RECENT ADVANCES

Advances in haemolytic uraemic syndrome

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The purpose of this review is to draw together recent advances in the pathogenesis of haemolytic uraemic syndrome (HUS) induced by verocytotoxin, including information from the successful Third International Symposium on Verocytotoxin Producing *Escherichia coli*, "VTEC'97", in Baltimore. However, the highly publicised outbreaks of verocytotoxin producing *Escherichia coli* (VTEC) infection in Japan and Scotland in 1996 demand a brief epidemiological preface.

These two epidemics were caused by the VTEC serotype O157. Reinforced by the recommendations of the Advisory Committee on the Biological Safety of Food in 1995, local public health laboratories in the UK have been routinely screening diarrhoeal faecal samples for this pathogen and the number of positive isolates reported to the Central Public Health Laboratory Service has soared. It is not known whether this reflects a true increase in VTEC related disease or is simply the result of more extensive investigation.

The family of verocytotoxins closely resembles shiga toxin, the exotoxin of *Shigella dysenteriae* type I. The terms shiga-like toxin and verocytotoxin are synonymous, although there is pressure from US groups to standardise to the former. VT-2 is the toxin most closely associated with HUS in Europe and North America. Unlike the shiga toxin itself, the verotoxins are plasmid encoded and thus this important pathogenic property can be transferred between *E coli* strains, with potentially serious epidemiological consequences. Experience suggests that this has not yet happened in the UK.

In the epidemiological survey conducted in Britain and Ireland in the late 1980s,^{1,2} serotype O157 was the main, but by no means the only, culprit, and other VTEC serotypes were also involved. Children presenting with diarrhoea associated HUS at the Birmingham Children's Hospital since 1990, however, have all had evidence of O157 either by stool culture or serology. Non-O157 serotypes are of major significance in other parts of the world—for example, O111 in Australia. Worryingly, Schmidt *et al* documented an outbreak of colitis and HUS at a German kindergarten in which the infective cause was a VT-2 producing *Citrobacter freundii* that had contaminated parsley used in a salad.³ It appears that the VT-2 plasmid can jump species!

For all these reasons a further period of surveillance in the UK is called for. From Febru-

ary 1997 for a period of three years paediatricians have again been asked to report cases of HUS to the surveillance unit of the Royal College of Paediatrics and Child Health using the monthly orange card scheme. This is linked to VTEC surveillance operated through public health laboratories. Because of the importance of recognising outbreaks early, paediatricians are also being encouraged to report immediately by telephone. Parallel studies are in progress in other countries.

Pathogenesis

Figure 1 gives an outline scheme of the pathogenesis of HUS, the starting point being verocytotoxin. The production of this exotoxin distinguishes the enterohaemorrhagic properties of VTEC from enteropathic *E coli*. Like enteropathic *E coli*, *E coli* O157, but not necessarily all VTEC serotypes, carries the *eaeA* gene responsible for a distinctive pattern of bacterial attachment to enterocytes with effacement of epithelial villi.⁴ This attachment, and the signalling events associated with it, induce the water and electrolyte loss which causes diarrhoea. VTEC serotypes do not normally invade the host, but their immediate proximity to the epithelium is likely to promote delivery of verocytotoxin to the mucosa. Polarised epithelial cells can transport toxin from the apical to the basolateral surface by transcytosis.⁵ Once in the mucosa, verocytotoxin itself is thought to be responsible for the microvascular injury leading to thrombosis, haemorrhage, and necrosis which appear clinically as bloody diarrhoea. Additionally, there is a dense neutrophil infiltrate in the submucosa.

Cytotoxicity

Verocytotoxin operates intracellularly. The verocytotoxin molecule consists of an A chain and five B chains. To gain entry to cells the B chain undergoes lectin binding to a specific terminal digalactose moiety of a ceramide based receptor, globotriaosylceramide (Gb3), also known as CD77 or blood group antigen Pk. The biological function of Gb3 is unknown. After receptor binding the holotoxin is internalised by a calcium and energy dependent mechanism via clathrin coated pits and incorporated into endosomes. From here it can be routed in a number of ways, including degradation by lysosomes, or it can be re-exported from the cell. To intoxicate, however, it has to gain access to the cytoplasm. This is achieved by routing to the trans-Golgi network and by

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Figure 1 Outline scheme of the pathogenesis of HUS.

retrograde transport to the endoplasmic reticulum, and, incidentally, via this to the nuclear envelope.⁵ In this process the A subunit is cleaved from the holotoxin by either furin or cytosolic calpain. The free A chain is itself an enzyme and cleaves adenine from ribosomal RNA at a point where aminoacyl transfer RNA is assembled.⁶ This causes peptide assembly and protein synthesis to be arrested. It is assumed that the cytotoxicity of verocytotoxin is caused by protein synthesis inhibition and that the receptor density on target cells, the intracellular toxin burden, intracellular routing, and the demand for protein synthesis by the cell are important factors.

Various cells behave differently in culture. Vero cells, an immortalised primate kidney cell line, are killed by low doses of verotoxin within a few hours through the process of apoptosis or programmed cell death.⁷ By contrast, primary cultures of human kidney epithelial cells, both tubular and glomerular, show increased sensitivity to the protein synthesis inhibitory effects of verocytotoxin and yet the cells do not appear apoptotic and die by necrosis. Similarly, primary cultures of human glomerular vascular endothelium, the assumed target of the toxin, do not show apoptosis, whereas other human microvascular cells do. These differences raise interesting questions about the cellular response to intoxication beyond the inhibition of protein synthesis.

The distinctive histological lesion of diarrhoea associated HUS is glomerular capillary thrombosis causing the glomeruli to appear engorged. Occasionally, there is propagation of the thrombus into the afferent arterioles.⁸ By ultrastructure the glomerular endothelial cells are swollen, vacuolated, and often separated from the basement membrane by amorphous or fibrillary material in the subendothelial space. It is a reasonable hypothesis that direct intoxication of glomerular capillary endothelial cells might lead to thrombosis, either by damaging the thromboresistant properties of healthy endothelium or by revealing prothrombotic subendothelial sites where cells become detached. Nothing is known about the transport and delivery of toxin from the gut to other organs, however.

In studying the effects of verocytotoxin on human glomerular microvascular endothelial cells (GMVEC) it is crucial to fully characterise these cells and determine their degree of confluence. With the method developed by van Setten *et al.*,⁹ GMVEC were over 99% pure and their endothelial origin was confirmed by the expression of von Willebrand factor, EN-4, PECAM-1 and V1E cadherin. VT-1 cytotoxicity was evaluated by determining the number of viable adherent cells and by assay of overall protein synthesis after exposure to varying concentrations of toxin. In unstimulated cells toxicity was inversely related to the degree and duration of confluence, proliferating cells being the most sensitive. In highly confluent GMVEC, VT-1 cytotoxicity required pre-exposure of the cells to inflammatory mediators, such as tumour necrosis factor- α (TNF- α), which induced an increase in the number of VT-1 receptors. Thin layer chromatography of extracted glycolipids from GMVEC showed binding of VT-1 to Gb3. There were no major differences in protein synthesis inhibition with equal concentrations of VT-1 or VT-2.

Inflammatory events

Following VTEC infection a peripheral blood neutrophilia occurs, the magnitude of which indicates both the likelihood of developing the complication of HUS¹⁰ and the severity of HUS once it has occurred.¹¹ This is a clinically useful predictor. Not only are monocytes¹² and neutrophils present in increased numbers, but there is also evidence that neutrophils are activated. For example, the plasma concentration of neutrophil elastase is increased disproportionately to the number of circulating neutrophils,¹³ and correlates with clinical outcome,¹⁴ and there is circumstantial evidence of oxygen free radical damage. In a necropsy study of D+ HUS increased numbers of neutrophils were found in the glomeruli, confirming earlier observations.⁸

These events are clearly important, but we do not know whether neutrophil activation occurs within the kidney, less still that it contributes directly to the endothelial lesion which is at the heart of HUS. Parallels can be drawn, however, with the generalised Schwartzman reaction in rabbits in which repeated or continuous bacterial lipopolysaccharide injection leads to glomerular thrombus and cortical necrosis, much like HUS.¹⁵ Early neutrophil engagement in glomeruli is observed consistently in such models and the thrombotic lesion is largely neutrophil dependent. In HUS induced by shigellosis, not only is there a leukaemoid event, but evidence that lipopolysaccharide is detectable in blood.¹⁶ No such data exist in VTEC infection, although lipopolysaccharide is perceived immunologically in that IgM and IgG antibodies are formed in the wake of infection. It is not known whether the absorption of lipopolysaccharide or other bacterial products such as *N* formylated peptides are sufficient to initiate the inflammatory cascade.

Given the compelling evidence for both toxic and inflammatory events, it is reasonable to

look for links between them. To be satisfying, concepts of the pathogenesis must encompass both pathways. Various points of contact have emerged. Both lipopolysaccharide and the cytokines TNF- α and interleukin (IL)-1 β stimulate human umbilical vein endothelial cells to increase the surface expression of verocytotoxin receptors, thus making them more vulnerable to cytotoxic effects.^{17, 18} We now know that a similar up regulation of receptors also occurs with primary cultures of human glomerular endothelium described above,⁹ although Obrig *et al*, using another microvascular kidney cell line, found high toxin sensitivity without cytokine prestimulation.¹⁹

Just as inflammatory events have the potential to increase cytotoxicity, so too is there a pathway by which verocytotoxin can induce an inflammatory event. Acute inflammation is coordinated by cytokines, notably IL-1 β , TNF- α , IL-6, and IL-8. Several studies have found high concentrations of these in the plasma or urine of children with VTEC infection or HUS.²⁰⁻²² Urinary concentrations of IL-8 and IL-6 exceed that in plasma, suggesting that these cytokines are produced within the renal tract. An interesting observation is that verotoxin itself is capable of acting directly on monocytes to switch on cytokine production.²³ Monocytes have a specific receptor for verocytotoxin which is different from Gb3. Van Setten *et al*²³ showed that verocytotoxin does not inhibit overall protein synthesis in monocytes, either under basal conditions or after stimulation with lipopolysaccharide, in contrast with endothelial cells. However, VT-1 induces the synthesis of IL-1 β , TNF- α , IL-6, and IL-8 in the absence of lipopolysaccharide.

Models of HUS and future research

More than a decade ago epidemiological studies pointed to verocytotoxin as the prime mover in this disease and yet it has taken a long time to recognise or develop animal models. Using FITS labelled verotoxin, Monnens (personal communication) was unable to show the presence of Gb3 in the glomeruli of young or adult mice, rats, guinea pigs, or rabbits. Gb3 was identified in the Java monkey. In baboons, Siegler *et al* found Gb3 in the colon and kidney, but not the glomeruli, and then went on to examine the effects of VT-1 free of lipopolysaccharide in this species.²⁴ Anaemia, thrombocytopenia, and acute renal failure occurred within 36 hours. Petechia, congestion, haemorrhage, and necrosis were seen in the intestine. Urinary fibrin degradation products, TNF- α and IL-6 excretion increased. Interestingly, the kidneys showed congestion, endothelial cell and tubular damage, but the microvascular thrombi were described as mild.

The plant toxin ricin has identical enzyme action on ribosomal RNA to the A chain of the verocytotoxin/shiga toxin family. Cell access is not receptor restricted, however, and all cells are vulnerable. Rats given intravenous ricin develop glomerular thrombosis within a few hours, preceded by oliguric renal failure and haemolysis (Taylor, *et al*, in press). Interestingly, the thrombotic microangiopathy, which

looks identical to the human disorder, is confined to the glomeruli, even though the toxin will have been widely distributed. This predilection for glomerular involvement suggests that there might be rheological or other determinants which target the glomerular microcirculation.

Other intriguing models include the naturally occurring VTEC related disease of greyhounds known as "Alabama rot". In addition to enteritis, these dogs develop demarcated ulcerating skin lesions, focal oedema (for example, in a limb) and acute renal failure with a microangiopathic haemolytic anaemia. On histology there are extensive glomerular microthrombi.²⁵ This appears much closer to HUS than "oedema disease" of piglets, another VTEC infection,²⁶ in which there is neurological, but not renal, disease.

It seems that at last the phenomenon of toxin mediated HUS can be reproduced in the laboratory and these models interrogated to provide details of the pathogenesis. This, in turn, will open up concepts of logical treatment. For the present time, however, it is salutary to note that there is no evidenced based primary treatment for VTEC induced HUS. It is generally agreed that prompt diagnosis and good supportive care of hydration, electrolyte balance, and nutrition are crucial; so too is management of the complications of HUS such as cerebral oedema, the surgical acute abdomen, myocardial dysfunction, and diabetes mellitus. Although the search for the pathogenesis and testable treatment is clearly valuable, the greatest benefit to children lies in the prevention of VTEC infection. It is here that public health measures based on sound epidemiology, microbiology, veterinary and food science are of prime importance.

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