Rapid modulation of electrolyte transport in Caco-2 cell monolayers by enteropathogenic *Escherichia coli* (EPEC) infection

G K Collington, I W Booth, S Knutton

Abstract

***Background and aims*—The pathophysiology of enteropathogenic *Escherichia coli* (EPEC) diarrhoea remains uncertain. EPEC adhere to enterocytes and transduce signals which produce a characteristic "attaching and effacing" (A/E) lesion in the brush border membrane. The present in vitro study was designed to determine whether signal transduction by EPEC also influences electrolyte transport.**

***Methods*—Caco-2 cell monolayers were rapidly infected with wild type EPEC strain E2348/69, or the signal transduction-defective mutant 14.2.1(1), and mounted in Ussing chambers.**

***Results*—Strain E2348/69 stimulated a rapid but transient increase in short circuit current (*I*sc) which coincided with A/E lesion formation; this *I*sc response was absent on infection with strain 14.2.1(1). While the initial rise in *I*sc induced by E2348/69 was partially (~35%) dependent on chloride, the remainder possibly represents an influx of sodium and amino acid(s) across the apical membrane.**

***Conclusions*—The study directly shows that, after initial adhesion, EPEC induce major alterations in host cell electrolyte transport. The observed *I*sc responses indicate a rapid modulation of electrolyte transport in Caco-2 cells by EPEC, including stimulation of chloride secretion, for which signal transduction to host cells is a prerequisite.**

(*Gut* 1998;**42**:200–207)

Keywords: enteropathogenic *Escherichia coli*; Caco-2 cells; Ussing chambers; electrolyte transport; signal transduction

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Accepted for publication
5 September 1997

Infections with enteropathogenic *Escherichia coli* (EPEC) are common in infants and young children in developing countries,[1 2] yet the pathophysiology of the resulting diarrhoea remains unclear. EPEC are not invasive and do not produce recognised enterotoxins.[3–5] It has generally been presumed that diarrhoea results from the direct interaction of EPEC with the small intestinal epithelium; EPEC adhere to enterocytes and produce a characteristic "attaching and effacing" (A/E) lesion in the brush border membrane.[6 7]

Current evidence suggests that A/E lesion formation proceeds in three stages[8]: (1) initial non-intimate attachment; (2) signal transduction to host cells leading to disruption of the brush border cytoskeleton and effacement of microvilli; (3) intimate adhesion of bacteria to the apical cell membrane and accumulation of host cell cytoskeletal elements beneath attached bacteria.

EPEC possess a large (55–70 MDa) plasmid encoding adherence factors which promote stage 1. Stages 2 and 3 involve chromosomal genes of the locus of enterocyte effacement (LEE).[9] Signal transduction to host cells requires EPEC secreted proteins (Esps); these are exported via a type III secretion system which involves genes within the LEE designated *sep* (for secretion of *E coli* proteins).[10 11] Signalling results in tyrosine phosphorylation of a 90 kDa host cell protein, Hp90.[12] Intimate EPEC attachment (stage 3) is mediated by intimin, a 94 kDa outer membrane protein, binding of which to Hp90 promotes accretion of cytoskeletal elements beneath attached bacteria to produce the mature A/E lesion.[13 14]

An assumption has been made that loss of functional microvillous surface area associated with A/E lesion formation leads to malabsorption and osmotic diarrhoea. A reduction in absorptive capacity has been shown in intestinal tissues using rabbit models of EPEC infection[15 16]; however, malabsorption alone would not explain the rapid onset of diarrhoea (as early as three hours) reported in experimental human infections.[17] This suggests the possibility of an EPEC induced secretory process, particularly in early infection. Given that no direct effect of EPEC infection on transepithelial electrolyte transport had been shown, we undertook to investigate this possibility in vitro.

Polarised cultured human intestinal cell lines—for example, Caco-2—grown on permeable supports and mounted into Ussing chambers can be used as in vitro models of transporting epithelia.[18 19] EPEC bind to Caco-2 cells, transduce signals, and produce A/E lesions identical with those seen in natural infections.[20] Using a rapid infection procedure we have studied the effects of EPEC infection on the transepithelial electrical parameters and ultrastructure of Caco-2 cells, from the onset of initial adhesion. The role of EPEC signal transduction in observed responses has been investigated using an EPEC class four mutant (*cfm*) that is unable to secrete signalling proteins, essential for A/E lesion formation, because of the interruption of a *sep* gene sequence.[10 11 21]

This study has been presented in part to the British Society of Gastroenterology.[22]

Table 1 Characteristics of EPEC strains used in this study

Strain	Origin	EPEC characteristics: Signal transduction	EPEC characteristics: "Attaching and effacing" adhesion	References
E coli (0127:H6)				
E2348/69	Infant diarrhoea outbreak, UK	+	+	12, 23
14.2.1(1)	Class four Tn*phoA* insertion mutant of E2348/69	–	–	12, 21

Methods

BACTERIA

Table 1 shows the origins and characteristics of the bacterial strains used in this study. Bacteria were grown routinely from frozen stocks subcultured into Mueller-Hinton broth (Oxoid Ltd, Basingstoke, Hants, UK) and incubated aerobically at 37°C for 18 hours. To facilitate expression of EPEC adherence factors, broth grown bacteria were subcultured (1:100 dilution) into bicarbonate buffered Dulbecco's modified Eagle's medium containing 25 mmol/l glucose (DMEM; Sigma Chemical Co, Poole, Dorset, UK) and incubated in an atmosphere of 5% CO_2 in air at 37°C for four to five hours.[24] The number of viable bacteria present in cultures was determined by plating serial dilutions of culture on to Mueller-Hinton agar, incubating aerobically at 37°C, and counting resultant colony forming units (CFU).

Supernatants of DMEM grown cultures were obtained by centrifugation at 10 000 *g* for 30 min, and sterilised by filtration through a 0.45 μm filter (Millipore Ltd, Watford, Herts, UK).

CELL CULTURE

Caco-2 cells were grown in DMEM supplemented with 1% non-essential amino acids, 2 mmol/l L-glutamine, and 10% fetal calf serum. Cells were maintained in a humidified atmosphere of 5% CO_2 in air at 37°C. Single-cell suspensions were obtained from confluent serial cultures by incubation with 0.25% trypsin and 0.02% EDTA in Ca^{2+}- and Mg^{2+}-free Hanks balanced salt solution. Caco-2 cells were seeded at a density of 5×10^4 cells/cm^2 on to Transwell polycarbonate microporous cell culture inserts (24.5 mm diameter, 0.4 μm pore; Costar Ltd, High Wycombe, Bucks, UK); monolayers were used a minimum of 21 days after seeding, by which time transepithelial electrical resistance (TEER) was stable (~150 Ω.cm^2) and cells exhibited a morphologically distinct apical brush border. All cell culture media, supplements, and reagents were purchased from Sigma.

INFECTION OF Caco-2 MONOLAYERS

Before infection with EPEC, Caco-2 monolayers growing on culture inserts were placed into prewarmed (37°C) DMEM without fetal calf serum and returned to routine incubation conditions for one hour. For rapid infection of monolayers, the culture insert was placed into a 30 mm diameter Petri dish and the DMEM bathing the apical cell surface was replaced with 1 ml DMEM grown bacterial culture (about 10^8–10^9 CFU); initial attachment of adherent bacteria to Caco-2 monolayers was facilitated by centrifugation on to the apical cell surface at 500 *g* for 4 min. After centrifugation, monolayers were rinsed in DMEM to remove non-adherent bacteria. Uninfected Caco-2 monolayers were subjected to all procedures involved in infection except exposure to bacteria.

MEASUREMENT OF TRANSEPITHELIAL ELECTRICAL PARAMETERS OF Caco-2 MONOLAYERS

For measurement of transepithelial electrical parameters, a culture insert with Caco-2 monolayer in situ was mounted between two Perspex half-chambers of a modified Ussing chamber. Each half-chamber was circulated continuously with 15 ml bathing solution (gassed with 5% CO_2/95% O_2) passing through a water-jacketed reservoir at 37°C. The exposed surface area of the monolayer was 3.8 cm^2. Monolayers were studied under short circuited conditions. The short circuit current (*I*sc) was passed through 3 mol/l KCl/agar bridges in series with Ag/AgCl electrodes connected to an automatic voltage clamp (WPI Inc, New Haven, CT, USA) which compensated for the resistances of the bathing solution and the culture insert. Transepithelial potential difference was detected using calomel electrodes in series with 3 mol/l KCl/agar bridges. Voltage pulses were used for determining TEER (Ω.cm^2) according to Ohm's law.

The bathing solution used in Ussing chambers was DMEM (prepared with the following composition (mmol/l): NaCl 104; KCl 5.4; $MgSO_4$ 0.8; NaH_2PO_4 0.9; $CaCl_2$ 1.8; $NaHCO_3$ 44; D-glucose 25) containing 1:25 dilution of Eagle's minimum essential medium (MEM) (×100) vitamin solution (M6895, Sigma) and 1:25 dilution of MEM (×50) amino acid solution (M7020, Sigma; L-amino acid composition (mmol/l): arginine 30, cystine 5, histidine 10, isoleucine 20, leucine 20, lysine 20, methionine 5, phenylalanine 10, threonine 20, tryptophan 2.5, tyrosine 10, valine 20). A nominally Cl^--free bathing solution was prepared by substituting SO_4^{2-} for Cl^-. In nominally Na^+-free bathing solution, NaCl, NaH_2PO_4 and $NaHCO_3$ were replaced by choline chloride, KH_2PO_4 and $KHCO_3$ respectively. Amino acid- and glucose-free bathing solutions were prepared by omission of the respective solutes. Osmolarity in all depleted media was maintained by addition of mannitol. Unless otherwise specified, all materials used in the preparation of bathing solutions were AnalaR grade (Merck Ltd, Poole, Dorset, UK). Additional reagents (amphotericin B and dibutyryl cyclicAMP (DbcAMP)) were purchased from Sigma.

BACTERIAL ADHESION

The level of adhesion of bacterial strains to Caco-2 cells was assessed after fixation in 70% methanol. Fixed preparations were Giemsa stained, and the proportion of cells with adherent bacterial colonies was determined by light microscopy.

For transmission electron microscopy, infected Caco-2 monolayers on culture inserts were fixed in 3% phosphate buffered (0.1

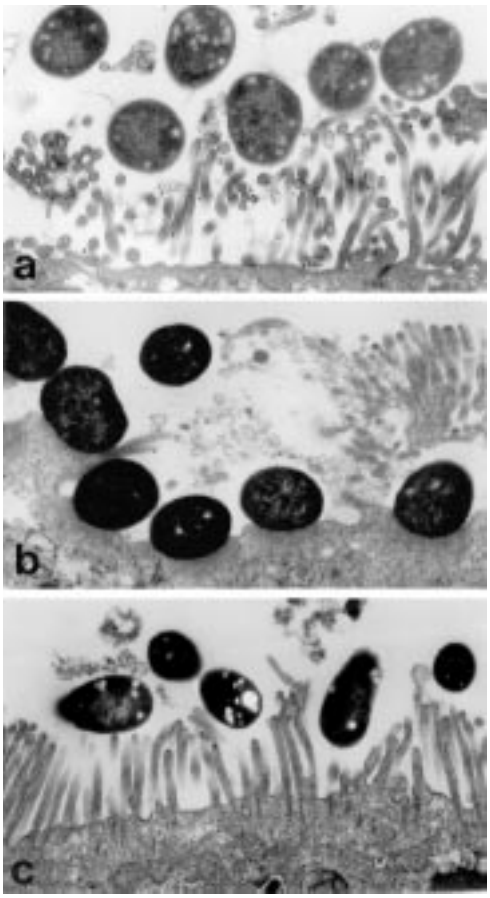

Figure 1 Transmission electron micrographs of EPEC adhesion to Caco-2 monolayers. On initial infection, both wild type strain E2348/69 (a) and cfm strain 14.2.1(1) adhered to intact brush border microvilli. After 15 minutes, E23438/69 were intimately attached to the apical membrane devoid of microvilli (b); however, even after 60 minutes, 14.2.1(1) did not intimately adhere to host cells (c). Original magnification: × 17 500.

mol/l, pH 7.4) glutaraldehyde. Segments of fixed monolayer, with supporting polycarbonate membrane, were post-fixed in 1% buffered osmium tetroxide, block stained in aqueous 1% uranyl acetate and embedded in Epon. Ultrathin sections were stained with uranyl and lead salts and examined using a Jeol 1200EX electron microscope.

STATISTICAL ANALYSES

Results are presented as mean (SEM). The data were analysed using Student's *t* test and $p<0.05$ was considered statistically significant.

Results

EPEC ADHESION TO Caco-2 MONOLAYERS

Caco-2 monolayers were infected with either the wild type EPEC strain E2348/69 or the *cfm* 14.2.1(1); the rapid infection method used produced synchronous initial attachment of bacteria to at least 75% of the cells in a monolayer.

To assess development of A/E lesions after rapid EPEC infection of Caco-2 cells, electron microscopy of monolayers was performed immediately after initial attachment of bacteria, and after 15, 30, and 60 minutes of subsequent incubation in Ussing chambers. At the onset of infection, both E2348/69 and 14.2.1(1) were seen attached to intact brush border microvilli (fig 1a illustrates initial adhesion of E2348/69). For strain E2348/69, characteristic A/E lesions were apparent by 15 minutes with bacteria intimately attached to the apical membrane devoid of microvilli (fig 1b); the failure of strain 14.2.1(1) to generate lesions (fig 1c), even after 60 minutes of infection, confirmed the lack of an A/E phenotype for this signal transduction-defective mutant.

EFFECT OF EPEC INFECTION ON TRANSEPITHELIAL ELECTRICAL PARAMETERS OF Caco-2 MONOLAYERS

Recordings of Caco-2 transepithelial electrical parameters were begun immediately after mounting monolayers into the Ussing chamber. Stable TEER values were obtained in uninfected monolayers within five minutes of mounting. Whereas uninfected Caco-2 monolayers maintained a constant *I*sc throughout the 60 minute study period, infection with EPEC strain E2348/69 induced a rapid increase in *I*sc (fig 2A) which reached a peak value after 10.5 minutes and which, despite subsequent decay, was still significantly higher than that in uninfected cells after 60 minutes. The distinctive *I*sc response induced by our wild type EPEC strain was not apparent when Caco-2 monolayers were infected with strain 14.2.1(1); infection with the signal transduction-defective mutant was associated with only a small sustained increase in *I*sc (fig 2a).

The peak *I*sc induced by E2348/69 was not associated with any significant change in the TEER of Caco-2 monolayers (TEER at 10 min: uninfected *v* E2348/69 infected; 152 (8) *v* 143 (6) $\Omega.cm^2$; n = 9, $p>0.05$). However, whereas the TEER of uninfected monolayers subsequently remained stable, E2348/69 infection caused a 20% reduction during the following 50 minutes, increasing to 40% by four hours (fig 2b). This observation confirms previous reports of a detrimental effect of EPEC infection on TEER of cultured epithelial cell monolayers.[25 26] In contrast, infection with strain 14.2.1(1) did not adversely affect monolayer TEER (fig 2b).

The influence of EPEC culture supernatant on transepithelial electrical parameters was examined by replacing bilaterally the Ussing chamber bathing solution with a sterile filtrate of DMEM grown E2348/69; no changes in *I*sc or TEER of uninfected Caco-2 cells were observed over the next 60 minutes (data not shown).

CHLORIDE DEPENDENCE OF E2348/69 INDUCED *I*SC RESPONSE

To examine the Cl^- dependence of the E2348/69 stimulated *I*sc response, nominally Cl^--free bathing solution was used in the Ussing chambers; treatment of Caco-2 monolayers and bacteria before mounting into chambers was otherwise unchanged. Under Cl^--free conditions the initial *I*sc increase caused by E2348/69 was reduced by up to 35% (fig 3), and the peak *I*sc was delayed by three minutes and significantly decreased from 22.3 (1.4) to 16.9 (1.3) $\mu A/cm^2$ (n = 9, $p<0.05$). The

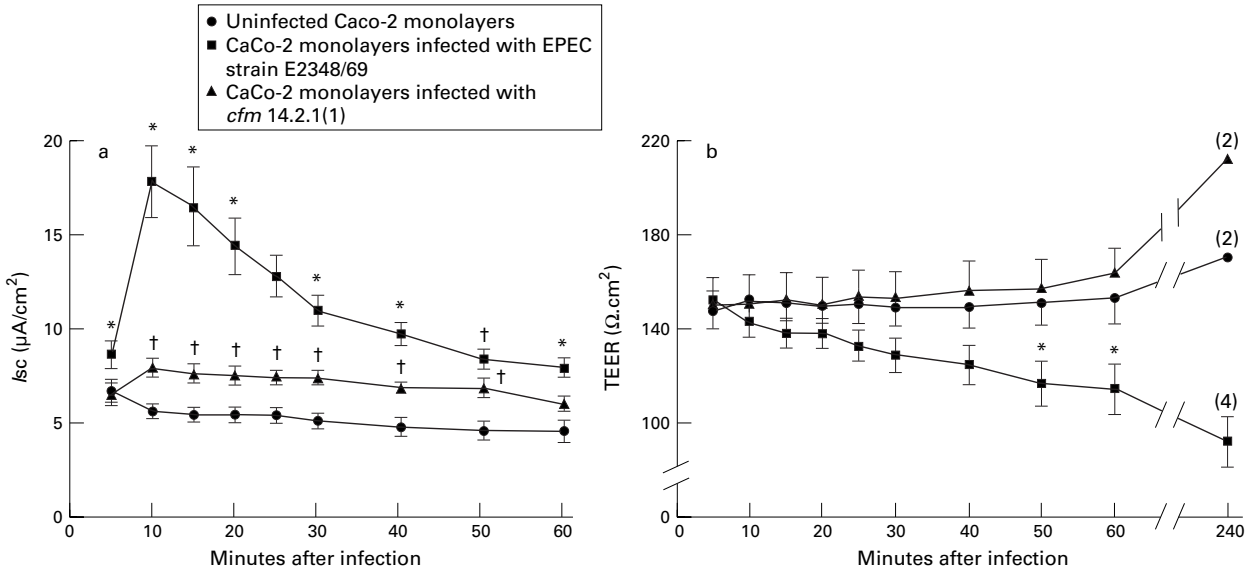

*Figure 2 (a) Short circuit current (Isc; μA/cm²) and (b) transepithelial electrical resistance (TEER; Ω.cm²) in uninfected Caco-2 monolayers, and those infected with EPEC strain E2348/69 or cfm 14.2.1(1). Each point represents the mean (SEM) from nine monolayers, unless otherwise indicated in parentheses. *p<0.05 v uninfected or 14.2.1(1) infected monolayers; †p<0.05 v uninfected monolayers only.*

absence of Cl^- did not affect the *I*sc of uninfected Caco-2 monolayers (fig 3), the TEER of uninfected or E2348/69 infected monolayers, or the fall in TEER associated with E2348/69 infection (data not shown).

The role of Cl^- in EPEC induced *I*sc was further studied by examining the sensitivity of uninfected and E2348/69 infected Caco-2 monolayers to the cyclic AMP derivative DbcAMP. Apical application of DbcAMP (1 mmol/l) to uninfected Caco-2 monolayers stimulated a maximum *I*sc increase of 6.7 (0.9) μA/cm² (n = 5, p<0.05) within two minutes; *I*sc fell within six minutes of peak response to a plateau of 2.4 (0.2) μA/cm² (n = 5, p<0.05) which was sustained. This response, which was dependent on the presence of Cl^- in bathing solutions, is characteristic of DbcAMP induced *I*sc in Caco-2 cells and indicative of electrogenic Cl^- secretion.[27] However, apical exposure of E2348/69 infected Caco-2 monolayers to 1 mmol/l DbcAMP, applied five or 40 minutes after the initial infection, did not further enhance the EPEC stimulated increase in *I*sc (data not shown).

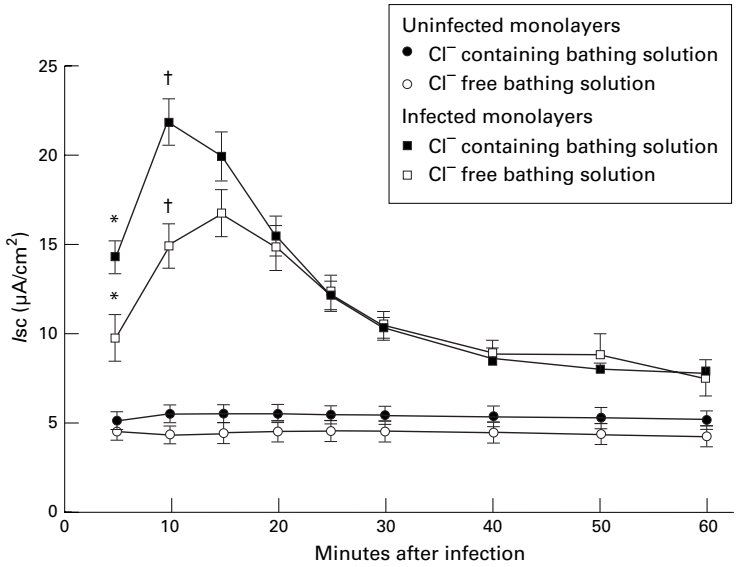

*Figure 3 Cl⁻ dependence of short circuit current (Isc; μA/cm²) in uninfected Caco-2 monolayers and those infected with EPEC strain E2348/69. The Isc of uninfected monolayers did not differ significantly between Cl⁻-inclusive and Cl⁻-free bathing solutions; each point represents the mean (SEM) of seven monolayers. The rapid increase in Isc induced by E2348/69 in Cl⁻-inclusive bathing solution was significantly reduced in Cl⁻-free solution; each point represents mean Isc (SEM) from nine monolayers. *p<0.05 v uninfected or 14.2.1(1) infected monolayers; †p<0.05 v uninfected monolayers.*

ROLE OF SODIUM IN E2348/69 INDUCED *I*SC RESPONSE

Bilateral removal of Na^+ from the Ussing chamber bathing solution abolished both the *I*sc of uninfected Caco-2 monolayers and the E2348/69 induced *I*sc response. In addition, the absence of Na^+ increased TEER of both uninfected and E2348/69 infected monolayers (TEER at 10 min: uninfected $+Na^+$ *v* $-Na^+$; 140 (9) *v* 253 (57) Ω.cm²; n = 5, p>0.05. E2348/69 infected $+Na^+$ *v* $-Na^+$; 118 (9) *v* 206 (26) Ω.cm²; n = 8, p<0.05). The Na^+ dependence of basal electrical polarity in Caco-2 cells[28] precluded any meaningful study under Na^+-free conditions. Therefore, the contribution of Na^+ to EPEC induced *I*sc was assessed by comparing the sensitivity of uninfected and E2348/69 infected Caco-2 monolayers to application of the Na^+ ionophore amphotericin B (10 μg/ml added to the apical bathing solution five minutes after monolayers were mounted into Ussing chambers). In uninfected monolayers, amphotericin B stimulated a progressive increase in *I*sc which reached a peak at 25 minutes (27.2 (1.3) μA/cm²; n = 3) (data not shown). In E2348/69 infected cells exposed to amphotericin B, *I*sc increased rapidly to a peak value which, although delayed from 10 to 15 minutes, was not significantly different from that caused by infection alone (26.5 (1.9) *v* 23.7 (2.1) μA/cm²; n = 3, p>0.05); both of these peak *I*sc responses were similar in magnitude to that induced by amphotericin B in uninfected monolayers.

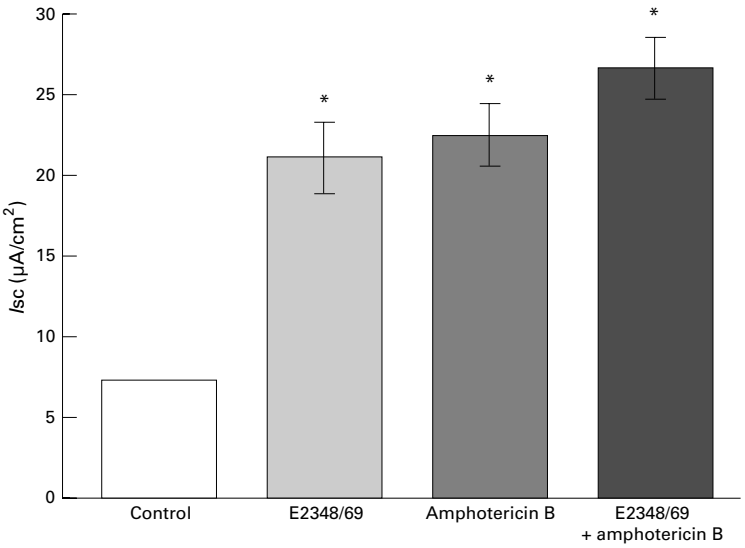

*Figure 4 Effect of amphotericin B (10 μg/ml apical application) on short circuit current (Isc; μA/cm²) in uninfected Caco-2 monolayers and those infected with EPEC strain E2348/69. Values presented were determined 15 minutes after initial infection. Although E2348/69 infection or amphotericin B treatment induced increases in Isc, these effects were not cumulative. Each bar represents the mean (SEM) from three monolayers. *p<0.05 v control (uninfected and untreated) monolayers.*

Moreover, no cumulative effect of E2348/69 infection and amphotericin B treatment was apparent during the initial increase in *I*sc (fig 4): after 15 minutes of study, the *I*sc increases induced by E2348/69 infection and amphotericin B treatment, respectively, were 13.6 (2.2) and 15.0 (1.9) μA/cm² (Δ*I*sc above uninfected/untreated monolayers; n = 3, p<0.05); however, these responses were not additive: the increase in *I*sc induced by coincident E2348/69 infection and amphotericin B treatment was only 19.1 (1.9) μA/cm² (Δ*I*sc at 15 min; n = 3, p>0.05 *v* E2348/69 infected or amphotericin B treated monolayers).

Irrespective of infection or amphotericin B treatment, TEER of Caco-2 monolayers was similar after 10 minutes of study. Subsequently, TEER in uninfected monolayers not exposed to amphotericin B was sustained (data not shown). In contrast, infection with E2348/69 or exposure to amphotericin B led to a progressive and similar fall in TEER, so that by 40 minutes TEER values were only 83 (6) and 88 (1)% (n = 3), respectively, of corresponding resistances at 10 minutes. Together, E2348/69 infection and amphotericin B treatment had an additive detrimental effect on TEER of monolayers, which by 40 minutes was reduced to 58 (4)% of that at 10 minutes (n = 3, p<0.05 *v* uninfected or E2348/69 infected monolayers).

In additional Ussing chamber studies, bathing solutions devoid of glucose or amino acids were used to explore the potential contribution of electrogenic Na^+/solute cotransport to generation of the *I*sc induced by E2348/69. The basal electrical parameters of uninfected Caco-2 monolayers were unaffected by the absence of glucose or amino acids. The glucose-free condition did not alter the *I*sc response stimulated by E2348/69 or the fall in TEER associated with infection (data not shown). However, the absence of amino acids caused a dramatic reduction in the E2348/69 induced *I*sc response, including a ~44% fall in the initial *I*sc increase (fig 5a). During 60 minutes of E2348/69 infection in the presence of amino acids, TEER of Caco-2 monolayers fell to 76 (5)% of that at 10 minutes (n = 5, p<0.05 *v* uninfected monolayers); however, there was no significant loss of TEER associated with

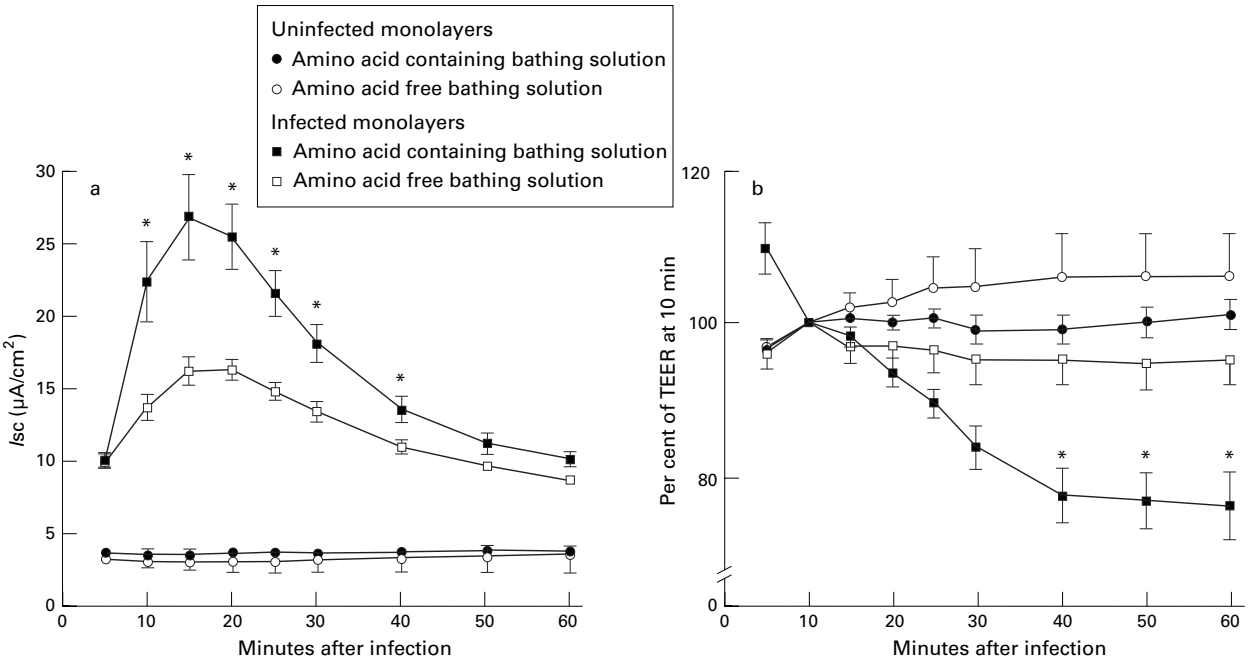

*Figure 5 Amino acid dependence of (a) short circuit current (Isc; μA/cm²) and (b) change in transepithelial electrical resistance (TEER; expressed as % of TEER at 10 minutes) in uninfected Caco-2 monolayers and those infected with EPEC strain E2348/69. The Isc of uninfected monolayers did not differ significantly between amino acid-inclusive and amino acid-free bathing solutions; each point represents the mean (SEM) from eight monolayers. The rapid and sustained increase in Isc induced by E2348/69 in amino acid-inclusive bathing solution was significantly reduced in amino acid-free solution; each point represents the mean (SEM) from five monolayers. Loss of TEER associated with E2348/69 infection did not occur under amino acid-free conditions. *p<0.05 v infected monolayers in amino acid-free bathing solution.*

E2348/69 infection under amino acid-free conditions (fig 5b).

Discussion

Using an infection method that produces rapid synchronous attachment of EPEC to Caco-2 cells, this study directly shows that, after initial adhesion, EPEC induce major alterations in host cell electrolyte transport. Furthermore, EPEC signal transduction to host cells is an absolute prerequisite for such changes in electrolyte transport.

Cells of the human intestinal line Caco-2 form a confluent polarised monolayer in culture and undergo spontaneous enterocytic differentiation.[28–30] In particular, they possess a well developed brush border membrane and a number of the polarised transport systems are present in mature small intestinal enterocytes—for example, Na^+-dependent solute cotransporters.[28 31–33] They also display electrogenic secretion of Cl^-.[27] Moreover, Caco-2 cells respond to EPEC infection with an A/E lesion that is ultrastructurally and biochemically identical with that seen in native small intestinal enterocytes.[12 20] These characteristics led us to conclude that Caco-2 cell monolayers constitute a useful model epithelium in which to study the pathophysiology of EPEC infection.

Growth of EPEC in DMEM, which stimulates expression of adherence factors,[24] facilitated rapid synchronous attachment of bacteria to Caco-2 cells once they were brought into contact. This infection technique synchronises the effect of EPEC on host cells, so optimising the generation of *I*sc, and allowed examination of *I*sc before development of A/E adhesion or any significant reduction in TEER.

The most striking electrical response to infection of Caco-2 cells with EPEC strain E2348/69 was a rapid ~3-fold increase in *I*sc which peaked about 10 minutes after infection and subsequently decayed toward basal levels. The initial *I*sc increase was not associated with any reduction in TEER, although infected cells exhibited a later fall. The transient nature of the initial *I*sc increase cannot be explained by this TEER loss; the decay in *I*sc after the peak of response was well established before any significant reduction in TEER was observed. However, if the initial rise in *I*sc represents increased activity of a specific ion channel or electrogenic transport system in the apical membrane, the transient nature of the peak response may well reflect EPEC induced loss of functional brush border membrane which occurs during A/E lesion formation.[6 7]

The coincidence of E2348/69 induced A/E lesion formation and initial *I*sc increase implicated EPEC signal transduction in the rapid modulation of Caco-2 electrolyte transport, and such a role was confirmed by the failure of the *cfm* strain 14.2.1(1) to stimulate the distinctive *I*sc response induced by the wild type. Recent studies have established that EPEC transduce signal to host cells by secreting specific proteins (Esps), encoded within the LEE, the export of which is dependent on *sep* genes.[10 11] These proteins are not secreted by the signal transduction-defective mutant 14.2.1(1)[10 11] and it may therefore be supposed that they are not only essential for A/E lesion formation, but also have a role in modulation of host cell electrolyte transport. This assumption is supported by a recent study which showed a reduction in the resting membrane potential of cultured epithelial cells, determined by whole cell current clamping, during EPEC infection which was dependent on signal transduction to host cells.[34]

Subsequent Cl^- substitution experiments indicated that at least one third of the initial *I*sc increase induced by strain E2348/69 was Cl^--dependent, consistent with stimulation of Cl^- secretion by EPEC. The apical Cl^- conductance of Caco-2 cells is reputedly sensitive to cyclic nucleotides and calcium[27 35–37]; however, there is some inconsistency in reported responses to these second messengers which may reflect differences in the maturity of the cells studied.[36] In our experimental system, application of the calcium ionophore A23187 does not affect *I*sc of Caco-2 cells (G K Collington, I W Booth and S Knutton, unpublished observation), yet DbcAMP stimulated a characteristic *I*sc increase.[27] Interestingly, the Cl^--dependent component of E238/69 induced *I*sc was similar in magnitude to the initial increase in *I*sc stimulated by 1 mmol/l DbcAMP, and infected Caco-2 monolayers were insensitive to the application of DbcAMP. Unfortunately, both the Cl^--dependent *I*sc increase induced by E2348/69 and the optimum *I*sc stimulated by DbcAMP were of relatively short duration, and these observations most probably reflect the limited capacity of Caco-2 cells to sustain the secretion of Cl^-.[27] As Caco-2 monolayers provide a poor model for the study of Cl^- secretion, further in vitro evaluation of this aspect of EPEC pathogenesis would be better undertaken in an alternative intestinal cell line—for example, T84 or HT-29—which possesses greater capacity for the sustained secretion of Cl^-.[18 19]

Although Caco-2 monolayers are not ideal for studying the secretion of Cl^-, they do constitute a useful model epithelium for the study of Na^+ conductance. The basal electrical polarity of Caco-2 monolayers is entirely dependent on Na^+, and cells maintain a high electrochemical gradient for this ion across their apical membrane.[28] In the present study, apical exposure of uninfected Caco-2 monolayers to the Na^+ ionophore amphotericin B induced a peak *I*sc increase similar in magnitude to that caused by EPEC infection. Furthermore, the failure of coincident amphotericin B application to enhance the initial *I*sc increase induced by EPEC infection suggests that the latter represents a Na^+ influx limited by the electrochemical gradient for this ion. As Caco-2 cells do not possess amiloride-sensitive apical Na^+ conductance,[28] it is unlikely that EPEC induced an influx of this ion by activation of specific channels. However, several Na^+-dependent cotransporters have been identified in the Caco-2 apical membrane, and the bathing solution contained a number of possible substrates for electrogenic Na^+

absorption including glucose[31] and amino acids.[28 32] In solute substitution experiments, the omission of glucose had no effect on the EPEC induced *I*sc; however, the response was highly dependent on the presence of amino acids. The precise subject(s) of this dependence and the mechanism of enhancement remain to be identified.

The adverse effect of EPEC infection on TEER of cultured cell monolayers was originally attributed to disruption of a transcellular pathway associated with intimate A/E lesion formation.[25] However, more recent evidence has shown that EPEC cause an increase in paracellular permeability, a defect attributed to disruption of the intercellular tight junctions.[26] Studies in intestinal epithelia have shown that a reduction in junctional integrity may be triggered by the activation of Na^+/solute cotransporters.[38 39] Correspondingly, during 60 minutes of EPEC infection we observed a reduction in TEER of Caco-2 monolayers which was abolished by omission of amino acids from the bathing solution. Furthermore, it became apparent during the course of our studies that the percentage of TEER lost between 10 and 60 minutes of EPEC infection increased with the magnitude of peak *I*sc stimulated (Pearson $r = 0.45$, $p<0.05$; $n = 22$). Although stimulation of absorption appears an unlikely trait for diarrhoeagenic bacteria, activation of Na^+/solute cotransport may contribute to EPEC pathogenesis by enhancing the detrimental effect of infection on tight junctional integrity.

In summary, the cause of diarrhoea during EPEC infection is considerably more complex than a loss of absorptive area consequent on A/E lesion formation. The data presented indicate that, after initial attachment, EPEC rapidly modulate host cell electrolyte transport. Most significantly, these changes are entirely dependent on EPEC signal transduction to host cells. In addition to stimulation of Cl^- secretion, which would contribute to diarrhoea during infection, EPEC possibly facilitate an influx of Na^+ and amino acid(s) into host cells; however, the pathophysiological significance of this effect remains unclear.

The authors thank Professor J-F Desjeux (INSERM, U290, Paris, France) for the design of the modified Ussing chamber and Dr Michael S Donnenberg (Division of Infectious Diseases, University of Maryland School of Medicine, Baltimore, MD, USA) for provision of EPEC strain 14.2.1(1). This work was supported by a grant from the Wellcome Trust (to SK).

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Call for Patients with Familial Pancreatic Disease: The EUROPAC Register

We are establishing a national UK register (EUROPAC) of families with hereditary pancreatitis, familial pancreatic cancer and where pancreatic cancer has occurred as part of a familial cancer syndrome. This collaboration in Liverpool is between the Department of Clinical Genetics (Dr Ian Ellis) and the Academic Department of Surgery (Professor John Neoptolemos). The data and samples are collected by behalf of ESPAC (the European Study Group for Pancreatic Cancer), Professor Markus Büchler, Berne, and Professor Hans Beger, Ulm. The study will collaborate with Dr David Whitcomb of the Midwest Multicenter Pancreatitis study group in the United States. We aim to recruit families who are prepared to donate blood for DNA studies. We hope to gain a clearer understanding of the genetic relationship between hereditary pancreatitis and familial pancreatic cancer, and develop screening protocols for individuals at risk.

Hereditary pancreatitis is associated with a mutation in the recently identified cationic trypsinogen gene. This mutation renders the enzyme active within the pancreas, leading to autodigestion. Individuals with recurrent pancreatitis have a greatly increased risk of developing pancreatic cancer, and there is some evidence that DNA analysis of cells from pancreatic fluid may be valuable in detecting premalignant changes which can predict the development of pancreatic adenocarcinoma.

The criteria for inclusion in the study are as follows:

- Hereditary pancreatitis: Three relatives with chronic pancreatitis in the absence of ethanol dependence, hypercalcaemia, or an obstructive cause.
- Familial pancreatic cancer: Two first degree relatives with pancreatic adenocarcinoma. Three or more relatives with pancreatic ductal adenocarcinoma. Pancreatic ductal adenocarcinoma in any two relatives where the sum of their ages is less than 110 years.
- Other familial cancer syndromes: A single documented pancreatic ductal adenocarcinoma in any family with an established familial cancer syndrome—for example, BRCA2, FAMMM, A-T, HNPCC, or FAP.

If you know of any suitable families who may be interested in joining the study, please contact: Fiona McRonald, Clinical Genetics, Alder Hey Children's Hospital, Eaton Road, Liverpool L12 2AP. Tel: 0151 252 5905.

Thank you for your help.