INTESTINAL MICROFLORA AND INFECTION

Intimin type influences the site of human intestinal mucosal colonisation by enterohaemorrhagic *Escherichia coli* O157:H7

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Gut 2002;**50**:180–185

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Accepted for publication 1 May 2001

Background: Enterohaemorrhagic (EHEC) and enteropathogenic (EPEC) *Escherichia coli* epithelial cell adhesion is characterised by intimate attachment, and attaching and effacing (A/E) lesion formation. This event is mediated in part by intimin binding to another bacterial protein, Tir (translocated intimin receptor), which is exported by the bacteria and integrated into the host cell plasma membrane. Importantly, EPEC (O127:H6) and EHEC (O157:H7) express antigenically distinct intimin types known as intimin α and γ, respectively. EHEC (O157:H7) colonises human intestinal explants although adhesion is restricted to the follicle associated epithelium of Peyer's patches. This phenotype is also observed with EPEC O127:H6 engineered to express EHEC intimin γ.

Aims: To investigate the influence of intimin on colonisation of human intestine by *E coli* O157:H7, and intimin types on tissue tropism in humans.

Methods: Human intestinal in vitro organ culture with wild type and mutant strains of O157:H7 were employed.

Results: Introducing a deletion mutation in the *eae* gene encoding intimin γ in EHEC (O157:H7) caused the strain (ICC170) to fail to colonise human intestinal explants. However, colonisation of Peyer's patches and A/E lesion formation were restored with intimin γ expression from a plasmid (ICC170 (pICC55)). In contrast, complementing the mutation with intimin α resulted in a strain (ICC170 (pCVD438)) capable of colonising and producing A/E lesions on both Peyer's patch and other small intestinal explants.

Conclusion: Intimin is necessary for human intestinal mucosal colonisation by *E coli* O157:H7. Intimin type influences the site of colonisation in a Tir type independent mechanism; intimin γ appears to restrict colonisation to human follicle associated epithelium.

Verocytotoxin (or Shiga toxin) producing *Escherichia coli* (VTEC or STEC) are an important example of an emerging group of microbial pathogens associated with food poisoning.[1] Certain VTEC, for example *E coli* O157:H7, can cause haemorrhagic colitis and haemolytic uraemic syndrome in humans and are termed enterohaemorrhagic *E coli* (EHEC).

EHEC, in common with enteropathogenic *E coli* (EPEC), can form characteristic attaching and effacing (A/E) lesions on mammalian cells growing in culture[2] and in animals.[3] Although EHEC O157:H7 was believed to colonise the human large intestinal mucosa,[1] association of EHEC and A/E lesion formation on human tissue had not been demonstrated until recently[4] when we showed, using in vitro organ culture (IVOC) of paediatric intestinal mucosa, that EHEC has a distinct tropism for the follicle associated epithelium (FAE) of ileal Peyer's patches where it causes A/E lesions. In contrast, no adhesion was evident on the mucosal surface of proximal or distal small intestine or colon.[4] This observation contrasted with the adhesion pattern exhibited by EPEC, which included the entire small intestinal mucosa with some colonic colonisation.[4]

The first gene to be associated with A/E activity was the EPEC *eae* gene encoding intimin, an outer membrane adhesion molecule essential for intimate bacterial attachment to eukaryotic host cells.[5] The *eae* gene has also been detected in EHEC O157[6] and other EHEC serogroups,[7] and has been shown to be required for colonisation of O157:H7 in animal models.[8 9]

The intimin encoding *eae* gene is part of a pathogenicity island found in EPEC and EHEC termed the locus of enterocyte effacement (LEE).[10 11] In addition to intimin, the LEE also encodes a type III secretion system (reviewed by Frankel and colleagues[12]) an intimin receptor (Tir/EspE),[13 14] and three secreted proteins EspA, EspB, and EspD, which are required for signal transduction in host cells and A/E lesion formation.[12 15] EspA is a structural protein and a major component of a large filamentous organelle that is transiently expressed on the bacterial surface and interacts with the host cell during the early stage of A/E lesion formation.[16 17] EspA filaments may contribute to bacterial adhesion but of greater significance is that they appear to be a component of a translocation apparatus and as such are essential for the translocation of EspB[16] and Tir[13] into host cells. Of note is the fact that there is a large amount of divergence between EHEC and EPEC LEE encoded gene products which interact directly with the host (that is, intimin, Tir, and the Esps),[11] and that the EPEC LEE is necessary and sufficient for A/E lesion formation[18] whereas EHEC LEE is necessary but not sufficient.[19]

At least five distinct intimin types, designated α, β, γ, δ, and ϵ, have been identified thus far.[20 21] Importantly, intimin α is specifically associated with one evolutionary branch of EPEC known as EPEC 1, intimin β is associated with both EPEC and

Abbreviations: EHEC, enterohaemorrhagic *Escherichia coli*; EPEC, enteropathogenic *E coli*; A/E, attaching and effacing; Tir, translocated intimin receptor; IVOC, in vitro organ culture; LEE, locus of enterocyte effacement; VTEC, verocytotoxigenic *E coli*; STEC, Shiga toxigenic *E coli*; FAE, follicle associated epithelium; Esp, EPEC secreted protein; FAS, fluorescent actin staining; SEM, scanning electron microscopy; PCR, polymerase chain reaction.

Table 1 Plasmids used in this study

Plasmid	Properties	Reference
pCVD444	pUC18 encoding intimin γ from EHEC O157:H7	Yu and Kaper[6]
pCVD438	pACYC184 encoding intimin α from EPEC O127:H6	Donnenberg and Kaper[29]
pICC55	A pCVD438 derivative encoding recombinant intimin γ	Hartland and colleagues[33]
pCVD438/01	pACYC184 encoding intimin α containing a C937A substitution	Frankel and colleagues[32]

EHEC belonging to their respective clone 2, whereas intimin γ is specifically associated with EHEC O157 and its related strain EPEC O55:H7.[22] Studies on the different intimins from EPEC and EHEC have shown that receptor binding activity is localised to the C terminal 280 amino acids (Int280).[23] A number of groups have reported that intimin can bind directly to uninfected host cells[23–25] and to Tir.[13 14 24] Binding to the host cell but not to Tir is dependent on a disulphide bridge at the carboxy terminus of Int280.[24] However, when expressed on the surface of EPEC, both of these binding activities of intimin are required for intimate bacterial adhesion and A/E lesion formation.

Recent results suggested that different intimin types might play a role in determining the pattern of colonisation and tissue tropism in the host. Among these are intimin exchange studies performed in piglets[26] and our recent investigation, using human intestinal explants, showing that while EPEC expressing intimin α colonised Peyer's patch as well as proximal and distal small intestinal tissue,[4] a restricted pattern of tissue tropism towards Peyer's patches was observed following expression of intimin γ from EHEC in the EPEC background.[27] The aim of this study was to test the importance of intimin in the colonisation of human intestinal mucosa by O157:H7 and to investigate the influence of intimin on tissue tropism when expressed in an EHEC background.

METHODS

Bacterial strains and plasmids

EHEC O157:H7, strain 85-170 expressing intimin γ, is a spontaneous Stx negative derivative of EHEC 84-289 which was originally isolated from a food handler in a Canadian nursing home.[28] CVD206 is an EPEC O127:H6 harbouring a deletion mutation in its *eae* gene encoding intimin α.[29] Prior to adhesion studies, bacteria were subcultured into brain heart infusion broth and incubated aerobically overnight at 37°C without agitation. When appropriate, chloramphenicol was added to a final concentration of 30 μg/ml. The plasmids used in this study are listed in table 1.

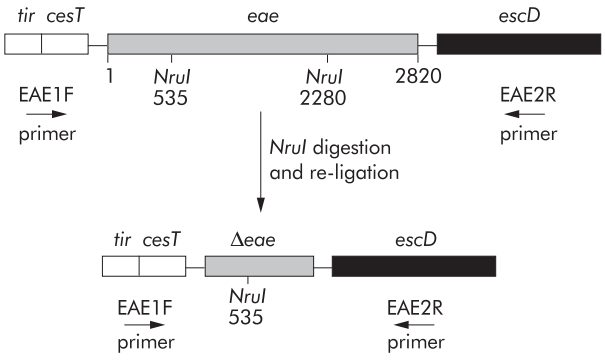

Figure 1 Schematic representation showing construction of the *eae* deletion of enterohaemorrhagic *Escherichia coli* (EHEC) strain 85-170. *Nru*I endonuclease digestion of plasmid pCVD444 was used to introduce deletion in the *eae* gene encoding intimin γ. Following re-ligation and polymerase chain reaction amplification, the DNA fragment containing the deleted form of the *eae* gene was cloned in the suicide vector pCVD442 and the deletion was introduced into EHEC by allelic exchange, as described in experimental procedures. The numbers represent nucleotides within the structural *eae* gene. The genes upstream (3′ end of *tir* and *cesT*) and downstream (*escD*) of *eae* are also indicated. (Not to scale.)

Construction of an *eae* deletion mutant of EHEC strain 85-170

In order to introduce a deletion mutation into the *eae* gene, we took advantage of two unique *Nru*I restriction sites in pCVD444[6] which are located in the coding region of the gene (fig 1). Following digestion and gel purification, the plasmid was self ligated, resulting in a plasmid which contains an 1873 bp deletion in the *eae* gene (fig 1). Using polymerase chain reaction (PCR) primers EAE1F (5′ TCTATTCCCGGGAATGAAAACAGATTGTGTTCTTTTGC 3′; positioned at 16 001 to 16 037 bp of the LEE region from EHEC O157:H7) and EAE2R (5′ AGAACATTCCCGGGTACATTTCAGCAGATATTTTTCCC 3′, positioned at 19 759 to 19 722 bp of the LEE region (accession number AF071034)), containing a 5′ *Sma*I site (underlined), the deleted *eae* gene and flanking DNA were recovered on a 1885 bp fragment. This fragment was gel purified and ligated into a *Sma*I digested suicide vector, pCVD442. The recombinant pCVD442 was then used to electroporate EHEC O157:H7 *eae* positive strain 85-170. Ampicillin resistant colonies were isolated and plated onto 10% sucrose L-agar plates, grown overnight at 30°C, to select for double crossover isolates. Ampicillin sensitive clones containing the *eae* deletion were identified by PCR amplification using primers EAE1F and EAE2R. A PCR product of 1885 bp, expected from the *eae* deleted clones, was obtained from over 50% of the sucrose resistant and ampicillin sensitive colonies. The rest of the colonies produced a PCR product of 3758 bp fragment similar to the wild type strain.

Detection of intimin expression by western blotting and fluorescent actin staining (FAS)

Expression of the intimin derivatives was determined by western blots. For immunodetection of intimin in whole cell extracts, stationary L-broth cultures were diluted 1:100 in Dulbecco's modified Eagle's medium and incubated for three hours at 37°C. An equivalent of an optical density 600 (OD_{600}) of 0.5 was loaded onto 7.5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis, as described previously. The electrophoresed polypeptides were transferred onto a nitrocellulose membrane and immunodetection of intimin was performed using a universal rabbit intimin antiserum, raised against a conserved region of intimin ($Int_{387–666}$), diluted 1:500 as described previously.[20 30] FAS test, to detect A/E lesion formation on infected HEp-2 cells, was performed as described previously.[2]

Tissue samples

Human tissue was obtained, with fully informed parental consent and local ethics committee approval, during routine investigation of patients for potential intestinal disorders. Mucosal biopsies of proximal small intestinal mucosa (fourth part duodenum), terminal ileum, Peyer's patches, and transverse colon were taken using a grasp biopsy forceps during routine endoscopy (Olympus PCF paediatric endoscope).

Table 2 Regional adherence of bacterial strains. Number of samples showing attaching/effacing lesions out of the samples tested

Strain used	Region of intestine tested			
	Duodenum	Terminal ileum	Peyer's patch	Colon
85/170	0/3	0/4	3/3	0/4
Age 85/170	43, 164, 212	24, 29, 127, 148	42, 46, 96	38, 82, 149, 198
ICC170	ND	ND	0/3	ND
Age ICC170			82, 82, 170	
ICC170 (pICC55)	0/4	ND	2/3	ND
Age ICC170 (pICC55)	61, 68, 113, 149		80, 133, 149	
ICC170 (pCVD438)	9/9 (4 adhesion only)	4/4 (2 adhesion only)	3/3	0/3
Age ICC170 (pCVD438)	68 (34–175)	24, 29, 38, 131	46, 46, 71	82, 116, 170
CVD206 (pCVD438)	9/9 (3 adhesion only)	ND	ND	ND
Age CVD206 (pCVD438)	68 (34–175)			
85/170 (pCVD438–01)	0/4	ND	0/3	ND
Age 85/170 (pCVD438–01)	66, 106, 131, 175		65, 142, 170	

Age in months, when more than four samples were tested the age is shown as median (range).
ND, not done.

Peyer's patches can be recognised and selectively biopsied during endoscopy, a technique made easier by the application of video endoscopy. Median ages and age ranges of the patients are shown in table 2. All endoscopic biopsies were taken from areas showing no obvious pathology or other abnormality, and all intestinal histology was reported to be normal in the material used in this study.

In vitro organ culture

In vitro organ culture (IVOC) was performed as described previously for eight hours.[31] Each bacterial strain was examined in human IVOC on at least three occasions using tissues from different children. An uninoculated specimen was included with each experimental culture to exclude the possibility of in vivo bacterial colonisation. After incubation with bacteria or appropriate control solutions, IVOC specimens were washed thoroughly three times to remove any non-adherent bacteria and then prepared for scanning electron microscopy (SEM), as described previously.[31] Samples were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer, post-fixed in 1% aqueous osmium tetroxide, and dehydrated in 2,2 dimethoxy-propane. Specimens were transferred to absolute ethanol, critically point dried using liquid carbon dioxide in an Emitech K850 apparatus, mounted on aluminium stubs, sputter coated with gold-palladium using a Polaron E5100 sputter coater, and viewed in a JEOL 5300 SEM at an accelerating voltage of 30 kV.

RESULTS

Construction of an *eae* deletion mutant of EHEC strain 85-170

In a previous study we have shown that EPEC expressing intimin α colonised any region of the IVOC small intestinal mucosa while EHEC expressing intimin γ showed restricted tropism towards the human Peyer's patch mucosa. The aim of this study was to determine the outcome of infection of different regions of the human gut with an EHEC *eae* null mutant (strain ICC170) and with ICC170 complemented with *eae* encoding either intimin γ or intimin α.

For this purpose, we introduced an *eae* deletion mutation in EHEC strain 85-170 (see methods and fig 1), generating strain ICC170. Confirmation of the deletion was achieved by PCR (see methods; data not shown). The *eae* mutation in ICC170 was complemented in trans with pCVD438, encoding intimin α from EPEC.[29] In addition, ICC170 was complemented, as controls, with pCVD438/01 encoding a biologically inactive form of intimin α in which Cys at position 937 was replaced with Ser,[32] or pICC55, a pCVD438 derivative containing the receptor binding domain of intimin γ on a cloned intimin α backbone.[27 33] Expression of the different intimins was determined by western blot analysis of whole cell lysates prepared from the ICC170 derivatives using a universal broad spectrum polyclonal intimin antiserum, reactive with all the different intimin types[30] (fig 2). Lysates from all the recombinant strains, but not from ICC170, reacted similarly with the antiserum (fig 2 and data not shown).

Interaction of the recombinant ICC170 strains with HEp-2 cells and human intestinal IVOC

Before the recombinant ICC170 strains were tested in the human intestinal IVOC model, the ability of the strains to mediate A/E lesion formation on HEp-2 cells was investigated. ICC170 (pCVD438) and ICC170 (pICC55) adhered to the cell monolayers and produced an FAS positive reaction, as did the parent 85-170 strain, while ICC170 and ICC170 (pCVD438/01) were unable to induce reorganisation of the host cell cytoskeleton (data not shown). The results show that ICC170 expressing both intimin α and intimin γ can mediate A/E lesion formation on cultured human epithelial cells.

In previous studies we have shown that the ability of EPEC to induce A/E lesions on human intestinal IVOC is dependent on surface expression of biologically active intimin[31] and that the type of intimin expressed determine tropism to different regions of the gut.[27] To determine the importance of intimin in colonisation and tissue specificity in an EHEC background, we investigated the ability of the recombinant ICC170 strains to mediate A/E lesion formation on mucosal surfaces, using endoscopically and histologically normal paediatric tissue.

The domed mucosal surface overlying individual lymphoid follicles within Peyer's patches can be easily recognised by SEM (fig 3A). Thus bacterial adhesion to FAE can be discriminated from other sites. We repeated experiments reported

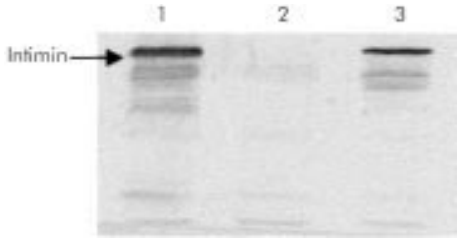

Figure 2 Western blot analysis of whole cell lysates prepared from enterohaemorrhagic *Escherichia coli* (EHEC) 85-170 and the ICC170 derivatives using a universal broad spectrum polyclonal intimin antiserum.[30] Lysates from the wild type (lane 1) and from ICC170 (pCVD438) (lane 3) strains reacted with the intimin antiserum, but not from ICC170 (lane 2).

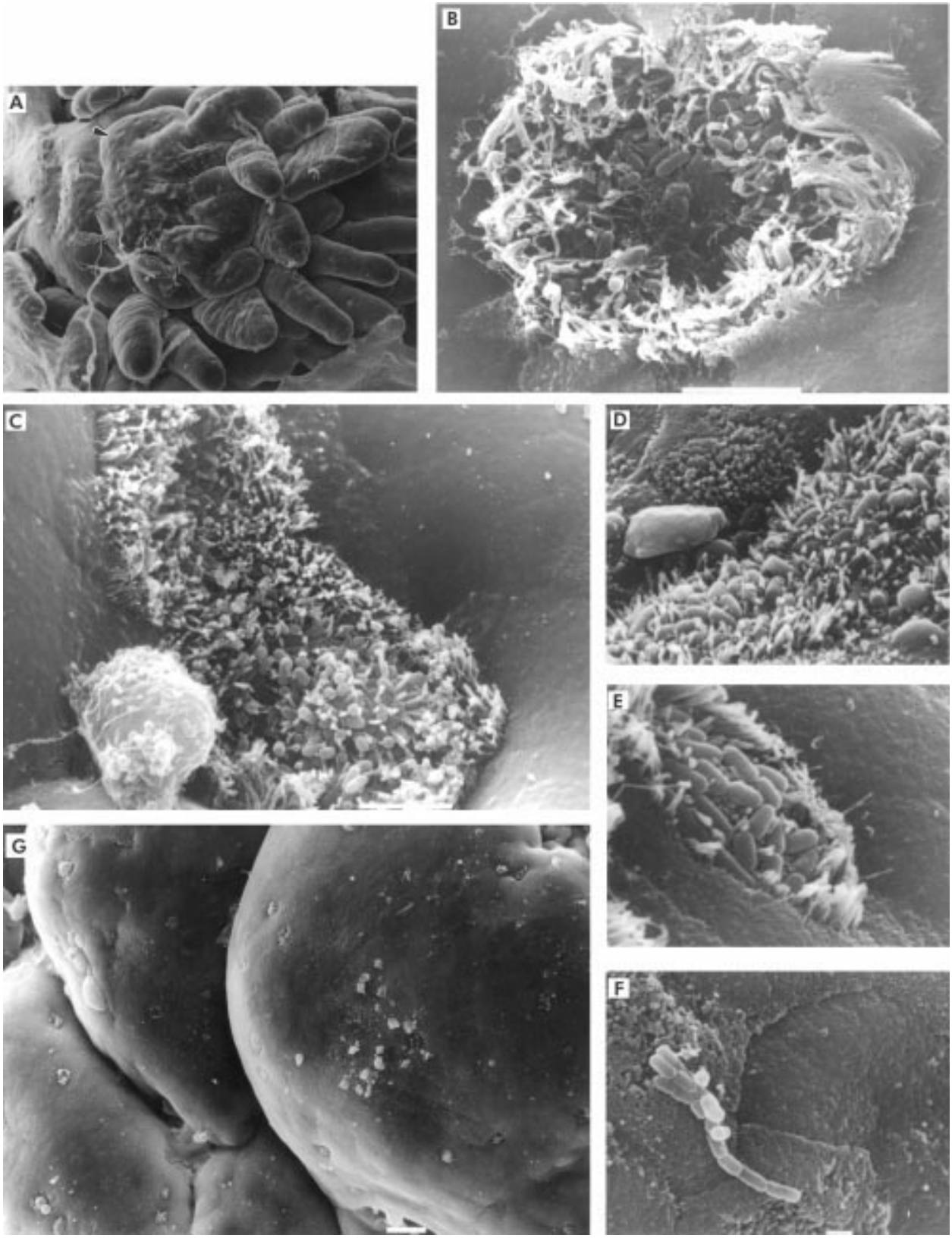

Figure 3 (A) Lymphoid follicles within Peyer's patch of distal ileum (arrow); bar=100 μm. (B) ICC170 (pICC55) adhering to follicle associated epithelium (FAE) and showing attaching and effacing (A/E) lesion formation; bar=5 μm. (C–E) ICC170 (pCVD438) A/E lesion formation on the FAE of the Peyer's patch, duodenum, and ileum, respectively; bar=5 μm, 1 μm, and 1 μm, respectively. (F) ICC170 (pCVD438) adhesion to the duodenum without A/E lesion formation; bar=1 μm. (G) Lack of adhesion of ICC170 (pCVD438/01) to the ileum; bar=10 μm.

previously[4] and again demonstrated a restricted pattern of tissue tropism for O157:H7 strain 85-170 to FAE of Peyer's patches. The *eae* deletion mutation strain ICC170 did not adhere to FAE whereas adhesion of the intimin γ complemented strain ICC170 (pICC55) was, in common with the parent 85-170 strain, limited to FAE (fig 3B; table 2). SEM results were typical of A/E lesion formation. In contrast, the intimin α complemented strain ICC170 (pCVD438) adhered to FAE of Peyer's patches as well as to proximal and distal small intestinal mucosa (fig 3 C–E, respectively). However, unlike E2348/69 expressing intimin α,[4 31] adhesion of ICC170 (pCVD438) (fig 3F) and CVD206 (pCVD438) (data not shown) to duodenum and terminal ileum was not always associated with A/E lesions (table 2). In contrast, and in agreement with previous reports,[31 32] ICC170 (pCVD438/01) (fig 3G), similar to ICC170 itself, did not adhere to intestinal IVOCs (table 2). These results show that intimin, as in EPEC, is essential for colonisation of human mucosa by EHEC and that expressing intimin α in EHEC allows the strain to extend colonisation to include the small intestine in humans.

DISCUSSION

The genetic basis of A/E lesion formation is well documented.[1 10 12 15] However, relatively little is known about the initial intestinal stage of EHEC infection involving colonisation of the gut. The only EHEC adherence factor that has been demonstrated to play a role in intestinal colonisation in vivo in animal models is the 94 to 97 kDa adhesion molecule intimin.[8 9] The importance of intimin in human disease has been shown for EPEC infection by volunteer studies in which an *eae* mutant was significantly attenuated compared with the wild type parent strain[34] and infection of human intestinal IVOC, in which the *eae* mutant was unable to colonise the tissue.[31] The importance of intimin in human disease is also supported by the presence of a high titre of serum intimin antibodies in individuals infected with EHEC[35] and in the colostrum of mothers in Brazil where EPEC infection is endemic.[36] In this study we introduced an *eae* deletion mutation into EHEC strain 85-170 (producing strain ICC170). The *eae* mutation rendered the strain incapable of colonising any region of the human intestinal IVOC mucosa. This result adds yet another layer of evidence for the contribution of intimin to EHEC infection in humans.

Recently, we used IVOC to demonstrate that EHEC O157:H7 shows a distinct tropism for the follicle associated epithelium (FAE) of ileal Peyer's patches where it caused A/E lesions, but no adhesion was evident on the mucosal surface of proximal or distal small intestine or colon.[4] This observation contrasts with the ability of EPEC to efficiently colonise any region of the human small intestinal mucosa and inefficiently the colonic mucosa.[4] The different tissue specificity exhibited by EPEC and EHEC on human intestinal IVOC prompted us to investigate the contribution of intimin types to tissue tropism. We addressed this question using isogenic EPEC derivatives expressing either intimin α or intimin γ and IVOC from different regions of the human gut. We showed that when an *eae* EPEC mutant CVD206[29] was complemented with *eae*α (strain CVD206 (pCVD348)), it efficiently colonised small, but not large, intestine in a similar manner to the intimin α expressing wild type EPEC (E2348/69). In contrast, complementing CVD206 with pBE310 or pICC55 encoding intimin γ resulted in strains which targeted the FAE of Peyer's patches, similarly to intimin γ expressing EHEC.[27] In this paper, we described further work in which ICC170 was complemented with pCVD438 encoding the EPEC intimin α. Using ICC170 (pCVD438) in combination with IVOC, we reported that the change in intimin type resulted in colonisation spreading from the FAE—that is, typical of the parent and ICC170 (pICC55) strains—to villous regions of the proximal and distal small intestine. Importantly, although in most cases adhesion to duodenum and terminal ileum was associated with A/E lesions, in some cases adhering bacteria were seen on the surface of the biopsy with no evidence of A/E lesions. This observation is the first example in which adhesion to the human mucosa can be separated from A/E lesion formation. The reason for this phenomenon is intriguing and is currently under investigation, although it might simply be due to the fact that the dynamics of A/E lesion formation observed in the current study are delayed and the eight hour duration of IVOC was not sufficient to allow the lesion to develop fully. This is supported by the observation that the intimin α expressing EPEC strain (CVD206 (pCVD438)) also showed a similar phenomenon. In addition, attachment of intimin positive bacteria without A/E lesion formation suggests that intimin contributes to initial adhesive events. Although definitive evidence to support this contention is lacking at the present time, the fact that intimin contributes to tissue tropism and host specificity, despite A/E lesions being end points in both instances, suggests that binding of intimin to a host cell receptor during ex vivo and in vivo infections occurs before intimin-Tir binding.[37]

EHEC adhesion to FAE of Peyer's patches appears to be a specific event, and several bacteria express adhesins promoting binding to these regions (for example, long polar fimbriae in *Salmonella*[38] and AF/R1 in RDEC-1[39]; strains not expressing these adhesins show reduced Peyer's patch adhesion and less virulence).[38 40] Indeed, the recently reported complete genome sequence of O157:H7 has identified several fimbrial operons, including with homology to long polar fimbriae,[41] which might therefore, in addition to intimin, contribute to Peyer's patch adhesion. Specialised cells within the FAE, termed M (microfold) cells (reviewed by Neutra and colleagues[42]) have been described as targets of bacterial cell adhesion, invasion, and damage in animals.[43] These cells are thought to function normally as antigen sampling cells, and bacterial pathogens appear to have evolved mechanisms to use this route as a means of colonisation and/or entry into the host. Differences in expression of apical membrane proteins (β-1 integrins[44]) and carbohydrates[45] on M cells in animal species in comparison with absorptive epithelium have provided explanations for this selective adhesion. However, these differences have not been fully established in human tissue[46] and their role in human infectious disease in general, and in *E coli* O157:H7 infection in particular, merits further study. More generally, the FAE has different characteristics to villous epithelium and these may be factors that influence bacterial adhesion. These include differences in the thickness of the glycocalyx,[47] reduction in goblet cell numbers, increased presence of intraepithelial lymphocytes, and the close proximity of lymphoid follicular cells. An important challenge for future work is to determine if any of these factors influence intimin mediated tissue tropism.

ACKNOWLEDGEMENTS

We are very grateful to Drs Simon Murch and Mike Thomson for their endoscopic skills in the provision of endoscopic biopsies, particularly from Peyer's patch areas. The work was supported by a joint BBSRC grant to ADP and GF.

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