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Vaccine 24 (2006) 2718–2731
Vaccine
www.elsevier.com/locate/vaccine

Review

Immunity and correlates of protection for rotavirus vaccines

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Received 23 May 2005; received in revised form 21 November 2005; accepted 22 December 2005
Available online 17 January 2006

Abstract

Rotaviruses are the most common cause of severe, dehydrating diarrhea in children worldwide. The tremendous global incidence of rotavirus gastroenteritis, especially in developing countries, emphasizes the need for vaccines to prevent associated morbidity and mortality. However, immunity to rotavirus is not completely understood. At this time, total serum RV IgA, measured shortly after infection, appears to be the best marker of protection against rotavirus. This review describes the current understanding of rotavirus immunity, including mechanisms of protection against rotavirus from selected animal models, and correlates of protection associated with natural infection or vaccination from humans.

Keywords: Rotavirus; Immunity; Vaccine

Contents

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doi:10.1016/j.vaccine.2005.12.048

1. Introduction

Since their discovery in 1973 [1] rotaviruses (RVs) have been identified as the most common cause of severe, dehydrating diarrhea in children worldwide [2,3]. RV immunity is not completely understood and issues like the relative role of serotype specific versus heterotypic immunity are controversial [4]. The role of serum antibodies in human immunity to RV has recently been reviewed [5]. Here, we will briefly review aspects of viral pathogenesis, serotype classification and epidemiology that shed light on RV immunity, followed by a discussion of the mechanisms of protection against RV in selected animal models (with emphasis on our results from the adult mouse model), and the correlates of protection associated with natural infection or vaccination in humans. We will emphasize total serum RV IgA and RV serum neutralizing antibodies as possible correlates of protection against disease.

2. Pathogenesis, serotype classification and epidemiology

RVs have characteristics that make them highly infectious pathogens very well adapted to their host (Table 1). RV belong to a large group of pathogens that will never be eradicated because they do not generate sterilizing immunity and because RV can infect animal hosts. Thus, reasonable goals of vaccination are probably to decrease or eliminate severe disease in children but not to prevent infection. The physiopathological mechanisms of RV induced diarrhea are multiple and not yet completely understood. These mechanisms have been reviewed previously [6] and are summarized in Table 2. Both substantial [7] or only minimal [8] histopathological findings have been reported in children with RV diarrhea and thus a direct relationship between the extent of histopathology and disease severity has not been well demonstrated. These divergent findings are compatible with the hypothesis that in children, both enterocyte cell death-independent and enterocyte cells death-dependent mechanisms seem to occur early and late, respectively, in the course of RV disease (Table 2). A better understanding of the mechanisms of RV diarrhea will probably improve our ability to understand immunity to RV illness and devise strategies to treat and prevent RV disease.

RVs are classified serologically into groups and serotypes, based on the antigenic specificities of the internal and outer capsid proteins [9]. They are divided into seven distinct serogroups (A–G), conferred predominantly by VP6, the major structural protein of the virus. The various serogroups are quite distinct genetically and are not capable of reassortment between groups. Viruses within a serogroup can undergo gene reassortment. Most human pathogens belong to groups A, B, and C. Group A viruses are, by far, the most common, and the only ones that will be addressed in this review [9]. Viruses within a serogroup are classified further into serotypes, which are defined by the two outer capsid proteins, VP7 (glycoprotein, G types) and VP4 (protease-sensitive, P types), both involved in virus neutralization. The assays and the rational for determining RV serotypes has been reviewed previously [4]. There are 15 G types

Table 1
Virus associated factors that probably contribute to reinfections

Characteristic	Comment	Reference
Short incubation period (1–2 days)	Does not give time for the recall of high levels of immune effector mechanisms	[9]
The entry cell is the same as the cell used for viral replication	Does not give time for the recall of high levels of immune effector mechanisms	[9]
Virus is excreted in high quantities (up to 10^{11} PFU/g of faeces)	High viral dissemination and innoculum	[9]
Up to 30% of children excrete antigen up to 57 days after onset of diarrhea	High viral dissemination and innoculum	[120]
High rate of viral mutation and gene reassortment	May permit the virus to evade the immune system. Currently unproven	[121]
Over 50% of infections are asymptomatic	RV are well adapted to the human host	[58]

Table 2
Mechanisms of RV induced diarrhea (may vary according to the animal species studied)

Mechanism	Comments	References
Action of NSP4 as a toxin induces a secretory diarrhea	Only demonstrated in rodents. Non-CFTR mediated. Occurs early in infection prior to cell death	[37,122]
RV stimulates the enteric nervous system (ENS) inducing a secretory diarrhea and increased intestinal motility	Drugs that inhibit the ENS are useful to treat RV diarrhea. Occurs early in infection prior to cell death	[123,124]
Altered metabolism of disacaridases and other enterocyte membrane proteins induces malabsortive/osmotic diarrhea	Occurs early in infection prior to cell death	[125,126]
Enterocyte cell death contributes to malabsortive/osmotic diarrhea	Late mechanism. In polarized intestinal epithelial cell lines and in vivo in murine enterocytes RV infected cells seem to die by apoptosis	[127,128]

(genotypes, determined by nucleic acid sequence similarity, and serotypes, determined by antigenic similarity as tested by neutralization assay, are generally equivalent for VP7), with G1, G2, G3, G4 and G9 constituting more than 90% of all human G serotypes detected globally. Additionally, there are 14 P serotypes with one P serotype (P1) representing more than 91% of circulating human RV strains [10]. An absolute relationship between P genotypes and serotypes does not exist. At least 23 P genotypes (P genotype numbers are denoted in brackets) P[1]–P[23] have been described. Importantly, the P[8], and P[4] genotypes correspond to two subtypes (P1A and P1B that share some cross-reactive epitopes) of P1 serotype [11]. Overall strain variability is reduced because worldwide human RV strains belonging to G1, G3, G4 and G9 serotypes are preferentially associated with P[8], while G2 serotype strains are most frequently associated with the P[4] genotype. The distribution of RV with different serotypes varies geographically: while P1A[8]G1 represents over 70% of RV infections in North America, Europe and Australia, these viruses only represent about 30% of the infections in South America and Asia, and 23% in Africa [10]. These variable frequencies could be due to differences of sanitary and climate conditions, or to closer contact of individuals with animal RVs in areas with more RV diversity or both [10]. Not withstanding these differences, approximately 90% and 65% of human RV strains circulating world wide share some cross-reactive epitopes on VP4 and VP7, the two proteins that are the target of protective antibodies (see below). It has been previously argued [4] that the mere existence of RV serotypes suggests a critical role for serotype specific antibodies in protection against RV. However, the relative limited diversity of the outer capsid proteins (specially VP4), that induce neutralizing antibodies, argues in favor of the hypothesis that the immune system is not exercising an important selective pressure against these RVs, and does not favor the appearance of RV with different serotypes or the appreciable antigenic drift of current serotypes.

The distribution of RV serotypes over time also supports a relatively moderate role for a selective pressure of serotype specific antibodies. In a given year, the incidence of individual serotypes can vary from region to region, and multiple G and P types can co-circulate within the same region [10]. In Australia, where surveillance has been maintained over a 28-year period (1973–2001), G1 serotype was the most prevalent strain [10]. This predominance of G1 was temporarily replaced by G2 and G4 strains between 1977–1979 and 1988–1989, respectively. Serotype G9 emerged in 1999–2000 and became predominant from 2001 to 2002 [10]. Thus, G1 RVs can persist over long periods of time in this population, and this seems incompatible with an exclusive role of serotype specific antibodies in protection. However, studies of clinical isolates collected over a 6-year period (1990–1995) in Melbourne, Australia, have suggested that subtype variations (e.g. G1a, G1b) could explain the persistence of serotype G1 RV [12]. During 1990–1993 the monotype G1a predominated, followed by predominance of monotype G1b during 1993–1995 [12]. It is not clear if these subtype variations were due to antigenic drift or not. Similarly to Australia, in Philadelphia, where yearly fluctuations of RV serotypes has been evaluated since 1992, G1 viruses predominate for 1 or more years, with intervals of G2, G3 or G4 predominance [13]. The appearance in 1995–1996 of a P[6]G9 virus (the G9 protein resembles a porcine VP7 [14]) into this well-studied community, permitted investigators to determine that this novel strain (that caused 50% of RV infections in this period), did not cause especially severe disease, or completely displace previously extant serotypes, although it did seem to target younger infants [13]. These findings support the existence of clinically important cross-reactive protective mechanisms against RV. A similar situation has been reported for a P[8]G5 RV (the G5 protein probably of porcine origin) in Rio de Janeiro [10]. However, in Belgium a P[6]G9 RV did cause an outbreak in a neonatal ward [15], suggesting that in this population serotype specific antibodies (obtained transplacentally) could be more important than in older children [16].

A special case of reinfection by RV is posed by virus outbreaks amongst school age children [17] and adults [18,19]. Many outbreaks of this type in the US and Japan have been due to G2 strains (mostly P1B[4] strains) [18,19]. Also, although a relationship between RV serotypes and virulence has not been clearly established [20], some studies have also associated more severe infections in children with G2 viruses, [21] and these viruses have been proposed to constitute a different genogroup from P1A[8], G1, G3, G4 and G9 viruses [22,23]. These studies suggest that infection with non P1B[4]G2 viruses will not induce substantial long lasting protection against the P1B[4]G2 viruses, and these viruses maybe a challenge for monovalent vaccines.

In conclusion, the epidemiological distribution of RV serotypes suggests that serotype specific immunity against RV plays an important, but not exclusive, role in protection along with a clear indication that heterotypic protection also occurs and is clinically significant. Further studies that address RV antigenic drift would help clarify this issue. The most important case in which serotype specific antibodies could play a dominant role in protection appears to be against the P1B[4]G2 viruses, that only share P subtype specific cross-reactive epitopes with most of human RV strains currently circulating.

3. RV immunity

3.1. Mechanisms of protection in animal models

Animal models have been instrumental for our understanding of immunity to RV. A comprehensive review of the literature of this subject is not intended, and with few exceptions, we will only refer to selected papers of the murine and the neonatal gnotobiotic pig models, that have been the most frequently used models. Each animal model offers certain

advantages and disadvantages. For example, the adult mouse model is an infection only model [24], while in the pig model, protection against diarrheal disease can be readily evaluated. In mice serum IgA is actively transported into the bile, while in humans this is not thought to occur [25]. Although gnotobiotic piglets seem to respond with an antiviral antibody response of similar onset and isotypes as piglets with conventional flora [26], it is unclear if the absence of normal intestinal flora may modulate the quality of the B cell [27], T cell and/or innate immune response. In addition, the window to evaluate vaccination of piglets is relatively short [26]. On the other hand, mice develop life long sterilizing immunity after primary homologous RV challenge. Thus, neither model fully mimics the human situation in which children frequently get naturally challenged with virus several months to years after vaccination. The great advantage of the mouse model is the large number of gene targeted immunodeficient animals and lymphocyte specific monoclonal antibody reagents available for study, making this model useful for undertaking mechanistic studies of immune effector mechanisms. In both mice and pigs, experiments can be done using homologous (murine or porcine) RV or heterologous (non-murine or porcine) RV. However, piglets are much more susceptible than mice to illness following heterologous human RV infection [28], and thus, the pig model is probably the one that most closely mimics the human situation in terms of the pathogenecity of human RV challenge.

Using B cell immunodeficient mice, it has been determined that B cells are absolutely necessary for long-term robust protection against RV [24]. In mice, both, a systemic (IgG and IgA expressing B cells present in spleen and bone marrow) and a mucosal (most probably polymeric IgA producing B cells in Peyer's Patches and the intestinal lamina propria (LP)) B cell immune response to RV occur [29]. This is in agreement with recent experiments that show that animals and children, during acute RV infection, undergo an important antigenemic, and in some species viremic phase [30–32]. Passive transfer of intestinally committed but not systemically committed B cells (B cells that express homing receptors specific for mucosal and systemic organs, respectively) into chronically RV infected T and B cell immunodeficient mice mediate an antiviral effect and clear ongoing infection [33]. These experiments suggest that the localization of the effector plasma cells to the intestine is important for their antiviral effect, and that systemic antibodies are not very efficient in protection in this model [33]. The superior antiviral capacity of the antibody secreting cells (ASC), localized to the LP, to mediate an antiviral effect, can be explained by their capacity to mediate expulsion (see below) and exclusion (avoid de novo infection of enterocytes) of RV, inside the enterocyte and in the gut lumen, respectively [34]. The possible mechanism of RV expulsion has been exemplified by the capacity of polymeric IgA anti-VP6 antibodies to mediate an antiviral effect [35]. It is hypothesized that these antibodies, during the trancytosis of polymeric IgA from the basolateral membrane to the gut lumen, bind virus VP6 and "expulse" it to the gut lumen. An alternative (non-mutually exclusive) model suggests that intracellular antibody inhibits RV transcription [36]. Since mainly crypt enterocytes express the polymeric Ig receptor, it is hypothesized that IgA can be loaded by these enterocytes. When the enterocytes reach the tip of the villi (site of RV infection) they still can have some IgA that can mediate the proposed viral expulsion. However, antibodies against VP6 that mediate RV "expulsion" have only been demonstrated in mice. Moreover, it is unknown if these antibodies can modulate RV induced diarrhea. Antibody to VP4 and/or VP7 can block enterocyte infection directly when present in the gut lumen (exclusion) and antibody to NSP4 may block diarrhea, but not infection, via its anti-enterotoxin effects [37]. Finally, although intestinal RV IgA seems to be the most efficient mechanism of protection against RV, virus specific IgG or IgM if present in the intestine, or in sufficient quantities in serum, can by incompletely understood mechanisms reach the intestine, and also mediate protection. This has been demonstrated in IgA deficient mice [38], in passive transfer studies of B cells from IgA KO mice into chronically infected Rag 2 KO mice [39] and recently in a monkey model [40]. A model that integrates and summarizes the above-mentioned experiments is presented in Fig. 1.

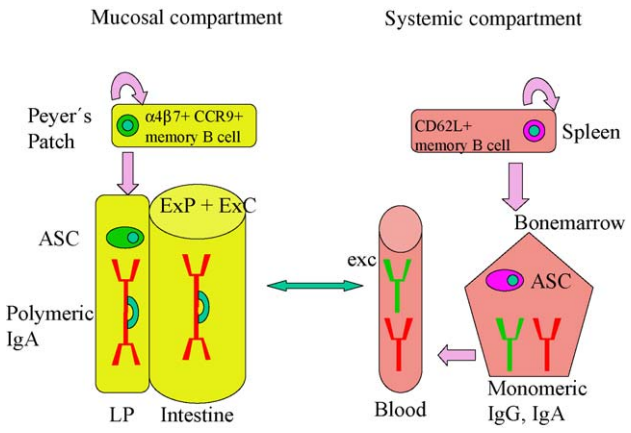

Fig. 1. Simplified model of human RV immunity: RV antigen stimulates two compartments of the immune system: RV antigen in Peyer's Patches generates memory B cells with homing receptors that permit these cells to circulate in blood and to return to Peyer's Patches, and ASC that home to the intestinal LP and secrete polymeric IgA. These antibodies are able to mediate viral expulsion (E × P) and exclusion (E × C) and fill the intestinal cavity. Systemic viral antigen stimulates memory B cells in spleen with homing receptors that permit these cells to circulate in blood and to return to the spleen, and ASC that home to the bone marrow and secrete monomeric IgA and IgG. These antibodies are the main source of serum antibodies. Nonetheless, as indicated by the arrow, antibodies from either compartment (serum and intestinal cavities) can spillover (by mechanisms not completely understood [25,34]) into the other compartment and thus, some systemic antibodies can potentially mediate low levels of intestinal viral exclusion (E × C) and serum IgA can be a correlate of intestinal IgA. For RV, this last situation is specifically seen up to 4 months after infection [65] (see the text for discussion). While the relevance of systemic antibodies that reach the intestinal compartment for anti-toxin protection has been documented [34], this has not been done for antiviral protection.

T cells are also important for antiviral immunity in mice: CD4+ T cells are essential for the development of more than 90% of the RV-specific intestinal IgA and thus, their presence seems critical for the establishment of protective long term memory responses [24]. In addition, it has been shown that, in the absence of T cells, a very low level of RV infection persists [41]. Moreover, murine RV-specific CD8+ T cells are involved in the timely resolution of primary RV infection and can mediate short-term partial protection against reinfection [24].

Both, VP6 [35,42] and NSP4 [37], have been shown to be targets of protective antibodies in adult mice and in mouse pups, respectively. In agreement with the proposed "expulsion" protective mechanisms of anti-VP6 antibodies (see above), these antibodies are not protective when administered passively to the intestinal cavity [35] and milk containing these antibodies is not protective to suckling mice [43]. In the adult mouse model, heterotypic immunity can be induced by heterologuos simian (RRV) and bovine RV [44]. Interestingly, if low doses of the RRV are given orally to mice, the animals respond with systemic, but no intestinal antibody and these animals are not protected. However, upon immunization with higher doses of RRV, both, systemic and mucosal antibodies, as well as protection are induced. High doses of bovine RV (that seem to replicate poorly in mice) were needed for the induction of stool RV IgA and protection in this model. A clear explanation for these findings is not available, but in the case of RRV it could be related to the fact that this virus can efficiently spread from the intestine at relatively low dose and can replicate systemically in mice [24].

In contrast to the adult mouse model of virus shedding, in the piglet model of RV diarrhea, active immunization with virus like particles that contain VP2 and VP6 do not induce protection [45]. Since immunization results in the development of VP6 specific intestinal ASC, these results suggest that in piglets, anti-VP6 antibodies are not protective [45]. However, vaccination with VP6, effectively boosted antibody responses and protection rates in piglets when administered as intranasal booster vaccines after initial priming with an attenuated human RV vaccine [46,47]. Also, in contrast to the findings in mice were passive transfer of milk antibodies against NSP4 are protective [37], in piglets after active immunization with selected RVs, no correlation was found between antibodies against NSP4 and protection [48]. In this model, sharing of VP4 or VP7 between the immunizing virus and the challenge virus seems to be required for protection (Fig. 2) [49,50]. It is difficult to know if children are more capable of inducing cross-reactive neutralizing antibodies than gnotobiotic neonatal piglets. However, since humans RVs exhibit relatively low VP4 diversity, one can conclude that after natural infection or vaccination with RV strains that express a P1 serotype, a relative cross-reactive protective response will likely be present. The direct demonstration of heterotypic cross-reactive human monoclonal antibodies isolated from adults has recently been documented and heterotypic monoclonal antibodies to VP7 have been shown to occur in mice [51].

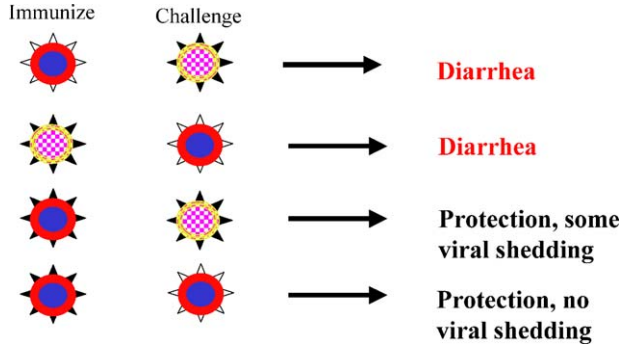

Fig. 2. Cartoon representation of experiments described in [48], illustrating that, in pigs, the immunizing and challenge RVs only need to share VP4 so protection against diarrhea is induced. Two homologous RVs that do not share VP4 (outer triangles) or VP7 (outer thick circle) fail to induce protection against each other (top two rows). A VP4 monoreasortant between the two viruses is able to induce protection against diarrhea induced by both parental viruses (bottom two rows). Sharing of VP7 and other viral proteins (inner circle) between the immunizing and challenge RV, results in protection against infection (lower row).

3.2. Adult human challenge studies

Two groups have performed immunological studies with adults experimentally challenged with homologous human RV. The earliest of these studied showed that the presence of pre-inoculation serum immunofluorescent antibody to the RV strain used for inoculation, or high levels of neutralizing antibody to selected RV strains, correlated with resistance to diarrheal illness [52]. On further analysis, the authors were also able to show a correlation between protection and pre-challenge serum antibodies directed at neutralizing epitopes on the serotype G1 and serotype G3 [53]. The relationship of pre-existing local neutralizing activity in intestinal fluid with protection was not strong [52], because only a few volunteers possessed these antibodies. Thus, for this group, the presence of serum neutralizing antibodies was the best correlate of protection, and jejunal neutralizing antibodies (not detected in infected individuals) were only present in some protected individuals.

In an initial adult challenge study, Ward et al. did not find a correlation between serum antibodies [54] or stool RV IgA [55] with protection. In a second study, the same authors tried to correlate pre-inoculation titers of serum neutralizing antibody, serum RVs IgA, serum RV IgG, jejunal neutralizing antibody, jejunal RV IgA, and stool RV IgA with protection [56]. Stool RV IgA titers could not be correlated to either infection or illness, but the mean titers of the other five antibodies were significantly or nearly significantly lower in individuals that became infected or developed diarrhea [56]. Of these antibodies, serum RV IgG best predicted the probability of infection, and jejunal neutralizing antibody best predicted the probability of illness [56]. Studies of the

rechallenge of a subset of these volunteers, gave further support to the conclusion that antibodies play a mayor role in protection against reinfection [57]. Nonetheless, protection observed was higher than that predicted, based on the levels of pre-inoculation serum antibodies, suggesting that other factors, in addition to antibodies, may also mediate protection [57]. In conclusion, as expected from the animal studies discussed above, the best correlate of protection against disease in these studies was jejunal neutralizing antibodies. An intriguing finding from these studies is the high levels of pre-inoculation stool RV IgA present in some adults that became infected [55]. The reason for this finding is not clear, but could be related to the fact that in the stool low affinity polyclonal IgA antibodies are present. These antibodies, either aggregated or not by the Fv protein could potentially give false positive results [34]. Whatever the explanation, these results suggest that fecal antibodies are not a good correlate of protection in adults.

3.3. Correlates of protection after natural infection of children

An important study that has evaluated correlates of protection against natural RV reinfection was performed by Velaquez et al. [58,59]. These authors followed a cohort of children from birth to 2 years of age, taking monthly serum samples and weekly stool samples. This study showed that first infections are generally the most severe, with severity decreasing as the number of infections increases. Protection conferred by infection was greatest against moderate-to-severe disease, less against mild illness, and least against asymptomatic infection. Asymptomatic infection conferred protection to a degree comparable to that achieved by symptomatic infection. The occurrence of two RV infections, whether symptomatic or asymptomatic, resulted in complete protection against moderate-to-severe illness. A single natural infection conferred protection generally comparable to successful RV vaccine candidates. Children with a total serum RV IgA titer >1:800 had a lower risk of RV infection and were protected completely against moderate-to-severe diarrhea. However, children with an IgG titer >1:6400 were protected against RV infection but not against RV diarrhea. Protective antibody titers were achieved after two consecutive symptomatic or asymptomatic RV infections. A strong trend was present for repeated infections with the same G serotype being less likely to occur, suggesting homotypic protection. However, this trend has not been clearly seen in other studies [60,61]. In addition, a single natural infection clearly offered some level of protection against severe disease from a heterotypic G strain which conclusively demonstrated the presence of heterotypic immunity as well.

Two studies in day care centers support conclusions from the previous cohort study by Velazquez and colleagues. In a Texas study, total serum RV IgA titers >1:200 and IgG titer >1:800 correlated with protection against infection and illness [62]. In a similar study in Denmark, total serum levels of IgA but not IgG correlated with less severe disease [63]. The authors of this last study provided an explanation as to why total serum RV IgA may correlate with protection: they found that the presence of total RV serum IgA correlated with the presence of serum immunoglobulin bound to secretory component. These antibodies are most probably polymeric IgA originating in the gut and reaching the serum as a "spillover" from the intestine (Fig. 1). At present a clear explanation as to how these antibodies are localized to the serum is not available [25] but their appearance seems to be common after mucosal infections [64]. The RV IgA antibodies specifically bound to secretory component disappeared from serum in less than 4 months after infection [63]. Moreover, the amounts of RV secretory component bound Ig found in serum about 1 week after the infection correlated to the amounts of RV secretory component in duodenal fluid [65]. In support of these findings, a more recent study correlated the presence of RV IgA secreting cells in the small intestine of children with total RV serum IgA [66]. Nonetheless, this correlation was not very strong probably because the children examined had been infected with RV long before the intestinal samples were available for study. Thus, total RV specific serum IgA within 4 months after infection seems to be a good correlate of intestinal IgA.

A cohort and a day care center study have addressed the role of stool IgA as a correlate of protection against natural RV infection in children [67,68]. In the day care center study, higher stool RV IgA antibody titers were associated with protection against infection and illness [67]. In the Australian cohort study, it was shown that frequent RV infection of children appears to stimulate production of sustained levels of fecal IgA, which correlates with protection against infection and disease [68]. An important finding from both studies was that, although a correlation between stool IgA and protection could be established, there were significant numbers of children with elevated pre-infection stool IgA titers that became infected. This finding is reminiscent of the adults challenge studies mentioned above [55]. However, in the infant children studies some of the stool IgA could have been of maternal milk origin, and perhaps this passively acquired IgA could have lower protective efficacy because it cannot mediate viral expulsion. Of note, while in some studies IgA in maternal milk has correlated with protection against RV diarrhea [69], in others, breast feeding only had a modest anti-viral effect [70] and this effect has been ascribed to its content of lactadherin and not RV IgA [71]. Irrespective of the explanation for the presence of the fecal IgA in infected children, these results suggest that fecal IgA is not a very good correlate of protection. An additional critical observation from the Australian study was that the fecal IgA in a subset of children was not long lasting (duration of a few weeks). Moreover, the children in whom the fecal IgA disappeared were the children most prone to reinfections and disease. In one of the adult challenge studies described above, a direct comparison between the longevity of serum and stool antibodies was performed. It was shown that stool antibodies decay more rapidly than

serum antibodies, but that both of these antibodies were able to persist for over a year [72]. Taken together these results suggest that intestinal antibodies, in both children and adults, seem to be shorter lived than serum antibodies. In addition, the explanation for their shorter duration in children as compared with adults, could involve other factors like immaturity of their immune system or simply the fact that their immune responses are primary or have been boosted less often than those of adults.

Several studies have analyzed the role of serum neutralizing antibodies in protection against RV. In an early study with children in an orphanage, protection against RV gastroenteritis seemed to be serotype specific and to be related to levels of antibody against homotypic virus [73]. A homotypic neutralizing antibody level of >1:128 appeared to be protective. However, seroconversions or concomitant antibody responses to G1 or G4 RV in most children with G3 RV infections, suggested that heterotypic immunity to RV can be induced [73]. In agreement with this last finding, a day care center study demonstrated that upon first exposures to RV G types, children develop predominantly homotypic antibody [74]. However, as the number of RV infections increase, children develop heterotypic antibody to G types at levels that correlate with broad protection against RV infection and illness, despite exposure to a restricted number of G types [74]. In a refinement of these studies, it was shown that higher IgA, IgG, and homotypic antibody levels to the antigenic site C of the G1 and G3 VP7s, correlated with protection against infection and illness, independently of total serum RV IgA or IgG titers [62].

In a case control study with 4–35-month-old children in Bangladesh, titers of both homologous and heterologous neutralizing antibody in acute serum specimens of children with RV infection were significantly lower than those of matched controls [75]. However, statistical analysis showed that only antibody titers to heterotypic RVs were independently associated with protection against RV disease [75].

In conclusion, total serum RV IgA and serum homotypic and heterotypic neutralizing antibodies seem to be better correlates of protection than total serum RV IgG induced by natural infection in children. In the context of the animal model results, the capacity of total serum RV IgA (that is mostly directed to the cross-reactive VP6 and thus only mediates viral explusion) to predict protection, can be understood because these antibodies generally reflect intestinal antibodies and can be a surrogate marker for the potentially "more" protective cross-reactive antibodies against VP4 and/or VP7 that mediate both expulsion and exclusion (see Figs. 1 and 2).

3.4. Correlates of protection after vaccination of children

Three types of live oral RV vaccines have been extensively tested in people: Jennerian or modified Jennerian bovine and simian RVs vaccines (attenuation based on host range restriction) and human attenuated vaccines (attenuation presumably based on multiple passages in cell culture). Since the correlates of protection as well as the basis for attenuation for these vaccines may be different, we will address them separately. We will address the capacity of each type of vaccine to induce homotypic/heterotypic protection and the role of serum IgA and neutralizing antibodies as correlates of protection.

3.4.1. Jennerian vaccines

3.4.1.1. RRV vaccines. Early studies with the monovalent P5B[3]G3 RRV vaccine showed that protection induced by low doses (10^4 PFU per child) of this vaccine were inconsistent, varying from 0 to 85% against moderate to severe diarrhea [76]. Moreover, in some of these studies, analysis of the RV detected suggests that the immunity induced by the vaccine was serotype specific, since significant protection was evident when serotype G3 RVs were circulating [77], but not in predominant seasons of G1 RVs [78]. However, studies in Finland with G3 monovalent RRV and monovalent RRV × human VP7 G1 reassortants showed that both vaccines induced fairly comparable immunity, that did not appear to be serotype specific [79]. In one of these studies, for example, a G1 and G2 RRV-human reassortant vaccines were comparable in inducing protection against a predominant G1 RV [80]. In a similar study in the US, in which the G3 monovalent RRV vaccine was compared to the G1 reassortant, the former induce a 58.5% heterotypic protection while the latter provided homotypic protection of 72.8%, over three seasons, against the predominant circulating serotype G1 RV [81]. Hence, in this study both homotypic and heterotypic immunity were observed, with homotypic immunity being somewhat greater. In addition, the presence of serum neutralizing antibodies to G1 RV or RV-specific IgA after vaccination, and before the first season, did not correlated with protection [81]. In a study in Peru [82], the G3 RRV monovalent vaccine was compared to the G1 and G2 RRV reassortants. Fifty percent of vaccinees developed an IgA ELISA seroresponse; however, a serotype-specific seroresponses were demonstrated in only 20% of the children against each of the three vaccine strains. Only the G3 RRV monovalent vaccine had protective efficacy (29%), against the circulating serotype G1 or G2 RV strains. Neither serotype G1 or serotype G2 RRV reassortant vaccines were protective against serotype G1 or G2 RV diarrhea. Thus, in this study, serum IgA was higher than the level of protection induced by the vaccine, and protection did not seem to be serotype specific or to correlate with the presence of neutralizing antibodies [82].

On this background, the tetravalent RRV (RRV plus monoreassortants that contain human G1, G2, or G4 and all other genes from RRV) vaccine was designed with the hope of broadening the diversity of neutralizing antibodies induced [76]. One low dose (10^4 PFU) [83] and two high dose (10^5 PFU) dose [84,85] studies compared the RRV-TV vaccine to the monovalent G1 RRV reassortant. These studies are probably the best available evidence supporting the utility of a polyvalent vaccine [4]. In the low dose study both vaccines protected against disease caused by serotype G1

RV during the first RV season. During the second RV season, the monovalent vaccine did not protect against any RV serotype, and the tetravalent vaccine reduced the incidence of disease caused by non-serotype G1 RV. Thus, investigators were unable to determine if the result represented serotype-specific protection or quantitative differences in the duration of protection induced by the two vaccines. The first high dose study, in a Native American population [84], showed that the protection against serotype G3 viruses induced by the RRV-TV was higher than that induced by the monovalent G1 vaccine [4]. In the third study, both vaccines protected against the predominant circulating serotype G1 viruses and there was a non-statistically significant trend for the RRV-TV to better protect against G3 viruses [85]. In conclusion, these three studies suggest that the monovalent G1 vaccine was less efficacious against G3 viruses than the RRV-TV. This evidence is not unambiguous, however, and evidence for the protective efficacy of the RRV-TV against G2 and G4 seems to be unavailable. Furthermore, the more consistent results of the RRV-TV vaccine cannot be historically compared to the inconsistent results of the monovalent or bivalent vaccine trials because in the former, mostly high doses and in the latter low doses of the vaccine were used. Thus, support for the need of polyvalent RV vaccines is reasonable but not conclusive.

In the three studies described above [83–85], as in the previously mentioned study of the monovalent RRV vaccine [81], total serum RV IgA and neutralizing antibodies were poor correlates of protection [86,87]. Interestingly, the reasons for the lack of correlation with protection for serum IgA and neutralizing antibodies seem to be different: in general, serotype specific conversions in neutralizing antibodies following vaccination with the RRV based vaccines has been low and substantially below the level of protective efficacies observed. On the other hand, the frequency of children demonstrating total serum RV IgA serconversion was generally higher than the protective efficacy. Of note, while in the low dose study no correlate of protection was observed, in the high dose study a correlation was found between total serum RV IgA and selected neutralizing antibody titers and protection. However, in the high dose study, serotype-specific immunity was no more significantly associated with protection than heterotypic immunity, and no specific titer of any antibody analyzed was a reliable indicator of protection [87]. This situation is reminiscent of the studies of mice vaccinated with high and low doses of RRV in which high doses produced both systemic and mucosal immunity, but low doses produced only systemic antibodies [44], and suggests that in the RRV vaccinated children (specially those receiving the low dose vaccine) some of the serum IgA may not be of mucosal, but rather of systemic origin (see Fig. 1). This interpretation is generally supported by other RRV-TV vaccines studies in Latin-America [88–90] and could explain why RRV induced serum IgA does not predict protection [91]. RRV-TV was licensed in the United States in 1998, but was withdrawn from the market 9 months later after reports of its association with intussusception [92].

3.4.1.2. Bovine vaccines. Two bovine vaccines RIT 4237 (withdrawn from trials in the 1980s) and WC3 based vaccines have been the most extensively tested for protection and the only ones we will address here. Of note, a third bovine based reassortants vaccine, UK bovine × human, has been developed by investigators at the NIH, shown to be safe and efficacious in a larger phase 2 trial in Finland, and is currently being licensed to several manufacturers in less developed countries [10].

Early studies of the RIT 4237 (NCDV-based P6[1]G6) vaccine were encouraging because the vaccine clearly showed substantial heterotypic protection as it shared neither G nor P types with human strains [93]. In a second study with this vaccine it provided 82% protection and IgG seroconversion (IgA seroconversion was not measured) was seen in 53% of the initially seronegative children [94]. Clinical protection correlated with IgG seroconversion, but the vaccinees who failed to seroconvert also had less RV diarrhea than the placebo recipients, suggesting that immunity may be mediated by factors other than or in addition to serum IgG. Heterotypic protection was seen in this study against G1, G2 and G3 viruses [94]. However, in later studies with RIT 4237, protection was not seen and the vaccine was discontinued [95]. Thus, as with the heterologous simian RRV vaccine, protective heterotypic efficacy was variable, but unlike that vaccine, the RIT 4237 vaccine seemed to induce a lower rate of seroconversion than protection.

Similar to the previous vaccine, the bovine WC3 (P7[5]G6) vaccine strain provided variable (0–76%) heterotypic protection against RV disease [96,97]. Serum neutralizing antibody titers to WC3 were high in vaccinees in many of these studies, but low to human RV serotypes and thus, neutralizing antibodies did not correlate with protection in most studies [96–98]. In a trial in which the vaccine did not protect, serum and stool IgA responses were present in an important fraction of children [98]. Thus, no clear correlate of protection was found for this vaccine formulation.

Analogously to the situation with the RRV vaccine, a human G1 reassortant with WC3 (WI79-9 P7[5]G1) was generated with the hope of inducing more neutralizing antibodies to human RVs [99]. This vaccine was shown to induce over 64% protection against all RV disease [99,100]. However, this reassortant was not very efficient at inducing G1 specific neutralizing antibodies, and neutralizing antibodies, again did not correlate with protection [101]. Moreover, in a limited number (nine) of children studied, no fecal IgA was induced by the vaccine [101], suggesting that stool IgA also did not correlate with protection. To broaden the antigens presented by this vaccine, a human G1 and G2 reassortants [102] and also a quadrivalent WC3 reassortant formulation, that contained four separate monovalent reassortants expressing human G1, G2, G3, and P1A[8], were tested. Both vaccines induced over 67.1% protection against

all RV disease [97,102,103]. However, since the predominant challenge virus for these studies were G1 strains, like for the RRV vaccines, the demonstration of the utility of the multivalent vaccine for protection against non-G1 strains is currently lacking in the literature, but seems likely [100]. Furthermore, similar to the situation with the RRV vaccines, comparison between the results of the polyvalent and monovalent WC3 vaccines is difficult because most studies administered only 1 or 2 doses of the monovalent vaccines while 3 doses have been administered of the polyvalent vaccines. No detailed analysis of the correlation between protection and the different antibodies (neutralizing, total serum or stool RV IgA) induced by the polyvalent WC3 vaccines has been presented. Nonetheless, neutralizing serum antibodies, serum or stool RV IgA have been reported at similar frequencies in the vaccinees, as the frequency of protection induced by the vaccine [102–105], and it will not be surprising if any of these antibodies results in a fairly good correlate of protection. A pentavalent formulation of WC3 reassortant vaccine is undergoing extensive phase three trials and additional data correlating type specific immune responses with protection from both G1 and non-G1 types is expected shortly [106].

3.4.2. Attenuated human vaccines

Although several neonatal human RV strains have been proposed as vaccines and some are still undergoing study, we will only refer here to studies of the monovalent tissue culture attenuated P1A[8]G1 vaccine derived from the 89-12 strain of wild type human RV [107]. This human RV has been passed multiple times in cell culture and is attenuated. In its first trial, the 89-12 vaccine induced 89% protection against any RV disease and 91% and 74% of the children developed serum RV IgA and neutralizing antibodies. The RIX4414 viral clone derived from 89-12 has been recently shown, in large multicenter studies, to induce serum IgA titers in 61–91% of vaccinated infants, and to significantly reduced RV gastroenteritis episodes (83–91% protection against moderate to severe episodes) and RV hospitalizations in vaccinated infants, compared with placebo recipients [108,109]. Vaccine efficacy was observed against severe RV gastroenteritis caused by G1 and non-G1 types, specifically G9 [108]. Thus, this vaccine seems to resemble natural asymptomatic infection, and it may well be that serum IgA will be a good correlate of vaccine induced protection. This vaccine has been recently approved for use in Mexico [110].

3.4.3. Other vaccine related studies

One study with the RRV reassortant vaccines has addressed the possibility that lack of correlation of serotype specific antibodies with protection could be due to G1 subtype variation in the circulating challenge strains compared to the G1 of the vaccine strain [111]. The authors compared post-vaccination neutralizing antibody titers of vaccinees against RV resembling the vaccine strain and G1 breakthrough strains, using sera from subjects who experienced breakthrough. Post-immunization neutralizing antibody titers to the vaccine-like strain, were greater than to the breakthrough strains. This difference did not, however, correlate with lack of protection, since similar differences in titer to the vaccine-like strain and breakthrough strains were found using post-vaccination sera from vaccinees who either experienced asymptomatic RV infections or no infections. Moreover, both breakthrough and control strains from the trial were in a lineage different from the serotype G1 vaccine strain. Thus, the results did not support the hypothesis that immune selection of antigenically distinct escape mutants led to vaccine breakthrough in subjects. However, it could not be excluded that vaccine failures could be partially due to antigenic differences in the VP7 proteins of currently circulating G1 strains [111].

4. Future studies to better understand immunity to RV and obtain better correlates of protection

From the above discussion, it is clear that we need a better understanding of immunity to RV in children. The nature of human cross-reactive antibodies against VP4, or VP7, is poorly understood. A recent study of human monoclonal neutralizing antibodies showed that cross-reactive neutralization epitopes recognized by humans may be distinct from those of mice. Moreover, the two human monoclonal antibodies evaluated were cross-reactive for at least two P genotypes [51]. Hence, humans, like mice, can clearly make antibodies that have heterotypic specificity. The cross reactivity of human monoclonal to VP7 has not yet been extensively investigated but murine monoclonals directed at VP7 can have heterotypic specificity [51]. Another important unresolved issue is why primary natural RV infection does not induce long-lived complete protective immunity. As mentioned previously, this is probably related to the fact that intestinal antibody responses in children are short lived. Moreover, this short lived response of protective intestinal antibodies could be related to the poor response of RV specific IFNγ secreting CD4+ T cells present in naturally infected adults and children [112,113]. Finally, studies with oral salmonella vaccines have evaluated blood circulating specific ASC as correlates of protection [114]. It is hypothesized that these circulating ASC have been stimulated by the vaccine antigen at the intestinal surfaces and they are on their way back to those sites for local antibody secretion. In this study, the number of ASC provided better correlates of protection than serum antibodies. This was probably because secondary immune responses, that produced no increase in serum antibodies, did produce a boost in the potentially mucosally committed ASC. The lack of stimulation of serum antibodies, by a secondary mucosal immune response, is reminiscent of studies that have proposed that coproconversion is more sensitive than seroconversion at detecting secondary RV infection [115]. Moreover, evidence exists that RV specific blood ASC in piglets [28] and humans [66] correlate with intestinal RV ASC. Based on these studies, we

have begun to study RV specific ASC in naturally infected children [116] as potential correlates of protection. RV-ASC are present in blood for only a short period of time and, as reported by others, are mainly of the IgM isotype [117]. For this reason, we have concentrated on the study of memory RV specific B cells that express intestinal homing receptors [116,118] as correlates of protection against RV. RV specific intestinal memory B cells have been shown to correlate with protection in the piglet model [119]. These cells seem to persist in circulation for more prolonged time than ASC and hopefully will reflect intestinal memory isotype-switched B cells.

5. Conclusions

a. Intestinal IgA is probably the most important mechanism for long-term protection against RV.
b. Total serum RV IgA measured shortly after infection generally reflects intestinal IgA levels, and may be the best (but imperfect) available marker that correlates with protection against RV. Total serum RV IgA can probably be used as a correlate of protection for many of the vaccines in development and in use.
c. Correlates of protection after vaccination may vary depending on the type (homologous versus heterologous) of vaccine studied: for some heterologous vaccines, serum antibodies may not provide adequate correlates of protection; for homologous vaccines serum IgA may reflect antiviral immunity.
d. The relative role played by serotype specific versus heterotypic antibodies against RV in children is still uncertain. Moreover, the need for polyvalent RV vaccines and for adjustment of the RV vaccines in accordance with the emergence of new RV serotypes is unknown.
e. Furthermore, doubts exist concerning the importance of subtype immunity to RV. Post licensure epidemiological studies following introduction of new vaccines maybe a good opportunity to address the effect of antigenic drift on immunity to RV.
f. The lack of a clear understanding of protective immunity to RV infection is an important limitation of current vaccine development.
g. Studies of children's RV immune response, and of the effects of maternal immunity on this response are needed.
h. The tremendous global incidence of RV infection, especially in developing countries, emphasizes the need for improved vaccines to prevent childhood deaths.

Acknowledgments

This work was supported by funds from the Pontificia Universidad Javeriana, Colciencias Grants 1203-04-12638 and 1203-04-16466, FIRCA Grants TWO5647-01 and TW005647-04 and by NIH Grants R01 AI21362-20 and Digestive Disease Center Grant DK56339 and a VA Merit Review Grant.

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