Rotavirus Nonstructural Protein 4 (NSP4)-Viral Enterotoxin with Multiple roles in Pathogenesis of Diarrhoea in Children

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ABSTRACT

Nonstructural protein 4 (NSP4) of Rotavirus has been designated as the first viral enterotoxin. Its role in viral replication is already well known. Intensive research over the past decade has shown the involvement of this protein in many cellular activities both in ‘expressed’ as well as ‘secreted’ form. It is responsible for increased intracellular calcium levels in the infected cell leading to a cascade of events which involve phospholipase C mediated secretion of Chloride ions. NSP4 also inhibits intestinal disaccharidases and sodium glucose symporter (SGLT1) so that complex sugars are retained resulting in malabsorption. It also alters actin in the villi resulting in their flattening and overall decreased absorptive area. NSP4 is associated with extracellular proteins and is hypothesized to have paracrine effects on neighbouring cells. Recent research has found it to be an activator of enteric nervous system too. All these factors contribute to the pathogenesis of diarrhoea which looks multifactorial and certainly very different from the bacterial toxin mediated diarrhoeas of E. coli and V. cholerae. We still don’t have the final word on this intriguing protein which is now a potential candidate for a vaccine against rotavirus. The aim of this review is to put forward the salient features of the research done to elucidate the functions of NSP4.

INTRODUCTION

Infectious acute diarrhoea causes considerable morbidity and mortality in infants and children less than five years of age. Bacterial agents of diarrhoea and their pathogenesis was elucidated in the first half of this century but it was not until the 1970s with the discovery of Norwalk virus by Kapikian et al., (1972) and subsequently of Rotaviruses by Bishop et al., (1973) that a headway was made regarding the viral etiology of diarrhoea. Since then Rotaviruses have been established as the most important viral cause of diarrhoea in the young of both humans and animals. Worldwide 611,000 deaths annually are attributed to rotavirus infection only. (Parashar et al., 2006) Rotavirus is a double stranded RNA virus belonging to the family Reoviridae. Mature viral particles are enveloped, 100 nm in diameter with icosahedral symmetry. A triple layered capsid surrounds a genome of 11 segments of double stranded RNA. The genome codes for six structural proteins VP1,VP2,VP3, VP4,VP6 & VP7 and six non-structural proteins NSP1,NSP2, NSP3, NSP4, NSP5 and NSP6. VP7 and VP4 constitute the outer most layer; VP6 forms the intermediate layer and VP2 is present in the innermost layer (Figure 2).

Differences in the antigen specific epitopes on the protein VP6 are the basis of classification of rotavirus into 7 groups (A-G). Group A rotavirus are the most frequent pathogens of humans and groups D-E have been found only in animals.

Group B rotaviruses have caused epidemics affecting only adults in China while group C is a relatively rare cause of diarrhoea in children. We are referring only to group A rotaviruses in this review.

Rotaviruses exhibit two distinct serological specificities namely subgroup and serotype. Subgroup I, II, I+II and non-I/ II are based on the different antigens of VP6. VP7 and VP4 possess neutralization antigens and form the basis of the current dual classification of group A rotavirus into G and P serotypes. (Estes & Kapikian, 2006)

Initially Non structural protein 4 (NSP4) was thought to play a role primarily in viral replication. But recently it has generated a lot of attention in viral research on being designated as the “first viral enterotoxin” and the major virulence factor responsible for the watery diarrhoea of rotavirus infection.
The underlying mechanisms have since been extensively researched and NSP4 has been found to have multiple effects on cell permeability, intracellular calcium levels, Sodium–glucose symporter (SGLT1), fibronectin and laminin among other parameters investigated in various study designs.

**NSP4 - MORPHOLOGY AND STRUCTURE**

Non structural protein 4 (NSP4), encoded by segment 10 is composed of 175 amino acids. The primary 20 kDa polypeptide is cotranslationally glycosylated to 29 kDa which is further processed to a 28 kDa protein. The amino terminal region of the protein contains an uncleaved signal sequence and three hydrophobic domains H1, H2, and H3. H1 domain contains two N-linked high mannose glycosylation sites and most of the residues float within the endoplasmic reticulum (ER) lumen. H2 is sandwiched within the ER membrane. H3 and rest of the residues up to the C terminus of the protein are hydrophilic and reside in the cytoplasm (Both et al., 1983; Chan et al., 1988 and Bergmann et al., 1989). Various functions have been attributed to different domains of the protein. The region between amino acid (aa) 55 and 72 has been found to possess plasma membrane destabilizing activity (Newton et al., 1997) while aa between 114-135 after cleavage from the parent protein has enterotoxic properties. The VP6, VP4, and tubulin binding sites correspond to NSP4 residues 161 to 175, 112 to 148, and 129 to 175, respectively. The C-terminus has been shown to bind double layered viral particles (DLPs). NSP4 residues 95 to 137 correspond to a oligomerization domain that has amphipathic properties (Figure 3).

**ROLE OF NSP4 IN VIRAL REPLICATION AND ASSEMBLY**

Rotaviruses being gastrointestinal pathogens the target of viral entry and replication was believed to be the differentiated enterocyte. Recent reports about extra intestinal spread of infection has changed this perception and other cells may be involved at some stage or other. The exact mechanisms of viral entry and uncoating are incompletely understood. Replication takes place in the cytoplasm with the ER playing a crucial role. As the double layered virus particles (DLPs) devoid of the outer layer composed of VP4 and VP7 are formed they start aggregating as ‘viroplasms’ in the cytoplasm.

Subsequently, DLPs bud into the ER through the interaction between VP6 and C terminal of NSP4, which acts as an ER resident receptor to transfer the viroplasm to the ER lumen (Au et al., 1983; Chan et al., 1988 and Bergmann et al., 1989). Various functions have been attributed to different domains of the protein. The region between amino acid (aa) 55 and 72 has been found to possess plasma membrane destabilizing activity (Newton et al., 1997) while aa between 114-135 after cleavage from the parent protein has enterotoxic properties. The VP6, VP4, and tubulin binding sites correspond to NSP4 residues 161 to 175, 112 to 148, and 129 to 175, respectively. The C-terminus has been shown to bind double layered viral particles (DLPs). NSP4 residues 95 to 137 correspond to a oligomerization domain that has amphipathic properties (Figure 3).
et al., 1993). During the budding process, the virion acquires VP7 and a temporary envelope. Once inside the ER, VP4 joins VP7 to complete the outer layer. The lipid envelope and NSP4 are shed by a process yet to be deciphered. Mature virions have no structural proteins and stay for a while in the ER before being finally released by cell-lysis (Taylor et al., 1992; Taylor et al., 1993).

Cells in which NSP4 expression has been stalled by using small interfering RNAs, viroposmation formation is inhibited as expected but additionally cellular distribution of some other proteins is also affected. Viral mRNA synthesis also seems to be altered (Lopez et al., 2005; Silvestri et al., 2005). Overall the role of NSP4 in replication needs to be researched further so that gaps in the existing knowledge could be filled.

SECRETION OF NSP4 PEPTIDE AND INTERACTION WITH EXTRACELLULAR MATRIX PROTEINS

The mature virion does not contain NSP4 or any of the other structural proteins. During active infection of the enterocyte NSP4 is translated and cleaved and a peptide (112 to 175) is actively secreted from the apical side. This peptide was hypothesized to have paracrine effects on the nearby cells (Zhang et al., 2000).

Other workers showed that NSP4 is released from the basal side of the infected epithelial cells also. They were able to demonstrate physiological interactions of NSP4 with laminin β-3 and fibronectin which are basically multifunctional proteins present as major constituents of basement membranes and thought to play a vital role in the assembly of extra cellular matrix. This was a very significant finding as till that time it was widely believed that NSP4 (aa 112 to 175) is actively secreted only from the apical side of infected epithelial cells via an atypical pathway that bypasses the Golgi apparatus and involves lipid microdomains called rafts (Jourdan et al., 1997; Sapin et al., 2002). After release from the apical side it was thought that it binds to a receptor and directly or indirectly triggers a signal transduction pathway to enhance chloride secretion, eventually resulting in diarrhea (Ball et al., 1996; Morris et al., 2001). This secretory mechanism, however, was proposed to occur in the intestinal crypts (Lundgren et al., 2000). The intestinal crypts have a continuous flow of secretions against which diffusion of NSP4 did not seem plausible.

The findings of Boshuizen et al., led to speculations that NSP4 travels towards the crypt region where it can induce fluid secretion via a route involving the ECM. Further findings by Ball and coworkers that NSP4 is able to cause chloride secretion when added to the submucosal (basal) surface of mouse mucosal sheets support the above view. It was also found that secreted NSP4 stays soluble in an aqueous environment as an oligomeric lipoprotein that has the property of binding to various cell types via an interaction with glycosaminoglycans. This broad cellular tropism exhibited by NSP4 is supported by findings that indicate that rotavirus infection is not confined to the intestine but instead leaves the intestine and enters the circulatory system (Blutt et al., 2003; Mossel et al., 2003). Rotavirus has been demonstrated in cerebral spinal fluid (Nishimura et al., 1993), liver and kidney, extraintestinal lymphoid tissue (Brown et al., 1998) and even in the heart, where it was mainly localized to endothelial cells (Mossel and Ramig 2003).

It has been proposed that rotavirus, enters the circulatory system through M cells that are present in the epithelium overlying Peyer’s patches (Blutt et al., 2003). Above findings also imply that NSP4 could get into the circulatory system, after being secreted into the extracellular matrix underlying the enterocytes.

Localization of NSP4 within the cell.

NSP4 seems to have three different addresses within the same cell depending on the level of expression and the time since infection. The first address is the ER where it can be found throughout the infection. Second place is the ER Golgi Intermediate compartment (ERGIC) where the cleavage and delivery of the peptide (114-135) takes place through a non classical pathway. A third address is in the cytoplasm within vesicular structures tagged with the autophagosomal marker LC3. Here NSP4 can be found as early as 6 hours after infection and is believed to recruit these vesicles to viroplasms. In all probability this association prevents fusion of lysosomes with viroplasms there by extending their lifespan and overall enhanced viral replication. Further research of the autophagy pathway are needed to confirm the above hypothesis (Berkova et al., 2003)

ASSOCIATION OF NSP4 WITH CAVEOLAE AND CELLULAR SIGNALLING

Parr et al., (2005) have shown that both NSP4 and the enterotoxic peptide (NSP4 114-135) show predilection for caveola-like model membranes. Caveolae are a type of lipid rafts that are rich in cholesterol and sphingomyelin and are found in most cells, including enterocytes. They contain the integral membrane protein caveolin-1 which functions mainly as a platform to organize signaling molecules at the plasma membrane. The apparatus of Ca2+ regulation and signaling consisting of Ca2+ ATPase, inositol trisphosphate receptors, tyrosine kinases, G proteins, and calmodulin is found in caveolae. In addition, caveolae are home to as much as 50% of cellular Phosphatidylinositol diphosphate (PIP2) and are the site of PIP2 hydrolysis in response to a ligand (Pike et al., 1996). The above authors demonstrated colocalization of NSP4 and caveolin-1 in NSP4-transfected and rotavirus-infected mammalian cells in ER, in the cytoplasm, and at the cell membrane using laser scanning confocal microscopy and proposed that the association of NSP4 and caveolin-1 contributes to NSP4 intracellular trafficking from the ER to the cell surface.

The binding site for caveolin-1 has been demonstrated in the hydrophobic region of the amphipathic helix (Ball et al., 2013). These findings also suggest that NSP4 can have a role in cellular signalling as discussed in the following sections.
ROLE OF NSP4 AS THE VIRAL ENTEROTOXIN AND PATHOGENESIS OF DIARRHOEA

Rotavirus infects the mature enterocytes in the mid and upper villous epithelium of the small intestine, which ultimately leads to cell death, villous atrophy, and diarrhea. Many workers have put forward various theories regarding the causative mechanism of diarrhea. These include enterocyte death, villus ischemia (Starkey et al., 1986), a toxin-like effect of the non-structural protein (NSP4) and activation of the enteric nervous system resulting in electrolyte and fluid secretion (Lundgren et al., 2000). Experiments in which NSP4 was administered intraperitoneally in infant mice resulted in diarrhoea in an age dependent manner (Ball et al., 1996; Horie et al., 1999). Ball et al., (2000) also demonstrated that a peptide sequence from aa 114 to 135 (crosslinked) of the 175 amino acid protein from SA11 strain was specifically responsible for diarrhoea in the mouse model. Another important finding was that diarrhoea associated with the early stages of rotavirus infection appeared to be mediated by enhanced fluid transport before gross histological changes in the mucosa occur.

In another study neonatal pigs inoculated with porcine rotavirus developed watery diarrhoea 8 h after infection, whereas a few histopathological lesions were found in jejunum segments after nearly two days of infection (Vellenga et al., 1992). Rotavirus-infected mouse cells when scanned under electron microscope were found to contain lots of swollen ER cisternae and vacuoles beneath the apical membrane (Starkey et al., 1986) but as such no significant gross morphological changes within the gastrointestinal lining were observed. These studies demonstrated that rotavirus-induced diarrhoea is not as a result of gross structural damages and disruption of microvilli leading to malabsorption as initially believed. As it was observed that induction of diarrhea and alterations in chloride secretion occurred within a period of about 3-8 h, homologous to those induced by the heat-stable toxin b of *Escherichia coli*, NSP4 was proposed to be a viral enteroxin.

The cascade of events leading to fluid secretion has been intensively researched and many crucial mechanisms have been deciphered. The basic theory that has been proposed is that NSP4 interacts with a receptor in the cells lining the GIT and sets in motion a signal transduction pathway involving phospholipase C which results in exit of chloride ions as well as water molecules across the plasma membrane. The resulting secretory diarrhoea was found to be calcium dependent (Ball et al., 1996 and Zhang et al., 2000).

Status of Calcium and interplay with NSP4

NSP4, when added ‘exogenously’ as well as when ‘expressed’ intracellularly, is supposed to be a key player in intracellular calcium regulation during rotavirus infection. Historically calcium levels have been shown to be vital for replication. In cells with depleted calcium, viral maturation and assembly was stalled after the DLPs enter the ER (Shahrabadi and Lee., 1986). Intracytoplasmic calcium ions (Ca$^{2+}$) are regulated by two main mechanisms working in opposite directions. First are the Ca$^{2+}$-ATPase pumps which actively push out Ca$^{2+}$ from cytoplasm to either the ECF or to the intracellular compartments such as ER and second are the Ca$^{2+}$ channels on the ER and cytoplasmic membrane which allow passive flow of Ca$^{2+}$ inside the cell. (Figure 4)

Michelangeli et al., (1991) reported that rotavirus protein synthesis in MA104 cells leads to elevated intracellular calcium levels which they proposed was as a result of increased permeability of plasma membrane.

**Fig. 4: Theories of diarrheal pathogenesis**

NSP4 = Nonstructural protein 4; ENS = Enteric nervous system; PLC = Phospholipase C; SHT = 5 Hydroxy tryptamine; SGLT1 = Na+-D-glucose symporter

It was observed that ‘expression’ of NSP4 is responsible for an increase in (Ca$^{2+}$) in *Spodoptera frugiperda* (Sf9) insect cells infected with baculovirus expressing the NSP4 gene (Tian et al., 1994). In another study the same authors investigated the underlying pathophysiology and measured the permeability of both plasma and ER membrane in the same setting and found that though NSP4 expression increases the basal Ca$^{2+}$ permeability of the ER membrane no significant changes were found in permeability of plasma membrane as previously proposed by Michelangeli et al. They also added purified NSP4 protein or a 22-amino-acid synthetic peptide consisting of residues 114 to 135 (NSP4 114–135) to noninfected Sf9 cells to assess the paracrine effects of secreted NSP4 on [Ca$^{2+}$]i. Both NSP4 and the NSP4 [114–135] peptide produced a time-dependent increase in (Ca$^{2+}$) that was reduced if phospholipase C was blocked with its inhibitor U-73122. Also in the same cells if ER Ca ATPase was inactivated with thapsigargin the increase in (Ca$^{2+}$) produced by NSP4 114–135 was completely abolished, but the peptide only partially reduced the change in (Ca$^{2+}$) produced by thapsigargin. No changes in (Ca$^{2+}$) were seen in cells treated with control peptides.
Effect of NSP4 on chloride transport also strengthens the hypothesis that NSP4 most probably involves signal transduction pathways to produce net chloride secretion at the onset of rotavirus diarrhoea (Lorrot et al., 2006). In a recent study chloride secretion induced by NSP4 was found to be related to increase in oxidative stress (Buccigrossi et al., 2014).

Effect on microvillar actin; intestinal enzymes & Na⁺-D-glucose symporter (SGLT1) - Malabsorption component of diarrhoea?

Like many other pathogens, rotavirus infection and replication leads to rearrangement of the cytoskeleton with disorganization of cytoskeleton elements such as actin and cytokeratin. Brunet et al., (2000) have demonstrated that in polarized human intestinal Caco-2 cells line, rotavirus infection induces calcium-dependent depolymerization of microvillin actin leading to disruption and flattening of microvilli as observed under electron microscopy. The immediate effect is that the surface area for absorption is drastically reduced resulting in malabsorption. In the same study it was also shown that a viral protein secreted into the medium had the same effects on microvillin actin in noninfected cells. The candidature of NSP4 as a likely player was proposed and further research showed that expression of NSP4, caused stabilization of long cellular projections in HEK 293 cell line. Quantification of filamentous actin (F-actin) content revealed an elevated F-actin content in NSP4 expressing and rotavirus-infected cells as opposed to that in nonexpressing and noninfected cells. Intracellular calcium levels were also vital for these changes in F-actin content as was an increased activation of the actin remodeling protein cofilin. Another point to notice is that actin framework of a cell is responsible for activities such as transport of ions, cell division and release of viral progeny and these activities could also be affected (Berkova et al., 2007).

Rotavirus infection in the mouse model has been found to decrease the activity of intestinal disaccharidases without any appreciable effect on the intestinal brush border membrane (Collins et al., 1990). Among the four brush border disaccharidases expressed in the small intestine, Sucrase isomaltase (SI), a enzyme complex which hydrolyzes maltose, maltotriose, and sucrose, has been extensively studied in vivo as well as in the enterocyte-like model Caco-2 cell line (Hauri et al., 1988). Jourdan et al., (1998) reported that both the activity and expression of SI in vitro in human Caco-2 cells were reduced by rotavirus infection without any histopathological lesions. They were also able to demonstrate that RRV infection did not affect SI production and tertiary structure but interfered with the enzyme’s delivery to the brush border membrane. Another evidence collected was that of Rotavirus infection inducing an important alteration of the brush border-associated cytoskeleton that correlates with decreased SI apical surface expression. As previously discussed NSP4 has already been implicated in inducing changes in cellular cytoskeleton (Berkova et al., 2007).
Rotavirus infection has a negative effect on NA+D-glucose symport (SGLT1) activity in both villus and crypt cell BBM of rabbit intestine. The active resorption of glucose was found to be higher for villi BBM in infected rabbits than for crypt cell BBM in control rabbits not exposed to rotavirus. There was no effect on SGLT1 protein expression, which was higher for villi than for crypt cells. This was an interesting finding as it became clear that enterocytes could not be crypt-like cells as proposed by a few workers in the crypt-cell invasion theory (Lorrot et al., 2004; Halaihel et al., 2000). In further experiments done on rabbit intestinal BBM, the NSP4–(114–135) peptide has been shown to directly and strongly inhibit SGLT1, but not Na+-L-leucine symport activities (Halaihel et al., 2000). To summarize NSP4 is definitely involved in glucose malabsorption during rotavirus infection in vivo. By inducing malabsorption of carbohydrates and their accumulation in the intestinal lumen as well as malabsorption of nutrients and a simultaneous inhibition of water reabsorption, which can lead to malabsorption component of diarrhea.

Effect on enteric nervous system and enterochromaffin cells

The research during the past decades has led to the observation that intestinal secretions induced by bacterial pathogens are partially due to activation of enteric secretomotor neurons. The same has been suspected for rotaviral gastroenteritis too. The enteric nervous system (ENS) consists of two layers within the intestinal wall, the myenteric plexus and the submucosal plexus. The functions are intertwined but the myenteric layer is more involved with muscle control and the submucosal with managing secretion. ENS is relatively huge given that it consists of 100 million neurons which are almost equal to those of spinal cord. Like the CNS, the ENS releases neurotransmitters such as serotonin, acetylcholine, and nitric oxide, and dopamine. Out of these serotonin is implicated to be the most important neurotransmitter. Stimulation of 5HT3 and 5HT4 receptors initiates peristalsis and facilitates secretion in the gastrointestinal tract (GIT). Particularly rich in serotonin are the enterochromaffin cells of the GIT mucosa which are present in the middle and upper segments of villi in the duodenum, jejenum and ileum and are classically associated with rotavirus replication and histopathological lesions. Enterochromaffin cells release mediators of endocrine signalling from the basolateral surface to activate afferent neuron endings within the lamina propria. They also appear to be sensitive to mechanical stimulation and may be involved in the peristaltic reflexes of the GIT (Bertand et al., 2000; Hansen and White, 2008). Kordasti et al., (2004) demonstrated that rotavirus infection results in stimulation of the ENS. They were also able to demonstrate that rotavirus diarrhea in mice can be attenuated with 5-HT3 receptor antagonists such as granisetron. Other researchers showed that crude and virus particle-free supernatants from rotavirus-infected MA104 cells stimulated 5-HT release within an hour in primary EC cells and carcinoma cells and cell lines (GOT1). Again recombinant and secretory NSP4 were able to release 5-HT from primary and GOT1 EC cell line (Hagborn et al., 2011).

Molecular epidemiology association with virulence

After sequencing of NSP4 gene from both human and animal strains and subsequent analysis five genotypes (A–F) were assigned initially. The Rotavirus Classification Working Group (RCWG) has assigned a total of 14 NSP4 genotypes (E1–E14) (Matthijnssens et al., 2008, 2011). As molecular sequencing database expands the number may rise in the future. The most common NSP4 genotypes in humans are E1 (Wa-like), E2 (Kun-like) and E3 (AU-1), which up till now were known as genotypes B, A and C respectively. Some other uncommon genotypes like E6 and E9 have also been reported from a few geographical areas (Khamrin et al., 2007; Rehman et al., 2007).

In one study mutation causing amino acid substitutions in the region between 130-141 aminocitc from two pairs of virulent and attenuated (tissue culture adapted) swine strains were found to have altered rotavirus virulence in mice (Zhang et al., 1998).

IMMUNE RESPONSE TO NSP4

Historically structural proteins VP4 and VP7 induced formation of type specific neutralizing antibodies has been considered to confer protection though the correlates for protection have not been clinically demonstrated. Studies on hepatitis C virus and some flaviviruses have shown that non structural proteins can induce protective immunity. In children suffering from non rotavirus diarrhoea antibodies against NSP4 were demonstrated in acute phase serum and may have conferred protection from rotavirus (Vizzi et al., 2005). Several workers have studied humoral immune response against NSP4 in detail. In human infants vaccinated with a live oral vaccine a good IgG and IgA response against NSP4 has been obtained which is not type specific or homotypic (Yuan et al., 2004). Ray et al., (2003) have reported significant heterotypic humoral response to NSP4 in a study done in children suffering from rotavirus gastroenteritis.

In another study gnotobiotic calves and piglets were first infected with bovine NSP4 A or porcine B genotype and subsequently challenged with recombinant NSP4 of various genotypes and derived from different animal and human strains. It was found that antibody production was more if the NSP4 was derived from the same species and also showed an association with species specific region mapping to aa131-141. The genotype of NSP4 did not make any difference to the overall response (Yuan et al., 2005). Orally given vaccines based on NSP4 have been found to confer protection in mouse model (Choi et al., 2006). There are no studies in humans as of now but are surely to follow suit.

CONCLUSION

To summarize research on the functions of NSP4 has been exhaustive to say the least. Regarding the enterotoxin function, there is agreement on a lot of hypotheses put forward by workers but gaps still remain in the final picture which is not as complete as that of bacterial enterotoxins. Various mechanisms are known but the exact contribution of secretory component vs the enteric nervous system vs malabsorption is not clear. What is clear
is the omnipresent association of NSP4 in various mechanisms investigated up till now. The immune response against this protein is also being studied.

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