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*J Immunol* 2002; 168:5716-5721; doi: 10.4049/jimmunol.168.11.5716

http://www.jimmunol.org/content/168/11/5716
Early Response to Rotavirus Infection Involves Massive B Cell Activation

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Rotavirus is an acute enteric pathogen which induces severe diarrhea in infants and children. To determine the immune response to rotavirus in vivo, we used a mouse model of rotavirus infection. We observed dramatic increases in the sizes of both Peyer’s patches and mesenteric lymph nodes, but not spleen, between 1 and 6 days after infection with a homologous strain of murine rotavirus, EC wild type. Histological analysis showed large increases in the numbers of lymphocytes in these same tissues in rotavirus-infected mice. Flow cytometric analysis confirmed the increase in numbers of lymphocytes and revealed a large increase in the percentage of activated B, but not T, lymphocytes in both Peyer’s patches and mesenteric lymph nodes of rotavirus-infected mice compared with control mice. Fragment cultures from these tissues established at 3–4 days postinfection contain rotavirus-specific IgM but not IgA Ab. A similar degree of lymphoid hyperplasia and percentage of activated B cells were observed in rotavirus-infected TCR knockout mice. Taken together, our findings show that rotavirus infection, in the context of a normal immune response, induces a large increase in the percentages of activated B cells in the absence of any detectable increase in the percentage of activated T cells, implicating a T cell-independent B cell response as the primary mechanism for initial rotavirus clearance. The Journal of Immunology, 2002, 168: 5716–5721.

Rotavirus (RV), a small intestinal pathogen, is one of the leading causes of severe dehydrating diarrhea in children under the age of five and causes the deaths of >600,000 children annually (1). A licensed tetravalent vaccine, RotaShield, successfully protected children from severe RV disease, but the vaccine was withdrawn from the market due to an increased incidence of intussusception in vaccinated children (2), leaving an urgent need for the development of safer as well as more efficacious vaccines. It is not known whether the immune response induced during clearance of a primary RV infection in children is responsible for the protective immunity against infection acquired by adulthood or whether immune mechanisms of clearance and protection occur through unrelated events. An understanding of the immune cells that mediate clearance of and protection from RV infection will aid in new RV vaccine development by focusing vaccine design toward stimulation of the immune cells necessary for protection.

RV infects and replicates in the epithelial cells of the small intestine. RV and rotaviral Ags are shed into the lumen of the intestine during an infection and are detectable by ELISA. It is thought that control of RV infection is luminal and dependent upon Ab. Ab, in particular intestinal IgA, correlates with protection from RV infection (3). Studies in mice have correlated the rise in fecal IgA levels after 7 days postinfection (dpi) with the decrease in rotaviral Ag shed (4). However, work in animal models has shown that protection from reinfection can also be established in the absence of IgA (5) and is thought to be modulated by IgG. Studies in infant mice, humans, and rabbits have determined that RV-specific IgM levels increase initially early (before 7 dpi) after infection and then decrease, while IgA and IgG levels increase (6–8). The correlation of Ab with protection implies a significant role for B lymphocytes in the mediation of immune responses to RV infection, but it is not known what exact contribution B lymphocytes and Ab production make to clearance of the infection.

Mice provide a good model for studying the immune response during a primary RV infection (9). Infant mice exhibit diarrhea starting 1 day after infection with homologous murine strains of RV (EC wild type (ECwt) and epizootic diarrhea of infant mice) and resolve infection by 9 days after infection (4). Infection of infant or adult mice with homologous strains of RV protects the mice against reinfection as adults (4). Naïve mice >14 days of age, infected with murine RV, shed detectable fecal RV Ag during a primary infection but do not exhibit diarrhea (10). The kinetics of infection in mice differs slightly from what is observed in humans. Unlike infant mice, human infants can get multiple RV infections up until approximately the age of 5 years (11), and there have been many reports of adult RV infections, particularly in the elderly (11). However, aside from these differences, studies of RV infection in mice can still provide valuable information on the induction of immune responses by the virus.

The mechanism of clearance of a RV infection in the mouse has been difficult to define. Recombinase-activating gene-2–/– and SCID mice (deficient in both B and T lymphocytes) are unable to clear RV infection (12, 13), suggesting that either B or T lymphocytes mediate RV clearance. TCR-deficient mice (lacking functional T cells) effectively clear a primary infection and produce RV-specific Ab, suggesting that clearance is T cell independent (14). However, adoptive transfer of CD8+ T cells into SCID mice results in clearance of RV with a short delay compared with normal mice (15), suggesting that clearance can occur in the absence of B cells or Abs and that control of RV infection can occur at the
cellular level. Further evidence for a B cell/Ab-independent clearance mechanism is provided by studies that demonstrated that RV infection in B cell-deficient JHD and μMT mice was efficiently cleared (16). Taken together, these studies in knockout (KO) mice have implicated both B and T lymphocyte subsets in rotaviral clearance, but within the context of an intact immune system the lymphocyte subsets critical for clearance have not been identified.

Local Ig responses in the small intestine of RV-infected mice are first detected 7 days after infection (6, 17), suggesting that there may be significant B lymphocyte activation in the gut-associated lymphoid tissues (GALT) before this time. Because RV Ag has been previously detected in the Peyer’s patches (PP) and mesenteric lymph nodes (MLN) of infected mice (17), we reasoned that the presence of virus in these tissues might stimulate early activation of lymphocytes at these sites and initiate the production of RV-specific Ab. We demonstrate in this work, in outbred CD-1 mice infected with a homologous murine RV, EC<sub>wt</sub>, that the earliest lymphocyte response to RV infection is a large increase in the percentage of activated B cells, and not T cells, in the PP and MLN. In addition, these B lymphocytes secrete RV-specific IgM and not IgA. The presence of activated B cells in RV-infected TCRKO mice demonstrates that the early response to RV infection is T cell independent.

Materials and Methods

Mice, RV inoculation, and fecal Ag quantitation

All mice were housed in microisolation cages and fed ad libitum. CD-1 female mice 6–8 wk of age (Charles River Breeding Laboratories, Wilmington, MA), C57BL/6J male and female mice, or TCRKO β and δ male and female mice (The Jackson Laboratory, Bar Harbor, ME) were inoculated orally with 100 μl of PBS or 100 μl of PBS containing 10<sup>6</sup> shedding doses (SD)<sub>50</sub> of murine EC<sub>wt</sub> as described previously (18). Fecal samples were collected daily 1–7 dpi and analyzed by ELISA for Ag shedding and RV-specific Ab against simian SA11 RV as described previously (18). Qualitative analysis of lymphocyte cell number

Five mice in each group were euthanized 1–7 dpi by administration of 100 μl of rodent combination anesthesia (37.5 mg/ml ketamine, 1.9 mg/ml xylazine, 0.37 mg/ml acepromazine) and cervical dislocation. PP, MLN, and spleens (SP) were removed and imaged using a Nikon Coolpix990 digital camera (Nikon, Tokyo, Japan). Part of histological analysis was performed by the Center for Comparative Medicine (Baylor College of Medicine, Houston, TX). Briefly, tissues were placed in 10% zinc buffered formalin, embedded in paraffin, sectioned, and stained with H&E following standard protocols. Histology images were recorded using an Olympus IX70 microscope (Olympus, Lake Success, NY).

Quantitative analysis of lymphocyte cell number

All small intestinal PP (typically 8–10) and MLN were harvested and placed in complete RPMI medium consisting of RPMI 1640 medium (Bio-Whittaker, Walkersville, MD) supplemented with 10% FBS (Summit Biotechnology, Fort Collins, CO), 200 μM l-glutamine, 10,000 U/ml penicillin, and 50 μM streptomycin, 5 mM 2-ME, and 5% NCTC-109 medium (Bio-Whittaker). Single cell suspensions were made by mechanically disrupting tissue by gently pressing through a 70-μm cell strainer (Fisher Scientific, Pittsburgh, PA). RBCs were lysed using ACK lysis buffer (Bio-Whittaker) and cell suspensions were washed and resuspended in complete RPMI 1640. Aliquots of cell suspensions were stained with trypan blue and viable cells were counted using a hemocytometer (BD Biosciences, San Diego, CA). Significant differences in cell numbers were determined using a two-way ANOVA with two between-group factors (treatment and dpi).

Flow cytometry

Aliquots of cell suspensions from SP, MLN, and PP containing 2 × 10<sup>6</sup> cells from each tissue were washed in PBS and centrifuged at 400 × g, and the cell pellets were suspended in 100 μl of PBS containing 2% FBS (Summit Biotechnology) and PE-labeled CD4 (0.06 μg/μl cells; BD Biosciences), CD8 (0.06 μg/10<sup>6</sup> cells; BD Biosciences), or CD19 (0.06 μg/10<sup>6</sup> cells; BD Biosciences) and FITC-labeled CD69 (0.06 μg/10<sup>6</sup> cells; BD Biosciences). Samples were incubated on ice for 30 min in the dark. Cells were washed once with 1 ml of 2% FBS in PBS and the resulting pellet was resuspended in 500 μl of 4% paraformaldehyde (pH 7; Fisher Scientific) and stored at 4°C until analysis. Samples were analyzed using a Coulter EPICS XL-MCL (Beckman Coulter, Hialeah, FL) flow cytometer. Significant differences between the percentages of labeled cells from control and infected groups were determined using a two-way ANOVA with two between-group factors (treatment and dpi).

Fragment cultures

Fragment cultures were performed as previously described (19). Briefly, all intestinal PP and MLN were removed and washed three times in HBSS-Ca-Mg (Life Technologies) supplemented with 25 mM HEPES (Sigma-Aldrich, St. Louis, MO) and 50 μg/ml gentamicin (Life Technologies). Tissues were suspended in 48-well plates containing 400 μl of GALT medium (H-Y medium (Sigma-Aldrich) containing 10 mM HEPES, 10% PBS, 4 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamicin, and 0.25 μg/ml amphotericin B). Samples were incubated for 4–6 days at 37°C with 95% humidity and 5% CO<sub>2</sub>. Supernatants were removed and specific Ab amounts were determined by ELISA (18). Differences in the amount of RV-specific Ab produced by each tissue were analyzed using a Student t test. A value of p < 0.05 was considered significant.

Results

RV infection causes hyperplasia of immune tissue

Quantitation of fecal RV Ag levels of CD-1 mice orally infected with a homologous strain of murine RV, EC<sub>wt</sub>, revealed that CD-1 mice shed RV Ag within 24 h after infection and rapidly resolved infection between 5 and 7 dpi (Fig. 1). This is in contrast to mice deficient in both B and T lymphocytes, which continually shed RV Ag, suggesting that lymphocytes mediate clearance of RV infection (12, 13). However, the phenotype and numbers of lymphocytes activated early during a primary RV infection have not been identified. Because the virus enters via the gastrointestinal tract, we examined PP and MLN, two sites of early inductive immune responses after exposure to enteric pathogens. Additionally, we examined the response in the SP, classically thought to be involved in systemic immune responses. Isolation of the three tissues revealed dramatic increases in the size of PP and MLN, but not SP, of EC<sub>wt</sub>-inoculated mice between 2 and 5 dpi compared with PBS-inoculated mice (4 dpi, shown in Fig. 2, A–C).

Increased numbers of lymphocytes in PP and MLN

Histological examination of PP and MLN sections from EC<sub>wt</sub>-inoculated mice revealed a hyperplasia of lymphocytes within both tissues without any detectable increase in germinal center formation (Fig. 2, D–G). The distribution and numbers of peanut agglutinin “CD19<sup>+</sup>” cells (germinal center B cells) did not differ between infected and uninfected sections of either tissue at 4 dpi.

FIGURE 1. Fecal RV Ag shedding in CD-1 outbred mice inoculated with EC<sub>wt</sub> murine RV. Mice were inoculated orally with PBS (○) or 10<sup>9</sup> SD<sub>50</sub> of EC<sub>wt</sub> in PBS (●). Fecal samples were collected from individual mice 1–7 dpi and analyzed for rotaviral Ag shedding. Results are reported as mean OD ± SD (n = 5).

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Additionally, tingible body macrophages indicative of Ag processing were observed in the PP sections from ECwt-inoculated mice at 2 and 4 dpi but not in PBS controls (data not shown). Because histological analysis indicated an increase in the number of lymphocytes, single cell populations were quantified from the SP, PP, and MLN from both control and infected groups. Anatomical and histological observations were confirmed; there were significant increases in total lymphocyte numbers in the PP and MLN of RV-inoculated mice (Fig. 3) compared with PBS-inoculated mice (Fig. 3; \( p < 0.05 \)). When taken together with the overall increase in lymphocyte number, the flow cytometry data indicate that, following a RV infection, there is an overall increase in the total number of each subset of lymphocytes in the PP and MLN but only a specific and large increase in the number and percentage of activated B cells.

A small but significant increase in the percentage of activated CD8\(^+\)T lymphocytes accompanied the increase in the percentage of activated B cells in the MLN at 4 dpi, but there was no increase in the percentage of CD8\(^+\)CD69\(^+\) T lymphocytes at any time point in the PP. No significant increases in the percentage of activated B or T lymphocyte populations was observed at any time point in SP or in the lamina propria from infected mice (data not shown). Similar results showed that the percentage of lymphocytes expressing two other activation markers, CD25 and CD44, also were increased in the CD19\(^+\) B cell, but not T cell, population early after primary infection (data not shown). These data indicate that murine RV infection of an immunologically intact mouse results in the activation of a significant percentage of B lymphocytes early after primary infection (data not shown). These data indicate that murine RV infection of an immunologically intact mouse results in the activation of a significant percentage of B lymphocytes early after primary infection (data not shown). These data indicate that murine RV infection of an immunologically intact mouse results in the activation of a significant percentage of B lymphocytes early after primary infection (data not shown).
B lymphocytes are activated in RV-infected TCRKO mice

To prove that T cells were not required for the early massive B cell activation we observed in the PP and MLN, but only the percentage of CD19+CD69+ lymphocytes is elevated. Lymphocytes were isolated from control-inoculated (PBS) and RV-infected (ECwt) mouse tissues and counted by hemocytometer. In the upper panels, the results are plotted as the average number of lymphocytes (n = 5) ± SD. *Significant differences in number of lymphocytes in RV-infected mice (p < 0.05) compared with PBS-inoculated mice at each time point by two-way ANOVA. †PBS-inoculated mice. ○ ECwt-inoculated mice. Isolated lymphocytes were then incubated with fluorescent Abs against cell surface lymphocyte phenotype markers CD4, CD8, or CD19 in combination with fluorescent Abs against the very early activation marker CD69. Single- and double-labeled cells were quantified using flow cytometry using a minimum of 10,000 events. In the lower panels, data are plotted as the percentage of positive lymphocytes of the total analyzed ± SD at 1–7 dpi. ○ PBS-inoculated mice (n = 5); †ECwt-inoculated mice (n = 5). Significant increases in the percentage of activated lymphocytes between RV- and PBS-inoculated mice at each time point were determined using a two-way ANOVA with two between-group factors (*, p < 0.05).

Detection of RV-specific Abs

To confirm RV specificity of the B cell response observed as early as 2 dpi, we tested fragment cultures from the MLN and PP for RV-specific Ab. Other groups have shown that, after infection with RV, a serum IgM response is observed as early as 3 dpi, while serum IgA is not detectable until 7 dpi (9). However, we and others have demonstrated that fecal samples from RV-infected mice have no detectable IgM but, like the serum response, IgA is present by 7 dpi (Refs. 4 and 9 and data not shown). TCRKO mice have detectable, albeit lower, levels of fecal IgA detectable by 7 dpi (14). To determine whether the increased percentage of activated B cells detected in the PP and MLN early after a primary RV infection in mice might be capable of locally producing either RV-specific IgM or RV-specific IgA, we used fragment culture followed by ELISA. PP and MLN fragments from outbred CD-1, C57BL/6J, and TCRKO mice collected at either 4 or 10 dpi were placed in culture for 4 days, and media were collected and tested for RV-specific IgM and IgA Ab. RV-specific IgM, but not IgA, was detected at 4 dpi in PP and MLN fragment cultures established from ECwt-inoculated CD-1 mice but was not detected in mock-inoculated mice (Fig. 5A). At 10 dpi, both RV-specific IgM and RV-specific IgA are present in the PP and MLN fragment cultures (Fig. 5B). Ileal intestinal segments were used as a control for IgA production. Similar results were observed at 4 dpi in the C57BL/6J strain (Fig. 5, C and D). RV-specific IgM was detected in fragment cultures of PP from ECwt-inoculated TCRKO mice 4 dpi (Fig. 5C); however, no IgM was detectable in the MLN (Fig. 5D). This can be attributed to our observation that there is a short delay in onset of Ag shedding in the TCRKO mice compared with their wild-type C57BL/6J controls (data not shown).

Discussion

This is the first report of early cellular immune responses induced by RV infection in an immunologically intact adult mouse. Infection of immunologically competent mice by a highly virulent homologous strain of RV (ECwt) results in large increases in the size of the PP and MLN, but not the SP. Based on histological analysis and quantification, the increased size of the PP and MLN results from a hyperplasia of lymphocytes. In this study, we examined immune responses to only one homologous murine strain of RV, ECwt, in CD-1 mice. Our preliminary data suggest that lymphoid hyperplasia is induced by a less infectious mouse RV (epizootic
diarrhea of infant mice) and heterologous simian and rhesus (RRV and SA11) RV strains in CD-1 mice. In contrast, neither a simian-human nor a bovine-human reassortant induced lymphoid hyperplasia in BALB/c mice (20). Therefore, further investigations will be necessary to determine whether the lymphoid hyperplasia induced by RV is dose, strain, or recipient mouse dependent.

Further analysis of the lymphocyte populations from the PP and MLN of ECwt-infected mice revealed a large increase in the percentage of activated CD8+ T lymphocytes in the PP and MLN, but not SP, during the first week following RV infection. There was an absence of concomitant detectable increases in the percentage of activated CD4+ and CD8+ T lymphocytes in these tissues during infection, with the exception of a small but significant increase in the percentage of activated CD8+ T lymphocytes in the MLN at one time point (4 dpi) (Fig. 3). Because our analysis is limited to 1–7 dpi, we do not know when or at what site CD4 or CD8 T cell responses occur. However, because RV-specific CD8 T cells are reported by others (15), activation of CD8 T cells must occur after 7 dpi. Together, the data suggest that there is an initial early T cell-independent B cell response possibly followed by a later T cell activation phase, and that the mechanism that likely mediates clearance of RV infection in the context of a immunocompetent mouse is a T cell-independent B cell response. It is possible that other gut-associated immunological sites harbor populations of activated T cells early in infection. However, this seems unlikely because the PP and MLN are thought to be the sites of induction of intestinal immune responses. Preliminary studies of the activation status of lamina propria lymphocytes (the effector site of the gut immune system) also failed to reveal any significant differences in activated T or B cell populations in RV-infected mice compared with uninfected mice (data not shown), suggesting that early activation of T cells is not occurring in the lamina propria.

Although flow cytometry is a widely accepted method for quantitating the activation status of lymphocytes, it is possible that the flow cytometry studies that we performed to detect activated lymphocyte populations after a murine RV infection might have missed small, but biologically important, increases in the percentages of activated T cell subsets. Therefore, we tested whether TCRKO mice, which have no T cells present in the PP and MLN, would develop the same increase in the percentage of activated B cells. TCRKO mice clear RV infection and produce RV-specific Ab after infection (14), suggesting that RV has the capability of activating B cells in the absence of T cells. Our detection of significant increases in the percentage of activated B lymphocytes in the MLN and PP from RV-infected TCRKO mice confirms that B cells in the PP and MLN do not require the presence of T cells to become activated in response to RV. However, the results in TCRKO mice do not eliminate the possibility that other immune cells, including dendritic cells or NK cells, are required to initiate B cell activation following a RV infection in the mouse.

We did not detect any significant differences in the percentages of CD4+, CD8+, CD19+, CD4+CD69+, and CD8+CD69+ present in the PP and MLN of RV-infected mice. However, the overall number of lymphocytes is increased in the PP and MLN by hemocytometer counting at many time points. When the overall number of lymphocytes in each tissue, obtained by hemocytometry, is multiplied by the percentage of each phenotypic subset examined in our study, obtained by flow cytometry, the PP and MLN from RV-infected mice had an increased number of each phenotypic subset of lymphocytes compared with the respective subset from control mice. This difference is not attributable to differences in percentages of each type (Fig. 3) but is due to the overall increases in lymphocyte numbers obtained by hemocytometry (Fig. 3). This overall increase in lymphocyte number in the PP and MLN results from one of two mechanisms: the local proliferation of PP and MLN lymphocytes or a general influx of naive lymphocytes into the PP and MLN. Currently studies are under way to test these hypotheses. Previous studies have demonstrated that trafficking mechanisms using the integrin $\alpha_4\beta_2$ have been shown to have an important role in targeting B lymphocytes to the intestine following RV infection (21, 22). Alternatively, many chemokines and cytokines are implicated as chemoattractants in B cell recruitment to specific sites (23) and could be possible factors that account for the increase in the percentage of activated B cells in the PP and MLN following RV infection.

We could not directly assess the RV specificity of the activated B cells because as activated B lymphocytes differentiate into RV-specific Ab-producing plasma cells they lose expression of both CD69 and the B cell marker CD19. Therefore, to confirm RV specificity of the B cell response, we tested fragment cultures for RV-specific Ab. We found that the B cells present in the PP and MLN at 4 dpi from RV-infected mice produced RV-specific IgM but not IgA (Fig. 5A). By 10 dpi, when RV shedding has ceased, RV-specific IgA can be detected (Fig. 5B). We do not observe increases in percentages of activated T cells, which are classically thought to control Ab isotype class switching from IgM to IgA, but Macpherson et al. (24) have recently demonstrated that some Ags can stimulate class switching to IgA in the intestine in the absence of T cell help. Our data suggest that RV may fall into this class of Ags. Further investigations into the immune responses after 7 dpi (at which time Ag is no longer detectable in fecal samples) will be necessary to conclude that RV does not induce any T cell activation, but our fragment culture data describing the local Ab response in two GALTs indicates that production of IgA in these
tissues occurs in the absence of T cell activation. The RV Ag specificity of the T cell-independent Ab response in CD-1 and TCRKO mice is not known. It is possible that all RV Ags can stimulate an Ab response, but Franco and Greenberg (14) hypothesized that the RV-specific fecal IgA is directed against the VP6 structural component of the virus. To more directly characterize the Ab produced by RV-specific B cells in the PP and MLN, ELISPOT studies will be necessary and are under way.

Ab responses to viral proteins are typically thought to be induced through primarily T cell-dependent pathways. However, studies on many viruses in T cell-deficient mouse models clearly indicate that many viruses can act as T cell-independent Ags and induce the production of Abs without T cell help. Polymavirus infection in T cell-deficient mice resulted in the production of virus-specific IgM and IgG, which was protective against the induction of disease (25). Several recent studies suggest that other viruses, such as vesicular stomatitis virus, lymphocytic choriomeningitis virus, Pinchinde virus, murine CMV, and vaccinia virus, can also induce IgM, followed by the production of isotype-switched IgG and IgA in the absence of T cell help (26). However, the ability of a virus to produce T cell-independent Ab responses in T cell-deficient mice does not necessarily mean that T cell-independent Ab responses are biologically relevant to the immune response to that same virus in a mouse that is immunologically intact. Our studies provide the first direct evidence in vivo that a T cell-independent B cell response likely mediates clearance of a primary RV infection within the context of an intact immunocompetent host.

The data presented in this work strongly suggest that the early Ab response to RV is T cell independent and is the first report of an enteric viral infection causing T cell-independent activation of B lymphocytes in vivo detectable very early after infection (within 48 h). Understanding the mechanisms involved in inducing this immune response will contribute significantly to the development of vaccines against RV and other viruses that infect across mucosal surfaces.

Acknowledgments

We thank Dr. Claudia Kozinetz for her assistance in performing the statistical analysis of the data, David Keeland for technical assistance, and Dr. Mary K. Estes and Dr. John Rodgers for helpful comments.

References