6

A VIRAL ENTEROTOXIN

A New Mechanism of Virus-Induced Pathogenesis

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1. SUMMARY

Acute infectious gastroenteritis is a major cause of infant morbidity in developed countries and of infant mortality in developing areas of the world. Rotavirus is recognized as the most important etiologic agent of infantile gastroenteritis, and studies of rotavirus serve as models to understand the complex interactions between enteric viruses and the multifunctional cells of the gastrointestinal tract. Understanding such interactions is significant for microbial pathogenesis because most (>80%) infections are initiated at mucosal surfaces. Rotaviruses are pathogens that infect the mature enterocytes of the villi in the intestine and infection appears to be limited to these highly differentiated cells in immunologically competent hosts. In such hosts, infections are generally acute yet diarrheal disease can be severe and life-threatening. Disease generally is resolved within 2–5 days after infection if affected hosts receive adequate rehydration. In immunocompromised hosts, virus infections persist, virus can be detected extraintestinally and virus excretion may be detected for extended periods of time (many months).

Rotaviruses infect almost all mammalian and some avian species and much of our understanding of rotavirus pathogenesis has come from studies in animal models,

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particularly in small animal models (mice and rabbits), but also in larger animals (cows and piglets). Studies in children are limited due to the difficulty and lack of clinical need of obtaining biopsies from infants and the inability to determine the precise time of natural infections. In all animal species where naïve animals can be infected, disease is age-dependent; for example, in mice and rabbits, diarrheal disease is the outcome of infections that occur only during the first two weeks of life (Ciarlet et al., 1998; Starkey et al., 1986; Ramig 1988; Ward et al., 1990; Burns et al., 1995), while animals remain susceptible to viral infection into adulthood. Rotavirus infections have been reported to occur repeatedly in humans from birth to old age, but the majority of infections after the first 2 years of life are asymptomatic or associated with mild gastrointestinal symptoms. The age-related resistance to rotavirus-induced diarrhea in humans is thought to be mediated primarily by acquired immunity, but it is not possible to directly test if humans also exhibit an age-dependent resistance to disease based on other factors such as intestinal development and maturation. Currently, our best understanding of the mechanisms of rotavirus pathogenesis rely on results obtained in animal models.

2. ROTAVIRUSES CAUSE DISEASE BY SEVERAL MECHANISMS

The outcome of an infection with rotavirus is clearly dependent on both host and viral factors. The host factors have been dissected by analysis of the outcome of infection in animals inoculated with well-defined viral strains. Both natural and experimental rotavirus infections are characterized by viral replication in enterocytes in the small intestine, with subsequent cell lysis and attendant villus blunting, depressed levels of mucosal disaccharidases, watery diarrhea and dehydration. A majority of studies have shown that rotaviruses can cause malabsorption secondary to destruction of enterocytes (Graham et al., 1984; Davidson et al., 1977). This mechanism is based on the observation that rotavirus infection results in histopathologic changes in the intestine. These changes are generally seen 24–36 hours after infection and the resulting blunting of the villi has been associated with reduced absorption.

Several observations suggest that malabsorption is not the entire basis of rotavirus pathogenesis. Most importantly, in several animal species, profuse diarrhea occurs *prior to* the detection of histologic changes in the intestine including prior to observations of villus blunting (Collins et al., 1989; Theil et al., 1978; McAdaragh et al., 1980; Mebus 1976; Pappenheimer and Enders, 1947; Ward et al., 1997). In addition, some animals exhibit diarrhea in the absence of clear histopathologic changes [neonatal mice infected with heterologous rotavirus strains (Burns et al., 1995)], and other adult rotavirus-infected animals (rabbits) show typical histologic changes in the intestine but do not get diarrhea (Ciarlet et al., 1998.) Finally, oral administration of epidermal growth factor to rotavirus-infected piglets can help restore intestinal mucosal dimensions and enzyme activities but such treatments do not hasten the resolution of diarrhea (Zijlstra et al., 1994). Thus, a clear association of mucosal damage and diarrhea is lacking. Explanations for this include the fact that the infection is patchy so one might observe mucosal changes in one part of the intestine but these would be insufficient to cause diarrheal disease. Vascular damage due to villus ischemia has been suggested to be involved but supporting evidence is limited. In other cases, animals with mucosal damage may release fluid into the intestinal lumen but compensatory physiologic

mechanisms (colonic reabsorption of fluid) decrease fluid loss so diarrhea is not observed. Finally, other mechanisms may be involved (see below).

Viral factors involved in virulence or pathogenesis have been dissected by several approaches. First, viral genes implicated in virulence have been examined by studies of reassortants that represent genetically characterized virus strains that contain a single gene from one parental virus that is virulent and all other ten genes from a second avirulent parental virus. Ideally the reciprocal single gene reassortant also is analyzed in which a virus contains a single gene from an avirulent parental virus and the other 10 genes from a virulent parental virus. The analysis of reassortants has led to the association of several rotavirus genes and virulence; these putative virulence genes code for both structural and nonstructural proteins (Burke and Desselberger, 1996). Structural genes implicated in virulence include those that code for the outer capsid proteins VP4 and VP7 that are present on the surface of virions and that are likely to be involved in virus stability and virus attachment and penetration into cells. In addition, an internal protein, VP3, that functions as the capping enzyme in the process of transcription of messenger RNA has been implicated in virulence. Finally, three nonstructural proteins, NSP1 and NSP2 whose function in the replication cycle remains unclear, and NSP4, a protein known to be involved in viral morphogenesis, have been found to be virulence genes (Broome et al., 1993; Hoshino et al., 1995). The association of NSP1 with virulence has varied depending on the host species studied; it is a virulence factor for mice but not for rabbits or piglets (Broome et al., 1993; Ciarlet et al., 1998; Bridger et al., 1998).

Recent experiments have revealed that NSP4 can function as a viral enterotoxin, and this may explain the association of this gene with virulence. The discovery that NSP4 functions as an enterotoxin was unexpected because no other viral enterotoxins have been described. The remainder of this article briefly reviews the salient features about this first viral enterotoxin.

2.1. How Does the Rotavirus Enterotoxin NSP4 Cause Disease?

The rotavirus enterotoxin is a nonstructural protein called NSP4 for nonstructural protein 4. This protein was initially identified as a nonstructural glycoprotein with a topology that spans the membrane of the endoplasmic reticulum (ER) and the cytoplasmic C terminus of NSP4 functions as an intracellular receptor in viral morphogenesis (Ericson et al., 1983; Au et al., 1989; Meyer et al., 1989; Chan et al., 1988; Taylor et al., 1993; Bergmann et al., 1989). The functioning of this protein initially was examined because the rotaviruses undergo a unique morphogenesis in which newly made subviral particles bud into the endoplasmic reticulum and during this process they obtain a transient membrane envelope that is subsequently lost within the lumen of the ER as particles mature. This process also involves calcium which is required to maintain the integrity of the two outer capsid proteins as well as specific conformations necessary for the correct association of proteins as virus matures in the ER. NSP4 mobilizes intracellular calcium ($[Ca^{2+}]i$) release from the ER and this may also affect viral morphogenesis (Tian et al., 1994). NSP4 also possesses a membrane destabilization activity when incubated with liposomes that simulate ER membranes, and it has been hypothesized that this activity may play a role in the removal of the transient envelope from budding particles (Tian et al., 1996). Finally, NSP4 may facilitate cell death and virus release from cells (Newton et al., 1997).

The discovery that NSP4 functions as an enterotoxin was made serendipitously during studies aimed at dissecting the molecular mechanisms by which NSP4 functions

Table 1. Properties of rotavirus NSP4 or NSP4 peptide 114–135

Functions in viral morphogenesis; mediating the acquisition of a transient membrane envelope as subviral particles bud into the endoplasmic reticulum (ER)	Au et al., 1989 Meyer et al., 1989
Mobilizes $[Ca^{2+}]_i$ release from internal stores (ER)	Tian et al., 1994; Tian et al., 1995 Dong et al., 1997
Associated with virulence based on studies of reassortants	Hoshino et al., 1995
Induces an age-dependent diarrhea in mice and rats when administered by intraperitoneal or intraluminal routes but not when given intramuscularly	Ball et al., 1996 Ball et al., 1998
Does not induce histologic changes in the intestine when diarrhea is present	Ball et al., 1996 Ball et al., 1998
Induces age-dependent chloride secretion in the intestinal mucosa of young mice	Ball et al., 1996
Alters plasma membrane permeability and is cytotoxic to cells	Tian et al., 1994 Newton et al., 1997
Mutations in NSP4 from virulent/avirulent pairs of virus are associated with altered virus virulence	Kirkwood et al., 1996 Zhang et al., 1998
NSP4 induced age-dependent diarrhea and age-dependent chloride permeability changes in mice lacking the cystic fibrosis chloride transductance resistance (CFTR) channel	Morris et al., 1999
Children make antibodies and cellular immune responses to NSP4	Richardson et al., 1993 Johanssen et al., 1999
Is a novel toxin? No primary sequence similarity to other known toxins	

in morphogenesis. A synthetic peptide of NSP4 that contains amino acid residues 114–135 was conjugated to a carrier and injected into mice to produce a new antiserum. Although the mice received peptide (and no virus), they got diarrhea. Pursuit of this observation showed that both the full-length protein and the 114–135 peptide share several properties that are consistent with NSP4 being an enterotoxin.

The protein induces diarrhea in pups with a DD_{50} of 0.56 nmols while the DD_{50} of the 114–135 peptide is more than 10 fold-higher, indicating that this peptide contains only a part of the active domain of the protein. Properties that define bacterial enterotoxins include their ability to stimulate net secretion in intestinal segments in the absence of inducing histologic changes. NSP4 was shown to possess these properties, confirming that it functions as an enterotoxin. Of interest was the observation that age-dependent chloride secretion was seen in the intestinal mucosa of young mice.

These early studies and in vitro analyses of the response of human intestinal (HT29) cells to exogenously added NSP4 have led to a working model for the mechanism of action of this enterotoxin.

It is hypothesized that in young mice, rotavirus replicates in cells and the rotavirus proteins, including the nonstructural protein NSP4 are produced. It is further hypothesized that NSP4 is released from virus-infected cells by a currently unknown mechanism (either by cell lysis or by secretion), and this extracellular NSP4 can activate a signaling pathway in secretory cells. Human intestinal cells exposed to exogenously added NSP4 initiate a signaling pathway that involves activation of phospholipase C, elevation in IP_3 and mobilization of $[Ca^{2+}]i$ (Dong et al., 1997). Crypt cells isolated from mice also respond in a similar manner but mobilization of $[Ca^{2+}]i$ occurs in both young and older mice (Morris et al., 1999).

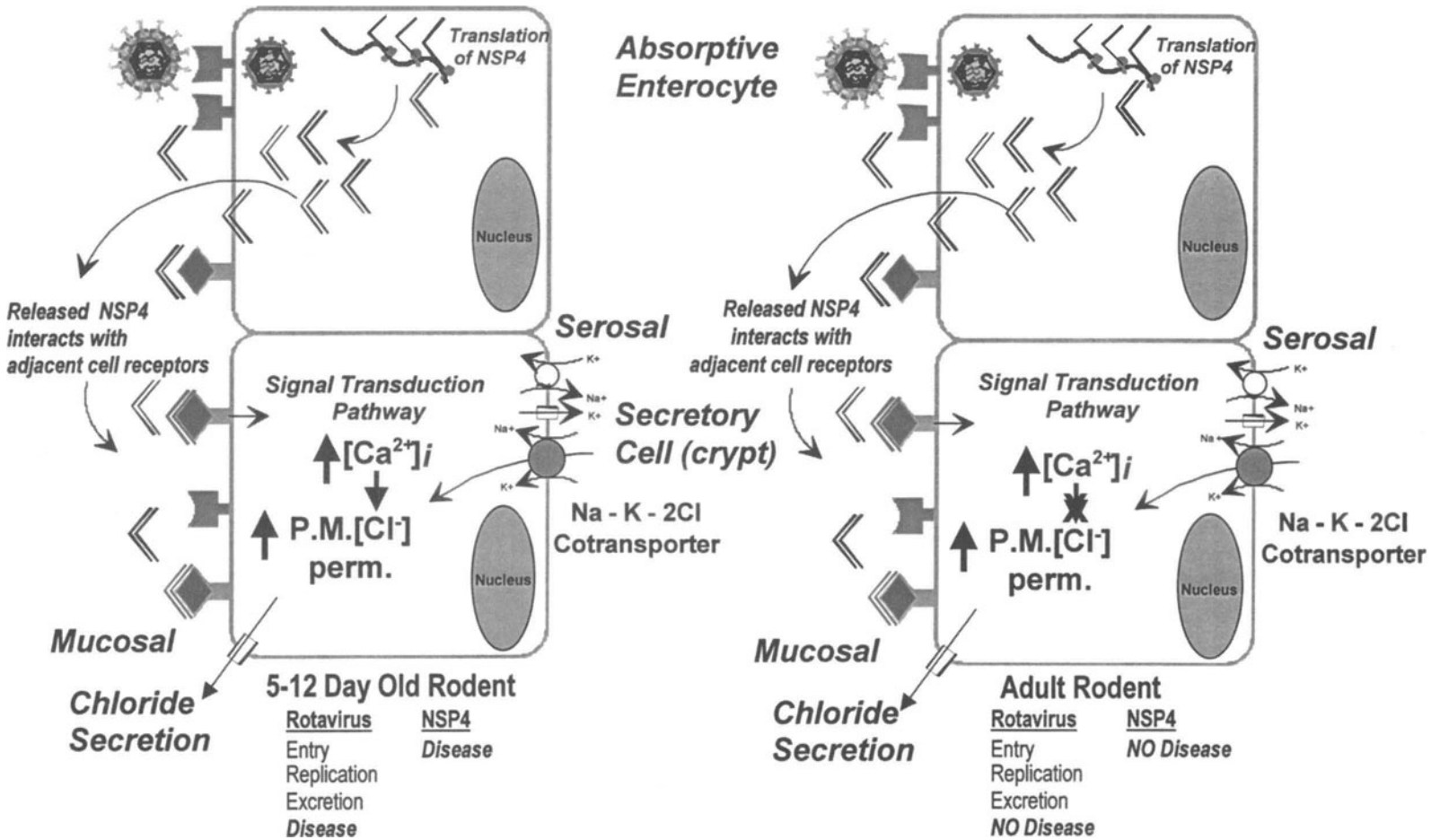

Figure 1. A proposed model of enterotoxin action. Rotavirus infected enterocytes produce virus-specific proteins including NSP4. NSP4 is released from cells by lysis or secretion and this NSP4 interacts with a receptor for NSP4 on adjacent cells. These secretory cells likely are crypt cells but might also be villus enterocytes. This triggers a signal transduction pathway that mobilizes intracellular calcium and results in increased plasma membrane chloride permeability. The plasma membrane permeability is age-dependent based on studies in CFTR knock-out mice (Morris et al., 1999).

It is hypothesized that this pathway then affects a calcium-activated chloride channel resulting in chloride secretion. Diarrhea occurs in mice lacking the cystic fibrosis transductance regulator (CFTR) channel following rotavirus infection or NSP4 treatment, indicating that a different chloride channel than CFTR mediates this effect (Angel et al., 1998; Morris et al., 1999). The chloride secretion is age-dependent in CFTR mice, indicating that age-dependent disease may result from an age-dependent induction, activation or regulation of this chloride channel (Morris et al., 1999). This pathway would be one that is activated when NSP4 is released from virus-infected cells, possibly by cell lysis, and the affected cells are hypothesized to be the secretory crypt cells. Other cell types including villus enterocytes may also be affected in a similar manner by NSP4.

An alternative pathway leading to changes in plasma membrane chloride permeability may be activated during viral infection when NSP4 is initially expressed as a transmembrane ER-specific glycoprotein within infected enterocytes. It is known that endogenous expression of NSP4 in cells can also mobilize $[Ca^{2+}]i$ from internal stores but this process occurs by a different process that is not affected by PLC inhibitors (Tian et al., 1995). Changes in the plasma membrane permeability are seen in virus-infected cells, but it remains unclear whether these effects result from expression of NSP4 or another viral protein (Michelangeli et al., 1991; Michelangeli et al., 1995).

2.2. Molecular Genetic Studies Support a Role for NSP4 in Diarrhea Induction

Confirmation of the proposed role for NSP4 in diarrhea induction by molecular genetic approaches has also been sought by studying pairs of virulent and avirulent viruses in which the avirulent virus was derived from the virulent parental virus by serial passage of virus in tissue culture. This approach was used because no reverse genetics system is available yet to allow one to construct rotaviruses containing specific genes or mutated genes by rescue of cloned genes. Comparisons of the sequences of NSP4 from two different pairs of avirulent/virulent viruses have shown that structural changes between amino acids 131 and 140 are important in pathogenesis (Zhang et al., 1998). Sequence changes in NSP4 were confirmed to have relevance to pathogenesis based on the expression and biologic testing of the virulent and avirulent NSP4 proteins and site-specific mutant forms of these proteins.

These results indicate that even a single mutation at amino acid 138 can affect the ability of NSP4 to mobilize intracellular calcium and to induce diarrhea in neonatal mice. Others have found a specific amino acid difference at position 135 in NSP4 of

Table 2. Summary of biological properties of NSP4 and NSP4 mutants

NSP4	$[Ca^{2+}]_i$ mobilization in insect cells	$[Ca^{2+}]_i$ mobilization in human intestinal epithelial cells	Diarrhea induction in neonatal mice
OSU-v NSP4	6-fold↑	10-fold↑	57%* (13/23)
OSU-a NSP4	1.4-fold↑	1.2-fold↑	16% (4/25)
OSU $NSP4_{P138S}$	No increase	No increase	0% (0/12)
OSU $NSP4_{D131-140}$	No increase	ND	0% (0/12)

*$P < 0.05$ Data from Zhang et al. (1998).

symptomatic and asymptomatic human rotavirus strains (Kirkwood et al., 1996). The location of amino acid changes in NSP4 of the virulent/avirulent virus pairs downstream from the C terminus of the initial peptide tested is consistent with the finding that the 114–135 peptide does not contain the entire active domain of the protein.

These results with the virulent/avirulent NSP4 proteins raise the question of what is the basis of avirulence. Does the avirulent form of the protein fail to bind to the putative receptor on cells or does it fail to induce the signaling required to effect diarrhea induction. Experiments to test these ideas are in progress.

2.3. Can Knowledge of the Mechanisms of Action of the NSP4 Be Used to Improve Methods of Treatment and Prevention of Disease?

The discovery that rotaviruses produce an enterotoxin raises the possibility that this protein could be useful to develop new methods to prevent or treat rotavirus-induced disease. One possibility is that this protein may be used to induce protective immunity against disease. This idea is feasible based on demonstrating that passive immunity to the 114–135 peptide of NSP4 can reduce both the incidence and severity of diarrhea in neonatal mice challenged with virulent rotavirus (Ball et al., 1996). Further studies are needed to know whether this immunity was provided by lactogenic immunity or by transplacentally transferred IgG, and whether immunity to the fully active protein would induce even greater protection.

An important question is what is the relative importance of enterotoxin action in rotavirus pathogenesis in children. This question is difficult to answer in children, but future studies can be designed to determine if induction of antibody correlates with protection from disease and whether vaccination strategies that induce immunity to NSP4 improve vaccine efficacy. Sequence analysis of over 50 genes that code for NSP4 in different rotavirus strains has recently shown that there are three genetic groups (Cunliffe et al., 1997; Horie et al., 1997; Kirkwood and Palombo, 1997). An interesting question is whether antibody to one genetic type confers protective immunity to challenge with viruses that encode a different genetic type of NSP4; that is, will a single NSP4 induce both homotypic and heterotypic protection?

Limited information about antibody responses to NSP4 in children is available. One study has shown that humoral antibody responses to NSP4 are detectable in children recovering from primary rotavirus infections, but the ability to detect such responses by immunoprecipitation depends on the virus strain used as antigen; homotypic responses are detected with the greatest sensitivity (Richardson et al., 1993). Humoral and cellular immune responses to NSP4 have been detected in children and adults following rotavirus infection, and preliminary data suggest that the presence of antibody to NSP4 may be associated with protection from natural infection (Johansen et al., 1999). Whether immunity to NSP4 will provide the long sought after correlate of protection for rotavirus remains an important issue that needs to be investigated.

Understanding the mechanism of action of NSP4 may lead to new treatments for rotavirus-induced diarrhea. For example, it is possible that antibody treatment or new drugs might be developed to treat children or animals with rotavirus infection and diarrhea, specifically immunocompromised children with chronic rotavirus diarrhea. Such potential new therapies may require a more detailed understanding of the receptor for NSP4 on cells, the structure of NSP4 and whether there are distinct signaling pathways for NSP4 action when the protein is expressed endogenously versus when cells are

exposed exogenously to NSP4. In the latter case, it remains to be determined how NSP4 is released from cells.

The discovery of the rotavirus enterotoxin raises interest in knowing whether other viruses encode enterotoxins. This is obviously important for understanding the mechanisms of pathogenesis for other gastroenteritis viruses such as astroviruses, caliciviruses, coronaviruses, and enteric adenoviruses. This question is also relevant for viruses such as HIV that cause a devastating enteropathy. Finally, these results emphasize the common mechanisms of pathogenesis shared among microbial pathogens.

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