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Pathogenicity of enteropathogenic *Escherichia coli*

T. J. BALDWIN

Institute of Infections and Immunity, School of Clinical Laboratory Sciences, University Hospital, Queen's Medical Centre, Nottingham NG7 2UH

Enteropathogenic *Escherichia coli* (EPEC) remain an important world-wide cause of diarrhoeal disease and mortality of infants and young children. Research programmes around the world have, in recent times, made enormous strides towards a better understanding of EPEC pathogenesis, yielding unique insights into the molecular intercourse between host and pathogen. Recombinant DNA and cell biology techniques have provided powerful tools, giving the first intriguing glimpses of a wealth of bacterial products mediating complex host: pathogen interactions involving the subversion of normal host signalling processes. Much has been discovered since 1945, when *E. coli* was first implicated as a cause of diarrhoea. However, many questions remain unanswered and many more remain unasked. Much remains to be discovered, especially in the area of molecular interactions between host and pathogen and how they relate to the manifestation of disease in the patient.

History of diarrhoeal disease of infants and young children

Perhaps the earliest record of severe diarrhoeal disease affecting a population is found in the Bible (II Chronicles, chapter 21, v 14–19). These verses give a graphic description of an epidemic that raged through the country of Judah around 900 BC during the reign of King Jehoram. From an examination of the narrative, it appears that the writer is describing classical dysentery affecting adults and children, described as 'having great sickness by disease of thy bowels' and 'thy bowels fall out by reason of the sickness day by day'. Although this example from some 3000 years ago appears stilted in this early 17th century English translation, it would be unintelligible for most readers in the original Hebrew. This example highlights some of the problems when looking at historical records. Firstly, conventions governing writing style change as language evolves; secondly, diverse terminology is used to describe similar events in different geographical regions; and thirdly, descriptions change as a greater scientific understanding of the event becomes available. A similar event occurring today would be reported very differently thanks to advances in scientific knowledge, and also because it would be contemporary to both reader and writer, and

would be reported to interested persons in a scientific journal.

Recent advances in our understanding of infantile diarrhoea come at the end of a long history of a disease that decimated the young population of Europe and the New World during the second half of this millennium. Records from the 17th century clearly convey the enormity of the problem [1], with parts of London showing more burials of infants than baptisms during the summer months of several years (baptismal registers giving a better indication of births than other official documentation). In London during the period 1667–1720, there were 328 231 infant deaths resulting from 'summer diarrhoea', an average of over 6000/year. In the first half of this period, the major cause of mortality was recorded in the death registers as 'gripping of the guts', an entry used for infants or young children that had died from diarrhoea with cholera-like symptoms [1]. The incidence of 'gripping of the guts' in death registers reduced dramatically after 1692 to be replaced by the entry 'convulsions'. The age range and seasonal incidence of the entries suggests a change in terminology rather than the decline of one disease and the coincident rise of another [1].

By the hot summers of 1717–1729, infant mortality was as high as ever, but the official registers showed almost complete replacement of 'gripping of the guts' by 'convulsions'. It should be noted that, without effective treatment, severe protracted diarrhoea in

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infants was associated with malnutrition, dehydration and fever, leading ultimately to convulsions and death [2]. By the middle of the 19th century, another switch in reporting practice had occurred, bringing clarity to the real cause of many of the childhood deaths; entries of 'death by diarrhoea' increased, while those under 'death by convulsions' showed a coincident decline.

Following the industrialisation of towns and cities in England and Wales, infantile summer diarrhoea and accompanying deaths rose to rival those of London. The ensuing concern and heightened awareness for public health led to the worst areas being described as the diarrhoeal districts of England and Wales. In many of these areas, the number of infant deaths/1000 infants under 1 year of age showed a correlation with the percentage of women employed in the industries of the town [1]. Cities such as Leicester and Preston, with high infant death rates attributed to diarrhoea, also had a large female workforce employed in local factories [1]. By necessity, working women placed their children in the squalid nurseries attached to the factories, where substandard hygiene and bottle feeding was the source of many infections [1]. Until the end of the 19th century, annual country-wide figures for infant deaths by diarrhoea averaged around 15 000. Notable exceptions were years with hot summers (1880, 1884, 1886 and 1893) when this figure doubled.

From 1900 to the beginning of the First World War, the number of infant deaths by diarrhoea/1000 live births halved [3]. This decline continued after the First War until, by the end of the Second World War, it had decreased to 0.8% of the pre-1900 value. There were still large numbers of cases, but the survival rate was much higher. By the 1960s, summer diarrhoea was still prevalent and a cause for serious concern, but with improved treatment and sanitation, death rates and incidence rates were much lower than had been previously known.

The causative agent of summer diarrhoea

When, in 1939, Dr John S.B. Bray started work at the Hillingdon Hospital, Middlesex, as an emergency medical service pathologist, his interest in infantile diarrhoea was aroused by the large number of bottle-fed infants among his subjects [4]. These children had died of severe gastro-enteritis or, as it was termed then, 'summer diarrhoea', 'cholera infantum', or 'disease of the season', and was characterised by apparently stable subjects becoming critically ill and on the point of death within hours. Bacteriological and pathological examinations revealed little difference between gastro-enteritis patients and healthy individuals, except for a characteristic seminal smell associated with more severe cases. Culture plates from these patients, with apparently normal faecal flora, also had this characteristic smell and were termed 'smeller' cultures.

Bray was able to raise antisera to one smeller strain and use it to evaluate isolates from healthy individuals and patients with diarrhoea. In his landmark paper of 1945, he was able to state conclusively that his antiserum was specific for '*Bacterium coli*' from human infants with severe protracted diarrhoea. These initial results demonstrated clearly that antigenically homogeneous *B. coli* strains were associated with outbreaks of summer diarrhoea among infants [5, 6]. Bray's antiserum agglutinated bacteria isolated from stool cultures of 95% of infants with severe summer diarrhoea, compared to only 4% of controls without diarrhoea.

At that time almost half of the summer diarrhoea cases encountered by Bray were considered to have been hospital-acquired. This caused considerable concern, not least because 28–30% of affected infants died, reflecting the severity of the disease and lack of effective treatments [5, 6]. Bray's findings were soon corroborated by groups all around the world, establishing *B. coli* or, as it is now known, *Escherichia coli* as the causative agent of summer diarrhoea in infants.

Restricted serotypes

Shortly after Bray's publication [5], two serogroups were identified as being associated with infantile diarrhoea, O55 and O111. Investigation of cultures from frozen faeces derived from nosocomial outbreaks of infantile diarrhoea during 1947 in New York State, revealed that the *E. coli* isolates were predominantly of serogroup O111. This gave the first indication that *E. coli* enteropathogens affecting infants and young children belong to a limited set of O serogroups [7]. Attempts to demonstrate the pathogenicity of these strains for young animals failed, leading some workers to utilise human subjects and volunteers. The morally dubious step of infecting a 2-month-old child suffering from multiple congenital defects with 10^8 cfu of *E. coli* belonging to serogroup O111 resulted in the development of severe diarrhoea, and convinced many workers of the pathogenicity of *E. coli* O111 for young children [8]. Healthy adults were also infected with *E. coli* of serogroup O111 isolated from cases of neonatal enteritis and the development of diarrhoea followed. These studies confirmed the pathogenicity of certain strains of this serogroup for man [9]. Larger studies, in which healthy adults were infected with strains of serogroups O55 and O111 isolated from cases of severe protracted infantile diarrhoea, showed that the severity of symptoms was related to the size of inoculum administered [10].

The term enteropathogenic *E. coli* (EPEC) was coined to describe strains associated with infantile diarrhoea [11]. By 1974 it was known that EPEC were associated with 13 O serogroups [12–17]. Between 1950 and 1961, it was realised that different serotypes within incriminated serogroups were not equally pathogenic,

and that the ability to cause disease in infants was associated with particular O:H serotypes [16].

Pathogenic mechanism of EPEC

The first ultrastructural examination of gut tissue derived from piglets infected with an O55:H7 EPEC isolate revealed a series of characteristic changes in the absorptive cells of the gut [18]. These included lengthening and loss of microvilli at sites of bacterial adherence, followed by invagination and thickening of the plasma membrane, with increased electron density in the adjacent cytoplasm [18]. Confirmation of these ultrastructural changes was made with rabbit ileal loops [19] and suckling rabbits [20, 21] as models of infection. To describe the ultrastructural appearance of this lesion, the term attaching and effacing (AE) lesion was introduced, and the incriminated strains were described as attaching and effacing *E. coli* [20, 22].

EPEC attach initially to enterocyte microvilli, causing their localised effacement. At these foci of colonisation, bacteria attach intimately to the plasma membrane of intestinal cell surfaces, leading to swelling of the plasma membrane around attached bacteria and

production of the characteristic pedestal structure [22, 23] (Fig. 1). Ultrastructural studies of biopsy material derived from human patients with EPEC diarrhoea confirmed the formation of the AE lesion *in vivo*, and that damage to the small intestinal mucosa resulted in destruction of the absorptive area of the gut, villus atrophy and thinning of the mucosal lining [22, 24–27]. In severe cases, regions of the lamina propria also displayed evidence of cellular infiltration by lymphocytes, plasma cells and eosinophils, but with virtual absence of polymorphonuclear leucocytes [26]. Similar events were seen in the colon as a consequence of bacterial overgrowth, with oedematous lamina propria and moderate lymphocyte and plasma cell infiltration [25]. Changes in cell organelles indicative of intracellular damage were also evident in heavily colonised enterocytes and were thought to precede the eventual death and loss of cells from the villus surface [26]. Biopsies infected *in vitro* with known diarrhoeagenic EPEC strains exhibited the same AE lesion, with extensive vesiculation of the microvillus membrane and pedestal formation identical to that seen in infected patients [23]. A significant advance in the study of EPEC virulence was made with the discovery that similar lesions and pedestal structures are produced on tissue culture cells when infected with these strains [28, 29].

Fig. 1. The attaching and effacing lesion. Human gut biopsy material infected with EPEC strain E2348/69 O127 and visualised by transmission electron microscopy. Reproduced with permission of S. Knutton, Institute of Child Health, Birmingham.

EPEC adherence

Identification of EPEC as an important enteropathogen prompted enormous interest in the diarrhoeagenic mechanism of these organisms, and yet no obvious virulence determinants could be identified [30]. Nevertheless, studies investigating the pathogenicity of EPEC strains for volunteers were convincing [10]. The apparent inability of EPEC to invade or produce any recognisable enterotoxin led some microbiologists to speculate that isolates may have lost virulence plasmids during storage [31]. Others suggested that EPEC utilise a diarrhoeagenic mechanism distinct from cell invasion or soluble toxin production [32]. The situation was finally resolved by showing that EPEC, including those that had been in storage for some time, were able to cause diarrhoea in adult volunteers without classic symptoms of invasive disease or apparent production of secreted enterotoxins, thus confirming their pathogenicity, and its independence of other established diarrhoeagenic mechanisms [33].

This lack of any recognised virulence determinants prompted investigation into the colonisation factors important for infection. A system developed by Cravioto *et al.* [34] to detect EPEC adherence to cultured human epithelial cells was extended to demonstrate different patterns of bacterial adherence [35–37]. Diarrhoeagenic *E. coli* isolates were shown to exhibit one of three distinct patterns of adherence to cultured epithelial cells. These were termed, respectively, localised adherence (LA), where the bacteria form clumps or microcolonies on the cell surface; diffuse adherence (DA), where bacteria adhere evenly over cells of the monolayer; and aggregative adherence (AA), where the bacteria adhere to the epithelial cells and the glass coverslip support in a stacked brick pattern [36, 38, 39] (Fig. 2).

The ability of diarrhoeagenic *E. coli* isolates of EPEC serogroups to form the characteristic AE lesion in gut tissue and cultured cells appeared initially to display a correlation with the LA phenotype, but recent studies have identified *E. coli* isolates that cause typical AE lesions but do not demonstrate normal localised adherence under standard assay conditions [40]. Moreover, some strains which were classed as poorly adherent or non-adherent to cultured cells under standard assay conditions have been shown to form lesions on gut biopsies and HEp-2 cells with extended infection times [41]. However, it is still widely agreed that the ability of isolates to form AE lesions on cultured cells predominates in isolates that display LA.

In a study where plasmid DNA from 32 *E. coli* isolates of EPEC serotypes was analysed, 31 were found to contain large 55–70 MDa plasmids [35, 42]. In the case of one of these strains, E2348-69 (O127), the genes for LA were found to be encoded on a large 60 MDa plasmid termed pMAR2. Curing this strain of

Fig. 2. Patterns of enterovirulent *E. coli* adherence to HEp-2 cells. HEp-2 cell monolayers infected with *c.* 10^8 bacteria were incubated at 37°C in CO₂ 5% in air for 3 h. After washing extensively to remove unattached bacteria, monolayers were stained with Giemsa and visualised by light microscopy: **a**, EPEC strain E2348/69 O127 showing typical localised adherence (LA), discrete micro-colonies are indicated by open triangle symbols; **b**, DAEC strain 135 O86 showing typical diffuse adherence (DA) with bacteria adhering evenly to the entire cell surface; **c**, EAaggEC strain JPN10 O44 showing typical aggregative adherence (AA) with bacteria adhering to cells and glass coverslip in a stacked brick pattern. Bar, 10 μm.

the large plasmid resulted in a variant that had lost the LA phenotype [43]. However, extended incubation of the variant with cultured cells revealed limited adherence and lesion formation [28]. A region of pMAR2 required for LA adherence was identified by deleting sections of a Tn801-tagged derivative plasmid,

pMAR7, isolating self-replicating regions carrying the *Tn801* antibiotic resistance and screening transformants for LA [44]. A 40-kb region of the original plasmid, representing a 60% deletion, was isolated (pMAR15) and shown to encode LA to HEP-2 cells. The genes for LA were localised to a region of pMAR15 spanning one of the two *SalI* restriction enzyme sites [44]. A 1-kb *BamHI*–*SalI* fragment from this region was developed and used as an EAF (EPEC adherence factor) DNA probe for screening clinical isolates for the LA determinant [43].

Visualisation of EPEC strains by electron microscopy and negative staining has demonstrated enormous variation in fimbria-like surface structures elaborated by different isolates [45]. It still remains to be determined which of these, if any, represent the adhesin responsible for the traditional LA phenotype. One fimbria-like structure has attracted considerable attention. Termed bundle forming pili (BFP), these structures form rope-like bundles that tether bacteria together in clumps [46]. The gene encoding the 18–21-kDa pilus subunit, termed *bfpA*, is carried by the EAF plasmid present in many EPEC isolates [47]. Functional expression of BFP requires 14 contiguous genes encoded on an 11.6-kb fragment of EAF plasmids [48]. In addition to these genes, the chromosomally encoded *dsbA* locus is also required for functional BFP. The *dsbA* gene encodes a periplasmic protein that catalyses the formation of disulphide bonds in exported proteins [49]. Interestingly, many EPEC isolates that clearly show LA or LA-like adherence under standard assay conditions do not express BFP under these same conditions. This raises the possibility that BFP may not be the only adhesin that can confer an LA phenotype.

The pedestal lesion and cytoskeletal disruption

The distinctive loss of microvilli, pedestal formation and other characteristic cytoskeletal changes seen during EPEC infection of human cells indicated substantial re-arrangements in cellular actin. Changes in the actin architecture of EPEC-infected cells could be evaluated with phalloidin, a fluorescein-conjugated phalloxin [50]. For the first time, this approach demonstrated clearly that major actin re-arrangements occurred in cells colonised with EPEC [51]. The region of the cell directly beneath the sites of bacterial intimate attachment showed intense fluorescence, indicating substantial actin polymerisation in the pedestal lesion [29]. In addition, by combining use of specific antibodies with immunofluorescence microscopy, other actin-associated proteins such as myosin, ezrin, talin and α -actinin were shown to be localised to the pedestal [52, 53]. The fluorescent actin staining (FAS) test was found to be specific for isolates of the EPEC and enterohaemorrhagic *E. coli* (EHEC) groups of enteropathogens, both of which form identical membrane lesions on gut tissue [29, 54]. Other *E. coli*

enteropathogens, such as enteroinvasive *E. coli* (EIEC), displayed only limited, diffuse actin accretion, while enteroaggregative and diffuse adhering *E. coli* (EAggEC and DAEC) gave no indication of any actin polymerisation under the conditions used [29, 55, 56].

Although EPEC-infected cells show substantial polymerisation of actin within pedestal structures, it is also clear that other actin structures within the cell, such as stress fibres, are not substantially affected (Fig. 3). Indeed, stress fibres directly beneath the sites of bacterial adhesion show little or no evidence of distortion or breakdown. This suggests that the EPEC-mediated actin polymerisation is localised and may not involve significant depolymerisation of established actin structures to generate the monomers required for the pedestal actin polymerisation.

This simple test for the AE lesion is perhaps one of the most important steps toward our present understanding of the processes of EPEC diarrhoea. The FAS test enabled the isolation of the first lesion mutants by insertion of transposons, which in turn enabled the genes involved in lesion formation to be cloned and sequenced, an area which will be described later.

Fig. 3. Altered actin distribution in HEP-2 cells infected with EPEC strain E2348/60 O127, visualised with phalloidin staining and UV light microscopy (FAS test): **a**, focuses on the actin accretion within the pedestal, directly beneath the attached bacteria; **b**, focuses on other actin structures of the cell, such as stress fibres, showing little or no breakdown of these structures in the vicinity of the attached bacteria.

Role of calcium

Elevated intracellular calcium is known to mediate activation of villin, an actin-severing protein, causing breakdown of the actin component of the microvillus core, leading to eventual loss of microvilli through membrane vesiculation similar to that seen during EPEC infection [57–59]. Intracellular calcium has been measured for numerous cell types with fluorescent calcium indicator dyes such as quin2, fura2 and indo1. These compounds undergo a shift in peak fluorescence intensity and wavelength when they bind calcium ions, and this can be measured to give an accurate estimate of calcium concentrations [60, 61].

The intracellular calcium concentration of EPEC-infected HEp-2 cell monolayers has been measured with the fluorescent calcium indicator quin2–2-[2-bis-(carboxymethyl)-amino-5-methylphenoxy]-methyl]-6-methoxy-8-bis-(carboxymethyl)-aminoquinoline [62]. This approach provided the first evidence that EPEC infection of HEp-2 monolayers caused a significant increase in intracellular free calcium from a basal level of 50–75 nM to 200–322 nM 4–6 h after infection [63]. The observed calcium rise was not dependent on extracellular calcium, as the use of calcium-free medium or EGTA-chelated medium did not affect the rise in intracellular calcium. Moreover, a significant reduction in the calcium rise could be achieved by pre-treating cells with the drug dantrolene, which is thought to inhibit mobilisation of calcium from caffeine-sensitive intracellular stores [64]. The intracellular location of elevated calcium was visualised with fluo3, a fluorescent dye with an emission peak in the visible part of the spectrum. These experiments indicated an elevated calcium concentration in the vicinity of the attached bacterial microcolonies. Together, these data suggested the involvement of specific pathways for the mobilisation of intracellular calcium stores, probably involving signal transduction pathways and the generation of second messenger molecules.

Release of calcium from stores in the endoplasmic reticulum is mediated by the intracellular second messenger 1,4,5-inositol triphosphate [64, 65], generated by the action of phospholipase C on the membrane phosphatidylinositol diphosphate (PIP₂) lipids. EPEC infection of human cells increases the hydrolysis of phosphatidylinositol lipids, suggesting the activation of a signal transduction pathway involving phospholipase C [63]. It was some time before these original findings were corroborated by other groups [66, 67], by which time it was clear that although all EPEC microcolonies were associated with actin polymerisation, not all were associated with calcium rises as visualised with fluo3. This raised the possibility that a significant rise in intracellular calcium was not essential for the generation of lesions on cultured epithelial cells; rather it may be involved

in the early effacement of microvilli from gut enterocytes. This view was supported by observations that chelation of intracellular calcium did not abolish actin polymerisation completely in the pedestal [68]. These findings, and the fact that chelation of intracellular calcium affected loss of viability of infected cells [68], suggested that the elevated calcium observed in cultured epithelial cells infected with EPEC may be a downstream event involved in the death of heavily colonised cells.

Activation of host protein kinases

In cells infected with EPEC, the generation of inositol phosphate second messengers and calcium release would be associated with other signalling events. These include the concomitant generation of the second messenger diacylglycerol (DAG), also in response to phospholipase C activity on PIP₂ [69]. The resulting activation of the phospholipid-dependent protein kinase C (PKC) by DAG, and calcium-dependent activation of protein kinases such as myosin light chain kinase (MLCK), would be expected to have profound effects on electrolyte secretion and cytoskeletal architecture of the gut enterocyte through the phosphorylation of target proteins.

EPEC infection of cultured epithelial cell monolayers and gut biopsy material revealed the phosphorylation of several host proteins, the most prominent of which were a group of *c.* five proteins in the 21–23 kDa region [70, 71] (Fig. 4). By N-terminal protein sequencing, one of these proteins was identified as myosin light chain (MLC) [52]. Phospho-amino acid analysis of MLC, purified from either the soluble or cytoskeletal fraction of infected cells, demonstrated that at least two protein kinases were involved in MLC phosphorylation at different times after infection [72]. During the early stages of infection, phosphorylated MLC could be purified only from the soluble cell fraction and was phosphorylated predominantly on threonine. Later in infection, MLC was phosphorylated predominantly on serine and was exclusively in the cytoskeletal cell fraction. Phosphopeptide mapping of cytosolic MLC demonstrated an identical pattern to that seen with MLC purified from cells treated with phorbol myristic acid (PMA), a potent activator of PKC. In contrast, MLC purified from cytoskeletal fractions gave similar phosphopeptide maps to those reported for involvement of myosin light chain kinase (MLCK) [72]. Therefore, it was proposed that early host cell events triggered by bacterial adherence involve PKC-mediated phosphorylation of MLC, causing its dissociation from polymerised actin and movement into the soluble cell fraction [72, 73]. The resulting destabilisation of actin structures in the microvillus core and villin activation would lead to calcium-mediated core breakdown and eventual loss of microvilli through vesiculation [72, 73]. Later, as the lesion develops, local actin polymerisation beneath the

Fig. 4. Altered kinase activity and protein phosphorylation in the intestinal cell line CaCo-2 cells infected with EPEC strain E2348/69 O127. Lane 1, numerous proteins phosphorylated in response to EPEC infection; ▲, position of phosphorylated MLC; △, position of the unknown 29-kDa protein phosphorylated on serine in response to EPEC infection and EGF treatment. Lane 2, CaCo-2 cell monolayers infected with the laboratory strain HB101 harbouring the adherence plasmid pMAR2.

sites of bacterial attachment would be stabilised by association with MLCK-phosphorylated MLC, leading to the development of pedestal structures. Antibodies specific for serine-phosphorylated MLC react strongly with pedestals, confirming the association of serine-phosphorylated MLC with the newly polymerised actin [72, 73].

It is a consistent feature of EPEC infections of volunteers, that the onset of diarrhoea is significantly faster than would be expected if the cytoskeletal changes associated with effacement of microvilli and reduced absorption were the only contributory factors. Indeed, observations that EPEC infection causes a severe watery diarrhoea seem more consistent with the involvement of a secretory component as well as the reduced absorption that has been suggested. An obvious candidate for the element responsible for early onset secretory type response is PKC [62, 70, 71, 73, 74]. PKC activation occurs early during EPEC infection and may promote a secretory response by phosphorylation of microvilli chloride transport proteins [74]. This would indeed promote a severe watery diarrhoea response similar to that seen with EPEC. There are many ramifications to the activation of PKC, but one that may be extremely pertinent to the development and persistence of diarrhoeal disease is that EPEC infection of cultured intestinal cell mono-

layers causes a reduced transmembrane electrical resistance, indicative of altered tight junctions between cells [74]. It has been suggested that PKC activity controls the structure of tight junctions, causing increased permeability between cells of the monolayer [74], which may go some way to explaining these observations.

Other host proteins are phosphorylated in response to EPEC infection, but many are still to be identified conclusively. One protein of *c.* 27–29 kDa, which is only seen in cells infected with EPEC or treated with epidermal growth factor (EGF), has been shown to be phosphorylated on serine and seems to predominate in the nucleus, but remains unidentified [62, 70, 71] (Fig. 4). Two other nuclear proteins are also phosphorylated, and from their size and physical characteristics it is likely that these are histones [62, 71]. A number of proteins are phosphorylated on tyrosine; recently one has been identified as phospholipase C- γ and may be the enzyme responsible for the production of the second messengers IP₃ and DAG [75]. A protein of 90 kDa, termed Hp90, is thought to be the intimin receptor requiring tyrosine phosphorylation for function, but also remains to be identified [75, 76]. Another tyrosine-phosphorylated protein of 85 kDa is the subject of present investigations and seems to be important for the actin polymerisation seen during EPEC infection.

EPEC-mediated loss of host cell viability

EPEC infection of gut tissue is characterised by major ultrastructural changes in the host cell [26], involving breakdown and redistribution of cytoskeletal actin [29]. In addition, prolonged infection of patients with EPEC causes gross histological damage to the intestinal mucosa resulting from extensive cell death in regions of colonisation [25, 27]. These observations suggest that a cytopathic effect is associated with infection by EPEC and it would, therefore, be expected that extended infection of tissue culture cells with EPEC isolates would be accompanied by loss of host cell viability. EPEC infection of tissue culture cells causes important biological changes, including cytoskeletal damage, alterations in local actin densities, and plasma membrane blebbing. These are also characteristic of cells that are in distress and may precede cell death. The viability of EPEC-infected tissue culture cells investigated by means of two vital staining procedures at different times after infection showed that moderate EPEC infection reduced the viability of the monolayer [68]. As infection times were extended, the viability of the monolayer reduced dramatically [68]. Chelation of extracellular calcium had no effect on viability loss, whereas chelation of intracellular calcium seemed to delay the onset of cell death by 2–3 h [68]. After this time, the dynamics of viability loss continued as normal, suggesting that the intracellular calcium chelator was being broken down in the cytoplasm,

thereby allowing death to proceed as normal [68]. As stated previously, although it seems reasonable that elevated calcium could be responsible for the initial effacement of microvilli from gut enterocytes, the elevated calcium observed in cultural epithelial cells infected with EPEC seems likely to reflect cell distress, preceding cell death.

Genetics of lesion formation

The development and availability of molecular genetic techniques has been essential in our understanding of the disease caused by EPEC. However, this would not have been possible without the development of simple phenotypic assays for the screening of mutants [77–81]. Perhaps the two most important phenotypic assays for the early isolation of mutants were the FAS test [29] and what has become known as invasion or uptake. It has always been recognised that ultra-thin sections of gut tissue and cultured monolayers invariably show a low number of bacteria inside cells [24–27, 80, 81], and that this does not represent invasion in the usual sense of the term. It has been suggested that this process resembles endocytosis and may be initiated by specific adhesins or triggered by irritation of the host cell prompting uptake before the pedestal forms [73, 80, 81].

The first EPEC mutants were isolated by screening *TnphoA* mutants for their inability to produce a FAS test. These mutants were still able to adhere to host cells non-intimately because of the presence of the EAF plasmid, but could not adhere intimately or produce normal lesions [82]. The disrupted gene was cloned, sequenced and found to have 50% similarity with the invasin protein of *Yersinia pseudotuberculosis*. The gene product was a 94-kDa outer-membrane protein involved in intimate adherence to cells. To reflect this function, the protein was termed intimin and the gene encoding it was termed *eaeA*, which stands for *E. coli* attachment and effacement [83]. Analysis of *eaeA* mutants for their ability to trigger familiar protein phosphorylation events in host cells clearly showed an almost identical pattern of protein phosphorylation after 3 h compared to wild-type strains. However, when infected cells were fractionated into soluble and cytoskeletal proteins at various time points, a very different picture emerged. Cells infected with wild-type EPEC showed a small amount of phosphorylated MLC in the soluble fraction after 0.5 h, increasing to a maximum by 1–1.5 h. After this time, the soluble phosphorylated myosin declined to uninfected levels by 3 h, while the cytoskeletal phosphorylated myosin increased. The level of cytoskeletal phosphorylated myosin remained at this level for at least 6 h. However, surprisingly, *eaeA* mutants triggered a much more rapid accumulation of soluble phosphorylated MLC, which by 1.5 h had returned to uninfected levels. A concomitant increase in cytoske-

letal phosphorylated MLC was observed but, unlike wild-type infections, this level was not sustained and by 4 h was almost at uninfected levels. The precise basis for these differences between wild-type EPEC and *eaeA* mutants is still unclear, but it appears that *eaeA* mutants can induce signals in host cells. This finding has since been confirmed by other groups who have demonstrated that *eaeA* mutants are still able to trigger tyrosine phosphorylation of Hp90 [76].

Screening *TnphoA* mutants for strains deficient in cell entry yielded a number of genes involved in EPEC pathogenicity [77]. There were several groups of 'non-invasive' mutants, of which one set had inserts located in the *bfbA* and *dsbA* loci that, as described earlier, are required together for functional BFP [78]. A second set comprising mutants that were unable to adhere intimately to host cells contained inserts in two distinct genes: *eaeA* encoding intimin, and a gene originally termed *eaeB*, but now re-named *espB* [84]. The *espB* gene encoded a 37–38-kDa protein with no discernible secretion signal peptide that was therefore considered to be a cytoplasmic protein. Another set of mutants, termed category 4 mutants or *cfm*, were completely deficient in the FAS test and induction of tyrosine phosphorylation. Cloning and sequencing the disrupted genes from *cfm* strains revealed homology to a type III secretion system [85]. It is now known that this region contains at least nine genes as part of the type III secretion system, now known as *sep* (secretion of EPEC proteins) genes [79]. Recently, it has been shown that the genes for *eaeA*, *espB* and *sep* are located within a 35-kb region of the *E. coli* chromosome. Termed LEE, for locus of effacing *E. coli*, this region is inserted into the *E. coli* chromosome at 82 min, adjacent to the *selC* gene encoding selenocysteine tRNA [86]. Interestingly, this is the same location at which the retronphage ϕ R73 and the 70-kb PAI region of uropathogenic *E. coli* are inserted, suggesting that this may be a hot spot for the insertion of virulence determinants [87]. It is now accepted that this region forms what has become known as the EPEC pathogenicity island [87].

Expression of *eaeA*, *espB* and several other genes involved in EPEC pathogenicity are regulated by the product of a gene encoded on the EAF plasmids. Termed *per*, for plasmid encoded regulator, its discovery brought a new level of complexity into our understanding of EPEC virulence regulation, by introducing the concept of EPEC virulence regulons [88].

Secreted proteins

As stated previously, no EPEC-secreted enterotoxin has ever been found. Recently, however, the secretion of a number of proteins from EPEC isolates under tissue culture conditions has been reported [89]. These proteins are also produced during the course of the

disease, as volunteers infected experimentally develop antibodies to the secreted proteins [85]. A 37-kDa secreted protein was purified and identified by N-terminal protein sequencing as the product of the *espB* gene [89], thought previously to be cytoplasmic because of the absence of a recognised secretion signal. Along with EspB, at least four other proteins of molecular size 110, 40, 39 and 20–25 kDa are also secreted, although some EPEC strains secrete up to eight additional proteins. Secretion of these proteins seemed, at first, to be regulated by CO₂ present in the tissue culture atmosphere and bicarbonate in the medium [89]. Around the same time, other groups confirmed these findings and investigated further the mechanisms of secretion, showing that it was dependent on the type III secretion system encoded within the LEE and that it was probably pH and osmolarity that controlled secretion, not CO₂ concentrations [85, 90]. The 39-kDa secreted protein showed strong homology to glyceraldehyde phosphate dehydrogenase (GAPDH), except that it contained an extra two amino acids at its amino terminus [90]. The gene encoding the 110-kDa secreted protein has been named *espC* and has been shown to have homology to genes encoding IgA proteases. In contrast, the gene encoding the 20–25-kDa protein termed *espA* seems to have little homology to anything in protein sequence data bases [91]. At least two of these secreted proteins, EspB and EspA, are essential for the production of signals in the host cell. There is preliminary evidence from this laboratory that the EspA protein is associated with the bacterial cell surface in many EPEC isolates, and that it can be purified simply by vortex mixing washed cells in isotonic buffer. The role in pathogenicity of the other secreted proteins at this time remains obscure.

Conclusions

The wealth of knowledge available on the pathogenic mechanism of EPEC has enabled Donnenberg and Kaper to propose an updated three-stage model of disease progression [78]. Stage 1 involves non-intimate adherence between bacteria and host cell *via* BFP (or possibly other LA adhesins): stage 2 sees the start of signal transduction in the host cell, with tyrosine kinase and PLC activation, calcium elevation, secretion of EspA and EspB, and activation of PKC; stage 3 involves intimate adherence of bacterium to epithelial cell *via* association of intimin with tyrosine-phosphorylated Hp90, actin polymerisation and pedestal formation. This model gives us perhaps the clearest picture to date of the processes involved in EPEC disease. However, if the rate of progression in this field in the future is anything like that in the past decade, then it can be expected that this model will soon need revising.

It is clear that our understanding of EPEC pathogenesis has come a long way since the work of Bray, and

even further since 900 BC, but there is still so much that remains unknown about the detail of the processes. How do the different host cell signalling components identified to date in infected cells interact together? What are the extracellular and intracellular signals that control expression of bacterial virulence factors? What host factors, if any, affect virulence expression in EPEC? Where did EPEC pathogenic determinants originate and how did they evolve? Some answers are close at hand, but others are years away.

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