treatment and Outcome

All the children were managed by dialysis for renal failure, control of hypertension and transfusion for anaemia, as necessary, but other aspects of their management varied over the years.

3.3.1 Pre-1980

(a) Fresh Frozen Plasma. Before 1980 no other specific treatment for D-HUS was offered, though fresh frozen plasma (FFP) was sometimes used for resuscitation. There were 9 children (6 males) in this group, with a mean age of 3.8 years. Seven required dialysis, 4 needed assisted ventilation and 4 received FFP.

(b) Outcome. The outcome for this group of children was poor (Table 3.2). Four (44%) died (nos.2,3,4,& 5), 3 in the acute phase of the disease with septicaemia and central nervous system (CNS) involvement in addition to renal failure and the fourth at the time of his first relapse. The fourth child (no.5) had CNS disease and the post-mortem showed cerebral oedema and a cerebral infarct; he is the only child in the pre-1980 group who had a relapse. Two children never regained renal function following presentation and progressed to end stage renal
failure (ESRF) (nos. 8 & 9). One received a successful cadaveric graft one year after presentation which is still functioning 16 years later. The other child lost her graft 4 years after transplantation with recurrence of primary disease. The first child did not receive cyclosporin A but the second did. Another child (no.16) had residual nephropathy with hypertension, proteinuria, and a glomerular filtration rate (GFR) of 77 ml/min/1.73m² SA. Only one girl (no.18) appears to have made a full recovery and had no residual nephropathy when investigated 12 years following her initial illness. One of the group has been lost to follow-up.

3.3.2 Post-1980

(a) Plasma Exchange. Since 1980 plasma exchange (PEx) with purified plasma protein fraction and an infusion of FFP at the end of each exchange has been used routinely in the management of children with D- HUS. Two different methods were employed: plasma filtration and centrifugal PEx. The choice of method depended on the patient's size; smaller children (<15 kg) had plasma filtration with a Gambro plasma filter and an AK10 blood monitor (Gambro Ltd), and the bigger children had centrifugal PEx using a COBE Spectra Apheresis system (COBE Laboratories Inc). The volume exchanged was twice
the child's estimated plasma volume, and was completed with 10 ml/kg FFP to replace the intrinsic clotting factors not present in plasma protein fraction and lost during filtration or centrifugation. The procedure was performed initially every day for 5 days if tolerated. In the 4 children who had more frequent relapses it was observed that as the frequency of PEx declined there was a tendency to relapse 6-8 weeks following the last PEx (Fig.3.4). Therefore for children with relapsing disease the initial run of PEx was followed by a programme of intermittent PEx performed at increasing intervals for several months after the last relapse. Between 1980 and 1991 eleven children (7 females) were managed in this way; their mean age was 4.7 years (range 0.2-13.5). Eight required dialysis for a median duration of 21 days (range 4-46) and 2 required assisted ventilation. The median number of PExs performed in each child was 38 (range 5-254).

(b) **Prostacyclin.** In addition, prostacyclin was administered to 7 of the children by constant intravenous infusion at a starting dose of 2 ng/kg/min, because of refractory hypertension or CNS disease. Two became hypotensive and another developed severe diarrhoea during the treatment. In all instances PGI₂ was used in conjunction with PEx, so no clear view on the efficacy of PEx could be formulated.
(c) **Outcome.** The outcome for the post-1980 group was variable (Table 3.2). Ten had further relapses of HUS, defined as an increase in the plasma creatinine concentration of 20% associated with thrombocytopenia. One died 3 years following onset during the third relapse (no.1), having received a total of 22 exchanges, with an intracranial haemorrhage. Two progressed to ESRF, one over a period of 9 months (no.7) and the other more gradually over a period of 5 years (no.6). Patient no.7 has now received 2 cadaveric grafts, both of which have failed within 3 weeks of transplantation with recurrence of the primary disease in the graft; this boy is now back on continuous ambulatory peritoneal dialysis. Patient no.6 received a successful cadaveric renal transplant 6 years following onset. Patient no.7 received cyclosporin A as part of his immunosuppressive protocol on both occasions, and no.6 did not. Seven of the group have significant residual nephropathy with proteinuria and hypertension, and of these 6 have a reduced GFR. Only one child (no.19) appears to have made a full recovery, she has now had 2 relapses and has received 19 PExs, yet is currently free of proteinuria, is normotensive and has a normal GFR.
3.3.3 Comparison of Outcome in Pre and Post-1980 Groups

There may be other variables in the year of presentation which affect outcome, nevertheless it appears that those children who received PEx (post 1980) had a lower mortality (1/11 vs 4/9; p<0.005, Exact test) than those who did not, but they had a higher relapse rate with 10/11 having further episodes of HUS compared to only 1/9 in the earlier group (p<0.005, Exact test). The overall recovery rate was however the same, with only one child from each group regaining normal renal function.

3.3.4 Overall Outcome

In this series of 20 children with idiopathic D-HUS, 5 (25%) died, 4 (20%) progressed to ESRF, 8 (40%) have residual nephropathy, and only 2 (10%) have apparently made a full recovery (Fig.3.5). Eleven (55%) have had one or more relapses, often associated with presumed viral respiratory tract infections and in many instances heralded by a deterioration in the control of hypertension. Two of the children with relapsing disease died during their first and third relapse respectively, and for the remaining 9 the median number of relapses was 3, with a maximum of 8 relapses in one case spread over a period of 6 years (no.11). Ten of these were amongst the 11 children who presented after 1980 and were treated by
PEx. In 6 the acute treatment was followed by a period of treatment with single PExs at increasing intervals for periods up to 18 months, and in 4 of these a relapse occurred within 2 months of the last PEx.

3.4 Discussion

Non-diarrhoea associated haemolytic uraemic syndrome is a heterogeneous yet distinct subgroup of HUS which differs from D+ HUS on epidemiologic, clinical, laboratory, histologic and prognostic grounds. In contrast to D+ HUS in which progress has been made in understanding the aetiology (52,53), the epidemiology (194), and the pathophysiology (195), the aetiology and pathogenesis of D- HUS remains unclear.

The prognosis for D- HUS is poor with a high mortality and morbidity. The incidence of terminal renal failure is high and it's management is problematic: all of the patients who went into ESRF have had renal transplants but only two have functioning renal grafts. The other 2 lost 3 grafts because of recurrence of HUS. In all these 3 instances cyclosporin A was used as part of the immunosuppressive protocol whereas the 2 children with functioning grafts did not receive CyA. A particular problem exists in relation to CyA, which causes a nephropathy that has many features in common with D- HUS,

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and it may be that its use is best avoided in these cases. Recurrence of HUS has been documented however in both CyA (80,196) and non-CyA treated patients (197-200). The onset of allograft rejection (201) and hypertension (202) may also trigger HUS and it can be very difficult to distinguish recurrence of primary disease from severe acute or chronic vascular rejection on the basis of histological examination of renal tissue.

Because of the poor outcome for D- HUS the practice at the HSC, GOS has been to treat these children aggressively in the hope that such an approach may improve ultimate prognosis. Early control of renal failure with prompt and aggressive management of hypertension is essential. All patients then embark on a course of PEx with plasma protein fraction completed with an infusion of FFP. Patients with relapsing disease are subsequently managed by a programme of intermittent PEx at increasing intervals for several months following the last relapse.

Plasma exchange has an established position in the management of TTP: a controlled trial conducted by the Canadian Apheresis Group (173), comparing PEx with plasma infusion in the treatment of TTP in adults found PEx to be more effective than plasma infusion in reducing mortality and morbidity. D- HUS in children is a very rare disorder and it would be difficult to accumulate a sufficient
number of cases to undertake a similar controlled study in them.

The rationale for the use of PEx is not clear. It has been suggested that a deficiency of PGI$_2$ synthesis plays an integral role in the pathogenesis of D- HUS, either because of a circulating inhibitor of PGI$_2$ production or because of a deficiency of a circulating factor necessary for PGI$_2$ synthesis (122,126,138,203). Walters et al (122) found a reduced support of PGI$_2$ production by endothelial tissue by the sera of 7/9 children with D- HUS (8 included in this review), whereas D+ HUS sera usually supported PGI$_2$ production normally. These phenomena have been part of the rationale for using FFP, PEx, and PGI$_2$ infusions in both TTP and D- HUS.

The experience from this series of the management of idiopathic D- HUS suggests that PEx improves the prognosis in this serious disorder, but it may be that its effect is to improve immediate survival only to reveal the underlying relapsing nature of the disease.

83
<table>
<thead>
<tr>
<th>Case (Gender)</th>
<th>Age (yr)</th>
<th>FH</th>
<th>Year</th>
<th>Prodrome</th>
<th>SBP</th>
<th>Hb</th>
<th>PMNL</th>
<th>Plat</th>
<th>PCr</th>
<th>Dialysis</th>
<th>Plasma exchange</th>
<th>Relapses</th>
<th>Cause of death</th>
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<tbody>
<tr>
<td>1(F)</td>
<td>13.5</td>
<td>-</td>
<td>1980</td>
<td>Vomiting</td>
<td>2.2</td>
<td>7.6</td>
<td>8.2</td>
<td>39</td>
<td>163</td>
<td>-</td>
<td>22</td>
<td>3</td>
<td>Cerebral haemorrhage</td>
</tr>
<tr>
<td>2(M)</td>
<td>0.7</td>
<td>-</td>
<td>1970</td>
<td>Vomiting</td>
<td>2.5</td>
<td>6.9</td>
<td>4.6</td>
<td>46</td>
<td>566</td>
<td>56</td>
<td>-</td>
<td>-</td>
<td>Septicaemia Peritonitis</td>
</tr>
<tr>
<td>3(M)</td>
<td>4.5</td>
<td>-</td>
<td>1970</td>
<td>Vomiting</td>
<td>2.7</td>
<td>5.6</td>
<td>5.7</td>
<td>32</td>
<td>659</td>
<td>49</td>
<td>-</td>
<td>-</td>
<td>Septicaemia</td>
</tr>
<tr>
<td>4(F)</td>
<td>4.5</td>
<td>-</td>
<td>1976</td>
<td>Vomiting</td>
<td>1.5</td>
<td>5.6</td>
<td>7.8</td>
<td>48</td>
<td>453</td>
<td>120</td>
<td>-</td>
<td>-</td>
<td>Septicaemia Peritonitis</td>
</tr>
<tr>
<td>5(M)</td>
<td>0.15</td>
<td>-</td>
<td>1979</td>
<td>Collapse</td>
<td>4.2</td>
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<td>15</td>
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<td>1</td>
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CAPD: Continuous ambulatory peritoneal dialysis; ESRF: End-stage renal failure; GFR: Glomerular filtration rate; Hb: Haemoglobin; FH: Family history; PCr: Plasma creatinine concentration; Plat: Platelets; PMNL: Polymorphonuclear leucocytes; SDS: Standard deviation score; SDS: Standard deviation score; SBP: Systolic blood pressure; URTI: Upper respiratory tract infection; UTI: Urinary tract infection.
<table>
<thead>
<tr>
<th>Case (Gender)</th>
<th>Age (yr)</th>
<th>FH Year</th>
<th>Prodrome</th>
<th>SBP SDS</th>
<th>Hb g/dl</th>
<th>PMNL x10^9/l</th>
<th>Plat l</th>
<th>PCr μmol/l</th>
<th>Dialysis (days)</th>
<th>Plasma exchange</th>
<th>Relapses</th>
<th>Outcome</th>
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<tbody>
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<td>+ 1981</td>
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<td>10.3</td>
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<td>846</td>
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<td></td>
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<td></td>
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<tr>
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<td></td>
<td>Vomiting</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
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</table>

FH+: Parents first cousins
*Never recovered renal function
For abbreviations see Table 3.1a
<table>
<thead>
<tr>
<th>Case (Gender)</th>
<th>Age (yr)</th>
<th>FH</th>
<th>Year</th>
<th>Prodrome</th>
<th>SBP SDS</th>
<th>Hb g/dl</th>
<th>PMNL x10^9/l</th>
<th>Plat</th>
<th>PCR μmol/l</th>
<th>Dialysis (days)</th>
<th>Plasma exchange</th>
<th>Relapses</th>
<th>Sequelae</th>
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<tr>
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<td>-</td>
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<td>4.0</td>
<td>4.8</td>
<td>55</td>
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<td>46</td>
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<td>6.2</td>
<td>3.7</td>
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<td>213</td>
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<td>67</td>
<td>4</td>
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<td>13(F)</td>
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<td>-</td>
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<td>5.9</td>
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<td>215</td>
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<td>58</td>
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<td>2.4</td>
<td>6.7</td>
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<td>95</td>
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<td>7.0</td>
<td>15.9</td>
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<td>2310</td>
<td>21</td>
<td>5</td>
<td>-</td>
<td>Hypertension GFR 98</td>
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FH+: Parents first cousins, 3/6 sibs affected.
For abbreviations see Table 3.1a

Table 3.1c Children with Idiopathic D-HUS with Residual Nephropathy
<table>
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<th>Case</th>
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<th>FH</th>
<th>Year</th>
<th>Prodrome</th>
<th>SBP SDS</th>
<th>Hb g/dl</th>
<th>PMNL x10^9/l</th>
<th>Plat μmol/l</th>
<th>PCr</th>
<th>Dialysis (days)</th>
<th>Plasma exchange</th>
<th>Relapses</th>
<th>Outcome</th>
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</thead>
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<tr>
<td>18(F)</td>
<td>2.1</td>
<td>-</td>
<td>1973</td>
<td>Fever, Vomiting, UTI</td>
<td>1.2</td>
<td>5.3</td>
<td>8.6</td>
<td>65</td>
<td>150</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>GFR 100</td>
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<td>19(F)</td>
<td>0.9</td>
<td>-</td>
<td>1986</td>
<td>Anaemia, Jaundice, URTI, Vomiting</td>
<td>3.4</td>
<td>4.9</td>
<td>13.6</td>
<td>86</td>
<td>111</td>
<td>5</td>
<td>19</td>
<td>2</td>
<td>GFR 103</td>
</tr>
<tr>
<td>20(M)</td>
<td>0.5</td>
<td>-</td>
<td>1973</td>
<td>Fever, Fits, URTI</td>
<td>2.5</td>
<td>9.9</td>
<td>4.1</td>
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<td>58</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</table>

For abbreviations see Table 3.1a
Table 3.2. D-HUS: Year of Presentation, Treatment with Plasma Exchange and Outcome.

<table>
<thead>
<tr>
<th>Year of presentation</th>
<th>Before 1980</th>
<th>After 1980</th>
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<td>Treatment</td>
<td>Conservative</td>
<td>Plasma Exchange</td>
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<tr>
<td>Number of patients</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>Early death</td>
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<tr>
<td>Early ESRF</td>
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<td>-</td>
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<tr>
<td>Relapse</td>
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</tr>
<tr>
<td>Nephropathy</td>
<td>1</td>
<td>7</td>
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<tr>
<td>Late ESRF</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td>Late death</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Lost to follow-up</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>Recovered</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

ESRF: End-stage renal failure; D-HUS: Non-diarrhoea-associated HUS.
Fig. 3.1: Age distribution of D- HUS cases (1966-92).
Fig. 3.2: Family tree of patient 10.
Fig. 3.3a: Glomerulus from patient 15 showing diffuse thickening of tuft capillary walls with 'double contouring' evident on silver staining.

Fig. 3.3b: Electron microscopy of glomerulus from patient 15. The glomerular capillary walls show marked widening of the subendothelial space which is filled with loose fibrillary and granular material. The capillary loop contains numerous platelets.
Fig. 3.4: Clinical course of patient 11 treated with a programme of plasma exchange. A relapse is shown by a simultaneous rise in plasma creatinine and fall in platelet count. The number of plasma exchanges are shown at the top.
Idiopathic D- HUS (1966-1992)

n=20

Death ESRF Nephropathy Recovered Lost to FU

5 (25%) 4 (20%) 8 (40%) 2 (10%) 1 (5%)

- cerebral sepsis
- Tx CAPD
- ↓GFR proteinuria hypertensive

ESRF end-stage renal failure
Tx transplanted
CAPD continuous ambulatory peritoneal dialysis
GFR glomerular filtration rate
FU follow-up

Fig. 3.5: Diagrammatic representation of outcome of D- HUS cases.
4.1 Introduction

This chapter examines the spectrum of neurological manifestations in children presenting with D+ HUS to the HSC, GOS, and describes a retrospective, nested, matched case control study to identify risk factors at presentation for central nervous system (CNS) disease. The significance of CNS involvement in HUS was recognized by Gasser and colleagues (1) in their original description of HUS in 1955. Since then many authors have emphasised the importance of such complications, which are reported as occurring in up to 50% of patients (17, 204-206). Focal and generalized seizures are the most common presentation of major CNS disease although other manifestations including irritability, drowsiness, coma, aphasia, psychosis, and visual defects have been reported. There is now increasing evidence that CNS disease is a major predictor of outcome and is associated with a high mortality and morbidity (17, 186, 204-209).
4.2 Patients and Methods

4.2.1 Selection of Patients with CNS Disease

The data for this study were obtained by the retrospective review of hospital records for children with a discharge diagnosis of D+ HUS during the period from January 1980 to November 1992. The criteria for diagnosis were a microangiopathic haemolytic anaemia, thrombocytopenia and acute renal failure in association with a diarrheal prodrome. CNS disease was defined to include generalized disturbances such as encephalopathy, coma and seizures, and more focal signs including hemiplegia, hemiparesis, visual defects and extrapyramidal signs. A total of 150 children presented with D+ HUS over this period, and CNS disease was identified in 23 of them (15.3%).

4.2.2 Selection of Matched Case Controls

Each child with CNS disease was matched with a patient without CNS disease. The matched controls were selected randomly from a group of patients nested within the cohort of children with D+ HUS, as defined above, who were of the same sex and comparable age, and presented within 18 months of the patients with CNS disease.
4.2.3 Clinical and Laboratory Data

The following data was recorded for each patient:

(i) CNS symptoms and signs;

(ii) use of antibiotics, antidiarrhoeal agents or antimitility drugs before admission;

(iii) systolic blood pressure at presentation, a standard deviation score (SDS) was derived by reference to age and sex matched 1987 Task Force Standards, using the formula $SDS = \frac{\text{measurement} - \text{mean}}{\text{SD}}$ where the mean and SD were those of normal sex and age matched population (189);

(iv) laboratory values for haemoglobin, polymorphonuclear leucocyte count (PMNL), and platelet count; plasma sodium, potassium, urea, creatinine, calcium, albumin, and magnesium concentrations at presentation.

4.3 Statistical Analysis

Frequencies were compared using Fisher's Exact Test for matched discontinuous data. The Student's paired t-test was used for matched continuous data.
4.4 Results

4.4.1 CNS Cases

The clinical and laboratory features of the 23 children with CNS complications are listed in Table 4.1.

(a) Clinical Features. Of the 23 children with neurological disease 12 were male and 11 female with a median age of 2.5 years (range 0.3-11.1). All were in renal failure and needed dialysis within 24 hours of admission. Fifteen (65%) required assisted ventilation and one of these had a device inserted for intracranial pressure monitoring. Seventeen (74%) had severe bloody diarrhoea at the time of presentation, 3 with associated rectal prolapse. One child had a sigmoidoscopy performed for suspected inflammatory colitis and was treated with steroids and antibiotics. A second patient had an exploratory laparotomy at the referring hospital for a presumed intussusception; petechial haemorrhages were noted along the small and large intestine and caecum, and the ascending colon appeared dusky and oedematous. Two required laparotomies at the HSC,GOS; one had an ischaemic stricture with perforation of the sigmoid colon which needed resection and the other had an ileostomy performed for severe colitis with functional obstruction.
(b) CNS Manifestations. All showed signs of irritability on admission and 18 (78%) had an alteration in level of consciousness. Eighteen had convulsions during the course of their illness, 11 at the time of presentation to hospital. Thirteen (57%) had generalized grand mal type convulsions and 4 presented with focal seizures; 3 in association with hemiparesis and facial weakness and one with an homonymous hemianopia and toe walking. One child presented with complex partial seizures associated with periods of twitching, staring and non-responsiveness. Eight (35%) more severely affected children had abnormal mouthing movements accompanied by teeth grinding and tremor of the lips and tongue. Two patients had papilloedema; fundal haemorrhages, cortical blindness, a convergent squint and a fixing defect were present in one case each. One child experienced vivid hallucinations and another presented with limb ataxia.

(c) CNS Imaging. Fifteen children had cranial computed tomographic (CT) scans, 6 of whom had 2 during the course of their admission. Seven had normal CT scans, 3 had normal first scans but when repeated showed cerebral oedema with atrophy (Fig. 4.1), 2 had successive scans showing cerebral oedema and then oedema with atrophy and 3 children had evidence of haemorrhagic infarction. Seven patients also had single photon emission computerized
tomograms (SPECT) of the brain using hexamethylpropylene amine oxime (HM-PAO) labelled technetium-99m; 3 studies were reported as normal, 3 showed areas of reduced cerebral perfusion (Fig. 4.2) and one was a technical failure. One child had magnetic resonance imaging (MRI) of the brain which showed multiple focal areas of high signal in the white matter of both hemispheres consistent with ischaemic lesions (Fig. 4.3).

(d) *Electroencephalograms (EEGs)*. Fourteen of these children had EEGs, only 2 of whom had normal traces. The rest showed irregular slow wave activity, with ill defined repetitive discharges, suggesting a diffuse metabolic encephalopathy.

(e) *Treatment*. All patients required dialysis for management of renal failure. Seventeen (74%) were treated with plasma exchange; the median number of 2 volume exchanges being 6 with a range of 1 to 13. Eleven of the 17 children exchanged also received infused prostacyclin at a starting dose of 2 ng/kg/min increasing as tolerated to 20 ng/kg/min.

(f) *Outcome*. Eight (35%) of the patients with CNS disease died, 7 as a consequence of cerebral and gastrointestinal complications and the 8th with severe ulcerative ischaemic proctocolitis. Six of these children
had post-mortem examinations, 5 of which showed evidence of cerebral oedema ranging from mild compression of the lateral ventricles to near coning with grooving of the cerebellar tonsils, with haemorrhagic infarction of the right anterior cerebral artery and an intracranial haemorrhage in the right frontal lobe in one case each. All 6 patients had histological evidence of severe ischaemic ulcerative proctocolitis, one had an associated acute myocarditis and another pancreatic involvement with thrombotic and fibrinoid changes and lung appearances as seen in adult respiratory distress syndrome. Three of the 15 with CNS disease who survived their acute illness have neurological sequelae: one has convulsions, one a mild hemiplegia and the third is developmentally delayed, has cortical blindness and a dense hemiplegia. One child who sustained pancreatic damage now has insulin dependent diabetes. Seven of the survivors have residual nephropathy with significant proteinuria and a GFR less than 80 ml/min/1.73 m² SA.

4.4.2 Comparison of CNS Cases and Matched Controls

(a) Clinical Features. The 23 patients with CNS disease were comparable to the 23 patients with D+ HUS and no CNS involvement in regard to age, sex ratio, mean presenting systolic blood pressure SDS, and use of antibiotics, antidiarrhoeal and antimitility agents before
admission to hospital (Table 4.2). There was no significant difference in the incidence of *Escherichia coli* 0157: H7 positive cases or rectal prolapse between the 2 groups.

(b) **Laboratory Investigations.** Haematological tests revealed that the mean PMNL count was significantly higher in patients with CNS disease than in the controls (25.8 vs 11.6 x10^9/L; p<0.001) but there was no significant difference in mean haemoglobin or platelet count (Table 4.2). Analysis of the biochemical data showed that those with CNS disease had significantly lower mean plasma sodium (124.2 vs 127.9 mmol/L; p=0.03) and calcium (1.98 vs 2.12 mmol/L; p=0.01) concentrations, and significantly lower mean urea (29.4 vs 45.8 mmol/L, p<0.001) and creatinine (339.5 vs 598.4 μmol/L; p<0.001) levels. No significant differences were found in mean plasma albumin, potassium or magnesium values (Table 4.2).

(c) **Outcome.** The mortality for those with CNS disease was significantly higher than for the controls (8 of 23 vs none of 23; p<0.05, Exact test). Seven of 15 survivors with CNS disease and 2 of 23 from the control group had renal sequelae and this difference in renal morbidity between the groups was also statistically significant (p=0.01).
4.5 Discussion

In this review there was a wide spectrum of neurological complications in patients with D+ HUS, varying from generalized disturbances, such as stupor, coma, and seizures, to more focal signs, including hemiplegia, hemiparesis, cortical blindness, and extrapyramidal signs. The incidence of neurological manifestations was 15.3% and the incidence of serious neurological sequelae was 2% of all patients. The incidence of serious neurological complications in other series has varied from 20% to 50% (204-206,210,211), these differences probably reflect the improvement of intensive care in recent years and earlier referral of more seriously affected patients.

Several mechanisms have been proposed to explain the neurological complications in haemolytic uraemic syndrome. Non specific metabolic derangements, hypertension, and renal failure may in part account for the relatively high incidence of seizures and alteration in consciousness. Although intravascular coagulation and microthrombi are the hallmarks of this syndrome in the renal system, these have not been consistently found in the central nervous system. Six patients in this series had neuropathological examinations; one had a normal brain and
5 evidence of cerebral oedema with haemorrhagic infarction and an intracerebral haemorrhage in one case each. Microthrombi and large vessel thrombi were not found. Rooney et al (206) examined the brains of 7 patients who had neurological complications and died of HUS; in these cases no microthrombi or distinctive pathological processes were found in the brains. In contrast, Upadhyaya et al (212) studied the brains of 3 patients with HUS and severe neurological symptoms and found microthrombi in the brains of 2 cases. Evidence of ischaemia with focal areas of infarction were noted in all 3. Trevathan et al (213) reported 3 cases of HUS with large vessel infarctions in the distribution of the middle and anterior cerebral arteries as demonstrated by CT. Post-mortem investigation, in one of the cases, revealed large vessel thrombi in the cerebral vasculature, while microthrombi were limited to the kidneys. Examination of the brain in 3 of 5 patients who died in the series reported by Hahn et al (210) showed extensive cerebral oedema with cerebellar tonsillar and tentorial herniations. No small or large vessel thrombi were noted in any of the cases. Histopathological studies have therefore failed to show evidence of a unifying cause for CNS disease in D+ HUS, with both small and large vessel disease, and cerebral oedema being found in different cases.
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There were 9 deaths in this series of 150 patients, 8 occurring in children who had neurological complications. Thus children with CNS disease had a significantly higher mortality than those without (8/23 vs 1/127; p<0.001). The presence of CNS disease in D+ HUS is therefore a significant mortality risk factor. In the past, the use of clinical features and admission laboratory values to predict subsequent neurological dysfunction have been limited (204-5,209-212,215). Sheth et al (204) associated elevated serum creatinine level with the presence of CNS disease based on admission laboratory data, and furthermore associated low serum $CO_2$, low serum calcium, and elevated serum creatinine levels with neurological illness when extremes of laboratory values were examined. Havens et al (208), found a low serum calcium value within the first 2 days of hospital admission to be predictive of patients who would have a bad outcome. Milford and Taylor (214) have associated hyponatraemia with an increased likelihood of convulsions and propose that inappropriate fluid and electrolyte administration may be the inciting event. Robson et al (209) proposed an association for an increased white cell count, thrombocytopenia, and anuria with a poor outcome for complicated patients in univariate analyses. Cimolai et al (211) using multivariate analyses found that female gender, prolonged use of an antimotility pharmacological agent and an increased haemoglobin level
Chapter 4: CNS Disease

were associated with an increased risk for development of a neurological manifestation.

In order to identify risk factors for serious neurological disease at presentation, the retrospective matched case control study described in this chapter was undertaken. Differences between cases with CNS involvement and those without were observed in the mean PMNL count which was significantly higher and the mean plasma sodium and calcium concentrations which were significantly lower. No significant differences were observed in haemoglobin, platelets, plasma potassium, magnesium or albumin concentrations, nor in systolic blood pressure SDS. Plasma urea and creatinine concentrations were significantly lower in those with CNS disease. The data from this study suggest that disease activity, reflected in a high PMNL count, and relative water overload, but not the degree of uraemia or hypertension, as significant risk factors for the development of CNS complications in D+ HUS. Possible explanations for the difference in renal function between the 2 groups are either the earlier referral of those with CNS disease, before the development of significant renal impairment, or a more insidious onset of renal failure in these patients.

There is now compelling evidence that serious CNS involvement is a major predictor of outcome. Severe CNS
disease is a feature of almost all fatal cases as evidenced by this review and other reports in the literature (204-7,210-1), and a substantial percentage of survivors are left with neurological sequelae (21,186,204-7), although there is potential for dramatic improvement even in patients with gross neurological deficits during the acute phase (215-6). A complex interaction of clinical and laboratory variables are likely to be responsible for the varied neurological complications of D+ HUS. This study serves to emphasise the importance of prompt diagnosis with judicious fluid and electrolyte administration and appropriately timed dialysis to prevent the problems of volume overload and metabolic disturbance, in an attempt to reduce the risk of CNS complications. It also stresses the importance of identifying patients presenting with high PMNL counts as being at greater risk of developing neurological disease; it may be that this group should be considered for more aggressive treatment with plasma exchange and infused prostacyclin, together with measures to reduce cerebral oedema if present, should there be any indication of CNS disease associated with a deterioration in clinical state.
<table>
<thead>
<tr>
<th>Case</th>
<th>Gender</th>
<th>Age (yr)</th>
<th>Systolic BP SDS</th>
<th>PMNL $\times 10^9$/l</th>
<th>Na mmol/1</th>
<th>Urea mmol/1</th>
<th>Creatinine $\mu$mol/1</th>
<th>Ventilation</th>
<th>Plasma Exchange</th>
<th>Outcome/sequelae</th>
<th>CNS etc</th>
<th>GFR</th>
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<td>3.0</td>
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<td>-</td>
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*This child also had cortical blindness and developmental delay.
BP: Blood pressure; CNS: Central nervous system; PMNL: Polymorphonuclear leucocytes; SDS: Standard deviation score.
Table 4.2. Characteristics of D+ HUS patients with and without CNS Involvement.

<table>
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<tr>
<th>Clinical Features</th>
<th>CNS disease</th>
<th>Controls</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cases</td>
<td>23</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>12/11</td>
<td>12/11F</td>
<td>ns</td>
</tr>
<tr>
<td>Median age (range, yr)</td>
<td>2.5(0.3-11.10)</td>
<td>2.8(0.9-12.3)</td>
<td>ns</td>
</tr>
<tr>
<td>Antimicrobial treatment</td>
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<td>7</td>
<td>ns</td>
</tr>
<tr>
<td>Antispasmodic treatment</td>
<td>4</td>
<td>1</td>
<td>ns</td>
</tr>
<tr>
<td>Antidiarrhoeal treatment</td>
<td>1</td>
<td>2</td>
<td>ns</td>
</tr>
<tr>
<td>Rectal prolapse</td>
<td>3</td>
<td>4</td>
<td>ns</td>
</tr>
<tr>
<td>Mean SBP SDS ±SD</td>
<td>1.21 ±1.54</td>
<td>1.34 ±1.21</td>
<td>ns</td>
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Laboratory Findings

<table>
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<th></th>
<th>CNS disease</th>
<th>Controls</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Haemoglobin (g/dl)</td>
<td>8.6 ±2.2</td>
<td>8.0 ±1.7</td>
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<tr>
<td>PMNL x10⁹/l</td>
<td>25.8 ±13.3</td>
<td>11.6 ±6.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Platelets x10⁹/l</td>
<td>59.8 ±29.7</td>
<td>76.6 ±42.4</td>
<td>ns</td>
</tr>
<tr>
<td>Sodium (mmol/l)</td>
<td>124.2 ±6.8</td>
<td>127.9 ±3.8</td>
<td>0.03</td>
</tr>
<tr>
<td>Potassium (mmol/l)</td>
<td>5.2 ±0.42</td>
<td>5.3 ±0.38</td>
<td>ns</td>
</tr>
<tr>
<td>Urea (mmol/l)</td>
<td>29.5 ±11.0</td>
<td>45.8 ±23.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Creatinine (µmol/l)</td>
<td>339.5 ±142.1</td>
<td>598.4 ±383.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Calcium (mmol/l)</td>
<td>1.98 ±0.4</td>
<td>2.12 ±0.1</td>
<td>0.01</td>
</tr>
<tr>
<td>Magnesium (mmol/l)</td>
<td>0.84 ±0.13</td>
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<tr>
<td>Albumin (g/l)</td>
<td>25.9 ±4.76</td>
<td>26.9 ±4.5</td>
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</tr>
<tr>
<td>E coli O157:H7 positive</td>
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Outcome

<table>
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</tr>
</thead>
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<tr>
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<td>0</td>
<td>&lt;0.05</td>
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<tr>
<td>GFR&lt;80 ml/min/1.73m²SA</td>
<td>7</td>
<td>2</td>
<td>0.01</td>
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</table>

Values for haematology and biochemistry are means ± standard deviation.
CNS: Central nervous system; PMNL: Polymorphonuclear leucocytes; SA: Surface area;
SBP: Systolic blood pressure; SDS: Standard deviation score.
Fig. 4.1: CT head scan showing cerebral oedema with atrophy.
Fig. 4.2: HMPAO brain SPECT scan showing focal defect in the L parietal lobe consistent with an area of reduced cerebral perfusion.
Fig. 4.3: MRI brain scan showing multiple focal areas of high signal in the white matter of both hemispheres consistent with ischaemic lesions.
D+ HUS with CNS involvement

(n=23)

Death  Neurological  Renal  Recovered

sequelae  sequelae

8 (35%)  3  5  7

+ nephropathy (n=2)  + diabetes (n=1)

Fig. 4.4: Diagrammatic representation of outcome of cases of D+ HUS with CNS involvement (1980-92)
CHAPTER 5: OUTCOME OF RENAL FUNCTION IN D+ HUS

5.1 Introduction

Over the past decade there have been significant advances in the understanding of both the aetiology and pathogenesis of D+ HUS (53,54,195) (Chapter 1), however the long-term outcome for those patients who survive the acute phase of the illness remains incompletely documented. This chapter is based on a study performed at the HSC, GOS and the Royal Free Hospital, London (RFH) reviewing the long-term outcome for renal function in 88 infants and children evaluated 5 to 21 years after the acute phase of D+ HUS (143). This study involves a number of children originally reviewed by Trompeter et al to identify prognostic criteria which was published in 1983 (60). A review of the literature on follow-up in children with HUS is presented in the first section.

5.2 Review of the Published Follow-Up Studies

Follow-up studies published over the years from different geographical regions have produced variable results with regard to renal outcome following an episode of HUS. A review of the literature on long-term follow-up is provided in Table 5.1.
5.2.1 Argentinian Study

The largest series is from Argentina (21) where HUS is the leading cause of chronic renal failure in childhood and adolescence and was published in 1973 prior to the widespread availability of acute dialysis. Gianantonio et al (21) produced detailed follow-up data on 124 survivors followed for at least 5 years. Only 60 children (48%) were reported to have recovered completely although hypertension was found in 10 of them. In 23 cases (18%) a progressive deterioration of renal function was observed. The authors concluded that 'survival during the acute phase of HUS has created a new chronic renal disease which is an important cause of chronic uraemia in Buenos Aires'.

5.2.2 European and North American Studies

A number of European and North American groups have published less gloomy results; however some of these studies suffer from too short follow-up periods, a large number of cases lost to follow-up, and the absence of data on glomerular filtration (114,186,217,218).

(a) Belgium. Binda ki Muaka et al (219) published a follow-up study on 42 surviving infants and children with HUS. Three patients (7%) had chronic renal failure. Normalization of renal function with no haematuria,
proteinuria, or hypertension was documented in 93%. No differences were found when patients were subdivided according to age, severity of disease, or whether or not peritoneal dialysis was required. The same centre reviewed 46 patients 10 years after the acute phase (180). In 3 patients significant late sequelae were discovered: proteinuria, arterial hypertension, and in one a low glomerular filtration rate. In an additional 14 former HUS patients, one or more mild abnormalities were found, so only 60% of the total population could be considered to have completely recovered.

(b) United Kingdom. In the series from London published in 1983 Trompeter et al (60) retrospectively analysed the records of 72 children with HUS in order to identify prognostic criteria. They identified youth, the presence of diarrhoea, and the occurrence in the summer months as favourable prognostic features. Furthermore, duration of the prodromal illness seemed to be important: long prodromes were associated with poor outcome. Finally of the 37 children who came off dialysis before 2 weeks, only one had residual renal disease, whereas of the 16 children needing dialysis longer, only one recovered completely.

More recently Coad et al (144) from Birmingham, analysing the changes in the postenteropathic form of the haemolytic uraemic syndrome in children presenting between
1970 and 1987, reported a mortality rate of 12%, development of sequelae in 15% and recovery in 73% of children following an episode of D+ HUS.

(c) France. The Paris experience reviewing the outcome of 67 patients presenting between 1974 and 1981 showed that children younger than 3 years, patients with oligo-anuria for less than 7 days, and patients with less than 40% of their glomeruli affected by microangiopathy did better in the long run (224). More recently Loirat et al (163) on behalf of the French Society of Paediatric Nephrology published results which suggested that previous authors may have underestimated the extent of renal sequelae following an episode of D+ HUS. As part of a multicentre randomised controlled trial on the treatment of childhood HUS with plasma, 54 of the 79 children were biopsied one month post presentation and 7 cases (7/54, 13%), all from the control group, showed evidence of diffuse cortical necrosis.
5.3 D+ HUS Follow-Up Study

The aim of this study was to assess renal function using more sensitive methods than had been used previously in a large cohort of children with a minimum follow-up of 5 years after an episode of D+ HUS to produce more reliable data on long-term prognosis.

5.3.1 Patients

From 1966 to 1985 120 infants and children with HUS were admitted to the HSC, GOS and the RFH, London. One hundred and three had a 'typical' enteropathic illness with a diarrhoeal prodrome (D+ HUS) and 17 an 'atypical' presentation without diarrhoea (D- HUS). A follow-up study was undertaken of the D+ HUS children. Ethical approval for this study was obtained from the Ethical Committee of the HSC, GOS. Of the original 103 with D+ HUS, 15 were not included in the study (Table 5.2). Eight had died, 5 during the acute phase of the illness, and 3 had progressed to end-stage renal failure following their initial presentation and died after dialysis or transplantation (Table 5.3). Seven other children were unavailable for study because they resided abroad (n=2), were unwilling to cooperate in the study (n=2), or could not be located (n=3). Of the 88 patients who attended for follow-up 40 were male and 48 female with a mean age of 11.6 years (range 5.3-22.6). The mean duration of follow-up was 8.5 years (range 5.1-21.3).
5.3.2 Methods

The children and young adults were reviewed as day cases, bringing with them an early morning urine sample in which the urine albumin/creatinine concentration ratio (UA/UC) was measured. Blood pressure was recorded using a random zero sphygmomanometer (225). Blood samples were taken for estimation of glomerular filtration rate (GFR) and plasma renin activity (PRA).

(a) Blood Pressure. Blood pressure was measured in the right arm with the child seated using a random zero sphygmomanometer, the arm being supported in a semi dependent position. No strenuous exercise was taken in the 2 hours preceding the measurements. After inflation of the cuff 3 readings of Korotkoffs sounds 1 and V were taken and the mean used for analysis. The fifth Korotkoff sound was used because it is more reliably heard (226). All measurements were made by the same observer throughout the study. Data were normally distributed and results were expressed as standard deviation scores (SDS) by reference to age and sex matched 1987 Task Force standards (189).
(b) **Albuminuria.** The early morning urine sample was collected in a plastic bottle containing 1:10,000 merthiolate to prevent bacterial growth. The urine creatinine concentration (UC mmol/L) was measured by an auto analyser Jaffe reaction, with an intra-assay coefficient of variation (CV) of 3%. Urine albumin concentration (UA mg/L) was measured by double antibody radioimmunoassay using a commercially available kit (Diagnostic Products Corporation, California, USA) with a sensitivity of 0.5 mg/L, an intra-assay CV of 4%, and an inter-assay CV of 5%. The UA/UC ratio was expressed in mg/mmol and was log transformed before statistical analysis; results were expressed as the geometric mean and range (±SD) calculated on logged data.

(c) **Glomerular Filtration Rate.** GFR was estimated from the plasma clearance of 51-chromium dianiminotetraacetic acid ($^{51}$Cr-EDTA) by single compartmental analysis (227) and the results expressed in ml/min/1.73m$^2$ surface area (SA).

(d) **Plasma Renin Activity.** One ml of venous blood was taken from the patients after they had rested supine for 2 hours. PRA was measured with a semi-micro radioimmunoassay (190) and expressed as ng A1/L/hour. Inter and intra-assay CV were 10% and 5% respectively. As for BP, SD scores were calculated for PRA compared to age matched controls (190).
5.3.3 Statistical Analysis

A one sample t-test was used to compare systolic and diastolic BP SDS with the notional value of zero. The geometric means of UA/UC were compared using an unpaired t-test on log transformed data. The interrelations between GFR, BP SDS, and UA/UC were examined by linear regression and Pearson's correlation coefficients. The ages and durations of dialysis for the children in the 2 outcome groups were compared using the Mann-Whitney test for comparison of 2 values. The geometric means of the total white cell counts in the 2 outcome groups were compared using unpaired t-test on log transformed data.

5.3.4 Results

(a) Mortality. The mortality during the acute phase of the disease was 3 of 35 (8.6%) before 1980 and 2 of 69 (2.9%) after 1980 (Table 5.3). The overall mortality between 1966 and 1985 was 8 of 104 (7.7%). Three children went into end stage renal failure without recovering function.

(b) Blood Pressure. The mean systolic BP SDS was 0.38 ±0.67 (SD) (n=88) significantly greater than zero (t=5.28, p<0.0001) but no child had a systolic BP SDS greater than 2.0 (Fig 5.1). The mean diastolic BP SDS was 0.10 ±0.76 which was not significantly different from zero (t=1.23, p=0.22).
Chapter 5: Outcome D+ HUS

(c) **Albuminuria.** The geometric mean UA/UC in the early morning urine sample from the D+ HUS children (n=88) was 1.27 mg/mmol (range 0.03-48.2), significantly higher than the value of 0.32 mg/mmol (range 0.05-1.95; t=5.97, p<0.0001) in 77 normal children (228) (Fig 5.3). Twenty seven of the 88 (31%) D+ HUS children had a UA/UC greater than 2 SD above the normal mean.

(d) **Glomerular Filtration Rate.** The mean $^{51}$Cr EDTA GFR ±SD was 95.1 ±22.7 ml/min/1.73m$^2$ SA (Fig 5.3). Data on these 16 is presented in Table 5.4. Nine of these children had both a GFR < 80 ml/min/1.73m$^2$ SA and a UA/UC > 2SD above the normal mean.

(e) **Plasma Renin Activity.** Data on PRA were available in only 41 children. The mean plasma renin SDS was 0.60 ±0.97 (SD) which was significantly greater than zero (t=3.9, p<0.001).

(f) **Correlations.** Significant negative correlations were found between GFR and UA/UC (r=-0.41, p<0.0001), GFR and systolic BP SDS (r=-0.48, p<0.0001), and GFR and white cell count at presentation (r=-0.36, p<0.001) (Fig 5.4, 5.5, & 5.6) and a significant positive correlation between UA/UC and systolic BP SDS (r=0.25, p=0.02) (Fig 5.7). No correlation was found between plasma renin SDS and any of the other parameters measured.
(g) **Comparison between Outcome Groups.** The patients were divided into 2 outcome groups; those who at follow-up had a GFR $\leq 80 \text{ ml/min/1.73m}^2$ SA ($n=16$), which is considered to be the lower limit of normal, or who had died having gone into end stage renal failure ($n=3$) (group 1, $n=19$) and those with a GFR $>80 \text{ ml/min/1.73 m}^2$ SA at the time of reinvestigation ($n=72$). There was no difference in the haemoglobin or platelet count at presentation, or the age of the children when compared between the 2 groups: in group 1 the mean age was 2.1 years (range 0.25-11.0) compared to 3.5 years (range 0.4-14.0) in group 2 ($z=0.86$, $p=0.38$). However the children in group 1 had a significantly higher total white cell count at presentation (geometric mean 20.9 x10$^9$/L, range 10.5-41.6) than those in group 2 (geometric mean 15.1 x 10$^9$/L, range 5.0-45.7; $t=-2.4$, $p=0.01$). The duration of dialysis was also significantly longer in group 1 (21.1 days ±17 ) than in group 2 (9.1 ±6.0, $z=2.91$, $p<0.001$); this analysis excludes from the total ($n=91$) the 22 children (24%) who did not have dialysis at the time of presentation. Eight (57%) of the 14 patients who were dialysed for 16 days or more had a reduced GFR whereas only 11 (14%) of the 74 dialysed for less than 16 days had a GFR $< 80 \text{ ml/min/1.73 m}^2$ SA at follow-up (Table 5.5).
5.3.5 Discussion

It is increasingly apparent from the literature and evidenced by this 5 year follow-up study, that contrary to a number of earlier reports, D+ HUS is associated with a substantial morbidity. In this series 3/104 (2.9%) of patients did not recover renal function after presentation and 34/88 (39%) had some abnormality of renal function at the time of follow-up. Eighteen per cent had a GFR < 80 ml/min/1.73m² SA and 31% had significant microalbuminuria. Three per cent of patients became hypertensive after their acute illness and were taking antihypertensive drugs at follow-up. The remainder had a systolic BP SD score < 2, but the mean systolic BP SD score for the group as a whole was significantly greater than zero. The mean PRA SD score was also greater than zero. There was an association between a lower GFR, microalbuminuria, and a higher systolic BP.

When the survivors were divided into 2 groups on the basis of GFR: <80 (group 1) and >80 ml/min/1.73m² SA (group 2), there was no significant difference in the haemoglobin or platelet count at presentation, or the age of the children when compared between the 2 groups. However patients in group 1 had a significantly higher white blood cell count on admission and a significantly longer duration of dialysis. Publications analysing prognostic features in HUS have produced variable results with regard to significance of age at presentation, duration of anuria or
dialysis, and presence of CNS involvement \((60, 142, 224, 229)\), but polymorphonuclear leucocytosis is consistently related to poor outcome \((142, 144, 194, 229)\).

Most of the patients in this study had been discharged from hospital follow-up and were not under regular medical review. However, in view of the substantial number with renal sequelae it may be important that children who have had an episode of D+ HUS be kept under general medical review with BP measurement and urine testing. Because of the positive correlation between UA/UC and systolic BP and the negative correlation between UA/UC and GFR then those patients found to have proteinuria on routine testing should be referred for further renal evaluation.
Chapter 5: Outcome D+ HUS

5.4 Prospective Ultrasound Study

The long-term follow-up study suggests that although most children with D+ HUS make a full recovery approximately 40% develop some form of renal sequelae. Patrquin et al from Montreal, Canada, in a report published in 1989 (230) proposed the use of resistive index (RI) to predict recovery from oliguria or anuria during the acute phase of D+ HUS. Following this observation, in an attempt to detect patients with renal impairment following an episode of D+ HUS a prospective study using duplex Doppler ultrasound in the evaluation of these children was undertaken.

5.4.1 Patients and Methods

This was a prospective single blind study, undertaken in collaboration with Dr F Gleeson from the Department of Radiology at the HSC,GOS, using Doppler ultrasound to assess renal and intrarenal blood flow in an attempt to recognize those children with impaired renal function following an episode of D+ HUS. Three groups of age and sex matched children were selected for study:

Group 1. Eight children with a history of D+ HUS and a GFR of greater than 80 ml/min/1.73m² SA.

Group 2. Eight children with a history of D+ HUS and a GFR less than 80 ml/min/1.73m² SA.

The ultrasound examinations were performed by two experienced sonographers, unaware of the medical history and renal function of the patient at the time of the examination. The renal and intrarenal arteries were examined with duplex Doppler ultrasound and a total of five measurements taken from each kidney. The RI was calculated from these measurements as peak systolic velocity minus end diastolic velocity divided by peak systolic velocity, which was calculated by the machine. A mean RI was calculated from the five readings. All examinations were performed with an Acuson 128 machine using real-time and pulsed Doppler scanning. The GFR was estimated from the plasma clearance of chromium-51 edetic acid (227).

5.4.2 Results

Thirty-five children were studied: 19 children in the normal group, and eight in each of the groups previously affected by D+ HUS. The results are summarized in Fig. 5.8. There was a large range of values measured in all three groups, with a range of 30.9% seen in the normal children, Group 3, and 31.1% seen in Group 2. There was considerable overlap in the RIs measured in all 3 groups. The lowest RI measured in all 3 groups was similar: Group 1, RI= 47.7%;
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Group 2, RI=47%; and Group 3, RI=45%. The highest RI, 78.1% was measured in a child with a GFR of 37 ml/min/1.73 m² SA, but measurements of 75.9% and 75.4% were obtained in the group of normal children. The means of the three groups were similar: Group 1 mean 56.9%, Group 2 mean 57.7% and Group 3 mean 59.9% (Fig. 5.8). The results were compared using Student's t-test, and showed no significant differences.

5.4.3 Discussion

The development of renal sequelae is a significant and serious long-term consequence of D+ HUS. The use of a non-invasive and easily reproducible means of detecting children with renal impairment secondary to D+ HUS would be a significant advance in their care, avoiding radioisotope injection, day case admission and venepunctures in young children. The paper by Patriquin et al (230) advocated the use of the RI in the acute phase, and demonstrated their ability to predict the time of recovery from the oliguric or anuric phase of the syndrome. The histopathological change seen in the acute phase involves an alteration of the endothelium, with swelling of endothelial cells effectively narrowing the capillary lumen. The presence of arteriolar occlusion increases the intrarenal vascular resistance, increasing pulsatility leading to a high RI (230). As the
kidney recovers, the intrarenal vascular resistance falls with a consequent fall in the RI. In those children with chronic renal impairment the histopathological changes are less specific; tubular atrophy, interstitial fibrosis and glomerular segmental sclerosis may all occur. These changes should also raise intrarenal vascular resistance resulting in a high RI. It would be reasonable therefore to expect a higher RI to occur in the children with the lowest GFRs, Group 2. The highest RI recorded in this series, 78.1%, was found in the child with the lowest GFR, 37 ml/min/1.73 m² SA. However her kidneys were sonographically abnormal, being small and of increased echogenicity. There were no other sonographically abnormal kidneys, and it may be that a significant elevation of RI only occurs in kidneys clearly abnormal on conventional real-time ultrasound in which measurement of RI would be of little benefit.

The initial enthusiasm for the use of RI in the assessment of renal and intrarenal blood flow has recently diminished with an increasing number of papers questioning its value (231,232). The normal range of RI and its alteration with age have not been established, and the wide range of values seen in the group of normal children questions the previous use of absolute measurements to predict disease. A significant change occurs with alteration of heart rate (233), and it is known that the other factors such as compression of the kidney by the ultrasound probe may
cause an elevation of RI (234). This pilot study suggests that RI is not as useful as was initially reported, and raises doubts about the ability of Doppler sonography to detect chronic renal impairment.
Table 5.1. Review of Literature on Long-term Follow-up of HUS in Children.

<table>
<thead>
<tr>
<th>Author (ref)</th>
<th>Year</th>
<th>Area/Country</th>
<th>Period of study</th>
<th>Number of cases</th>
<th>Deaths n (%)</th>
<th>Follow-up Years Number</th>
<th>GFR/CCr</th>
<th>Hyper-tension</th>
<th>Proteinuria</th>
</tr>
</thead>
<tbody>
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<td>Argentina</td>
<td>1957-72</td>
<td>679</td>
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<td>124</td>
<td>73</td>
<td>51</td>
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<tr>
<td>Donckerwolke (220)</td>
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<td>1960-69</td>
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<td>1957-72</td>
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<td>1976</td>
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<td>1969-75</td>
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<td>1969-80</td>
<td>72</td>
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<td>69</td>
<td>50</td>
<td>9</td>
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<td>1972-88</td>
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<td>7(9)</td>
<td>2-18</td>
<td>59</td>
<td>44</td>
<td>9</td>
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CCr: Creatinine clearance; GFR: Glomerular filtration rate (ml/min/1.73m²SA).
Table 5.2. Children with D+ HUS Included/unavailable for Follow-up Study.

<table>
<thead>
<tr>
<th>Year</th>
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<th>Died</th>
<th>Unavailable</th>
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<td><strong>88</strong></td>
<td><strong>8</strong></td>
<td><strong>7</strong></td>
</tr>
<tr>
<td>Gender</td>
<td>Year</td>
<td>Age (yr)</td>
<td>PMNL x10^9/1</td>
<td>Dialysis duration (days)</td>
</tr>
<tr>
<td>--------</td>
<td>------</td>
<td>----------</td>
<td>--------------</td>
<td>--------------------------</td>
</tr>
<tr>
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<td>1985</td>
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<td>22.9</td>
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</table>

PMNL: Polymorphonuclear leucocytes.
Table 5.4 Characteristics of 16 Patients with GFR ≤80 ml/min/1.73m²SA at Review.

<table>
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<tr>
<th>Gender</th>
<th>Age at onset (yr)</th>
<th>PMNL x10^9/l</th>
<th>Dialysis (days)</th>
<th>Age at follow-up (yr)</th>
<th>GFR ml/min/1.73m²SA</th>
<th>UA/UC (mg/mmol)</th>
<th>Systolic BP SDS</th>
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<td>14.4</td>
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<td>17.2</td>
<td>16</td>
<td>8.0</td>
<td>52</td>
<td>81.3</td>
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<td>14</td>
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<td>12.6</td>
<td>80</td>
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</tbody>
</table>

BP: Blood pressure; GFR: Glomerular filtration rate; PMNL: Polymorphonuclear leukocytes; SA: Surface area; SDS: Standard deviation score; UA/UC: Urine albumin/creatinine concentration ratio.
### Table 5.5 GFR and Duration of Dialysis in Patients with D+ HUS

<table>
<thead>
<tr>
<th>Duration of dialysis (days)</th>
<th>Total</th>
<th>GFR &lt; 80 ml/min/1.73m²SA</th>
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<tr>
<td>1-5</td>
<td>12</td>
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</tr>
<tr>
<td>6-10</td>
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<td>7</td>
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<td>16-20</td>
<td>8</td>
<td>3</td>
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<td>21-30</td>
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<td>3</td>
</tr>
<tr>
<td>&gt;30</td>
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</tbody>
</table>

GFR: Glomerular filtration rate; HUS: Haemolytic uraemic syndrome; SA: Surface area.
Fig. 5.1: Systolic BP SDS at follow-up.
Fig. 5.2: UA/UC (mg/mmol) in D+ HUS and normal controls.
Fig. 5.3: GFR at follow-up.
Fig. 5.4: Correlation between GFR and UA/UC.

$r = -0.41$
$p < 0.0001$
Fig. 5.5: Correlation between GFR and systolic BP SDS.

$r = -0.48$
$p < 0.0001$
Fig. 5.6: Correlation between GFR and white blood cell count at presentation.

$r = -0.36$

$p < 0.001$
Fig. 5.7: Correlation between UA/UC and systolic BP SDS.
Fig. 5.8: Resistive indices in the 3 patient groups -
GFR >80, GFR <80 and normal controls.
CHAPTER 6:

NEUTROPHIL-MEDIATED ENDOTHELIAL INJURY IN D+ HUS

6.1 Introduction

The second half of this thesis details three laboratory studies undertaken to examine the potential role of polymorphonuclear leucocytes in the pathogenesis of D+ HUS. There is now increasing evidence that PMNLs are implicated in the pathogenesis of HUS induced by both VTEC and Shigella dysenteriae type 1. In view of the association of infectious agents with D+ HUS, it is not surprising that a neutrophil leucocytosis has been noted in this disorder (17,51). Indeed in D+ HUS, a high neutrophil count at presentation indicates a poor prognosis (142-145), suggesting that the neutrophil may participate in the pathogenesis. In the first study neutrophil adherence and subsequent injury to the endothelium using neutrophils and plasma from children with acute D+ HUS has been examined, and it is demonstrated that endothelial damage can be prevented in certain cases by inhibiting neutrophil adherence to endothelium. This work was undertaken in collaboration with Dr K D Forsyth, then a research fellow in the Department of Immunology at the Institute of Child Health,
London, and formed a part of his thesis on 'Neutrophil-endothelial interactions'(235).

6.2 Patients and Methods

6.2.1 Patients

Twelve children presenting to the HSC,GOS, with acute D+ HUS between July 1988 and May 1989 were included in this study. Their ages ranged from 9 months to 6 years 8 months. There were 9 girls and 3 boys. The clinical and laboratory features of the patients studied are presented in Table 6.1. Ethical approval for the study was given by the Hospital Ethical Committee. Plasma and neutrophils were collected sequentially from these subjects at the time of presentation. As the neutrophil studies were performed within 2 hours of venepuncture, 12 healthy laboratory staff were used as controls. The plasma and neutrophils from the controls were collected and processed in an identical manner to the HUS specimens.

6.2.2 Preparation of Plasma and Neutrophils

At the time of presentation to the hospital blood was collected into EDTA, and the plasma separated by centrifugation within 30 minutes. The remaining blood was diluted with an equal volume of Dulbecco's phosphate-
buffered saline, and underlayered with 'Ficoll-Faque' (Pharmacia, Uppsala, Sweden). After centrifugation at 400 x g for 20 min, the pellet containing red cells and neutrophils was isolated. Most of the red cells were removed by sedimentation with 3% dextran (Fisons, Loughborough) for 30 min and those remaining were removed by hypotonic lysis. Neutrophils prepared this way were more than 99% pure. For the neutrophil adhesion assay, neutrophils were reconstituted to 1 x 10^6 cells/ml in RPMI 1640 without phenol red, containing 1% fetal calf serum, and used immediately. For analysis of the neutrophil effect on fibronectin architecture, neutrophils were used at 2 x 10^6/ml in RPMI 1640, with 20% plasma. Siliconised plasticware was used at all stages of cell preparation.

6.2.3 Preparation of Endothelial Cells

Endothelial cells were obtained from human umbilical cord veins by digestion for 20 min with collagenase type 2 (Sigma, St Louis, Missouri, USA) 0.1% reconstituted in Dulbecco's minimum essential medium (Gibco, Paisley, Scotland). Cells were cultured in endothelial cell growth medium, consisting of RPMI 1640 (Gibco), supplemented with 10% fetal calf serum and 10% newborn calf serum (Gibco), gentamicin 40 U/ml (Roussel, Dublin, Republic of Ireland), 5 U/ml preservative free sodium heparin (Payne and Byrne,
Greenford, Middlesex), and 15 μg/ml endothelial cell growth supplement (Sigma). Cells were grown to confluence in 25 cm² tissue culture flasks (Becton, Dickinson), and passaged by exposure to 0.05% trypsin and 0.02% EDTA in modified Puck's saline A (Gibco) for 3 min at 37°C. Endothelial cells were used at the second to third passage. Before use they were transferred to Nunc (Denmark) 96-well tissue culture grade microtitre plates for use in the adhesion assay, or onto glass coverslips for study of fibronectin morphology, and confirmed to be confluent by inversion microscopy; confluence generally occurred 2-3 days after seeding. Cells were identified as endothelial cells by characteristic 'cobblestone' morphology when confluent, and by immunoperoxidase staining with an endothelial cell monoclonal antibody (FD44).

6.2.4 Neutrophil Adherence to Endothelium

The number of neutrophils adherent to endothelium was counted by alkaline phosphatase enzyme linked immunosorbent assay (ELISA) (236,237). 100 μl neutrophil suspension (1x10⁶ cells/ml) was added to confluent endothelium grown on 96-well microtitre plates (Nunc). The plates were incubated for 30 min at 37°C, washed to remove non-adherent neutrophils, and 100 μl buffer added to each.
Thus the wells contained only adherent neutrophils. To quantify the adherent cells, a standard curve of neutrophil numbers was constructed; 100 μl of the original neutrophil suspension was added to unused wells and doubling dilutions of the original suspension were made in duplicate. Neutrophils were then counted by means of an alkaline phosphatase ELISA.

(a) Measurement of Adherent Cells. The measurement in vitro of cells adherent to plastic or endothelium generally relies on radioisotopic methods. The alkaline phosphatase activity of adherent neutrophils can be measured easily, does not require previous cell isolation and labelling, and has been extensively validated as a method of counting adherent cells (238). This method relies on the endogenous alkaline phosphatase present within neutrophils. 100 μl 1% p-nitrophenyl phosphate disodium (Sigma, St Louis, USA) in diethanolamine buffer (1mol/l, pH 9.8) was added to each well, and incubated at 30°C for 3-4 hrs. The optical density of each well was measured at 405 nm in a Titretek microplate scanner. Adherent cell numbers were estimated by interpolation from the standard curve. The standard curves obtained were consistently straight lines through the origin.
6.2.5 Model of Fibronectin

Forsyth and colleagues (235) have found a characteristic architecture of fibronectin on the surface of endothelial cells, with disruption of this by degranulated neutrophils. Two IgG mouse monoclonal antibodies raised against human fibronectin were used (FN3 and FN4). FN3 is specific for cellular fibronectin and does not cross react with plasma fibronectin (239). The epitope recognized by FN3 is lost from cellular fibronectin after limited proteolysis with \( \alpha \)-chymotrypsin. FN4 recognises both cellular and plasma fibronectin (239). In addition, a rabbit polyclonal antibody for fibronectin (JMBI) was studied.

6.2.6 Assessment of Fibronectin Architecture

Neutrophils in 20% plasma from controls or patients were added in a checkerboard pattern for 60 min to endothelial cells grown on coverslips. After 60 min incubation at 37°C the coverslips were removed from the wells, washed in medium, and processed by an immunoperoxidase technique. Endogenous peroxidase was blocked by incubation with 1.6% hydrogen peroxidase in absolute methanol for 20 min. The slides were washed 3 times in 'tris' hydrochloric acid buffer, 0.05 mmol/l, pH 7.6, between each step. Fc receptors were blocked by
incubation with normal goat serum diluted 1/50 in normal saline for 20 min. The monoclonal antibodies were applied for 1 hr at room temperature, followed by the secondary antibody (either against mouse IgG or against rabbit IgG for JMBI) for 30 min and the complex developed with 0.05% diaminobenzadine hydrochloride (Sigma) and 0.01% hydrogen peroxide for 10 min. Nuclei were counterstained with Mayer's progressive haematoxylin for 30 sec. The endothelial cells stained for fibronectin were scored according to the extent of fibronectin degradation: 0=normal fibrillar architecture; 1= fibrillar strands of fibronectin with focal lumps; 2= patchy loss of fibrils, some free immunostaining material; 3= extensive dissolution, no recognisable fibrillar structure.

6.2.7 Effect of Leucocyte Integrin Antibody

In an attempt to inhibit neutrophil adhesion to endothelium, the effect of co-incubation of a monoclonal antibody that recognises the common \( \beta \) chain of the leucocyte integrins (240) (MHM23) with neutrophils from HUS patients was assessed for its effect on fibronectin morphology of endothelium. Neutrophils plus CD18 antibody in control plasma were added to endothelium grown on coverslips. These were incubated at 1 hr at 37°C, the coverslip removed and washed, and the endothelium immunostained for fibronectin.
Chapter 6: Neutrophil-Mediated Endothelial Injury

6.3 Results

6.3.1 Clinical Details

The clinical and laboratory characteristics of the patients studied are given in Table 6.1.

6.3.2 Neutrophil Adherence to Endothelium

All 12 HUS subjects showed a higher percentage of their neutrophils adhering to endothelium compared with the control of the day (Fig. 6.1). Control adherence was 14% ±11 (mean ±SD), and for the HUS subjects, 30% ±12. This difference (by paired t-test) was highly significant (p<0.0005).

6.3.3 Endothelial Fibronectin Architecture

In 10 of the HUS subjects fibronectin degradation by neutrophils and plasma incubated with endothelium was assessed. On all occasions HUS neutrophils induced varying degrees of destruction of the normal endothelial fibronectin architecture. Patterns of fibronectin degradation under the various conditions tested are listed in Table 6.2. With the exception of 2 subjects whose neutrophils caused minor changes only, control neutrophils in 20% control plasma did not affect fibronectin
architecture. However plasma from 8 of the 10 HUS patients incubated with control neutrophils caused fibronectin breakdown (mean score 1.7; that is mild/moderate disruption of the fibronectin architecture). Control plasma plus HUS neutrophils from all 10 patients produced fibronectin breakdown, with greater disruption of the normal architecture than occurred with HUS plasma and control neutrophils (mean score 2.3, indicating moderate fibronectin breakdown). The greatest degree of fibronectin degradation occurred when neutrophils and plasma from HUS patients were combined on the endothelium (mean score 2.8, indicating extensive fibronectin degradation). Although neutrophils from HUS subjects induce fibronectin degradation, there is an important contribution to fibronectin degradation from HUS plasma, as shown by the highest score when plasma and neutrophils from HUS are combined. The scoring system however hides the heterogeneity amongst the subjects. In patients 1, 2, 5, and 6 there is little fibronectin degradation induced by HUS plasma incubated with control neutrophils whereas in the remainder the HUS plasma appears to induce more fibronectin breakdown.
6.3.4 Effect of Co Incubation of Neutrophils with CD18 Antibody

Because of the increased neutrophil adherence and subsequent fibronectin breakdown in the HUS patients, the ability of a CD18 antibody to inhibit the fibronectin breakdown was studied. In the 4 patients with minimal plasma effect, incubation of HUS neutrophils with CD18 inhibited completely the fibronectin degradation seen without the antibody (Table 6.2). In the remainder the CD18 antibody was completely ineffective.

6.4 Discussion

Activated neutrophils are known to be able to damage endothelium (146-8), and neutrophil release products can damage the glomerular basement membrane (149). Attachment of neutrophils is the key event in inducing release of granule contents by these cells (241). A rabbit model of HUS has shown neutrophil-induced renal damage (242). In view of this, the role of the neutrophil and plasma from HUS patients and their ability to damage the endothelium was studied, using in vitro methods of neutrophil adhesion and fibronectin degradation of cultured endothelium.

The study has demonstrated that double the number of neutrophils from HUS patients will adhere to endothelium in culture compared with control neutrophils. In addition these neutrophils can degrade the extracellular matrix and
integrin molecule fibronectin, indicating endothelial cell damage. There is no relationship between clinical or laboratory indices in these patients and the extent of fibronectin breakdown or augmented neutrophil adhesion to endothelium.

Neutrophils from HUS patients are activated, as double the number of neutrophils from these patients compared to controls have adhered to the endothelium. Neutrophils use leucocyte integrin molecules CR3 (complement receptor 3 CD18/CD11b), LFA-1 (leucocyte function associated antigen-1 CD18/CD11a), and to a lesser extent p150,95 CD18/CD11c to adhere to endothelium (240). An increase in function of these molecules, producing hyperadhesive states, is seen after stimulation of neutrophils - for example, by bacterial analogues such as FMLP (formyl-met-leu-phe) or inflammatory mediators such as tumour necrosis factor - causing the cytoplasmic granules containing preformed integrin molecules to fuse with the neutropil plasma membrane, rapidly increasing surface CR3 and p150,95 (243). The ligand on endothelium for LFA-1 induces ICAM-1 (intercellular adhesion molecule-1 CD45 and possibly ICAM-2 (244,245). The former is expressed at low levels on endothelium, and levels increase after stimulation by interleukin-1 or interferon-gamma treatment of the endothelium (246,247). CR3 and
p150, 95, however use an Arg-Gly-Asp sequence for adherence, and hence bind through the extracellular matrix molecules, such as fibronectin, which are rich in this sequence (248, 249).

The finding of degraded fibronectin after hyperadherence of neutrophils to endothelium from patients with HUS is therefore of fundamental importance. Forsyth and colleagues (235) have found that fibronectin degradation is an important marker of endothelial cell damage. It is likely that the activated hyperadherent neutrophils degranulate onto the endothelium, both causing degradation of the fibronectin by local release of proteases, and in certain cases allowing free granule constituents into the plasma. This degradation of fibronectin has important pathophysiological consequences, with the potential to induce an inflammatory amplification loop. The activity of fibronectin can be modulated by breakdown of its structure. Neutrophils will adhere more avidly to degraded fibronectin, and can degrade fibronectin to enhance their own adherence (250). For instance proteolytic digests of fibronectin, but not intact fibronectin, can induce concentration dependent degranulation, resulting in release of neutrophil elastase and lactoferrin (251). The cytoadhesive Arg-Gly-Asp sequence of fibronectin appears to be the element of degraded fibronectin that is both stimulatory and degranulatory for neutrophils (251). If
Chapter 6: Neutrophil-Mediated Endothelial Injury

The fibronectin within the extracellular matrix of the endothelial cells becomes degraded by the action of stimulated neutrophils, further neutrophil adhesion is induced, leading to further neutrophil degranulation and fibronectin degradation.

In an attempt to interrupt this amplification loop, the ability of a monoclonal antibody (CD18) directed against the common $\beta$ chain of the three leucocyte integrin molecules to inhibit neutrophil mediated fibronectin degradation of the endothelium has been assessed. Such antibodies to the $\beta$ chain of the leucocyte integrins partially block neutrophil adherence to endothelium (236). In 4 of 10 HUS patients the fibronectin degradation was primarily a neutrophil phenomenon, with little stimulatory effect from the plasma. In this group CD18 was able to inhibit completely fibronectin degradation, whereas in the group of patients whose plasma had a significant effect, the antibody could not prevent endothelial injury.

Utilising antibodies directed against the leucocyte integrins to modulate adhesion and leucocyte induced endothelial damage has been shown to be effective in a rabbit and canine model of neutrophil-mediated vascular injury (252,253). The CD18 antibody used here was effective in reducing endothelial damage in the less severe cases.
Table 6.1  Clinical and Laboratory Characteristics of HUS Patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yr)</th>
<th>Gender</th>
<th>--Diarrhoea--</th>
<th>Anuria Days</th>
<th>Duration of illness*</th>
<th>Hb g/dL</th>
<th>PMNL --x10^9/ℓ--</th>
<th>Plat µL</th>
<th>Urea mmol/L</th>
<th>PCr pmol/L</th>
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<td>1</td>
<td>10.1</td>
<td>20.6</td>
<td>21</td>
<td>20.8</td>
</tr>
</tbody>
</table>

*Days from presentation to local hospital with HUS to collection of blood specimens at GOS.

PMNL: polymorphonuclear leucocyte; Plat; platelets; PCr: plasma creatinine concentration.
Table 6.2. Disruption of Fibronectin Architecture on Cultured Endothelium and Effect of CD18 Antibody.

<table>
<thead>
<tr>
<th>Patients</th>
<th>Control PMNL/ control plasma</th>
<th>Control PMNL/ HUS plasma</th>
<th>HUS PMNL/ control plasma</th>
<th>HUS PMNL/ HUS plasma</th>
<th>HUS PMNL/control plasma/CD18</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<td>2</td>
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<tr>
<td>3</td>
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<td>0</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>Mean</td>
<td>0.2</td>
<td>1.7</td>
<td>2.3</td>
<td>2.8</td>
<td>1.5</td>
</tr>
</tbody>
</table>

A scoring system to quantitate the degree of fibronectin dissolution was used based on a subjective morphological assessment: 0 = normal fibronectin architecture; 1 = mild disruption; ranging to 3 = extensive disruption with no recognisable fibronectin architecture remaining.

Neutrophils (PMNLs) from HUS patients degraded endothelial fibronectin in all cases. In patients 1, 2, 5 and 6 where there was minimal fibronectin degradation with plasma, but co-incubation of PMNLs with a CD18 antibody inhibited the fibronectin degradation.

CD18 - monoclonal antibody directed against common β chain of adhesion molecules on PMNLs.
Fig. 6.1: % neutrophils adhering to endothelium from HUS compared with control of the day.
CHAPTER 7: ELASTASE AND NEUTROPHIL ACTIVATION IN HUS

7.1 Introduction

The observations in Chapter 6 and other experimental studies (242,254) suggest that the neutrophil is of prime pathophysiological importance in disorders, such as HUS, associated with endothelial cell damage. Activated neutrophils are capable of endothelial injury (146-8), and their release products can damage glomerular basement membrane (149). Elastase is the major lysosomal proteinase liberated by activated polymorphonuclear leucocytes (PMNLs) (255), and is complexed in the circulation by antiproteinases of which the most important is α1-antitrypsin (α1-AT) (256). In this study PMNL activation in HUS is evaluated by the measurement of free and α1-AT complexed elastase in plasma.

7.2 Patients and Methods

7.2.1 Patients

Fifty-six children with HUS treated at the HSC,GOS were studied. The criteria for diagnosis were a microangiopathic haemolytic anaemia, thrombocytopenia, and acute renal failure. They were divided into two groups
on the basis of the presence (n=47) or absence (n=9) of a diarrhoeal prodrome. Samples were taken within 24 hours of admission to hospital, and 5 children also had samples taken throughout the acute phase of the illness.

7.2.2 Controls

Blood samples were also obtained from:

1. Thirty-five healthy children being followed up after routine surgical procedures such as hernia repair or being investigated for short stature.

2. Twenty-one inpatients identified by the haematology laboratory as having a PMNL count greater than $10 \times 10^9/L$ and defined as high PMNL count controls; they either had a septicaemic illness or other infection and all had a normal plasma creatinine concentration, blood samples were obtained within 48 hours of admission and prior to or within 24 hours of instituting therapy.

3. Thirty-five children with chronic renal failure who had a glomerular filtration rate less than 15 ml/min/1.73 m² SA.
Chapter 7: Elastase and Neutrophil Activation

7.2.3 Methods

Blood samples were collected into potassium EDTA (ethylenediaminetetraacetic acid) tubes, and the plasma separated by centrifugation at 2000g within 30 minutes and stored at -70°C until assayed.

(a) *α1-AT Complexed Elastase Enzyme-Linked Immunosorbent Assay (ELISA).* A modified assay for the measurement of α1-AT complexed elastase was used (257). The first antibody was sheep anti-human elastase antibody (ICN Immuno biologicals, High Wycombe, England) which specifically recognises leucocyte elastase found in the azurophilic granules of human neutrophils. The antiserum was produced by immunising sheep with purified protein obtained from the peripheral leucocytes of a patient with chronic myeloid leukaemia. Microtitre plates (Nunc-Immunoplate Gibco BRL, Paisley, Scotland) were coated with 100 μL antiserum (10 μg/ml) in bicarbonate buffer (50 mM, pH 9.6) by incubation overnight at 4°C. All subsequent incubations were carried out at room temperature with volumes of 100 μl and the plates were always washed four times with 150 mM Dulbecco's phosphate-buffered saline pH 7.2 (PBS) containing 0.1% 'Tween 20' (BDH Limited, Porte, England) between stages. Standards or samples diluted 1:80 in PBS were then added and incubated for two hours. The second antibody, horseradish-peroxidase-conjugated sheep
anti-human α1-AT (The Binding Site Limited, Birmingham, UK) diluted 1:1000 in PBS, was added to each well and incubated for two hours. The substrate (1.0 mg/ml O-phenylenediamine and 0.03% H₂O₂ in 100 mM citrate-phosphate buffer, pH 4.0) was added and incubated for 20 minutes. The reaction was stopped by the addition of 50 µl of 2 M H₂SO₄. The absorbance was measured at 492 nm in Titertek multiscan plus (Flow Laboratories Limited, Rickmansworth, England) ELISA reader. Standards were obtained by adding increasing amounts of reconstituted elastase 2.5 µg/ml in PBS to a constant volume of control plasma, giving final elastase concentrations of 50-16,000 ng/ml. A typical standard curve obtained is shown in Fig. 7.1. The control plasma was harvested on a single occasion, aliquoted into small portions, and frozen at -70°C. The concentration was expressed as a function of the added elastase. The units obtained by these standards are arbitrary because of the unknown concentration of α1-AT initially present in the control plasma.

(b) Free Elastase. Free elastase activity was measured enzymatically using a method adapted from Johnson et al (258,259). The synthetic peptide methoxysuccinyl-ala-ala-pro-val-p-nitroanilide (MSAAPV-pNA) (Sigma Ltd, Porte, England) was used as substrate.
This substrate is specific for human leucocyte elastase and does not recognise pancreatic elastase. Sample activities were determined by reference to a standard curve obtained with human leucocyte elastase (Sigma) made up in a 100 μl of 100 mM HEPES (N₂-hydroxyethylpiperazine-N₂-ethanesulphonic acid) buffer pH7.5 containing 0.5 M NaCl and 10% dimethylsulphoxide added to 1 ml of 1 mM substrate made up in the same buffer. One unit was defined as the change in absorbance of the optical density at 410 nm of 0.001/min at 25°C over 10 minutes. The elastase standard concentrations ranged from 1 to 400 ng/ml. A concentration of elastase of 1 ng/ml produced a change in optical density of 0.16/10 minutes, which was significantly greater than 0.04/10 minutes of the blank buffer.

7.2.4 Statistical Analysis

Data were tested for normal or log normal distribution using normal probability plots. Means were compared by Student's t-test and regression analysis was performed using an ordinary least square regression using one independent variable for a linear model.
7.3 Results

7.3.1 α1-AT Complexed Elastase Levels in Different Groups

Plasma α1-AT complexed elastase in children was log normally distributed with a geometric mean of 0.29 μg/ml and range (± 2 SD) of 0.11-0.76 μg/ml (Table 7.1 & Fig 7.2). Complexed elastase in D+ HUS was 2.54 (0.62-10.5) μg/ml and in D- HUS 1.13 (0.14-9.0) μg/ml, both significantly greater than the control group (D+ p<0.0001; D- p<0.0001). Mean values for D+ and D- HUS patients were not significantly different, but the D- patients were heterogeneous: 3 had markedly raised complexed elastase with levels above 1.0 μg/ml, while 6 had levels which were within the normal range. Children with a raised PMNL count had a geometric mean bound elastase of 0.57 (0.14-2.30), significantly less than in D+ HUS (p<0.0001) and not significantly higher than normal controls. In children with chronic renal failure the mean α1-AT complexed elastase was 0.29 μg/ml, similar to normal controls.

7.3.2 Serial α1-AT Complexed Elastase Levels and PMNL Counts

Serial measurements of α1-AT complexed elastase were undertaken in 5 patients with D+ HUS following their admission in acute renal failure. On day 1 the geometric mean of the complexed elastase in these patients was 2.55
Chapter 7: Elastase and Neutrophil Activation

μg/ml. The level peaked at a geometric mean of 4.25 μg/ml on day 2 and subsequently fell to 2.23 μg/ml on day 3 and 1.36 μg/ml on day 5. From day 7 onwards the values were within the normal range (Fig 7.3a). The mean PMNL count was 18.3 \times 10^9/l on day 1 and 17.3 \times 10^9/l on day 2, and remained elevated over a longer period than the complexed elastase: on day 5 the mean PMNL count was still markedly raised (20.4 \times 10^9/l) and only returned to the normal range at day 11 (Fig 7.3b).

7.3.3 PMNL Counts in Different Groups

The children with D+ HUS had a raised PMNL count at presentation, confirming the finding of Walters et al (142). Their mean PMNL count was 11.7 ±6.6 \times 10^9/l (SD), significantly higher than the PMNL count of 6.0 ±1.2 \times 10^9/l in normal controls (p<0.001) and 5.1 ±1.9 \times 10^9/l in D- HUS (p<0.001) (Fig.7.4).

7.3.4 Correlation between α1-AT Complexed Elastase and PMNL Counts

There was a significant positive correlation between α1-AT complexed elastase and PMNL count in the D+ HUS group (r=0.74, p<0.01) but not in any of the other groups (Fig.7.4).
7.3.5 Free Elastase Activity

The change in absorbance over 10 minutes produced with plasma from 20 D+ HUS children ranged from 0.04 to 0.12, and from 20 normal children ranged from 0.04 to 0.10. These results were below our lower limit of detection of 1 ng/ml, confirming that no free elastase could be detected in these children.

7.4 Discussion

There is increasing evidence for PMNL-mediated endothelial injury in HUS (150,242,260). The collaborative work reported in Chapter 6 demonstrated that HUS neutrophils have greater adherence to endothelial cells in culture than control neutrophils, and that these neutrophils can induce endothelial injury assessed morphologically by degradation of endothelial fibronectin. It is postulated that activated PMNLs degranulate onto endothelium causing damage by local release of proteases such as elastase (261). Neutrophils can oxidatively inactivate the main elastase inhibitor in plasma, α1-antitrypsin, and so allow rampant elastase activity despite adequate plasma levels of the inhibitor (262).

In this study PMNL elastase was measured, as a marker of neutrophil activation, in HUS. Free elastase
activity could not however be detected in the plasma of patients in the acute phase of the disease. In contrast, Kaplan et al (260), using an enzymatic method, reported raised elastase activity in D+ HUS serum compared to normal controls, but they used N-succinyl-ala-ala-ala-p-nitroanilide as substrate, which is not specific for leucocyte elastase and may also be broken down by pancreatic elastase. It is unlikely that neutrophil elastase, which is a very potent proteinase, exists free in the circulation, as it rapidly becomes bound to cell surfaces or plasma inhibitors, primarily α1-AT.

α1-AT is the major component of the α1 electrophoretic band of human plasma proteins and is an inhibitor of proteolytic enzymes. It is primarily a defence protein whose function is to protect the tissues from released proteolytic enzymes. It is synthesised in the liver and secreted into the plasma. As an acute phase reactant its normal plasma concentration can rise fourfold in response to the general stimulus of inflammation (263). It may be that free elastase exists early in the disease process before the upregulation of α1-AT production, but there is no evidence for this hypothesis. When measured in its α1-AT complexed form, elastase levels were found to be markedly raised in HUS. There was a significant increase in complexed elastase in both D+ and D- HUS relative to the 3 control groups - normal children, those in chronic
renal failure and those with high PMNL counts secondary to other febrile illnesses. A positive correlation between the complexed elastase levels and the PMNL counts was found in the D+ HUS group of children. This correlation is of particular importance when considering that the outcome of this disorder is associated with the PMNL count at presentation (142-4). Milford et al (264) using a radioimmunoassay for human neutrophil elastase, also found raised levels; their assay was such that both free and bound elastase would have been measured.

Some of the D- HUS patients also had elevated α1-AT complexed elastase levels, yet their PMNL counts were within the normal range. The explanation for this is unclear, and reinforces the heterogeneity of this subgroup.

The sequential data on the 5 patients with D+ HUS show that high complexed elastase levels at presentation fall to normal within a few days. The corresponding PMNL counts, however, remain elevated for a longer period. This suggests that neutrophil activation occurs early in the disease process and that it is not the leucocytosis per se which reflects disease activity. There is nevertheless some correlation between the PMNL count and bound elastase levels in the D+ HUS group. A significant correlation was also observed when the normal and high PMNL controls were
analysed together; the slope of the regression line obtained was not significantly different from that of the D+ HUS group but its origin was several orders of magnitude less. Thus, although α1-AT complexed elastase may reflect the PMNL count, there is a clear upward shift of this relationship in D+ HUS. This shift may reflect the markedly elevated α1-AT levels in D+ HUS as demonstrated by Bergstein et al (127) and Kaplan and Mills (260).

The evidence supporting PMNL involvement in the pathogenesis of D+ HUS thus far can be summarised as follows:

(1) the raised peripheral blood PMNL count at presentation (142);

(2) the association of a high PMNL count with the development of neurological complications as shown in Chapter 4;

(3) the association of a high PMNL count at presentation with acute mortality and residual nephropathy as shown in Chapter 5;

(4) the evidence that HUS PMNLs are hyperadherent to endothelium and cause endothelial damage as illustrated in Chapter 6;

(5) and the finding reported in this Chapter that α1-AT complexed elastase levels are raised at presentation in HUS reflecting neutrophil activation and degranulation.
<table>
<thead>
<tr>
<th>Group</th>
<th>Number</th>
<th>PMNL $\times 10^9$/l</th>
<th>$\alpha_1$-AT complexed elastase ($\mu$g/l)</th>
<th>Geometric mean</th>
<th>Range (±2SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>D+ HUS</td>
<td>47</td>
<td>11.7±6.6</td>
<td>2.54*</td>
<td>0.62-10.5</td>
<td></td>
</tr>
<tr>
<td>D- HUS</td>
<td>9</td>
<td>5.1±1.9</td>
<td>1.13*</td>
<td>0.14-9.0</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>35</td>
<td>6.0±1.2</td>
<td>0.29</td>
<td>0.11-0.76</td>
<td></td>
</tr>
<tr>
<td>High PMNL</td>
<td>21</td>
<td>17.1±4.4</td>
<td>0.57</td>
<td>0.14-2.30</td>
<td></td>
</tr>
<tr>
<td>CRF</td>
<td>35</td>
<td>-</td>
<td>0.29</td>
<td>0.10-0.85</td>
<td></td>
</tr>
</tbody>
</table>

AT: Antitrypsin; CRF: Chronic renal failure; D+: Diarrhoea-associated; D-: Non-diarrhoea-associated; HUS: Haemolytic uraemic syndrome; PMNL: Polymorphonuclear leucocyte; SD: Standard deviation.

*p<0.001 vs normal.
Fig. 7.1: Standard curve $\alpha$1-AT complexed elastase ELISA.
Fig. 7.2: Plasma α1-AT complexed elastase levels in different groups.
Figs. 7.3 a & b: Serial α1-AT complexed elastase and PMNL counts in 5 patients with D+ HUS following admission.
Fig. 7.4: Correlation between α1-AT complexed elastase and PMNL count in the different groups.
CHAPTER 8: INTERLEUKIN-8 AND NEUTROPHIL ACTIVATION IN HUS

8.1 Introduction

In this chapter, possible mechanisms of PMNL involvement in D+ HUS are explored in a study measuring potential mediators and known markers of PMNL activation in the serum and plasma of children with D+ HUS. Two key participants in the cytokine cascade: tumour necrosis factor α (TNF α) and interleukin-8 (IL-8) were measured. TNF α is a proinflammatory cytokine and a proximal mediator in the cascade having the ability to induce gene expression for other cytokines and receptors such as IL-1, IL-6, IL-2 receptor and IL-8 (265,266). IL-8 is a cytokine which is produced by monocytes and is primarily defined as a selective activator and chemoattractant of PMNLs; it stimulates the release of lysosomal enzymes and superoxides from them (267). In this study, the release of IL-8 into the serum in the acute phase of D+ HUS was investigated and its relationship to the peripheral blood PMNL count; PMNL α-AT-E was measured as a marker of PMNL degranulation (256,268,269) and antineutrophil cytoplasmic antibody (ANCA) which is present in other vasculopathies (270-73).
Chapter 8: IL-8 and Neutrophil Activation

8.2 Patients and Methods

8.2.1 Patients

Thirty-four children with HUS treated at the HSC, GOS were studied. The criteria for diagnosis were a microangiopathic haemolytic anaemia, thrombocytopenia, and acute renal failure. They were divided into two groups on the basis of the presence (D+ HUS; n=25) or absence (D- HUS; n=9) of a diarrhoeal prodrome. Three of the children with D+ HUS died within four days of admission to the Unit. Blood samples were taken within 24 hours of admission, and nine children also had serial samples taken throughout the acute phase of their illness.

8.2.2 Controls

Blood samples were also obtained from:

(1) seventeen healthy control children admitted for routine surgical procedures or investigation of short stature; and

(2) fifteen sick children identified by the haematology laboratory as having a PMNL count > 10 x 10⁹/1, designated high PMNL count controls, they had a septicaemia or other infection, and all had a normal plasma creatinine concentration. Blood samples were obtained within 48 hours of admission.
Ten children with chronic renal failure were included, all of whom had a glomerular filtration rate (GFR) less than 15 ml.min/1.73m² SA.

8.2.3 Samples

Blood samples were collected for measurement of IL-8, TNF α, and ANCA into plain tubes with no anticoagulant and for α1-AT-E into potassium EDTA tubes. The samples were separated by centrifugation at 2000g within 30 minutes and serum and plasma stored at -70°C until assayed.

8.2.4 Methods

(a) Serum IL-8 Immunoassay. IL-8 was measured by solid phase enzyme linked immunoassay (ELISA; Quantikine, Research and Diagnostic Systems, Minnesota, USA). A monoclonal antibody specific for IL-8 was coated onto the microtitre plates provided. Standards with known amounts of IL-8 and samples were pipetted, in duplicate, into the wells, and any IL-8 present bound by the immobilised antibody. For detection, an enzyme linked polyclonal antibody specific for IL-8 was applied. A substrate solution containing tetramethylbenzidine and H₂O₂ was added, and the colour developed was proportional to the amount of IL-8 bound in the initial step. A standard curve
was drawn of optical density (OD) against IL-8 concentration (Fig. 8.1). The lower limit of detection in this assay was 50 pg/ml. This was determined by adding 2 standard deviations to the mean optical density value of 5 zero standard replicates and calculating the corresponding concentration from the standard curve. The intra-assay coefficient of variation (CV) was 8% (n=10) and the interassay CV was 9.3% (n=5).

(b) Serum TNF α Immunoassay. TNF α was measured by solid phase ELISA (Quantikine). A monoclonal antibody specific for TNF α was coated onto the microtitre plates provided. Standards with known amounts of TNF α and samples were pipetted, in duplicate, into the wells. For detection, an enzyme-linked polyclonal antibody specific for TNF α was applied. A substrate solution was added, as above, and a standard curve drawn of OD against the concentration of TNF α in the standard wells. The lower limit of detection for this assay was 10 pg/ml. The intra-assay CV was 5.4% (n=10) and the inter-assay CV 4% (n=5).

(c) Human α1-AT Complexed Elastase Immunoassay. Plasma α1-AT-E was measured by a modified ELISA as described in Chapter 7 (Section 7.2.3 (a)).
(d) Immunoassay for ANCA against an Acid Extract of Neutrophil Cytoplasm. IgM and IgG ANCA were measured in serum by an ELISA technique using acid extracted neutrophil cytoplasm antigen prepared as described by Savage et al (274). For each preparation, the optimal dilution for coating was determined using negative control sera and positive patient sera. The antigen was coated onto microtitre plates (Nunc). Test or control serum samples were added in duplicate to neutrophil antigen-coated plates. For detection, peroxidase conjugated anti-human IgG and IgM (Sigma) were applied, and after incubation and washing a substrate was added. After further incubation the reaction was stopped and ODs measured using an ELISA reader. The OD values of the standard sera at various dilutions were used to construct a standard curve of arbitrary units, from which the ANCA content of each test serum could be estimated. The arbitrary units were set as 64 and 32 for the IgG class ANCA-positive standard serum and IgM class ANCA-positive standard serum, respectively. The normal range for IgM for this assay is 0.5 to 9.1 units and for IgG 0.5 to 9.0 units.
8.2.5 Statistical Analysis

Fisher's Exact test was used to determine the significance of the IL-8 results in the different groups compared to normals. The Mann-Whitney test was used as a test of significance of difference between the medians of the different groups. The correlations between IL-8 and α1-AT-E, and IL-8 and PMNL count were examined non-parametrically using Spearman's rank correlation coefficient, α1-AT-E and PMNL counts were log-normally distributed, and geometric means were compared using Student's t-test on log-transformed data.

8.3 Results

8.3.1 Serum IL-8

IL-8 was not detected in the serum of 17 normal healthy children. It was, however, detected and was significantly elevated in 20 of 25 children with D+ HUS (median value: 305 pg/ml, range: non-detectable (ND) - 3300, p<0.005), and in three of nine D- HUS children (p<0.05; Fig 8.2). The median IL-8 value in D+ HUS was significantly higher than in D- HUS (p<0.005). Only two of ten children in chronic renal failure (CRF) had detectable IL-8 levels, and this did not reach significance (p>0.05).
Children in the high PMNL control group had significantly elevated IL-8 with a median value of 460 pg/ml (range 150 to 5200, p<0.005), and these results were not significantly different from those obtained in the D+ HUS group (p>0.05).

### 8.3.2 Serum TNF α

When 40 normal donor sera were evaluated using this assay (Quantikine) the maximum observed value was 25.8 pg/ml. TNF α was detected in the serum of only 1 of 16 of the children with D+ HUS, in whom the level was 1800 pg/ml. Moderately elevated levels were detected in three of 15 high PMNL controls with values of 27, 28 and 30 pg/ml recorded, and no significant activity was detected in any of the normal children or those with D- HUS.

### 8.3.3 Serum ANCA

No IgM ANCA was detected in any of the 10 D+ HUS or 9 D- HUS sera tested. One of the D- HUS group had a weakly positive IgG ANCA of 13.1 units; the remaining 8 D- and all the D+ HUS children had negative IgG ANCA results.
8.3.4 Serial IL-8 and α1-AT-E

Serial measurements of IL-8 were undertaken in nine children with D+ HUS following their admission in acute renal failure, and in seven of them α1-AT-E was also measured. This data is graphically illustrated in Figs. 8.3 and 8.4. In this group of patients the serum IL-8 reached a maximum on day 3 with a mean of 8,193 pg/ml, and then fell sharply to mean values of 272 and 312 pg/ml on days 4 and 5, respectively. α1-AT-E peaked on days 3 to 4 with mean values of 5,100 and 4,200 ng/ml, respectively, but then declined more gradually. The highest values for both IL-8 and α1-AT-E were seen in the three children who died in the acute phase of the disease (Figs. 8.3 and 8.4). A significant positive correlation was found between the IL-8 and the circulating α1-AT-E levels (r=0.50, p<0.05) in these children (Fig. 8.5).

8.3.5 Polymorphonuclear Leucocyte Counts

The children with D+ HUS had a raised PMNL count at presentation with a geometric mean of 14.0 x10^9/l (range 7.6 to 24.3). Their mean PMNL count was significantly higher than the PMNL count of 4.9 x10^9/l (2.7 to 6.8) in normal controls (p<0.001) and 5.7 x10^9/l (4.6 to 7.2) in D- HUS (p<0.001). Serial PMNLs on eight of the nine patients are illustrated in Fig. 8.6; they remain elevated
over a longer period than both the IL-8 and the α1-AT-E, returning gradually into the normal range by days 11 to 12.

8.3.6 Correlation Between IL-8 and α1-AT-E

A significant positive correlation was found between the PMNL count and the IL-8 level at presentation in the D+ HUS group \( r=0.63, p<0.005 \), but not in the high PMNL group \( r=0.34, p>0.05 \) (Fig. 8.7).

8.4 Discussion

As discussed in Chapter 1, the aetiology of D+ HUS is more clearly understood now that an association has been found with toxin-producing enteric pathogens, in particular Verocytotoxin-producing \textit{Escherichia coli} (VTEC) (53,54). It is also well recognised that damage to the glomerular endothelium is the main site of injury in HUS, and there is now increasing evidence suggesting that the PMNL has an important role in the pathogenesis. HUS PMNLs have been shown to have increased adhesion to endothelium in vitro (Chapter 6), α1-AT complexed elastase levels are increased in the acute phase of the syndrome (Chapter 7), and by electron microscopy peripheral blood neutrophils appear degranulated (264).
It is not known whether the neutrophil response which occurs is attributable to the action of verocytotoxin or to endotoxin, the lipopolysaccharide (LPS) derived from the bacterial cell wall. Endotoxin has been suggested as having a pathogenic role in D+ HUS but has not yet been studied in VTEC disease. It alters the surface characteristics of endothelial cells via a cascade of cytokines which renders them adhesive for neutrophils (275,276).

Significant TNF α was detected in only one of 16 D+ HUS children tested; this child was admitted in the early phase of her disease before the onset of acute renal failure. She subsequently went into renal failure and developed neurological complications. She survived her acute illness but has residual nephropathy with proteinuria and a reduced GFR. TNF α is a proximal mediator in the cytokine cascade, appearing in the circulation of several species as a brief, early peak after infusion of bacteria or bacterial LPS (265). The majority of patients in this study were referrals from other hospitals admitted to the Unit several days after the onset of their illness, which may account for the inability to detect TNF α in the others at the time of their admission.
Chapter 8: IL-8 and Neutrophil Activation

IL-8 is a recently described 6-10 kDa protein known for its in vitro leucocyte chemoattractant and activation properties (277-284), and is a potential mediator of host response to injury and infection. It has several properties that suggest that it plays a role in mediating some of the inflammatory responses of neutrophils induced by endotoxin, IL-1 and TNF α (267). It is known to cause shedding and upregulation, respectively, of the two important neutrophil adhesion molecules: leucocyte adhesion molecule 1 (also known as LAM-1, LECAM-1 and L-selectin) and CD11b/CD18 (285-287). Adherence via the latter has been shown to mediate neutrophil priming for the respiratory burst (288) and degranulation (289). It has been shown that IL-8 appears in the circulation of primates in vivo during septic shock, sublethal endotoxaemia, and after administration of IL-1 α (290); more recently increased levels have been detected in normal humans as part of the acute inflammatory response to intravenously administered endotoxin (291).

IL-8 was significantly elevated in the serum of both D+ and D- HUS patients and in the high PMNL count controls. IL-8 levels were significantly higher in D+ than in D- HUS. This study therefore demonstrates that IL-8 is produced in vivo as part of the inflammatory response occurring in humans during the acute phase of D+ HUS. The sequential data on nine children with D+ HUS shows that
IL-8 circulates in blood with peak levels occurring at 72 hours.

Serial measurements of α1-AT-E were undertaken on seven of the nine patients. Plasma α1-AT-E levels peaked at 72 to 96 hours and declined more slowly than the corresponding IL-8 levels. From this data IL-8 appears to peak more transiently in the circulation, achieving a maximum level just before the more protracted burst of complexed elastase. The correlation of IL-8 with α1-AT-E suggests that IL-8, which is known to stimulate degranulation of adherent neutrophils, may be promoting the release of this proteolytic enzyme in these patients. A positive correlation was also found between IL-8 and the PMNL count at presentation in the D+ HUS group. This correlation is of particular importance when considering that the outcome is associated with the PMNL count at presentation (142-144), and indeed the higher levels of IL-8 were recorded in three patients who died during the acute phase of the disease.

From this data it cannot be concluded whether the observed release of IL-8 into serum in D+ HUS is induced by Verocytotoxin or high LPS levels. It is also unknown whether serum IL-8 represents a pathogenetically important mechanism or is a concommitant marker of disease activity. There is, however, increasing evidence suggesting that IL-
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8 promotes leucocyte adhesion in vivo and leads to recruitment of PMNLs to sites of tissue inflammation (285,286). IL-8 has previously been shown to induce granulocytosis upon systemic injection and skin reaction upon local injection in experimental animals (292). The recruitment of PMNLs to inflammatory sites is also dependent on other chemotactic factors generated in tissue, such as the cleavage products of complement activation C5a and C3b. Elevated elastase levels do indicate that granulocytes become highly activated in this disease and may represent a source of endothelial cell damage. The findings in this study are consistent with the hypothesis that IL-8 contributes to the dynamics of circulating PMNLs and acts as a mediator of the PMNL activation which occurs. The conclusion of this Chapter is that IL-8 participates in the complex cascade of cytokine responses to infectious and inflammatory stimuli, and plays a significant role in the pathophysiology of D+ HUS.

Significant ANCA activity was not detected in HUS serum and there is therefore no evidence here to implicate a role for the antibody in this disease.
Fig 8.1: Serum IL-8 immunoassay standard curve.
Fig. 8.2: Serum IL-8 levels in the different groups.
Figs. 8.3 & 8.4: Serial IL-8 and α1-AT complexed elastase levels in D+ HUS.
Fig. 8.5: Correlation between IL-8 and α1-AT complexed elastase.
Fig. 8.6: Serial PMNL counts in 8 D+ HUS cases.
Fig. 8.7: Correlation between IL-8 and PMNL count in D+ HUS and high PMNL count controls.
9.1 Principle Observations

In this thesis the 208 children with haemolytic uraemic syndrome presenting to the Hospital for Sick Children, Great Ormond Street, between 1966 and 1992 have been reviewed (Chapter 2). One hundred and eighty six (89%) of them had an illness associated with a diarrhoeal prodrome (D+ HUS) with a mortality of 8%. Central nervous system involvement was evident in 23 of 150 (15%) D+ HUS children presenting between 1980 and 1992 and was associated with a raised PMNL count, an increased mortality and a greater renal morbidity (Chapter 4). Evidence of impaired renal function was identified in 34/88 (39%) D+ HUS children investigated between 5 and 21 years after presentation (Chapter 5). Twenty children with idiopathic HUS not associated with a diarrhoeal prodrome (D- HUS) presented since 1966. The prognosis for this group was bad, although treatment with plasma exchange provided some evidence of benefit (Chapter 3).

Evidence of PMNL involvement in D+ HUS first came from a study by Walters et al (142) of a subset of patients from this series, in which a raised PMNL count was associated with a poor outcome. In Chapter 4 of this
thesis the relevance of PMNL count to CNS disease in D+ HUS was confirmed. A study undertaken in collaboration with Dr KD Forsyth (Chapter 6) tested the hypothesis that PMNLs from HUS patients are activated and damage endothelium through release of their intracellular contents. The proportion of HUS PMNLs adhering to endothelium was twice as high as for PMNLs from controls. In addition, HUS PMNLs induced endothelial injury, assessed morphologically by degradation of endothelial cell fibronectin.

Evidence of PMNL activation with degranulation and release of proteases was demonstrated in Chapter 7 with the finding that α1-AT complexed elastase was raised at presentation in HUS. In Chapter 8 data were presented suggesting that PMNLs in HUS are recruited by IL-8 and that this cytokine plays a key role in the PMNL activation in HUS.

9.2 Outstanding Issues in D+ HUS

In Chapter 1 the association of D+ HUS with verocytotoxin was reviewed and possible mechanisms of endothelial cell injury discussed. The observations and data in this thesis raise several questions with regard to pathogenesis.

Is verocytotoxin alone sufficient explanation for the development of HUS? Not all individuals infected develop the disease. Does this therefore represent a dose
effect? Given the age distribution of the disease, it may be that antibody protection plays a part. Other toxins, specifically endotoxin, the lipopolysaccharide (LPS) derived from the bacterial cell wall, may be involved in the process and indeed the potential role of endotoxin is an important area for further research.

The evidence for PMNL involvement, as opposed to a 'bystander reaction' in D+ HUS is compelling, but the precise role of PMNLs in pathogenesis remains unclear. PMNLs are activated via a complex system of interactions between cell surface glycoproteins, cleavage products of complement activation, and components of the cytokine cascade. The main stimulus for PMNL production in this disorder is unknown: LPS and verocytotoxin have a potential role and our data suggest that IL-8 also plays a part. A major role for IL-8 has now been proposed in the regulation of PMNL transendothelial migration (286) and thus, potentially, the control of PMNL-mediated tissue injury. Endotoxin and the proinflammatory cytokines TNFα and IL-1 may be involved in this process at an earlier stage than we have been able to detect with our studies to date.

The consequences of PMNL margination and activation in HUS remain unclear. It is known that endothelial functional integrity, which is disrupted in this disorder, can be damaged by release from adherent PMNLs of proteases such as elastase, which can degrade collagen, elastin,
proteoglycan and fibronectin (293). The relationship between PMNL activation, platelet activation, and intravascular thrombosis which characterise HUS is not understood, though many pathogenic pathways are possible.

9.3 Therapeutic Possibilities

The studies reported in this thesis directly implicate PMNLs in the endothelial damage in HUS. It is unlikely that the findings of PMNL-mediated vascular injury are unique to HUS. It is probable that in other acute inflammatory states where there is neutrophil activation, such as sepsis, part of the vascular damage is attributable to the PMNL.

The investigation of the molecular and cellular basis of other acute inflammatory states, such as meningococcal disease, has led to novel approaches to treatment based on the modulation of the inflammatory process. A number of experimental strategies have been pursued, which include:

1) The administration of antibodies directed against the lipid A and core polysaccharide components of endotoxin (294).

2) The application of agents which directly bind endotoxin such as taurolin and polymixin B (295).

3) The use of antibodies and soluble receptors to block the effects of the important inflammatory mediators.
4) The development of drugs aimed at reducing the effects of cytokine stimulation of PMNLs such as pentoxiphylline (297).

5) The modulation of leucocyte-endothelial adhesion by antibodies which block the common β2-integrin sub-unit, CD18 (298).

While all of these approaches have shown promise in experimental models, and small numbers of patients, only antibodies to endotoxin have been tested in formal trials (294,298). Human monoclonal immunoglobulin M (Ig M) to the lipid A domain of endotoxin derived from a mutant strain of *E. coli* has recently been shown to reduce mortality in adult patients with Gram-negative septicaemia. It is essential that these and other monoclonal antibodies are subjected to rigorous clinical trials before their widespread introduction.

Controlled trials of therapy in HUS have been notoriously difficult, in part because of failure to distinguish between D+ and D- subgroups, but more particularly because of the need to exclude from such trials those patients with the likelihood of a good outcome. Potential trials have therefore been blighted from both a clinical point of view and in terms of the statistical requirement to increase the power of the study. Despite these reservations, and although there is still much experimental work yet to do, our studies
suggest that targeting PMNL function and adhesion to endothelium early in the course of this disease before extensive PMNL activation and degranulation have occurred, may have important therapeutic possibilities.

The data reported here emphasise the important prognostic significance of a raised PMNL count at presentation and its association with neurological complications, a higher mortality and residual renal morbidity. The therapeutic use of fresh frozen plasma, prostacyclin and plasma exchange do not appear to have altered the prognosis for this group of patients, but agents now being developed which are capable of altering PMNL function may have a therapeutic role in children with HUS in the future.
9.4 D- HUS

Idiopathic D- HUS is a much less common disorder than D+ HUS. Systematic studies have been and remain difficult to undertake because of the rarity and heterogeneity of the disease. In contrast to D+ HUS in which considerable progress has been made, the aetiology and pathogenesis of D- HUS remains unclear. It is most likely to be genetically determined. The prognosis in D- HUS remains poor with high mortality and morbidity. Moreover, our experience of plasma exchange in this disease suggests that its effect in improving immediate survival only postpones the inevitable relapsing nature of the condition. What is more, the use of plasma exchange is not unproblematic and carries with it the possibility, albeit slight, of infection with contaminated blood products.
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Atypical (non-diarrhea-associated) hemolytic-uremic syndrome in childhood

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We describe the clinical and laboratory features of 20 children who were seen during the past 20 years with idiopathic nondiarrhea-associated hemolytic-uremic syndrome. There was no seasonal variation in time of onset; a genetic predisposition seemed likely in two of the cases. The prodromal illness was nonspecific and by definition did not include diarrhea. Hypertension was a major problem in the majority of the patients. Five died, three during the initial illness; four are in end-stage renal failure, and all but two of the survivors have residual nephropathy. Eleven patients had a "relapsing" course; up to eight additional documented episodes of hemolytic-uremic syndrome occurred in individual patients. Of the nine children treated before 1980, three died shortly after onset, two never recovered function after the initial illness, one had a relapsing course and died later, and one had residual nephropathy. Plasma exchange was introduced for the management of non-diarrhea-associated hemolytic-uremic syndrome in 1980; since then, all of the 11 patients have recovered function after the initial episode, but 10 of them had relapses. It appears that with the introduction of plasma exchange there has been an improved outcome in the initial phase, but the survivors tend to have relapses. Atypical (non-diarrhea-associated) hemolytic-uremic syndrome is a heterogeneous yet distinct subgroup of hemolytic-uremic syndrome that differs from diarrhea-associated hemolytic-uremic syndrome on epidemiologic, clinical, laboratory, histologic, and prognostic grounds. (J Pediatr 1993;122:532-7)

Evidence from clinical, epidemiologic, pathologic, and pathophysiologic studies suggests that hemolytic-uremic syndrome is not a single disease entity but a clinical syndrome resulting from a variety of disease processes. Two principal subgroups have been identified in children: typical cases with a diarrheal prodrome (sudden onset in young children and good prognosis) and atypical cases with an insidious onset (no antecedent diarrhea, a tendency to relapse, and a worse prognosis). This latter subgroup is itself heterogeneous: some are familial, and some are clinically and pathologically indistinguishable from thrombotic thrombocytopenic purpura. There is evidence of prostacyclin deficiency in some of these patients.

In this article we describe the clinical and laboratory
features of 20 children who were seen during the past 20 years at the Hospital for Sick Children, London, with idiopathic non-diarrhea-associated HUS.

**METHODS**

**Patients.** Between 1968 and 1991 inclusive, 192 children with HUS, defined as the simultaneous occurrence of a microangiopathic hemolytic anemia, thrombocytopenia, and acute renal failure, were referred to the Hospital for Sick Children, London. Of these children, 169 (88%) had prodromal diarrhea and 23 (12%) did not. Three of the patients with D^- HUS had documented infections in association with the disease that excluded them from the idiopathic group. One patient had a fatal illness with Streptococcus pneumoniae pneumonia and bacteremia and thus was in the subgroup of neuraminidase-associated HUS.12-14 The second patient had a Mycoplasma pneumoniae infection complicated by an anti-p autoimmune hemolytic anemia, and the third had a group B beta-hemolytic streptococcal septicemia. The clinical and laboratory features of the remaining 20 children with idiopathic D^- HUS, constituting 11.7% of the total population with HUS, have been tabulated.10 Ten patients were boys and 10 were girls, with a median age of 4.5 years (range, 0.2 to 13.5 years). Seven of the group (35%) were less than 1 year and 6 (30%) were more than 5 years of age at presentation. Five patients (25%) were from Asian (n = 4) or African (n = 1) families, all but one of whom were residents in the United Kingdom at the time of onset of the illness. Only one of the patients had affected siblings; this child and one other had consanguineous parents. In none was there a history of the disorder in preceding generations. In contrast to diarreha-associated HUS, which is more common in the summer months,5,15,16 there was no seasonal variation in the incidence of D^- HUS; nine of the cases (45%) were encountered between May and September inclusive.

**Clinical features and laboratory investigations**

**Prodrome.** The prodromal symptoms were vomiting (n = 15), anemia (n = 8), convulsions (n = 6), fever (n = 6), a maculopapular rash (n = 6), apparent upper respiratory tract infection (n = 6), malaise (n = 5), and jaundice (n = 4). One child had tuberculous adenitis and was receiving treatment with rifampin and isoniazid, and two had Escherichia coli urinary tract infections.

**Renal function.** The median plasma creatinine concentration at onset was 215 μmol/L (range, 58 to 2310 μmol/L) (2.4 mg/dl; range, 0.65 to 26 mg/dl). Five of the children did not require dialysis at any stage; the remaining 15 underwent dialysis for management of renal failure, but in five dialysis was not required until after a mean of 14 days (range, 3 to 31 days) from the time of admission because the development of renal failure was insidious. Of the 15 children who required dialysis, five died while still undergoing dialysis on days 49, 56, and 120, respectively; two cases progressed to end-stage renal failure without recovery of renal function, and in the remaining 10 the mean duration of dialysis was 20 days (range, 4 to 46 days).

**Hypertension.** The mean (±SD) systolic blood pressure standard deviation score, derived by reference to age- and sex-matched 1987 Task Force standards,17 at the time of admission to the Hospital for Sick Children was 2.5 ± 0.9. Fifty patients had hypertension, with a systolic BP SDS greater than 2.0. The mean diastolic BP SDS was 1.9 ± 1.1; 11 patients had a diastolic BP SDS greater than 2.0. Of these 15 patients, 13 required antihypertensive medication in addition to volume depletion to control the blood pressure, suggesting that the hypertension was renin mediated. Data on plasma renin activity were available for only 12 children. The mean PRA SDS, calculated by comparing PRA with age-matched control values,18 was 2.5 ± 1.4; six children had a PRA SDS greater than 2.0. There was no correlation between the systolic BP SDS and the PRA SDS in the 12 children for whom both results were available (p > 0.05, Spearman rank correlation coefficient). In 5 of the 11 children with "relapsing" disease, recurrence was associated with an exacerbation of hypertension. Of the 14 children followed, excluding two who underwent successful renal transplantation and two who are currently receiving continuous ambulatory peritoneal dialysis, eight have significant hypertension and require substantial antihypertensive medication.

**Neurologic involvement.** Six of the children (30%) had CNS involvement when they were first seen, with convulsions and an alteration in the level of consciousness; in all children the symptoms persisted despite correction of hypertension and the metabolic complications of renal failure.

**Hematologic data.** There was a marked degree of anemia at onset with a microangiopathic hemolytic anemia and erythrocyte fragmentation. The mean hemoglobin concentration was 6.3 ± 1.5 g/dl, but there was no correlation between the hemoglobin concentration at onset and the severity of renal failure as expressed by the plasma creatinine concentration (r = -0.02; p = 0.90). In contrast to D^+ HUS, which is associated with a raised peripheral polymorphonuclear leukocyte count at presentation,19-21 the mean circulating PMNL count was 7.1 ± 3.6 X 10^9/L. There was no apparent difference in PMNL count between those who died (8.1 ± 3.7 X 10^9/L; n = 5), those who were in ESRF (6.2 ± 3.1 X 10^9/L; n = 4), those with residual nephropathy (6.3 ± 3.9 X 10^9/L; n = 8), and the two who...
recovered (8.6 and 13.6 x 10^9/L). The mean platelet count at presentation was 67.9 x 10^9/L (range, 15 to 136 x 10^9/L). There was no correlation between the severity of thrombocytopenia and the plasma creatinine concentration (r = 0.30; p = 0.30). Data on the coagulation profile were obtained at admission of 14 of the children. The mean (± SD) prothrombin time was 13.5 ± 1.2 seconds, kaolin partial thromboplastin time was 34.5 ± 4.9 seconds, and thrombin time was 19.8 ± 8.6 seconds. The kaolin partial thromboplastin time was significantly shorter than control values (paired t test, p = 0.02), and the thrombin time was significantly longer (p < 0.001) but the prothrombin time was not significantly different from control values (p = 0.60).

Renal disease. Histologic studies of 7 of the 20 children, all of whom had relapsing disease, were available. The predominant histologic findings were extraglomerular arteriolar proliferative changes involving endothelial and smooth muscle cells that produced changes resembling the “onion skin” proliferative lesions of malignant hypertension12,23 (Figure). In all cases blood vessels showed marked muscular medial hypertrophy with intimal proliferation. In the four children who underwent biopsy later in the disease course, many glomeruli were sclerosed and there were widespread areas of tubular necrosis and atrophy.

RESULTS

All the children were managed by control of hypertension, dialysis for renal failure, and transfusion for anemia as necessary, but other aspects of their management varied over the years.

Before 1980. Before 1980 no other specific treatment for HUS was offered, although fresh-frozen plasma was sometimes used for resuscitation. There were nine children (six boys) in this group, with a mean age of 3.8 years. Seven patients required dialysis, four needed assisted ventilation, and four received FFP. The outcome for this group of children was poor (Table). Four patients (44%) died, three in the acute phase of the disease of septicemia and CNS involvement in addition to renal failure and the fourth at the time of the first relapse. The fourth child had CNS disease and the postmortem examination showed cerebral edema and a cerebral infarct; he is the only child in the pre-1980 group who had a relapse. Two children never regained renal function and progressed to ESRF. One child received a successful cadaveric graft 1 year after onset but is still functioning 16 years later. The other child’s graft failed 4 years after transplantation because of recurrence of primary disease. The first child did not receive cyclosporine but the second did. Another child has residual nephropathy with hypertension, proteinuria, and a GFR of 77 ml/min per 1.73 m2.

Figure. Characteristic histologic features associated with D+ HUS. Contracted glomerulus with adjacent small interlobular artery shows marked intimal thickening caused by myointimal cells. (Periodic acid-Schiff stain; original magnification, x250.)
Only one girl appears to have made a full recovery; she had no residual nephropathy when her case was investigated 12 years after the initial illness. One of the group has been lost to follow-up.

After 1980. Since 1980, plasma exchange with purified plasma protein fraction and an infusion of FFP at the end of each exchange was used routinely in the management of children with D+ HUS. Two different methods were employed: plasma filtration and centrifugal PEx. The choice of the method depended on the patient's size; smaller children (<15 kg) underwent plasma filtration with a Gambro plasma filter and an AK10 blood monitor (Gambro Ltd.), and the larger children underwent centrifugal PEx with a Cobe Spectra Apheresis system (Cobe BLT, Inc., Lakewood, Colo.). The volume exchanged was twice the child's estimated plasma volume and was completed with 10 ml/kg FFP to replace the intrinsic clotting factors not present in purified plasma fraction and lost during filtration or centrifugation. Initially the procedure was performed every day for 5 days if tolerated. In the four children who had more frequent relapses, it was observed that as the frequency of PEx declined there was a tendency to relapse 6 to 8 weeks after the last PEx. Therefore, for children with relapsing disease, the initial run of PEx was followed by a program of intermittent PEx performed at increasing intervals for several months after the last relapse.

In addition, PG12 was administered to seven of the children by constant intravenous infusion at a starting dose of 2 ng/kg per minute and increased gradually to a maximum of 20 ng/kg per minute, because of refractory hypertension or CNS disease. Two children became hypotensive and another had severe diarrhea during the treatment. In all instances PG12 was used in conjunction with PEx, so no clear view on its efficacy could be formulated.

Between 1980 and 1991, 11 children (seven girls) with D+ HUS were managed in this way; their mean age was 4.7 years (range, 0.2 to 13.5 years). Eight children required dialysis for a median duration of 21 days (range, 4 to 46 days) and two required assisted ventilation. The median number of PExs performed in each child was 38 (range, 5 to 254). In addition to PEx, seven children also received an infusion of PG12. The outcome for this group was variable (Table). Ten children had further relapses of HUS, defined as an increase in the plasma creatinine concentration of 20% associated with thrombocytopenia. One child died 3 years after onset, during the third relapse, having received a total of 22 exchanges, of an intracranial hemorrhage. Two children progressed to ESRF, one during a period of 9 months and the other more gradually during a period of 5 years. The first of these has now received two cadaveric grafts, both of which failed within 3 weeks of transplantation, with recurrence of the primary disease in the graft; this boy has now resumed continuous ambulatory peritoneal dialysis. The other child underwent successful cadaveric renal transplantation 6 years after onset. The first child received cyclosporine as part of the immunosuppressive protocol on both occasions, and the second child did not. Seven of this group have significant residual nephropathy with proteinuria and hypertension, and of these, six have a reduced GFR. Only one child appears to have made a full recovery; she has now had two relapses and has received 19 PExs and yet is currently free of proteinuria, is normotensive, and has a normal GFR.

There may be other variables in the year of onset that affect outcome, but it appears that those children who received PEx had a lower mortality rate (1/11 vs 4/9; p < 0.005, exact test) than those who did not. However, the relapse rate was higher in the children who received PEx, 10 of 11 having further episodes of HUS compared with only 1 of 9 in the earlier group (p < 0.005, exact test). The overall recovery rate was the same, however; only one child from each group regained normal renal function.

Overall outcome. In this series of 20 children with idiopathic D+ HUS, 5 (25%) died, 4 (20%) progressed to ESRF, 8 (40%) have residual nephropathy, and only 2 (10%) have apparently made a full recovery. Eleven children (55%) have had one or more relapses, often in association with presumed viral respiratory tract infections and in many instances heralded by a deterioration in the control of hypertension. Two of the children with relapsing disease died during the first and third relapses, respectively, and for the remaining nine the median number of relapses was three, with a maximum of eight relapses in one case spread over a period of 6 years. Ten of these children were among the 11 children who were first seen after 1980 and were treated by PEx. In six children the acute-phase treatment with PEx was followed by a period of treatment with single PExs at increasing intervals for periods up to 18 months.

Table. Study results

<table>
<thead>
<tr>
<th></th>
<th>No. PEx (before 1980; n = 9)</th>
<th>PEx (after 1980; n = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.</td>
<td>%</td>
</tr>
<tr>
<td>Early death</td>
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<td>33</td>
</tr>
<tr>
<td>Early ESRF</td>
<td>2</td>
<td>22</td>
</tr>
<tr>
<td>Relapse</td>
<td>1</td>
<td>11</td>
</tr>
<tr>
<td>Nephropathy</td>
<td>1</td>
<td>11</td>
</tr>
<tr>
<td>Late death</td>
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<td>1*</td>
</tr>
<tr>
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<td>2</td>
</tr>
<tr>
<td>Unknown</td>
<td>1</td>
<td>11</td>
</tr>
<tr>
<td>Recovered</td>
<td>1</td>
<td>11</td>
</tr>
</tbody>
</table>

*Late death occurred after relapse.
and in four a relapse occurred within 2 months of the last PEx.

**DISCUSSION**

Non-diarrhea-associated hemolytic-uremic syndrome is a heterogeneous yet distinct subgroup of HUS that differs from D+ HUS on epidemiologic, clinical, laboratory, histologic, and prognostic grounds. In contrast to D+ HUS, in which progress has been made in understanding the cause,24 epidemiology,25 and pathophysiology,26 the cause and pathogenesis of D− HUS remain unclear.

The prognosis for D− HUS is poor; mortality and morbidity rates are high. The incidence of terminal renal failure is high and its management is difficult: all of our patients have undergone renal transplantation but only two have functioning grafts. In the other two children, three grafts failed because of recurrence of HUS. In all three instances, cyclosporine was used as part of the immunosuppressive protocol, whereas the two children with functioning grafts did not receive cyclosporine. A particular problem exists in relation to cyclosporine that causes a nephropathy with many features held in common with D− HUS, and its use may best be avoided in these cases. Recurrence of HUS has been documented, however, in both cyclosporine-treated 27, 28 and non-cyclosporine-treated patients.29-32 The onset of allograft rejection 33 and hypertension34 may also trigger HUS, and it can be difficult to distinguish recurrence of primary disease from severe acute or chronic vascular rejection on the basis of histologic examination of renal tissue.

Because of the probable poor outcome of D− HUS, we have treated it aggressively in the hope that such an approach will improve ultimate prognosis. Early control of renal failure with prompt and aggressive management of hypertension is essential. We then embark on a course of PEx with purified protein fraction, complete with an infusion of FFP. Patients with relapsing disease are subsequently managed by a program of intermittent PEx at increasing intervals for several months after the last relapse.

Plasma exchange has an established position in the management of thrombotic thrombocytopenic purpura: a controlled trial conducted by the Canadian Apheresis Group,35 comparing PEx with plasma infusion in the treatment of thrombotic thrombocytopenic purpura in adults, found PEx to be more effective than plasma infusion in reducing mortality and morbidity rates. In children D− HUS is a rare disorder, and it would be difficult to accumulate a sufficient number of cases to undertake a similar controlled study.

The rationale for the use of PEx is not clear. It has been suggested that a deficiency of PG12 synthesis plays an integral role in the pathogenesis of D− HUS, because of either a circulating inhibitor of PG12 production or a deficiency of a circulating factor necessary for PG12 synthesis.7, 9-11 Walters et al.10 found reduced support of PG12 production by endothelial tissue in the sera of seven of nine children with D− HUS (eight included in this report), whereas D+ HUS sera usually supported PG12 production normally. These phenomena have been part of the rationale for using FFP, PEx, and PG12 infusions in both thrombotic thrombocytopenic purpura and D− HUS, but the pathophysiology of these conditions needs reevaluation in the light of recent evidence on the importance of nitric oxide in vascular and platelet function.36

Our experience in the management of idiopathic D− HUS leads us to believe that PEx may indeed improve the prognosis in this serious disorder, but it may be that the effect of PEx is to improve immediate survival rates only to reveal the relapsing nature of the condition.

**REFERENCES**


Long term renal outcome of childhood haemolytic uraemic syndrome

Margaret M Fitzpatrick, Vanita Shah, Richard S Trompetter, Michael J Dillon, T Martin Barratt

Abstract

Objective—To evaluate the long term outcome of renal function in infants and children after diarrhoea associated haemolytic uraemic syndrome.

Setting—The Hospital for Sick Children, Great Ormond Street, and the Royal Free Hospital, London.

Subjects—103 children with the syndrome who presented between 1966 and 1985; 88 attended for follow up investigations (40 male, 48 female) with a mean age 11·6 (range 5·2-22·6) years and a mean duration of follow up of 8·5 (range 5·1-21·3) years.

Main outcome measures—Blood pressure, ratio of early morning urine albumin to creatinine concentration, glomerular filtration rate, and plasma renin activity.

Results—The mean (SD) systolic blood pressure standard deviation score was 0·38 (0·67) and diastolic blood pressure SD score was 0·10 (0·76). The geometric mean ratio of overnight urine albumin to creatinine concentration was 1·27 (range 0·63-48·2), significantly higher than the value observed in 77 normal children (0·32 (0·05-1·95), p<0·0001). Glomerular filtration rate estimated from the plasma clearance of chromium-51 EDTA was 95·1 (22·7) ml/min/1·73 m² surface area, and 16 children had a rate of <80 ml/min/1·73 m². Significant negative correlations were found between glomerular filtration rate and urinary albumin to creatinine ratio (r= 0·41, p<0·0001) and glomerular filtration rate and systolic blood pressure SD score (r= 0·48, p<0·0001). A significant positive correlation was found between urinary albumin to creatinine ratio and systolic blood pressure SD score (r= 0·25, p=0·02).

Conclusions—After an acute episode of diarrhoea associated haemolytic uraemic syndrome 31% (27/88) of children had an increased albumin excretion, 18% (16/88) had a reduced glomerular filtration rate and 10% (9/88) had both, in association with a higher systolic blood pressure, indicating considerable residual nephropathy in this group.

Introduction

The haemolytic uraemic syndromes are a heterogeneous group of disorders characterised by haemolytic anaemia, thrombocytopenia, and renal failure. Two main subgroups are now recognised: the first is associated with a diarrhoeal prodrome and is a major cause of acute renal failure in children in Britain, whereas the second, which is rare in childhood, has no antecedent diarrhoea. During the past decade, with the discovery that Escherichia coli producing verocytotoxin are an important cause of diarrhoea associated haemolytic uraemic syndrome, there have been considerable advances in understanding the aetiology and pathogenesis of this disorder. The long term outcome for renal function in those patients who survive the acute phase of the illness is, however, incompletely documented. This paper reviews the long term outcome of renal function in 88 infants and children evaluated five to 21 years after the acute phase of the syndrome.

Methods

From 1966 to 1985 a total of 120 infants and children with haemolytic uraemic syndrome were admitted to the Hospital for Sick Children, Great Ormond Street, and the Royal Free Hospital, London. One hundred and three had a "typical" enteropathogenic illness with a diarrhoeal prodrome and 17 an "atypical" presentation without diarrhoea. A follow up study was undertaken of the children whose illness had the diarrhoeal prodrome. Ethical approval for this study was obtained from the ethics committee of the Hospital for Sick Children, Great Ormond Street.

Of the original 103 children with diarrhoea associated haemolytic uraemic syndrome (table I), 15 were not included in the study. Eight had died, five during the acute phase of the illness, and three had progressed to end stage renal failure after their initial presentation and died after dialysis or transplantation (table II).
syndrome

Table II—Characteristics of eight patients with diarrhoea associated haemolytic uraemic syndrome who died

<table>
<thead>
<tr>
<th>Sex</th>
<th>Year of presentation</th>
<th>Age (years)</th>
<th>White blood cell count (× 10^9/L)</th>
<th>Duration of dialysis (days)</th>
<th>Time after presentation (months)</th>
<th>Glomerular filtration rate (ml/min/1.73 m²)</th>
<th>Cause of death</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>1966</td>
<td>0.6</td>
<td>27.6</td>
<td>14</td>
<td>0.5</td>
<td>114.7</td>
<td>Cerebral</td>
</tr>
<tr>
<td>F</td>
<td>1966</td>
<td>1.1</td>
<td>12.2</td>
<td>4</td>
<td>1.6</td>
<td>138.5</td>
<td>Septicaemia</td>
</tr>
<tr>
<td>F</td>
<td>1968</td>
<td>0.2</td>
<td>17.2</td>
<td>26</td>
<td>0.2</td>
<td>113.6</td>
<td>Cerebral</td>
</tr>
<tr>
<td>F</td>
<td>1971</td>
<td>0.5</td>
<td>20.1</td>
<td>56</td>
<td>0.6</td>
<td>83.3</td>
<td>Cardiac</td>
</tr>
<tr>
<td>M</td>
<td>1973</td>
<td>1.1</td>
<td>22.9</td>
<td>28</td>
<td>5.5</td>
<td>139.8</td>
<td>Septicaemia</td>
</tr>
<tr>
<td>M</td>
<td>1980</td>
<td>2.7</td>
<td>22.9</td>
<td>6</td>
<td>0.7</td>
<td>115.8</td>
<td>Cerebral</td>
</tr>
<tr>
<td>F</td>
<td>1985</td>
<td>3</td>
<td>22.9</td>
<td>9</td>
<td>0.3</td>
<td>115.8</td>
<td>Cerebral</td>
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Table III—Standard deviation scores for systolic blood pressure in 88 patients with diarrhoea associated haemolytic uraemic syndrome

<table>
<thead>
<tr>
<th>Standard deviation score</th>
<th>No of patients</th>
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<tr>
<td>-1.0</td>
<td>7</td>
</tr>
<tr>
<td>-0.5</td>
<td>22</td>
</tr>
<tr>
<td>0.0</td>
<td>21</td>
</tr>
<tr>
<td>0.5</td>
<td>19</td>
</tr>
<tr>
<td>1.0</td>
<td>13</td>
</tr>
<tr>
<td>1.5-2.0</td>
<td>6</td>
</tr>
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</table>

Table IV—Characteristics of 16 patients with glomerular filtration rate ≤80 ml/min/1.73 m² surface area at review

<table>
<thead>
<tr>
<th>Sex</th>
<th>Age (years)</th>
<th>White blood cell count (× 10^9/L)</th>
<th>Duration of dialysis (days)</th>
<th>Time after follow up (months)</th>
<th>Glomerular filtration rate (ml/min)</th>
<th>Urinary albumin to creatinine ratio (mg/mmol)</th>
<th>Systolic blood pressure (SD score)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>1.3</td>
<td>27.6</td>
<td>12</td>
<td>14.6</td>
<td>15</td>
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<td>1.87</td>
</tr>
<tr>
<td>F</td>
<td>0.6</td>
<td>20.0</td>
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<td>14.6</td>
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<tr>
<td>F</td>
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<td>17</td>
<td>8.5</td>
<td>56</td>
<td>139.8</td>
<td>1.50</td>
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<td>22.0</td>
<td>24</td>
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<td>5</td>
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<td>63</td>
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<td>F</td>
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<td>25.3</td>
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<td>10.7</td>
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<td>139.8</td>
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<td>8</td>
<td>11.5</td>
<td>72</td>
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<tr>
<td>M</td>
<td>3.0</td>
<td>24.9</td>
<td>15</td>
<td>11.9</td>
<td>74</td>
<td>139.8</td>
<td>1.50</td>
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<tr>
<td>M</td>
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<td>12</td>
<td>18.8</td>
<td>74</td>
<td>139.8</td>
<td>1.50</td>
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<td>12</td>
<td>12.6</td>
<td>80</td>
<td>139.8</td>
<td>1.50</td>
</tr>
</tbody>
</table>

deviation were those of the normal sex and age matched population.

Urine—The early morning urine sample was collected in a plastic bottle containing merthiolate 1:10 000 to prevent bacterial growth. The urine creatinine concentration (mmol/l) was measured by an autoanalyser Jaffe reaction with an intra-assay coefficient of variation of 3%. Urine albumin concentration (mg/l) was measured by double antibody radioimmunoassay with a commercially available kit (Diagnostic Products Corporation, Los Angeles, California, USA) with a sensitivity of 0.5 mg/l, an intra-assay coefficient of variation of 4%, and an interassay coefficient of 5%. The ratio of urinary albumin to creatinine concentration was expressed as mg/mmol and was log transformed before statistical analysis; results were expressed as the geometric mean and range (2 SD) calculated on log data.

Glomerular filtration rate was estimated from the plasma clearance of chromium-51 EDTA by single compartmental analysis and the results expressed in ml/min/1.73 m² surface area.

Plasma renin activity—One ml of venous blood was taken from the patients after they had rested supine for two hours. Plasma renin activity was measured with a semimicro radioimmunoassay and expressed as ng angiotensin I 1/hour. Interassay and intra-assay coefficients of variation were 10% and 5% respectively.

As for blood pressure, SD scores were calculated for plasma renin activity compared with age matched controls.

Statistical analysis—A one sample t test was used to compare systolic and diastolic blood pressure SD score and plasma renin SD score with the notional value of zero. The geometric means of the urinary albumin to creatinine ratio were compared by an unpaired t test on log transformed data. The interrelations between glomerular filtration rate, blood pressure, SD score, and urinary albumin to creatinine ratio were examined by linear regression and Pearson's correlation coefficients. The ages and durations of dialysis for the children in two outcome groups (defined according to glomerular filtration rate) were compared with the Mann-Whitney test for comparison of two values, and the geometric means of the total white cell counts in the two groups were compared by using an unpaired t test on log transformed data.

Results

The mortality during the acute phase of the disease was 3/35 (9%) before 1980 and 2/68 (3%) after 1980 (table I). The overall mortality between 1966 and 1985 was 8/103 (8%). Three children went into end stage renal failure without recovering function. Two children had neurological sequelae: one had a left sided hemiparesis and the other had spastic diplegia and cortical blindness.

In the 88 children followed, the mean (SD) systolic blood pressure SD score was 0.38 (0.67) significantly greater than zero (t=5.28, p<0.001), but no child had a score greater than 2.0 (table III). The mean diastolic score was 0.10 (0.76), which was not significantly different from zero (t=1.23, p<0.22). The geometric mean ratio of albumin to creatinine in the early morning urine sample was 1.27 (range 0.03-48.2), significantly higher than the value of 0.32 (0.05-1.95) (t=5.97, p<0.0001) in 77 normal children (fig 1). Of the 88 children with diarrhoea associated haemolytic uraemic syndrome 27 (31%) had a ratio >2 SD above the normal mean. The mean glomerular filtration rate was 95.1 (22.7) ml/min/1.73 m² surface area (range 15-160 ml/min/1.73 m²), and 16 of the patients had a rate ≤80 ml/min/1.73 m² (table IV). Nine of these children had both a glomerular filtration
Diarrhoea associated haemolytic uraemic syndrome is one of the major causes of acute renal failure in childhood. The immediate prognosis has improved substantially over the past two decades as short term dialysis has become a widely available and safe form of treatment. Most recent series report an acute fatality rate of 5-10% in our study the mortality during the acute phase was 5.0%. The long term consequences of haemolytic uraemic syndrome are, however, less well documented, with various outcomes being reported from different centres and regions.

The aim of this study was to assess renal function in a large group of children with a minimum follow up of five years, after an acute episode and to provide more reliable data on the long term prognosis of the condition.

In this series 3/103 (3%) of patients did not recover renal function after presentation and 34/88 (39%) had some abnormality of renal function at the time of follow up. Eighteen per cent had a glomerular filtration rate <80 ml/min/1.73 m² and 31% had significant microalbuminuria. Three per cent of patients became hypertensive after their acute illness and were taking antihypertensive drugs at follow up. The remainder had systolic blood pressure SD score <2, but the mean systolic blood pressure SD score for the group as a whole was significantly greater than zero. The mean plasma renin activity SD score was also greater than zero. We found that a lower glomerular filtration rate, microalbuminuria, and a higher systolic blood pressure were associated.

We divided our survivors into two groups on the basis of glomerular filtration rate: <80 ml/min/1.73 m² (group 1) and >80 ml/min/1.73 m² (group 2). There was no difference in the age at presentation between the two groups, but patients in group 1 had a significantly higher white blood count at presentation and a significantly longer duration of dialysis. Recent publications analysing prognostic features in haemolytic uraemic syndrome have produced variable results with regard to the significance of age at presentation, the duration of anuria or dialysis, and the presence of central nervous system involvement, but polymorphonuclear leucocytosis is consistently related to poor outcome.

Discussion

Diarrhoea associated haemolytic uraemic syndrome is one of the major causes of acute renal failure in childhood. The immediate prognosis has improved substantially over the past two decades as short term dialysis has become a widely available and safe form of treatment. Most recent series report an acute fatality rate of 5-10%. In our study the mortality during the acute phase was 5.0%. The long term consequences of haemolytic uraemic syndrome are, however, less well documented, with various outcomes being reported from different centres and regions.

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Follow up studies published over the past years have produced conflicting results with regard to renal outcome after an episode of haemolytic uraemic syndrome. The largest series, published before the widespread availability of acute dialysis, is from Argentina, where haemolytic uraemic syndrome is common. Of 124 survivors followed for a minimum of five years, only 60 (48%) made a full recovery; 23 (18%) progressed to end stage renal failure. More recent data have pointed to a better outcome. Van Dyck et al added their findings to those from studies in Utrecht, London, and Paris, and in a total of 258 patients from western Europe found an acute mortality of 9%, progression to end stage renal failure in 9%, chronic renal failure in 4%, late sequelae in 12%, and complete recovery in 66%. The data from these different centres are, however, difficult to interpret. In these series no clear distinction has been made between haemolytic uraemic syndrome with and without the diarrhoeal prodrome and it is now known that most children with no antecedent diarrhoea have a poor prognosis. Different methods of assessing renal function were used in the different centres and the duration of follow up was inadequate in some instances. In comparison with Van Dyck et al.'s results we found a lower acute mortality (5%) and fewer children progressing to end stage renal failure (3%) but a higher proportion of children with renal sequelae (39%). In a recent publication from the Argentinian group, Perelstein et al found that children with a history of haemolytic uraemic syndrome and normal renal function, as determined by standard diagnostic procedures, had a loss of normal renal functional reserve after a protein load when compared with normal controls. This suggests that the extent of residual renal problems following diarrhoea associated haemolytic uraemic syndrome may have been underestimated.

Most of our patients had been discharged from hospital follow up and were not under regular medical review. In view of the substantial number of patients with renal sequelae we suggest that children who have had an episode of diarrhoea associated haemolytic uraemic syndrome be kept under medical review with blood pressure measurement and urine testing. Because of the poorly understood correlation between microalbuminuria and systolic blood pressure and the negative correlation of microalbuminuria and glomerular filtration rate, patients with noticeable microalbuminuria on routine testing should be more closely monitored, with measurement of glomerular filtration rate. Milford et al have suggested measurement of the ratio of protein to creatinine in an early morning sample of urine as an effective means of monitoring the progress of patients after an episode of diarrhoea associated haemolytic uraemic syndrome. They examined the prognostic value of changes in these ratios in 40 children one year after diagnosis and found that 57% of those who seemed to have fully recovered had normal ratios, compared with none of those with poor outcomes.

Diarrhoea associated haemolytic uraemic syndrome is now emerging as an important clinical and public health problem, and there is evidence that its incidence is increasing, particularly in young children. This study shows it is associated with substantial morbidity, and a considerable number of patients have evidence of occult nephropathy at follow up. The abnormalities in many patients are subtle and the long term implications unclear; some may show further decline in renal function or become hypertensive. This disease may consequently have an increasing impact in adult nephrology.


(Accepted 3 July 1991)

ONE HUNDRED YEARS AGO

A Handbook to London has been presented to all the members of the Congress. The English and French versions appear side by side on the same page. The information supplied appears to be of a thoroughly reliable and practical character, and the maps must have been of great assistance to persons unfamiliar with London; in addition to the ordinary guidebook maps, there are others showing the situations of the hospitals of London, of the cemeteries and prisons—a lugubrious collocation—and, out of compliment to the demographers, a chart is added showing the gross present population of the metropolitan parish, with the number of persons per acre.

(British Medical Journal 1891;3:391)
Duplex Doppler ultrasound in the investigation of occult nephropathy following haemolytic uraemic syndrome

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Keywords: Doppler, Nephropathy, Haemolytic uraemic syndrome

Abstract. Duplex doppler ultrasound has been reported to be of value in the detection of raised vascular resistance, particularly in the renal tract. A prospective single blind study investigating the use of duplex Doppler ultrasound to measure resistive index (RI) in patients with impaired renal function and a history of diarrhoea-associated haemolytic uraemic syndrome (D+ HUS) was performed. There was considerable overlap in the range of RIs, with RIs greater than 70% in children with normal renal function and in those with renal impairment following D+ HUS. There was no significant difference in the mean RI between the groups studied. We feel that the RI is not of value in predicting the presence of occult nephropathy following haemolytic uraemic syndrome.

The haemolytic uraemic syndromes (HUS) are a heterogeneous group of disorders characterized by haemolytic anaemia, thrombocytopenia and renal failure (Gasser et al, 1955). Two main subgroups are now recognized: the first is associated with a diarrhoeal prodrome, D+ HUS, and is a major cause of acute renal failure in Britain; the second is rare in childhood and has no antecedent diarrhoea, D- HUS (Levin & Barratt, 1984). Most children with D+ HUS make a full recovery, but 15% or more may develop some form of renal impairment (Milford & Taylor, 1990). Patriquin et al (1989) have recently proposed the use of resistive index (RI) to predict recovery from oliguria or anuria during the acute phase of D+ HUS. This observation led us to attempt to detect those children with chronic renal impairment following an episode of D+ HUS using duplex Doppler ultrasound.

Patients and methods

This was a prospective single blind study using Doppler ultrasound to assess renal and intrarenal blood flow in an attempt to recognize those children with impaired renal function following an episode of D+ HUS. Three groups of age and sex matched children were selected for study.

Group 1. Eight children with a history of D+ HUS and a glomerular filtration rate of less than 80 ml/min/1.73 m² surface area (SA).

Group 2. Eight children with a history of D+ HUS and a glomerular filtration rate greater than 80 ml/min/1.73 m² SA.


The ultrasound examinations were performed by two experienced sonographers, unaware of the medical history and renal function of the patient at the time of the examination. The renal and intrarenal arteries were examined with duplex Doppler ultrasound and a total of five measurements taken from each kidney. The RI was calculated from these measurements as peak systolic velocity minus end diastolic velocity divided by peak systolic velocity, which was calculated by the machine. A mean RI was calculated from the five readings. All examinations were performed with an Acuson 128 machine using real-time and pulsed Doppler scanning. The glomerular filtration rate (GFR) was estimated from the plasma clearance of chromium-51 edetic acid (Chantler & Barratt, 1972).

Results

Thirty-five children were studied: 19 children in the normal group, and eight in each of the groups previously affected by D+ HUS. The results are summarized in Fig. 1. There was a wide range of values measured in all three groups, with a range of 30.9% seen in the normal children, Group 3, and 31.1% seen in Group 1. There was considerable overlap in the RIs measured in all three groups. The lowest RI measured in all three groups was similar: Group 1, RI = 47.0%; Group 2, RI = 48.6%; and Group 3, RI = 45.0%. The highest RI, 78.1%, was measured in a child with a GFR of 37 ml/min/1.73 m² SA, but measurements of 75.9% and 75.4% were obtained in the group of normal children. The means of the three groups were similar: Group 1 mean 57.1%, Group 2 mean 56.9% and Group 3 mean 59.9% (Table I). The results were compared using paired Students' t-test, and showed no significant differences.

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changes should also raise intrarenal vascular resistance in chronic renal impairment the histopathological changes in the RI (Patriquin et al, 1989). In those children with intrarenal vascular resistance falls with a consequent fall capillary lumen (Levin et al, 1989). The presence of arteriolar occlusion increases the intrarenal vascular resistance, increasing pulsatility leading to a high RI. As the kidney recovers, the resistance, increasing pulsatility leading to a high RI. It would be reasonable therefore to expect a higher RI to occur in the children with the lowest GFRs, Group 1. The highest RI recorded in our series, 78.1%, was found in the child with the lowest GFR, 37 ml/min/1.73 m² SA. However, his kidneys were sonographically abnormal, being small and of increased echogenicity. There were no other sonographically abnormal kidneys, and it may be that a significant elevation of RI only occurs in kidneys clearly abnormal on conventional real-time ultrasound in which measurement of RI would be of little benefit.

The initial enthusiasm for the use of the RI in the assessment of renal and intrarenal blood flow has recently diminished with an increasing number of papers questioning its value (Genkins et al, 1989; Kelcz et al, 1990; Drake et al, 1990; Grant & Perrella, 1990). The normal range of RI and its alteration with age have not been established, and the wide range of values seen in our group of normal children questions the previous use of absolute measurements to predict disease. A significant change occurs with alteration of heart rate (Mostbeck et al, 1990), and it is known that other factors such as compression of the kidney by the ultrasound probe may cause an elevated RI (Pozniak et al, 1988). We suggest that RI is not as useful as was initially reported, and raise doubts about the use of RI in detecting chronic renal impairment.

### Table I. Ranges and means of the RIs in the three groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Range (%)</th>
<th>Mean RI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>47.0–78.1</td>
<td>57.1</td>
</tr>
<tr>
<td>2</td>
<td>48.6–65.0</td>
<td>56.9</td>
</tr>
<tr>
<td>3</td>
<td>45.0–75.9</td>
<td>59.9</td>
</tr>
</tbody>
</table>

**Figure 1.** RIs measured in the three groups of children.

**Discussion**

Chronic renal impairment is the most serious long-term consequence of D+ HUS. The development of a non-invasive and easily reproducible means of detecting children with renal impairment secondary to D+ HUS would be a significant advance in their care, avoiding radioisotope injection, day admission and two venepunctures in young children. The recent paper by Patriquin et al (1989) advocated the use of the RI in the acute phase, and demonstrated their ability to predict the time of recovery from the oliguric or anuric phase of the syndrome. The histopathological change seen in the acute phase is an alteration of the endothelium, with swelling of endothelial cells effectively narrowing the capillary lumen (Levin et al, 1989). The presence of arteriolar occlusion increases the intrarenal vascular resistance, increasing pulsatility leading to a high RI (Patriquin et al, 1989). As the kidney recovers, the intrarenal vascular resistance falls with a consequent fall in the RI (Patriquin et al, 1989). In those children with chronic renal impairment the histopathological changes are less specific. Tubular atrophy, interstitial fibrosis and glomerular segmental sclerosis may all occur. These changes should also raise intrarenal vascular resistance resulting in a high RI. It would be reasonable therefore to expect a higher RI to occur in the children with the lowest GFRs, Group 1. The highest RI recorded in our series, 78.1%, was found in the child with the lowest GFR, 37 ml/min/1.73 m² SA. However, his kidneys were sonographically abnormal, being small and of increased echogenicity. There were no other sonographically abnormal kidneys, and it may be that a significant elevation of RI only occurs in kidneys clearly abnormal on conventional real-time ultrasound in which measurement of RI would be of little benefit.

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**References**


**Duplex Doppler US in nephropathy**


Percutaneous transluminal angioplasty: pain during balloon inflation

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Keywords: Arteries, Transluminal angioplasty, Angiography, Complication, Arteries, Stenosis

Abstract. The pain during the balloon dilatation of angioplasty was evaluated prospectively to assess its clinical significance. In 54 angioplasties, no pain was observed in 54%, mild pain in 20%, moderate pain in 11% and severe pain in 15%. Moderate or severe pain was observed in 39% of 28 iliac angioplasties and in 7% of 14 femoral angioplasties. There was a significant difference between the two groups. We did not find any significant correlation between the severity of pain and stenotic ratio before angioplasty. Severe pain may be a warning of severe dissection; in our study, all severe dissections were accompanied by severe pain without arterial rupture.

Patients often experience some degree of pain during inflation of balloons for angioplasty, not associated with clinical problems. However, some authors have suggested that severe pain may be an important warning of arterial rupture (Jensen et al, 1985; Chong et al, 1990). The frequency and severity of pain during balloon inflation has not been reported. We have evaluated prospectively the pain experienced by patients to determine its clinical significance.

Materials and methods

The study comprises 54 angioplasties in 44 patients with atherosclerosis: 28 iliac, 14 femoral and 12 renal arterial dilatations. Technical failures such as subintimal passage of the guidewire or catheter were excluded in this study. Angioplasties were performed in a routine fashion under local anaesthesia (Korogi et al, 1987).

The balloon required was selected by measuring the diameter of the contralateral artery or the adjacent patent portion of the vessel on the conventional angiogram. The diameters of the balloons used were 6–8 mm in iliac angioplasty and 4–6 mm in femoral or renal angioplasty. An oversized balloon was never used. The balloon was inflated by hand twice for 30 s, or several times until the “waist” of the balloon disappeared under fluoroscopy. We did not use a pressure gauge because of loss of feeling at inflation and dilatation of the stenosis. However, the syringe size used was larger than 10 ml capacity to avoid excessive pressure.

Patients were asked to report any pain they experienced, and angiographers recorded the patient’s distress and motion during balloon inflations. The data were scored from zero to 3 by the following criteria: (0) no pain; (1) mild pain (no distress or motion); (2) moderate pain (face being distorted or moving slightly); and (3) severe pain (giving a cry or major movement).

The site of dilatation, the stenotic ratio before dilatation and the luminal diameter after dilatation were correlated with severity of the pain. Severe dissection after dilatation was defined as an intimal dissection compromising flow and requiring further therapy, although intimal dissection itself was a common finding at the dilatation site. The unpaired t-test was used in the statistical evaluations.

Results

In 54 angioplasties, no pain was observed in 29 dilatations (54%), mild pain in 11 (20%), moderate pain in six (11%), and severe pain in eight (15%) dilatations. The pain was observed only during balloon inflation. In few patients, however, pain continued for several to 10 s after deflation of the balloon.

No dilated artery ruptured in this study. However, severe dissection occurred in three dilatations (two iliac and one femoral), and was associated with severe pain. In these two patients with iliac angioplasty, no other symptoms related to severe dissection were present. They were referred for bypass surgery because of unsuccessful re-dilatation, which also produced severe pain. Another patient with femoral angioplasty complained of temporary pain in the lower leg after occurrence of severe dissection, and immediate re-dilatation, which did not produce severe pain, was performed successfully. Data of the re-dilatations are not included in this evaluation. No overdistension was observed in our series.

Analysis of the data regarding the site of dilatation demonstrated a significant difference between iliac and femoral angioplasties (p < 0.05) (Table I). Eleven of 28 iliac angioplasties (39%) had moderate or severe pain against only one of 14 femoral angioplasties (7%). There was still a significant difference between the two when the three cases of severe dissection were excluded from analysis (p < 0.05). Although the mean score was...
**NEUTROPHIL-MEDIATED ENDOTHELIAL INJURY IN HAEMOLYTIC URAEMIC SYNDROME**

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Departments of Immunology1 and Nephrology2, Institute of Child Health, University of London, London WC1

**Summary**

Neutrophil leucocytosis is associated with a poor outcome in the haemolytic uraemic syndrome (HUS). This study tested the hypothesis that neutrophils from HUS patients are activated and through release of their intracellular contents damage endothelium. The proportion of neutrophils adhering to endothelium in culture was twice as high for HUS patients' neutrophils as for control neutrophils (n = 12). In addition, these neutrophils induced endothelial injury, assessed morphologically by degradation of endothelial cell fibronectin. In an attempt to inhibit neutrophil adhesion and subsequent endothelial damage the hyperadhesive neutrophils from HUS patients were incubated with a CD18 antibody directed against the common beta chain of the leucocyte integrin molecules. The CD18 antibody was able to abrogate endothelial damage in four of the ten subjects studied. These observations suggest that the neutrophil is of prime pathophysiological importance in HUS, and that methods aimed at reducing neutrophil adhesion and neutrophil-mediated endothelial damage are likely to be beneficial.

**Introduction**

The haemolytic uraemic syndrome (HUS) is characterised by the triad of microangiopathic haemolytic anaemia, thrombocytopenia, and nephropathy. There are two main subgroups of HUS—the typical diarrhoeal form and the atypical non-diarrhoeal form.1 The aetiology of HUS is probably multifactorial, although infectious agents such as verocytotoxin-producing *Escherichia coli*, *shigella*, and *salmonella*,1 and viruses have been implicated. In view of the association of infectious agents with many cases of HUS, reports of neutrophil leucocytosis in HUS are not surprising.2,3 Indeed, in the typical form of HUS, a high neutrophil count at presentation indicates a poor prognosis, suggesting that the neutrophil may have a role in the pathogenesis.4

In this study we examined neutrophil adherence and subsequent injury to the endothelium with neutrophils and plasma from children who had the acute diarrheal form of HUS.

**Patients and Methods**

Twelve children presenting sequentially to the Hospital for Sick Children, Great Ormond Street, with the acute diarrheal form of HUS (microangiopathic haemolytic anaemia, thrombocytopenia, and acute renal failure) between July, 1988, and May, 1989, were included in this study. Their ages ranged from 9 months to 6 years 8 months. There were 9 girls and 3 boys. Clinical details of the patients are given in table 1, and laboratory indices at presentation in table II. All patients required dialysis for recovery from renal failure, except patients 9 and 10, whose renal involvement was mild. The study was approved by the hospital ethical committee. Blood samples for preparation of plasma and neutrophils were collected from subjects at the time of presentation. Since the neutrophil studies were carried out within 2 h of venepuncture, no control children were available and twelve healthy adult laboratory staff were used as controls. Their blood was collected and processed in an identical manner.

Blood was collected into EDTA, and the plasma separated by centrifugation within 30 min. The remaining blood was diluted with an equal volume of Dulbecco's phosphate-buffered saline, and underlayered with 'Ficoll-Faque' (Pharmacia, Uppsala, Sweden). After centrifugation at 400 x g for 20 min, the pellet containing red cells and neutrophils was isolated. Most of the red cells were

**TABLE I—CLINICAL CHARACTERISTICS OF HUS PATIENTS**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>Diarrhoea</th>
<th>Duration (days) of Diarrhoea</th>
<th>Anuria</th>
<th>Days since presentation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>55</td>
<td>F</td>
<td>Moderate</td>
<td>10</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>43</td>
<td>F</td>
<td>Moderate</td>
<td>7</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>47</td>
<td>M</td>
<td>Moderate</td>
<td>7</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>67</td>
<td>F</td>
<td>Moderate</td>
<td>2</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>58</td>
<td>M</td>
<td>Moderate</td>
<td>6</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>69</td>
<td>F</td>
<td>Moderate</td>
<td>9</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>08</td>
<td>F</td>
<td>Moderate</td>
<td>5</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>8</td>
<td>56</td>
<td>F</td>
<td>Severe</td>
<td>21</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>9</td>
<td>23</td>
<td>F</td>
<td>Severe</td>
<td>21</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>10</td>
<td>49</td>
<td>M</td>
<td>Severe</td>
<td>20</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>11</td>
<td>15</td>
<td>M</td>
<td>Severe</td>
<td>20</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>12</td>
<td>19</td>
<td>F</td>
<td>Moderate</td>
<td>8</td>
<td>12</td>
<td>1</td>
</tr>
</tbody>
</table>

*Days from presentation to local hospital with HUS to collection of blood specimens at Hospital for Sick Children.

**K. M. DE COCK AND OTHERS: REFERENCES**

removed by sedimentation with 3% dextran (Fisons, Loughborough) for 30 min and those remaining were removed by hypotonic lysis. Neutrophils prepared in this way were more than 98% pure. For the neutrophil adhesion assay, neutrophils were reconstituted to 1 x 10^6 cells/ml in RPMI 1640 without phenol red, containing 1% fetal calf serum, and used immediately. For analysis of the neutrophil effect on fibronectin architecture, neutrophils were used at 2 x 10^5/ml in RPMI 1640, with 20% plasma. Silicified plastic ware was used at all stages of cell preparation.

Endothelial cells were obtained from human umbilical cord veins by digestion for 20 min with collagenase type II (Sigma, St Louis, U.S.A.) for 30 min and those remaining were removed by sedimentation with 3% dextran (Fisons, Ireland), and 15 pg/ml preservative-free sodium heparin (Payne and Byrne, Greenford, Middlesex), and 15 pg/ml endothelial cell growth medium, consisting of RPMI 1640 (Gibco), growth supplement (Sigma). Cells were grown to confluence in 25 cm^2 tissue culture flasks (Becton Dickinson), and passaged by reconstitution to 1 x 10^6 cells/ml in RPMI 1640 without phenol red, containing 1% fetal calf serum, and used immediately. For analysis of the adhesion assay, or onto glass coverslips for study of fibronectin morphology of endothelium. Neutrophils plus CD18 monoclonal antibody that recognises the common beta chain of the leukocyte integrins (MHH23) with neutrophils from HUS patients was assessed for its effect on fibronectin morphology of endothelium. Neutrophils plus CD18 antibody in control plasma were added to endothelium grown on coverslips. These were incubated for 1 h at 37°C, the coverslip removed and washed, and the endothelium immunostained for fibronectin.

**Results**

The percentage of neutrophils adhering to endothelium was higher for all twelve HUS subjects than for the control of the day.

![Percentage of neutrophils adhering to endothelium from HUS subjects compared with control of the day.](image-url)
TABLE III—DISRUPTION OF FIBRONECTIN ARCHITECTURE ON CULTURED ENDOTHELIUM AND EFFECT OF CD18 ANTIBODY

<table>
<thead>
<tr>
<th>Patient</th>
<th>CN/CP</th>
<th>CN/HP</th>
<th>HN/CP</th>
<th>HN/HP</th>
<th>HN/CP plus CD18</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>1</td>
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CN = control neutrophils; CP = control plasma; HN = HUS neutrophils; HP = HUS plasma; CD18 = monoclonal antibody directed against common \( \beta \) chain of adhesion molecules on neutrophils.

incubated with endothelium. Neutrophils from all ten induced varying degrees of destruction of the normal endothelial fibronectin architecture. Patterns of fibronectin degradation under the various conditions tested are listed in table III. With the exception of two subjects whose neutrophils caused minor changes only, control neutrophils in 20% control plasma did not affect fibronectin architecture. However, plasma from eight of the ten HUS patients incubated with control neutrophils caused fibronectin breakdown (mean score 1.7; i.e., mild-moderate disruption of the normal fibronectin architecture). Control plasma plus HUS neutrophils from all ten patients produced fibronectin breakdown, with greater disruption of the normal architecture than occurred with HUS plasma and control neutrophils (mean score 2.3, indicating moderate fibronectin breakdown). The greatest fibronectin degradation occurred when neutrophils and plasma from HUS subjects were combined on the endothelium (mean score 2.8, indicating extensive fibronectin degradation). Although neutrophils from HUS subjects induce fibronectin degradation, there is clearly an important contribution to fibronectin degradation from HUS plasma.

The scoring system, however, hid the heterogeneity among the patients. In subjects 1, 2, 3, and 6 little or no fibronectin degradation was induced by HUS plasma incubated with control neutrophils, whereas in the remainder the HUS plasma seemed to induce more fibronectin breakdown.

Because of the greater neutrophil adherence and subsequent fibronectin breakdown in the HUS subjects, we studied whether a CD18 antibody could inhibit the fibronectin breakdown. Incubation of neutrophils from the four subjects whose plasma had little effect on fibronectin breakdown with CD18 inhibited completely the fibronectin degradation seen without the antibody (table III). In the remainder, the CD18 antibody was completely ineffective.

**Discussion**

Activated neutrophils can damage endothelium, and neutrophil release products can damage the glomerular basement membrane. Attachment of neutrophils is a key event in inducing release of granule contents by these cells. A rabbit model of HUS has shown neutrophil-induced renal damage. We studied the role of neutrophils and plasma from HUS subjects, and their ability to damage endothelium.

We showed that the proportion of neutrophils adhering to endothelium in culture was twice as high for HUS patients as for controls. In addition, these neutrophils could degrade the extracellular matrix and integrin molecule fibronectin, indicating endothelial cell damage. There was no relation between clinical or laboratory indices in these patients and the extent of fibronectin breakdown or augmented neutrophil adhesion to endothelium.

Neutrophils from HUS subjects must be activated. Neutrophils use leukocyte integrin molecules CR3 (complement receptor 3 [CD18/CD11b]), LFA-1 (leucocyte function associated antigen-1 [CD18/CD11a]), and to a lesser extent p150,95 (CD18/CD11c) to adhere to endothelium. An increase in function of these molecules, producing hyperadhesive states, is seen after stimulation of neutrophils—for example, by bacterial analogues such as FMLP (formyl-met-leu-phe) or inflammatory mediators such as tumour necrosis factor—causing the cytoplasmic granules containing preformed integrin molecules to fuse with the neutrophil plasma membrane, rapidly increasing surface CR3 and p150,95. The ligand on endothelium for LFA-1 includes ICAM-1 (intercellular adhesion molecule-1 [CD54]) and possibly ICAM-2. The former is expressed at low levels on endothelium, and levels increase after stimulation by interleukin-1 or interferon-y treatment of the endothelium. The ligand for CR3, p150,95, however, use an Arg-Gly-Asp sequence for adherence, and hence bind through the extracellular matrix molecules, such as fibronectin, which are rich in this sequence.

The finding of degraded fibronectin after hyperadherence of neutrophils to endothelium from subjects with HUS is therefore of fundamental importance. We have found that fibronectin degradation is an important cell marker of damage to the endothelium (unpublished). It is likely that the activated hyperadherent neutrophils degranulate onto the endothelium, both causing degradation of the fibronectin by local release of proteases such as elastase, and in certain cases allowing free granule constituents into the plasma. Neutrophils can oxidatively inactivate the main elastase inhibitor of plasma, \( \alpha-1 \) antitrypsin, and so allow rampant elastase activity despite adequate plasma levels of the inhibitor. This degradation of fibronectin has important pathophysiological consequences, with the potential to induce an inflammatory amplification loop. The activity of fibronectin can be modulated by breakdown of its structure. Neutrophils will adhere more avidly to degraded fibronectin, and can degrade fibronectin to enhance their own adherence. For instance, proteolytic digests of fibronectin, but not intact fibronectin, can induce concentration-dependent neutrophil degranulation, resulting in release of neutrophil elastase and lactoferrin. The cytoadhesive Arg-Gly-Asp sequence of fibronectin appears to be the element of degraded fibronectin that is both stimulatory and degranulating for neutrophils. If the fibronectin within the extracellular matrix of the endothelial cells becomes degraded by the action of stimulated neutrophils, further neutrophil adherence is induced, leading to further neutrophil degranulation and fibronectin degradation.

In an attempt to interrupt this amplification loop, we added the monoclonal antibody CD18, directed against the common \( \beta \) chain of the three leukocyte integrin molecules and studied its ability to inhibit neutrophil-mediated fibronectin degradation of the endothelium. Such antibodies to the \( \beta \) chain of the leukocyte integrin can partially block neutrophil adherence to endothelium. In four of the ten HUS patients the fibronectin degradation was primarily
a neutrophil phenomenon, with little stimulatory effect from the plasma. In this group CD18 was able to inhibit neutrophil adhesion to endothelium by an LFA-1 dependent mechanism. This study was supported by the Arthritis and Rheumatism Foundation.

The value of hyperbaric oxygen in the treatment of acute carbon monoxide intoxication was assessed in 629 adults who had been poisoned at home in the 12 h before admission to hospital. In patients without initial impairment of consciousness (group A) the effect of 6 h of normobaric oxygen (NBO) (group A0, n = 170) was compared with that of 2 h of hyperbaric oxygen (HBO) at 2 atmospheres absolute (ATA) plus 4 h NBO (group A1, n = 173). At the 1 month follow-up 66% of A0 and 68% of A1 patients had recovered. In patients with initial impairment of consciousness the effect of one session of HBO (group B1, n = 145) was compared with that of two sessions (group B2, n = 141); all group B patients also received 4 h of NBO. At 1 month of follow-up 54% group B1 and 52% group B2 patients had recovered. The 7 patients left with neuropsychiatric sequelae (3 B1, 4 B2) and the 4 who died (2 B1, 2 B2) had all presented with coma. HBO was not useful in patients who did not lose consciousness during carbon monoxide intoxication, irrespective of their carboxyhaemoglobin level, nor were two sessions of HBO in patients who sustained only a brief loss of consciousness. The prognosis is poorest for those presenting with coma; the trial needs to be pursued in this group.

TRIAL OF NORMOBARIC AND HYPERBARIC OXYGEN FOR ACUTE CARBON MONOXIDE INTOXICATION

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Summary

The value of hyperbaric oxygen in the treatment of acute carbon monoxide intoxication was assessed in 629 adults who had been poisoned at home in the 12 h before admission to hospital. In patients without initial impairment of consciousness (group A) the effect of 6 h of normobaric oxygen (NBO) (group A0, n = 170) was compared with that of 2 h of hyperbaric oxygen (HBO) at 2 atmospheres absolute (ATA) plus 4 h NBO (group A1, n = 173). At the 1 month follow-up 66% of A0 and 68% of A1 patients had recovered. In patients with initial impairment of consciousness the effect of one session of HBO (group B1, n = 145) was compared with that of two sessions (group B2, n = 141); all group B patients also received 4 h of NBO. At 1 month of follow-up 54% group B1 and 52% group B2 patients had recovered. The 7 patients left with neuropsychiatric sequelae (3 B1, 4 B2) and the 4 who died (2 B1, 2 B2) had all presented with coma. HBO was not useful in patients who did not lose consciousness during carbon monoxide intoxication, irrespective of their carboxyhaemoglobin level, nor were two sessions of HBO in patients who sustained only a brief loss of consciousness. The prognosis is poorest for those presenting with coma; the trial needs to be pursued in this group.
Neutrophil activation in the haemolytic uraemic syndrome: free and complexed elastase in plasma

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Abstract. There is evidence of neutrophil involvement in the pathogenesis of the haemolytic uraemic syndrome (HUS), and neutrophil release products are thought to cause endothelial cell damage. Elastase is the major lysosomal proteinase liberated by activated neutrophils. In this study we measured both free and complexed elastase. No free elastase activity could be detected in the plasma of patients with diarrhoea-associated (D+) HUS using a specific substrate. However, there was a marked increase in a 1-antitrypsin (a 1-AT) complexed elastase as measured by a newly developed enzyme-linked immunosorbent assay not only in D+ HUS, but also in non-diarrhoea-associated (D-) HUS. This finding is independent of either a high polymorphonuclear leucocyte count or renal failure. This increase in bound elastase together with our sequential data which demonstrate raised a 1-AT complexed elastase levels early in the disease process further support the theory that neutrophil activation is one of the key events in the pathophysiology of this disorder.

Key words: Haemolytic uraemic syndrome - Neutrophils - Free and complexed elastase

Introduction

The haemolytic uraemic syndromes (HUS) are a heterogeneous group of disorders [1, 2]. In children two main subgroups are recognised - the "typical" form with a diarrhoeal prodrome (D+ HUS) and the "atypical" non-diarrhoeal form (D- HUS) [3]. A neutrophil leucocytosis is characteristic of D+ HUS [4–6]. The peripheral blood polymorphonuclear leucocyte (PMNL) count at presentation is significantly higher in D+ than in D- HUS, and within the D+ group a high PMNL count is associated with a poor outcome [7, 8]. Endothelial cell damage plays a central role in the pathogenesis of HUS and experimental studies have implicated PMNLs [9–12]. Activated PMNLs are capable of endothelial injury [13–15], and their release products can damage glomerular basement membrane [16]. Elastase is the major lysosomal proteinase liberated by activated PMNLs [17], and is complexed in the circulation by antiproteinases of which the most important is a 1-antitrypsin (a 1-AT) [18]. In this study we have evaluated PMNL activation in HUS by measurement of free and a 1-AT complexed elastase in plasma.

Patients and methods

Patients

Fifty-six children with HUS treated at the Hospital for Sick Children, Great Ormond Street, London, were studied. The criteria for diagnosis were a microangiopathic haemolytic anaemia, thrombocytopenia, and acute renal failure. They were divided into two groups on the basis of the presence (n = 47) or absence (n = 9) of a diarrhoeal prodrome. Samples were taken within 24 h of admission to hospital, and 5 children also had samples taken throughout the acute phase of the illness.

Blood samples were also obtained from:

1. Thirty-five healthy children being followed up after routine surgical procedures such as hernia repair or being investigated for short stature, who were used as normal controls.

2. Twenty-one inpatients identified by the haematology laboratory as having a PMNL count greater than 10 x 10^9/1 and defined as high PMNL count controls; they either had a septicemia or other infection and all had a normal plasma creatinine concentration, blood samples were obtained within 48 h of admission and within 24 h of instituting therapy.

3. Thirty-five children with chronic renal failure who had a glomerular filtration rate less than 15 ml/min per 1.73 m^2 SA.

Methods

Blood samples were collected into potassium-EDTA (ethylenediaminetetraacetic acid) tubes, and the plasma separated by centrifugation at 2000 g within 30 min and stored at −70°C until assayed.

a 1-AT complexed elastase enzyme-linked immunosorbent assay (ELISA). A modified assay for the measurement of a 1-AT complexed elastase was used [19]. The first antibody was sheep anti-human elastase antibody (ICN Immuno biologicals, High Wycombe, England) which
specifically recognises leucocyte elastase found in the azurophilic granules of human neutrophils. The antisera was produced by immunising sheep with purified protein obtained from the peripheral leucocytes of a patient with chronic myeloid leukaemia. Microtitre plates (Nunc-ImmunoPlate Gibco BRL, Paisley, Scotland) were coated with 100 μl antisera (10 μg/ml) in bicarbonate buffer (50 mM, pH 9.6) by incubation overnight at 4°C. All subsequent incubations were carried out at room temperature with volumes of 100 μl and the plates were always washed four times with 150 mM Dulbecco’s phosphate-buffered saline pH 7.2 (PBS) containing 0.1% “Tween 20” (BDH Limiteé, Porte, England) between stages. Standards or samples diluted 1:80 in PBS were then added and incubated for 2 h. The second antibody, horseradish-peroxidase conjugated sheep anti-human α1-AT (The Binding Site Ltd, Birmingham, UK) diluted 1 in 1000 in PBS, was added to each well and incubated for 2 h. The substrate (1.0 mg/ml O-phenylendiamine and 0.03% H2O2 in 100 mM citrate-phosphate buffer, pH 4.0) was added and incubated for 20 min. The reaction was stopped by the addition of 50 μl of 2 M H2SO4. The absorbance was measured at 492 nm in Titertek multiscan plus (Flow Laboratories Limited, Rickmansworth, England) ELISA reader. Standards were obtained by adding increasing amounts of reconstituted elastase, 2.5 μg/ml in PBS, to a constant volume of control plasma, giving final elastase concentrations of 50–16,000 ng/ml. The control plasma was harvested on a single occasion, aliquoted into small portions, and frozen at −70°C. The concentration was expressed as a function of the added elastase. We acknowledge that the units obtained by these standards are arbitrary because of the unknown concentration of α1-complexed elastase initially present in the control plasma.

Free elastase. Free elastase activity was measured enzymatically using a method adapted from Johnson et al. [20, 21]. The synthetic peptide methoxysuccinyl-ala-ala-pro-val-p-nitroanilide (MSAAPV-pNA) (Sigma Ltd, Porte, England) was used as substrate. This substrate is specific for human leucocyte elastase and does not recognise pancreatic elastase. Sample activities were determined by reference to a standard curve obtained with human leucocyte elastase (Sigma) made up in 100 μl of 100 mM HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid) buffer pH 7.5 containing 0.5 M NaCl and 10% dimethylsulphoxide added to 1 ml of 1 mM substrate made up in the same buffer. One unit was defined as change in absorbance of the optical density at 410 nm of 0.001/min at 25°C over 10 min. The elastase standard concentrations ranged from 1 to 400 ng/ml. A concentration of elastase of 1 ng/ml produced a change in optical density of 0.16/10 min, which was significantly greater than 0.04/10 min of the blank buffer.

Statistical analysis
Data were tested for normal or log normal distribution using normal probability plots. Means were compared by Student’s t-test and regression analysis was performed using an ordinary least-square regression using one independent variable for a linear model.

Results

α1-AT complexed elastase
Plasma α1-AT complexed elastase in children was log-normally distributed with a geometric mean of 0.29 μg/ml and range (±2 SD) of 0.11–0.76 μg/ml (Table 1). Complexed elastase in D+ HUS was 2.54 (0.62–10.5) μg/ml and in D− HUS 1.13 (0.14–9.0) μg/ml, both significantly greater than the control group (D+ P <0.0001; D− P <0.0001). Mean values for D+ and D− HUS patients were not significantly different, but the D− HUS patients were heterogeneous: 3 had markedly raised complexed elastase with levels above 1.0 μg/ml, while 6 had levels which were within the normal range. Children with a raised PMNL count had a geometric mean bound elastase of 0.57 (0.14–2.30), significantly less than in D+ HUS (P <0.0001) and not significantly higher than normal controls. In children with chronic renal failure the mean α1-AT complexed elastase was 0.29 μg/ml, similar to normal controls. Serial measurements of α1-AT complexed elastase were undertaken in 5 patients with D+ HUS following their admission in acute renal failure. On day 1 the geometric mean of the complexed elastase in these patients was 2.55 μg/ml. The level peaked at a geometric mean of 4.25 μg/ml on day 2 and subsequently fell to 2.23 μg/ml on day 3 and 1.36 μg/ml on day 5. From day 7 onwards the values were within the normal range (Fig. 1a). The mean PMNL count was 18.3 ×10^9/1 on day 1 and 17.3 ×10^9/1 on day 2, and remained elevated over a longer period than the complexed elastase: on day 5 the mean PMNL count was still markedly raised (20.4 ×10^9/1) and only returned to the normal range at day 11 (Fig. 1b).

The children with D+ HUS had a raised PMNL count at presentation, confirming the finding of Walters et al. [7]. Their mean PMNL count was 11.7 ± 6.6 ×10^9/1 (SD), significantly higher than the PMNL count of 6.0 ± 1.2 ×10^9/1 in normal controls (P <0.001) and 5.1 ± 1.9 ×10^9/1 in D− HUS (P <0.001) (Fig. 2).

There was a significant positive correlation between the PMNL count and the α1-AT complexed elastase level in the D+ HUS group (r = 0.74, P <0.01) but not in any of the other groups (Fig. 2).

Free elastase activity
The change in absorbance over 10 min produced with plasma from 20 D+ HUS children ranged from 0.04 to 0.12, and from 20 normal children ranged from 0.04 to 0.10. These results were below our lower limit of detection of 1 ng/ml, confirming that no free elastase activity could be detected in these children.

Discussion
An association between toxin-producing enteric pathogens and D+ HUS is now established in the majority of cases

### Table 1. Plasma α1-antitrypsin complexed elastase levels and polymorphonuclear leucocyte counts

<table>
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<tr>
<th>Group</th>
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<th>PMNL ×10^9/1</th>
<th>α1-AT complexed elastase (μg/ml)</th>
<th>Geometric mean</th>
<th>Range (±2 SD)</th>
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<tr>
<td>D+ HUS</td>
<td>47</td>
<td>11.7±  6.6</td>
<td>2.54*</td>
<td>0.62–10.5</td>
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<td>D− HUS</td>
<td>9</td>
<td>5.1±  1.9</td>
<td>1.13*</td>
<td>0.14–9.00</td>
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<td>Normal</td>
<td>35</td>
<td>6.0±  1.2</td>
<td>0.29</td>
<td>0.11–0.76</td>
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<tr>
<td>High PMNL</td>
<td>21</td>
<td>17.1±  4.4</td>
<td>0.57</td>
<td>0.14–2.30</td>
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<tr>
<td>CRF</td>
<td>35</td>
<td>0.29</td>
<td>0.10–0.85</td>
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* P <0.001 vs controls

D+ HUS, Diarrhoea-associated haemolytic uraemic syndrome (HUS); D− HUS, non-diarrhoea associated “atypical” HUS; PMNL, polymorphonuclear leucocyte count; CRF, chronic renal failure
There is increasing evidence for PMNL activation and PMNL-mediated endothelial injury in HUS [10–12]. Forsyth and colleagues [11] have demonstrated that HUS neutrophils have greater adherence to endothelial cells in culture than control neutrophils, and that these neutrophils can induce endothelial injury, assessed morphologically by degradation of endothelial fibronectin. It is postulated that activated PMNLs degranulate onto endothelium causing damage by local release of proteases such as elastase [25]. The involvement of PMNLs in the pathogenesis of HUS is further substantiated by the finding that a high PMNL count at presentation is associated with a poor outcome in D+ HUS [7], and that those patients with Shigella infections who develop HUS have significantly higher PMNL counts than those who do not [26].

We attempted to measure PMNL elastase as a marker of PMNL activation in HUS. However, we could not detect elastase activity in the plasma of patients in the acute phase of HUS. In contrast, Kaplan et al. [12], using an enzymatic method, reported raised elastase activity in D+ HUS serum compared to normal controls, but they used N-succinyl-alal-alal-alp-nitroanilide as substrate, which is not specific for leucocyte elastase and may also be broken down by pancreatic elastase. It is unlikely that neutrophil elastase, which is a very potent proteinase, exists free in the circulation, as it rapidly becomes bound to cell surfaces or plasma inhibitors, primarily α1-AT.

α1-AT is the major component of the α1 electrophoretic band of human plasma proteins and is an inhibitor of proteolytic enzymes. It is primarily a defence protein whose function is to protect the tissues from released proteolytic enzymes. It is synthesized in the liver and secreted into the plasma. As an acute phase reactant its normal plasma concentration can rise fourfold in response to the general stimulus of inflammation [27]. It may be that free elastase exists early in the disease process before the upregulation of α1-AT production, but we found no evidence of this. When measured in its α1-AT complexed form, elastase levels were found to be markedly raised in HUS. There was a significant increase in complexed elastase levels in both D+ and D– HUS relative to the three control groups – normal children, those in chronic renal failure and those with high PMNL counts secondary to other febrile illnesses. A positive correlation between complexed elastase levels and PMNL counts was found in the D+ HUS group of children. This correlation is of particular importance when considering that the outcome of this disorder is associated with the PMNL count at presentation [7]. Milford et al. [28], using a radioimmunoassay for human neutrophil elastase, also found raised levels: their
assay was such that both free and bound elastase would have been measured. Some of the D+ HUS patients also had elevated α1-AT complexes elastase levels, yet their PMNL counts were within the normal range. The explanation for this is unclear, and it appears that the D+ HUS group is itself heterogeneous.

The sequential data on 5 patients with D+ HUS show that high complexed elastase levels at presentation fall to normal within a few days. The corresponding PMNL counts, however, remain elevated for a longer period. This suggests that neutrophil activation occurs early in the disease process and that it is not the leucocytosis per se which reflects disease activity. There is nevertheless some correlation between the PMNL count and elastase levels in the D+ HUS group. A significant correlation was also observed when the normal and high PMNL controls were analysed together; the slope of the regression line obtained was not significantly different from that of the D+ HUS group, but its origin was several orders of magnitude less. Thus, although, α1-AT complexed elastase may reflect the PMNL count, there is a clear upward shift of this relationship in D+ HUS. This shift may reflect the markedly elevated α1-AT levels in D+ HUS as demonstrated by Bergstein et al. [29] and Kaplan and Mills [12].

The evidence supporting PMNL involvement in the pathogenesis of D+ HUS can be summarised as follows: (1) the raised peripheral blood PMNL count at presentation [7]; (2) the association of a high PMNL count at presentation with acute mortality and residual nephropathy [7, 30]; (3) the evidence that HUS PMNL are hyperadherent to endothelium and cause endothelial damage [11]; (4) the finding that α1-AT complexed elastase is raised at presentation in HUS, reflecting neutrophil activation. These observations suggest that the acute mortality and renal sequelae of HUS may be ameliorated by agents which suppress PMNL activation.

Acknowledgements. We would like to thank Dr. M. Levin for useful discussion. M. F. and V. S. were supported by the Kidney Research Aid Fund, and G. F. was supported by the Deutsche Forschungsgemeinschaft via Medizinische Hochschule.

References

Interleukin-8 and polymorphonuclear leucocyte activation in hemolytic uremic syndrome of childhood

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Interleukin-8 and polymorphonuclear leucocyte activation in hemolytic uremic syndrome of childhood. Polymorphonuclear leucocytes (PMNLs) are implicated in the pathogenesis of diarrhea-associated hemolytic uremic syndrome (D+ HUS). We investigated mechanisms of PMNL involvement by measuring tumor necrosis factor α (TNFα) and the novel cytokine, interleukin-8 (IL-8), a potent activator of neutrophils, together with α1-antitrypsin-complexed elastase (α1-AT-E) as a marker of neutrophil degranulation, and anti-neutrophil cytoplasmic antibodies (ANCA). IL-8 was not detected in the 17 normal children, but was significantly elevated in 20 of 25 D+ HUS children (P < 0.005), and in three of nine children with non-diarrhea-associated (D−) HUS. Sequential data showed that IL-8 peaked transiently in the circulation, reaching a maximum just before a more protracted burst of α1-AT-E. The IL-8 levels correlated significantly with circulating α1-AT-E concentrations (r = 0.50, P < 0.05). In D+ HUS IL-8 levels also correlated with the PMNL count (r = 0.63, P < 0.005), and the highest values were seen in those children who died in the acute phase of the disease. TNFα was raised in only 1 of 16 D- HUS children and in no patients were ANCA detected. The data suggest that PMNLs in HUS are recruited by IL-8, that this cytokine plays a key role in the PMNL activation which occurs, and that agents which suppress this recruitment and activation might play a therapeutic role in this disorder.

There is now substantial evidence pointing to PMNL involvement in the pathogenesis of D+ HUS. This disorder, which is the most common cause of acute renal failure in children in the United Kingdom, is characterized by hemolytic anemia, thrombocytopenia, and renal failure [1, 2]. Two broad subtypes are recognized: the first is associated with diarrheal prodrome (D+) whereas the second, which is rare in childhood and has a worse prognosis, is not (D−)[2]. The peripheral blood PMNL count is raised at presentation in D+ HUS [3], and there is an association between the height of the PMNL count, acute mortality, and residual nephropathy [3-6]. There is evidence that activated PMNLs cause endothelial injury [7-9] and that their release products can damage the glomerular basement membrane [10]. In D+ HUS children, PMNLs have been found to adhere more avidly to endothelium and to damage the endothelial cell [11], and alpha 1-antitrypsin complexed elastase (α1-AT-E) levels are raised at presentation, reflecting PMNL activation and degranulation [12].

The aim of the present study was to investigate mechanisms of PMNL involvement by measuring mediators and markers of PMNL activation in the serum and plasma of children with D+ HUS. We measured two key participants in the cytokine cascade: tumor necrosis factor α (TNFα) and interleukin 8 (IL-8). TNFα is a proinflammatory cytokine and a proximal mediator in the cascade having the ability to induce gene expression for other cytokines and receptors such as IL-1, IL-6, IL-2 receptor and IL-8 [13, 14]. IL-8 is a cytokine which is produced by monocytes and is primarily defined as a selective activator and chemoattractant of PMNLs; it stimulates the release of lysosomal enzymes and superoxides from them [15]. In this study we investigated whether IL-8 was released into serum in the acute phase of D+ HUS and its relationship to peripheral blood PMNL count; we measured PMNL α1-AT-E which is a marker of PMNL degranulation [16-18] and anti-neutrophil cytoplasmic antibody (ANCA) which is present in other vasculopathies [19-22].

Methods

Patients

Thirty-four children with HUS treated at the Hospital for Sick Children, Great Ormond Street, London, were studied. The criteria for diagnosis were a microangiopathic hemolytic anemia, thrombocytopenia, and acute renal failure. They were divided into two groups on the basis of the presence (D+ HUS; N = 25) or absence (D− HUS; N = 9) of a diarrheal prodrome. Three of the children with D+ HUS died within four days of admission to the Unit. Blood samples were taken within 24 hours of admission, and nine children also had serial samples taken throughout the acute phase of their illness.

Blood samples were also obtained from: (1.) seventeen healthy control children admitted for routine surgical procedures or investigation of short stature; and (2.) fifteen sick children identified by the hematology laboratory as having a PMNL count >10 × 10^9/liter, designated high PMNL count controls, they had a septicaemia or other infection, and all had a normal plasma creatinine concentration. Blood samples were obtained from them within 48 hours of admission. (3.) Ten children with chronic renal failure were included, all of whom
who had a glomerular filtration rate (GFR) less than 15 ml/min/1.73 m² SA.

**Methods**

**Serum IL-8 immunoassay**

IL-8 was measured by solid phase enzyme linked immunoassay (ELISA; Quantikine, Research and Diagnostic Systems, Minneapolis, Minnesota, USA). A monoclonal antibody specific for IL-8 was coated onto the microtiter plates provided. Standards with known amounts of IL-8 and samples were pipetted, in duplicate, into the wells, and any IL-8 present bound by the immobilized antibody. For detection, an enzyme-linked polyclonal antibody specific for IL-8 was applied. A substrate solution containing tetramethylbenzidine and H₂O₂ was added, and the color developed was proportional to the amount of IL-8 bound in the initial step. A standard curve was prepared of OD versus IL-8 concentration (Fig. 1). The lower limit of detection in this assay was 50 pg/ml. This was determined by adding 2 standard deviations to the mean optical density value of 5 zero standard replicates and calculating the corresponding concentration from the standard curve. The intra-assay coefficient of variation (CV) was 8% (N = 10) and the interassay CV was 9.3% (N = 5).

**Serum TNFα immunoassay**

TNFα was measured by solid phase ELISA (Quantikine). A monoclonal antibody specific for TNFα was coated onto the microtiter plates provided. Standards with known amounts of TNFα and samples were pipetted, in duplicate, into the wells. For detection, an enzyme-linked polyclonal antibody specific for TNFα was applied. A substrate solution was added, as above, and a standard curve drawn of OD against the concentration of TNFα in the standard wells. The lower limit of detection for this assay was 10 pg/ml. The intra-assay CV was 5.4% (N = 10) and inter-assay CV 4% (N = 5).

**Human α1-AT complexed elastase immunoassay**

Plasma α1-AT-E was measured by a modified ELISA as previously described [12]. A sheep anti-human elastase antibody (ICN Immunobiologicals, High Wycombe, UK) specific for leucocyte elastase was coated onto microtiter plates (Nunc, Roskilde, Denmark). For detection, horseradish-peroxidase-conjugated sheep anti-human α1-AT (The Binding Site Ltd., Birmingham, UK) was applied. A substrate was added and the color developed in proportion to the amount of α1-AT complexed elastase in the standard wells and by comparing the OD of the samples to the standard curve of the concentration of α1-AT complexed elastase in the unknown samples determined.

**Imunoassay for ANCA against an acid extract of neutrophil cytoplasm**

IgM and IgG ANCA were measured in serum by an ELISA technique using acid extracted neutrophil cytoplasm antigen prepared as described by Savage et al [23]. For each preparation, the optimal dilution for coating was determined using negative control sera and positive patient sera. The antigen was coated onto microtiter plates (Nunc). Test or control serum samples were added in duplicate to neutrophil antigen-coated plates. For detection, peroxidase conjugated anti-human IgG and IgM (Sigma) were applied, and after incubation and washing a substrate was added. After further incubation the reaction was stopped and ODs measured using an ELISA reader. The OD values of the standard sera at various dilutions were used to construct a standard curve of arbitrary units, from which the ANCA content of each test serum could be estimated. The arbitrary units were set as 64 and 32 for the IgG class ANCA-positive standard serum and IgM class ANCA-positive standard serum, respectively. The normal range for IgM for this assay is 0.5 to 9.1 units and for IgG 0.5 to 9.0 units.

**Statistical analysis**

Fisher's Exact test was used to determine the significance of the IL-8 results in the different groups compared to normals. The Mann-Whitney test was used as a test of significance of difference between the medians of the different groups. The correlations between IL-8 and α1-AT-E, and IL-8 and PMNL count were examined non-parametrically using Spearman's rank correlation coefficient. α1-AT-E and PMNL counts were log-normally distributed, and geometric means were compared using Student's t-test on log-transformed data.
Results

Serum IL-8

IL-8 was not detected in the serum of 17 normal healthy children. It was, however, detected and was significantly elevated in 20 of 25 children with D+ HUS [median value: 305 pg/ml, range: non-detectable (ND) - 3300, P < 0.005], and in three of nine D− HUS children (P < 0.05; Fig. 2). The median IL-8 value in D+ HUS was significantly higher than in D− HUS (P < 0.005). Only two of ten children in chronic renal failure (CRF) had detectable IL-8 levels, and this did not reach significance (P > 0.05). Children in the high PMNL control group had significantly elevated IL-8 with a median value of 460 pg/ml (range 150 to 5200, P < 0.005), and these results were not significantly different from those obtained in the D+ HUS group (P > 0.05).

Serum TNFa

When 40 normal donor sera were evaluated using this assay (Quantikine) the maximum observed value was 25.8 pg/ml. TNFa was detected in the serum of only 1 of 16 of the children with D+ HUS, in whom the level was 1800 pg/ml. Moderately elevated levels were detected in three of 15 high PMNL controls with values of 27, 28, and 30 pg/ml recorded, and no significant activity was detected in any of the normal children or those with D− HUS.

Serum ANCA

No IgM ANCA was detected in any of the 10 D+ HUS or 9 D− HUS sera tested. One of the D− HUS group had a weakly positive IgG ANCA of 13.1 units; the remaining 8 D− and all the D+ HUS children had negative IgG ANCA results.

Serial IL-8 and α1-AT-E

Serial measurements of IL-8 were undertaken in nine children with D+ HUS following their admission in acute renal failure, and in seven of them α1-AT-E was also measured. This data is graphically illustrated in Figures 3 and 4. In this group of patients the serum IL-8 reached a maximum on day 3 with a mean of 8,193 pg/ml, and then fell sharply to mean values of 272 and 312 pg/ml on days 4 and 5, respectively. α1-AT-E peaked on days 3 to 4 with mean values of 5,100 and 4,200 ng/ml, respectively, but then declined more gradually. The highest values for both IL-8 and α1-AT-E were seen in the three children who died in the acute phase of the disease (Figs. 2 and 3). We found a significant positive correlation between the IL-8 and the circulating α1-AT-E levels (r = 0.50, P < 0.05) in these children.

Polymorphonuetrophleucocyte counts (PMNLs)

The children with D+ HUS had a raised PMNL count at presentation with a geometric mean of $14.0 \times 10^9$/liter (range 7.6 to 24.3). Their mean PMNL count was significantly higher than the PMNL count of $4.9 \times 10^9$/liter (2.7 to 6.8) in normal controls (P < 0.001) and $5.7 \times 10^9$/liter (4.6 to 7.2) in D− HUS (P < 0.001). Serial PMNLs on eight of the nine patients are illustrated in Figure 5; they remain elevated over a longer period than both the IL-8 and the α1-AT-E, returning gradually into the normal range by days 11 to 12.

There was a significant positive correlation between the PMNL count and the IL-8 level at presentation in the D+ HUS group (r = 0.63, P < 0.005), but not in the high PMNL group (r = 0.34, P > 0.05).

Discussion

The etiology of D+ HUS is more clearly understood now that an association has been found with toxin-producing enteric
pathogens, in particular Verocytotoxin-producing Escherichia coli (VTEC) [24, 25]. It is also well recognized that damage to the glomerular endothelium is the main site of injury in HUS, and there is now increasing evidence suggesting that the PMNL has an important role in the pathogenesis. There is a significant relationship between the PMNL count at onset and adverse outcome [3–6]. HUS PMNLs show increased adhesion to endothelium in vitro [11], α1-AT complexed elastase levels are increased in the acute phase of the syndrome [12], and by electron microscopy peripheral blood neutrophils appear degranulated [26].

It is not known whether the neutrophil response which occurs is attributable to the action of verocytotoxin or to endotoxin, the lipopolysaccharide (LPS) derived from the bacterial cell wall. Endotoxin has been suggested as having a pathogenic role in D+ HUS but has not yet been studied in VTEC disease. It alters the surface characteristics of endothelial cells via a cascade of cytokines which renders them adhesive for neutrophils [27, 28].

Significant TNFα was detected in only one of the 16 D+ HUS children tested; this child was admitted in the early phase of her disease before the onset of acute renal failure. She subsequently went into renal failure and developed neurological complications. She survived her acute illness but has residual nephropathy with proteinuria and a reduced GFR. TNFα is a proximal...
mediator in the cytokine cascade, appearing in the circulation of several species as a brief, early peak after infusion of bacteria or bacterial LPS [13]. The majority of our patients were referrals from other hospitals admitted to the unit several days after the onset of their illness, which may account for our inability to detect TNFα in the others at the time of their admission.

IL-8 is a recently described 6-10 kDa protein known for its in vitro leukocyte chemotactant and activation properties [29-36], and is a potential mediator of host response to injury and infection. It has several properties that suggest that it plays a role in mediating some of the inflammatory responses of neutrophils induced by endotoxin, IL-1 and TNFα [15]. It is known to cause shedding and up-regulation, respectively, of the two important neutrophil adhesion molecules: leucocyte adhesion molecule 1 (also known as LAM-1, LECAM-1 and L-selectin) and CD11b/CD18 [37-39]. Adherence via the latter has been shown to mediate neutrophil priming for the respiratory burst [40] and degranulation [41]. It has been shown that IL-8 appears in the circulation of primates in vivo during septic shock, sublethal endotoxia, and after the administration of IL-1α [42]; more recently increased levels have been detected in normal humans as part of the acute inflammatory response to intravenously administered endotoxin [43].

We detected significantly elevated IL-8 in both D+ and D− HUS and in high PMNL controls. IL-8 levels were significantly higher in D+ than in D− HUS. Our study therefore demonstrates that IL-8 is produced in vivo as part of the inflammatory response occurring in humans during the acute phase of D+ HUS. The sequential data on nine children with D+ HUS shows that IL-8 circulates in blood with peak levels occurring at 72 hours.

Serial measurements of α1-AT-E, a known marker of PMNL activation and degranulation [16-18], were undertaken on seven of the nine patients. Plasma α1-AT-E levels peaked at 72 to 96 hours and declined more slowly than the corresponding IL-8 levels. From this data IL-8 appears to peak more transiently in the circulation, achieving a maximum level just before the more protracted burst of complexed elastase. The correlation of IL-8 with α1-AT-E suggests that IL-8, which is known to stimulate degranulation of adherent neutrophils, may be promoting the release of this proteolytic enzyme in these patients. A positive correlation was also found between IL-8 and the PMNL count at presentation in the D+ HUS group. This correlation is of particular importance when considering that the outcome is associated with the PMNL count at presentation [3-6], and indeed the higher levels of IL-8 were recorded in three patients who died during the acute phase of the disease.

From our data we cannot conclude whether the observed release of IL-8 into serum in D+ HUS is induced by Verocytotoxin or high LPS levels. It is also unknown whether serum IL-8 represents a pathogenetically important mechanism or is a concomitant marker of disease activity. There is, however, increasing evidence suggesting that IL-8 promotes leukocyte adhesion in vivo and leads to recruitment of PMNLs to sites of tissue inflammation [37, 38]. IL-8 has previously been shown to induce granulocytosis upon systemic injection and skin reaction upon local injection in experimental animals [44]. The recruitment of PMNLs to inflammatory sites is also dependent on other chemotactic factors generated in tissue, such as the cleavage products of complement activation C5a and C3b, which we are currently measuring prospectively in children with HUS. Elevated elastase levels do indicate that granulocytes become highly activated in this disease and may represent a source of endothelial cell damage. Our findings in this study are consistent with the hypothesis that IL-8 contributes to the dynamics of circulating PMNLs and acts as a mediator of the PMNL activation which occurs. We conclude that IL-8 participates in the complex cascade of cytokine responses to infectious and inflammatory stimuli, and plays a significant role in the pathophysiology of D+ HUS. These findings raise the possibility that agents which suppress PMNL recruitment and activation may have a therapeutic role in this disorder.

We were unable to detect significant ANCA activity in HUS serum and conclude that this antibody is not implicated in this disease.

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