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Mechanisms of diarrhoea

I. W. BOOTH
A. S. McNEISH

INTRODUCTION

Acute infections of the gastrointestinal tract are by far the commonest cause of diarrhoea in adults and children worldwide. Acute diarrhoea in early life can be devastating. Young children have a relatively large surface area in relation to body weight, a high basal requirement for water, electrolytes and nutrients, and immature renal function with a relative inability to conserve fluid and electrolytes. The problem is further compounded in developing countries, where vulnerable infants and young children are exposed to environmental contamination, malnutrition and inadequate medical care. High mortality and prolonged morbidity are the results (Mata and Guerrant, 1988).

The World Health Organization has estimated that three to five billion episodes of diarrhoea per annum occur in developing countries. Of these, one billion occur in children under 5 years of age. Diarrhoea accounts for five million deaths per year, of which half are in children under 5 years of age. Seven per cent of all children in this age-group die of diarrhoea (Snyder and Merson, 1982). In contrast, acute diarrhoeal disease in industrialized countries, although still a common cause of hospital admission in childhood, produces less morbidity and mortality (Guerrant et al, 1990). In adults, infective diarrhoea is more commonly a social inconvenience than a life-threatening disorder. The ease of world travel has led to the emergence of travellers' diarrhoea as an important problem (Steffen and Boppart, 1987).

Microbial diarrhoea results from induction of active chloride secretion (often with inhibition of sodium chloride absorption), or from the osmotically induced movement of water into the intestinal lumen because of non-absorbed solute in the lumen. Characteristically, organisms which switch on fluid and electrolyte secretion (enterotoxigenic) attach to the intestinal mucosa but do not invade. They induce secretion by the production of enterotoxins which interact with intracellular mediators of intestinal electrolyte transport (*Vibrio cholerae*, toxigenic *Escherichia coli*). Other organisms invade and damage the mucosa and may elaborate cytotoxins and/or enterotoxins (*Salmonella*, *Shigella*, *Campylobacter jejuni*). In order to provide a framework for discussing the mechanisms whereby diarrhoea-inducing organisms can disturb water and electrolyte transport, we have included a brief outline of the regulation of intestinal ion transport.

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INTRACELLULAR REGULATION OF INTESTINAL ELECTROLYTE TRANSPORT

Absorption and secretion are probably spatially separated in the small intestinal epithelium, with absorption in villous cells, and chloride secretion coming mainly from crypt cells (Sullivan and Field, 1990). The hypothesis that crypt cells are the site of secretion has recently been supported by observations on the cell-specific expression of the cystic fibrosis transmembrane conductance regulator (CFTR), which acts as a regulated chloride channel (Trezise and Buchwald, 1991). CFTR mRNA was determined by in situ hybridization in rat small intestine. CFTR was maximally expressed in the intestinal crypts, and least in villous cells, a distribution consistent with crypt cells being the site of chloride secretion.

Net small intestinal secretion, which is therefore an exaggeration of a physiological process, arises usually from a combination of a failure to reabsorb the large amount of endogenous secretions which enter the gut each day, and active chloride secretion.

Sodium and chloride absorption (Figure 1)

Net sodium and chloride absorption take place through two linked exchanges in the brush border membrane. Sodium is absorbed in exchange

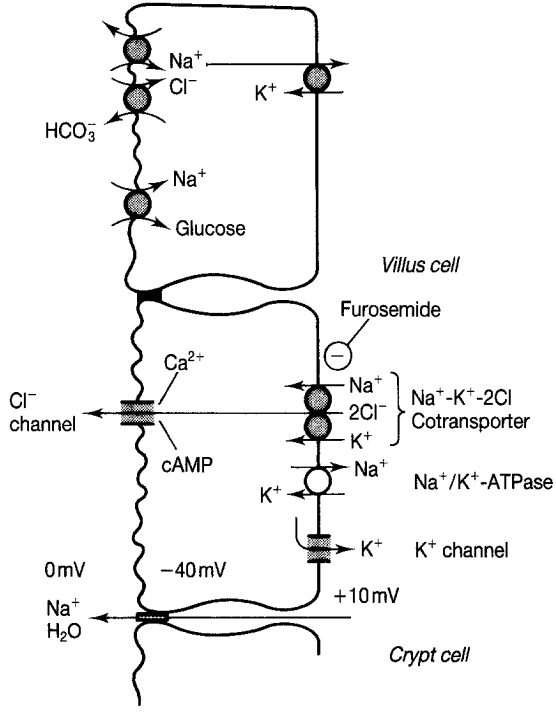

Figure 1. An overview of electrolyte absorption and secretion in the small intestine.

for secreted protons, and chloride in exchange for bicarbonate (Turnberg et al, 1970; Liedtke and Hopfer, 1982; Booth et al, 1985). Sodium is also cotransported with glucose or amino acids, a mechanism of particular clinical importance. These symports usually remain intact during infective secretory diarrhoea, and therefore represent a maintained route for sodium absorption (Editorial, 1981).

The energy for sodium translocation across the brush border membrane comes from the activity of Na^+/K^+-ATPase in the basolateral membrane of the enterocyte. Extrusion of three sodium atoms in exchange for two of potassium establishes a low intracellular activity of sodium, thereby maintaining an appropriate electrochemical gradient for the entry of sodium in exchange for protons, or by co-transport with glucose.

Active chloride secretion (Figure 1)

The maintenance of a low intracellular sodium concentration by Na^+/K^+-ATPase provides the driving force for the cotransport of chloride with sodium across the basolateral membrane. The cotransporter probably has a stoichiometry of 1 sodium:1 potassium:2 chloride, and is therefore electrically neutral.

The brush border membrane is relatively impermeable to chloride ions, but chloride channels, which open in response to intracellular cAMP and calcium, permit the exit of chloride (Li et al, 1988). A strong electrical gradient across the brush border membrane (−40 to −60 mV relative to the lumen) promotes the secretion of chloride into the lumen. Potassium-selective channels in the basolateral membrane allow potassium entering the cell with sodium and chloride to escape back into the serosal extracellular fluid (Shorofsky et al, 1983).

The entry of chloride into the intestinal lumen provides an electrical and osmotic gradient for the paracellular movement of sodium and water through cation-selective intercellular tight junctions.

Intracellular mediators of secretion

The opening of chloride channels and the inhibition of sodium chloride absorption in the brush border membrane take place in response to a number of intracellular second messengers: cAMP, cGMP, calcium–calmodulin, phosphatidylinositol metabolites and guanosine triphosphate-dependent regulator proteins (G-proteins). In general, intracellular mediators activate protein kinases which phosphorylate carrier proteins or carrier-associated regulator proteins, leading to a conformational change and an alteration in transport function.

Cyclic adenosine monophosphate (cAMP)

Cyclic AMP is formed from ATP by the activation of adenylate cyclase, an integral basolateral membrane protein. A guanyl nucleotide-binding protein (G-protein) is an important intermediary in the process and links receptor activation of adenylate cyclase (Figure 2) (Gilman, 1987). The

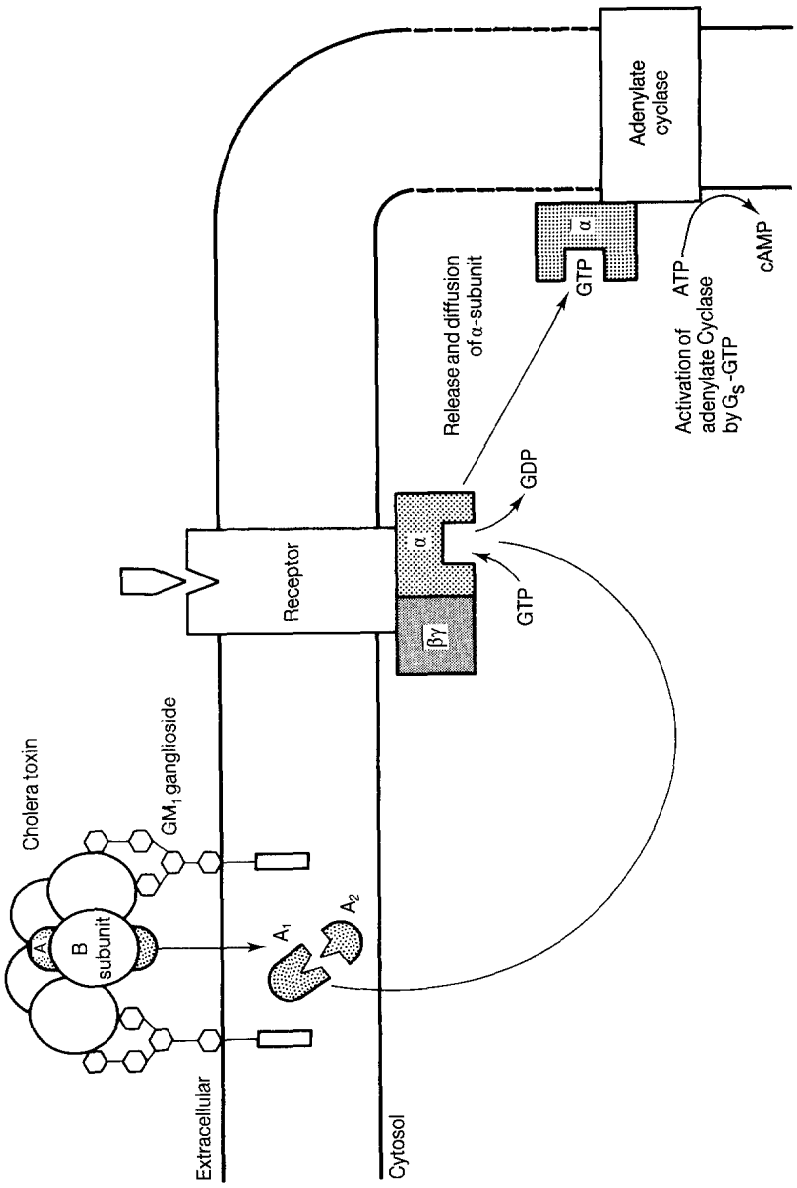

Figure 2. Activation of adenylate cyclase by cholera toxin in the enterocyte. The catalytic subunit of adenylate cyclase is in the basolateral membrane but activation of the α-subunit of the G-protein probably takes place in the brush border membrane.

G-protein comprises α-, β- and γ-subunits. It interconverts between an inactive GDP form and an active GTP form which activates adenylate cyclase. Receptor activation triggers the exchange of GTP for bound GDP, and the α-subunit-bearing GTP dissociates from the β–γ subunits. The stimulatory G-protein (Gs) is mobile within the cell membrane, and may move from the luminal membrane to activate adenylate cyclase, which is restricted to the basolateral membrane. There is the possibility that it also crosses the cytoplasm directly.

The G-protein is also an ATPase, and GTP bound to the α-subunit is slowly hydrolysed to GDP, thereby switching off the activation of adenylate cyclase (Figure 3). Adenylate cyclase is activated by vasoactive intestinal peptide and by certain prostaglandins. In contrast, α_2-adrenergic agonists and somatostatin activate inhibitory G-proteins (Gi) which inhibit adenylate cyclase.

Cyclic guanosine monophosphate (cGMP)

Cyclic GMP is formed following the activation of guanylate cyclase, which exists in particulate and soluble forms, predominantly in the apical membrane of epithelial cells and the adjacent cytoplasm. Like adenylate cyclase, it comprises a catalytic subunit and G-proteins, and there is a specific receptor. The particulate form in the small intestine is activated by heat-stable *E. coli* enterotoxin (Rao et al, 1980) and guanylin (Currie et al, 1992).

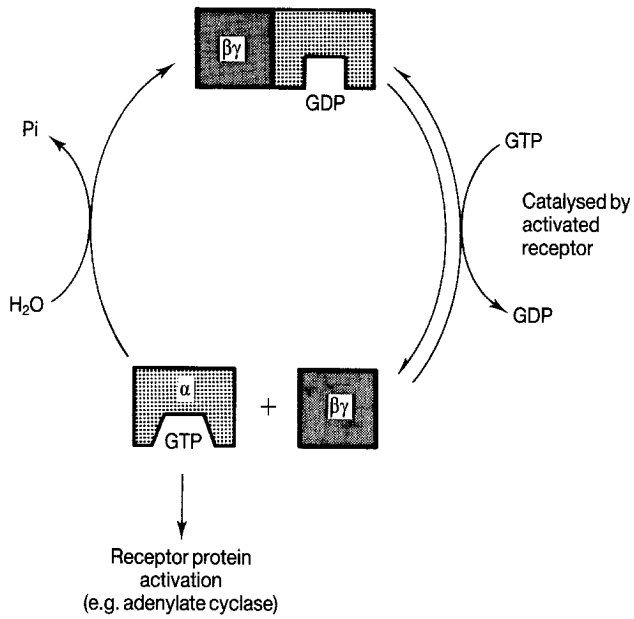

Figure 3. G-protein converts between inactive GDP and active GTP forms. Gα-GTP is the active form and activates adenylate cyclase.

Cyclic nucleotide-dependent protein kinases

Virtually all the known effects of cyclic nucleotides are mediated by protein kinases (Nishizuka, 1986). The binding of cAMP to the regulatory (R) subunit of protein kinases leads to dissociation of two active catalytic (C) subunits which phosphorylate serine and threonine residues in target proteins. A number of intestinal membrane proteins are phosphorylated by cyclic nucleotide-dependent protein kinases, but the precise relationship between these events and alterations in membrane transport, e.g. chloride secretion, is not known.

Calcium

The calcium ion is an intracellular messenger in many signal-transducing cells, including enterocytes. Secretion can therefore occur without the involvement of cyclic nucleotides, in response, for example, to 5-hydroxytryptamine (5-HT) and acetylcholine. Under these circumstances,

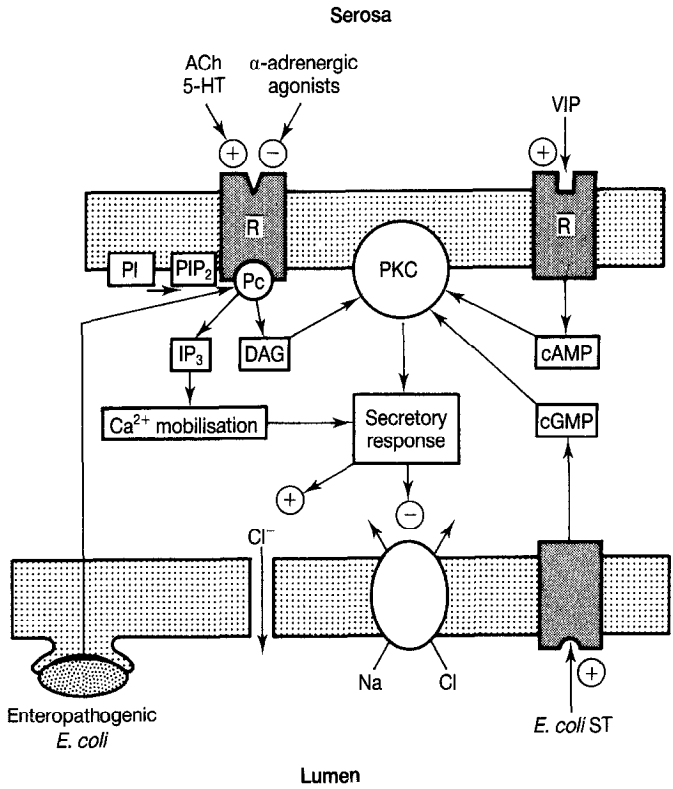

Figure 4. The interaction of the phosphoinositide cascade, calcium and protein kinase c: ACh, acetylcholine; 5-HT, 5-hydroxytryptamine; VIP, vasoactive intestinal polypeptide; PIP_2, phosphatidylinositol 4,5-biphosphate; Pc, phospholipase C; IP_3, inositol 1,4,5-triphosphate; DAG, diacylglycerol; ST, heat-stable *E. coli* toxin; R, receptor.

secretion occurs in response to an increase in intracellular calcium, which is often released from intracellular stores within the endoplasmic reticulum in response to initiation of the phosphoinositide cascade (Berridge, 1987).

Like adenylate cyclase, the phosphoinositide cascade converts extracellular signals into intracellular ones (Figure 4). The binding of a hormone, e.g. 5-HT, to a cell surface receptor leads to the activation of the membrane-bound enzyme phosphoinositidase (phospholipase C) by activation of a G-protein. Phosphoinositidase hydrolyses phosphatidyl inositol 4,5-bisphosphate (PIP_2), a membrane phospholipid. Two messengers are produced: inositol triphosphate (IP_3), which opens calcium channels in the endoplasmic reticulum, and diacylglycerol (DAG), which activates protein kinase C, leading to phosphorylation of membrane-bound proteins.

Liberated calcium binds to calmodulin, a 17-kDa protein, which in turn activates calmodulin-dependent protein kinases, regulates cyclic nucleotide metabolism and affects the behaviour of cytoskeletal proteins. Calcium can also activate protein kinase C, independently of calmodulin, an effect which is potentiated by DAG.

NEUROHUMORAL AND INFLAMMATORY MEDIATORS

Neurohumoral factors

The small intestine is richly supplied with cholinergic, adrenergic and peptidergic neurones. Endocrine cells are also present in abundance. It is against this background that a potentially key role for the enteric nervous system in mediating the effects of cholera toxin (CT) has been proposed. CT-induced secretion can be blocked in the cat in vivo by a variety of nerve and ganglion blockers, suggesting that CT may activate neuronal reflexes as part of a secretory reaction (Cassuto et al, 1981). There is also evidence that 5-HT is involved. For example, a combination of 5-HT_2 and 5-HT_3 receptor blockade totally abolishes CT-induced secretion without any influence on the concurrent increase in cAMP (Beubler and Horina, 1990). A model has been suggested in which CT induces release of 5-HT from enterochromaffin cells, causing prostaglandin E_2 formation via 5-HT_2 receptors and activation of neuronal structures by 5-HT_3 receptors.

5-HT has also been implicated in diarrhoea caused by *Entamoeba histolytica*. Cell-free supernatants stimulate calcium-dependent chloride secretion in the colon. Amoebic lysates were found to contain high concentrations of 5-HT and the secretory response could be inhibited by the 5-HT antagonist bufotenine (McGowan et al, 1983).

Eicosanoids

It is well recognized that prostaglandins, particularly the E and F series, can produce diarrhoea by inhibiting transepithelial ion transport and stimulating net secretion (Rask-Madsen, 1986). A number of pieces of evidence implicate prostaglandins and other inflammatory mediators in the pathogenesis

of some forms of acute infective diarrhoea. For example, aspirin, a prostaglandin synthetase inhibitor, will decrease intestinal fluid loss in children with acute gastroenteritis (Gracey et al, 1984). Indomethacin will also inhibit the cAMP-mediated secretory response to *Salmonella typhimurium* infection without altering the degree of mucosal inflammation (Gots et al, 1974). Prostaglandin E_2 and $F_2 \alpha$ concentrations in plasma and stool are also increased during rotavirus gastroenteritis in childhood (Yamashiro et al, 1989), and leukotriene and prostaglandin E_2 production are increased following instillation of *Clostridium difficile* toxin into rabbit ileal loops (Triadafilopoulos et al, 1989).

Mast cell degranulation

There is now convincing evidence that degranulating mast cells mediate the chloride secretory response to intestinal anaphylaxis (Perdue et al, 1991). It seems likely that this mechanism is the basis of the secretory response seen during parasitic infection with *Trichinella spiralis* (Russell, 1985).

MECHANISMS OF DIARRHOEA (Table 1)

Many ways have now been identified by which organisms produce diarrhoea. Until recently, it was thought that each organism had one main pathogenic mechanism. This is now known not to be the case. *Vibrio cholerae*, for example, was thought to induce secretory diarrhoea by producing cholera toxin (CT). It is now known that at least three toxins are produced, that the enteric nervous system is important in mediating the response, and that 5-HT is probably involved. Similarly, the notion that *Yersinia enterocolitica* produces diarrhoea solely by damaging the small intestinal mucosa is incorrect. The organism possesses at least one further, and very subtle, virulence mechanism.

The attractive past classification of organisms on the basis of their main pathogenic mechanism has therefore become progressively harder, and is no longer possible. In what follows each organism is considered singly.

VIBRIO CHOLERAE

Cholera toxin (CT)

Cholera is the archetype of toxigenic diarrhoeas. Bacteria colonize the surface of the small intestine and secrete soluble enterotoxins. Severe secretory diarrhoea is the hallmark of the disease. Infected patients may pass up to 20 units of stool per day, with sodium and potassium concentrations similar to those in plasma. Despite massive secretion, there is no histological evidence of epithelial damage. The structure and mechanism of cholera toxin are now well understood, although new insights continue to be obtained. After colonization of the small intestine, *V. cholerae* releases an

Table 1. Mechanisms of infective diarrhoea.

Enterotoxin production	
Vibrio cholerae	Cholera toxin
	Zonula occludens toxin (ZOT)
	Accessary cholera toxin (ACE)
Enterotoxigenic *E. coli*	Heat-labile toxin (LT)
	Heat-stable toxin (STa)
Clostridium difficile	Toxin A
Shigella spp.	Shiga toxin
Aeromonas spp.	
Cytotoxin production	
Shigella	Shiga toxin
Clostridium difficile	Toxin B
Aeromonas spp.	
Enterohaemorrhagic *E. coli*	Shiga-like toxins (Vero toxins)
Increased intracellular calcium	
Enteropathogenic *E. coli* (from IP_3-sensitive intracellular stores)	
Enteroaggregative *E. coli* (from external calcium)	
Brush border damage	
(attachment and effacing lesion)	
Enteropathogenic *E. coli*	
Enterohaemorrhagic *E. coli*	
Epithelial invasion	
Shigella	
Salmonella	
Yersinia enterocolitica	
Disorganized enterocyte renewal and migration	
Rotaviruses	
Yersinia enterocolitica	
Neurohumoral inflammatory mediators	
V. cholerae, *Entamoeba histolytica*	5-Hydroxytryptamine
Rotavirus, *Salmonella*, *Clostridium difficile*	Eicosanoids
Trichinella spiralis	Mast cell degranulation
Protein tyrosine phosphatase activity	
Yersinia enterocolitica	

84-kDa enterotoxin (CT) comprising five identical B subunits and the catalytically active A subunit (A_1 peptide linked to an A_2 peptide by a disulphide bond; Figure 2). The B chains of CT bind irreversibly with GM_1 ganglioside on the microvillous membrane and remain there, while the A_1 peptide

enters the cell and activates adenylate cyclase. The A_2 subunit may position the A_1 peptide within the B-subunit ring structure and/or enable passage of the A_1 unit across the cell membrane.

V. cholerae produces a neuraminidase which catalyses the conversion of higher-order gangliosides to GM_1. Neuraminidase may therefore produce locally high concentrations of GM_1 receptor, thereby enhancing the binding and the pathological effects on water and electrolyte fluxes (Galen et al, 1992). It seems likely that the lag of up to 60 min between B-subunit attachment and changes in ion transport is accounted for by the time taken for the A_1 subunit to penetrate the cell membrane (Fishman, 1980).

After gaining entry to the cell, the 23-kDa A_1 subunit covalently modifies the Gs protein. A_1 catalyses the transfer of the ADP-ribose moiety from nicotinamide adenine dinucleotide (NAD) to an arginine side chain of the α-subunit of Gs (Figure 5). This ADP ribosylation inhibits the GTPase activity of Gs and thereby inhibits its inbuilt deactivation. Consequently, the Gs α subunit remains permanently in the active form and adenylate cyclase is continually activated (Cassel and Selinger, 1977). Toxin-catalysed ADP ribosylation is stimulated in vitro by a family of guanine nucleotide-binding proteins, termed ADP-ribosylation factors (Moss and Vaughan, 1991), which serve as allosteric activators of the toxin. As a result of adenylate cyclase activation, apical chloride channels open and sodium chloride absorption is inhibited. Glucose-stimulated sodium uptake remains unaffected, providing a continuing route for oral rehydration with glucose–electrolyte solutions during cholera.

This series of events implies that when CT binds to the microvillous membrane, either: (a) the A_1 subunit moves from the brush border to the basolateral membrane (where the catalytic subunit of adenylate cyclase is located), to ADP ribosylate the Gs subunit; or (b) the regulatory subunits

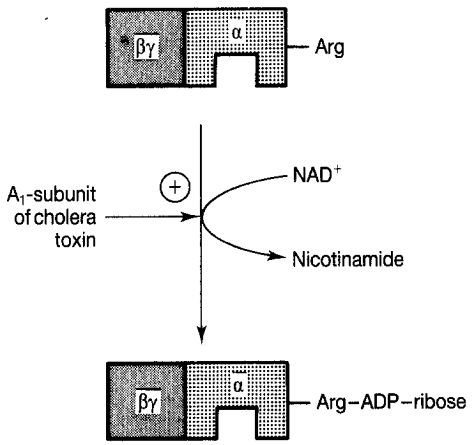

Figure 5. The A_1 subunit of CT catalyses the ADP-ribosylation of the Gs protein, inhibiting its GTPase activity.

are modified by toxin on the luminal side, and they then move to the catalytic subunits in the basolateral membrane. Recent evidence suggests that the latter is the case, and that brush border membrane contains high levels of regulatory subunits of adenylate cyclase which can be ADP ribosylated without the catalytic subunits (Dominguez et al, 1987).

Zonula occludens toxin (ZOT)

Depite removal of the *cfx* A gene encoding the A subunit of CT from recombinant *V. cholerae* vaccine strains, human volunteers continued to get mild to moderate diarrhoea after vaccination (Levine et al, 1988). Although the response was much less than with the parent strain, the continuing diarrhoea led to the search for a further toxin. This has now been identified (Fasano et al, 1991) and its gene has been cloned (Baudry et al, 1992). Culture supernatants of CT-negative *V. cholerae* strains were found to contain a toxin which increased small intestinal permeability by altering intracellular tight junctions (zonula occludens) (Figure 6). The ability to produce toxin (ZOT) correlates with diarrhoeagenicity in humans. The toxin is reversible, heat-labile and sensitive to protease digestion. The gene encoding ZOT has now been cloned. The DNA sequence predicts a molecular mass of 44.8 kDa. The ZOT gene encoding the new toxin is immediately upstream of the operon encoding CT (Baudry et al, 1992).

ZOT is important on two counts. It has a very unusual mode of action and may be the first bacterial product capable of reversibly altering tight junctions. It also has implications for oral vaccine development, as ZOT may well be the cause of continuing symptoms seen during vaccine trials to date.

Accessory cholera toxin (ACE)

A sterile supernatant from a chromosonal region located upstream of ZOT was shown to increase short-circuit current in rabbit epithelium in Ussing chambers in vitro. Enterotoxic activity was also confirmed in rabbit ileal loops (Fasano et al, 1992).

V. cholerae therefore produces two toxins in addition to CT—ZOT and ACE—which are localized in the same chromosomal core region as the CT gene.

ESCHERICHIA COLI (SEE ALSO CHAPTER 3)

Escherichia coli are a normal part of the gut flora, but worldwide are the commonest identifiable cause of diarrhoea, particularly amongst travellers. There are a number of well-recognized mechanisms whereby *E. coli* may cause disease, but there have been, and continue to be, difficulties in distinguishing pathogenic *E. coli* from commensals. New groups of *E. coli* with added virulence traits or toxins continue to be described. This seems set to continue.

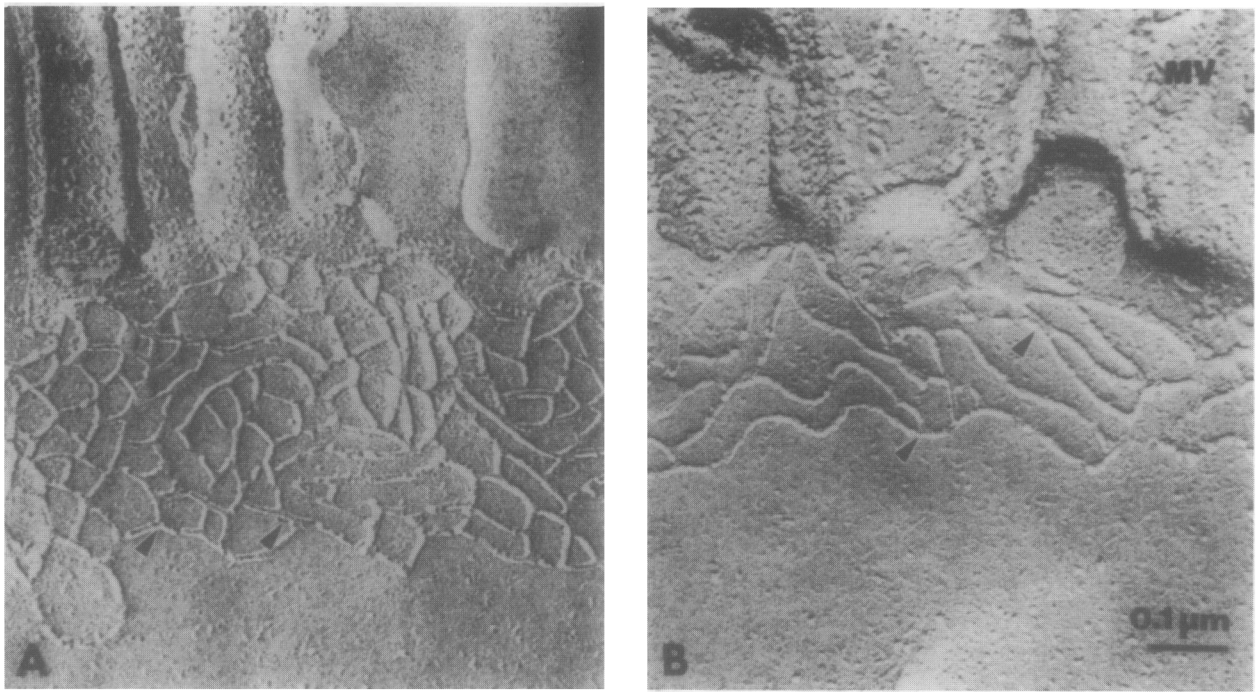

Figure 6. Freeze-fracture studies of rabbit ileal tissue exposed to culture supernatants of *V. cholerae* for 60 min. (A) An intact zonula occludens (ZO) with numerous intersections (marked) between junctional strands; MV, microvilli. (B) An affected ZO from ileal tissue exposed to *V. cholerae* 395; the reticulum appears simplified due to greatly decreased incidence of strand intersections (Fasano et al, 1991).

Enterotoxigenic *Escherichia coli*

Enterotoxigenic *E. coli* (ETEC) produce one or more secretory toxins which are capable of producing diarrhoea but without histological damage. The toxins belong to two major classes: heat-labile (LT) and heat-stable (ST). The genes encoding most of these toxins reside on transmissible plasmids, which explains why more than one toxin can be produced by the same strain of *E. coli*.

Heat-labile enterotoxin (LT)

This was the first toxin identified in *E. coli* and it belongs to the same family of structurally and functionally related protein enterotoxins as CT. Like CT, it comprises five B subunits and a single A subunit. It induces intestinal secretion by activating adenylate cyclase in an NAD-dependent reaction. Although CT and LT bind to GM_1 ganglioside, there are 8–10 times more binding sites for LT than for CT, suggesting that LT binds to additional sites, possibly proteins, not recognized by CT (Griffiths and Crichley, 1991).

Heat-stable enterotoxin (STa)

The second group of toxins produced by ETEC is smaller (molecular weight about 2000) and less antigenic, and stimulates intestinal secretion by a different mechanism from LT and CT. The toxins are made up of 18 or 19 amino acid residues and share a highly conserved C-terminal end, rich in cysteine residues. The gene encoding STa predicts a larger polypeptide of 7–8 kDa, suggesting that extensive post-translational modification takes place.

STa stimulates particulate guanylate cyclase in the brush border to produce cyclic guanosine monophosphate (cGMP) (Guandalini et al, 1982). Guanylate cyclase activation occurs rapidly without a lag-phase and, in contrast to CT and adenylate cyclase, the activation is reversible. Chloride secretion and inhibition of sodium chloride absorption take place, leading to a secretory diarrhoea.

Until recently, it was suggested that the STa binding site and the guanyl cyclase activity were on separate proteins coupled by cytoskeletal components (Waldman et al, 1986). However, following the cloning and expression of cDNAs encoding cell surface receptors for the atrial natriuretic peptides (ANPs), the concept of receptor-guanylyl cyclases has emerged. It is now clear that in the case of ANP, binding sites for hormones and the guanylyl cyclase catalytic activity reside on the same protein separated by a single transmembrane domain (Chang et al, 1989). De Sauvage et al (1991) reported the first identification of a human STa receptor. The receptor retains the same overall structure as ANP receptors, but does not itself respond to ANP. Cells overexpressing the STa receptor bind STa with high affinity and respond with a 50-fold increase in cellular cGMP levels. As a number of natriuretic hormone receptors have now been identified, it seems possible that there may also be other STa receptors.

The identification of the STa receptor leads to therapeutic opportunities in which antibodies could be produced which block STa-receptor binding, but without guanylyl cyclase activation. In fact, only 13 amino acids out of the 19 or 20 are necessary for a secretory effect. A large number of small peptides, conotoxins, have been purified from sea snail venoms and found to have sequence similarities with this active, cysteine-rich portion of STa. Unfortunately, one of the most homologous conotoxins (GI) fails to bind to the STa receptor in rat intestine.

Until recently, an indigenous activator of intestinal guanylate cyclase had not been identified. Using T84 cells, a human colon carcinoma-derived cell line, as a bioassay, Currie et al (1992) have purified and sequenced a 15 amino acid peptide (guanylin) from rat jejunum which activates guanylate cyclase. Analysis of the peptide revealed a high degree of homology with STa and synthetic guanalin is capable of displacing STa bound to T84 cells. This peptide may therefore have a physiological role in modulating intestinal fluid and electrolyte transport in the small intestine. At present, the specific cell sources of guanylin are not known.

Enteropathogenic *Escherichia coli* (EPEC)

Until recently, EPEC were defined on the basis of exclusion; those diarrhoea-associated serotypes of *E. coli* which did not produce a recognizable toxin, were not invasive and which did not have colonizing fimbriae were considered to be EPEC. They are an important cause of acute and persistent diarrhoea in both developed and developing countries (Hill et al, 1991; Rothbaum et al, 1982; Robins-Brown, 1987) and are discussed further in Chapter 3.

EPEC colonize the small intestine and produce a characteristic attaching and effacing lesion (Figure 7). Initial non-intimate attachment to enterocytes is followed by localized vesiculation of brush border microvilli and then intimate adherence to the plasma membrane (Knutton et al, 1987). Disruption of the brush border cytoskeleton allows the enterocyte cell membrane to distort around the bacterium in a cup-like pedestal structure containing a dense plaque of short filaments at the cytoplasmic surface of the plasma membrane. A morphologically identical lesion is produced by EPEC in cultured cell lines, and, using fluorescein-labelled phallotoxin, these filaments have been identified as actin. This observation forms the basis of a new, inexpensive test for the identification of EPEC using fluorescent actin staining (FAS) (Knutton et al, 1989).

Few data exist on the effects of EPEC on intestinal water and electrolyte transport, but recent studies in a rabbit model suggest that electroneutral sodium chloride absorption in the caecum is inhibited, but without stimulation of chloride secretion (Tai et al, 1989). Major insights have recently been gained into the mechanism of the attaching and effacing lesion in enterocytes which follows EPEC colonization. EPEC stimulate protein kinase activity in enterocytes, leading to phosphorylation of myosin light chain, an integral cytoskeletal component (Baldwin et al, 1990; Manjarrez-Herrendez et al, 1992). This phosphorylation leads to alterations in cell

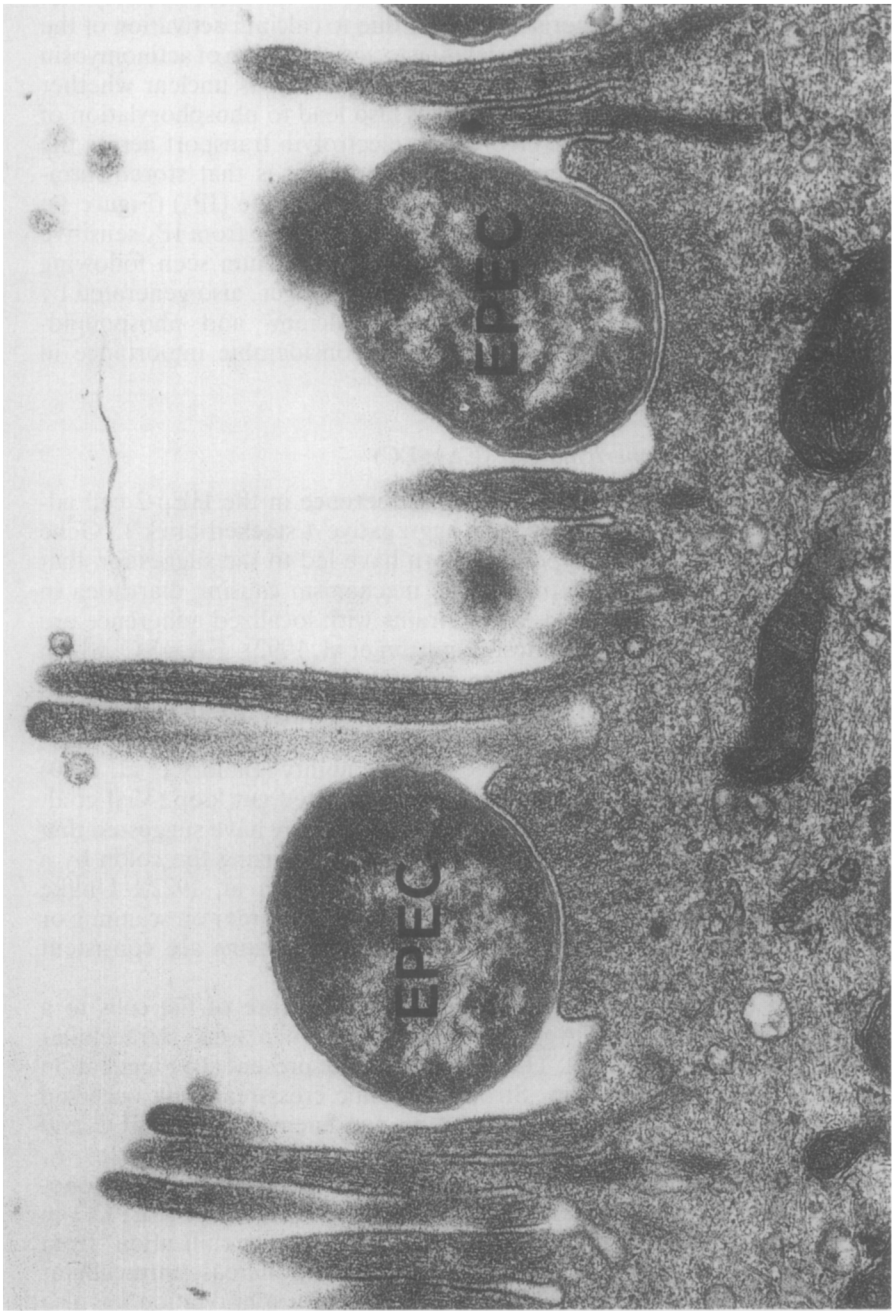

Figure 7. Electronmicrograph showing human intestinal mucosa infected with a classical enteropathogenic *Escherichia coli* (EPEC). EPEC produce a characteristic 'attaching and effacing' lesion characterized by intimate bacterial attachment, localized destruction of brush border microvilli and accretion of a dense plaque of cytoskeletal filaments beneath adherent bacteria; ×45 000. Courtesy of Dr S. Knutton.

shape and in actin organisation (Knutton et al, 1989). EPEC infection also leads to raised intracellular calcium concentrations, and it seems likely that the formation of the characteristic lesion is due to calcium activation of the actin depolymerizing protein villin, leading to accumulation of actinomyosin within the lesion (Baldwin et al, 1991). At present it is unclear whether raised intracellular calcium concentrations also lead to phosphorylation of other cell membrane proteins involved in electrolyte transport across the brush border membrane. One plausible hypothesis is that stored intracellular calcium is released by inositol 1,4,5-triphosphate (IP_3) (Figure 4). For example, dantrolene, an inhibitor of calcium release from IP_3-sensitive stores, will also abolish the increase in cytosolic calcium seen following EPEC infection. Moreover, another second messenger, also generated by phospholipase C, diacylglycerol, activates calcium- and phospolipid-dependent protein kinase C, an enzyme of considerable importance in regulating ion fluxes (Figure 4).

Enteroaggregative *Escherichia coli* (EAggEC)

Escherichia coli shows three patterns of adherence in the HEp-2 cell adhesion assay: localized, diffuse and aggregative ('stacked-brick'). Gene sequences coding for each type of pattern have led to the suggestion that each is related to a specific pathogenic mechanism causing diarrhoea in humans (Cravioto et al, 1991). Most strains with localized adherence are EPEC and give a positive FAS test (Knutton et al, 1992). EAggEC, which do not give a positive FAS test, are associated with persistent diarrhoea (Bhan et al, 1989), which in addition may be bloody (Cravioto et al, 1991), suggesting that there are two mechanisms whereby EAggEC cause diarrhoea. Some aggregative strains have invasive ability (Baudry et al, 1990) and cause a haemorrhagic lesion in the rabbit ligated gut loop (Vial et al, 1987). Studies using a human gut tissue adhesion assay have suggested that that EAggEC may be a large bowel pathogen, colonizing the colon by a fimbrially mediated adhesion mechanism (Knutton et al, 1922). Unlike EPEC, EAggEC do not appear to damage the brush border on scanning or on transmission electron microscopy. These observations are consistent with a toxin-mediated mechanism of disease.

Infection of HEp-2 cells with EAggEC, or exposure of the cells to a culture supernatant of EAggEC, significantly increased intracellular calcium (Baldwin et al, 1992). The effects were not present after incubation with heat-treated supernatant. Strong antigenic cross-reactivity occurred between a protein of 120 kDa secreted by non-haemolytic EAggEC and antibodies raised against the C-terminal region of *E. coli* haemolysins. *E. coli* haemolysin is transiently inserted into lipid membranes, forming monomeric ion-specific pores that allow influx of calcium. In contrast to EPEC, in which intracellular calcium is increased following mobilization from intracellular stores, EAggEC haemolysin appears to increase intracellular calcium from external calcium. Myosin light-chain phosphorylation was also produced by culture with a supernatant of EAggEC strains, while heating again abolished this activity. It seems likely that the observed phospho-

rylation is due to calcium-dependent kinases, such as myosin light-chain kinase. Extensive pore formation by *E. coli* haemolysin is likely to cause cell death, which may be a factor in the persistent diarrhoea seen in association with EAggEC infection (Baldwin et al, 1992).

Enteroinvasive *Escherichia coli* (EIEC)

Enteroinvasive *E. coli* produce a dysenteric illness in humans which tends to occur in food-associated outbreaks (Chapter 1). Man appears to be the principal reservoir. Pathogenesis of EIEC diarrhoea is similar to shigellosis, in that they possess the ability to invade and multiply within epithelial cells in the distal small intestine and colon. This invasive property depends on expression of several outer membrane proteins, structural genes for which are found on 140-MDa enteroinvasive plasmids that are almost identical to the plasmids in *Shigella flexneri* (Harris et al, 1982). Watery diarrhoea frequently precedes dysentery in EIEC infection, and may occur in the absence of bloody diarrhoea. Recently it has been shown that EIEC also produce low levels of Vero cell cytotoxins that are distinct from Shiga-like toxin I or II of enterohaemorrhagic *E. coli* (EHEC). Secretory activity in EIEC culture supernatants was also demonstrated in rabbit ileal loops and in Ussing chambers. Curing the 140-MDa invasiveness plasmid from an EIEC strain did not diminish enterotoxin production. The enterotoxin is 68–80 kDa in size and is distinct from EIEC cytotoxin (Fasano et al, 1990).

Enterohaemorrhagic *Escherichia coli* (EHEC)

EHEC infection is associated with haemorrhagic colitis, haemolytic uraemic syndrome and thrombotic thrombocytopenic purpura (Chapter 3). So far, only one serotype, O157:H7, has been associated with food-borne disease, and most outbreaks have been linked to consumption of undercooked ground (minced) beef or unpasteurized milk.

The organism can adhere to human small intestinal epithelial cells, where it produces the attachment and effacement lesions visible on electron microscopy which are also characteristic of EPEC infection (Knutton et al, 1989). All wild-type O157:H7 strains also produce large quantities of one or all of three types of bacteriophage-encoded cytotoxins: Shiga-like toxin (SLT) I, II and III variants, know as verotoxins (because of their effects on Vero tissue culture cells) (Brown et al, 1989). The genes coding for these three proteins have been cloned so that EHEC strains can now be identified on this basis. SLT-I is probably identical to Shiga toxin, with a 99% sequence homology. Both SLT-I and SLT-II are enterotoxic for rabbit ileal loops, paralytic and lethal for rabbits and mice, and cytotoxic for HeLa and Vero cells.

In haemorrhagic colitis, the extent of the disease is variable, but the ascending colon is usually involved. The mucosal surface is ulcerated and the wall thickened by submucosal haemorrhage. The presence of patchy inflammation and coagulative necrosis in association with capillary thrombi

is very suggestive of O157:H7 colitis. All of the histological changes seen in *Clostridium difficile* infection may also be seen during EHEC infection.

SHIGELLA

Penetration of colonic epithelial cells and subsequent tissue invasion are crucial steps in the pathogenesis of *Shigella* dysentery (Sansonetti, 1991). Enterocyte penetration is mediated by a 120–140-kDa plasmid, and its four protein products are expressed on the surface of the organism (Hale et al, 1983). These proteins (invasion plasmid antigens; ipa A–D) seem to be responsible for bringing the bacterium and colonocyte into close contact and for triggering part of the bacterial signal for the induction of phagocytosis. The invasion genes are controlled by both positive and negative regulatory systems. A chromosomal locus *virR* codes for a repressor, which regulates the expression of virulent plasmid through a plasmid locus *virF*. An additional plasmid locus *virB*, which is positively regulated by *virF*, produces a 35-kDa protein which in turn upregulates the expression of the invasion antigens ipa A–D.

Cytochalasins B and D, which block actin polymerization, also block penetration by *Shigella*, and actin polymerization and myosin accumulation can be seen at the site of bacterial penetration.

Plasmid genes are required for the efficient intracellular multiplication of bacteria, which takes place independently of Shiga toxin production. Early lysis of the phagocytic membrane occurs, a process which is dependent on the presence of a plasmid-encoded contact haemolysin. Bacteria therefore come to live free in the cytoplasm of infected cells. Intra- and intercellular spread is crucial for successful invasion, and this property is determined by an ability to polymerize actin. A plasmid locus *virG* is necessary for reinfection of adjacent cells. Subsequently, the host cell is killed by *Shigella*, in a process which involves a rapid fall in intracellular ATP and an increase in pyruvate, both consistent with a block in cellular respiration. Surprisingly, Shiga toxin, a potent cytotoxin, does not seem to play an important role in the early killing of infected cells.

The pathogenesis of the watery diarrhoea which may precede dysentery remains uncertain, although one clinical study indicated that diarrhoea could be accounted for on the basis of normal ileal effluent overwhelming a grossly dysfunctional colon (Buttler et al, 1986).

Shiga toxin, a protein produced by *Shigella dysenteriae* I, causes enterotoxic, cytotoxic and neurotoxic effects in rabbit intestine, epithelial cells lines and certain mammalian species, respectively (Eiklid and Olsnes, 1985). The receptor is present on the brush border membrane and is a glycolipid (globotriaosylceramide; Gb_3) (Mobassaleh et al, 1989). Structurally, Shiga toxin is similar to CT, with five B subunits with a binding function, and a single A subunit which produces its biological effects by inhibition of protein synthesis and induction of cell death (Keusch Donohue-Rolf and Jacewicz, 1985). The importance of Shiga toxin in the pathogenesis of shigellosis is uncertain, and there is evidence that dysenteric infection will

occur in volunteers infected with invasive, toxin-negative strains (Gemski et al, 1972). However, *Shigella dysenteriae* I, which elaborates large amounts of toxin, tends to produce a more severe illness, and Shiga-like toxin is associated with haemolytic uraemic syndrome and post-dysenteric microangiopathic haemolytic anaemia as is seen following EHEC infection.

SALMONELLA

The mechanisms underlying the pathogenesis of *Salmonella* infection are poorly understood. Infecting organisms adhere to and destroy the brush border membrane or target cells and are associated intracellularly with polymerization of actin. However, in contrast to *Shigella*, which use the epithelium as a multiplication site, *Salmonella* use the epithelium as a route to gain access to the lamina propria. Here they are taken up by reticuloendothelial cells and transported to Peyer's patches and mesenteric lymph nodes.

Some strains produce a Shiga-like toxin and others a cholera-like toxin (Finkelstein et al, 1983). The part played by these toxins in salmonellosis is unclear.

CAMPYLOBACTER JEJUNI

Campylobacter infection can present as a dysenteric infection with a colitis, or alternatively with a syndrome characterized by watery diarrhoea. Understanding the mechanisms of the diarrhoeal disease has been hampered by the lack of a good animal model. The macaque monkey has recently been shown to produce a colitis in response to infection, with clinical and pathological characteristics similar to those of the human disease (Russell et al, 1989). *Campylobacter jejuni* is known to produce two toxins, an enterotoxin and a cytotoxin (Johnson and Lior, 1984). The enterotoxin is similar to CT in many ways (Ruiz-Palacios et al, 1983) and activates adenylate cyclase. At present, the role of these toxins in human disease is uncertain, but it has been suggested that differing toxin production may predict the major clinical manifestations of infection (Klipstein et al, 1985). The subject has been reviewed by Walker et al (1986).

CLOSTRIDIUM DIFFICILE

It is now well recognized that the severe disease which may accompany antibiotic administration is often due to a pseudomembranous colitis caused by *C. difficile* infection (Bartlett, 1978). *Clostridium difficile* produces four toxins which affect the gut: toxin A, an enterotoxin; toxin B, a cytotoxin; a heat-labile toxin (Banno et al, 1984); and a motility-altering factor (Justus et al, 1982). The gene for toxin A has now been cloned and sequenced (Dove et al, 1990).

Toxin A is responsible for changes in intestinal ion transport, reduced nutrient absorption from the small intestine and the induction of marked electrolyte secretion (Triadafilopoulos et al, 1987). The secretory changes are not associated with increased tissue concentrations of cyclic nucleotides (Guandalini et al, 1988), although the changes may be calcium-dependent (Triadafilopoulos et al, 1989). Toxin A also has cytotoxic properties: instillation into rabbit ileal loops leads to enterocyte death and infiltration of the lamina propria with neutrophils, features which distinguish it from cholera toxin (Triadafilopoulos et al, 1987). There is some evidence that toxin A may kill cells by altering the organization of microfilaments and microtubules (Fiorentine et al, 1990). Toxin B is probably responsible for the pseudomembranous exudate which characterizes the infection clinically (Bartlett, 1978; Viscidi and Bartlett, 1981).

AEROMONAS

Aeromonas spp. were first proposed as enteric pathogens in humans more than 30 years ago (Martinez-Silva et al, 1961) and many subsequent clinical reports support the association. However, acceptance of the role of *Aeromonas* in human diarrhoeal disease (Khardori and Fainstein, 1988) has not been universal, partly because of the complexity of the taxonomy of aeromonads and their precise identification, and the use of inappropriate procedures for their isolation (Janda and Duffey, 1988) and methods to establish their diarrhoeagenicity (Morgan et al, 1985). The strongest evidence in support of *Aeromonas* spp. as enteric pathogens comes from adequately controlled epidemiological studies, e.g. in Western Australia, where 11% of children with diarrhoea had enterotoxigenic *Aeromonas* detected in comparison with less than 1% in the control group (Burke et al, 1983). There are now many reports of patients with diarrhoea in whom *Aeromonas* was the only recognized pathogen and several reviews on *Aeromonas*-associated diarrhoea are available (Freij, 1984; Agger et al, 1985; Agger, 1986; Burke and Gracey, 1986; Sack et al, 1988; San Joaquin and Pickett, 1988).

Aeromonas usually causes mild, self-limiting watery diarrhoea but can cause dysenteric symptoms (bloody diarrhoea with mucus) or persistent diarrhoea. The disease has a summer peak and may be water-borne. Differences in biotype and virulence characteristics between environmental and clinical isolates suggest that only some strains which occur in water are potentially pathogenic (Burke et al, 1984). The taxonomy of *Aeromonas* spp. has been revised recently and the genus now includes ten species (*A. hydrophila, A. sobria, A. caviae, A. salmonicida, A. media, A. veronii, A. schubertii, A. eucrenophila, A. jandaei* and *A. trota*) (Carnahan et al, 1991), most of which have been associated with disease in humans (Altwegg et al, 1990). These micro-organisms are capable of producing a wide range of toxins, which is compatible with the scope of gastrointestinal and extra-intestinal illness which *Aeromonas* infections can cause (Rolston, 1988). Recognized *Aeromonas* toxins include cytotonic and cytotoxic enterotoxins

and cytolytic haemolysins (Burke and Gracey, 1986). The determinants of enterotoxic, cytotoxic and haemolytic activity are believed to be located at three different segments of the *A. hydrophila* chromosome (Chakraborty et al, 1984). The mechanism(s) of action of *Aeromonas* enterotoxins are not known. The cytotonic toxin increases steroid secretion and cAMP in Y1 cells and rabbit intestinal cells (Ljungh et al, 1982); however, mucosal damage from the cytotoxic effects may also play a role. Although invasiveness does not occur in the guinea-pig, keratoconjuctivitis (Serény) test, invasion of the subepithelial space in rabbit ileal loops (Pitarangsi et al, 1982) and invasion in tissue culture cells in vitro have been reported (Lawson et al, 1985), which may have relevance to those patients who have dysenteric illnesses. *Aeromonas* spp. can adhere to intestinal brush borders (Levett and Daniel, 1988), and agglutination of erythrocytes or yeast cells has been reported (Adams et al, 1983) although the pathogenic significance of this is uncertain.

YERSINIA ENTEROCOLITICA

Yersinia enterocolitica most commonly causes an acute gastroenteritis. It can also cause a chronic ileocolitis resembling Crohn's disease. It is an invasive organism; after oral administration to mice it preferentially penetrates the epithelium overlying Peyer's patches and proliferates within the follicles. Organisms subsequently spread into the lamina propria of the villi, where they are engulfed by macrophages (Hanski et al, 1989). Invasiveness is encoded by a chromosomal *inv* gene which specifies a 103-kDa protein, invasin. The *inv* gene is not expressed by non-pathogenic strains, which are unable to penetrate intact epithelial cells. An additional chromosomal locus, the *ail* locus, specifies attachment and invasiveness (Millan et al, 1988, 1989). Enterotoxin production is frequently identified in virulent strains (Okamoto et al, 1980) but is not essential for disease production.

A rabbit model has been used to study the effects of *Y. enterocolitica* on intestinal structure and function (O'Loughlin et al, 1990). Patchy microabscess formation occurred in both small and large intestine. Crypt hypertrophy with diffuse brush border damage and reduced disaccharidase activities occurred in the jejunum and ileum. Glucose-coupled sodium absorption was impaired, but there was no active chloride secretion. Abnormal epithelial cell renewal, as seen in viral gastroenteritis, did not seem to be the cause of these changes. Rather, they were due to a direct effect of the organism or its products. Colonic transport was enhanced, as a compensatory salvage phenomenon in response to increased delivery of ileal effluent. Diarrhoea, therefore, seems to result from nutrient malabsorption and not from a secretory process.

It has recently become clear that the pathogenesis of *Yersinia* infection is linked closely to intracellular tyrosine phosphorylation and dephosphorylation. Indeed, bacteria may well interact with host cells in a much more sophisticated way than previously recognized (Clemens et al, 1991). Tyrosine kinases are a family of enzymes responsible for tyrosine phosphorylation, the extent of which plays an integral part in the regulation of the cell

cycle and the transduction of growth signals. For example, the binding of insulin to its receptor switches on tyrosine kinase activity and the multiple effects of insulin are probably mediated by the phosphorylation of tyrosine residues on target proteins. Similarly, the epidermal growth factor receptor contains a cytosolic tyrosine kinase domain; many oncogenes also act through tyrosine kinase.

Since tyrosine phosphorylation plays such a key role, cells must regulate precisely the extent of this phosphorylation, and they achieve this through protein tyrosine phosphatases (PTPases). Recent observations have shown that *Yersinia* spp. express outer membrane proteins (Yops) which are not only virulence factors, but which also demonstrate tyrosine phosphatase activity. Moreover, conversion of a cysteine residue to alanine in the catalytic domain of YopH abolished PTPase activity in *Y. pseudoturberculosis* and significantly reduced its virulence in a mouse model (Bliska et al, 1991). Infection of macrophages by *Yersinia* also causes the dephosphorylation of several phosphotyrosine-containing proteins. It therefore seems likely that PTPase disables one or more classes of mononuclear cells, thereby crippling the ability of the immune system to respond to bacterial infection.

ROTAVIRUS

Rotavirus diarrhoea is considered to be the commonest cause of gastroenteritis in children under the age of 2 years in both developing and developed countries (see Chapter 5). Characteristically, stools in rotavirus diarrhoea contain modest electrolyte concentrations (sodium and chloride 30–50 mmol/l). Non-absorbed carbohydrate is frequently present in stools. The proximal small intestine is the usual site of infection; the stomach and the colon are not involved.

Most of the information about the pathophysiology of rotavirus diarrhoea comes from studies in a piglet model infected with a coronavirus, the transmissible gastroenteritis virus (TGE) (Hamilton, 1990). A number of similarities exist between the response of infants to rotavirus and that of the piglet to TGE. The piglet therefore seems to be a suitable model for studying human rotavirus infection.

In the piglet, TGE virus infection triggers a response which is characterized by grossly disorganized enterocyte renewal and migration. Crypt cell proliferation accompanies an increase in enterocyte renewal and shedding of infected cells into the small intestinal lumen. Rapid enterocyte migration is accompanied by incomplete differentiation, so that villous enterocytes have the characteristics of undifferentiated, secretory-type crypt cells rather than those of differentiated absorptive cells. These abnormalities appear to be the major determinants of viral diarrhoea (Table 2). Perfusion studies in vivo have not supported a secretory state in this model.

In contrast to these observations, studies of murine rotavirus diarrhoea using an in vitro perfusion system of infant mouse intestine (Starkey et al, 1990a) suggested a secretory component in the infective response (Starkey et al, 1990b) associated with changes in the microcirculation of intestinal villi

Table 2. Characteristics of villous enterocytes in transmissible gastroenteritis (TGE) diarrhoea in piglets (Hamilton, 1990).

Increased thymidine kinase activity
Reduced disaccharidase activities
Defective sodium-coupled glucose uptake
Reduced Na^+/K^+-ATPase activity
Decreased NaCl absorption
Impaired alanine-stimulated sodium uptake

(Osbourn et al, 1991). At present, it is difficult to reconcile these observations with those made in the TGE piglet model. Recent studies in rotavirus-infected tissue culture cells suggest that an increase in intracellular calcium is a potential mediator of cell death. Although viral replication was not affected, cell death was preventable by lowering the calcium in the tissue culture medium (Michelangeli et al, 1991).

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