Association of Gamma Interferon and Interleukin-17 Production in Intestinal CD4+ T Cells with Protection against Rotavirus Shedding in Mice Intranasally Immunized with VP6 and the Adjuvant LT(R192G)∗

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Mucosal immunization of mice with chimeric, Escherichia coli-expressed VP6, the protein that comprises the intermediate capsid layer of the rotavirus particle, together with attenuated E. coli heat-labile toxin LT(R192G) as an adjuvant, reduces fecal shedding of rotavirus antigen by >95% after murine rotavirus challenge, and the only lymphocytes required for protection are CD4+ T cells. Because these cells produce cytokines with antiviral properties, the cytokines whose expression is upregulated in intestinal memory CD4+ T cells immediately after rotavirus challenge of VP6/LT(R192G)-immunized mice may be directly or indirectly responsible for the rapid suppression of rotavirus shedding. This study was designed to identify which cytokines are significantly upregulated in intestinal effector sites and secondary lymphoid tissues of intranasally immunized BALB/c mice after challenge with murine rotavirus strain EDIM. Initially, this was done by using microarray analysis to quantify mRNAs for 96 murine common cytokines. With this procedure, the synthesis of mRNAs for gamma interferon (IFN-γ) and interleukin-17 (IL-17) was found to be temporarily upregulated in intestinal lymphoid cells of VP6/LT(R192G)-immunized mice at 12 h after rotavirus challenge. These cytokines were also produced in CD4+ T cells obtained from intestinal sites specific to VP6/LT(R192G)-immunized mice after in vitro exposure to VP6 as determined by intracellular cytokine staining and secretion of cytokines. Although genetically modified mice that lack receptors for either IFN-γ or IL-17 remained protected after immunization, these results provide suggestive evidence that these cytokines may play direct or indirect roles in protection against rotavirus after mucosal immunization of mice with VP6/LT(R192G).

Rotaviruses are the primary etiologic agents of severe infantile gastroenteritis and are estimated to cause >600,000 deaths worldwide each year (39, 40). Vaccines developed to combat rotavirus disease that have been evaluated in clinical trials have all been live rotaviruses that are delivered orally. Their development is based on the high levels of protection against severe rotavirus disease stimulated by natural infections (14). Protection elicited by these vaccines has been variable, and none has consistently provided complete protection against severe disease even in developed nations (12). Furthermore, the first rotavirus vaccine licensed in the United States was withdrawn in less than 1 year due to a rare but detectable association with intussusception (27, 36). Therefore, potentially safer and more efficacious rotavirus vaccines not composed of live viruses have been developed and evaluated in animal models. One such candidate is the VP6 protein, which comprises the intermediate capsid layer of the triple-layered rotavirus particle. When expressed in Escherichia coli as a fusion protein with either maltose-binding protein (MBP) or a histidine hexapeptide (6XHis) as required for purification and administered either intranasally or orally to adult mice along with genetically attenuated E. coli heat-labile toxin LT(R192G) as an adjuvant, this candidate vaccine provided nearly complete protection against fecal rotavirus shedding (9, 10).

The mechanisms by which this VP6/LT(R192G) vaccine elicits protection appear to be distinct from those suggested after live rotavirus immunization (20, 31). That is, protection is not dependent on B or CD8+ T cells but, instead, CD4+ T cells are the only lymphocytes required for protection, and this protection remains fully intact for many months, probably for the lifetime of the mouse (9, 10, 34). Although there is evidence that CD4+ T cells may also be the only lymphocytes needed for protection against other viruses (11, 22, 23, 35, 37, 41, 54), antibodies and CD8+ cytotoxic T cells are the most common effectors of protection against viral diseases, and the role of CD4+ T cells is typically restricted to their helper functions. Since cytokines made by CD4+ T cells, such as gamma interferon (IFN-γ), have antiviral activities (11, 35), one mechanism by which mucosal immunization of mice with VP6/LT(R192G) may elicit protection is through the direct or indirect activities of cytokines whose production is stimulated in rotavirus-specific CD4+ T cells after intestinal rotavirus infection.

The first purpose of the present study was to identify, through microarray analyses, cytokines whose production is specifically upregulated in intestinal lymphoid tissues within the first days after rotavirus challenge of VP6/LT(R192G)-immunized mice, during which time intestinal production of
rotavirus is rapidly suppressed. The next purpose was to determine whether the cytokines identified in vivo are also upregulated after in vitro VP6 stimulation of intestinal memory CD4<sup>+</sup> T cells using fluorescence-activated cell sorting (FACS) analysis. Using these methods, interferon-17 (IL-17) and IFN-γ were the only cytokines identified whose production was specifically upregulated within the intestinal CD4<sup>+</sup> T cells of VP6/LT(R192G)-immunized mice after both in vivo and in vitro rotavirus stimulation. Thus, CD4<sup>+</sup> T-cell production of IL-17 and IFN-γ may play a pivotal role in protection against rotavirus shedding after mucosal immunization of mice with VP6/LT(R192G).

MATERIALS AND METHODS

**Virus.** The murine strain of rotavirus (EDIM) used in the present study was initially acquired from M. Collins (Microbiological Associates, Bethesda, MD). The production of wild-type (wt), unpassaged (i.e., grown only in infected neonatal mice) EDIM used to challenge mice after vaccination was described previously (34).

**Mice.** Six-week-old BALB/c mice were purchased from Harlan Laboratories (Indianapolis, IN). Breeding pairs of IL-17 receptor (IL-17R) knockout (KO) mice on a C57BL/6 background were obtained from Aigen (Sagett, WA) through Taconic (Hudson, NY). The animals were bred, and the pups were used after reaching 6 weeks of age. Six-week-old IFN-γ receptor/IFN-γ receptor double-KO mice (IFN-γR) and wt controls (129Sv/ev background) were obtained from B&K Universal (East Yorkshire, England). All mice were housed in microisolation cages and shown by an enzyme-linked immunosorbent assay (ELISA) to have no detectable rotavirus antibody prior to use. All animal procedures were conducted in accordance with the Cincinnati Children’s Hospital Research Foundation Institutional Animal Care and Use Committee.

**Construction, expression, and purification of the recombinant rotavirus VP6 protein.** All steps involving the construction of a recombinant plasmid containing the coding sequence for the chimeric EDIM VP6:6XHis protein, the expression of the VP6:6XHis protein in E. coli, and the purification of this protein have been described elsewhere (47).

**Adjuvant.** The attenuated E. coli heat-labile toxin LT(R192G) was used as adjuvant in these studies. LT(R192G) carries a mutation in the proteolytic site of the A subunit at amino acid 192 that abrogates cleavage and attenuates the toxicity of the protein (15).

**Immunization of mice using expressed VP6 protein and the adjuvant LT(R192G).** Under light sedation with isoflurane (Abbott Laboratories, Chicago, IL), 6-week-old mice were intranasally administered, either individually or together, two doses of EDIM VP6:6XHis (4 µg) and LT(R192G) (10 µg) separated by 2 weeks.

**Challenge of mice with EDIM and detection of viral shedding.** Four weeks after the second immunization, groups of six to eight unimmunized (naive) or VP6/LT(R192G)-immunized mice were orally challenged with 4 x 10<sup>4</sup> focus-forming units (10,000 50% shedding doses [SD<sub>50</sub>]) of wt EDIM. To measure rotavirus shedding, two stool pellets were collected from each mouse for 7 days after challenge and stored at −20°C in 1 ml of Earle’s balanced salt solution. The collected samples were thawed and mixed well before debris was removed by centrifugation (1,000 x g, 10 min). Rotavirus antigen was then quantified by using an ELISA as previously described (32). The results were expressed as nanograms of rotavirus antigen per milliliter of stool sample. Protection was determined by comparing the mean quantity of viral antigen shed in immunized animals relative to that shed in unimmunized animals during the 7 days after EDIM challenge.

**Isolation of lymphocytes for mRNA quantitation and cytokine determination.** At various time points after EDIM challenge (0, 12 h, 1 day, 2 days, 4 days, 6 days, and 8 days), intestinal lymphocytes (IL), Peyer’s patches (PP), and mesenteric lymph nodes (MLN) were harvested from naive, LT(R192G)-immunized, and VP6/LT(R192G)-immunized mice for gene expression analysis. Each group consisted of pooled lymphocyte populations from two to four animals. The mice were sacrificed, and their intestines were harvested. Mesenteric and connective tissues were removed from the intestines, and the PP were excised. The MLN and PP were prepared as single-cell suspensions by disruption between sterile glass frosted slides and passage through a 70-µm-pore-size filter (BD Biosciences, Bedford, MA) and then collected in complete RPMI medium containing 10% fetal bovine serum, 2 mM l-glutamine, 1 mM sodium pyruvate, 100 µg of penicillin/ml, 100 µg of streptomycin/ml, and 100 µM minimal essential medium nonessential amino acids, 55 µM 2-mercaptoethanol (Gibco/Invitrogen Corp., Grand Island, NY), and 2.5 µg of amphotericin B (Cellgro Mediatech, Herndon, VA)/ml.

To prepare IL containing both intraepithelial lymphocytes (IEL) and lamina propria (LP) cells, the intestines were cut open longitudinally, washed twice in complete RPMI, and placed in a petri dish containing complete RPMI. The intestines were then cut into small pieces and added to 6 to 8 wells of a 24-well tissue culture plate. A total of 1 ml of Liberase CI (Roche, Indianapolis, IN) diluted in RPMI was added to each well at a final concentration of 0.5 mg/ml and allowed to incubate for 50 min at 37°C. After incubation, 500 µl of complete RPMI plus 0.05% DNase I (Sigma, St. Louis, MO) was added to each well. The digested intestinal pieces were strained sequentially through 100- and 70-µm-pore-size filters (BD Biosciences) while rinsing with fresh complete RPMI plus 0.05% DNase I. The medium containing IL was collected and held on ice. The IL and PP cell suspensions were each pelleted at 1,000 x g for 10 min. The cell pellets of both tissues were resuspended in 40% Percoll (Amer sham Pharmacia Biotech, Uppsala, Sweden) and layered onto a 70% Percoll cushion. The gradients were centrifuged (30 min, 1,500 x g), and lymphocytes were isolated from the interface. The cells were washed with RPMI, pelleted (1,000 x g, 10 min), washed again in RPMI, and resuspended in phosphate-buffered saline. Prior to RNA isolation, lymphocytes from MLN, PP, and intestines were quantified by using trypan blue exclusion.

To determine T-cell responses and cytokine secretion after in vitro stimulation, MLN, PP, LP, and IEL lymphoid cells were isolated from naive, VP6-immunized, LT(R192G)-immunized, or VP6/LT(R192G)-immunized mice at the time of challenge. To obtain enough cells for in vitro stimulation, each group consisted of pooled lymphocytes from four mice. All intestinal cell populations of IEL and LP lymphocytes followed the previously described method (31), except that the LP fraction was incubated with Liberase CI twice prior to Percoll gradient centrifugation. MLN and PP isolations used the method described above. The lymphocyte populations from each site were then further enriched by using high-affinity negative selection columns specific for CD3<sup>+</sup> cells as described by the manufacturer (R&D Systems, Minneapolis, MN).

**RT-PCR.** RNA was isolated from intestinal RNA isolation. The IL, MLN, and PP lymphocyte populations isolated above were pelleted at 1,000 x g for 7 min. The supernatants were removed, and 5 to 10 x 10<sup>6</sup> cells were lysed in 1 ml of TRIzol (Invitrogen, Carlsbad, CA) for 5 min at room temperature. A total of 200 µl of chloroform was then added, followed by shaking, and the mixture was allowed to sit for 3 min. The samples were centrifuged (15 min, 12,000 x g, 4°C). The aqueous upper phase was obtained, and 600 µl of 70% ethanol was added, the mixture was transferred onto an RNeasy Mini column (QIAGEN, Valencia, CA). The remainder of the RNA isolation procedure, i.e., washes and elutions, followed the Qiagen RNeasy protocol as supplied by the manufacturer. Total RNA was eluted in 30 µl of RNase-free distilled water and stored at −80°C. The quality and quantity of the RNA in the samples were determined through their 260/280-nm absorbance ratios (recommended value, >1.8), respectively. The quality of the extracted RNA was also determined by evaluating the intensities of the 28S and 18S rRNA bands after agarose gel electrophoresis and ethidium bromide staining.

**cDNA microarray.** The GEArray Q series mouse common cytokine gene array (SuperArray Bioscience Corp., Frederick, MD) was used to determine mRNA expression of cytokines in all samples. The 112 gene-specific cDNA fragments, including housekeeping genes, are printed in a tetra-spot format onto a nylon membrane. The biotin-labeled cDNA probe was synthesized according to the protocol of the AmpoLabeling-LPR kit (SuperArray Bioscience Corp.). This kit uses a nonspecific reverse transcription step, followed by a gene-specific PCR step. We used 1 µg of total RNA from each sample for analysis. The gene array protocol for chemiluminescent detection was followed as specified by the manufacturer.

The membranes were exposed to X-ray film (Kodak X-OMat FS-1) for 5 min. An Epson scanner was used to convert the image into a grayscale TIFF file. The tetra-spot images were then converted into uncorrected mean pixel intensities by using the Scanalyze software (Michael Eisen, Stanford University). The data were analyzed by using the GEArray analyzer software provided by SuperArray. This software matches the data with the gene list for the specific gene array used.

All intensity values underwent background subtraction using the signal of a blank control. The data were also normalized to the average signal of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene. The final corrected mean intensity values were then used to compare the quantities of cytokine mRNA in the samples. Genes with a substantial normalized intensity (∼1:2 ratio of gene of interest to housekeeping gene) were further analyzed.
Therefore, a normalized mean intensity of 0.5 became the cutoff. If a gene met this requirement for at least one time point in any group it was kept for further analysis. To further analyze the data at various time points after infection, differential expression (DE) values were calculated. The time zero (prechallenge) value for each gene in each group served as the control. To calculate the DE values, the corresponding time zero intensity was subtracted from the intensity of each subsequent time point. Therefore, the DE signifies the difference in gene expression between the time points after infection and their uninfected control. To allow only the most significant changes in gene expression to be observed, a DE value of ≥2 was chosen to represent upregulation of expression.

Measurement of T-cell responses and cytokine secretion from isolated lymphocytes. CD3+ lymphocytes from the LP, IEL, MLN, and PP isolated as described above were obtained from naive, VP6-immunized, LT(R192G)-immunized, and VP6/LT(R192G)-immunized mice at 4 weeks after the second immunization, the normal time of challenge. The cells (10^7 cells/ml) were placed in 24-well tissue culture plates containing naïve BALB/c bone marrow-derived dendritic cells (10^6 dendritic cells/well) as described by Inaba et al. (24). The dendritic cell populations were activated with lipopolysaccharide (Sigma) at a concentration of 1 μg/ml for 18 h prior to addition of the lymphocyte populations. All cultured cells were either stimulated with virus (with EDIM VP6::6XHis antigen (4 μg/ml)) or left unstimulated for 18 h. During the final 4 h of culture, brefeldin A (Pharmingen, San Diego, CA) was added to the cells to inhibit extracellular cytokine transport.

For the determination of cytokine secretion, the supernatants from the cultures were harvested and frozen. To measure the cytokines secreted during VP6 stimulation, ELISA kits for IL-4, IL-5, IFN-γ, and IL-17 were purchased from R&D Systems, and protocols were followed as instructed by the manufacturers. Cytokine levels measured were expressed as picograms/milliliter.

To measure intracellular cytokine production after in vitro stimulation, the cells were incubated with purified antibody to block Fc receptors (2.4G2) and then stained with fluorescein isothiocyanate-conjugated antibody to CD4 (clone RM4-4), PerCP-conjugated CD8a (clone 53-6.7), Allophycocyanin-conjugated CD44 (clone IM7), and isotype controls from Pharmingen. The cells were fixed and permeabilized by using a GolgiPlug kit (Pharmingen). Using phycoerythrin-conjugated antibodies (Pharmingen) for either IL-4 (clone 11B11), IL-5 (clone TRFK5), IL-17 (clone TC11-18H10), or IFN-γ (clone XMG1.2), the cells were stained for the presence of accumulated cytokine. The samples were analyzed on a FACSCalibur (BD Biosciences, San Jose, CA). The data were acquired by counting 100,000 events. Cells were first gated on the lymphocyte population by forward and side scatter and then gated on CD4+ and CD8+ populations. The percentage of CD4+ or CD8+ T cells staining for CD44hi and the specific cytokines were determined. The data were analyzed by using CellQuest software (BD Biosciences). The data were obtained from three independently performed experiments.

RESULTS

Intranasal immunization of BALB/c mice with VP6/LT(R192G) protects against fecal shedding of EDIM after challenge. We previously reported that when adult BALB/c mice were administered two intranasal doses of a fusion protein composed of MBP and the VP6 protein of murine rotavirus strain EDIM, along with LT(R192G) as adjuvant, and then orally challenged with EDIM 4 weeks after the second dose, fecal shedding of rotavirus antigen was consistently reduced by ≥95% relative to that found in unimmunized control mice (9, 10). MBP was included to facilitate the purification of VP6 after its expression in E. coli. To avoid potential complications associated with the use of MBP, whose large size is comparable to the immunogen of interest (42.7 kDa for MBP versus 45.0 kDa for EDIM VP6), a new fusion protein was generated composed of EDIM VP6 linked at its carboxyl terminus to a histidine hexapeptide and purified by nickel affinity chromatography (8). After 2 intranasal immunizations with this new VP6 fusion protein, along with LT(R192G), fecal shedding of rotavirus antigen was reduced 93% relative to naive or unimmunized control mice (Fig. 1), a value that was comparable to that found after EDIM challenge of mice immunized with MBP::EDIM VP6. Furthermore, as was also found after immunization with MBP::EDIM VP6, essentially no rotavirus shedding was found by 3 days after challenge in EDIM VP6::6XHis-immunized mice. No significant reduction in fecal rotavirus shedding after EDIM challenge was found after intranasal immunization with either EDIM VP6::6XHis or LT(R192G) administered individually (data not shown).

VP6/LT(R192G) immunized mice display a rapid upregulation of the Th1 cytokine IFN-γ and the T-cell cytokine IL-17 in intestinal lymphoid tissue after EDIM challenge. To determine whether mRNA production from any cytokine gene is specifically upregulated after intestinal EDIM challenge either in tissues adjacent to the site of infection (i.e., intestinal lymphoid tissue) or in secondary lymphoid organs (i.e., PP or MLN) due to prior VP6/LT(R192G) immunization, the quantities of cytokine mRNAs present in these sites were determined by microarray analysis as a function of time after challenge in VP6/LT(R192G)-immunized, LT(R192G)-immunized, and naive adult BALB/c mice. First, we identified genes on the array whose mRNAs were produced in substantial amounts at some time during the 8-day observation period. For the purpose of the present study, a signal of at least 50% of the amount found for the GAPDH housekeeping gene was defined as a substantial amount. Second, DE, or the difference in gene expression at the time of EDIM challenge (time zero) and at each time point after challenge, was used to determine the effects of EDIM infection on this expression in both immunized and in the naive groups of mice. Genes whose normalized intensity DE values differed by ≥2 relative to time zero were defined as significantly upregulated. During the 8-day observation period, 12 genes met the criteria of upregulation in at least one site at some point in time in ≥1 group of mice (Table 1).
TABLE 1. Differential expression values determined from gene expression in IL, MLN, and PP lymphocyte populations isolated from naïve, LT(R192G)-immunized, and VP6/LT(R192G)-immunized mice at specific time points after EDIM challenge

<table>
<thead>
<tr>
<th>Group</th>
<th>Cell type</th>
<th>Gene</th>
<th>12 h</th>
<th>1 day</th>
<th>2 days</th>
<th>4 days</th>
<th>6 days</th>
<th>8 days</th>
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<td>Naive</td>
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<td>IFN-α1</td>
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<td>IFN-α1</td>
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<td>IFN-αβ</td>
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<td></td>
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<td></td>
<td>CD40L</td>
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<tr>
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* DE values were determined by subtracting the corresponding (prechallenge) time zero intensity from the intensity of each subsequent time point (12 h and 1, 2, 4, 6, and 8 days after EDIM challenge). A ΔE value of ≥2 represents a significant upregulation of expression. Only DE values that met this criteria are shown.

No significant upregulation of any cytokine gene was observed at any lymphoid site examined within the first 12 h after EDIM infection of naïve mice. Furthermore, the only lymphoid sites in naïve mice where any gene was upregulated before 6 days after challenge were the PP and MLN (Table 1). In LT(R192G)-immunized mice, two genes, IFN-γ and TRAIL, were upregulated but only in the IL at 24 h after challenge. In VP6/LT(R192G)-immunized mice, no significant gene upregulation was found after EDIM challenge in the MLN. In contrast, cytokine genes were upregulated during the first 12 h after EDIM challenge in the intestinal lymphoid sites (PP and IL) of these animals in conjunction with the rapid suppression of intestinal rotavirus shedding (Table 1).

Based on the rapid production of IFN-γ mRNAs within IL by 12 h after challenge of VP6/LT(R192G)-immunized mice and the complete lack of measurable upregulation of genes associated with Th2 responses, such as IL-4 and IL-5, the responses observed in these animals were primarily Th1. Besides IFN-γ, the only other proinflammatory cytokine rapidly upregulated in intestinal lymphoid sites of VP6/LT(R192G)-immunized mice was IL-17, a cytokine whose production is primarily associated with T cells and is typically associated with its own helper T-cell subset, i.e., Th17 (29). Interestingly, when examining the mean pixel intensity of the genes for IFN-γ and IL-17, the pattern of upregulation for IL-17 (Fig. 2B) paralleled that of IFN-γ in IL throughout the 8-day period after EDIM challenge (Fig. 2A). These results were verified by using real-time reverse transcription-PCR analysis of the mRNAs for these two cytokine genes (data not shown).

In vitro stimulation of memory T cells retained in the intestinal lymphoid cells of VP6/LT(R192G)-immunized mice produce IFN-γ and IL-17. The previous results suggest that memory T cells that can be induced to rapidly produce IFN-γ and IL-17 mRNAs after EDIM challenge were generated after intranasal immunization with VP6/LT(R192G), and these cells were retained within the IL, the primary effector site involved in intestinal immunity. It follows, therefore, that the production and secretion of these two cytokines may be detectable in T cells obtained from intestinal sites in these animals after in vitro stimulation with VP6. To verify this, 4 weeks after the second intranasal immunization, lymphoid cells obtained from the LP, IEL, MLN, and PP of naïve, VP6-immunized, LT(R192G)-immunized, and VP6/LT(R192G)-immunized mice were stimulated in vitro with VP6 to determine which, if any, memory T-cell subset would secrete IFN-γ and IL-17. The percentages of lymphocytes producing these cytokines were determined by flow cytometry after in vitro stimulation and intracellular cytokine staining. Cells were stained for CD4 and CD8 surface markers in addition to the memory marker CD44. Representative dot plots of unstimulated and VP6-stimulated LP cells showing staining for IFN-γ and IL-17 are shown in Fig. 3 and 4, respectively. Isotype controls were stained for each cytokine measured, and their values were ≤0.03. Values obtained from unstimulated cultures were subtracted from the values obtained from VP6-stimulated cultures and a difference of ≥0.1% between the stimulated and unstimulated cells was considered significant. This experiment was repeated three times and representative
data from a single experiment are shown in Table 2. IL-17 production was almost solely associated with memory CD4+ T cells of VP6/LT(R192G)-immunized mice, whereas IFN-γ-producing cells were found within the memory CD4+ T-cell population but were primarily associated with memory CD8+ T cells. All groups of the immunized animals had measurable IFN-γ-producing memory CD8+ T cells in their intestinal lymphoid tissues, but the VP6/LT(R192G)-immunized animals had the greatest percentage (Table 2). The largest populations of IL-17-producing memory CD4+ T cells were detected in the LP and IEL subsets of IL (2.80 and 2.11%, respectively) from VP6/LT(R192G)-immunized mice. Only background levels of cells staining for either cytokine were detected in the naive animals. With the exception of IL-5 secretion in the LP of VP6/LT(R192G)-immunized mice, essentially no stimulation of any of the Th2 cytokines evaluated was detected (data not shown).

FIG. 3. Representative dot plot showing staining for IFN-γ in LP cells isolated from VP6/LT(R192G)-immunized mice. Cells were isolated, stimulated, and stained as described in Materials and Methods. For analysis, cells were gated on the lymphocyte population by forward and side scatter and then gated on CD4+ or CD8+ positive cells. The percentage of CD4+ T cells staining for CD44high and IFN-γ was determined in unstimulated (A) and VP6-stimulated (C) cells. The percentage of CD8+ T cells staining for CD44high and IFN-γ was also determined in unstimulated (B) and VP6-stimulated (D) cells.

FIG. 4. Representative dot plot showing staining for IL-17 in LP cells isolated from VP6/LT(R192G)-immunized mice. Cells were isolated, stimulated, and stained as described in Materials and Methods. For analysis, cells were gated on the lymphocyte population by forward and side scatter and then gated on CD4+ or CD8+ positive cells. The percentage of CD4+ T cells staining for CD44high and IL-17 was determined in unstimulated (A) and VP6-stimulated (C) cells. The percentage of CD8+ T cells staining for CD44high and IL-17 was also determined in unstimulated (B) and VP6-stimulated (D) cells.

Both IFN-αβγR KO mice and IL-17R KO mice are protected from EDIM shedding after VP6/LT(R192G) immunization. The results presented suggest that IFN-γ and IL-17 may have roles in suppression of EDIM antigen shedding after challenge of VP6/LT(R192G)-immunized mice. In an attempt to further define the roles of each of these cytokines in protection in this model, two knockout mouse strains (IFN-αβγR KO and IL-17R KO) were immunized and challenged with EDIM. It should be noted that we had previously reported that IFN-γ KO mice were protected as well as immunologically normal control animals after VP6/LT(R192G) immunization (46). However, in light of the new observations we felt it was prudent to test IFN receptor KO mice in the present study. After administration of two intranasal doses of VP6/LT(R192G) and challenge with EDIM 4 weeks later, fecal shedding of rotavirus antigen in IFN-γ KO mice and IL-17R KO mice was reduced 96 and 99%, respectively, compared to unimmunized control mice (Table 3). These values were essentially identical to those found after EDIM challenge of BALB/c mice immunized with VP6/LT(R192G). Although this result does not negate the possible roles of these two cytokines in protection in this model, it does indicate that neither, by itself, is required.
tion after live virus immunization was largely dependent on model, it was found that protection against rotavirus reinfec-
ted after oral immunization with live rotaviruses, the only
deciphering the mechanisms by which active immunity is elic-
production specifically in intestinal lymphoid tissues of VP6/
kine mRNAs in intestinal and secondary lymphoid tissues as a
utilized this model to identify significantly upregulated cyto-
mechanisms of protection associated with this new vaccine, we
tranasally or orally, has been developed and evaluated in the
expressed VP6 protein and adjuvant, administered either in-

Cells were analyzed by gating on forward and side scatter to determine the percentage of CD4
played a smaller role in long-term protection against reinfec-
responsible for resolution of most rotavirus shedding but

Lymphocytes were isolated and either unstimulated or stimulated with VP6 for 18 h, the last 4 h of culture in the presence of brefeldin A, and then stained with monoclonal antibodies to cell surface markers and intracellular cytokines. Cells were analyzed by gating on forward and side scatter to determine the lymphocyte population and then by gating on CD4$^+$ or CD8$^+$ T cells. The percentage of CD4$^+$ or CD8$^+$ cells staining for CD44high and the cytokine being measured was determined. Isotype control values were ≤ 0.03 and were sub-
tracted from the cytokine-specific values. The percentage of unstimulated cells staining for each cytokine was subtracted from the percentage of VP6-stimulated cells. –, Values of < 0.1%.

**TABLE 2. Percentages of CD4$^+$ and CD8$^+$ T cells isolated prior to challenge (time zero) from the MLN, PP, IEL, and LP of naive, VP6-immunized, LT(R192G)-immunized, and VP6/LT(R192G)-immunized mice that were stimulated to produce cytokines as determined by FACS**

<table>
<thead>
<tr>
<th>Group</th>
<th>Cell type</th>
<th>% CD4$^+$ T cells IL-17</th>
<th>% CD4$^+$ T cells IFN-γ</th>
<th>% CD8$^+$ T cells IL-17</th>
<th>% CD8$^+$ T cells IFN-γ</th>
<th>% CD8$^+$ T cells IL-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive</td>
<td>IEL</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>LP</td>
<td>–</td>
<td>0.30</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>MLN</td>
<td>–</td>
<td>0.45</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>PP</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>VP6/LT(R192G) immunized</td>
<td>IEL</td>
<td>2.11</td>
<td>–</td>
<td>0.35</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
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<td>2.80</td>
<td>0.16</td>
<td>1.54</td>
<td>0.28</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>MLN</td>
<td>0.14</td>
<td>–</td>
<td>–</td>
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</tr>
<tr>
<td></td>
<td>PP</td>
<td>0.71</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>VP6 immunized</td>
<td>IEL</td>
<td>–</td>
<td>0.10</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>LP</td>
<td>0.14</td>
<td>0.13</td>
<td>0.95</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>MLN</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>PP</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>LT(R192G) immunized</td>
<td>IEL</td>
<td>–</td>
<td>0.16</td>
<td>0.18</td>
<td>0.59</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>LP</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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</tr>
<tr>
<td></td>
<td>MLN</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>PP</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

* Lymphocytes were isolated and either unstimulated or stimulated with VP6 for 18 h, the last 4 h of culture in the presence of brefeldin A, and then stained with monoclonal antibodies to cell surface markers and intracellular cytokines. Cells were analyzed by gating on forward and side scatter to determine the lymphocyte population and then by gating on CD4$^+$ or CD8$^+$ T cells. The percentage of CD4$^+$ or CD8$^+$ cells staining for CD44high and the cytokine being measured was determined. Isotype control values were ≤ 0.03 and were sub-
tracted from the cytokine-specific values. The percentage of unstimulated cells staining for each cytokine was subtracted from the percentage of VP6-stimulated cells. –, Values of < 0.1%.

**DISCUSSION**

A new rotavirus vaccine candidate composed of E. coli-
expressed VP6 protein and adjuvant, administered either in-
trasally or orally, has been developed and evaluated in the
adult mouse model (9, 10). In order to help elucidate the
mechanisms of protection associated with this new vaccine, we
utilized this model to identify significantly upregulated cyto-
kine mRNAs in intestinal and secondary lymphoid tissues as a
function of time after rotavirus challenge and to quantify their
production specifically in intestinal lymphoid tissues of VP6/
LT(R192G)-immunized mice.

The adult mouse model was originally developed to aid in
deciphering the mechanisms by which active immunity is elic-
ated after oral immunization with live rotaviruses, the only
vaccines that have been evaluated in humans. Using this
model, it was found that protection against rotavirus reinfection after live virus immunization was largely dependent on antibodies based on loss of protection in B-cell-deficient JpD and μMT mice (19–21, 31). CD8$^+$ T cells were shown to be
responsible for resolution of most rotavirus shedding but
played a smaller role in long-term protection against reinfection (19, 20, 31), whereas CD4$^+$ T cells appeared to play their
traditional helper role in B-cell activation and maturation (31, 33). In a subsequent study it was found that intranasal immuniza-
tion of mice with either triple- or double-layered inacti-
vated EDIM rotavirus particles elicited protection that was
only partially dependent on antibody based on the incomplete
loss of protection against EDIM shedding in B-cell-deficient
mice (32). No greater loss of protection was observed when these immunized mice were depleted of CD8$^+$ T cells just
prior to EDIM challenge, thus indicating that the remaining
protection was not due to CD8$^+$ T cells. Because CD4$^+$ T cells were the only memory cells unaccounted for in that study, it was suggested that they played a direct role in protection after immunization with inactivated rotavirus particles.

More recently, it was found that intranasal immunization
with the VP6 protein plus the adjuvant LT(R192G) provided
equal protection against rotavirus shedding in normal BALB/c
and B-cell-deficient BALB/c mice (9). Therefore, the presence

**TABLE 3. Protection against EDIM shedding after VP6/LT(R192G)
immunization in IL-17R KO mice and IFN-α/βR KO mice**

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>Mean amt of antigen shed (ng/mouse/day) ± SD</th>
<th>% Protection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Naive mice</td>
<td>VP6/LT(R192G)-immunized mice</td>
</tr>
<tr>
<td>C57BL/6 IL-17R KO</td>
<td>989 ± 826</td>
<td>8 ± 22</td>
</tr>
<tr>
<td>129Sv/ev IFN-α/βR KO</td>
<td>4,128 ± 4,865</td>
<td>179 ± 387</td>
</tr>
</tbody>
</table>

* Groups of six mice from each strain were either not immunized or immu-
nized with two doses of VP6/LT(R192G). At 4 weeks after the last immunization, the mice were orally challenged with 10,000 SD$_0$ of wt EDIM, and rotavirus shedding was measured for 7 days after challenge.

* That is, the mean quantity of rotavirus antigen shed per day for 7 days after EDIM challenge.

* The percent protection represents the percent decrease in the mean quantity of rotavirus antigen shed per day during the 7 days after challenge in the
immunized groups compared to the nonimmunized or naive group.

FIG. 5. Cytokine secretion after in vitro stimulation of lymphocytes isolated prior to challenge (time zero) from the MLN, PP, IEL, and LP of naive, VP6-immunized, LT(R192G)-immunized, and VP6/
LT(R192G)-immunized mice. Lymphocytes isolated from 12 animals were pooled and placed in culture and stimulated with VP6 or left unstimulated for 18 h. Supernatants were harvested, and the cytokine concentrations were determined by ELISA. Values are expressed as the amount of cytokine secreted in the supernatants of the stimulated cultures minus the amount secreted in the unstimulated cultures (in pg/ml). The data are representative of one of three independent experiments.
of antibody was not essential to VP6/LT(R192G)-induced protection. Depletion of CD8+ T cells had no effect on protection in VP6/LT(R192G)-immunized, B-cell-deficient mice, but depletion of CD4+ T cells in these animals resulted in a complete loss of protection (34). These studies showed that CD4+ T cells were the only lymphocytes necessary for protection in VP6/LT(R192G)-immunized mice but did not rule out the involvement of antibody or CD8+ T cells if present. Antibody to VP6 has been shown to be protective in a backpack model (7, 17), and CD8+ T cells have been shown to be involved in rotavirus clearance (20, 31). From these studies, it was concluded that the role of CD4+ T cells in protection against rotavirus in mice varied greatly as a function of the immunogen, the adjuvant, and the route of immunization.

Lymphocytes, such as CD4+ T cells, are known to secrete cytokines in response to viral infection and memory T cells that recognize epitopes on the infecting virus are rapidly activated to produce cytokines (6, 16). Because rotavirus shedding is quickly suppressed after EDIM challenge of VP6/LT(R192G)-immunized mice and CD4+ T cells were found to be critical to VP6/LT(R192G)-induced protection, it is possible that one or more of these cytokines is directly or indirectly responsible for this protection. It was of interest, therefore, to determine which cytokine genes are uniquely expressed in intestinal lymphoid sites after EDIM challenge of VP6/LT(R192G)-immunized mice and which of these are primarily expressed in CD4+ T cells.

Using microarray analysis to measure the relative quantities of mRNAs for the different cytokine genes expressed after EDIM challenge of naive mice, biphasic transcriptional responses were observed in PP and MLN, initially at 24 h after EDIM challenge and then centered around 6 days after challenge. In contrast, no significant cytokine mRNA response was detected in the IL of the naive mice. This is not surprising since PP are the primary intestinal inductive sites and lymphocytes activated at these sites migrate to the draining MLN, where they continue to divide and differentiate prior to their entry into the blood and eventual dispersal to the LP for final differentiation (28). Thus, the bursts in cytokine mRNA production after EDIM infection of naive mice correspond well with the expected times of initial induction and subsequent final differentiation of lymphocytes, as seen in the inductive (PP) and early maturation (MLN) sites in these animals.

The early-response genes upregulated in the inductive sites of naive animals were lymphotixin B (LtB), CD40L, and IFN-α. Because PP and MLN contain a large population of immunoglobulin A (IgA)-producing cells that are critical for a gut-associated innate immune response against viral infection (13, 51), it is not unexpected to see an upregulation of CD40L and LtB. CD40L has been shown to be involved in B-cell activation (48), and LtB is required for the proper homing of IgA precursors in the gut (25). Furthermore, there are numerous encoding genes for IFN-α, and these genes can be expressed from virtually any cell type in response to appropriate stimulation; infections with viruses are known potent stimulators (5).

In the other control group, i.e., mice immunized with LT(R192G) only, a very different temporal pattern of cytokine gene upregulation was observed. That is, no significant upregulation of any cytokine gene was observed during the 8-day observation period at the inductive (PP) and early lymphocyte maturation (MLN) sites, and the cytokine burst at 24 h post-challenge observed in the MLN and PP of naive mice was seen only in the effector IL site and involved a different set of cytokines (IFN-γ and TRAIL). Thus, immunization with the adjuvant alone had a dramatic effect on the site and timing of cytokine upregulation after the subsequent rotavirus challenge distinct from that seen in naive mice but, since the mice were not protected against rotavirus shedding in either group, the cytokine response patterns observed in both control groups were insufficient for protection.

In the VP6/LT(R192G)-immunized mice that are expected to retain VP6-specific memory cells in lymphoid tissues, a very rapid cytokine mRNA response was observed in the IL, an effector site that is in direct contact with villus enterocytes, the targets for rotavirus infection. However, as observed in inductive sites after EDIM infection of naive mice, the initial burst of cytokine mRNA production in the IL was short-lived, and a second wave of cytokine mRNAs was generated in this effector site at approximately 6 days after infection corresponding to the time of final differentiation of new effector cells. Because the very early cytokine mRNA response was not observed in the IL after infection of naive mice and was both delayed and did not include IL-17 upregulation in the IL of LT(R192G)-immunized mice, this very early response in the VP6/LT(R192G)-immunized group was hypothesized to be associated with the rapid inhibition of rotavirus shedding.

Based on the observed simultaneous upregulation of the IL-17 and IFN-γ genes in association with suppression of rotavirus shedding, it was important to next examine T-cell responses and cytokine secretion in lymphocyte populations specific to the LP, IEL, MLN, and PP of naive, VP6-immunized, LT(R192G)-immunized, and VP6/LT(R192G)-immunized mice. Prior methodology of lymphocyte isolation made it extremely difficult to measure T-cell responses in LP and IEL fractions due to low viability of the cells after isolation. After enrichment of the lymphocyte fractions with high-affinity negative selection columns specific for CD3+, the cells could be stimulated and then stained for surface phenotype and the intracellular accumulation of cytokines. Examination of the intracellular cytokine staining data revealed that IFN-γ-producing cells were primarily present in the memory CD8+ LP fraction of all groups of immunized animals. However, VP6/LT(R192G)-immunized animals contained a greater percentage of memory CD8+ T cells staining for IFN-γ, and a small but measurable fraction of IFN-γ-producing memory CD4+ T cells were found only in the LP of the VP6/LT(R192G)-immunized mice. The production of IL-17 was found primarily in memory CD8+ T cells of VP6/LT(R192G)-immunized mice, with the largest amounts residing in intestinal LP and IEL. Therefore, the expression observed for the IL-17 gene in vivo was comparable to the T-cell responses measured in vitro, thus further suggesting an association with VP6/LT(R192G)-induced protection against rotavirus shedding.

To further investigate the roles of IFN-γ and IL-17 in protection against shedding of rotavirus antigen, two KO mouse strains (IFN-α/β−/− KO and IL-17R KO) were immunized with VP6/LT(R192G) and challenged with EDIM four weeks later. Fecal shedding of rotavirus antigen in both IFN-α/β−/− KO mice and IL-17R KO mice was reduced 96 and 99%, respec-
tively, compared to their naive control groups. Because these values were essentially identical to that found after EDIM challenge of wt BALB/c mice immunized with VP6/LT(R192G), protection was not shown to be individually associated with either IFN-γ or IL-17. Similarly, Stat1−/−, Ifnar2−/−, and IFN-γ−/− mice have been found to be protected from rotavirus shedding after VP6/LT(R192G) immunization (46), thus supporting the conclusion that IFN-γ was not required for protection. Based on these observations with KO mice, it appears that IFN-γ or IL-17 alone is required for protection after VP6/LT(R192G) immunization or that factors other than these two cytokines, which were not detected in the present study, are sufficient for protection.

Although IFN-γ and IL-17 may not be independently necessary for protection in this model, their abundant presence suggests they play some role. For example, IFN-γ is an antiviral cytokine that is secreted primarily by activated T cells and NK cells, is associated with Th1 responses, and has been reported to block rotavirus replication in vitro (4). The rapid upregulation of IFN-γ gene expression in intestinal effector cells by 12 h after rotavirus infection of immunized mice and the lack of detectable upregulation of cytokine genes in these cells associated with Th2 responses, such as IL-4 and IL-5, suggests that memory T cells are retained within this site after VP6/LT(R192G) immunization, and these belong primarily to the Th1 subtype. Using a new method for in vivo retention of cytokines within activated T cells, rapid production of IFN-γ by 12 h after a secondary viral infection was recently reported in splenic CD8+ T cells of lymphocytic choriomeningitis virus (LCMV)-immunized mice and, as also observed after EDIM challenge of VP6/LT(R192G)-immunized mice, IFN-γ synthesis was rapidly suppressed by 24 h after LCMV challenge (30). In contrast, peak synthesis of IFN-γ occurred 5 days after primary LCMV infection, a time at which T-cell expansion was still taking place. The authors of that study noted that this very rapid on-off synthesis of IFN-γ after secondary viral infection was an unexpected result (30). We now have made the same observation concerning IFN-γ gene expression in the IL of VP6/LT(R192G)-immunized mice.

IL-17 was discovered in 1993 (43), and its production has been reported to be triggered by the dendritic-cell-secreted cytokine, IL-23, primarily in memory T cells (1, 38, 52). Although IL-17 has not been categorized as solely a Th1 or Th2 cytokine, its expression appears to be specific to activated CD4+ and CD8+ T cells, whereas the IL-17R has been found on many cell types (26, 45, 52, 53). IL-17 is a proinflammatory cytokine and has been shown to induce the production of chemokines, such as IL-8, MCP-1, and Gro-α (18, 49, 50). This stimulation of chemokines allows the recruitment of neutrophils and monocytes to the site of infection. More specifically, IL-17 has been shown to induce chemokine secretion in human fetal and rodent intestinal epithelial cells (2, 3). Enhanced secretion of chemokines, such as IL-8 and MCP-1, was demonstrated when human intestinal epithelial cells were stimulated with both IFN-γ and IL-17. This combination of IFN-γ and IL-17 also generated enhanced NF-κB-binding activity (2). Furthermore, IL-8 secretion and NF-κB activation in intestinal epithelial cells has been shown to occur in a primary response to rotavirus infection (42, 44). Therefore, IL-17 may play a role in enhancing the stimulation of such chemokines.

The data collected using microarray technology provide a direct measure of the timing of cytokine gene expression at different sites after EDIM infection specifically associated with prior VP6/LT(R192G) immunization. Cytokine expression unique to the site of infection in VP6/LT(R192G)-immunized animals provides an insight into the mechanisms that lead to protection from rotavirus. The main conclusions of the present study were that protection after intranasal immunization with VP6/LT(R192G) was associated with a strong intestinal Th1 response and that the expression of IL-17, which was shown to be mainly secreted by CD4+ T cells, may have an important function in protection. Therefore, future studies should focus on the roles of both IL-17 and IFN-γ in protection in this model.

REFERENCES


