Natural Immunity to Rotavirus Infection in Children

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Annual deaths in infants and young children due to rotavirus (RV) infection are around 100,000 in India and about 600,000 globally. Development of a vaccine for this disease is a high priority. The protective mechanisms for RV diarrhea in human are not fully understood, but it is known that children develop natural immunity against RV. Early exposure to RV results in most severe episode of diarrhea and subsequent infections are milder or asymptomatic. Of the immune responses measured during natural infection, RV-specific antibodies have been well documented, whereas data on cellular immunity in humans are sparse. It is generally thought that two outer capsid proteins VP4 and VP7 play a critical role in protective immunity by stimulating production of neutralizing antibodies. While serotype-specific protection mediated by antibodies directed against the outer capsid proteins may be a mechanism of protection, such a correlate for protection has been difficult to demonstrate in humans during clinical trials. Increasing evidences suggest that viral proteins that lack a capacity of eliciting neutralizing antibody response also induce protective immunity. Limited efforts have focused on the role of non-structural proteins in protective immunity. This review describes current understanding of antibody responses in children with focus on responses specific to viral antigens with their possible role in protective immunity. We have also briefly reviewed the responses elicited to non-antibody effectors during RV infection in human subjects.

Keywords: Rotavirus, Diarrhea, Children, Immune response, IgA, IgG, Antibodies, T cell, Cytokines, Natural infection

Introduction

Rotavirus (RV) is the most common cause of diarrhea worldwide and diarrheal deaths in children in developing countries. It accounts for 5% of all deaths in children younger than 5 years in developing countries. Mortality rates are highest in south Asia and sub-Saharan Africa, with more than 0.1 million deaths annually in India alone.

RV is transmitted by the fecal-oral route and attaches to the proximal small intestine, if not neutralized by stomach acidity. It has an incubation period of 18-36 h, during which it enters epithelial cells and possibly generates a potent enterotoxin-non-structural protein 4 (NSP4), as suggested from studies in murine models. It damages epithelial surface, leading to blunted villi causing massive shedding of the virus in stools. It is hypothesized that secreted NSP4 or other effecter molecules released from infected cells may also stimulate the enteric nervous system (ENS). The outcome is watery diarrhea of 2-7 days duration, resulting in loss of fluids and electrolytes, which may lead to severe or fatal dehydration. RV infection is believed to be localized exclusively in small intestine, but different studies recently have reported extra-intestinal spread in human subjects.

RV is characterized by a triple layered structure with 11 segments of double-stranded RNA that encode for six structural and six non-structural proteins. Two proteins that form the outer capsid: VP7 a glycoprotein (G protein) and VP4, a protease-sensitive protein (P protein) represent prime targets for the immune system to mount neutralizing antibody response and are the key antigens to characterize strains. Serotype classification based on G and P proteins have identified 15 VP7 serotypes and 14 VP4 serotypes. G1, G2, G3, G4 and G9 VP7 serotypes and P1 VP4 serotype are the most common RV strains present globally. Additionally, recent studies have shown emergence of G12 strains worldwide.
including India\textsuperscript{16,17}. Diversity of RV strains and high prevalence of mixed infections are unique features of RV epidemiology in India\textsuperscript{18-20}. P6 strains of probable bovine origin and natural reassortants have also been detected in the subcontinent. A novel strain P-type 11 human RV, strain 116E has been isolated from neonates in Delhi and its VP4 is related to the bovine serotype G10 P[11] B223 and VP7 to human serotype G9 strain\textsuperscript{21}. Another neonatal strain G10 P[11] has also been reported from Bangalore\textsuperscript{22}.

Studies of natural history of RV infection have demonstrated that protective immunity is induced from early infections; first infections after neonatal period are symptomatic and repeat disease is uncommon after second infection\textsuperscript{23-25}. This protection by natural infection forms the scientific rationale behind development of live oral vaccines. Both monovalent strains and multivalent animal strains based vaccines have demonstrated efficacy as candidate vaccines. Two vaccines Rotarix (monovalent) and Rotateq (polyvalent) have recently completed clinical trials and are licensed in Europe and USA respectively and several other countries\textsuperscript{1}. In India, the neonatal strains 116E and I321 have recently been developed as live oral vaccines and based on early clinical study, the former is being pursued as a candidate vaccine\textsuperscript{26}.

Despite significant research efforts and development of oral vaccines during last three decades, the natural immune mechanisms controlling and preventing RV disease in humans are not completely understood. It is generally thought that two outer capsid proteins VP4 and VP7 are the key components in conferring protection against RV diarrhea, primarily by stimulating production of neutralizing antibodies. While serotype-specific protection mediated by antibodies directed against the outer capsid proteins may be mechanism of protection, such a correlate for protection has been difficult to demonstrate in humans. Instead, vaccine trials with bovine and rhesus RV strains have suggested that serotype cross reactive immunity plays an important role\textsuperscript{27}. Increasing evidences also suggest that viral proteins that lack a capacity of eliciting neutralizing antibody response also induce protective immunity\textsuperscript{5,28}. Limited efforts have focused on the role of non-neutralizing proteins in protective immunity. Therefore, for development of new vaccines, two major issues need consideration. It is important to identify and evaluate other targets involved in antibody-mediated protection, apart from neutralization antibodies to VP4 and VP7. Secondly, the role of other non-antibody effectors in protection needs further elucidation.

Here, we briefly review the existing data on natural immunity, following RV infection in children and have attempted to provide a better understanding of the role of antibody and non-antibody effectors in protection against RV infection.

**Rotavirus immunity**

**Generation of immune response**

The virus replicates in the mature epithelial cells present in the villi of the small intestine\textsuperscript{4}. These epithelial cells during enteric infections play an important role in the initiation of the host defense by releasing certain cytokines/chemokines that further trigger antigen-specific immunity by mediating B and T-cell responses. During RV infection, the mechanism of immune regulation by the cytokines secreted by epithelial cells is yet to be clearly determined. However, there is evidence for the enhanced expression of NF-kB and interferon (IFN) transcriptional elements and release of several C-C and C-X-C chemokines and IFN from the epithelial cells, which probably play a role in orchestration of immune responses\textsuperscript{29}.

From the current understanding of RV pathogenesis, a model for the generation of effector responses has been proposed. Though small intestine is the primary site for RV infection and replication, recent reports indicate escape of RV from GI tract, resulting in antigenemia and possible viremia, therefore resulting in generation of both systemic and mucosal responses\textsuperscript{7,9,27}. It is proposed that Peyer’s patches (PP) serve as the major site for induction of immune response in the intestine. The viral progeny generated on the mucosal surface attaches and enters M cells and via M cells viral antigens are transported to antigen presenting cells (APC). Antigen presentation by APC leads to activation of Th cells, followed by expansion of B and T cell responses. Virus-specific antibody secreting B-cell and memory B-cells generated in the Peyer’s patches enter the blood stream and traffic via homing receptors. Antibody secreting cells (ASC) migrate to lamina propria, the site for maturation of effector B-cells, whereas memory B-cells home to Peyer’s patches. ASC residing in the lamina propria secrete polymeric IgA, which is eventually transported to the intestinal
lumen. This model is supported from study in human subjects, where B cells that express RV-specific surface Ig predominantly, also express α4β7, indicating trafficking of primed cells to the intestine.30

Further, the systemic viral antigen also stimulates formation of memory B cells and ASC in the spleen. ASC, home to bone marrow and secrete monomeric IgG and IgA, which is the primary source of serum antibodies, whereas memory B-cells circulate in blood and return to the spleen. Of late, presence of memory RV-specific B cells with both gut and systemic trafficking have been reported in children convalescing from RV infection, supporting the systemic stimulation of RV.31 However, serum IgA response during RV infection correlates to mucosal IgA response, therefore, the mechanism of spillage between the two components is not clear.27

Similar to B-cells, virus-specific CTLp leave PP circulates in blood and traffic back to other PP or to the intestinal lamina propria. This is mediated, in part by interactions between integrin α4β7 expressed on T-cells and the cell adhesion molecule MadCAM1 expressed on the vascular endothelium of the post-capillary venules in the intestine. In support of this, RV-specific memory CD8+ T-cells that have in vivo antiviral effect have been shown to express the intestinal homing receptor.32,33

**Antibody responses in RV infected children**

RV infections induce protective immunity, the evidence of which is well documented from studies in Australia, India and Mexico.23-25

- Ruth Bishop and co-workers in a longitudinal follow-up of 81 neonates prospectively for 3 years in Australia found that the first infections are symptomatic in neonates and provide protection against severe disease caused by re-infection.23

- Later, Raul Valazquez and colleagues confirmed and extended the above study. They reported that first RV infection in infants provide 87% protection against moderate to severe disease caused by second infection and two infections are 100% protective.

- Bhan and colleagues analyzed a cohort of newborns in New Delhi who were nosocomially infected with RV during their first days of life and followed twice weekly for 14-23 months. Asymptomatic infection occurred in 60% by the fourth day of life. The 148 children with neonatal RV infection had 46% fewer attacks of RV diarrhea in the follow-up period than the 56 infants without nosocomial infection.25

The extensive epidemiological observations of natural infection in infants show that children develop natural immunity against RV after early and repeated illness.

RV infection results in both serum (IgG and IgA) and intestinal antibody (IgA) responses. Protective effect of naturally acquired serum IgG and IgA antibodies against RV infection and illness has been studied extensively. In children with RV gastroenteritis or asymptomatic infection, preexisting levels of serum IgA, but not IgG correlate with protection as children with IgA responses develop less severe disease and less vomiting. On the contrary, in another study in rural Bangladesh, significantly low serum IgG titers have been reported in children with symptomatic RV diarrhea than in healthy control subjects. Thus, IgG response correlates with protection against clinical illness. These conflicting results could not be explained due to lack of information regarding previous exposure to RV infections in the subjects analyzed.

Velazquez and colleagues addressed this issue with a better approach in a cohort study of 200 Mexican infants who were monitored from birth to 2 years of age. Children with IgG titer >1:6400 were protected against RV infection, but not disease, whereas children with total IgA titer >1:800 had lower risk of RV infection and were completely protected against moderate to severe diarrhea. Protective antibody titers were achieved after two consecutive symptomatic or asymptomatic rotavirus infections. Thus, findings from this study indicated serum IgA to be a marker of protection against RV infection and moderate to severe diarrhea. However, in animal studies, as well as in adult volunteers, local antibodies were found to be the better predictors of protection against RV illness.

Protection has also been associated with fecal IgA response in few studies, but evidence is not conclusive. In 14 adult volunteers, similar kinetics for stool and serum RV IgA response have been observed, suggesting that serum IgA may be a good indicator of local antibody responses. Further, serum IgG and IgA responses were studied in correlation to secretory IgA response in duodenal fluid of Danish children, following RV gastroenteritis. High levels of serum IgA and secretory IgA were recorded during
first ten days of RV gastroenteritis. Elevated IgA response was detected in feces, 5-7 months post-infection and was absent during acute phase. The amount of secretory Igs in duodenal fluid correlated to that detected in serum. RV IgG was not detected in duodenal fluids or feces.

Thus, when the studies are reviewed together, it is difficult to conclude whether serum or local antibodies or both mediate protection. However, serum IgA, measured shortly after infection represents a good correlate of intestinal IgA and a marker for protective immunity. It is of considerable interest to further define the viral proteins capable of eliciting protective antibody responses during natural RV infection.

**Antibody response to RV neutralizing antigens**

Proteins VP4 and VP7 comprise the outer capsid of RV. VP4 forms the spikes, is the viral attachment protein, and is cleaved by trypsin into VP8* and VP5*, whereas VP7 is a glycoprotein and the major constituent of outer protein layer. Both proteins induce neutralizing antibodies (NAb) that can block enterocyte infection directly, when present in lumen and have been shown to be protective. Virus neutralization mechanisms are poorly understood. However, VP4 (VP8*) has been proposed to neutralize infectivity by inhibiting binding of virus to the cell, whereas VP7 neutralizes by inhibiting virus decapsidation. Rotaviruses display complex serotype diversity and neutralizing antibody responses, which have shown to be both homotypic (serotype-specific) and heterotypic (cross-reactive). In early studies, protection against illness was observed to be associated with preexisting homotypic neutralizing antibody. Chiba et al. first observed this relationship in an orphanage in Japan and reported NAb titers ≥ 128 in serum protected against subsequent illness. Further, in another study, development of predominant homotypic antibody response was also demonstrated upon first exposure to RV; the protection correlated to the high levels of antibody in children; high titers resulted in complete protection. Homotypic immune response was also reported to primary RV infection, particularly with G1 RV serotype; it was observed that with repeated exposures, children developed heterotypic antibody responses to G types and were protected against wide range of RV serotypes. It was also observed that low NAb titers in mothers predisposed the children to infection with that serotype, if the serotype was in circulation. All these data indicate NAb (homotypic) responses to RV are associated with protection against disease (Table 1).

In a cohort study in Bangladesh, Ward et al. reported that protection against natural RV infection was not dependent on serotype-specific neutralizing antibody. They analyzed acute phase sera of 156 RV cases versus 312 control cases of children aged 4-35 months. Titers of both homologous and heterologous neutralizing antibodies in acute samples of cases were significantly lower than in controls. Further, only heterotypic antibody production was associated with protection, suggesting that immunity to RV disease might be mediated by other factors.

The relative contribution of VP4 and VP7 in induction of homotypic as well as heterotypic antibodies has been investigated recently. A study in infants using human animal reassortants detected homotypic and heterotypic responses induced by both VP4 and VP7 during primary RV infection. Infants seroconverted more frequently to VP4, but the titers of VP7 seroconversion were higher than VP4. G1 infection elicited homotypic (G1) and heterotypic (G3) response, while G2 and P1A induced homotypic response only. Recently, Gorrell and Bishop analyzed VP4 and VP7 specific NAb response in sera of children with primary versus secondary RV infection. After primary infection, response to VP7 was observed to be serotype-specific, whereas to VP4 was heterotypic. However, upon reinfection, VP7 was found immuno-dominant, in terms of cross-reactive neutralizing antibody production and neutralizing antibody titer.

Thus, specific role of VP7 and VP4 in production of cross-reactive neutralizing antibodies and protection is unclear. The current vaccine development efforts are, therefore, based on correlation of broad immunity by vaccines that are monovalent as well as polyvalent.

**Antibody response to non-neutralizing antigens**

Structural proteins constituting the inner capsid (VP6), the core (VP1, 2, 3) as well as NSP1-6 represent the non-neutralizing antigens. Very few studies have evaluated immunogenecity of these antigens during RV infection. Richardson et al. evaluated immunogenecity of these antigens during RV infection.
examined 20 young children with primary RV infection and detected serum IgG responses to rotavirus core antigens VP2, VP3, VP6 and to non-structural proteins NS35 and NS26, in addition to the outer capsid neutralization antigen in majority of patients. Similarly, recent study of the antibody responses in 11 infants with severe RV gastroenteritis to individual RV polypeptides by radioimmunoprecipitation assay (RIPA) also showed IgG and/or IgA responses to structural viral proteins VP2 and VP6 and the non-structural protein NSP2. Interestingly, fecal sIgA in convalescent samples reacted strongly toward NSP2 and VP6

Thus, this data indicate antigenic importance of RV proteins, other than VP4 and VP7. Supportive evidence from mice studies reinforces immunodominance of VP2, VP6, and NSP2 other than VP4 and VP7 during RV natural infection. However, data are not conclusive, and future studies are required to extensively evaluate the role of individual proteins in immunity during natural RV infection.

Recent animal studies have provided increasing evidence for the role of VP6 and NSP4 in protective immunity. Immunization studies of mice with chimeric VP6, together with attenuated E. coli heat-labile toxin LT(R192G) as an adjuvant have shown reduced fecal shedding of RV antigen by >95% after murine rotavirus challenge. Furthermore, a plant based multi-component vaccine containing NSP4 is found to mediate protection in mice. Thus, these two proteins VP6 and NSP4 constitute potential targets for a sub-unit vaccine design — as an alternate vaccine strategy, but their role in protective immunity merits further confirmation in human studies.

The non-neutralizing RV inner capsid protein VP6 induces protective immunity in mice after DNA vaccination and after immunization with
virus-like particles containing VP6. In addition, a monoclonal antibody directed at VP6 induces protective immunity in adult SCID mice against RV. Recent findings suggest that well-conserved immunodominant VP6 protein can function as a target for heterotypic antibodies and protective immunity. Anti-VP6 IgA antibodies confer protection in vivo by inhibiting viral transcription at the start of intracellular phase of viral replication cycle.

Recently, it is demonstrated that VP6 RV protein interacts with a large fraction of naive B-lymphocytes in adults and neonates. The naive B-cell VP6 interaction may influence the strength and quality of acquired immune response and should be considered for elaborating RV vaccine strategies. Studies during natural infection indicate humoral response to VP6. In RV infected diarrheal children, 72% seroconversion is reported versus 13% in non-RV infected diarrheal children and 53% children with baseline antibodies to VP6 do not develop diarrhea. In a previous study, all convalescent sera from RV infected Swedish children and adults have been found to contain IgG antibodies against rVP6. Thus, VP6 elicits serum antibody responses, but its role in protection needs to be further evaluated.

A non-structural protein of RV, NSP4 functions as an intracellular receptor during sub-viral particle morphogenesis. Evidence for its role in pathogenesis was first shown in gnotobiotic piglet model and later, it was identified as an enterotoxin on the basis of its ability to induce diarrhea in infant mice. Passively acquired antibodies to NSP4 reduce both the incidence and severity of diarrhea in infant mouse pups challenged with virulent RV, suggesting that immune response to NSP4 probably could also modulate RV diarrhea in humans.

The relative importance of immunity to NSP4 in humans remained poorly understood for long, as preliminary studies in children were done with small number of subjects and showed variable levels of immunogenicity. The inconsistency between the studies could be related to differences in antigens used or other assay conditions. Antigenic differences between NSP4s from different infecting strains might also affect immune response after infection as seen in animal studies. Serotype-specific NSP4 antibody response was also reported in children. A low IgG antibody response to NSP4 after RV infection was documented, indicating poor immunogenicity. However, a comprehensive study by our group indicated significant NSP4-specific IgG and IgA antibody response for the first time in large number of RV infected children.

The phyletics of NSP4 revealed the presence of five distinct NSP4 alleles (A, B, C, and D, E); the majority of human strains have been classified as either genotype A or B. In one of the study, the response to NSP4 was not significantly influenced by NSP4 genotype of the infecting strain and about 50-70% of RV infected children were seroconverted (IgG/IgA) when various recombinant NSP4 (A, B and C) were used as antigen. In another study, heterotypic protection was also observed against RV diarrhea in mice. Two recent studies have demonstrated NSP4 response is partially heterotypic (Table 1). Taken together, data show that NSP4-specific antibody responses occur during natural infection and the response is broad and not genotype-specific.

Cellular immunity

Non-antibody effectors such as NK cells, cytotoxic T-cells (CTL’s) and cytokines confer protection against disease or mediate antiviral activity in humans and animals. Studies in mice have shown appearance of CTL’s in the intraepithelial lymphocytes 6 days after oral inoculation with RV. Passive transfer of Thy-1+ CD8 + intraepithelial lymphocytes in severe combined immunodeficient (SCID) mice ablates RV shedding from the intestinal tract, indicating protection against disease in absence of neutralizing antibodies. Also, RV-specific CTL’s from adult mice mediated passive protection in suckling mice against murine RV induced disease on re-challenge.

T-cell responses have been extensively reported in murine model and the key features are summarized:

a) MHC class I restricts CD8 + αβ T-cells play a major role in resolution of primary RV infection. After primary infection, CD8 + T-cells mediate almost complete protection (up to 2 weeks) or partial protection (3 months or less) from re-infection; however, this protection diminishes 8 months after primary infection.

b) Perforin, Fas or INFγ are not essential in an anti-RV activity mediated by CD8 +
T-cells and CD8+ T-cells expressing α4β7 marker are more efficient in mediating RV clearance.

c) Th1/Th2 pattern of cytokine responses occur, post-immunization with both heterologous or homologous murine RV and RV 2/6 virus-like particles (2/6-VLPs) in mice.

d) In infant and adult mice, RV-specific T-cell responses (both the CD4+ and CD8+) peak on days 5 to 7 after infection and then decline rapidly in suckling mice. Two peaks in the CD8 response on days 7 and 14 post-infection occur in infant mice, indicating the biphasic pattern of RV shedding in infant mice, whereas a single peak occurs in adults.

e) Protection studies done after intranasal immunization with a chimeric VP6 protein and the adjuvant LT (R192G) have demonstrated that CD4+ T cells are the only lymphocytes needed to protect mice against RV shedding and do not require IgA and association of γ-interferon and interleukin-17 production in intestinal CD4+ T cells with protection against RV shedding.

f) Mucosal delivery of NSP4 fusion protein has been shown to elicit Th1 response. Oral immunization with a shiga toxin B subunit: rotavirus NSP4(90) fusion protein is observed to protect mice against gastroenteritis.

Thus, in animal models, role of T-cells in active and passive immunity and in protection from RV disease have been demonstrated.

In humans, it is difficult to study intestinal antigen specific T-cells in situ; studies aimed at characterizing these cells have been limited to identification of circulating T-cells after antigen priming. Lymphoproliferative assays have demonstrated presence of circulating T-cells specific to RV. A positive correlation of lymphoproliferative response with previous RV exposure has been reported in children and adults. RV-specific T-cell responses decline briefly after a serologically-confirmed RV infection in children, whereas healthy adults develop strong and consistent lymphoproliferative responses, implicating role in viral clearance and protection.

The evidence in immuno-deficient children about the role of cell-mediated immune response (CMIR) is concurrent with observations in animal studies.

CMI response may protect against re-infection through secretion of cytokines. Cytokines are secreted primarily by T-cells and macrophages during the activation and effector phases of innate and specific immunity and have an array of critical functions in both immunity and pathogenesis of infectious diseases. They play a role in antiviral defense operating through inhibition of virus infection or by modulating wide range of immune responses. Cytokines such as IFN-γ and TNFα are critical to contain or clear the virus and protect host in the acute phase till other immune responses such as serum antibody responses are elicited. In recent in vitro findings, IFN-γ is shown to inhibit entry of RV into human intestinal cell lines Caco-2 and HT-29.

Little is known about cytokine response and its function in children during RV diarrhea. Few recent studies have demonstrated elevated levels of several cytokines, including IFN-α, IFN-γ, IL-10 and TNF-α in the serum or plasma of children with RV diarrhea. Higher IFN-γ level has been found to be associated with reduced diarrhea severity particularly with vomiting. In a recent study, designed to assess the role of various cytokines in response to virulent or attenuated human RV in pigs, a significantly higher level of IFN-γ conferring protection has been observed in pigs immunized with virulent human rotavirus (HRV), as compared to the attenuated HRV. Thus, these studies implicate role of cytokine responses in protection, but require further evaluation. Recently, there is increasing evidence to show that IFN-γ responses serve as an important tool for evaluation of CMIR to RV. In adults, both CD4+ and CD8+ IFN-γ responses to RV have been detected, whereas in children predominantly CD8+ IFN-γ response is reported.

Conclusions

The mechanisms involved in induction of protective immunity, following RV infection and disease are not well understood. Since, RV infects the small intestine, protection against disease is believed to be mediated by IgA antibodies at the mucosal surface. Serum IgA response is considered as a correlate, reflecting RV-specific mucosal response,
however, not with an accuracy that helps assess various candidate vaccines for clinical trials. The targets of protective immunity are believed to be VP7 and VP4, but data in animal studies have also demonstrated the role of VP6 and NSP4 in mediating protective immunity. The issue remains unresolved in human subjects, though evidence for heterotypic antibody responses to VP6 and NSP4 is documented during natural RV infection. Future studies need to delineate targets for protective, antibody responses in children.

Further, lack of studies to address the role of cellular immunity has resulted in poor understanding of RV immunity. Cellular responses both CD4 and CD8-mediated in murine model have demonstrated role in viral clearance and protection against reinfection. Of late, evidence for cytokine responses and protective immunity mediated by CD4+ T-cells is implicated. However, T-cell responses in human subjects, studied with circulating peripheral cells have shown that responses in children are transient and decline after RV infection. Whereas repeated exposures to RV lead to development of stronger response in adults, implicating their role in viral clearance and protection. Few studies have evaluated cytokine profile, but their role in protection and/or pathogenesis is not conclusive. Thus, considering these limitations future studies to assess cellular responses are required in children during natural RV infection. The efforts for developing and commercializing new vaccines are progressing very rapidly on somewhat temporal basis, but can be strengthened further by an improved understanding of RV immunology.

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