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I, 2. Physiology and pathophysiology of the gut in relation to viral diarrhea

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Introduction

The epithelium of the gastrointestinal tract forms a barrier between the outside world and the host. The intestine is lined by a folded continuous monolayer of columnar polarized epithelial cells joined together by tight junctions, dispersed enteroendocrine cells and M cells. The epithelium is permeant to small solutes and water which can cross via paracellular or transcellular pathways. Intestinal epithelial cells originate from anchored stem cells localized near the base of intestinal crypts and are subject to constant renewal, turning over every 4-7 days. Differentiation gives rise to columnar secretory and absorptive epithelial cells, as well as mucus-secreting goblet cells, Paneth cells and enteroendocrine cells (Madara, 1999). Epithelial proliferation may be enhanced under pathological conditions. This may have effects on the balance of functions by different epithelial cells along the crypt-villus axis (Barrett and Keely, 2000; Karam, 1999; Podolsky, 1993). The surface area of the intestinal epithelium is largely amplified (about 600-fold in the small intestine) by the presence of folds, villi, and cell microvillar structures. This enhances the capacity of the intestine to transport nutrients, electrolytes and water.

Many advances have been made in the understanding of intestinal electrolyte transport from the molecular to the whole-tissue level. This chapter emphasizes molecular mechanisms of intestinal epithelial ion transport processes, as well as the intra- and extracellular factors involved in their regulation, as a framework for the understanding of virus-induced gastroenteritis.

Abbreviations used in text and figures: 5HT: 5-hydroxytryptamine, serotonin; AC: adenylylate cyclase; ACh: acetylcholine; AQP: water channels of the aquaporin family; CaCC: Ca\(^{2+}\)-activated chloride channel; cAMP: adenosine 3’-5' cyclic monophosphate; CF: cystic fibrosis; CFTR: cystic fibrosis transmembrane conductance regulator, chloride channel; cGMP: guanosine 3’-5’ cyclic monophosphate; ENS: enteric nervous system; ER: endoplasmic reticulum; GC: guanylate cyclase; GLUT2: facilitated sugar
Normal intestinal physiology

In the intestine, Na⁺, K⁺, HCO₃⁻, and Cl⁻ are transported actively determining fluid movements to maintain isotonicity between the intestinal lumen, the cell and the internal milieu. The intestine also contains ion-dependent mechanisms for the transport of peptides, amino acids, sugars, and bile acids (Lee et al., 1994; Lentz, 1995; Mailliard et al., 1995). In addition to the absorption of ions, nutrient uptake may collaborate in generating fluid movement by solvent drag (Pappenheimer et al., 1994; Turner and Madara, 1995). The function of the intestine as a transporting machine is guaranteed by its structural and functional asymmetry at the cellular and crypt-villus axis levels and along the gut from duodenum to distal colon. The intestinal cells transport electrolytes and nutrients in a vectorial manner, generating electrochemical and osmotic gradients to drive absorption and/or secretion. This transport is made possible by the structural and functional polarity of the enterocyte. Transport proteins and receptors for regulatory mediators and toxins are asymmetrically distributed in the apical and basolateral cell membranes.

The diverse epithelial cell types are arranged so that there is a functional heterogeneity in electrolyte, fluid, and nutrient transport along the crypt-villus axis. It was a dogma for a long time that absorption and secretion took place in villus and crypt cells, respectively (Montrose et al., 1999). This paradigm has been challenged since secretory mechanisms have been observed in some villus cells (Kockerling and Fromm, 1993; Stewart and Turnberg, 1989), and colonic crypt cells are able to absorb Na⁺ and water in the absence of secretory stimuli (Naftalin and Pedley, 1999; Naftalin et al., 1995; Singh et al., 1995; Thiagarajah et al., 2001). The small intestine is a leaky epithelium absorbing large volumes of fluid against small electrochemical gradients whereas the distal colon is a semi-tight epithelium capable of absorbing Na⁺ against a large gradient.

Fluid balance in the intestine

The intestine handles large amounts of fluid containing water, nutrients and salts. Net fluid transport through and across the gut results from food intake, secretory and absorption processes and final excretion. On average, about two liters of water are
ingested daily, together with nutrients and electrolytes. In addition, 7 liters of fluids are secreted by different parts of the gut to aid digestion (saliva, gastric and pancreatic juices, bile and intestinal secretion). In total, over 98% of these fluids are absorbed back into the body in the small and large intestine, accompanying nutrients and salts. Of these, about 5 liters are absorbed in the duodenum and jejunum, 2 liters in the ileum and 2 liters in the colon, remaining only 100 ml excreted in the stools in the healthy person. Normally, the ileo-cecal flow is approximately 2 liters but the colon may be able to absorb as much as 5-6 liters per day. Water movements in secretory and absorptive epithelia are secondary to electrolyte and nutrient transport and may occur through transcellular and/or paracellular pathways (Montrose et al., 1999). Diarrhea occurs when secretory processes exceed the absorptive capacity of the intestine, from an impairment of nutrient and water absorption and/or from an increase in the gut motility inducing an accelerated transit.

**Extracellular regulation of intestinal function**

Intestinal transport is regulated by a number of extracellular stimuli, which may act on the luminal or serosal sides of the epithelium. These include endocrine, paracrine and neurocrine mediators (Fig. 1). The regulation of epithelial function is the result of the integrated response to all these systems. Enteroendocrine cells in the crypt and villus region release peptides and other substances such as serotonin (5HT), neurtensin, secretin, somatostatin, guanylin etc, across their basolateral or apical membranes [Madara and Trier, 1994]. The diffusion of these bioactive substances locally regulates the transport functions of neighboring cells (i.e., paracrine regulation) and signal to more distant cells along the crypt villous axis by stimulation of dendritic terminals of ENS neurons. These substances together with bloodborne peptides are involved in endocrine regulation of distant regions of the epithelium. Neurotransmitters such as VIP, 5HT, substance P and ACh, released from enteric nerve endings regulate epithelial functions of enterocytes (absorption and secretion) and enteroendocrine cells as well as motility and microcirculation. Adding to the complexity of regulatory mechanisms, the mast cells, neutrophils (PMN), macrophages and T lymphocytes from the immunological system and localized in the lamina propria, can also release substances (histamine, cytokines, adenosine, etc) that regulate the epithelium (immune regulation). On the other hand, molecules such as bile salts, fatty acids, and microbial toxins found in the luminal contents can also affect electrolyte and nutrient transport, acting directly on epithelial cells or indirectly by way of stimulation of enteroendocrine cells. The myofibroblasts form a continuous sheath underlying the basement membrane of the epithelial layer that release prostaglandins induced by serotonin, histamine, and TNF-α. Released prostaglandins may stimulate epithelial Cl⁻ secretion (Berschneider and Powell, 1992; Kandil et al., 1994; Lundgren, 1992; Sears and Kaper, 1996).
Fig. 1. Regulation of intestinal functions by the enteroendocrine, immune and enteric nervous systems. The intestinal epithelium is a continuous monolayer containing different cell types, mainly villus cells, crypt cells and enteroendocrine cells. Epithelial cells release cytokines, prostaglandins and nitric oxide (NO) in response to luminal stimuli. Enteroendocrine cells secrete mediators such as 5HT, neurotensin, substance P, secretin, somatostatin, and guanylin. The myofibroblast sheath releases prostaglandins, cytokines and NO. Other cells present in the lamina propria, such as mast cells, polymorphonuclear leucocytes (PMN) and T lymphocytes from the immunologic system let out a variety of chemical mediators (histamine, cytokines, prostaglandins, NO etc). The enteric nervous system releases VIP, 5HT, substance P, ACh and NO. All three systems interact with each other at different levels to finally regulate transport function by enterocytes (secretion by crypt cells and absorption by villus cells), paracellular permeability, gut motility and microcirculation. Enteric viruses have a distinct predilection for different cell types in the intestinal epithelium. Rotavirus, TGEV, adenovirus, calicivirus and astrovirus infect the villus cells whereas parvovirus infects exclusively crypt cells. Coronavirus and torovirus infect basal villus cells and crypt cells. See text for discussion and references.

**Intestinal absorption**

**Sugar transport**

Nutrient absorption in the villus enterocyte depends to a large extent on the presence of a Na⁺ gradient between the intra- and extracellular media generated by the Na⁺/K⁺
ATPase. This pump provides the driving force for the uphill transport of sugars and amino acids. On the other hand, both Na⁺ and water absorption are largely dependent on the presence of nutrients in the apical side, especially glucose. Transport mechanisms are schematized in Fig. 2.

Fig. 2. Ion and nutrient transport mechanisms in the intestine. For the sake of clarity absorption mechanisms are represented in villus cells (upper part) and secretion mechanisms in crypt cells (lower part). **Villus cells:** A Na⁺ gradient across the apical and basolateral membranes is generated by the operations of the basolateral Na⁺/K⁺-ATPase. Glucose and Na⁺ are taken up across the apical membrane of villus cells by the SGLT1 cotransporter driven by the Na⁺ gradient. Fructose enters the cells driven by the chemical gradient through a facilitated diffusion pathway in the apical membrane (GLUT 5). Glucose and fructose exit through a facilitated diffusion pathway in the basolateral membrane (GLUT2). Amino acids (AA) enter the cell by Na⁺-dependent or Na⁺-independent transport mechanisms and exit across the basolateral membrane through another type of Na⁺-independent mechanisms. Apical NaCl entry through Na⁺/H⁺ (NHE-3) and Cl⁻/HCO₃⁻ exchange drive electroneutral NaCl absorption in the small intestine and colon. Na⁺ exits the cell through the basolateral Na⁺/K⁺-ATPase, but the route of Cl⁻ efflux is not known. The H⁺ gradient created across the apical membrane by the Na⁺/H⁺ exchange drives the cotransport of peptides. Different chemical mediators coming from enteroendocrine (EC), immune (Im) and enteric nervous (ENS) systems act by binding to specific receptors in the basolateral membrane coupled to adenyl cyclase or phospholipase C which result in increases in the second messengers Ca²⁺, diacylglycerol (DG) and cAMP. These regulate the activity of transporters, mainly SGLT1, by PKA and PKC mediated phosphorylation. **Crypt cells:** Chloride secretion in the small intestine and colon is depicted in the lower cell. Cl⁻ enters cells at the basolateral membrane through Na⁺/K⁺/2Cl⁻ cotransport and leaves cells

(cont. on p 28)
Sugars are transported across the apical membrane by different transport proteins. The Na+/glucose cotransporter (SGLT1) accumulates glucose and galactose inside the enterocyte. The facilitated sugar transporter GLUT5 transports fructose down its chemical gradient. These sugars are then transported out of the cell by GLUT2, a facilitated transporter located in the basolateral membrane. The SGLT1 cotransporter has a stochiometry of 2 Na⁺ and one glucose molecule per cycle. Sodium entering the cell by this way is extruded by the Na⁺/K⁺ pump with a concomitant transport of Cl⁻ and HCO₃⁻, and the osmotic flow of water. These mechanisms provide the rational for oral rehydration therapy used in secretory diarrheas [Bass, Section I, Chapter 5 of this book].

The regulation of intestinal sugar absorption via Na⁺/glucose cotransporters occurs over short (minutes) or long term (days). In oocytes expressing rabbit SGLT1, the activation of protein kinase A (PKA) increased the maximum rate of Na⁺/glucose cotransport by 30%, and the activation of protein kinase C (PKC) decreased the maximum rate of transport by 60%. Changes in maximum transport rates are accompanied by proportional changes in the membrane area and in the number of cotransporters through the regulation of exocytosis and endocytosis by PKA and PKC (Wright et al., 1997).

Amino acid and peptide transport

The small intestine is the main site for the absorption of protein digestion products. This process involves the participation of transport mechanisms for amino acids and small peptides. Large peptides resulting from luminal digestion by proteases are hydrolyzed to amino acids and di- and tripeptides by peptidases present in the brush border membrane. Amino acids are transported by group-specific transport systems localized in the apical as well as in the basolateral membrane. For most transport systems (B, B°,+, Imino, b, XAA, ~) the driving force is the Na⁺ gradient across the apical membrane. For the Na⁺ independent transporters (systems y°, b°,~), the driving force is the electrical potential across the apical membrane. Absorbed amino acids cross
the basolateral membrane into the circulation through Na⁺-independent transporters, different from those present in the apical membrane (Ganapathy et al., 1994).

Peptides are transported across the apical membranes by H⁺/peptide cotransporters. The H⁺ gradient across the apical membrane required to drive transport is assured by the existence of a Na⁺/H⁺ exchanger, partly converting the Na⁺ gradient that exists across the brush border membrane into a H⁺ gradient. Most of the peptides entering the enterocyte are digested to free amino acids by intracellular peptidases. A small fraction of peptides enters the blood across the basolateral membrane by H⁺/peptide cotransport systems driven by the peptide concentration gradient. Short term regulation of amino acid transport is controlled by several hormones and neurotransmitters acting through the different second messengers (Ganapathy et al., 1994).

**Electroneutral NaCl absorption**

A significant proportion of the Na⁺ absorbed in the intestine occurs independently of nutrient transport. Part of the Na⁺ is absorbed together with Cl⁻ without net charge transfer. Evidence supports a dual-exchanger model where the operation in parallel of Na⁺/H⁺ and Cl⁻/HCO₃⁻ exchangers assures electroneutral NaCl transport (Montrose et al., 1999). Supply of H⁺ and HCO₃⁻ is dependent on the activity of intracellular carbonic anhydrase. The functioning of the system is driven by the Na⁺ gradient across the apical membrane which in turn is maintained by the activity of the Na⁺/K⁺ ATPase located in the basolateral side. This mechanism is important in the small intestine and the proximal colon. In the distal colon, an electrogenic amiloride-sensitive Na⁺ absorption regulated by aldosterone accounts for about 50% of the Na⁺ reabsorbed. In this case Na⁺ crosses the apical membrane through a rheogenic channel. Basolateral K⁺ channels would recycle K⁺ across the basolateral membrane. The paracellular flux of Cl⁻ driven by Na⁺ movement would restore electroneutrality between apical and basolateral sides.

**Intestinal secretion and its regulation**

The small and large intestine are able to secrete a vast amount of fluid, mainly constituted by an isotonic secretion of NaCl. Chloride is transported from the plasma to the lumen through a transcellular pathway. The transport of Cl⁻ in mammalian secretory epithelia is not due to a Cl⁻ pump but results from the concourse of distinct transmembrane transport mechanisms asymmetrically located in polarized cells. The Cl⁻ secretory mechanism in crypt cells of the small and large intestine has been well characterized (Fig. 2; for review see Barrett and Keely, 2000; Montrose et al., 1999; Morris, 1999). Chloride enters the cell across the basolateral membrane via an electroneutral Na⁺/K⁺/2Cl⁻ cotransport mechanism. Operation of the cotransport is mainly driven by the Na⁺ concentration gradient established by the ouabain-sensitive basolateral Na⁺/K⁺ ATPase. Potassium taken by the pump and by the cotransporter exits the cell and recycles through the opening of basolateral K⁺ channels. These three mechanisms operate to effectively accumulate Cl⁻ intracellularly above its electrochemical equilibrium. The opening of regulated rheogenic Cl⁻ channels in the apical membrane allows Cl⁻ to diffuse into the lumen, down its elec-
trochemical gradient. The outflux of Cl and K⁺ at the apical and basolateral membranes, respectively, contributes to generate a lumen negative transepithelial electrical potential. This voltage drives the passage of Na⁺ through the paracellular pathway. The accumulation of NaCl in the lumen in turn drives the osmotic flow of water for net secretion of fluid.

Chloride transport is mainly regulated by the opening of apical Cl⁻ and basolateral K⁺ channels, as a result of changes in intracellular second messengers such as cAMP and Ca²⁺. More recent evidence suggests that the Na⁺/K⁺/2Cl⁻ cotransporter is also subject to regulation (Haas and Forbush, 2000). The main apical chloride pathway in the intestine is the CFTR (cystic fibrosis transmembrane conductance regulator) chloride channel activated mainly by cAMP-dependent phosphorylation, resulting from adenylate cyclase activation by secretagogues such as VIP (Barrett and Keely, 2000). CFTR is an integral membrane protein with two membrane spanning domains, each including 6 putative transmembrane regions which form the channel pore (McCarty, 2000). On the cytoplasmic side, this protein presents two nucleotide-binding domains (NBD1, NBD2) that bind and hydrolyse ATP and a regulatory (R) domain. The latter is phosphorylated by PKA, but also by PKC. Both ATP hydrolysis by NBD and phosphorylation of the R domain are required for channel activation (Gadsby and Nairn, 1999). The CFTR channel can also be activated by cGMP dependent protein kinase II and, to a lesser extent, by Ca²⁺ and PKC (French et al., 1995). Phosphorylation by PKC alone does not seem to open the channel but rather to sensitize the channel to subsequent phosphorylation by PKA, resulting in a potentiation of secretion (Jia et al., 1997). Another level of possible control of CFTR activity is the regulation of the number of transporters at any time present in the membrane. This may occur by changing the rate of insertion or retrieval of channels containing vesicles, modulated by the concentrations of cAMP and/or Ca²⁺ (Morris, 1999).

In the intestine, Ca²⁺-dependent secretory agonists such as ACh, histamine, bradykinin and neurotensin, evoke a small transient response even in the continuous presence of the agonist (Morris, 1999). This response contrasts with the sustained increase in chloride secretion induced by cAMP-dependent secretagogues. Secretion induced by the combination of the agonists acting via Ca²⁺ and cAMP is synergistic (Vajanaphanich et al., 1995). On the other hand, patients with cystic fibrosis or mice with a targeted deletion of the CFTR gene do not have a chloride secretory response induced by cAMP- or Ca²⁺-mediated agonists (Berschneider et al., 1988; Grubb and Gabriel, 1997; Morris et al., 1999; Taylor et al., 1988). At this point, the presence of a Ca²⁺-activated chloride channel (CaCC) involved in chloride secretion is somewhat controversial (Barrett and Keely, 2000). A Ca²⁺-activated chloride conductance has been observed in intestine in situ and in cultured intestinal cell lines (Anderson et al., 1992; McEwan et al., 1994; Merlin et al., 1998; Morris and Frizzell, 1993). Elevation of intracellular Ca²⁺ concentration does not appear to activate the CFTR channel directly. Instead, the regulatory effect of Ca²⁺ may be due to PKC-dependent phosphorylation of the channel and/or Ca²⁺ activation of the basolateral K⁺ channel. In this case, the chloride electrochemical gradient would be increased, and chloride would be secreted through the apical membrane via chloride channels (CFTR or not) open at any given time. Calcium-activated Cl⁻ channels may be a property of the devel-
oping intestine. Morris et al. (1992) have shown that HT29 cells exhibit Ca^{2+}-activated Cl^- conductance that is lost as cells become polarized.

Functional and pharmacological characterization indicates the intervention of at least two types of basolateral K^+ channels involved in chloride secretion by intestinal cells. One channel can be activated by muscarinic agonists like carbachol, is activated by Ca^{2+}, insensitive to Ba^{2+} and blocked by clotrimazol and charibdotoxin (Devor and Duffey, 1992; Greger et al., 1997; Jensen et al., 1998; Lomax et al., 1996; Vandorpe et al., 1998). This channel may correspond to the recently described hIK1 potassium channel (Ishii et al., 1997; Jensen et al., 1998; Vandorpe et al., 1998). The second one seems to be activated by cAMP (Greger et al., 1997; McRoberts et al., 1985).

The Na^+/K^+/2Cl^- cotransporter appears to be under regulation by phosphorylation stimulated by a decrease in intracellular chloride. The nature of the protein kinase involved is not yet known. Thus, an apical chloride efflux would indirectly stimulate basolateral chloride uptake, providing a positive feedback for secretion (Lytle and Forbush, 1992; Lytle and Forbush, 1996; Torchia et al., 1992). The activity of this cotransporter may be also regulated by insertion and retrieval of membrane transporter molecules modulated by the concentration of cAMP, and cytoskeletal dynamics (D'Andrea et al., 1996; Hecht and Koutsouris, 1999; Karczewski and Groot, 2000; Matthews et al., 1992; Matthews et al., 1997; Matthews et al., 1995).

**Water transport**

The intestine is capable of transporting large amounts of fluid even in the absence of any external hydrostatic or osmotic driving forces. The rate of water absorption is proportional to the rate of solute absorption (NaCl, amino acids and sugars); the fluid transported is isotonic to the saline bathing the epithelium. However, the molecular mechanisms of water transport (absorption or secretion) across the intestinal epithelia are not yet clear. It is a dogma that active salt and nutrient transport generates local osmotic gradients within the epithelium that drive water transport (Diamond, 1979, Diamond and Bossert, 1967; Fromter and Diamond, 1972). This transport may be carried out across paracellular and/or transcellular pathways. In the duodenum and proximal jejunum water movement across the epithelium is believed to occur by a paracellular pathway. In the colon the situation is different. It is a tight epithelium with a lower paracellular permeability, and water may be transported against an effective osmotic gradient. Naftalin et al. (1999) have proposed that the myofibroblast-reticular sheath in the distal colon crypts serves as an additional diffusion barrier in series with the basal membrane, in order to generate a localized hypertonic Na^+ gradient to accomplish uphill water transport capable of dehydrating feces (Naftalin and Pedley, 1999; Naftalin et al., 1999).

The paracellular flow of fluid and solutes is regulated by the permeability of tight junctions. These are characteristic of polarized absorptive and secretory epithelia. Tight junctions are dynamic structures that may adapt to a variety of situations, by mechanisms that begin to be elucidated. A number of enteric pathogens including rotavirus may affect tight-junction function (Sears, 2000). Numerous extracellular mediators including
hormones, neurotransmitters, biogenic amines and cytokines participate in a variety of physiological and pathological processes modulating tight junction permeability across epithelia (Nusrat et al., 2000). These agents induce increases of intracellular calcium and adenosine 3′-5′cyclic monophosphate (cAMP) and may involve mechanisms such as the contraction of the cytoskeleton and the perijunctional ring. Other effectors include protein kinase C, myosin light-chain kinase, and phosphatidylinositol 3-kinase (Karczewski and Groot, 2000). Other factors such as glucose, when present in the apical side, appear to regulate tight junction permeability. It has been proposed that initiation of Na+/glucose cotransport leads to activation of a Na+/H+ exchange, increased phosphorylation of myosin light chain, contraction of the perijunctional actomyosin ring, and, ultimately, increased permeability of intestinal tight junctions (Nusrat et al., 2000).

The transcellular route for water movements involves the presence of molecular entities both at the brush border and the basolateral membranes of enterocytes. Water channels of the aquaporin family (AQP) have been identified in the small intestine (AQP2 and 7) and in the colon (AQP3, 4 and 8), located in villus and crypt cells (Ma and Verkman, 1999). A new aquaporin (AQP10) was identified in human small intestine and localized in absorptive duodenal and jejunal epithelial cells (Hatakeyama et al., 2001).

Recently, based on experiments in Xenopus oocytes expressing SGLT1, Wright and Loo (2000) have proposed that this cotransporter may function as a water channel. Moreover, SGLT1 may couple the transport of Na+ and sugar to water translocation, in this way functioning as a Na+/glucose/H2O cotransporter. The physiological role of these different transporters remains to be elucidated as well as their participation in pathological processes.

Pathophysiology of virus-induced diarrhea

Mechanisms of diarrhea

At least four mechanisms have been implicated in diarrheal syndromes: increased secretion, decreased absorption (ions and/or solutes and water), altered motility and increased permeability (Fig. 3). Viral infection induces the synthesis of viral proteins, cell lysis and inflammatory responses. Cell lysis permits the release of viral progeny, which in turn infect neighboring cells. The cytolytic infection leads to a decrease in the digestive and absorptive capacity of the intestine, generating a malabsorption component of diarrhea. Cell and viral proteins will act upon immune cells to release a variety of chemical mediators. The interaction between the immune enteroendocrine and enteric systems will lead to increases in intracellular second messengers Ca2+ and cAMP. Direct or mediated stimulation of enterocytes by viral protein (e.g., the rotaviral NSP4 enterotoxin) [see Estes, Section II, Chapter 6 of this book] could increase second messengers. These messengers may a) activate Cl− channels inducing hypersecretion, b) act upon the cytoskeleton and the tight junction to increase paracellular permeability, c) derange intestinal motility and, d) activate transporter function or decrease enzyme expression to effectively impair nutrient and water absorption.
Usually, infectious agents, including viruses, cause diarrhea by more than one of these mechanisms. Viral pathogens have developed strategies to colonize different environments in the small intestine, disrupting the normal fluid balance of the gut and causing diarrhea. These enteric viruses are stable at low pH and resistant to digestive enzymes, important characteristics in their infective capacity. The viruses causing diarrhea in humans and animals may belong to different families (Table 1; for review see Glass et al., 2001; Saif, 1999; Parashar and Glass Section I, Chapter 1 of this book). Among these viruses, rotaviruses, caliciviruses (including noro- and sapoviruses), enteric adenoviruses and astroviruses are the main agents inducing diarrhea in humans as well as in animals. In addition, picobirnaviruses and HIV have been associated with diarrheal syndromes in humans. Also, viruses belonging to the Coronaviridae (TGEV, PEDV, Torovirus), and Parvoviridae infect the intestine and are responsible for diarrhea in animals.
| Virus                  | Family           | Host          | Site of infection                                          | Mechanism of action                                                                 | Type of diarrhea                                                                 |
|------------------------|------------------|---------------|------------------------------------------------------------|-------------------------------------------------------------------------------------|----------------------------------------------------------------------------------|
| Rotavirus              | Reoviridae       | human, animal | middle and tip villus enterocyte of jejunum and ileum      | cytolytic infection, villus atrophy, crypt hyperplasia, NSP4 enterotoxin, activation of ENS, increased paracellular permeability, decreased digestive enzyme expression | mixed: primary secretory; enhanced permeability, increased motility, malabsorption at late stage; mild/severe |
| Calicivirus norovirus  | Caliciviridae    | human, animal | villus enterocyte of duodenum and jejunum; patchy mucosal lesions. | cytolytic infection, villus atrophy, crypt hyperplasia, malabsorption of fat and xylose, diminished activity of disaccharidase enzymes, and delayed gastric emptying | mixed: primary malabsorption, secondary secretory; moderate                         |
| Parvovirus             | Parvoviridae     | animal        | primary systemic, basolateral pole of crypt enterocyte of jejunum and ileum | cytolytic infection of crypt enterocytes, severe villus atrophy, mucosal collapse, hemorrhagic diarrhea | malabsorption; severe, hemorrhagic.                                                |
| Virus                          | Family          | Host             | Site of infection                                                                 | Mechanism of action                  | Type of diarrhea                                   |
|-------------------------------|-----------------|------------------|-----------------------------------------------------------------------------------|--------------------------------------|---------------------------------------------------|
| Transmissible Gastroenteritis Virus (TGEV) | *Coronaviridae* | animal           | entire villus enterocytes in multiple stages of differentiation, of duodenum, jejunum and ileum | cytolytic infection, pronounced villus atrophy, crypt hyperplasia | mixed: primary malabsorption, secondary secretory; severe |
| Porcine Epidemic Diarrhea Virus (PEDV) | *Coronaviridae* | animal           | base and side of villus and crypt enterocytes of duodenum, jejunum, ileum and proximal colon | cytolytic infection, moderate villus atrophy | mixed: primary malabsorption, secondary secretory; moderate |
| Torovirus                     | *Coronaviridae* | animal, human    | crypt and basal villus enterocytes of jejunum, ileum and colon                    | cytolytic infection, villus atrophy, crypt hyperplasia | role in human disease unknown                      |
| Picobirnavirus                | *Picobirnaviridae* | animal, human | unknown                                                                          | unknown                              | role in human disease unknown                      |
The mechanisms by which viruses induce diarrhea are ill-defined. Most of them infect the small intestine enterocytes with a distinct tropism for different cell types (villus/crypt) and intestinal segment (duodenum, jejunum, ileum and colon). The majority of the viruses mentioned above penetrate the cell through the apical membrane, except for parvovirus where a systemic infection seems to precede penetration of crypt cells through the basolateral membrane (Saif, 1999). Once inside the cell, virus replication usually causes cell lysis and the release of viral progeny which in turn infects neighboring cells. The cytolytic infection often leads to villus atrophy and a decrease in the digestive and absorptive capacities of the intestine, generating a malabsorption component of diarrhea. As undigested and non-absorbed nutrients reach the colon, microbial fermentation leads to an accumulation of osmotically active solutes, adding an osmotic component to the syndrome. Secondary to villus enterocyte loss, a crypt hyperplasia follows, inducing a secretory contribution to diarrhea (Moon, 1994). This type of pathogenic mechanism may be responsible for the induction of diarrhea following infection by calicivirus, enteric adenovirus, TGEV and astrovirus. In the case of parvovirus, the lytic infection of the crypt enterocyte leads to a reduction of villus enterocyte production and induces a malabsorption type of diarrhea.

The extent of histopathological lesions often does not explain the intensity of diarrhea, suggesting that the mechanisms outlined might be a simplistic view of the process. In many cases, viral diarrhea may be a multifactorial disease where impaired digestion and absorption may be the major factors, with osmotic activity, secretion and hypermotility complicating the process. However, this sequence does not appear to operate for other viruses such as rotavirus, where a secreted viral protein (NSP4) seems to act as an enterotoxin triggering a simultaneous pleiades of complex effects (Estes et al., 2001).

**Rotavirus as a model for the genesis of viral diarrhea**

Due to the clinical importance of rotavirus diarrhea and the fact that cell culture and animal models of the disease have been developed, our knowledge of the pathogenesis has greatly advanced during the past few years (for review see Estes et al., 2001; Estes and Morris, 1999; Lundgren and Svensson, 2001; Morris and Estes, 2001; Ruiz et al., 2000; Estes Section II, Chapter 6 of this book). Rotaviruses infect mature enterocytes of jejunum and ileum, inducing cell lysis, which will release the new rotavirus progeny and synthesized viral proteins into the lumen of the intestine. After several viral replication cycles, extensive cell death provokes shortening of microvilli and a reduction of the absorptive surface of the intestinal epithelium resulting in a late malabsorption component of diarrhea. Shortening of villi also induces crypt hyperplasia and hypersecretion, further increasing the syndrome (Burke and Desselberger, 1996; Greenberg et al., 1994). However, these histopathological lesions appear at a late stage of infection when diarrhea is already installed. On the other hand, changes in ion homeostasis of the infected cell, in particular Ca<sup>2+</sup>, have provided new insights into the pathogenic mechanisms that eventually lead to diarrhea.

**Rotavirus infection of cultured cells induces changes in the homeostasis of both mono- and divalent cations that appear to be mediated by the synthesis of viral proteins**
Among these perturbances is a progressive increase in Ca\(^{2+}\) membrane permeability which leads to an elevation of cytosolic Ca\(^{2+}\) concentration and sequestered pools, without an apparent failure of regulatory mechanisms in virus-infected MA104, HT29 and Caco-2 cells (Brunet et al., 2000a; Michelangeli et al., 1991; Perez et al., 1999). In parallel to the increase in plasma membrane permeability and cytosolic Ca\(^{2+}\) concentration, there is an enhancement of ER sequestered Ca\(^{2+}\) stores (Brunet et al., 2000a; Michelangeli et al., 1995; Perez et al., 1999). This effect is likely to be due to the activation of ER Ca\(^{2+}\) pumps.

The nonstructural protein NSP4, expressed constitutively during rotavirus infection as well as added exogenously to noninfected cells, has been implicated in disturbances of Ca\(^{2+}\) homeostasis. The expression of recombinant NSP4 protein in insect cells induced an increase in cytosol Ca\(^{2+}\) concentration (Tian et al., 1994). This effect was proposed to be due to the release of Ca\(^{2+}\) from intracellular stores without increases in plasma membrane Ca\(^{2+}\) permeability and linked to a membrane destabilizing activity of NSP4 (Tian et al., 1995; Tian et al., 1996).

Later during infection, another permeability pathway to Ca\(^{2+}\) is induced by a secreted or released viral product acting from the outside of the cell in Caco-2 cells (Brunet et al., 2000a). This depends on the activation of the phospholipase C-IP\(_3\) cascade, resulting in store depletion, which in turn may activate capacitative Ca\(^{2+}\) entry. It is reasonable to think that NSP4 is responsible for these effects. It is known that exogenously added recombinant-produced NSP4, or a cleaved form of NSP4 which is secreted from infected cells, increases [Ca\(^{2+}\)]\(_i\) in HT29 cells (Zhang et al., 2000).

These changes in [Ca\(^{2+}\)]\(_i\), caused by infection or by exogenous NSP4 will have important consequences on the enterocyte physiology. These include actions on the cytoskeleton, ionic and nutrient transport, expression of digestive enzymes and eventually cell death which will be addressed below. These numerous modifications will lead to stimulation of secretion, diminished absorption, enhanced paracellular permeability and perhaps hypermotility which will participate in the pathogenesis of diarrhea from the early stages of the disease. We will analyse the different components of rotavirus diarrhea in the context of these four mechanisms.

**Secretory component**

That a primary secretory component of rotavirus diarrhea might exist was first proposed based on the effect of rotavirus on Ca\(^{2+}\) homeostasis of infected cells (Michelangeli et al., 1991). The finding that the endogenous expression or the exogenous addition of the nonstructural protein NSP4 is capable of increasing [Ca\(^{2+}\)]\(_i\), (Dong et al., 1997; Tian et al., 1994; Tian et al., 1995) triggered a number of studies that lead to the hypothesis that this protein may act as a viral enterotoxin to induce secretion and diarrhea (Estes et al., 2001; Estes and Morris, 1999; Morris and Estes, 2001). There is a series of evidence that supports this hypothesis: a) intraperitoneal or intraluminal injection of NSP4 or NSP4 peptide fragments cause age-dependent diarrhea in mice (Ball et al., 1996; Sasaki et al., 2001); b) NSP4 and/or a fragment of this protein are released from infected cell monolayers as a consequence of secretion and apparently not cell lysis (Zhang...
et al., 2000); c) NSP4 or the synthetic NSP4_{114-135} peptide potentiated cAMP-dependent Cl\(^{-}\) secretion in isolated mouse intestine mounted in Ussing chambers, similarly to carbachol (Ball et al., 1996); d) exogenous addition of NSP4 or the NSP4_{114-135} peptide to noninfected cells induced the release of Ca\(^{2+}\) in HT29 cells through the activation of the phospholipase C-IP\(_3\) cascade and a secondary increase in plasma membrane Ca\(^{2+}\) permeability, similarly to cholinergic agents (Dong et al., 1997). [For further details see Estes, Section II, Chapter 6 of this book].

The relationship between diarrhea, Ca\(^{2+}\) and NSP4 has been confirmed studying mutations in NSP4, which are associated with altered virus virulence. There is an association between the capacity of this protein to induce changes in intracellular Ca\(^{2+}\) in HT29 cells and the induction of diarrhea by the mutated NSP4 in mice. This mutation has been mapped between amino acids 131-140 (Zhang et al., 1998). However, NSP4 may not be the only virulence factor as attenuation of rotavirus in culture cells appears to be unrelated to mutations in the NSP4 gene (Angel et al., 1998; Ward et al., 1997) and NSP4 sequences in both diarrheal and asymptotically-infected kittens were closely related to each other (Oka et al., 2001). Also, there was no apparent correlation in the deduced amino acid sequences corresponding to the proposed enterotoxic peptide region between rotaviruses recovered from children with and without diarrhea (Lee et al., 2000). [For discussion of these questions see also Estes, Section II, Chapter 6 of this book].

The target cell for the NSP4 protein action is not yet known. NSP4 could act directly on uninfected crypt cells or mature enterocytes, or both, to induce secretion. In search of the chloride secretion pathway activated by NSP4, studies have been performed in CFTR knockout mice, which do not respond to Ca\(^{2+}\) and cAMP dependent secretagogues (Morris et al., 1999). Rotavirus particles, NSP4, or its active NSP4_{114-135} peptide can overcome secretory inhibition and elicit diarrhea when administered to CF mouse pups. The evidence was interpreted as showing that NSP4 induces diarrhea in neonatal mice through the activation of an age- and Ca\(^{2+}\)-dependent Cl\(^{-}\) secretory mechanism different from the CFTR (Morris et al., 1999).

Within this attractive hypothesis of direct stimulation of crypt cells, several points remain to be clarified: a) the molecular identification of the putative Ca\(^{2+}\) dependent Cl\(^{-}\) channel; b) the reason why carbachol is not able to stimulate this Cl\(^{-}\) channel related to diarrhea in CFTR knockout mice; c) the identity and localization of the putative NSP4 receptor; d) the correlation between the Ca\(^{2+}\) dependent component of secretion and diarrhea. In this sense, the changes in short circuit current (Cl\(^{-}\) transport) in isolated mucosal sheets of mice intestine induced by NSP4_{114-135} peptide or carbachol represent a small fraction of that induced by the cAMP dependent secretagogue, forskolin. Also, Cl\(^{-}\) influx measured in isolated crypts of CFTR\(^{-}\) pup mice induced by carbachol or NSP4 is small in comparison to forskolin as to explain the diarrheal syndrome. However, NSP4 and carbachol could potentiate CFTR-dependent secretion indirectly through the activation of the Ca\(^{2+}\)-stimulated basolateral K\(^{+}\) conductance, hyperpolarization and Cl\(^{-}\) uptake via the cotransport (Morris, 1999). Another experimental complication is that only a small percentage (20\%) of the isolated crypts responded to either carbachol or NSP4 with increases in [Ca\(^{2+}\)]. Furthermore, it is not explained how NSP4 elicits these changes in isolated crypts if a luminal receptor is postulated and how NSP4 can reach deep into the
lumen of a secreting crypt \textit{in vitro} and \textit{in vivo} intestine. The lumen of isolated crypt can be collapsed at the tip after isolation as it occurs in isolated gastric glands (Perez \textit{et al.}, 2001), which would imply that NSP4 acted on basolateral receptors. Also, we should consider the sweeping out effect produced by the net flow of water accompanying crypt secretion. In this case, the water flow going out of the crypt would oppose the diffusional entry of NSP4. This effect has been shown for other secretory epithelia [Moody and Durbin, 1969]. These considerations do not at this point invalidate the hypothesis of NSP4 acting as an enterotoxin. However, they may cast doubts as to its role in the direct stimulation of crypt cells to induce Cl\textsuperscript{-} secretion.

The enteric nervous system is an integrative network of neurons that regulate gastrointestinal functions and might be the link between NSP4 and the secretion of water and electrolytes involved in rotavirus diarrhea. Pharmacological blockade of nervous transmission is able to inhibit secretion in the intestinal mucosa and to attenuate diarrhea induced by rotavirus infection (Lundgren \textit{et al.}, 2000; Lundgren and Svensson, Section I, Chapter 3 of this book). Released or secreted NSP4 could be responsible for the activation of enteric neurons directly or indirectly through the stimulation of peptide and amine secretion from paracrine cells. In addition to NSP4, inflammatory mediators released from myofibroblasts and inflammatory cells during infection may be capable of regulating secretion and absorption directly or through the activation of the enteric nervous network and the paracrine system (Cooke, 2000; Montrose \textit{et al.}, 1999; Moon, 1994; Powell \textit{et al.}, 1999). Inflammation may contribute to fluid loss and diarrhea in other enteric viral infections for which an enterotoxin has not yet been described.

\textit{Malabsorption component}

Rotavirus induces a lytic infection of the villus enterocyte leading to shortening of microvilli and a reduction of the absorptive surface of the intestinal epithelium resulting in a late malabsorption component of diarrhea (Burke and Desselberger, 1996; Greenberg \textit{et al.}, 1994). Rotavirus infection provokes cell death of MA104 cells by necrosis in a Ca\textsuperscript{2+} dependent pathway (Perez \textit{et al.}, 1998). The events triggered by the increase in cytosolic Ca\textsuperscript{2+} which lead to cell death in rotavirus infected cells are yet unknown. Calcium may participate in the generation of these alterations by activating Ca\textsuperscript{2+} dependent enzymes such as phospholipases A and C, proteases and endonucleases. On the other hand, the expression of NSP4 alone in MA104 cells induced cell death. Analysis of NSP4 deletion mutants indicates that a membrane-proximal region located within the cytoplasmic domain may mediate cytotoxicity (Newton \textit{et al.}, 1997). Newly synthesized particles would be released into the medium after cell lysis and induce new infectious lytic cycles. However, we should note that an alternative model for the release of rotavirus particles before lysis of Caco-2 cells in a secretion-like process has been reported (Jourdan \textit{et al.}, 1997). The importance of these findings in the \textit{in vivo} infection remains to be further studied.

A reduction of electrolyte and nutrient absorption may be the consequence of other processes occurring earlier during infection before cell lysis. The Na\textsuperscript{+} gradient across the
membrane is collapsed early during infection due to an increase in [\(\text{Na}^+\)] (del Castillo et al., 1991). If this occurred in the villus enterocyte, it would impair electroneutral NaCl absorption and Na\(^+\) linked nutrient absorption with a consequent fluid loss and diarrhea.

The mechanism of rotavirus diarrhea may involve a generalized inhibition of Na\(^+\)/solute cotransport systems. A strong inhibition of both Na\(^+\)/glucose (SGLT1) and Na\(^+\)/leucine symport activities have been found in brush-border membrane vesicles isolated from rotavirus infected rabbits (Halaihel et al., 2000a). The viral effect on this symport appears to be direct and perhaps linked to NSP4. The NSP4\(_{114-135}\) peptide is a specific and noncompetitive inhibitor of SGLT1 (Halaihel et al., 2000b).

Another mechanism that may be involved in a decrease of nutrient absorption during rotavirus infection is the reduction of digestive enzyme expression at the apical brush border membrane. During the course of infection of suckling mice and piglets with rotavirus, the activities of alkaline phosphatase, lactase, sucrase and maltase decreased (Collins et al., 1988; Davidson et al., 1977; Shepherd et al., 1979). Furthermore, rotavirus infection of Caco-2 cells induces a decreased apical surface expression of sucrase-isomaltase which correlated with important alterations of the cytoskeleton (Jourdan et al., 1998), perhaps due to an increase in [Ca\(^{2+}\)] (Brunet et al., 2000a; Brunet et al., 2000b).

As pointed out above, a decrease in nutrient, electrolyte and water absorption due to reduction in the absorptive surface is the major and primary cause of diarrhea induced by other enteric viruses. Coronavirus (TGEV) has been used extensively to study enteritis-associated changes in nutrient and electrolyte transport. A decreased Na\(^+\)/glucose transport has been shown in isolated piglet intestines mounted in Ussing chambers (Shepherd et al., 1979; Telch et al., 1981) as well as in brush border membrane vesicles (Keljo et al., 1985). Also, Na\(^+\) coupled alanine transport was reduced during TGEV infection in piglet intestine brush border membranes (Rhoads et al., 1989).

**Paracellular permeability component**

The role of the paracellular pathway and the integrity of the tight junction in the barrier function and fluid balance in the intestine have been long recognized. A number of disease states, including inflammatory and diarrheal diseases, may be caused by primary defects in epithelial barrier function (Bjarnason et al., 1995). Pathogens may act at different levels such as direct cleavage of tight junctional structural proteins, modification of the actin cytoskeleton, activation of cellular signal transduction and, triggering transmigration of polymorphonuclear cells across the epithelial cell barrier (Sears, 2000).

Profound changes in the permeability characteristics of the intestine of children during acute rotavirus diarrhea have been found (Stintzing et al., 1986). Experiments performed in rotavirus infected Caco-2 and/or MDCK cell monolayers showed a disruption of tight junctions and decrease of transepithelial resistance (Dickman et al., 2000; Obert et al., 2000; Svensson et al., 1991). This was accompanied by increased transepithelial permeability to macromolecules (Dickman et al., 2000; Obert et al., 2000) and altered distribution of tight junction proteins claudin-1, occludin, and ZO-1 (Dickman et al., 2000). These changes did not appear to be related to the observed
changes in the cytoskeleton (Brunet et al., 2000b; Obert et al., 2000) but perhaps due to changes in cell metabolism caused by infection (Dickman et al., 2000). The modification of paracellular permeability might be induced by the endogenous expression of NSP4 in infected cells or released NSP4 acting as an enterotoxin on non-infected cells. In MDCK cell monolayers, apical addition of NSP4 induced a reduction in transepithelial electrical resistance, a redistribution of filamentous actin, an increase in paracellular permeability to extracellular markers and the targeting of the tight junction associated protein ZO-1 (Tafazoli et al., 2001). These effects may be mediated by an increase in [Ca$^{2+}$]. Similar changes might be evoked by inflammatory mediators released during infection in intact tissue either directly or mediated by an action of the enteric nervous system (Lundgren and Svensson, 2001; Moon, 1994).

**Altered motility**

Deranged motility has been recognized as an important factor in the genesis of the diarrhea (Moon, 1994). The enteric nervous system is critical for the integration of intestinal electrolyte transport with motility. The enterotoxins produced by various bacteria induce effects on intestinal motility via an activation of the ENS. The effects of rotavirus enteritis on intestinal motility indicate that intestinal transit time is shortened. The possible involvement of the ENS in this response is not known (Lundgren and Svensson, 2001). However, again NSP4 may be participating in this response, either directly or indirectly, through the release of chemical mediators.

Changes in microcirculation have been also proposed as participating in the genesis of diarrhea induced by virus infection [Osborne et al., 1991; Stephen, 1989; Stephen and Osborne, 1988]. According to this hypothesis, infection of villus cell by rotavirus would trigger the release of "neuroactive/hormonal substances" which would cause ischaemia by vasoconstriction and, in turn, villus shortening and a decrease in absorptive capacity. In addition, ischaemia induces hyperplasia [Stephen and Osborne, 1988] of immature cells and hypersecretion. These effects did not seem to be virus specific. The extent and participation of this component in virus-induced diarrhea remains to be assessed.

**Conclusions**

Based on the current knowledge of the effects of rotavirus infection on the physiology of the intestine at different levels of organization, a working model for the pathogenesis of rotavirus diarrhea is presented in Fig. 4. Rotavirus particles would bind to a functional receptor constituted by a complex of several molecules (sialic acid-dependent and -independent) on the apical membrane of the villus enterocyte (for review see Arias et al., 2001; López et al., Section II, Chapter 2 of this book). For internalization into the cytoplasm, a Ca$^{2+}$-dependent endocytosis mechanism has been proposed (Ruiz et al., 2000). During the first viral replication cycle, proteins and particles are synthesized. The enterotoxigenic viral protein NSP4 would be secreted. At the end of the cycle, a large number of newly synthesized viral particles are released after cell lysis, concomitantly with viral proteins.
The viral progeny will infect neighboring downstream cells, and the released NSP4 would trigger a series of events that would lead to diarrhea. Binding to putative apical receptors, NSP4 would induce an increase of [Ca\textsuperscript{2+}] through a phospholipase C-IP3 pathway in villus and perhaps crypt enterocytes, and enteroendocrine cells. At the villus cell level, the enterotoxin would inhibit Na\textsuperscript{+}/nutrient cotransport and digestive enzyme activities decreasing water and solute transport. Concomitantly, the [Ca\textsuperscript{2+}] increase would disrupt the cytoskeleton and the tight junction, leading to an enhanced paracellular permeability. Chloride secretion by crypt cells would also be stimulated. This would occur through a direct or indirect activation of the enteric nervous system and the release of chemical mediators.

Fig. 4. Pathophysiological model of mixed type diarrhea induced by rotavirus infection. During the first cycle of infection the synthesis of rotaviral proteins in the infected cell leads to increase in plasma membrane permeability to Ca\textsuperscript{2+}, activation of regulatory mechanisms, and increase in Ca\textsuperscript{2+} content in the ER. The increase of cytosolic Ca\textsuperscript{2+} concentration in the infected cell provokes the activation of Ca\textsuperscript{2+} dependent enzymes, which in turn will induce cell lysis and release of viral proteins and viral progeny. NSP4 may act as a viral enterotoxin to induce secretory diarrhea by: a) Ca\textsuperscript{2+} dependent secretion of amines and peptides to induce stimulation of the enteric nervous system (ENS); b) activation of crypt cell Cl\textsuperscript{−} secretion by the release of neurotransmitters; c) Ca\textsuperscript{2+} dependent secretion by crypt cells or mature enterocytes by activation of Cl\textsuperscript{−} channels. NSP4 may also reduce nutrient and ion absorption. In parallel, released virus will infect downstream absorptive cells, leading to cell death and reduction of the absorptive surface generating an osmotic component of diarrhea. In addition, NSP4 will alter the integrity of the tight junction (tj), opening a paracellular permeability pathway and increasing secretion by solvent drag or exudation. Changes in motility may also occur in response to the activation of the ENS.
A direct stimulation of the crypt cell by NSP4, although unlikely, may also occur. As infected cells die in a calcium-mediated necrosis process, the absorptive surface is reduced further increasing the malabsorption component which is superimposed on the secretory one, resulting in a mixed type diarrhea. The understanding of the pathogenic processes of viral diarrheas may serve as the basis for a rational approach in the design of novel therapeutic strategies and the search for new antiviral drugs.

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