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II, 11. Human adaptive immunity to rotaviruses: A model of intestinal mucosal adaptive immunity

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Introduction

Rotaviruses (RV) are the single most important cause of severe diarrhea in young children, and there is an urgent need to develop effective vaccines against them (Franco and Greenberg, 2001). Because RV replication is highly restricted to enterocytes in vivo, the immune response against them originates in and exhibits its effector function directly at the intestinal mucosa. For this reason, RV infections are an excellent model to study intestinal mucosal immunity. Here we will review the human adaptative immune response to RV, first placing the immune response to RV in the context of the immune response to other mucosal viruses, and then highlighting studies of both RV-specific T and B cells.

Since children with T and/or B immunodeficiencies can develop chronic RV infection, prolonged symptoms and extraintestinal infection (Saulsbury et al., 1980; Wood et al., 1988; Gilger et al., 1992) it is clear that both T and B cells are important for immunity to RV. Nonetheless, the T and B cells induced by natural RV infection are not sufficient in many cases to prevent symptomatic or asymptomatic reinfections in children (Velazquez et al., 1996) or adults (Rodriguez et al., 1987; Nakajima et al., 2001). The severity and number of RV infections diminish with age, and life threatening infections are generally limited to the primary infection, suggesting that adaptive protective immune responses to RV develop gradually. Reinfection with many other viruses that replicate only at mucosal surfaces is also frequent (Murphy, 1999). This is in contrast to viruses that spread and replicate systemically for which the development of long lasting protective immunity is the rule (Murphy, 1999). The various reasons proposed to explain absence of complete immunity to mucosal viruses such as RV, following primary infection, include:

1) A short incubation period after viral exposure. Since the ultimate cellular target for replication of viruses like influenza viruses, rhinoviruses or RV is the same cell as...
used for entry, these viruses do not require a period of recirculation through the organism. For this reason after secondary viral exposure, the immune system does not have time to amplify the protective specific responses against these viruses. Sustained high levels of antiviral effector mechanisms at the mucosal surface are thus necessary to achieve protection against these viruses (Roitt, 1997).

2) Difficulty in maintaining a high level of protective antibody at respiratory and gastrointestinal mucosal surfaces. This problem is due to the high volume, rapid transit time and hostile environment (for example proteases that can inactivate the antibodies) of these surfaces (Murphy, 1999).

3) A short lived protective humoral mucosal immune response. Although the serum antibody response to systemic viruses (e.g. mumps virus, rubella virus, or HBV) lasts for over a decade, the mucosal antibody responses to influenza virus, respiratory syncytial virus, coronavirus and RV last from 3 to 30 months (Doherty and Ahmed, 1997; Murphy, 1999). For a given virus (RV included) to which both systemic and mucosal antibody responses develop, the former seem to be more long lasting than the latter (Coulson et al., 1990; Nishio et al., 1990).

4) Viral mutations that permit the virus to escape the effect of protective neutralizing antibodies. Although this factor is certainly not exclusive to mucosal viruses, it probably plays an important role in conjunction with factors 2 and 3 mentioned above. Protection from reinfections with rhinoviruses seems to be highly dependent on the presence of serotype-specific antibodies but the existence of over 100 viral serotypes makes subsequent infections with viruses of different serotypes highly likely (Doherty and Ahmed, 1997). Susceptibility to severe influenza disease is also serotype dependent: the appearance of a completely new viral serotype (antigenic shift) causes pandemics. More frequently, minor changes that do not change the serotype of the hemagglutinin (antigenic drift) of a circulating virus seem to be responsible for the susceptibility of individuals to reinfections with influenza virus in each winter epidemic (Doherty and Ahmed, 1997). For this reason the design of annual influenza vaccines takes into considerations drift mutations in circulating viruses for a given period. RV-specific immunity seems to be, to some extent, serotype-specific (Kapikian et al., 2001), and reinfections in children appear to be preferentially (but not exclusively) due to viruses of a different serotype (Velazquez et al., 1996). However, serotype-specific immunity for RVs seems to be less important than for some other mucosal viruses such as rhinoviruses or influenza viruses. Unlike the multitude of serotypes found for rhinoviruses, RV of only five major serotype combinations circulate in most parts of the world (Desselberger et al., 2001) suggesting that variation in viral serotype alone can not account for viral reinfections. In addition, as opposed to influenza virus, the appearance of a new RV serotype (e.g. emerging by reassortment with an animal RV) followed by wide spread disease in a population immune to RV of a different serotype seems to be a very rare event if it exists at all. The appearance of limited mutations in the neutralizing antigens that do not change the viral serotype (like influenza antigenic drift) have been proposed as a factor that could be favoring RV reinfection, but this hypothesis remains unproven at present (Jin et al., 1996; Coulson, 1998).
Since serotype-specific immunity cannot completely explain protection against RV, other factors probably play a role in protection against this pathogen. For example, studies in animal models have suggested that non-neutralizing antibodies against other viral proteins (VP6 and or NSP4 (Ball et al., 1996; Burns et al., 1996)) or other mechanisms (like T cells) can also play a role in immunity (Kapikian et al., 2001).

The above discussion underscores the importance of studying RV-specific memory T and B lymphocytes, and of investigating the ways these memory cells localize to the intestinal mucosal surfaces where they are required to mediate their protective effect. The understanding of RV-specific CD4+ T cell responses is a key issue because most of RV-specific antibody responses in animal models seem to be dependent on CD4 help (Franco and Greenberg, 1997). Studies of the T cell response to RV have used lymphoproliferation as a readout after in vitro restimulation with viral antigen (Table 1). It is generally assumed that results obtained using this method reflect the presence of CD4+ antigen specific T cells. These studies have shown that most, but not all, healthy adults and children and children in the convalescent phase of RV-induced diarrhea have RV-specific CD4+ T cells. The percentage of healthy elderly persons and healthy children below the age of six months that have a lymphoproliferative responses to RV antigen is lower than the percentage of healthy adults and children older than six months of age that have a lymphoproliferative responses to RV antigen (Totterdell et al., 1988a; Offit et al., 1992). The T cells that proliferate in response to RV most probably recognize cross-reactive epitopes since restimulation in vitro using simian and bovine RVs as antigens seems to be as efficient as human RV (Table 1). The fine specificity of this T cell response, either at a protein or epitope level, has not been thoroughly investigated. In fact, only one study has formally demonstrated that the proliferative responses were due, at least in part, to CD4 cells (Rott et al., 1997).

Table 1
Studies of in vitro proliferative responses to RV antigen

| Primary study population | Antigen used for in vitro restimulation of cells | Reference |
|--------------------------|-----------------------------------------------|-----------|
| Healthy adults           | Tissue culture supernatant of simian RV       | Totterdell et al., 1988b |
| Elderly adults with and  | Tissue culture supernatant of simian RV       | Totterdell et al., 1988a |
| without RV diarrhea      |                                               |           |
| Healthy adults           | Infectious and inactivated human RV and       | Yasukawa et al., 1990 |
|                          | infectious bovine RV                          |           |
| Healthy children and     | Purified human RV and simian RRV              | Offit et al., 1992 |
| adults                   |                                               |           |
| Children with RV induced | Purified human RV                             | Offit et al., 1993 |
| diarrhea                 |                                               |           |
| Healthy adults           | Purified inactivated simian RV*               | Rott et al., 1997 |

* This study used purified subsets of CD4+ T lymphocytes and is thus the only one for which the lymphoproliferative activity can directly be attributed to CD4 T cells.
As mentioned above, an important issue to address with RV-specific lymphocytes is the mechanism that permits these lymphocytes to localize and accumulate in the intestinal mucosa where they are required to perform their antiviral effect. Mature naive lymphocytes recirculate between various secondary lymphoid organs until they encounter the antigen that stimulates their specific receptor. After antigen-specific activation, the lymphocyte migration pattern changes, redirecting them preferentially to the tissue where the activating antigen was originally encountered (Butcher et al., 1999). This tissue-specific migration provides lymphocytes with the best chance of re-encountering the antigen that originally stimulated them. The homing of lymphocytes stimulated by antigens first encountered in intestinal Peyer’s patches back to the same or other Peyer’s patches or the intestinal lamina propria is mediated by interactions between the integrin α4β7, expressed on B and T lymphocytes and the cell adhesion molecule MadCAM 1, expressed on the vascular endothelium of the postcapillary venules in the intestine (Butcher et al., 1999). To date, only one study has addressed the issue of the potential capacity of RV-specific CD4+ T cells from humans to localize to the intestine based on the presence or absence of the intestinal homing receptor (the integrin α4β7+) on these lymphocytes: Rott et al. studied the ability of α4β7+ and α4β7- CD4+ T cell subsets of memory phenotype (CD45RA-) (purified by flow cytometry) to proliferate in response to RV. α4β7+ CD45RA- cells proliferated more than 2.3 fold over α4β7- CD45RA- CD4+ T cells to RV antigen (Rott et al., 1997). In contrast, α4β7- memory cells were the predominant population responsive to mumps antigen after intramuscular vaccination. These findings suggest that RV-specific T cells, but not T cells specific for a viral antigen administered parenterally, have the capacity to migrate to the intestinal mucosa (Rott et al., 1997).

The studies that have used lymphoproliferation to study the T cell response to RV have the limitations that they do not directly quantify or establish the phenotype (with the exception of the study by Rott et al., 1997) of the cells responding to the RV antigen. We have recently adapted a flow cytometry based assay (Waldrop et al., 1997) for the study of RV-specific T cells that detects the intracellular accumulation of cytokines after short term in vitro antigen stimulation (Jaimes et al., 2002). Because this assay allows to analyse individual cells it permits the simultaneous quantification and characterization of the phenotype of the RV-specific T cells. Using this assay we have been able to characterize, for the first time, human RV-specific CD8+ T cells, and directly determined the frequencies of RV-specific CD4+T cells that secrete IFN-γ and IL-13 and CD8+ T cells that secrete IFN-γ in children and adults with RV-induced diarrhea and in healthy adults. A representative plot of a flow cytometric analysis of an adult in the convalescent phase of RV induced diarrhea is shown in Fig. 1. Table 2 shows the frequencies of IFN-γ secreting CD4 and CD8+ RV-specific T cells in healthy adults, and adults and children with RV induced diarrhea: while adults with RV induced diarrhea have greater numbers of RV-specific T cells than healthy adults, children with RV induced diarrhea have very low levels of RV-specific T cells. Using this assay we expect to be able to identify, quantitate and evaluate differences between mucosally primed RV T cells and T cells specific for viruses that replicate systemically. Some future studies will investigate: 1) the pattern and quantity of cytokines
secreted by RV-specific lymphocytes; 2) the expression of various molecules involved in lymphocyte migration (Rott et al., 1997) and 3) the kinetics of appearance and circulation in blood of RV-specific lymphocytes after RV infection.

![Graph showing frequencies of CD8+ cells in an adult with RV induced diarrhea.](image)

**Fig. 1.** Frequencies of CD8+ cells in an adult with RV induced diarrhea. Cell were restimulated *in vitro* with cesium chloride density gradient purified RF RV (right) or the supernatant of mock infected cells (left) in the presence of brefeldin A to permit the accumulation of intracellular cytokines. Cells were then fixed, permeabilized and stained with fluorochrome labeled monoclonal antibodies against CD8 and CD69 (an early T cell activation marker) and interferon gamma. The events in the graphs are gated on CD8+ lymphocytes. The percentage of RV-specific T cell (events that express both CD69 and interferon gamma) are shown in the upper right quadrant.

### Table 2

Frequencies of IFN-γ secreting RV-specific CD4 and CD8 T cells in healthy adults and children and adults with RV diarrhea

| Study population             | Mean % RV specific CD4 T cells (SEM)* | Mean % RV specific CD8 T cells (SEM)* |
|------------------------------|--------------------------------------|--------------------------------------|
| Adults with RV diarrhea (n= 7) | 0.18 (0.10)                          | 0.28 (0.11)                          |
| Healthy adults (n= 8)         | 0.02 (0.006)                          | 0.03 (0.01)                          |
| Children with RV diarrhea (n= 12) | 0.01 (0.009)                        | 0.02 (0.006)                        |

* The percentages of RV-specific CD4 and CD8 T cells were calculated as indicated in the legend to Fig. 1.

Studies of the human antibody response to RV have highlighted the importance of serum IgA (Velazquez et al., 2000) and particularly the intestinal IgA response in pro-
tection against RV (Coulson et al., 1992). As for T cells, the detailed characterization of the B cells that produced these IgA antibodies may shed more light on the quantitative and qualitative aspect of intestinal mucosal B cells. Published studies that have characterized these B cells have been limited to the study of effector antibody secreting cells (ASC) identified by ELISPOT. Blood circulating ASC detected in children after acute natural infection or vaccination have been shown to secrete predominantly the IgM isotype (Kaila et al., 1992; Isolauri et al., 1995). Our current model to explain the detection of these RV-specific ASC in human peripheral blood is that these B cells, originally stimulated by RV antigen in Peyer’s patches, are detected in blood during their transit from Peyer’s patches, through the circulation and back to the intestine. A recent study supported this model by showing in older children without documented recent RV infection that there exists a correlation between the presence of blood circulating RV-specific ASCs and intestinal ASCs (Brown et al., 2000). Furthermore, we have recently performed studies similar to those of Rott et al. (1997) but using purified α4β7+ and α4β7- subsets of peripheral blood B cells and then testing in an ELISPOT assay. As expected, RV-specific ASC were predominantly present in the α4β7+ subpopulation (Gonzalez et al., 2002).

We have also recently developed a flow cytometry assay in the mouse model that identifies antigen activated (IgD-) B cells (CD19+) expressing surface RV-specific immunoglobulin (Kuklin et al., 2001; Youngman et al., 2002). With this assay we are able to detect both RV-specific ASCs and memory murine B cells. The principal of this assay consists of the specific binding of a fluorescent RV antigen to a B cell that expresses RV-specific Ig. As an antigen we chose recombinant virus-like particles (VLP) made from RV VP6 protein and a fusion protein consisting of green fluorescent protein (GFP) fused to the N terminus of RV VP2 deleted of its first 92 aminoacids (Charpilienne et al., 2001). In addition to the RV/GPF-VLPs, the B cells are also stained with fluorochrome labeled monoclonal antibodies directed against a B cell marker (CD19), IgD (to distinguish antigen activated from naive B cells), and the intestinal homing receptor α4β7 and then analyzed in a flow cytometer. We have recently applied this assay to detect RV-specific B cells in both children and adults with an acute RV infection (Gonzalez et al., 2002). An example of one of these experiments is shown in Fig. 2. Like for RV-specific ASCs, these studies have shown that B cells that express RV-specific surface immunoglobulin from both children and adults predominantly express α4β7 on their surface. These results open the possibility of characterizing in detail the phenotype of mucosal effector and memory B cells and in particular the expression of other molecules in addition to the integrin α4β7 that have been implicated in migration of lymphocytes to the intestine (Cook et al., 2000).

Acknowledgments

This work was supported by funds from the Pontificia Universidad Javeriana, ECOS/ICFES/COLCIENCIAS/ICETEX, Fundación para la Promoción de la Investigación y la Tecnología, Banco de la República grant 1103, Colciencias grants 1203-04-151-98
Fig. 2. Frequencies of CD19+ IgD-, α4β7 +/-, GFP-VLPS+ cells in an adult with RV induced diarrhea. PBMC were stained with fluorochrome labeled monoclonal antibodies against CD19, IgD, α4β7 and with GFP-VLPS and acquired in a flow cytometer. a) forward and light scatter plot showing two populations of cells (R1 small cells R2 large cells). b) Dot plot of Large cells (R2 in a) showing staining of CD19 and IgD and the creation of a gate (R3) of the IgD- CD19+ cells. Notice that CD19 low cells are not acquired for the experiment. c) Dot plot gated in R3 showing expression of the intestinal homing receptor α4β7 and binding of GFP-VLPS. The percentage of RV-specific B cells are shown in the left quadrants.

and 1203-04-966-98 and by ICGEB grant COL99-01(a1); by a grant from the National Institutes of Health R37AI21632 and by VA grants to H.B.G.. We would like to thank the subjects that participated in our study for their generosity and the personnel of the pediatrics department of the Hospital San Ignacio for their help in identifying children with diarrhea.

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