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Rotavirus

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ABSTRACT Rotaviruses (RV) are ubiquitous, highly infectious, segmented double-stranded RNA genome viruses of importance in public health because of the severe acute gastroenteritis they cause in young children and many animal species. They are very well adapted to their host, with symptomatic and asymptomatic reinfections being virtually universal during the first 3 years of life. Antibodies are the major arm of the immune system responsible for protecting infants from RV reinfection. The relationship between the virus and the B cells (Bc) that produce these antibodies is complex and incompletely understood: most blood-circulating Bc that express RV-specific immunoglobulin (Ig) on their surface (RV-Ig) are naive Bc and recognize the intermediate capsid viral protein VP6 with low affinity. When compared to non-antigen-specific Bc, RV-Bc are enriched in $CD27^+$ memory Bc (mBc) that express IgM. The Ig genes used by naive RV-Bc are different than those expressed by RV-mBc, suggesting that the latter do not primarily develop from the former. Although RV predominantly infects mature villus enterocytes, an acute systemic viremia also occurs and RV-Bc can be thought of as belonging to either the intestinal or systemic immune compartments. Serotype-specific or heterotypic RV antibodies appear to mediate protection by multiple mechanisms, including intracellular and extracellular homotypic and heterotypic neutralization. Passive administration of RV-Ig can be used either prophylactically or therapeutically. A better understanding of the Bc response generated against RV will improve our capacity to identify improved correlates of protection for RV vaccines.

INTRODUCTION

Rotaviruses (RV) are ubiquitous highly infectious double-stranded RNA viruses of importance in public health because of the severe acute gastroenteritis (GE) they cause in young children and many other animal species. They are very well adapted to their host, causing frequent symptomatic and asymptomatic reinfections. Antibodies are the major component of the immune system that protects infants against RV reinfection. The relationship between the virus and the B cells (Bc) that produce these antibodies is complex and incompletely understood (1). In this review, the following basic aspects of RV-specific Bc (RV-Bc) will be addressed: (i) ontogeny; (ii) use of immunoglobulin (Ig) genes; (iii) differential distribution (compartmentalization) in the intestinal and systemic immune systems; (iv) specificity of RV-Ig produced and the mechanisms by which it mediates protection; and finally, (v) practical applications for the use of RV-Ig, including RV-Ig as a prophylactic or therapeutic agent and as a correlate of protection. The immune response generated against RV vaccines has been recently reviewed (2, 3) and will only be briefly discussed. The focus of this review is antibodies induced by natural RV infection in humans, but reference to studies of the murine and porcine animal models of RV infection will be made when necessary.

THE ANTIBODY RESPONSE AGAINST RV

(i) Ontogeny of RV-Bc and RV-Ig

A newborn receives important quantities of RV-IgG and RV-IgM transplacentally. High levels of these transplacentally transmitted Igs correlate with lower numbers of RV infections in young children and probably mediate protection against severe RV disease (4). In addition,

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maternal antibodies are also transferred to the infant through breast milk. It has been postulated that colostrum secretory IgA (sIgA) can be systemically absorbed to some degree (5), but most antibodies in the serum of neonates are transplacentally acquired and do not contain RV-IgA. Although breast milk antibodies are thought to have a local intestinal antiviral effect, this effect is relatively modest when it comes to preventing severe RV disease (1). In animal models, high-titered serum maternal antibodies (similar to what is observed in children from low-income countries) have some protective effect and can also inhibit the response to an RV vaccine (6). In contrast, low-titered maternal antibody in serum (as seen in children from high-income countries) did not protect against infection. The experimental conditions that induced low-titered maternal antibody in serum had a complex effect on subsequent immune response, which included an increase in the numbers of RV antibody-secreting cells (ASC) in the intestine induced upon infection in a porcine model system (7). In children, the presence of high-titered maternal antibodies in both serum and milk is thought to partially explain the lower immunogenicity of RV vaccines in low-income countries (3). The effect of maternal antibody levels and quality on RV vaccine immunogenicity in children needs to be further investigated (3).

Fortunately, RV is one of a few microbial antigens for which a flow cytometry assay to identify and characterize Bc expressing RV-Ig at the membrane surface has been developed. Bc that bind green fluorescent protein (GFP)-containing RV virus-like particles (VLPs) made from viral core protein VP2 and intermediate capsid protein VP6 (an immunodominant antibody target) are considered RV-Bc. Figure 1 shows an example of a typical experiment using RV GFP-containing VLPs, and Table 1 gives a summary of the subsets of RV-Bc that have been shown to specifically bind GFP-containing VLPs using flow cytometry-based assays.

Unexpectedly, children are born with a high number (approximately 1 to 2%) of RV-Bc with a naive phenotype (8). These Bc secrete antibodies that have a low affinity to VP6 (9). Intriguingly, by 2 months of age, children will have a high number of RV-Bc that express IgM, IgD, and CD27 (generally considered a marker constellation for memory Bc [mBc]), irrespective of whether the children have had an RV infection, as evidenced by the presence of RV-IgA in serum (10). It is uncertain at present if these RV-mBc have or have not been induced by asymptomatic or symptomatic RV infections (either enteric or systemic) that, for some reason, do not induce serum RV-IgA. However, they may be

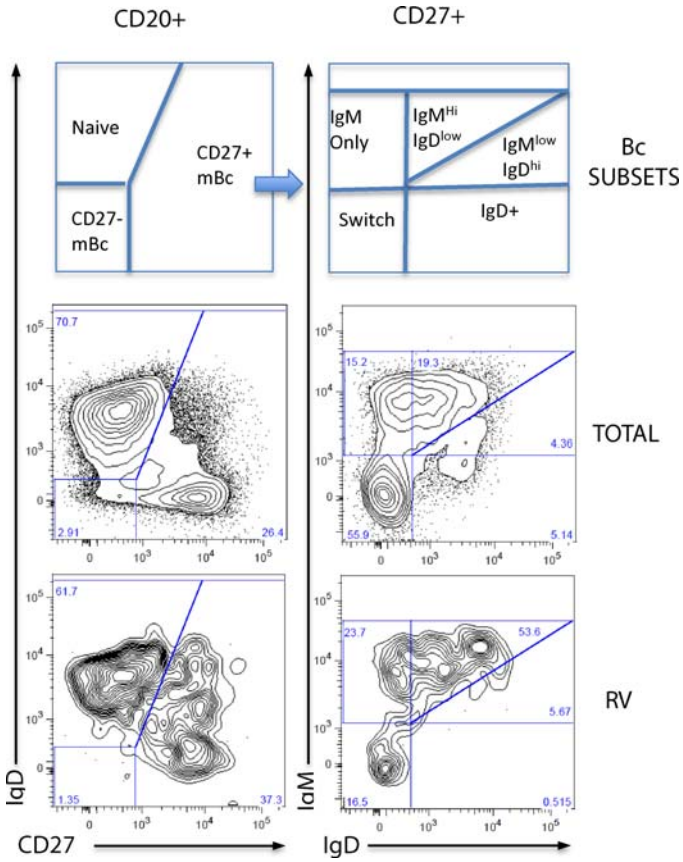

FIGURE 1 Flow cytometry experiment to characterize and compare RV-Bc to total Bc subsets (from reference 11 with modifications). Top row plots illustrate the Bc subsets considered. Middle row dot plots are gated on total $CD20^+$ Bc. Bottom row plots are gated on $CD20^+$ Bc that bind fluorescent RV VLPs (RV-Bc). As a first step in this analysis, Bc are evaluated for the expression of IgD and CD27 (left dot plots). IgD^+ $CD27^-$ Bc are naive cells, and IgD^- $CD27^-$ (IgM, IgG, or IgA^+) are a low-frequency subset of mBc. The $CD27^+$ Bc of the left panels are further analyzed for the expression of IgD and IgM (right dot plots). IgM^- IgD^- cells are classical switched mBc; IgM^+ IgD^- cells are called IgM-only mBc and resemble in many ways the switched mBc. IgD^+ IgM^- cells are a poorly characterized subset of mBc. Double-positive IgM^+ IgD^+ mBc are a heterogeneous population of mBc; in the present experiment, they are further subdivided into IgM^{hi} IgD^{low} (a phenotype that resembles marginal zone Bc) and IgM^{low} IgD^{hi}. doi:10.1128/microbiolspec.AID-0011-2013.f1

playing a role in RV immunity. In experiments in which human IgM-mBc (mostly IgD^+) are passively transferred to RV-infected immunodeficient mice, they are able to switch to IgG ASC and mediate immunity against RV antigenemia and viremia (11). Moreover, RV-mBc from adults are enriched in the IgD^{low} IgM^{hi} $CD27^+$ subset of Bc (11) (Fig. 1, Table 1). This phenotype is reminiscent of spleen marginal zone Bc, a subset that has been

postulated to develop (by an unknown mechanism) a prediversified Ig repertoire and to participate in "innate" Ig responses to pathogens (12).

Between 6 months (when maternal antibodies wane) and 3 years of age, almost every child will have been infected by RV, and approximately 50% of them will have had at least one symptomatic infection that consists primarily of diarrhea and vomiting (3). The primary Bc response in the peripheral blood of these children is characterized acutely by the presence of circulating RV-IgM ASC (13). Between 1 and 2 weeks after infection, the classical IgD^- $CD27^+$ mBc appear in blood (14), and concomitantly, RV-IgA and IgM can be detected in serum (15) and stool (16). Importantly, in the serum of these children, secretory antibodies (antibodies that have been secreted to the intestinal lumen) can also be identified (17). RV sIgs present in the stool and serum originate from intestinal lamina propria RV ASCs that secrete polymeric antibodies. These antibodies are transported by the polymeric Ig receptor, present on the basolateral membrane of enterocytes, to the lumen of the intestine. In the lumen, proteases cleave the receptor, leaving part of it covalently attached to the Ig. This portion of the receptor, known as the secretory component, serves as a marker of sIg and protects the Igs from degradation in the harsh intestinal lumen. By an unknown mechanism, some of these sIg antibodies are retrotranscytosed and reach the systemic circulation (18). Four months after viral infection, RV-IgA persist in the circulation of children but the RV-sIg have disappeared (17). This finding has been associated with the fact that the production of local intestinal antibodies is short lived compared to antibodies (especially RV-IgG) present in serum (see below for discussion on the persistence of RV-Ig) (19).

Children and adults can be reinfected with RV, with the majority of reinfections being less symptomatic or asymptomatic compared to the first one or two. When caring for children with RV infections, up to 50% of parents become infected and half of them will have mild GE symptoms. Among the elderly, it is not uncommon for symptomatic RV outbreaks to reoccur, presumably due to waning immunity and or immunesenescence in this population (1).

(ii) Ig Gene Usage of RV-Bc

The relatively high number of circulating RV-Bc and the availability of a flow cytometry assay (Fig. 1) to detect them have permitted extensive studies of the Ig genes used by these cells in human peripheral blood and, to a much lesser degree, the intestine (9, 20, 21). The general strategy to perform these studies has been to clone and express the genes of single RV-Bc sorted (using the same strategy used to identify RV-Bc by flow cytometry described above and in Fig. 1) and in some cases expanded in vitro. These studies are robust because the genes amplified by PCR come from single cells and the specificity and functionality of the antibody they produce can be further tested.

Initial findings showed that, compared to non-antigen-specific Bc, RV-Bc of healthy adults have a biased usage of VH genes (21). In addition, the RV-Bc VH, D, JH, VL, and JL segment usage, extent of junctional diversity, and mean H chain complementarity-determining region 3 length of adults and infants were found to be similar (21). The genetic resemblance between cells from infants and adults was unexpected, since it was thought that children's Bc would have similarities to Bc present during the fetal period, which have a particular repertoire of VH and VL gene usage and lack junctional diversity. These results suggested that the Bc repertoire is not a limiting determinant of the quality of antibody responses to RV in children (21). Nonetheless, a subsequent report showed that VH sequences of RV-Bc from children with acute RV GE have a lower number of mutations than those of the corresponding adult sequences (22). Besides, further studies that concentrated on VP6-specific antibodies encoded by the VH1-46 gene segment (an immunodominant gene in RV-Bc of both children and adults) showed that the mutations detected in adults conferred functional advantages to these antibodies (9). For instance, somatic mutations in the H chain CDR2 region of these antibodies generated a prolonged off-rate in VP6 binding and increased antiviral activity in an in vitro intracellular neutralization assay. Of note, using three-dimensional cryoelectron microscopy, investigators demonstrated that these antibodies bind VP6 where this protein forms viral type 1 channels, suggesting that the mechanism of intracellular neutralization could involve inhibition of viral RNA release during replication (9).

More recently, the Ig gene repertoire of VP6-Bc in circulating naive ($CD19^+$ IgD^+ $CD27^-$) or mBc subsets ($CD19^+$ IgD^+ $CD27^+$ IgM mBc or $CD19^+$ IgD^- $CD27^+$ switched mBc that in these experiments include IgM-only mBc) from healthy adults has been evaluated (20). As previously stated, compared to non-antigen-specific mBc, RV-mBc had an increased frequency of IgM mBc (Table 1). Also, IgM RV-mBc had a shorter CDR3 length than naive and RV-switched mBc. This could be explained by both lower numbers of N and P nucleotide additions and shorter D segment length, due to increased

exonuclease activity. A comparable finding was observed for total non-antigen-specific IgM-mBc, suggesting that IgM RV-mBc are probably selected by the same unidentified mechanism (20). In addition, the authors observed that switched RV-mBc were enriched in IgM-only mBc at the expense of IgA mBc (Table 1) and had a lower frequency of somatic mutations. These findings suggest that RV-mBc have undergone less extensive maturation in germinal centers (where switching and somatic hypermutation occur). Although the IgM mBc and switched mBc subpopulations of RV-mBc thus seem to be selected by different mechanisms, they share a very intriguing difference from naive RV-Bc. While the VH1 family is the dominant VH gene family used by naive RV-mBc, the VH3 family is dominant in both subtypes of RV-mBc. Particularly, the dominance of the VH1-46 gene segment usage is high in naive RV-Bc (28.6%) compared with IgM and switched RV-mBc (7.8 and 8.3%, respectively). In contrast, the VH3 family is the predominant VH family used by naive mBc and the two subtypes of mBc in total non-antigen-specific Bc. Altogether, these findings indicate that, unexpectedly, the dominant naive RV-VH1-expressing Bc do not have an advantage in the two selection processes that give rise to IgM and switched RV-mBc. Hence, more studies are necessary at both the ontological and functional levels to understand the relationship between the RV-specific naive and memory repertoires of Bc.

The investigations described above have been performed with blood-circulating Bc, and as previously stated, RV-Bc are probably concentrated in the intestine (see below for discussion on the compartmentalization of RV-Bc). Accordingly, two recent studies addressed the genes used by intestinal RV-Bc in healthy adult subjects (23, 24). In the first report, IgG or IgM plasma cells from the small bowel ($CD138^+$ $CD27^+$ that still express surface Ig of the corresponding isotype) capable of binding 2/6 RV-VLPs were purified by cell sorting (23). Ig genes from Bc derived from three donors were cloned, sequenced, and expressed. The VH genes of 26 Bc clones obtained (22 IgA, 4 IgM) were highly mutated and preferentially expressed the VH4 family, which is not dominant among peripheral blood RV-Bc (23). Ten of the IgA antibodies were expressed, and 8 were shown to bind RV but not a control antigen. In the second report, the genes from total non-antigen-specific IgA and IgG plasmablasts ($CD38^+$ $CD27^+$ cells still expressing the corresponding Ig isotype) from the lamina propria of the terminal ileum of healthy adult volunteers were cloned and expressed in vitro (6). Although the IgG plasmablasts were approximately 10 times less frequent than the IgA plasmablasts, the IgH, IgK, and IgL chain gene repertoire of both types of plasmablasts were similar to each other and to their blood-circulating counterparts. The reactivity profile of Ig produced by the Bc clones against a large panel of self-antigens, intestinal bacteria, and RV was characterized. Approximately 30% of intestinal IgA and IgG plasmablast antibodies were polyreactive, and the majority of these recognized RV 2/6 VLPs. Only 1 of 137 IgA and 2 of 85 IgG plasmablast clones were exclusively specific for the RV VLPs (24). Thus, the majority of intestinal plasmablasts that recognize RV (most probably VP6) are polyreactive. This observation underscores the necessity to characterize the reactivity of RV-Ig obtained from blood-circulating Bc in the studies previously described. Hence, future

TABLE 1 Blood-circulating Bc subsets in healthy adults and their enrichment or not in RV-Bc

Bc subset common name (phenotype)	Function and/or relationship of the Bc subset to disease	Enrichment in RV-Bc	Reference(s)
Naive ($CD27^-$ IgD^+ IgM^+)	Bc that have emigrated from the bone marrow and express the preimmune repertoire of Ig	No	53
IgM mBc ($CD27^+$ IgD^+ IgM^+)	Bc that probably represent a heterogeneous subpopulation, an important part of which has a prediversified repertoire of Ig; the $CD27^+$ IgD^{low} IgM^{hi} subset resembles marginal zone Bc and are particularly enriched in RV-mBc	Yes	11, 20, 53
$CD27^-$ mBc ($CD27^-$ IgD^- IgM or IgG or IgA^+)	Atypical mBc that do not express CD27 and that are enriched in patients with autoimmune diseases such as lupus	Yes in the IgG^+ subset	53
IgM-only mBc ($CD27^+$ IgD^- IgM^+)	A minor mBc population that expresses IgM and seems to be related to switch mBc	Yes	20
Switch mBc ($CD27^+$ IgD^- IgM^- IgG or IgA^+)	Classical mBc subset that expresses IgG or IgA	Decreased frequency	11, 20, 53

investigations to directly compare the blood and intestinal RV-Bc repertoires seem warranted.

(iii) Compartmentalization of RV-Bc

Although the largest amount of RV replication in both animals and humans occurs in the intestinal tract, infection is generally accompanied by antigenemia and viremia. Low levels of systemic RV replication commonly occur in animals, and many children have elevated liver enzyme levels, suggesting that mild hepatitis is a common feature of RV infection (2). With this pattern of infection, it is not surprising that the distribution of RV-Bc in animals is biased to intestinal localization but also has an important systemic component (25). For this reason, the RV model has been useful to test the hypothesis that the immune system functions in compartments (1). According to this hypothesis, an immune response will be tailored to best function at the anatomical niche where it develops, and lymphocytes specific for a specific pathogen will concentrate in the compartment of entry of that pathogen. For RV, this is clearly the case. During an acute infection, most viral reactive Bc are concentrated in the intestine (25). However, concomitant with viremia, RV-Bc also appear in the spleen and bone marrow (25). Nine months after primary RV infection in a mouse, effector ASC predominantly secrete RV-IgA (the isotype suited to survive in the harsh environment of the intestinal lumen), rather than RV-IgG, and are concentrated in the small intestinal lamina propria and, to a lesser extent, in Peyer's patches (Fig. 2, top panel). In contrast, equal numbers of IgG and IgA RV-ASC are present in the spleen and bone marrow (Fig. 2, top panel). When comparing RV-mBc (characterized by flow cytometry) at these late time points after infection, it is estimated that a mouse will have approximately the same number of RV-mBc in the spleen and in the Peyer's patches (for technical reasons, RV-mBc were not evaluated in the intestinal lamina propria) (Fig. 2, middle panel). However, the frequency of RV-mBc as a fraction of total cells in each organ is higher in the Peyer's patches (Fig. 2, bottom panel), indicating a selective accumulation in these organs. An important prediction of the compartmentalization hypothesis is that RV-Bc that have been primed in a given Peyer's patch will have the capacity to migrate to the blood and, from there, selectively to other Peyer's patches, lamina propria, and other parts of the intestinal compartment to provide a thorough protection against the pathogen. This prediction has been directly demonstrated for RV (26), and the compartmentalized migration of the RV-Bc has been shown to depend on the expression of the integrin α4β7 (the intestinal homing receptor) and the chemokine receptor 9

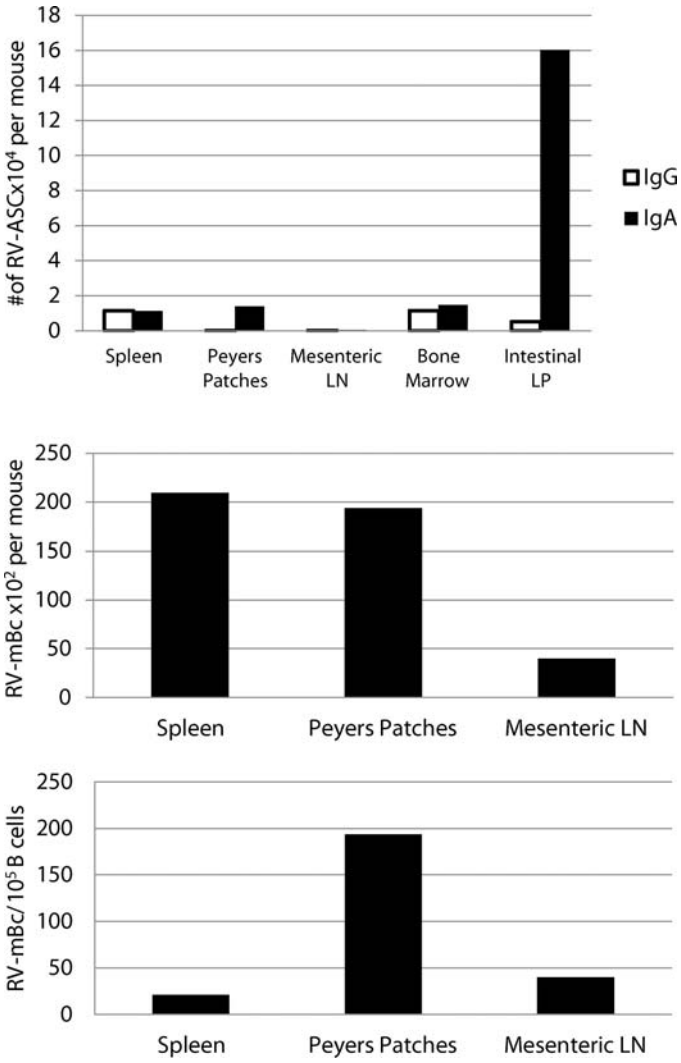

FIGURE 2 Distribution of RV-Bc 9 months after primary oral infection of mice in selected organs (data from experiments reported in reference 25). The top panel shows numbers of IgG and IgA RV ASC per mouse, in different organs, evaluated by enzyme-linked immunospot assay. The middle panel shows numbers of RV-mBc (small IgD^- Bc) that bind RV VLPs per mouse, in different organs, evaluated by flow cytometry. The bottom panel shows the same data as the second panel but expressed as RV-mBc per 10^5 cells of the respective organ. LN, lymph node; LP, lamina propria. doi:10.1128/microbiolspec.AID-0011-2013.f2

(CCR9) (1). Of note, relatively high levels of α4β7 are expressed by intestinally committed mBc/ASC compared to naive Bc that also express this receptor (1). Since the CCR9 ligand, the chemokine TECK, is selectively expressed in the small intestine, RV-Bc that express CCR9 will home specifically to this portion of the intestine, where RV predominantly replicates. In humans, a direct relationship has been shown between circulating RV-ASCs

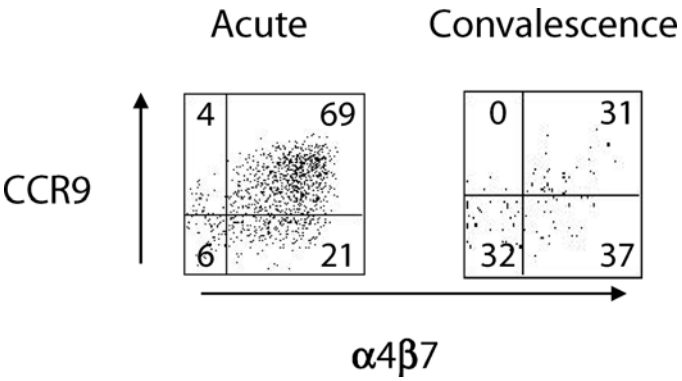

FIGURE 3 Expression of the integrin α4β7 (the intestinal homing receptor) and CCR9 (chemokine receptor 9 whose ligand, TECK or CCL25, is selectively expressed in the small intestine) on RV-Bc (Bc that bind fluorescent RV VLPs) in children with acute (left dot plot)- or convalescent (right dot plot)-phase RV infection. The figure is from reference 14, with modifications. doi:10.1128/microbiolspec.AID-0011-2013.f3

and the presence of RV-specific ASCs in the human small intestinal lamina propria (27). Moreover, in children with an acute RV infection, the great majority of blood-circulating RV-ASC express both intestinal homing receptors (Fig. 3, left panel) (14). In the convalescent phase of viral infection, approximately one-third of RV-mBc express both receptors and are presumably targeted to the small intestine. Another third only express α4β7 (presumably targeted to other parts of the intestine and other mucosal surfaces), and the final third express neither receptor (presumably targeted to the spleen and other systemic organs) (Fig. 3, right panel) (13, 14). This result (Fig. 3) is consistent with a compartmentalized mBc response to RV in humans, as observed in animals (Fig. 2).

(iv) Mechanisms of Protection and Specificity of RV-Ig

Although in mice RV-Bc can play a contributory role in viral clearance (28), they are primarily necessary for preventing reinfection (29). The localization of RV-Bc to the intestine seems to be critical for these cells to be able to mediate their antiviral effect, since in immunodeficient mice chronically infected with RV, the transfer of $\alpha4\beta7^+$ mBc is associated with an antiviral response in the intestine and viral clearance, while the transfer of $\alpha4\beta7^-$ mBc is associated with a serum response and viral persistence (30). In this model, both wild-type and IgA-deficient Bc expressing the intestinal homing receptor mediated viral immunity, although the latter did so with somewhat lesser efficiency (31). This suggets that intestinal localization and, to a lesser extent, Ig isotype are critical factors in the protection mechanism. Another factor that seems critical in determining the capacity of a Bc to protect against RV is the age at which the mice are immunized. The age of immunization is, itself, related to the capacity of the mouse to develop $CD4^+$ T cells. Mouse pups immunized orally with a heterologous, poorly replication-competent simian RV were less protected than adult mice and produced less RV intestinal IgA and neutralizing antibodies. This finding was associated with the immature T-cell response in the pups (32). In fact, more than 90% of the intestinal IgA in adult mice is $CD4^+$ T-cell dependent (29). Although the T-cell-independent response may aid in viral clearance (28), the mechanism of how the virus stimulates it is not completely clear. In T-cell-deficient mice, RV can induce a strong Bc polyclonal activation (33), and this phenomenon has been linked, in one study, to the capacity of VP7 to stimulate Bc via Toll 4 receptor (34). In vitro, RV has been shown to induce activation and differentiation of human Bc, present in peripheral blood mononuclear cells, into ASC (35). However, this effect was not observed with purified Bc, suggesting the participation of other cells in activating the Bc; most likely dendritic cells that produce alpha interferon. In children, intestinal IgA responses are frequently short lived (16), while systemic IgG responses tend to persist (19). It has also been shown that children, like mice, have an age-related delay in the development of neutralizing Ig (54). A primary infection in young children thus develops in the context of an immature immune system, with a poor capacity to stimulate memory $CD4^+$ T cells and with the development of a T-cell-independent/innate Bc response that may, in some cases, not persist.

The great majority of RV-Igs both in animals (36) and humans (1) are directed against VP6. The role played by most of these antibodies in RV immunity is incompletely understood, but it has been postulated that some of these antibodies mediate intracellular neutralization (37). It is hypothesized that during the transcytosis of polymeric IgA from the basolateral membrane of enterocytes to the gut lumen, these Ig bind virus VP6 and "expulse" it to the gut lumen. Loading of the sIgA may occur in the crypt enterocytes that express the polymeric Ig receptor. When these enterocytes reach the tip of the villi (site of RV infection), they still may have some IgA that can mediate the proposed viral expulsion. This model has been supported by in vitro experiments in which neutralization of RV can be achieved if the anti-VP6 dimeric IgA antibodies are delivered intracellularly to infected cells (38). Furthermore, in mice, protection by these antibodies is dependent on transcytosis of mucosal Igs by the

polymeric Ig receptor (39) and not mediated via immune exclusion (see below) (40). Of note, several recent reports have identified llama VP6-directed monoclonal antibodies with substantial traditional neutralization activity (41, 42). The mechanistic and structural basis of these findings has not yet been determined, and numerous attempts to find comparable VP6 antibodies in mice and humans have thus far been unsuccessful.

The classical mechanism of Ig-mediated protection against RV involves viral exclusion (block of enterocyte infection) by neutralizing antibodies directed against the virus outer proteins VP4 and VP7 (1). The mechanism of neutralization for both types of antibodies has been described in detail. Based on structural studies, two main neutralizing epitopes have been described for VP7. The VP7-1 epitope lies at the three corners of VP7 trimers and involves at least two VP7 molecules. Antibodies against this epitope neutralize the virus by stabilizing the capsid and preventing viral uncoating. The VP7-2 epitope is in the center of the protein, and the mechanism of neutralization is unknown (43, 44). VP4, the virus spike protein, is cleaved in two proteins, VP5*, which constitutes the stalk of the spike, and VP8*, localized on the tip of the stalk, which mediates initial cellular attachment. At least four structurally defined epitopes have been identified in VP8*, and antibodies against them mediate neutralization by blocking virus attachment to cells (43). Five structurally defined epitopes have been identified for VP5* (43). The VP5-1 epitope is located in the apical hydrophobic loops of the protein and seems to block the association of VP5* with membranes, probably blocking membrane penetration (44). The mechanisms of neutralization of antibodies directed to the other epitopes are not completely clear at present.

THERAPEUTIC AND PROPHYLACTIC APPLICATIONS OF RV ANTIBODIES

RV-Ig can have therapeutic/prophylactic applications and has been used as a correlate of protection for RV vaccines. The latter have been recently reviewed (3) and will only be briefly mentioned here.

Passive administration of RV-Ig can shorten the duration of RV infection in animals (1). Oral administration of commercial human Ig preparations, with high titers of neutralizing RV-Ig, have been shown to be of clinical benefit in children with RV GE (45). This type of treatment may be helpful for children with primary and acquired immunodeficiency and prolonged RV infection (1, 46). Careful kinetic studies of children suffering primary immunodeficiency, who presented with chronic diarrhea and RV excretion, confirmed that RV-Ig can survive passage in the gastrointestinal tract and temporarily reduce viral excretion (46). However, preparations of human Ig are expensive, and multiple alternatives have been investigated. Treatment with Igs extracted from colostrum from immunized bovines was shown to reduce stool frequency and to accelerate viral clearance in children with RV GE (47). An even less expensive strategy has been to treat children with Ig extracted from eggs of chickens immunized with human RV (48). Further investigation of strategies to improve the efficacy of orally administered antibodies includes studies of single-chain antibody fragments expressed from llama antibody genes (42, 49). The advantages of this approach are that the antibodies are relatively small, soluble, acid- and heat-resistant, and easy to express in multiple vectors, including lactobacilli. Surprisingly, in two such studies, antibodies directed to RV VP6 possessed broad neutralizing activity in vitro and conferred protection against diarrhea in mice (41, 42). It is possible that the small size of the VHH antibodies may enable access to VP6 on the complete triple-layered viral particle that is covered by VP4 and VP7. Testing of these VHH constructs in a clinical setting and further evaluation of the preparations mentioned before (bovine colostrum and chicken IgY) are necessary to determine their practical utility. Furthermore, testing the value of human Ig in relevant clinical settings is necessary; for example, a recent Cochrane Database systematic review concluded that no randomized controlled trials assessing the effectiveness or safety of oral immunoglobulin preparations for the treatment of RV diarrhea in hospitalized low-birth-weight infants have been published (50).

Two safe and effective RV vaccines, the monovalent G1P[8] Rotarix vaccine (RV1) and the bovine reassortant pentavalent RotaTeq vaccine (RV5) are recommended by WHO for worldwide use (3). Nonetheless, they are clearly less efficacious in the poorer countries of Africa and Asia, where they would be most useful, than in the Americas and Europe. For this and other practical reasons, these vaccines need to be improved and/or better ones developed (3). Our poor understanding of the mechanisms of protection induced by RV vaccines, and in particular the lack of good correlates of protection, hamper this process, as does our lack of insight as to why the vaccines perform less effectively in the poorest countries. Total serum RV-IgA measured shortly after vaccination is the correlate of protection currently most widely used (1). It is very unlikely that total RV IgA is actually the effector of protection but rather a surrogate marker of other more specific immune effectors. Given

this indirect measure, it should not be unexpected that some vaccinees with serum RV-IgA responses develop mild RV GE, whereas in others, the protection provided by the vaccine can exceed the levels predicted by the serum RV-IgA response (2, 3). The first incongruity can be best explained because of the induction of serum RV-IgA without intestinal RV-IgA. This occurs in mice infected with some heterologous RV, for which the viral dose that induces antigenemia is lower than the dose that induces intestinal infection. When low doses of these RV are used for infection, serum RV-IgA can be detected in the absence of intestinal RV-IgA and these mice are susceptible to infection with a homologous RV (1). In children, this has been hypothesized to occur with the vaccines based on rhesus RV (1). A similar situation may exist with the RV5 bovine reassortant vaccine, for which the level of serum RV-IgA exceeds the level of protection in several clinical studies in developing countries (2). The second incongruity, the excess actual protection observed when compared to serum RV-IgA responses (or neutralization responses), occurs most frequently with homologous vaccines, like RV1. In this case, it is possible that the excess protection is explained by an antibody response in the intestine (where the vaccine virus has been shown to replicate) that is not "strong" enough to be reflected in the circulation.

Importantly, RV1 and RV5 have similar efficacy against severe RV GE in countries where a high diversity of strains cocirculate, supporting the conclusion that immunity to RV has a substantial heterotypic component (3). For this reason, the specificity of the Bc response is probably only a secondary aspect of an ideal correlate of protection and probably explains why serum RV-IgA is a better correlate than the homotypic neutralization response. Thus, efforts to identify stronger correlates of protection may have to concentrate on strategies to better evaluate the intestinal RV-Ig responses that have the capacity to persist over time. Although stool RV-IgA has been shown to be a good correlate of protection for natural RV infection (16), this parameter may not be useful for evaluating protection induced by RV vaccines because of the interference in the measurement of vaccine-induced RV-Ig by maternal antibodies from breast-feeding (10). This is particularly true in developing countries, where the frequency of breast-feeding is high and the basal levels of preimmune stool RV-IgA mask vaccine-induced coproconversions. As an alternative, measurement of blood-circulating intestinally induced RV-Bc has been proposed (3). In a gnotobiotic pig model, for example, both systemic and intestinal RV ASCs have been shown to be a good correlate of protective immunity to human RV challenge (51). In spite of this, it is not clear if measurement of RV ASC can predict long-term persistence of immunity, and alternative efforts have concentrated on the study of RV-mBc. As previously mentioned, during an acute RV infection in children, circulating IgD^- RV-mBc express intestinal-homing receptors ($\alpha4\beta7^+$, $CCR9^+$) and thus probably reflect mucosal immunity (14). In agreement with this finding, a correlation between protection from disease and plasma RV-IgA and $\alpha4\beta7^+$ $CCR9^+$ IgD^- circulating RV-mBc was found in a trial of the attenuated human RV1 vaccine precursor. However, the correlation coefficients with protection for both parameters were low, making them of little practical use (10).

FUTURE CHALLENGES

RV-Bc have been studied at many levels, opening several fruitful areas of basic research on the interaction between Bc and an enteric human viral pathogen. Critical questions that remain to be addressed include (i) the relationship between the preimmune repertoire and the mBc repertoire, especially in the intestine. The studies described above, which take advantage of the high numbers of RV-naive Bc, make RV one of the few human pathogens in which this question has started to be addressed and need to be pursued. (ii) Currently, immunologists are puzzled by the mechanism of selection, function, and biological relevance of the IgM mBc (12). Since RV-mBc are enriched in this subset, RV antigen may be used as a tool to unravel the mystery of IgM mBc. Further studies of this mBc subset and of the innate and T-cell-independent antibody response against RV will likely expand our understanding of RV immunity. (iii) The study of RV-Bc may help clarify the extent and mechanisms that determine compartmentalization of the immune system. As discussed, limited studies of intestinal RV-Bc suggest that they use a different Ig gene repertoire than RV-Bc in circulation, and a direct comparison of both compartments would help confirm that this is the case.

From a practical point of view, ongoing clinical studies are evaluating whether llama-derived single molecule antibodies against VP6 can provide broad protection against RV, as predicted from animal studies. If this is the case, future studies should be aimed at examining the possibility that humans are also able to generate such antibodies and that this phenomenon might help explain heterotypic immunity. An alternative option for passive protection could be to design antibodies against conserved heterotypic neutralizing

epitopes on VP7 and/or VP4. In this respect, antibodies against the VP5-1 epitope seem appealing because of their conserved nature and essential functional role of the VP5 domain in viral penetration.

Concerning correlates of protection for RV vaccines, it is possible that formal statistical analysis of serum RV-IgA responses from children in the large clinical trials that have been performed to date will establish this parameter as a practical surrogate marker of protection. Even if so, as discussed above, the development of better correlates seems desirable, and in this respect, two areas of further study seem promising. (i) The characterization of serum RV-sIg induced by RV vaccines may be a useful way to obtain an indirect measure of the immune response generated in the intestine. Preliminary studies have shown that this is possible (J. Angel and M. Franco, unpublished data). However, an important number (approximately one-third) of placebo recipients appear to have plasma RV-sIg in the absence of detectable RV-IgA. This can be explained because, at this age, RV-sIg is mostly composed of IgM RV-sIg (Angel and Franco, unpublished). The best (but not exclusive) explanation for these RV-sIg (presumably IgM) responses in non-vaccinated children are low-level asymptomatic infections: at the age of RV vaccination (2 to 3 months), RV RNA may be detected by a sensitive PCR in the stools of up to one-third of healthy children (52). (ii) Further studies of circulating RV-mBc (particularly those primed in the intestine) and their relationship with levels of intestinal and serum Ig (10, 53) may also be helpful to identify improved correlates of protection. Our poor understanding of IgM RV-mBc, and the fact that they are probably present in children that have not been infected with RV (defined by the absence of serum RV-IgA), hinder these studies.

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