Mice Develop Effective but Delayed Protective Immune Responses When Immunized as Neonates either Intranasally with Nonliving VP6/LT(R192G) or Orally with Live Rhesus Rotavirus Vaccine Candidates

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Rotavirus vaccines are delivered early in life, when the immune system is immature. To determine the effects of immaturity on responses to candidate vaccines, neonatal (7 days old) and adult mice were immunized with single doses of either *Escherichia coli*-expressed rotavirus VP6 protein and the adjuvant LT(R192G) or live rhesus rotavirus (RRV), and protection against fecal rotavirus shedding following challenge with the murine rotavirus strain EDIM was determined. Neonatal mice immunized intranasally with VP6/LT(R192G) were unprotected at 10 days postimmunization (dpi) and had no detectable rotavirus B-cell (antibody) or CD4⁺ CD8⁺ T-cell (rotavirus-inducible, Th1 [gamma interferon and interleukin-2 {IL-2}]-, Th2 [IL-5 and IL-4]-, or ThIL-17 [IL-17]-producing spleen cells) responses. However, by 28 and 42 dpi, these mice were significantly ($P \geq 0.003$) protected and contained memory rotavirus-specific T cells but produced no rotavirus antibody. In contrast, adult mice were nearly fully protected by 10 dpi and contained both rotavirus immunoglobulin G and memory T cells. Neonates immunized orally with RRV were also less protected ($P = 0.01$) than adult mice by 10 dpi and produced correspondingly less rotavirus antibody. Both groups contained few rotavirus-specific memory T cells. Protection levels by 28 dpi for neonates or adults were equal, as were rotavirus antibody levels. This report introduces a neonatal mouse model for active protection studies with rotavirus vaccines. It indicates that, with time, neonatal mice develop full protection after intranasal immunization with VP6/LT(R192G) or oral immunization with a live heterologous rotavirus and supports reports that protection depends on CD4⁺ T cells or antibody, respectively.

Rotaviruses cause severe gastrointestinal illness that accounts for ca. 600,000 deaths worldwide in young children each year (54). Because natural rotavirus infections provide protection against subsequent rotavirus disease, particularly against severe disease, vaccines evaluated in clinical trials to date have all been comprised of live, attenuated rotavirus strains that are delivered orally to mimic natural infection. Protection after natural rotavirus infection has been associated with both serum and intestinal rotavirus antibodies (11, 18, 19, 41, 65, 68). Even though similar associations have been difficult to establish after live virus immunization (69), very recent evidence suggests a strong correlation between the presence of serum rotavirus IgA and protection following immunization with Rotarix, a candidate vaccine that was licensed in Mexico in 2004 (20). Even with this candidate vaccine, however, it is unclear whether antibodies are responsible for protection or their presence merely correlates with the true effectors of protection.

Two live rotavirus vaccine candidates are in the process of being licensed internationally (57, 66), but the safety and efficacies of these vaccines will be fully determinable only through postli-

censure studies. This has warranted the development of second-generation, nonliving rotavirus vaccine candidates, several of which have been evaluated in animal models, primarily the adult mouse model designed for studies of active immunity (70). One candidate vaccine evaluated with this model is *Escherichia coli*-expressed VP6 protein. When administered either intranasally or orally together with attenuated *E. coli* heat-labile toxin LT(R192G), the expressed VP6 protein of the murine rotavirus strain EDIM provided nearly complete protection against EDIM shedding in adult mice (13, 16). The mechanisms by which VP6/LT(R192G) elicits protection appear to differ from those for live virus immunization in this model. That is, protection is reduced after live virus immunization of B-cell-deficient mice (27, 42) but is fully retained for extended times after mucosal immunization with VP6/LT(R192G) (48).

Although human infants typically do not experience severe rotavirus disease during the first months of life, severe disease is common in young children when the first rotavirus infection occurs after ca. 3 to 6 months of age (5). Therefore, a rotavirus vaccine should be administered and able to elicit protective immune responses prior to this age in order to be fully effective. Since natural rotavirus infections of neonates have been reported to provide some protection against severe rotavirus disease (6, 8), the development of neonatal rotavirus vaccines is under serious consideration.

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The ability of a neonatal mouse or human to generate sufficient effectors of protection after immunization is dependent, among other possible variables, on its state of immunological maturity. Specific immune cell functions mature in neonatal mice through the weaning period, while the numbers of immune cells in inductive and effector sites increase (2, 61). The human neonate is more mature at birth than the neonatal mouse; however, the immune development of the mouse accelerates after birth. In contrast, immune development in the postnatal infant is prolonged through 2 years of age. An understanding of the neonatal immune response to either a live virus or viral protein/adjuvant immunization could influence the development of appropriate rotavirus vaccine strategies for this target population.

The purpose of this study was to determine whether protection against rotavirus shedding in mice elicited by intranasal immunization with VP6/LT(R192G) or oral immunization with live rotavirus was dependent on the age of the mice when vaccinated or on the elapse of time between vaccine administration and rotavirus challenge. Therefore, neonatal and adult mice were immunized by either of these two methods and then challenged with EDIM at various time points after immunization. Protection was determined by reductions in rotavirus shedding relative to that found in mock-immunized control mice of the same ages. Associations between protection and both humoral (antibody) and cellular (T-cell) responses were determined for each group of immunized mice.

MATERIALS AND METHODS

Viruses. The rhesus rotavirus (RRV) strain was used for live virus immunization in this study because it is still a potentially viable vaccine candidate as part of the tetravalent RRV vaccine and because it will infect and elicit antibody responses in mice, as it does in humans after intestinal infection. The RRV strain used in this study was obtained from Harry Greenberg (Stanford University, Palo Alto, CA). It was grown in the African green monkey kidney MA104 cell line, and its titer was determined using a fluorescent focus assay of the same cells. Infectious RRV obtained from cell culture lysates was used for live virus immunization and to measure neutralizing antibody titers in sera obtained from immunized mice. Preparations of this virus were also purified as previously described (45, 47), and infectious triple-layered particles were used to hyperimmunize mice and for in vitro stimulation of cultured spleen cells. The murine rotavirus strain EDIM, originally obtained from M. Collins (Microbiological Associates, Bethesda, MD), was used unpassaged as the challenge virus in these studies and was also used for oral immunization of mice. Unpassaged EDIM (wt) was obtained from the stools of infected neonatal mice and was processed and characterized as previously described (16). This virus was also passaged in MA104 cells, and triple-layered particles obtained after the 35th passage were purified and used to hyperimmunize mice. This tissue culture-grown virus was also used to measure neutralizing antibody titers. The D × RRV rotavirus, which has a VP7 gene from the human G1 rotavirus D strain, was obtained from Wyeth-Ayerst Research (Radnor, PA). It is one component of the tetravalent RRV vaccine. The G1 Wa strain of human rotavirus was originally obtained from Richard Wyatt (NIH, Bethesda, MD). Both D × RRV and Wa were grown in MA104 cells, and lysates of these viruses were used to measure neutralizing antibody titers. In addition, purified triple-layered particles of these viruses were used to hyperimmunize mice.

Mice. Pregnant (mid- to late gestational age) and nonpregnant (5 to 6 weeks of age) female BALB/c mice were purchased from Harlan-Sprague-Dawley (Indianapolis, IN). Pregnant mice were housed individually, and the other adult mice were housed (4/cage) in sterile microisolation cages. Weaning of suckling pups occurred at 21 days of age. Alternatively, pups immunized and challenged prior to weaning remained with their dams or surrogate dams until 25 days of age. Breeding pairs of J chain knockout (KO) mice backcrossed 10 to 12 generations into the BALB/c strain (Taconic Laboratories, Germantown, N.Y.) were obtained from Barbara Hendrickson (University of Chicago Children's Hospital, Chicago, Illinois). In these mice, transport of dimeric IgA by the intestinal

polymeric receptor is impaired, and they lack intestinal dimeric IgA (33). Mice were bred in our facility and used at 6 to 12 weeks of age. For the experiments using J chain KO mice, age-matched wt BALB/c mice were obtained from Taconic Laboratories. All procedures were performed in accordance with protocols reviewed and approved by the Children's Hospital Research Foundation Institutional Animal Care and Use Committee.

Construction, expression, and purification of recombinant rotavirus VP6 protein. A bacterial expression vector, pET/EDIM VP6::6XHis, for expressing recombinant VP6 protein was constructed by inserting amplicons generated by PCR encoding the EDIM VP6::6XHis protein into the EcoRV cloning site of pETBlue-1 (Novagen, Madison, WI). The PCR amplicons were generated using oligonucleotide primers P1 (5'-ATGGATGTGCTGTACTCTATCTACGTCACGTACACTG-3') (positive sense, nucleotides 1 to 33) and P2 (5'-TTAATGATGATGATGATGATGCTTTACCAGCATGCT-3') (negative sense, nucleotides 1200 to 1214 and six histidine codons [underlined]), with the previously described plasmid pcDNA1/EDIM6 as the template (15). The PCR mixture contained template DNA (200 ng), the deoxynucleoside triphosphates dATP, dCTP, dGTP, and dTTP (0.3 mM [each]), MgSO₄ (1 mM), primers P1 and P2 (0.3 μM [each]), and Platinum Pfx DNA polymerase (2.5 units; Invitrogen, Carlsbad, CA) in 50 μl of Pfx amplification buffer (Invitrogen). The reaction was carried out by denaturation of the template (94°C, 2 min), 35 PCR cycles (94°C, 15 s; 55°C, 30 s; 68°C, 3 min), and a final incubation step (68°C, 7 min) to ensure complete synthesis of the amplicons. The recombinant pET/EDIM VP6::6XHis plasmid was transformed into *E. coli* DH5α (Invitrogen), and cells were grown on Luria-Bertani (LB) agar plates (tryptone, 10 g; yeast extract, 7 g; NaCl, 10 g; D-glucose, 10 g; carbenicillin, 100 μg; and Bacto agar, 15 g [in 1 liter of H₂O]). Resistant colonies were grown on replicate plates in the presence or absence of IPTG (isopropyl-β-D-thiogalactopyranoside; BioVectra, Charlottetown, Canada) and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Promega, Madison, WI). Colonies on replicate plates corresponding to positive, white colonies on IPTG-containing plates were selected. The orientation of the inserts was determined by PCR using primers P1 and pETBlueDOWN (Invitrogen). The authenticity of the cloned VP6 gene was ascertained by sequencing of the DNA insert using the pETBlueUP and pETBlueDOWN primers. For IPTG-inducible expression, pET/EDIM VP6::6XHis was transferred into the Rosetta (DE3)/pLacI host strain (Novagen). The protocols for induction and purification of recombinant VP6 in the presence of 8 M urea using six-His-binding Talon resin (Clontech) have already been described (12). The concentration of the EDIM VP6::6XHis recombinant protein was determined by the Bradford method (Amresco, Solon, OH) as described by the manufacturer. The protein was characterized using Western blot analysis as described previously (14). Rabbit anti-polyhistidine antibody (Santa Cruz Biotechnology, Santa Cruz, CA) detection was used to confirm the specificity and molecular weight of the recombinant protein. The final product was found to be free of lipopolysaccharide (LPS).

Adjuvant. The attenuated *E. coli* heat-labile toxin LT(R192G) was used as the adjuvant in these studies. LT(R192G) carries a glycine-for-arginine substitution in the proteolytic site of the A subunit at amino acid 192 which abrogates cleavage of LT(R192G) and attenuates the toxicity of the protein (21).

Immunization of mice using VP6/LT(R192G). Neonatal (7 days old) and adult (≥6 weeks old) mice were administered a single intranasal (i.n.) dose of 4 μg of the VP6 protein described above mixed with 2 μg of LT(R192G). Adult mice were lightly anesthetized with isoflurane (IsoFlo; Abbott Laboratories, North Chicago, IL) prior to intranasal immunization.

Immunization of mice with live RRV, D × RRV, or EDIM. Neonatal mice received 1 × 10⁷ focus-forming units (FFU) of RRV in 25 μl administered by oral gavage at 7 days of age and were observed for 10 days postimmunization (dpi). During this period, daily evaluation for the presence of illness (defined as having diarrheal stool after gentle palpation of the abdomen) was performed for each animal, and results were reported as a percentage of ill animals out of the total. The severity of diarrhea was scored daily by assigning numeric values to the color (yellow = 3; yellow-brown = 2; and brown = 1), degree of soiling (very soiled = 4; somewhat soiled = 1; and no soiling = 0), and consistency (very liquid = 4; liquid = 3; and solid = 1) of the stool. The diarrhea score was calculated by dividing the total severity score by the total number of animals on each day after RRV immunization. To determine the quantity of antigen and infectious virus in the neonates, the small intestine from one mouse was harvested daily, weighed, homogenized in Earle's balanced salt solution (EBSS), and stored at -20°C until assayed. RRV-immunized neonatal mice were cross-fostered to naïve dams prior to challenge to eliminate any possible effects of maternal antibody. Adult mice were orally immunized by gavage with 1 × 10⁷ FFU of RRV. For experiments involving J chain KO mice, 1 × 10⁷ to 10 × 10⁷ FFU of D × RRV was used to immunize the mice prior to EDIM challenge. Fecal pellets were collected from each mouse daily for 8 days postimmunization to ensure resolution of RRV or

D $\times$ RRV antigen shedding prior to EDIM challenge. RRV, D $\times$ RRV, or wt EDIM was also used to immunize mice orally in order to determine serum neutralizing antibody titers. The doses of RRV and D $\times$ RRV used were as just listed, and the dose of wt EDIM was 8×10^5 FFU.

Challenge of mice with EDIM. Neonate and adult mice were orally challenged at 10, 28, or 42 dpi with 10^5 50% shedding doses of wt EDIM.

Detection of rotavirus shedding. Stool specimens were collected daily from each animal for 7 days after EDIM challenge. Two or three fecal pellets were collected in 1.0 ml of EBSS, and specimens were stored at -20°C . Prior to rotavirus antigen quantification, samples were thawed, homogenized, and centrifuged ($1,500 \times g$, 5 min, 4°C) to remove debris. The amount of rotavirus antigen shed was determined in the collected supernatants by an enzyme-linked immunosorbent assay (ELISA), using previously described methods (45). Antigen shedding was determined in ng of rotavirus antigen/ml of stool specimen. The limit of detection for this assay was 5 ng of rotavirus antigen/ml. When calculated values were used for analyses, the amount of rotavirus antigen shed, as determined in 1-ml samples, was used as the quantity produced by each mouse on that day, and the results were averaged in ng/mouse/day.

Rotavirus antibody determinations. Prior to EDIM challenge, blood specimens were collected by retro-orbital capillary plexus puncture, and stool samples were collected in EBSS as described above. Serum samples were heat inactivated (56°C , 30 min) and analyzed for rotavirus IgA and IgG. Stool samples were analyzed for fecal rotavirus IgA. Quantities of antibody were determined by ELISA as described previously and expressed in ng of rotavirus-specific antibody/ml (43). Neutralization antibody titers for serum specimens obtained from RRV- or mock-immunized mice prior to EDIM challenge were determined against RRV and EDIM by previously described methods (36). Neutralizing antibody titers were also determined for sera obtained from mice hyperimmunized by two subcutaneous injections (separated by 2 weeks) with 5 μg of purified triple-layered particles of RRV, EDIM, D $\times$ RRV, or Wa virus together with Ribi adjuvant (Corixa Co., Hamilton, MT) used according to the manufacturer's specifications. Finally, neutralizing antibody titers were determined after oral immunization of mice with either RRV, wt EDIM, or D $\times$ RRV.

In vitro stimulation of spleen cells and determination of cytokine production. Spleens from naive, VP6-immunized, and live RRV-immunized neonatal and adult mice were harvested on days 10 and 28 postimmunization, immediately prior to EDIM challenge. Lymphocytes were isolated from the spleens of six mice per group and were pooled in order to provide a representative sampling of each group. Single-cell suspensions of spleen cells were made, and red blood cells were lysed with 8.3 mg/ml of ammonium chloride in 0.01 M Tris, pH 7.4. Cells were washed and resuspended in complete RPMI medium (Gibco, Inc., Grand Island, NY) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 2.5 $\mu\text{g}/\text{ml}$ amphotericin B, 100 μM RPMI medium with nonessential amino acids, and 55 μM 2-mercaptoethanol. Cells were resuspended at 1×10^7 cells per ml and distributed into 24-well cell culture plates. Lymphocyte cultures from mock- or VP6/LT(R192G)-immunized mice were either left unstimulated or stimulated with 4 μg of VP6 protein per well of cells. Spleen cultures from RRV-immunized mice were also either left unstimulated or stimulated with 10 μg of purified triple-layered RRV particles per well. The quantities of VP6 and RRV used for stimulation were the lowest quantities needed for maximum stimulation. In vitro stimulation was continued for 18 h at 37°C , at which time a BD GolgiPlug containing brefeldin A (BD Biosciences Pharmingen) was added to cell cultures for the remaining 4 h of incubation. Stimulated and unstimulated cell cultures were harvested, the cells were pelleted by centrifugation and used to determine intracellular cytokine accumulation, and the cell supernatants were stored at -20°C for determining cytokine secretion during the 18-h incubation prior to the brefeldin A addition. Cytokine quantities in the supernatants obtained after in vitro stimulation were determined by ELISAs, using kits for mouse interleukin-4 (IL-4), IL-5, and gamma interferon (IFN- γ) (Pierce Endogen, Rockford, IL) and for mouse IL-2 and IL-17 (R&D Systems, Minneapolis, MN) as described by the manufacturers. Cytokine concentrations were expressed in pg per 1×10^6 cells.

For determinations of intracellular cytokine staining, cells collected after in vitro stimulation were resuspended in phosphate-buffered saline plus 2% FBS and stained as follows. Spleen cells were divided into aliquots at 0.5×10^6 to 1.0×10^6 cells/100 μl in 96-well V-bottomed culture plates. Fc receptors were blocked by the addition of 1.25 μg of anti-mouse CD16/CD32 (2.4G2). Cells were then stained at 4°C for 30 min for surface antigens using, in combination, fluorescein isothiocyanate-conjugated anti-mouse CD4 (RM4-4), peridinin chlorophyll a-conjugated anti-CD8a (53-6.7), and allophycocyanin-conjugated anti-CD44 (IM7), using the manufacturer's suggested dilutions. Cells were washed twice in phosphate-buffered saline–2% FBS. Cells were permeabilized for 15 min at 4°C in 100 μl of BD Cytofix/Cytoperm solution. Cells were washed twice in BD

Perm/Wash solution. Antibodies to mouse IL-2 (JES6-5H4), IL-4 (11B11), IL-5 (TRFK5), IL-17 (TC11-18H10), and IFN- γ (XMG1.2), all conjugated to R-phycoerythrin, were used to stain for the presence of intracellular cytokine accumulation. Isotype fluorescent conjugate-matched antibodies were used for negative control staining. All antibodies and staining solutions were obtained from BD Biosciences Pharmingen (San Diego, CA). Data were acquired using a FACSCaliber flow cytometer and analyzed by CellQuest software (BD Biosciences, San Jose, CA). Cells were gated on the lymphocyte population by forward and side scatter determinations, and 200,000 cells were acquired. Gating was then done on the CD4 or CD8 population. The percentages of either CD4 $^+$ or CD8 $^+$ cells staining for CD44 and specific cytokines were determined. Measurements of T-cell responses were performed in two independent experiments.

Statistical methods. All analyses were performed using the statistical software package SAS, version 9.1.3. The level of significance was 0.05 for two-sided tests. For comparing the amount of shedding across the groups, two measures were used, the mean rotavirus shedding amounts for 7 days after EDIM challenge and the percent decrease in rotavirus shedding from that of the mock-immunized group. The statistical methods used were a two-sample *t* test when two groups were compared and analysis of variance for situations with more than two groups being compared. When the number of groups being compared was greater than two, the Bonferroni adjusted multiple comparison procedure was used. Rotavirus antibody levels are not normally distributed, so log transformation was performed, and all analyses were done using the log-transformed variables. The two statistical methods performed were a two-sample *t* test if there were two groups compared and analysis of variance when more than two groups were compared. Again, the Bonferroni adjusted multiple comparison procedure was used to control the level of significance to 0.05.

RESULTS

Protection against EDIM shedding after intranasal immunization of neonatal or adult mice with VP6/LT(R192G). We previously reported that when adult BALB/c mice were administered two i.n. immunizations of chimeric EDIM VP6 protein combined with the adjuvant LT(R192G) 2 weeks apart and challenged 4 weeks later with EDIM, fecal shedding of rotavirus antigen was reduced $>99\%$ for at least 12 months relative to that found for unimmunized animals (13, 16). We also reported that a single immunization was as effective as two or three in these animals. Therefore, our first experiment was to determine if the same degree of protection could be attained when a single i.n. dose of VP6/LT(R192G) was administered to immunologically immature neonatal BALB/c mice and if the time after immunization required for effective protection was affected by the immaturity of these animals. A single i.n. dose of 4 μg of recombinant EDIM VP6::Hisx6 and 2 μg of LT(R192G) was administered to neonatal mice at 7 days of age, and these mice, along with a group of unimmunized, age-matched control animals, were challenged at 10, 28, and 42 dpi with 10^5 50% shedding doses of wild-type unpassaged EDIM. Groups of adult mice were immunized and challenged according to the same schedule. No reduction in the amount of rotavirus shedding was found in the neonatal mice when they were challenged at 10 dpi (Table 1); in fact, there was a significant ($P = 0.007$) increase in shedding in the immunized mice. In contrast, a high level of protection (88.6%) was found in adult mice by this time. At 28 dpi, however, protection in mice immunized as neonates improved (77.6%) and was not significantly ($P = 0.19$) different from that found in the adult mice (97.8%). Finally, by 42 dpi, the level of protection in mice immunized as neonates was equal to that found in those immunized as adults (98.5% versus 98.3%).

Protection against EDIM shedding after oral immunization of neonatal or adult mice with live RRV. It has been reported that oral immunization of 4- to 7-day-old BALB/c mice with

TABLE 1. Protection against EDIM shedding after i.n. immunization with VP6/LT(R192G) in neonatal and adult BALB/c mice challenged at 10, 28, or 42 dpi

Group ^a	Time of challenge (dpi)	n	Quantity of antigen shed (ng/mouse/day) ^b	% Protection ^c
Mock-immunized neonates	10	8	2,424 ± 4,843	0
VP6-immunized neonates		8	7,166 ± 17,497 ^{d,f}	
Mock-immunized neonates		7	1,817 ± 2,414	
VP6-immunized neonates	28	8	426 ± 2,074 ^e	76.6
Mock-immunized neonates		9	4,072 ± 5,399	
VP6-immunized neonates	42	7	63 ± 224 ^e	98.5
Mock-immunized adults		8	2,555 ± 3,820	
VP6-immunized adults	10	8	290 ± 747 ^{e,g}	88.6
Mock-immunized adults		8	4,384 ± 6,161	
VP6-immunized adults	28	8	97 ± 262 ^e	97.8
Mock-immunized adults		8	3,063 ± 4,370	
VP6-immunized adults	42	7	51 ± 137 ^e	98.3

^a Neonates were immunized at 7 days of age, and adults were immunized at ≥6 weeks of age.

^b Mean quantity of rotavirus antigen shed ± standard deviation (SD) over 7 days postchallenge (ng/mouse/day).

^c Percent protection represents the percent decrease in mean quantity of rotavirus antigen shed during the 7 days after challenge compared to that for mock-immunized mice.

^d Significantly ($P = 0.007$) greater than mock-immunized mice.

^e Significantly ($P \leq 0.003$) less than mock-immunized mice.

^f Significantly ($P \leq 0.001$) greater than immunized neonatal mice challenged at 28 or 42 dpi or immunized adult mice challenged at 10, 28, or 42 dpi.

^g Significantly ($P \leq 0.04$) greater than immunized adult mice challenged at 28 or 42 dpi.

10^7 PFU of the heterologous simian rotavirus strain RRV induced nearly complete protection against rotavirus shedding when mice were challenged 6 weeks later with the homologous murine rotavirus strain EDIM or EC_{wt} (23, 43). However, protection after RRV immunization was not determined in association with development of the immune systems of the neonatal mice. Therefore, protection against EDIM shedding was examined as a function of time after RRV immunization of neonatal and adult mice.

BALB/c mice were immunized by a single oral administration of 1×10^7 FFU of RRV, and protection against EDIM shedding was measured at the same time periods (i.e., 10, 28, and 42 dpi) and with the same methods as those used after VP6/LT(R192G) vaccination. Although RRV induced no illness in adult mice, neonatal mice experienced illness for 6 days after RRV inoculation (Fig. 1A). However, rotavirus antigen and live rotavirus were detected in the intestines of individual neonatal animals during only the first 4 days after infection (Fig. 1B). RRV antigen was also detected in the stools of adult mice between 2 and 7 dpi but was cleared by 8 dpi (Fig. 1C), 2 days prior to the first time point of EDIM challenge. Although the quantities of RRV shed in the adult animals relative to those shed in neonates could not be directly compared because of the different methods of collection (stools versus whole intestines), peak intestinal rotavirus antigen production per gram of stool was greater in the adult mice.

Because RRV infects adult as well as neonatal mice, it is possible that the dams of infected pups became infected themselves, which could result in passive protection of pups against EDIM challenge by immunological effectors in their milk, specifically rotavirus antibody (51, 59, 62). To avoid this possibility, the RRV-immunized pups were transferred to rotavirus-negative, lactating dams prior to EDIM challenge.

Immunization of neonatal mice with RRV stimulated significant ($P = 0.01$) protection (91.7%) against rotavirus shedding when the mice were challenged at 10 dpi (Table 2), and protection was nearly complete (99.7%) when the EDIM challenge was done at 28 dpi. The same high level of protection was found at 42 dpi (results not shown). The low levels of rotavirus antigen found after EDIM challenge in stools of highly protected mice were due to virus replication and not merely to “pass-through” challenge virus. This is based on the finding that even these low levels were not detected in mice on the days after inoculation with the same quantities of inactivated EDIM as those used for the live virus challenge. Although the protection generated within 10 days after oral immunization of neonatal mice with live RRV was very substantial, this protection was significantly ($P = 0.01$) lower than that found by this time in immunized adult mice (99.8%), when maximal protection was already attained (Table 2).

Rotavirus antibody responses elicited in neonatal and adult mice after oral immunization with live RRV or i.n. immunization with VP6/LT(R192G). Rotavirus antibody has been correlated with subsequent protection from rotavirus disease after both natural infection and, to a lesser degree, live virus immunization of humans (68, 69). Rotavirus antibody was also shown to be at least partially responsible for the sterilizing immunity against rotavirus infection observed in adult or neonatal mice after oral immunization with live murine rotaviruses (27, 42, 49). In contrast, CD4 T cells, but not antibody, were required to maintain maximal protection against EDIM shedding after i.n. immunization with VP6/LT(R192G) (48), thus indicating that the mechanisms of protection differ between these two methods of immunization. Based on these observations, it was of interest to determine whether the protection against EDIM shedding generated after either oral, live RRV, or i.n. VP6/LT(R192G) immunization of neonatal and adult mice correlated with the presence of rotavirus antibodies.

(i) Oral, live RRV immunization. Neonatal mice immunized with RRV at 7 days of age had already generated large quantities of serum rotavirus IgG by the time of EDIM challenge at 10 dpi, and these titers increased another 2.4-fold ($P = 0.007$) by day 28 dpi (Fig. 2A). Serum and stool rotavirus IgAs were also detectable by 10 dpi and increased 1.9-fold (not significant) and 5.7-fold ($P = 0.0002$), respectively, by 28 dpi (Fig. 2B and C). Serum rotavirus IgG and stool rotavirus IgA responses 10 days after RRV immunization of adult mice were larger ($P \leq 0.003$) than those observed after neonatal immunization, and these titers did not increase significantly between 10 and 28 dpi (Fig. 2A to C). The lower quantities of serum rotavirus IgG and stool rotavirus IgA found at 10 dpi in mice immunized as neonates (compared to the levels found at either 28 dpi in mice immunized as neonates or 10 dpi in mice immunized as adults) corresponded to the significantly reduced level of protection observed in these mice (Table 2).

Because an association was found between the levels of rotavirus antibodies and active immunity after RRV immunization both in this study and in previously reported studies (23, 43), it was of interest to determine whether these antibodies were capable of neutralizing the challenge EDIM strain. Therefore, the titers of serum neutralizing antibodies against both RRV and EDIM were measured in these immunized mice on the day of challenge. By 10 dpi, mice immunized with

FIG. 1. Diarrheal illnesses and quantities of intestinal/fecal rotavirus detected following oral, live RRV inoculation of neonatal or adult BALB/c mice. (A) Neonatal mice were inoculated with RRV (1×10^7 FFU) at 7 days of age and monitored daily for evidence of diarrhea (■) and diarrheal scores (◆). (B) One mouse was also sacrificed each day, and its intestines were evaluated for quantities of rotavirus antigen (■) and infectious RRV (◆). (C) Adult mice (≥ 6 weeks of age) were

TABLE 2. Protection against EDIM shedding after oral immunization with live RRV in neonatal and adult BALB/c mice challenged at 10 or 28 dpi

Group ^a	Time of challenge (dpi)	n	Quantity of antigen shed (ng/mouse/day) ^b	% Protection ^c
Mock-immunized neonates		7	14,205 $\pm$ 40,180	
RRV-immunized neonates	10	10	1,183 $\pm$ 3,201 ^{d,e}	91.7
Mock-immunized neonates		8	2,904 $\pm$ 3,439	
RRV-immunized neonates	28	8	8 $\pm$ 42 ^d	99.7
Mock-immunized adults		8	5,624 $\pm$ 8,789	
RRV-immunized adults	10	8	11 $\pm$ 22 ^d	99.8
RRV-immunized adults	28	8	13 $\pm$ 24 ^d	99.8

^a Neonates were immunized at 7 days of age, and adults were immunized at ≥ 6 weeks of age.

^b Mean quantity of rotavirus antigen shed $\pm$ SD over 7 days postchallenge.

^c Percent protection represents the percent decrease in mean quantity of rotavirus antigen shed during the 7 days after challenge due to immunization.

^d Significantly ($P \leq 0.01$) less than mock-immunized mice.

^e Significantly ($P \leq 0.01$) greater than immunized neonatal mice challenged at 28 dpi or immunized adult mice challenged at 10 or 28 dpi.

RRV as either neonates or adults had moderate to high titers of neutralizing antibody against RRV (Fig. 3A) but no detectable neutralizing antibody against EDIM (Fig. 3B). Therefore, protection did not appear to be due to neutralizing antibody, a suggestion already reported from results obtained after live rotavirus immunization of adult mice (71). It was also noted that the titers of neutralizing antibody against RRV decreased ($P \leq 0.005$) in both groups of mice between 10 and 28 dpi (Fig. 3A), even though the levels of serum rotavirus IgG and IgA and stool rotavirus IgA increased during this period (Fig. 2A to C). This decrease, however, resulted in no loss of protection, and in the case of mice immunized as neonates, it was associated with an increase in protection (Table 2).

It should be noted that even though RRV infection of mice did not produce detectable neutralizing antibodies against the challenge murine EDIM rotavirus strain in this study, RRV and EDIM belong to the same VP7 or G genotype, which suggests that they are also serotypically related (22). In fact, we have reported that antisera obtained from guinea pigs hyperimmunized with RRV neutralized EDIM sufficiently to be included within the same serotype (71). Therefore, it was of interest to determine if another rotavirus strain that is serotypically unrelated to EDIM could be shown to provide antibody-dependent protection against EDIM shedding that was unrelated to neutralizing antibodies following live oral inoculation. It has been reported (25) that an RRV reassortant strain that contains the G1 VP7 gene from the D strain of rotavirus (i.e., D $\times$ RRV) provided excellent protection in mice after oral inoculation. We confirmed this finding and found protection of BALB/c mice against EDIM shedding after oral immunization with 10^8 FFU of D $\times$ RRV to be essentially 100% (Table 3). This protection was highly dependent on secretory antibodies because mice lacking the J chain that were unable to transport dimeric IgA antibodies into the

inoculated with the same quantity of RRV (1×10^7 FFU) and monitored daily for fecal shedding of rotavirus antigen. Error bars represent standard deviations.

FIG. 2. Geometric means (GMT) of levels of serum rotavirus IgG (A), serum rotavirus IgA (B), and stool rotavirus IgA (C) in specimens from BALB/c mice orally immunized with live RRV (1×10^7 FFU) as neonates or adults collected on the day of EDIM challenge (10 or 28 dpi). Seven to 10 mice were included in each group of mock- and RRV-immunized mice for each time point. The lower limits of detection were 100 ng/ml for serum rotavirus IgG and IgA and 5 ng/ml for

FIG. 3. Geometric mean titers (GMT) of neutralizing antibody against RRV (A) or EDIM (B) in serum specimens from BALB/c mice orally immunized as neonates or adults with 1×10^7 FFU of RRV collected on the day of EDIM challenge (10 or 28 dpi). The lower limit of detection was 20. Error bars represent standard deviations. *, antibody levels in mice immunized with RRV as either neonates or adults were significantly ($P \leq 0.005$) less at 28 dpi than at 10 dpi.

intestinal lumen had a significantly ($P < 0.001$) reduced ability to protect against EDIM shedding after D $\times$ RRV inoculation (Table 3). The J chain KO mice were shown to produce large quantities of both serum rotavirus IgG and IgA but no detectable stool rotavirus IgA (results not shown). Furthermore, serum antibodies generated in BALB/c mice after subcutaneous immunization with the G1P[3] D $\times$ RRV reassortant strain readily neutralized RRV and the G1 Wa strain of rotavirus but were unable to neutralize the G3P[16] EDIM strain (Table 4). Likewise, serum antibodies generated after subcutaneous immunization of mice with EDIM were able to neu-

stool rotavirus IgA. Error bars represent standard deviations. *, antibody levels in mice immunized as adults were significantly ($P \leq 0.003$) higher than those found in mice immunized as neonates.

TABLE 3. Protection against EDIM shedding after oral immunization of adult immunologically normal (wt) or J chain knockout BALB/c mice with 10^8 FFU of D $\times$ RRV strain of rotavirus^a

Group	<i>n</i>	Quantity of rotavirus antigen shed (ng/mouse/day) ^b	% Protection ^c
Unimmunized wt mice	6	2,084 $\pm$ 3,241	
D $\times$ RRV-immunized wt mice	5	0.2 $\pm$ 1.0	99.99
Unimmunized J chain KO mice	6	6,149 $\pm$ 6,157	
D $\times$ RRV-immunized J chain KO mice	6	4,261 $\pm$ 5,480	30.70 ^d

^a Groups of mice were challenged with wt EDIM 28 days after D $\times$ RRV immunization.

^b Mean quantity of rotavirus antigen shed ($\pm$ SD) for 7 days after EDIM challenge.

^c Percent protection represents the percent decrease in mean quantity of rotavirus antigen shed during the 7 days after challenge due to immunization.

^d Significantly ($P < 0.001$) less than in immunologically normal mice.

tralize EDIM but were unable to neutralize either the D $\times$ RRV, RRV, or Wa strain of rotavirus (Table 4). The neutralizing antibodies generated after oral inoculation with these viruses, although having quite low titers, were also serotype specific where detectable (Table 4). Thus, protection against EDIM shedding after oral immunization with live D $\times$ RRV appears to be highly dependent on nonneutralizing intestinal antibodies.

(ii) Intranasal VP6/LT(R192G) immunization. Possible relationships between rotavirus antibody levels and protection were also examined after i.n. immunization of neonatal and adult mice with VP6/LT(R192G). By 10 dpi, neonatal mice had no detectable serum anti-VP6 IgG, and only one of eight and one of seven mice had detectable levels on the days of EDIM challenge, at 28 and 42 dpi, respectively (Fig. 4). No serum or stool anti-VP6 IgA was detected in any of the mice immunized as neonates at any of these times after immunization (results not shown). In spite of this, protection against EDIM shedding increased steadily following immunization and was equivalent to that found in mice immunized as adults by 42 dpi (Table 1). Adult mice also developed no detectable serum or stool anti-VP6 IgA at any time after VP6/LT(R192G) immunization, but their levels of serum anti-VP6 IgG increased greatly ($P < 0.001$) between 7 and 28 dpi (Fig. 4). Based on these combined results, no consistent association was found between rotavirus IgG or IgA levels and protection in mice immunized with VP6/LT(R192G).

CD4⁺ and CD8⁺ T-cell responses to rotavirus antigen in spleen cells collected from mice as a function of time after oral RRV or i.n. VP6/LT(R192G) immunization of neonates or adults. Although T cells have not been proven to have direct effector roles in extended protection following oral immunization with live rotaviruses of humans or animals, CD8⁺ T cells were shown to be responsible for the initial resolution of rotavirus shedding in immunologically normal mice (27, 42). Furthermore, the production of rotavirus antibody, an established effector of protection after live rotavirus immunization of mice, was shown to be dependent on helper CD4⁺ T cells (46). Following i.n. immunization of mice with VP6/LT(R192G), CD4⁺ T cells were found to be the only lymphocytes required for protection, and this protection remained fully intact for at

TABLE 4. Neutralizing antibody titers in sera obtained from hyperimmunized or orally immunized mice determined against homotypic and heterotypic rotaviruses^a

Rotavirus strain used for immunization	GMT of neutralizing antibody against specific rotavirus strain ^b			
	D $\times$ RRV	RRV	Wa	EDIM
D $\times$ RRV	>2,500 (156)	1,570 (38)	1,071 (<20)	<20 (<20)
RRV	>2,500 (77)	>2,500 (72)	<20 (<20)	<20 (<20)
Wa	30	<20	403	<20
EDIM	<20 (<20)	<20 (<20)	<20 (<20)	100 (<20)

^a Groups of six mice each were either hyperimmunized with purified rotaviruses or orally immunized with lysates of these viruses by the procedures described in Materials and Methods. Serum specimens collected from these animals were tested for neutralizing antibody levels against the specified viruses, and the geometric mean titers (GMTs) of these sera were calculated. The lowest titer measurable was 20, and titers below this value were set at 20 to calculate GMTs.

^b Titers not in parentheses are for sera collected from hyperimmunized mice; titers in parentheses are for sera from orally immunized mice.

least 1 year (48). It was therefore of interest to characterize the memory T-cell populations in mice as a function of time after either oral immunization with RRV or i.n. immunization with VP6/LT(R192G) of neonates or adults and to determine whether these T-cell populations quantitatively correspond to the levels of protection against EDIM shedding generated in the immunized animals.

To quantify memory T cells in immunized mice, spleens were obtained on the days of EDIM challenge (i.e., 10 and 28 days after immunization) and stimulated *in vitro* with rotavirus antigen. The cells were then labeled with fluorescent antibodies that recognized lymphocyte surface antigens or intracellular cytokines. CD4⁺ or CD8⁺ T lymphocytes displaying the memory surface antigen CD44 (CD44^{high}) were analyzed by flow cytometry for their intracellular cytokine accumulation in response to rotavirus antigen stimulation. This entailed gating on

FIG. 4. Geometric means (GMT) of levels of serum anti-VP6 IgG in mice immunized with VP6/LT(R192G) as either neonates or adults, as determined at either 10, 28, or 42 dpi. Seven or eight mice were included in each group of mock- and VP6/LT(R192G)-immunized mice for each time point. The lower limit of detection was 100 ng/ml. Error bars represent standard deviations. *, antibody levels found in mice immunized as adults were significantly ($P \leq 0.02$) higher than those found in mice immunized as neonates.

TABLE 5. Percentages of CD4⁺ CD44^{high} and CD8⁺ CD44^{high} T cells obtained from the spleens of mock-, VP6/LT(R192G)-, and RRV-immunized neonatal or adult mice that were stimulated to produce cytokines at 10 or 28 dpi, as determined by fluorescence-activated cell sorting^a

Age at immunization	Immunization protocol	Time (dpi)	% of CD4 ⁺ CD44 ^{high} cells with cytokine			% of CD8 ⁺ CD44 ^{high} cells with cytokine	
			IL-2	IL-17	IFN- γ	IL-17	IFN- γ
Neonate	Mock ^b	10	— ^d	—	—	—	—
	VP6/LT(R192G) ^b	10	—	—	—	—	—
	RRV ^c	10	—	—	—	—	—
	Mock ^b	28	—	—	—	—	—
	VP6/LT(R192G) ^b	28	0.10	0.34	0.16	—	0.40
	RRV ^c	28	—	—	0.12	—	0.60
Adult	Mock ^b	10	—	—	—	—	—
	VP6/LT(R192G) ^b	10	0.36	0.35	.013	—	0.54
	RRV ^c	10	—	—	—	—	—
	Mock ^b	28	—	—	—	—	—
	VP6/LT(R192G) ^b	28	0.53	0.33	—	—	1.50
	RRV ^c	28	—	—	0.33	—	0.53

^a Values represent percentages of cells stained in stimulated cultures minus those stained in unstimulated cultures.

^b Cultures were stimulated with 4 μ g of EDIM VP6::6XHis.

^c Cultures were stimulated with 10 μ g of purified RRV particles.

^d —, values of <0.1%.

lymphocytes followed by gating on CD4⁺ or CD8⁺ cells. The percentage of CD4⁺ or CD8⁺ cells expressing CD44 combined with IL-2 or IFN- γ (Th1 response cytokines), IL-4 or IL-5 (Th2 response cytokines), or IL-17 (ThIL-17 response cytokine) was determined. The data are expressed as differences between stimulated and unstimulated cell populations. The values found for the unstimulated cells were consistently <0.1%, and only differences of $\geq 0.1\%$ between the stimulated and unstimulated cells were considered significant. Although the data from only one of two experiments are presented, both gave similar results.

Although incubation of spleen cells from previously mock-immunized mice with a nonspecific stimulator (concanavalin A) resulted in the activation of both Th1 and Th2 CD4 T cells based on their production of IL-4 and IFN- γ (the positive control for this assay), rotavirus antigen stimulated no detectable CD4⁺ CD44^{high} or CD8⁺ CD44^{high} T cells expressing IL-4 or IL-5 in either naïve, VP6/LT(R192G)-immunized, or RRV-immunized neonatal or adult mice (results not shown). Furthermore, no significant numbers of CD4⁺ CD44^{high} or CD8⁺ CD44^{high} T cells were stimulated to produce the cytokines measured in any mock-immunized animal (Table 5). Ten days after immunization of neonatal mice with VP6/LT(R192G), rotavirus antigen also did not stimulate a significant number of memory T cells to express these cytokines. However, by 28 dpi for neonatal mice, the expression of IL-2, IFN- γ , and IL-17 was stimulated in $\geq 0.1\%$ of the CD4⁺ CD44^{high} cells, and IFN- γ production was stimulated in 0.44% of CD8⁺ CD44^{high} cells. The percentages of splenic CD4⁺ CD44^{high} and CD8⁺ CD44^{high} cells that were stimulated to produce cytokines at both 10 and 28 dpi for adult mice were very similar to those observed at 28 dpi for neonatal mice. Thus, there was an excellent relationship between the presence of memory cells capable of producing these Th1 (IL-2 and

TABLE 6. Quantities of Th1, Th2, and ThIL-17 cytokines released from spleen cells obtained from mock- or vaccine-immunized mice after in vitro stimulation^a

Age at immunization	Immunization protocol	Time (dpi)	Quantity (pg/10 ⁶ cells) of cytokine			
			IL-2	IFN- γ	IL-5	IL-17
Neonate	Mock ^b	10	<1	101	<1	<1
	VP6/LT(R192G) ^b	10	<1	159	<1	<1
	RRV ^c	10	2	892	<1	<1
	Mock ^b	28	<1	101	<1	<1
	VP6/LT(R192G) ^b	28	26	3,204	2	36
	RRV ^c	28	6	1,540	<1	2
Adult	Mock ^b	10	<1	431	<1	5
	VP6/LT(R192G) ^b	10	79	5,139	<1	220
	RRV ^c	10	3	990	<1	2
	Mock ^b	28	<1	<1	<1	<1
	VP6/LT(R192G) ^b	28	98	12,629	121	121
	RRV ^c	28	<1	8,004	<1	<1

^a Quantities of cytokines represent differences between in vitro-stimulated and unstimulated cultures.

^b Cultures were stimulated with 4 μ g of EDIM VP6::6XHis.

^c Cultures were stimulated with 10 μ g of purified RRV particles.

IFN- γ) and ThIL-17 cytokines and protection from EDIM shedding in VP6/LT(R192G)-immunized mice.

A very different cytokine expression pattern was observed after in vitro stimulation of spleen cells obtained from RRV-immunized mice (Table 5). Even though rotavirus shedding was found to be reduced 91.9% when mice immunized as neonates were challenged with EDIM at 10 dpi, <0.1% of splenic memory cells from these mice were stimulated to produce the cytokines examined at that time. By 28 dpi for neonatal mice as well as by both 10 and 28 dpi for mice administered RRV as adults, significant numbers of CD4⁺ CD44^{high} and CD8⁺ CD44^{high} cells could be stimulated to produce the Th1 cytokine IFN- γ . At no time, however, did spleen cells obtained from either group of RRV-immunized mice express significant amounts of IL-17, a cytokine produced by helper T cells and associated with immune stimulation.

Memory T cells in the spleens of mice present as a function of time after immunization with RRV or VP6/LT(R192G) were also characterized by the cytokines they released into the culture supernatants during the first 18 h of stimulation with rotavirus antigens. Th1 (IFN- γ and IL-2), Th2 (IL-5 and IL-4), and ThIL-17 cytokines were included in these measurements, as was done with the fluorescence-activated cell sorting analyses. Stimulation of spleen cells from mock-immunized mice of both ages resulted in the production and release of small quantities of IFN- γ , but little, if any, of the other cytokines evaluated (Table 6). The source of IFN- γ in these supernatants may be attributed to non-CD4/CD8-expressing T cells or natural killer cells and most likely represents an innate or primary response to rotavirus antigen. Stimulation of spleen cells obtained from mice at 10 dpi with VP6/LT(R192G) as neonates also resulted in the release of only small quantities of IFN- γ . In contrast, when spleen cells obtained from this same group of mice were stimulated at 28 dpi, they released large amounts of IFN- γ along with substantial amounts of IL-2 and IL-17, but very little IL-5 (Table 6) and no detectable IL-4 (results not shown). Even more of the Th1 cytokines and IL-17 were released from spleen cells obtained from adult mice when the cells

were stimulated both 10 and 28 days after VP6/LT(R192G) immunization. The magnitudes of the IFN- γ , IL-2, and IL-17 responses corresponded to the levels of protection found in these mice as a function of time after immunization. In contrast, spleen cells obtained both 10 and 28 days after immunization of either neonatal or adult mice with live RRV released substantial quantities of IFN- γ , but little, if any, of the other cytokines examined, when stimulated *in vitro* with rotavirus antigens (Table 6). The quantities of cytokines released into the culture supernatants after *in vitro* stimulation of spleen cells obtained from immunized mice are in agreement with the results obtained by fluorescence-activated cell sorting.

DISCUSSION

Because severe rotavirus disease in a full-term infant typically occurs when the child is ≥ 3 months of age, there is a window period after birth in which to vaccinate in order to protect against such illness. However, for a vaccine to be effective when administered within 3 months of birth, young children within this age range must be able to effectively respond to vaccination. Although factors such as maternal antibodies may block vaccine responsiveness during the first months of life, it is probable that the immaturity of the young child's immune system will also limit the full effectiveness of vaccination. The extent to which responsiveness to live rotavirus as well as nonliving rotavirus vaccine candidates is compromised due to immunological immaturity has not been determined. The purpose of this study was to attempt to make this determination for both types of vaccine candidates by using a mouse model.

The neonatal period for humans is 28 days, while that for mice is ≤ 7 days (61). Thus, even though the newborn mouse is less immunologically mature than the newborn infant, the time required for the immune system to mature is greatly accelerated in mice compared with that for human infants (2, 61). It has been suggested that murine models of early-life immunization reproduce the main known characteristics of infant vaccine antibody responses when immunization is initiated in mice at ≥ 7 days of age (61). Based on this suggestion, we chose to compare responses to both live and nonliving rotavirus vaccines in mice when administered at 7 days and 6 weeks of age, at which times the mice were immunologically immature and fully mature, respectively.

The immunogens used in this study [VP6/LT(R192G) and RRV] were selected because both are vaccine candidates (16, 35), both elicit nearly full protection against EDIM shedding when mice are challenged 6 weeks after vaccination (13, 16, 23, 43), and both are delivered via mucosal surfaces. Intranasal immunization of 7-day-old mice with a single dose of VP6/LT(R192G) elicited no protection against EDIM shedding when the mice were challenged prior to weaning at 17 days of age. Following the same protocol, adult mice were nearly fully protected by 10 days after vaccination. The lack of protection in the mice immunized as neonates and challenged at 10 dpi mirrored the total lack of detectable B- or T-cell responses to the vaccine determined on that day of challenge. In contrast, the mice immunized as adults developed both B- and T-cell responses by 10 dpi.

It is well established that antibody responses in neonatal

mice or humans differ from those found in adults (2, 61). Among these differences is the finding that neonatal responses have a delayed onset. The reasons for this are still being determined but include, among others, underlying differences between neonatal and adult B cells, the scarcity of B cells, and the scarcity and immaturity of T helper cells. One outcome of the last factor is the dramatic difference between the numbers of VP6-specific T cells (both CD4⁺ and CD8⁺) found in the spleens of mice at 10 dpi for VP6/LT(R192G) immunization of neonates versus adults. It is known that the spleens of neonatal mice are particularly lymphopenic and that lymph nodes in neonatal animals are phenotypically and functionally more mature than the spleens (3, 39). Even so, CD4⁺ T cells in the lymph nodes of neonatal mice are highly deficient in the ability to develop Th1 responses and are strongly biased toward Th2 responses (1, 29). Since VP6-specific Th2 cells were undetectable at 10 dpi for either adult or neonatal mice immunized with VP6/LT(R192G), it is not surprising that anti-VP6 antibody responses as well as protection were undetectable at 10 dpi for neonatal mice.

Although mice immunized with VP6/LT(R192G) as neonates were unable to mount detectable antibody or T-cell responses by 10 dpi, their T-cell responses approached those of animals immunized as adults by 28 dpi. This is an important finding because it suggests that even though T-cell responses are delayed after a single immunization of VP6/LT(R192G) in neonates, they do eventually develop and, in this case, are associated with protection. Corresponding increases in anti-VP6 antibody responses were not detected, even by 42 dpi, indicating that these antibodies are not required for protection after intranasal VP6/LT(R192G) immunization, as previously reported (48). Since mice given the same immunization as adults generated considerable anti-VP6 IgG responses beginning by 10 dpi, and since these levels expanded until at least 42 dpi, the ability to generate T-cell responses after immunization with VP6/LT(R192G) in neonates did not carry over to B-cell responses. This suggests that the T-cell responses generated with time after a single immunization of neonatal mice were not due merely to retention of antigen but, instead, were due to delayed responses that were initiated near the time of immunization.

The lack of rotavirus antibody production, even with time, after a single VP6/LT(R192G) immunization of neonatal mice was not due to an inability of neonatal mice to generate antibody responses because mice immunized at the same age with live RRV generated considerable rotavirus antibody, even by 10 dpi. These antibody responses were also associated with nearly complete protection against EDIM shedding within that short time after immunization. The differences in antibody responses generated after live, oral RRV versus intranasal VP6/LT(R192G) immunization were due either to the routes of immunization or to the immunogens. RRV replicates in intestinal epithelial cells, and rotavirus antigens generated in this process are expected to be taken up through M cells into the Peyer's patches (5), the primary intestinal inductive sites for immune responses. The subsequent course of development of mature B and T cells and the generation of circulating and secretory antibodies have been well described in numerous reviews. In contrast, VP6, along with LT(R192G), is presumed to be assimilated into the bronchiole-associated lymphoid tis-

sue, where it triggers the activation of local B and T cells which eventually traffic to other mucosal sites, including the gut, where they mature into antibody-producing plasma cells or fully effective T helper or cytotoxic T cells (7, 73). Protection associated with these events requires the presence of the adjuvant, whose mechanism of stimulation has been investigated but remains unclear (28). Memory T cells that recognize VP6 are eventually generated after VP6/LT(R192G) immunization of neonatal mice, but B-cell development appears to be blocked, which does not occur after live RRV immunization. Murine models assessing neonatal responses to human infant vaccines indicate that limited germinal center reactions probably result from the delayed development of follicular dendritic cells and limit plasma cell differentiation (38, 55).

In contrast to the results obtained after VP6/LT(R192G) immunization, the generation of rotavirus antibody was associated with protection against EDIM shedding after live RRV immunization. The importance of secretory antibody in protection after live virus immunization was verified following immunization of J chain KO mice with an RRV reassortant that contained the VP7 gene of a G1 human rotavirus. Protection against murine rotaviruses elicited by immunization with this fully heterotypic strain was found to be equal to that against RRV in immunologically normal BALB/c mice (25). However, we found that little of this protection was retained in $D \times$ RRV-immunized J chain KO mice in the same genetic background. This implied that the transport of dimeric IgA into the intestine played a major role in protection against EDIM shedding after $D \times$ RRV immunization. Antibodies have been recognized as important effectors of protection after live virus immunization of adult mice for over a decade (27, 42), but we were unable to associate this protection with neutralizing antibody against the challenge rotavirus strain (71). Since the $D \times$ RRV strain elicited no detectable neutralizing antibody against EDIM, even after hyperimmunization of mice, these results further support the concept that protection after live rotavirus immunization of mice can be elicited by non-neutralizing antibodies. One mechanism for protection suggested for nonneutralizing anti-VP6 secretory IgA is intracellular blockage of rotavirus replication in infected intestinal enterocytes during transcytosis into the intestinal lumen (9, 24).

We have reported that the only lymphocytes required for protection after VP6/LT(R192G) immunization of adult mice are $CD4^+$ T cells (48). This conclusion was supported by the finding that J chain KO mice that were unable to transport IgA to their intestines were protected against EDIM shedding after intranasal immunization with either VP6/LT(R192G) or VP6/cholera toxin (44). In contrast, others have reported that protection against murine rotavirus shedding after intranasal immunization with virus-like particles (VLPs) and cholera toxin was substantially less in J chain KO mice than in immunologically normal animals (60). This suggested that the importance of intestinal antibody was less crucial for protection after VP6 immunization than after VLP immunization under otherwise similar conditions. Although the finding that $CD4$ T cells are the only lymphocytes needed for protection after intranasal immunization with VP6/LT(R192G) is unusual, it is not without precedent. Others have reported similar results for mengovirus (50), Friend virus complex (32), Sendai virus (34), respiratory syncytial virus (56), and herpes simplex viruses (17, 40).

The protection elicited in neonatal mice after VP6/LT(R192G) immunization in the present study was also associated with T-cell responses, particularly those of T cells that were stimulated to produce Th1- and ThIL-17-specific cytokines. Cytokines such as IFN- γ have direct antirotavirus activity (4), while IL-17 has not been found to be directly antiviral, but instead is a proinflammatory cytokine and has been shown to induce the production of chemokines (26, 64, 72). This stimulation of chemokines allows the recruitment of neutrophils and monocytes to the site of infection. IL-17 has been reported to play a protective role in defense against microbial diseases (30, 31) but has also been suggested to play an active role in inflammatory diseases, autoimmune diseases, and cancer (37). However, IL-17 is the cytokine that we have found to be most consistently associated with protection after VP6/LT(R192G) immunization (K. L. Smiley et al., unpublished data). It should be noted that although IFN- γ may play a direct role in protection when produced at the intestinal mucosa after rotavirus infection of VP6/LT(R192G)-immunized mice, the numbers of rotavirus-specific memory $CD4^+$ and $CD8^+$ T cells in the spleens of RRV-immunized mice that could be stimulated to secrete this cytokine did not correlate with the high levels of protection observed (Table 5). Thus, the role of IFN- γ in protection against rotavirus shedding in mice remains to be defined.

The other neonatal model for studies of active immunity against rotavirus is the gnotobiotic piglet. Using this model, it was reported that oral administration of an attenuated human rotavirus (Wa strain) to piglets at 3 to 5 days of age provided partial protection against fecal rotavirus shedding and diarrhea following challenge with virulent Wa (75), while intranasal immunization with a VLP/LT(R192G) vaccine elicited little or no protection against either viral shedding or disease (74). There are multiple differences between the piglet and neonatal mouse models, one of which is that the piglets are gnotobiotic and intestinal immune responses are modified in germfree animals (10, 58, 63). In addition to differences in the levels of protection induced in piglets after VLP immunization and in neonatal mice after VP6 immunization, there were also differences in intestinal rotavirus IgA responses detected after immunization. Rotavirus-specific antibody-secreting cells were readily detectable in intestinal specimens obtained from immunized piglets (74), while rotavirus IgA was not detected in stool specimens obtained from immunized mice. In fact, the levels of intestinal rotavirus-specific antibody-secreting cells appeared to be higher after intranasal administration of VLP/LT(R192G) to piglets than after oral administration of live, attenuated Wa virus (74, 75).

The comparative results obtained with the neonatal piglet and mouse models discussed so far have been for piglets intranasally immunized with VLP/LT(R192G) and mice immunized with VP6/LT(R192G). Others have intranasally immunized adult mice with VLPs and either cholera toxin (53, 60) or LT(R192G) (52) as an adjuvant, and protection against fecal rotavirus shedding obtained after challenge with a murine rotavirus was comparable to that found after immunization of mice with VP6/LT(R192G). Several explanations were provided to explain the differences in protection found when VLPs were used as immunogens in piglets and adult mice, including the relatively small doses of both antigen and adjuvant deliv-

ered to the piglets compared to those given to mice based on body sizes (74). Certainly, VP6 and VLPs are not identical, and possible differences in the importance of CD4 T cells and intestinal antibodies in protection after immunization of mice with these two immunogens have already been discussed here and elsewhere (44). However, it is possible that the difference in protection found after immunization of mice with VP6 and of neonatal piglets with VLPs is primarily due to the ages of the animals at the time of challenge. No protection of mice was observed when they were immunized at 7 days of age and challenged at 10 dpi. Even when challenged at 28 dpi, when the mice were 35 days of age, shedding of the challenge virus was reduced only 76.6%. By 42 dpi, however, protection was identical to that found in mice immunized as adults. Although neonatal mice are quite immunologically immature, their immune systems develop rapidly compared to those of humans (2, 61) and, presumably, piglets. Therefore, the lack of protection in piglets immunized with the VLP/LT(R192G) vaccine may have been due to the immaturity of their immune systems, and protection against fecal virus shedding may have occurred if the time of challenge were delayed beyond 4 to 6 weeks of age, a time when the piglets are expected to be too old to be susceptible to rotavirus disease (67). Although both the neonatal mouse and piglet models have considerable merit in evaluations of rotavirus vaccine candidates, in the end, the predictive value of each will only be determinable after the vaccines are evaluated in humans.

Taken together, the results obtained in this study indicate that neonatal mice do have delayed immune responses to potential rotavirus vaccine candidates. In contrast to results obtained with adult mice, where both B- and T-cell responses were readily detected at 10 dpi with VP6/LT(R192G), no rotavirus-specific B- or T-cell response was detected by 10 dpi for neonatal mice immunized with this nonliving vaccine candidate. However, VP6-specific memory T cells were clearly present by 28 dpi in neonatal mice after a single immunization. It appeared that B-cell responses were not required for protection after immunization with this vaccine candidate, as previously suggested (48), because even by 42 dpi no significant level of anti-VP6 antibody was detected in the face of nearly complete protection. Thus, the immaturity of the immune system of the neonatal mouse did not appear to affect the ability of the VP6/LT(R192G) vaccine to eventually elicit protection. Antibodies were, however, found to play an important role after live, oral RRV immunization of mice. In fact, antirotavirus antibody levels generated after RRV immunization of neonatal mice, although significantly lower than those found in adult mice by 10 dpi, appeared to be sufficient to provide nearly complete protection. Based on this result, the lack of immune system maturity in the neonatal mouse did not prevent the development of a fully protective immune response in neonatal mice that was comparable to that generated in mice immunized with RRV as adults. Thus, if these results are translatable to the human infant, neither vaccine candidate evaluated in this study should be affected, within weeks after its administration, by the immature immune system of the young child if the vaccines are administered during the neonatal period.

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